Biomarkers for tuberculosis in the context of HIV/AIDS in Ethiopia

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Colofon

Illustration on the cover: by the author of this dissertation, depicting the multidimensional approach for the identification of Biomarkers

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Biomarkers voor tuberculose in het kader van HIV/AIDS in Ethiopië

(met een samenvatting in het Nederlands)

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Desta Kassa Misgina

Geboren op 26 December 1968 te Tigray, Ethiopia

Promotors: Prof. dr. A.I.M. Hoepelman
Prof. dr. L. Meyaard

Co-promotors: Dr. D. van Baarle

To my father: **Kassa Misgina Bayreu,** and

My mother: **Yehanesu Woldegebriel Gebrezgiabher**

"መንገዲ እንተጠፊኡካ፣ ጓል መንገዲ ድለ"

Tigringa proverb

beoordelingscommissie : **Prof.dr. M.J.M. Bonten**

Prof. dr. T.H.M. Ottenhoff
Prof. dr. R.A. Coutinho
Prof. dr. F. Miedema
Dr. RE Barth

This study is part of the longitudinal study entitled *"Biomarkers of protective immunity against tuberculosis in the context of Human Immunodeficiency Virus/Acquired immunodeficiency Syndrome (HIV/AIDS) in Africa",* which consists of 15 consortium partner institutions (7 from Africa, 5 from Europe, and 3 from the United States) , and supported by the Bill & Melinda Gates Foundation as a part of the Grand Challenges in Global Health Initiative (GCGHI), grant no. 37772 through GC6-74.

The studies presented in this thesis were conducted at the Ethiopian Health and Nutrition Research Institute in Addis Ababa, Ethiopia, and at the Department of immunology, University Medical Center Utrecht, Utrecht, The Netherlands.

Table of contents

Chapter 1

General introduction

1.1. Tuberculosis

Tuberculosis (TB) is a chronic infectious disease caused primarily by *Mycobacterium tuberculosis* (*Mtb*); and occasionally by other closely related members of *Mycobacterium tuberculosis* complex (MTBC): *M africanum, M bovis, M canetti* and rarely *M microti* (1, 2). *Mtb*, which is a rod shaped intracellular bacillus, is described as neither gram positive nor gram negative, but as acid-fast bacillus (AFB) (3).

It is presumed that the genus Mycobacterium originated more than 150 million years ago (4), and the members of MTBC emerged 40,000 years ago from its progenitor in East Africa (5). Around 460 BC, Hippocrates was the first to describe *phthisis* (to refer TB), while Sh¨onlein used the term "tuberculosis" in 1830 for the first time. 52 years after, in March 1882, Robert Koch discovered *M tuberculosis* as the etiological agent of TB (the tubercle bacillus) (6).

1.1.1. Epidemiology

Despite that nearly 20 million lives have been saved in the past 17 years (7) ,TB is still a major public health threat, particularly in developing countries(**7**). Nearly two billion of the world's population are latently infected with Mtb (2 ,8); and 9 million incident TB cases, and 1.4 million deaths from TB occurred worldwide in 2011. Especially Sub Saharan African is highly affected by TB, where 75% of the TB cases worldwide are found (7).

Ethiopia (population of 84 million) is one of the African countries highly affected by TB. In 2011, the estimated prevalence, incidence of TB, and mortality due to TB (per 100 000 population) was 237, 258, and 18, respectively. The estimated cases with MDR-TB in new-TB cases and previously treated TB cases in 2011 in Ethiopia were 1.6% and 12%, respectively (7). A standardized TB prevention programme and the Directly Observed Treatment, Short Course (DOTS) was established in 1976 and in 1992, respectively. Currently TB treatment service is available in 2773 public DOTS centers in Ethiopia (9).

1.1.2. TB pathogenesis

The clinical outcome of *Mtb* infection depends on the balance between the host immune response to eliminate the bacilli, the survival strategies of the bacilli, and the extent of tissue necrosis and fibrosis. Although the pathogenesis of TB is not fully understood, there are three main chronological events that occur: Inhalation of the bacilli, cell-mediated immunity and granuloma formation, and reactivation of latent infection (10, 3,11) **(Fig 1)**.

Inhalation (transmission) of the bacilli

Except *M bovis*, transmission of the bacilli occurs via aerosols. Once within the lungs, *Mtb* is taken up by alveolar macrophages (AMs) and dendritic cells (DCs), and establish infection by

avoiding phagosome maturation and delivery to the lysosome, which is referred to as **primary infection (Fig 1A)**.

Subsequently, either bacterial killing by the activated macrophages , or successful containment of the infection, or progression to active disease **(referred as primary TB)** occurs. After 7-21 days post infection, the bacilli replicate virtually within macrophages, which induces the production of inflammatory mediators that activate macrophages to kill the bacilli via generation of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (12).

Initial containment of the *Mtb* infection is dependent on host genetic factors that affect progression from infection to disease (e.g. a tuberculin skin test locus (TST-1 and -2) on chromosomal region 11p14 and 5p15, and variants of Toll-like receptors, TLRs); the inflammatory microenvironment at the site of the infection (accumulation of immune cells, differences in cytokine profiles, induction of immunoregulatory mechanisms, and granuloma formation); and the strain of the inhaled *Mtb* (ability to survive within macrophages by triggering of an anti-inflammatory response, blocking ROIs and RNIs production, and reducing the acidification of the *Mtb* containing phagosomes) (10).

It is estimated that, *Mtb* infection is established in 30% of the individuals exposed to the bacilli (close contacts), of whom 12% will develop primary TB and 18% will contain the infection as latent TB (13).

Cell-mediated immunity and Granuloma formation

By 2-8 weeks post infection, a cell-mediated immune response is initiated, and granuloma formation is established due to local inflammatory responses (14). The granuloma formation comprises two parts: the necrotic central core, which is composed of infected AMs and epithelial cells that provide nutritional support to *Mtb*; and the dense cellular wall, which is composed of macrophages, T cells and neutrophils that restrict the spread of *Mtb*. The establishment of granulomas marks the persistence stage of *Mtb* infection, **referred to as latent TB infection (LTBI**) **or latency (Fig 1B).**

Reactivation of latent infection (post-primary TB)

The third phase of TB pathogenesis emerges when the latent infection (dormant bacilli) reactivates, which is **referred to as reactivation or post-primary TB (Fig 1C)**. Approximately 10% of the HIV negative persons latently infected with *Mtb* progress to active TB during their life time (15,16). The main risk factors for the reactivation of LTBI are a decline in the host's immunity due to genetic cause, HIV infection, stress, old age, alcohol abuse, malnutrition and corticosteroid usage (17). Active TB is predominantly a disease of the lung, referred as pulmonary TB (PTB) and accounts for 85% of cases. But also affects organs other than lung,

referred as extrapulmonary (EPTB) that accounts 15% of the TB cases in HIV negative people (18).

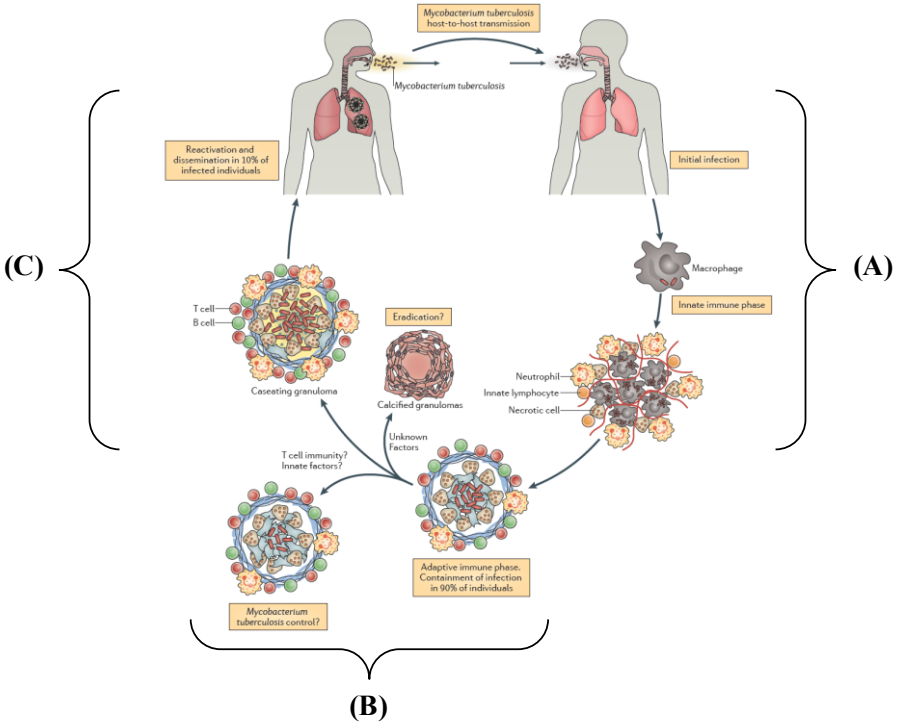

Figure 1: TB pathogenesis.
TB infection and pathogenesis can be divided into three well-defined chronological events. After inhalation of *Mtb* (transmission), the bacilli will be internalized by the alveolar macrophages and **primary infection** is established**.** This initiates innate immune response, which in some cases destroys the bacilli via activated macrophages, and also induce various cytokines and chemokines productions which facilitates recruitment of other immune cells to the site of the infection **(A).** Within 2 to 8 weeks post infection, **cellular immunity develops**; **granuloma,** which consists of infected macrophages , neutrophils, lymphocytes and necrotic cells is established; and the infection is controlled, which is referred as **latent TB infection (LTBI)** (**B).** After years or months, when the immune system failed, the dormant bacilli reactivates, which results in death of the infected macrophages, and formation of necrotic zone (caseum) in the centre of the granuloma. Ultimately, this will be followed by the disintegrating of the granuloma structure, which causes damage to nearby bronchi and more lesions, which is referred as **post primary TB**; and facilitates spreading of the *Mtb* to other areas of the lung and to others(**C**). *(Figure adapted from Nunes-Alves C et al, Nat Rev Microbiol. 2014;12(4):289-99).*

1.1.3. Host immune response against *Mtb* infection

The major host immune response components against *Mtb* infection are innate immunity, T-cell mediated immunity, and production of cytokines and chemokines (10).

Robust innate immune immunity is an essential prerequisite to limit the bacterial growth in the initial phase of *Mtb* infection. The first cells to encounter *Mtb* are alveolar macrophages and tissue DCs, and polymorphonuclear neutrophils (PMN). The first host defense against *Mtb* infection is initiated by alveolar macrophages, where immune mediators such as interferon gamma (IFN-γ) released from infected macrophages, activate the macrophages and promote bacterial killing. IFN-γ is predominantly secreted by natural killer (NK) and T cells upon activation by interleukin 12 (IL-12) and IL-18 produced by DC and monocytes. Moreover, studies have shown an influx of PMN to the site of infection in acute PTB patients. NK cells and PMN have also been reported to directly lyse *Mtb*-infected monocytes and macrophages *in vitro* (19,20).

T-cell mediated immunity is critical to control *Mtb* infection. Following *Mtb* infection, macrophages and DCs initiate T-cell immunity by presenting mycobacterial antigens, costimulatory signals, and cytokine production. Between 8 to 12 days, post-infection, *Mtb* infected DCs migrate to the lymph nodes under the influence of chemokines (CCL19 and CCL21) to activate T cells and drive naive T cell differentiation toward Th1 phenotypes. Between 2 to 8 weeks post-infection, CD4+ and CD8+ T cells have differentiated to effector/memory T cells, and migrate back to the lungs to fight the bacilli.

The T helper (Th)-1 subset of the CD4+ T cells in particular, plays central role to control *Mtb* infection by releasing IFN-γ, which activated macrophages to eliminate the intracellular bacilli, and by facilitating the formation and maintenance of the granuloma by releasing additional pro-inflammatory cytokine. Th-2 CD4+ T cells, via the production of Interleukin (IL)-4, IL-5, IL-10 and transforming growth factor β (TGF-β), either enhances disease progression by counter-acting the protective role of Th1 cells and macrophages; or controls the excessive immunopathologic effect of Th1 inflammatory responses (10,20, 21,22). The more recently identified subset of CD4+T cells, Th17 cells, also play protective role against *Mtb* infection (21). CD8+ T cells play important role in control of *Mtb* infection by secreting IFN-γ and TNF, and by direct lysing *Mtb* infected cells through the production of granulysin (23).

Although, it has been well-established that T-cell effector mechanism rather than B cells (humoral immune response) are required to control the infection (10), there is emerging evidence that B cells can regulate the level of granulomatous reaction, cytokine production, and T cell response (24). It has been demonstrated that antibodies specific to surface antigens of *Mtb* mediate protective effects against *Mtb* infection by limiting the establishment of *Mtb* infection to

other cells, enhance phagosome-lysosome fusion, neutralize bacterial products or toxins, and cellular- or complement-mediated bacterial lysis (25).

The outcome of *Mtb* infection is determined by a complex interplay of the cytokines and chemokines released at different time points of the infection (26, 27). The main cytokines which are shown to be important in the occurrence, progression and control of *Mtb* infection are INF-γ, TNF-α, IL-12, IL-2, IL-6, IL-17, IL-10, IL-4 and TGF-β) (10,25).

Following *Mtb* infection, alveolar macrophages and other non immune cells such epithelial cells and fibroblasts produce chemokines, which leads to early recruitment of innate immune cells to the site of infection, which results in granuloma formation and limits *Mtb* infection (25). However, it is not well defined whether chemokines are indicative of TB disease severity or not (28).

1.1.4. Diagnosis of tuberculosis

According to modeling studies, 400000 lives could be saved each year with the introduction of TB diagnostic with sensitivity greater than 85% and specificity greater than 97% (29). Patients with a persistent cough for more than 2 weeks should be assessed for TB. In children, the important clues for TB diagnostic are a history of exposure to an individual with TB or evidence of TB infection, a positive TST (30,9). The approaches to diagnose active TB include detailed medical history and clinical examination, and bacteriological, histopathological, radiological, and immunological investigations.

Bacteriological methods for TB diagnosis

About 57% of global TB patients receive bacteriological diagnosis for TB that includes microscopy, culture, and molecular methods (31). The sputum smear microscopy, in which the tubercle bacilli in sputum samples are examined under a microscope, which is developed more than 100 years ago, is the gold standard to diagnose TB in most settings where TB is endemic and most severe. However, light microcopy, which requires high bacterial load (3000 –5000 AFB/mL) to detect *Mtb*, has less sensitivity (50 -70%) to diagnose TB (32), especially in individuals coinfected with HIV (~ 35%) (31) and children (<50%) (33).

Culture of *Mtb* from clinical specimens is the gold standard for definite diagnosis of TB, species identification, and drug susceptibility testing (DST). Culture allows to differentiate between dead and live bacilli and genotyping for epidemiologic purposes. The culture method is 20-30% times more sensitive than sputum smear microscopy. However, sputum culture is often expensive, requires high-level technical expertise, and takes at least two weeks to get results, which makes it complex for routine use (34, 32).

Molecular tests are proven technologies for direct detection, identification and susceptibility testing of *Mtb*, and for comparison of *Mtb* strains (genotyping) in clinical specimens. However, molecular tests are expensive, require highly trained expertise, and may not differentiate dead and live bacilli (35, 34)

Radiologic diagnosis of TB

Chest X-ray (CXR), abnormalities suggestive of active TB include upper lobe infiltrates (bi-lateral or uni-lateral), cavitation, and nodular shadows around the cavity. However, CXR findings suggestive for TB are rarely conclusive, particularly in HIV infected patients, and can only complete the clinical presentation and history to constitute arguments of TB (18).

Immunological methods for TB Diagnosis

Immunodiagnostics for TB is increasingly used. Although detection of antibodies to *Mtb* in the blood is a relatively simple and cost-effective, the existing serological tests for TB are inaccurate to diagnosis PTB as well as EPTB. Thus, the WHO issued a policy statement against the use of serological tests for the diagnosis of active TB (31,36). Another approach to diagnose TB is to detect circulating antigens that *Mtb* secretes in clinical specimens such as serum, sputum, urine, CSF, and pleural fluid (37). However, an antigen detection test using urine specimen would be particularly attractive with children and HIV patients who may have difficulty in providing sputum, and in patients suspected of EPTB which might prevent the use of more invasive tests (38).

Cellular immunodiagnostics, including tuberculin skin test (TST) and Interferon-Gamma Release Assays (IGRAs) have been used to diagnose LTBI. The TST is the only universally accepted test for the diagnosis of LTBI. However, sensitivity of TST can be compromised in individuals with immunodeficiency; and specificity can be affected by previous BCG vaccination and exposure to non-tuberculosis mycobacterium. Furthermore, subjectivity of its interpretation, the need for a return visit, and the fact that TST does not distinguish latent and active TB limits the usefulness of TST (39,40). Compared to TST, IGRAs have several advantages including superior specificity and sensitivity, requirement of a single visit, and it does not cause bias to read the test result (40). LTBI is characterized by a positive TST, normal chest X-ray, negative sputum smears and culture, no clinical signs and symptoms, and being non-infectious (41).

1.1.5. Treatment of TB

To achieve the goals of TB treatment (cure the patient, prevent relapse, prevent death, stop TB transmission, and prevent drug resistance), long duration of therapy with a combination of drugs is required. The current recommended regimen for newly diagnosed cases of TB include Isoniazid (H), Rifampin (R), Ethambutol (E), and Pyrazinamide (P) to be taken daily for 8 weeks **(intensive phase)**, followed by Isoniazid and Rifampin to be taken three times weekly for 4

months **(continuation phase)** [2HRZE/4(HR) $_3$]. For re-treatment TB cases, the standard treatement is a combination of five drugs to be taken for the first 8 weeks, followed by four drugs for the next 4 weeks; followed by treatment with a combination of three drugs to be taken for 5 months (42, 9).

There are problems with regard to the current therapies of TB including compliance, treatment interruption, toxic effects, and pharmacokinetic interactions particularly with ART in patients with HIV (43, 16). In addition, treatment of drug-resistant TB is very costly and lasts a minimum of 18 months, and it is associated with high rates of drug related toxic effects (3).

1.2. Human immunodeficiency virus/ Acquired immunodeficiency syndrome (HIV/AIDS)

1.2.1. The etiologic agent

HIV, a member of the genus Lentivirus (slowly replicating retrovirus) and family Retroviridae, was discovered as a causative agent of AIDS in 1983 (44,45).

HIV has two lineages, HIV type 1 (HIV-1) and type 2 (HIV-2), each of which can be subdivided into groups and subtypes. The HIV-1 epidemic involves three distinct groups called M (major), O (outlier), and N (non-M, non-O) (46), but a new group called P was also identified in Cameroon (47). Group M, the most successful group, diversified into nine genetic subtypes (named A-D, F-H and J-K) (48). Whereas, subtype B accounts for 70% of HIV-1 infections in newly diagnosed patients living in Europe (49), in Ethiopia, like in the Southern African countries, Sudan, Somalia and India, the HIV epidemics is exclusively caused by subtype C (50).

1.2.2. The HIV epidemic

Nearly 30 million people died of HV/AIDS worldwide in the past 30 years, and 34.0 million people were living with HIV at the end of 2011. Sub-Saharan Africa, accounts for 23.5 million (69%) of the people living with HIV worldwide, and 70% of all the people dying from AIDS in 2011 (51).

Since the detection of the first two AIDS cases in 1986 in Ethiopia, HIV/AIDS remains to be one of the sever infectious diseases in the country. In 2012, the HIV prevalence in the adult population in Ethiopia was 1.3%. The number of people living with HIV/AIDS was 759,268; the number of AIDS associated deaths was 41, 000, and the number of new HIV infections was estimated to be 20,158. The incidence of HIV infection is reduced by 50% between 2001 and 2011 in Ethiopia (52, 51).

1.2.3. The advent of Highly Active Antiretroviral Therapy (HAART)

The introduction of HAART in the mid 90's, has reduced HIV associated morbidity and mortality, and HIV transmission globally. At the end of 2011, the majority (>55%) of people eligible for ART in low- and middle-income countries and in sub-Saharan Africa receiving ART

(51). In Ethiopia, of the 398000 patients eligible for ART by 2012, about 274805 (69%) were actually treated (53).

There are several challenges associated with HAART including early mortality, incomplete immune-virologic responses, variations in HAART outcomes, lack of universal consensus to define treatment failures, and time to switch regimens, drug resistance , and lost to follow-ups (54, 55). Infections with TB, hepatitis viruses-B (HBV) and C (HCV), cytomegalovirus, or herpes simplex virus also reduce successful HAART outcomes (56). Although, HAART reduces TB related morbidity and mortality (57); restoration of immune responses specific to *Mtb* remained impaired in TB/HIV patients on HAART (58). This suggest the need of longitudinal cohort studies on HAART.

1.3. TB and HIV/AIDS

Mtb and HIV infections aggravate the pathogenesis of one another. In 2011, 1.1 million (13%) of the 8.7 million people who developed TB worldwide were HIV-positive, and there were 0.4 million HIV-associated TB deaths (7). The rate of reactivation of LTBI in HIV negative individuals is 10% in their lifetime while it is 5-10% in HIV infected individuals per year (59). TB accounts for 13% of all HIV/AIDS related adult mortality (60,7).

The severity of TB and HIV infection is exacerbated in Africa, where 79% of the HIV-positive TB cases in the world in 2011 were in this Region (**7).** In Ethiopia, 17% of the incident of TB cases in 2011 were HIV positive (7).

1.4. The challenges to TB control

To eliminate TB by 2050 (incidence of new cases < 1 per million), the incidence of TB must decrease by 16% yearly over the next 40 years (61). However, it is estimated that incidence would only decrease around 6% yearly, indicating that TB would not be eliminated in 2050 (3). The factors that impair the success of TB control include lack of effective diagnostics and therapeutics, the emergence of multi-and extensively drug resist TB (MDR-and XDR-TB) and HIV infection, social and economic factors, and the complex pathogenesis of *Mtb* infection which is not fully understood (62, 63). Thus, in order to control TB at global level successfully, there is an urgent need to develop simple and accurate diagnostics, effective vaccines, and new anti-TB drugs that have shorter duration of therapy, easier to deliver, safe, and low in cost.

The fact that an estimated 90% of individuals infected with *Mtb* remains asymptomatic, while 5-10% develop active TB, shows the opportunity to identify multiple biomarkers for TB that correlates with the clinical stages of TB, which could play crucial role in the development of effective diagnostics and therapeutics (64, 63, 65).

1.5. Biomarkers for TB

The goal of biomarker research is to discover candidate biomarkers and use them to develop novel diagnostic, prognostic, and therapeutic tools for patients (66).

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (66); or any factor whose measured value is consistently associated with changing health or disease states (64**).**

1.5.1. The biomarker research process

As shown in **Fig. 2,** the essential process components for the development of novel biomarker includes **study design**; **candidate biomarker discovery** through differential expression analysis; **qualification**, the process by which candidate biomarkers identified are translated to methods and materials suitable for verification; **validation,** the process by which, the robustness of the candidate markers is tested against a level of biological variability in a larger more heterogonous target populations; and **assay implementation**, where the assays can be either chromatography or antibody-based, both of which can be optimized to provide a robust, sensitive and quantitative assay (67, 68,69).

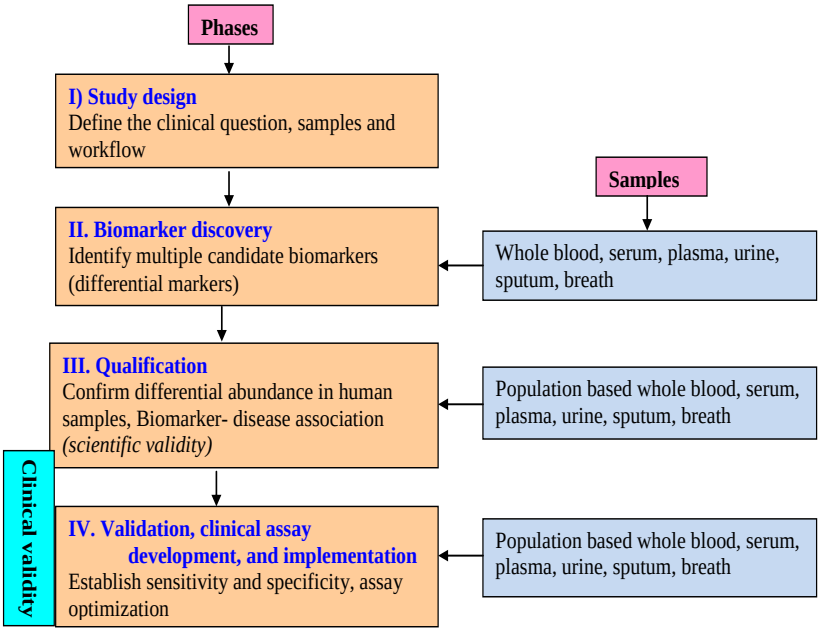

Figure 2. Processes for the development of novel biomarkers for tuberculosis

1.5.2. The aapproaches for TB biomarker discovery

Candidate biomarkers for TB should be able to differentiate people with active TB from healthy individuals, normalized with therapy, and reproducibly predict clinical outcomes in diverse patient populations (63,70).

The potential applications of biomarkers for TB include predicting treatment outcomes in active TB patients, predicting reactivation risk in LTBI individuals, provide a surrogate marker for disease severity, discriminate different types of TB disease including EPTB; and to differentiate active TB case, individuals with LTBI, or neither (63, 70).

TB biomarker discovery is based on three principles: **clinically,** where *Mtb* infection presents with spectrum of clinical stages that are poorly separated; **histopathologically**, where host-pathogen interactions results in changes of immune cell phenotypes and immune mediators they produce; and **immunologically**, where levels of immune markers changes overtime depending on bacilli antigen composition and burden (71). Candidate biomarkers for TB can be measured at the level of immunologic, proteomics and metabolomics (71, 70).

1.6. Scope of the thesis

Tuberculosis remains to be one of the severe infectious diseases, especially in developing countries. Universal and specific biomarkers for TB could accelerate the development of novel diagnostics and therapies for TB, which are urgently needed to control the epidemic effectively.

As part of the "biomarkers for TB" study project *"Biomarkers of protective immunity against tuberculosis in the context of Human Immunodeficiency Virus/Acquired immunodeficiency Syndrome (HIV/AIDS) in Africa"*, which consists of 15consortium partner institutions (7 from Africa, 5 from Europe, and 3 from the United States) , and supported by the Bill & Melinda Gates Foundation through the Grand Challenges in Global Health Initiative (GCGH), grant no. 37772; a prospective cohort study was set up in Ethiopia within this project; with the objectives to identify candidate host biomarkers for TB in the context of HIV infection and therapy.

This thesis consists three parts (Part I, -II and –III).

Part-I, which consist of Chapters 2, 3 and 4, addresses the outcomes of HAART in HIV patients with TB. Both, the success as well as the potential challenges in the era of rapid expansion of access to ART in resource-limited settings where TB and HIV are endemic is reviewed in **Chapter 2.** In **Chapter-3,** a detailed description of the recruitment, diagnostic and follow-up procedures used in this prospective cohort study, as well as the baseline characteristics of the study population are presented. Since studies regarding HAART outcome in HIV patients with TB have been conflicting, in **Chapters 3 and 4,** we addressed the immuno-hematological response, viral suppression, CD4+ T cell count recovery, and TB specific immune function recovery in HIV patients with either latent or active TB on HAART.

Part-II which includes Chapters 4, 5 and 6, focuses on the identification of a panel of cytokines and chemokine biomarkers in response to *Mtb* specific antigens which is essential to develop effective diagnostic and therapeutic tools for TB. In Chapter 4, the profile of cytokine/chemokine responses against a wide range of *Mtb* antigens including secreted, latency, reactivation, and resuscitation-promoting factor (RPF) antigens is investigated. In Chapter-5, the effect of geographical locations and ethnicity on the cytokine/chemokine responses against *Mtb* antigens is presented. In Chapter-6, the effect of HAART and TB treatment on the cytokine/chemokine response to *Mtb* antigens was addressed.

In chapters 8 and 9 of **Part-III**, the pattern of whole blood host gene expression across different ethnic groups in Africa with different HIV and TB infection status are investigated.

In **Chapter-10,** the studies are summarized and discussed in light of current of literature knowledge.

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PART-I

Outcomes of HAART in HIV patients with latent and active TB

Chapter 2

The pattern of immunologic and virologic responses to Highly Active Antiretroviral Treatment (HAART): Does success bring further challenges? Review Article

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Abstract

Background: Since the advent of HAART, there is significant reduction in Opportunistic Infections (OIs), morbidity, mortality and HIV transmission. However, the low Antiretroviral Therapy (ART) coverage in resource-limited countries (42%) and the presence of globally 500-800 thousand patients on first-line required switching to second-line drugs in 2010 are some concerns. Other challenges related to HAART includes lifelong therapy, failed treatment response, optimal time to start treatment and switching regimens, drug interaction, toxicity, cardiovascular risks, drug resistance, lost to follow-up, immune reconstitution Inflammatory syndrome (IRIS), early mortality, and lack of restoration of solid immunity against HIV. To achieve the goals of ART, national ART programmes focus on the vital patient monitoring systems including clinical, immunologic, virologic, adherence, lost to follow-up and mortality.
Objectives: This review is aimed to address the profile of immunovirological responses to HAART and the factors associated with, with a special emphasis on the draw backs of immunologic assessment to diagnose virologic failures.
Major findings: WHO recommends clinical and immunological assessments as surrogates of plasma viral load (VL) to identify first-line treatment failures in resource-poor settings. However, immunological tools have poor sensitivity (20-30%) and specificity (86-90%) to identify virologic failures that may lead to continue with failed regimen or to unnecessary switch of regimen which could result in more complex profile of resistance. There are three major types of immunovirologic responders in clinical practice: Concordant responders (40-60%); concordant non-responders (12-27.3%); and discordant responders which included lack of CD4+ increases despite viral suppression (7-48%), and optimal CD4+ responses in the absence of viral suppression (5-23.8%), whereby the risk of morbidity and mortality is higher in the concordant non-responders and discordant responders.
Conclusions: ART benefits substantial number of HIV patients even in resource-poor settings. Since clinico-immunological assessments have lower performance in diagnosing virologic failures, moving towards the availability of VL testing to confirm treatment failures, if not pre-HAART resistance testing, is a logical and timely approach for resource limited countries like Ethiopia where the long-term effect of the roll-out ART is not well investigated. However, high cost and technical demand of VL testing, lack of experience of health professionals, weak infrastructure and health care system, unavailability and high costs of second-line drugs could be the major challenges during expansion of VL testing. Moreover, longitudinal studies on long-term effects of HAART, and surveys focused on transmitted or acquired HIV drug resistance, and Early Warning Indicators are highly pertinent

Introduction

HIV/AIDS remains to be global challenge since its discovery (1). At the end of 2008, 33.4 million people were living with HIV, 2.7 million newly infected, and 2 million deaths occurred due to HIV/AIDS worldwide (2). In Ethiopia, HIV prevalence of the adult population in 2007 is estimated to be 2.1% (Urban 7.7%, Rural 0.9 %), and the number of people living with HIV is 977,394, including 64,813 children (3).

Despite the absence of curative therapy for HIV/AIDS, Highly Active Antiretroviral therapy (HAART) reduces OIs, HIV transmission, morbidity and mortality at global level (4, 5). Mean survival of those on HAART is estimated to be 13 years (6), although the high rates of deaths among lost to follow-ups should be considered for accurate estimate of survival (7). Moreover, since outcome of HAART at population level depends on time of starting ART, uptake, adherence, pre-treatment and co-infections, survival following HAART might not be uniform in all HIV infected groups (8,9).

Since the launching of "3 by 5" global initiative (10), > 4.7 million people were on ART worldwide at the end of 2008, although only 42% of the 9.5 million people in need of ART had an access in the resource limited countries (5). In Ethiopia, where 3880 patients were getting free ART in three sites before the start of free ART in 2005, >167,000 (~53%) of the adults requiring ART were getting the service in 517 health facilities as of October 2009 (11). Of those on ART in Ethiopia, 99% were on first line regimens with the retention rate of 74% (11), while retention rate in other African countries was 75% and 67% at 12 and at 24 months, respectively, (5).

With all the success stories of HAART, there are challenges which could compromise the goals of ART including failed/incomplete treatment responses (12), drug interaction and toxicity (13), drug resistance (14), lost to follow-up (5), and early mortality (15). Moreover, the presence of 500-800 thousand patients that require switching to second-line drugs in 2010 (5), and where this number could increase gradually with the expansion of ART services, indicates another challenge in terms of availability and increment of cost of the second-line therapy per patient (16)

In summary, whereas the positive impact of ART is remarkable, in view of the patient monitoring system in resource-poor countries which is exclusively dependent on clinico-immunological methods, which lacks sensitivity/specificity to detect virological failures, accurate diagnosis and management of treatment failures could be a major challenge in the era of rapid ART expansion (17). The focus of the current review is therefore to highlight the profile of immunologic and virologic responses to HAART and the risk factors associated with treatment failures, with special emphasis to the limitations of the immunologic based patient monitoring.

Methods

This review comprises published articles in Ethiopia, developing and the developed world which are related to HIV/AIDS infection, ART and the treatment outcomes as measured by immunological and Virologic responses with special emphasis to the limitations of the immunological based monitoring of patients on ART. The review process was done through desk review and online search with more focus to publications of the past five years.

Results and discussion

Monitoring of treatment responses in patients on HAART

Although, the primary goal of HAART is to suppress plasma HIV-1 RNA level (viral load, VL) below the level of detection within three to six months of starting therapy and to maintain it for the rest of the patient's life (**18),** there are other important goals of HARRT including to restore and preserve immunologic function, reduce HIV-related morbidity and mortality, to improve quality of life and to reduce vertical transmission (17).

Since the discovery of Zidovudine (ZDV) in 1987 (19), more than 30 ARV drugs have been made available (20). Although it varies in different guidelines, according to WHO (21), first-line ART includes at least two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) analog; while second-line ART includes two NRTIs boosted with protease inhibitors, preferably Ritonavir. The preferred first line regimens in Ethiopia includes Tenofovir (TDF) + Emtricitabine (FTC) + Efavirenz (EFV); ZDV+ Lamivudine (3TC)+ EFV; or ZDV+3TC+ Nevirapine (NVP) (21).

Although, it varies in different settings, ART should be started when CD4+ count is 201-350, or <= 200/µl for developed and resource-poor settings, respectively (21). However, given the observed lower morbidity, mortality and fewer adverse events associated with the initiation of HAART at higher CD4 cell counts (23, 24), WHO recommends CD4+ count <= 350 cells/µl to initiate ART as a universal guideline (5).

Times of initiating, replacing and stopping therapy are the most critical questions during ART (21). Based on the basics that progression of HIV infection is affected by the synergetic effect of immunological and virological along with host factors (race, genetics, age, gender, mode of transmission, co-infections, nutrition, pregnancy, psychosocial factors) (25), CD4+ count and VL measurements are golden prognostic biomarkers of HIV/AIDS disease progression (26). In settings where measurements of CD4+ count and VL level are limited, total lymphocyte count, hemoglobin (Hgb) and body mass index (BMI) are recommended as simple markers for disease progression (25, 27).

Therefore, taking resource constraints into account, and that virological failures precede immunological failures, then comes clinical failure, WHO guidelines recommend clinical and immunological (CD4+ count) assessments as surrogates for VL to monitor patients on ART in resource limited settings, unlike to that in high income countries where VL is done three to four times a year (21). However, the sequential nature of treatment failure which is not strongly evidence based and may take years to happen is the major drawback of clinicoimmunological assessment based patient monitoring (17). Evidence from models showed an average of five years from the first evidence of virological failure until 50% of patients progress to WHO stage III (3). Likewise, CD4+ counts correlate with the level of VL at group level but not at individual level (28). Thus, immunologic markers have poor sensitivity (20%-33%), specificity (86%-90%), with 21% and 91% positive and negative predictive values, respectively, to identify virologic failures which could lead to continue to treat patients with failed regimen or to unnecessary switch of regimens (29, 30). Thus higher morbidity, mortality and more complex profile of resistance were observed in settings where virologic assessment is not available (31).

Definitions of ART failures

The criteria to define ART failures are not uniform. According to WHO (21), there are three definitions: clinical failure when there is a recurrent WHO stage 4; immunologic failure when CD4 falls to or below the pre-therapy baseline, or below 50% from the on- peak value, or is persistently < 100 cells/mm; Virologic failure when plasma VL >10000 copies/ml; Virologic success when VL is < 400 or 50 copies/ml (depending on the type of the assay) after six months of treatment (21). According to a recent WHO guideline which recommends VL to be done every six months, treatment failure is defined as persistent VL > 5000 copies/ml (5). Although not well defined, VL cut-off > 10000 copies/ml to define treatment failures is linked with subsequent decline in CD4+ cell count (32) and clinical progression (33).

Others defined immunologic failure as an increase of CD4+ cells/ul < 50 at 6-12 months (34); < 100 at 12-24 months (35, 36), or < 500 at 4-5 years (37) irrespective of viral suppression. Virologic failure was defined as a primary failure where VL does not decrease to < 50 copies/ml on two different occasions after six months on ART; and secondary failure (viral rebound) where there is VL >50 copies/ml confirmed (21).

Putting together, the variation in defining the cut-off values of treatment failures indicates the need of research and programmatic data to better understand the profile of immunovirological responses to HAART which might differ in different countries.

The profile of immunologic and virologic responses to HAART

Immunologic responses (CD4+ recovery)

Without therapy, the average decline rate of CD4 cells/μl ("CD4 slope") is estimated to be 50 cells per year, and the average VL level ranges from 30,000 to 50,000 copies/ml (26). CD4+ recovery following HAART which is due to redistribution of the cells from tissues, regeneration of naïve T cells, or due to the reduction of immune activation mediated cell death (apoptosis) (37), occurs as a two phase process: In the first phase of the two months on ART, rapid increase of CD4+ cells occurs; and in the second phase of the third month and onwards on ART, CD4+ count increase slows down but persists over time (38). Overall, the long-term shape of CD4+ count after HAART depends on the baseline CD4+ count, control of viral replication overtime, the stage of the disease at baseline, duration on treatment (39, 40), as well as on baseline patient factors including higher HIV RNA level, co-morbidities, presence of drug resistant viruses, sub-optimal pharmacokinetics, and potency of the ARV regimen (17). The time required to reach to normal value of CD4+ counts ranges from two to eight years (24, 41).

Immunological failure and the risk factors

Complete immune recovery following HAART is not observed in all patients. Absent or modest improvements in CD4+ counts occurs in 5–27% of the patients on HAART who achieve plasma HIV-1 RNA suppression (42,43,44) which has clinical implications. Higher relative risk of progression to AIDS; and AIDS and non-AIDS related mortalities were reported among discordant responders as compared to those virologic and immunologic concordant responders (45). Whereas there is no clear understanding on how to assess immunologic failures with regard to time after HAART, questions such as the clinical risks and the possible treatment for immunological failures would be a concern for the health care workers in the ART clinic (46).

Of the risk factors for the failure or incomplete immune recovery includes the degree of CD4+ decline before and at the initiation of the treatment (the steeper the decline the steeper the rise), the rate of decline in viral-load (47), older age (17, 47), co-infection (e.g. HCV, HIV-2, HTLV-1, HTLV-2), medications (ZDV, TDF+DDI), and persistent immune activation (17). However, others have reported no differences in immunological response according to baseline viral load, HIV risk factor, sex, HCV co-infection and HAART regimen (24). Several explanations have been given about the mechanisms by which inadequate immune CD4+ recovery occurred in response to HAART. These included, myelosuppressive effects of ARV drugs (e.g. ZDV) (48), thymic involution related to older age (49), and abnormal cell death (apoptosis) due to higher immune activation related to higher background risk of endemic infections (50)

Virologic responses

VL level is an excellent indicator of the degree of viral replication in the immune system, progression to AIDS, morbidity, mortality, and HIV transmission. VL level predicts also treatment success/failure faster than CD4+ counts and also resolves discordant clinico-immunological responses, so that it is an important tool to protect 1st and 2nd line regimens from unnecessary switch whereby reduces resistance risk. Due to all these factors, therefore, VL measurement has been considered as a golden standard to monitor patients on ART (51). However, whereas CD4+ level which measures the strength of the immune system is the best biomarker of when to start treatment (5), VL test is less necessary before initiating ART as it rarely informs when to start ART (21).

Even though not always true, the minimum change in VL after treatment to be considered statistically significant (2 standard deviations) is a threefold or a 0.5 $\log_{10}$ copies/ml change (52). Virologic response has been reported therefore to decrease at week 72 and disappeared after 96 weeks of treatment (53). It has been observed also that 75-90.7% of treatment-naïve patients reached undetectable viral load by 12 months on ART, while it was reduced to 72% after 24 months (53). The proportion of treatment naïve patients with viral rebound was 9.4% after one year, and 20.1-20.6 % after 2 years, while it was 35·7–40·1% after 2 years of pretreated patients (54,55).

Virological failure and the risk factors

The risk factors for virological failure includes sex (although reports are controversial) (19, 56), older age, poor adherence, previous exposure to ART, lower base line CD4+ count, OIs, TB after ART, persistent lower VL, insufficient CD4+ cell gain, clinical symptoms, lower weight than baseline, and emergence of drug resistant viruses (57,58). Digestive symptoms and poor adherence to ART were also reported as risk factors for low ARV plasma concentrations (59), which in turn results in sub-optimal virlogical responses.

In summary, besides that WHO recommends a single second line regimen for Virologic failures in poor settings (21), identification and managing of virologic failures could be more challenging in resource limited settings due to the limitation of trained health professionals, preparedness of the health facilities, and limited availability of second line drugs.

Discordant/Concordant Immunovirological responses

Besides the independent immunologic and virologic failures (12), concordant/discordant responses are another challenge during ART. Although the frequency of concordant/discordant immunovirologic responders depends on the definition (cut-off values) of immunologic and virologic responses, there are three immunovirological responders in clinical practices: 1)

Concordant responders ($VL^+/CD4^+$) (40-60%), 2) Concordant non-responders ($VL^-/CD4^-$) (12-27.3%), and 3) discordant responders which is sub divided as immunological non-responders (lack of CD4 increases despite viral suppression ($VL^+/CD4^-$), (7%-48%), and immunological responders (good CD4+ responses in the absence of viral suppression, $VL^-/CD4^+$) (5%-18%) (15, 51, 59, 61, 62).

Whereas discordant results complicate the interaction between virological and CD4+ count response (61), and cause more challenges to the health care providers during patient management and monitoring (51), higher risk of clinical progression and mortality was observed in discordant responders as compared to complete response (62,63).

The risk factors for discordant/concordant ART responses

The mechanisms of discordant response ($VL^+/CD4^-$, $VL^-/CD4^+$) are not fully understood (36). Among the risk factors for $VL^+/CD4^-$ were lower baseline CD4+ count (50-100/µl), higher baseline VL (100,000 copies/ml), HAART composed of three NRTIs, the use of lamivudine (3TC)/zidovudine (ZDV), didanosine/tenofovir (DDI/TDF), poor adherence, advanced age, and being ARV naïve (63). The factors which contribute for $VL^-/CD4^+$ includes sexual transmission of HIV, absence of clinical progression, lower baseline CD4 counts, higher baseline VL, low-level viral rebound during the first year after achieving undetectable VL, younger age, pretreatment and saquinavir regimen (63), use of 3TC/ZDV, ddI/3TC, or ddI/stavudine, ritonavir-boosted protease inhibitor-(PI) based regimen (36, 64), and treatment compliance (53).

Evidences showed that the frequency and risk factors for discordant responses to HAART in developing and developed countries were comparable (64). However, the studies are different in terms of study design, inclusion/exclusion criteria, ethnicity, ART experience, sample size, ARV regimen, length of follow-up, and the definitions, which results in the variations of results related to the factors associated with discordant responses. Therefore, longer follow-up studies are highly pertinent to assess the pattern as well as the long-term impact of concordant/discordant responses treatment outcomes and the risk factors associated with in the context of local settings (64).

HAART and TB/HIV co-infection

Co-infection with TB/HIV complicates pathogenesis, epidemiology, clinical presentation, diagnosis, treatment, prevention aspects of one or the other. Whereas 11% of all HIV/AIDS - related adult mortality accounts to TB, 39% of all TB related deaths is attributable to HIV (64).

Patients from TB endemic areas present themselves to the health facilities for TB/HIV diagnosis and simultaneous treatment. Even though the optimal interval between the starting of TB

treatment and ART remains to be determined (65), the objectives for initiating early ART in patients on anti-TB treatment are to reduce risk of HIV related morbidity/mortality and improved sputum smear conversion; while the factors for differing ART includes high pill burden, poor adherence, impaired tolerability, drug-drug interactions, toxicity, and morbidity and mortality due to TBIRIS (66).

According to WHO guidelines (21), ART should be initiated within 2 to 8 weeks of anti-TB treatment in those with CD4+ count < 200 cells/ul; in the continuation phase of anti-TB treatment in those with CD4+ cell counts 200-350 cells/ul; but with great urgency in those highly immunocompromised patients. However, in cases a person needs TB and HIV treatment concurrently, first line treatment options include ZDV/3TC or d4T/3TC plus either an NNRTI or ABC (5).

Overall, HAART restores host immune response specific to *Mycobacterium tuberculosis (MTB)*, reduces incidence of TB, and improves survival, while anti-TB treatment in TB/HIV co-infected patients on the other hand minimizes the negative effects of TB on the course of HIV and reduces the transmission of MTB (59, 67, 68, 69).

Whereas the long term impact of HAART on TB control is dependent in part on the rate and extent of *MTB* specific immune restoration (68), TB disease prevalence at baseline, and incidence rate during the initial months of ART are higher on those enrolled for ART, which results in higher morbidity, mortality and complicates the delivery of ART (Fig 1) (68, 70, 71).

Specific strategy is required to reduce the impact of TB in the era of ART.WHO has recommended the '3Is' strategy which incorporates intensified case finding, infection control and isoniazed preventive therapy to reduce the burden of TB in people living with HIV (72). However, the scenario of intensive case finding is also greatly affected by the screening strategy applied, immunodeficiency and the diagnostic tests available (64). In this regard, the fact that most diseases are sputum smear negative and culture-positive (73) is also a major challenge.

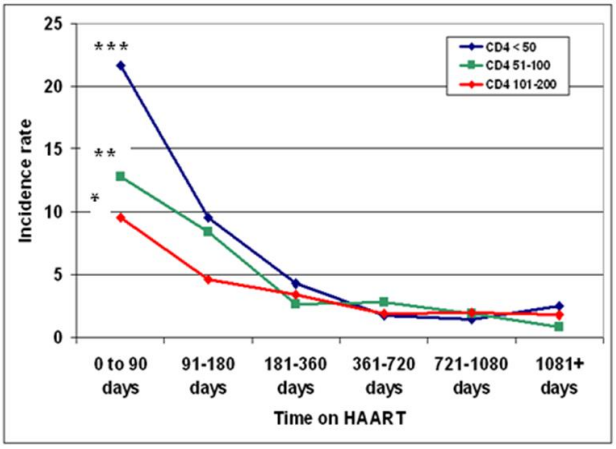

Fig 1. The incidence of TB in HIV infected patients on HAART relative to duration on HAART (days) and CD4+ counts (*: CD4+ < 50; **: CD4+ 51-100; *: CD4+ 101-200 (adapted from 63)**

Furthermore, whereas concurrent treatments with anti-TB and HAART improves survival of the patients (74), there are also reported complications during the dual treatment including drug interactions, increased risk of treatment interruptions, high pill burden, shared toxicities, and paradoxical TB Induced Reconstitution inflammatory Syndrome (TBIRIS) (65,75). Nevirapine concentrations are frequently sub-therapeutic in patients on rifampicin-based TB treatment, which may result in inferior virological outcomes (65); while others have reported virological responses in TB/HIV patients to be similar with those who did not have TB (70).

Putting together, during their simultaneous treatment with anti-TB and ART, awareness creation of the health professionals in the TB/HIV clinics is highly essential in areas where TB and HIV are prevalent. Furthermore, future directions of clinical and basic researches should be conducted to improve TB diagnosis in HIV-infected, in children and extrapulmonary TB, to improve treatment strategy in TB/HIV, for better understanding of the pathophysiology of IRIS, and develop new preventive measures.

Summary

HAART restores host immune responses, decreases risk of OI, morbidity and mortality globally. The un-resolved questions related to HAART comprises variations in the treatment outcomes (countries, Ethnic), cut-off values for immunovirological failures, discordant results; drug resistance, toxicity, drug interactions, and early mortality.

Since clinical and immunological assessments lack sensitivity/specificity to diagnose virologic failures, complications in the patient monitoring in the era of extensive ART expansion might

occur in the resource poor settings, where patient monitoring is dependent on clinico-immunological parameters. Incorporating VL testing to confirm treatment failures, as well as genotyping testing for treatment failed samples, if not pre-HAART resistance testing, should be an immediate plan. Furthermore, considering the rapid expansion of ART where the long-term effect of which is not well investigated, and that research data on ART are predominantly from the developed world, local research data from well defined cohorts of patients on long-term HAART which can complement data from randomized clinical trials (76) are highly pertinent and timely. Likewise, routine national surveys for transmitted and acquired ARV drug resistance and Early Warning Indicators (EWI) should also be implemented in parallel to the rapid expansion of ART.

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Chapter 3

Clinical, hematologic and immunologic characteristics of Mycobacterium tuberculosis (Mtb) patients with and without HIV-1 infection: Responses to six month TB treatment.

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Abstract

Comprehensive clinical management of TB/HIV patients is a challenge in endemic areas. Clinical, immunologic and hematologic parameters are important for better patient management and further understanding of TB/HIV interactions. We characterized symptoms for TB, immunologic, hematologic, HIV RNA load (VL), and IFN-γ response to the *Mtb* specific antigens (ESAT-6/CFP-10) in five clinical groups before and after TB treatment (TT).

Adults of both sexes (n= 224) included 132 TB cases [HIV-TB+=80; HIV+TB+=52], 67 LTBI [HIV-TST+=43; HIV+TST+=24], and 25 Controls (HIV-TST-). Cough and weight loss were the common symptoms in HIV-TB+. There was >70% overlap of cough with CD4+<200 cells/µl in HIV+TB+ patients, indicating late onset of symptoms, or late presentation of the patients. There were lower CD4+, TLC, Hgb, and BMI, but higher WBC and Neutrophil values in HIV-TB+ ($p < 0.05$), which all normalized after TT. HIV coinfection (HIV+TB+) further perturbs the immunohematologic values, and no significant increase in CD4+ count, decrease in VL, or normalization of any hematologic values after TT was observed. IFN-γ production was elevated in HIV-TST+ but impaired in HIV-TB+ *($p = 0.003$)*, severely impaired in HIV+TB+ (*$p = 0.07$)* and HIV+TST+ (*$p = 0.002$).* In addition, IFN-γ production reconstituted with TT in HIV-TB+ *($p = 0.02$)*, but not in HIV+TB+ patients, which was correlated with CD4+ counts (*$r=0.76$, $p = 0.006$)*. In conclusion, the distinctive pattern of CD4+, TLC, Hgb, WBC, Neutrophil, and BMI in TB and TB/HIV patients shows they may serve as prognostic markers and response to TB therapy. Likewise, the distinctive profile of IFN-γ during active TB and LTBI confirms the central role of IFN-γ to control *Mtb* infection, and its potential to serve as a correlate of protective immunity and response to therapy. The higher mortality and the impaired response in CD4+, VL and IFN-γ to TT in TB/HIV patients indicates severe imunosuppression at diagnosis, and stress the need of early intervention, supporting early initiation of HAART for TB/HIV patients.

Introduction

Mycobacterium tuberculosis (*Mtb*) causes severe morbidity and mortality next to HIV/AIDS [1]. Ethiopia, with a population of 79.3 million, ranks 7th among the 22 high TB burden countries in the world [2].

Although the host immunity against *Mtb* is not fully understood, the CD4+ cell mediated cellular immune response plays a central role [3]. Likewise, neutrophils which are recruited earlier into infected sites upon inflammatory signals, are known to induce microbicidal activities as well as granuloma formation during *Mtb* infection [4]. TB disease, however, is known to perturb a diverse set of blood cells. Both an increase in total white blood cell (WBC) and neutrophil counts [5] as well as a decrease in total lymphocyte count (TLC) and CD4+ cells [6] have been found. In addition, a decrease in hemoglobin (Hgb) and in body mass index (BMI) [7] was reported during TB disease. However, whether TB induces depletion of CD4+ T cells and whether alteration of WBC populations is associated with immune dysfunction specific to TB is not well understood. In this study, we assessed the correlation between the dynamics of CD4+ T cells, and the level of IFN-γ production upon stimulation with *Mtb* antigens during TB disease and treatment.

HIV coinfection is suggested to further alter blood cell populations and changing the Th1/Th2 dichotomy [8] which influences the course of pulmonary TB [3], clinical presentation [9] and clinical signs and symptoms [10], leading to misdiagnosis or delay in diagnosis of TB [10]. Discontinuation and failure of TB treatment [11] and drug resistant TB [12] are also higher during TB/HIV coinfection. Moreover, quality of life of TB/HIV coinfected patients was reported to be lower as compared to HIV infected patients without active TB [13].

Furthermore, although it is known that TST results can be affected by several confounding factors [14], diagnosis and treatment of latent TB still depends largely on TST [15]**.** As reported by others [16, 17], TST reactivity was also affected by higher viremia and lower CD4+ count in patients infected with HIV.

The severity of TB infection could be exacerbated in Africa where dual infection is high [2]. In Ethiopia, the HIV infection rate among TB patients was estimated to be 19% (30% in urban and 15% in rural areas) [2, 18], and 19% in smear positive and 26% in smear negative pulmonary TB patients [18]. Earlier studies done in Ethiopia have also shown that TB affects all occupational groups, but most severely affects the age group from 20-30 which is primarily dying of AIDS [19].

Comprehensive management of TB/HIV coinfected patients is a real challenge in settings like in Ethiopia where TB/HIV coinfection is endemic and resources including well-trained health care personnel and advanced laboratory technologies are limited. Thus, although locally established

epidemiological, clinical, and immunehematologic indices are helpful for accurate differential diagnosis and better patient management, there is no sufficient data related to TB/HIV coinfection in Ethiopia.

This study aimed to characterize immunologic (CD4+ count), virologic (HIV RNA level) and hematologic (WBC, TLC, neutrophil, and Hgb) parameters. In addition, presentation of symptoms and BMI were analyzed. Furthermore, we measured IFN-γ production to the *Mtb* specific antigen Early Secretory Antigenic Target-6/Culture Filtrate Protein-10 (ESAT-6/CFP-10). These analyses were performed in five clinical groups including pulmonary TB patients [(HIV+TB+, HIV-TB+)]; Tuberculin Skin Test positive (TST+) subjects [(HIV+TST+, HIV-TST+)], and apparently healthy controls (HIV-TST-), before and after TB treatment.

Methods and materials

Study population

This descriptive and longitudinal type of study was conducted from April 2007-January 2009 at St Peter Specialized Referral TB Hospital, Akaki and Kality Health centers, in Addis Ababa, Ethiopia. 224 adults of both sexes were enrolled from the health facilities after informed and written consent was sought. These included: 132 pulmonary TB cases [(HIV+TB+=52; HIV-TB+=80)]; 67 latent TB infected (LTBI) groups [(HIV+TST+=43; HIV-TST+=24)]; and 25 Controls (HIV-TST-). After enrolment, HIV-TB+ and HIV+TB+ patients were scheduled for a 6th month follow-up visit. Subjects with diabetes mellitus, chronic bronchitis, on steroid therapy, previously or currently on ART or TB treatment, and with pregnancy were excluded at enrolment.

At recruitment, all subjects were interviewed using a standard questionnaire, and detailed physical and clinical examinations, anthropometric and demographic data were recorded. 20 ml heparinized venous blood was collected from all subjects at diagnosis, and at 6th month follow-up. Samples were processed within three hours at the National HIV Referral Laboratory, of the Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa.

Laboratory tests

Laboratory examinations of blood were performed by automated machines. Hematological values were determined using Cell Dyn, *Abbott laboratories, Abott Par IC Jl 60064, US*; CD4+ T cell counts were determined using BD FACSCalibur (Becton Dickinson, San Jose, USA); and Plasma HIV RNA load was assessed using the NucliSens EasyQ NASBA diagnostic 2007/1 (Organon, Teknika) which quantifies HIV-1 with a linear dynamic range from 50-3,000,000 copies /ml. Measurement of IFN-γ in the 7th day whole blood culture supernatant stimulated with *Mtb* specific antigen (ESAT-6/CFP-10) were measured by xMAP multiplex technology (Luminex, Austin TX, USA), using Biosource reagents (Biosource, Camarillo, USA), and

analyzed with the STarStation v2.0 software (Applied Cytometry Systems, United Kingdom) as described previously [20]. HIV-1 infection screening was done using rapid tests: Determine HIV1/2 (Abbott Laboratories, Japan), Capilus HIV-1/2 (Trinity Biotech, Ireland); and Unigold HIV-1/2 Recombinant/Synthetic (Trinity Biotech, Ireland) [21].

Diagnosis and treatment of pulmonary tuberculosis (PTB)

TB diagnosis and treatment were given at the study sites according to the national protocol [21]**.** Diagnosis of PTB was based on both clinical and bacteriological evidence. At least two sputum smears stained by the Ziehil-Neelsen direct method were required to be positive for Acid Fast Bacilli (AFB) with microscopy**.** Confirmed TB patients were put on Directly Observed Treatment Short Course (DOTS) with the regimen including Rifampicin (R), Isoniazed (H), Pyrazinamide (Z) and Ethambutol (E) for two months of intensive phase, and H and E for the continuation phase for six months**.**

Mantoux Tuberculin Skin Testing (TST)

Except for active TB patients, cutaneous test for tuberculin was done for all participants by an experienced nurse according to the national guidelines [21]. A diameter of skin induration with ≥ 10 mm in HIV uninfected, and ≥ 5 mm in HIV-infected individuals was graded as TST positive (TST+), the remaining were recorded as TST negatives (TST-). For further comparative analysis, anergic reaction was defined as TST size "0 mm".

Definition of clinical and laboratory abnormalities

BMI [weight (kg)/height (m) ^{2}], was defined as being normal when BMI ≥ 18.50 to 24.99 kg/m^2); and it was defined as malnutrition when BMI <18.50 kg/m^2) [22]. Hgb (<12g/dl), total WBC (<4000/μl), neutrophil (<2200/μl), TLC (<1200/μl) and CD4+ T cell <200/μl) were used to define anemia, leucopenia, Neutroponia, lymphopenia, and CD4+ T cell lymphocytopenia, respectively. Fever was defined as axillary temperature ≥ 37.5 ^{0}C [23].

Statistical analysis

Entered data were double-checked for discrepancies. A descriptive analysis including counts and frequencies for categorical variables, and mean (standard deviation, SD) or median (interquartile range, IQR) for continuous variables were computed. Results were compared between and within groups using Pearson's chi square for categorical variables, and the non-parametric (*Mann-Whitney U test* or *Wilcoxon Rank-Sum test*), or parametric tests (Student's *t* test or ANOVA) for continuous variables. The level of significance was set at $p < 0.05$ and all *p* values were two-tailed. Analysis was done using Intercooled STATA version 10.0 (College Station, Texas, USA).

Ethical considerations

This study was approved by the Research and Ethical Clearance Committee (RECC), EHNRI; and by the National Health Research Ethics Review Committee (NHRERC) of the Ethiopian Science and Technology Agency (ESTA), Ethiopia.

Results

1. *Characteristics of the study participants*

A summary of the demographic characteristics and clinical features of the 224 study participants are shown in Table 1. The mean ± SD of age of the study population at enrolment was 30.8 ±8.7 years, and 101 (45.1%) were males.

Clinical features

As shown in Table 1, the common symptoms in HIV-TB+ patients in descending order were cough, weight loss, chest pain, fatigue, fever, night sweating, difficulty of breathing, and cough up blood. Frequency of these symptoms was similar in the HIV+TB+, except that difficulty with breathing was lower in HIV+TB+ than in HIV-TB+ patients ($p = 0.014$).

Considering cough for more than two weeks as the common clinical symptom [24], symptoms which were co-expressed with cough in the HIV+TB+ groups were night sweating (90.7%) weight loss (83.7 %), fatigue (81.4%), fever (63.3%) and cough with blood (55.8%). Moreover, there was more than 70% overlap between the incidence of the symptoms of TB (coughing, cough up blood, fever, night sweatiness and fatigue) and imunosuppression (CD4+ < 200 cells/µl) in the HIV+TB+ patients, which might be related to the late onset of the symptoms and/or late presentation of the patients to the health facilities.

BCG scar presence and TST responsiveness

A BCG scar was observed in 28.1% of all the study populations. Observed scar frequency was almost 10% lower in that of TB cases (20.4%), followed by TST negative groups (31.3%), while it was highest in the TST positive groups (39.5%). Scar prevalence was also moderately lower in the HIV positive than the HIV negative groups (Table 1).

We also observed a lower TST reaction size ($p = 0.008$) and higher frequency of patients with anergic TST induration ($\chi^2 = 10.8$; $p = 0.001$) in HIV infected (HIV+TST+) than in HIV un-infected (HIV-TST+) groups. TST reactivity was diminished in subjects with CD4+ T cells below 200 cells/µl and with VL > 100000 copies/ml.

Table1: Pretreatment distribution of demographic and clinical features of the study groups (n=224) enrolled from three health facilities in Addis Ababa, Ethiopia.

Parameters	HIV+TB+ (n=52)	HIV-TB+ (n=80)	HIV+TST+ (n=43)	HIV-TST+ (n=24)	Controls (n=25)	p[a]
Demographic data						
Sex: Male: 101(45.1)	24 (46.2)	42 (52.5)	17 (39.5)	9 (37.50)	9 (36)	0.17
Age, yrs: 30.8± 8.7	33.7 (8.4)	28.7 (9.8)	35.0 (8.0)	26.6 (7.4)	24.6 (6.6)	
Clinical features						
Coughing ≥ 2 weeks	43 (80.7)	72 (90)	4 (9.3)	3 (12.5)	0	**
Cough up blood	12 (23.1)	20 (25.0)	2 (4.7)	0	0	**
Difficulty of breathing	29 (55.8)	61 (76.3)	3 (7.0)	2 (8.3)	1 (4.0)	**
Fever (> 37.5 0C)	42 (81.8)	65 (81.3)	14 (32.6)	3 (12.5)	0	**
Night sweating	46 (88.5)	65 (81.3)	13 (30.2)	3 (12.5)	0	**
Weight loss	44 (84.6)	69 (86.3)	14 (32.6)	1 (4.2)	0	**
Chest pain	42 (80.8)	66 (82.5)	6 (14.0)	3 (12.5)	0	**
Fatigue	42 (84.3)	65 (81.3)	12 (27.9)	1 (4.2)	0	
Never smoking	42 (80.8)	73 (91.3)	34 (79.1)	23 (95.8)	24 (96.6)	
BCG scar presence and TST responsiveness						
BCG: 86(28.1) [§]	10 (19.2)	17 (21.2)	16 (37.2)	10 (41.7)	8 (32.0)	
TST induration (mm):	NA	NA	6.7 (10.9) [c]	9.9 (9.3)		*
TST anergy, n(%)	NA	NA	82 (68.9)	20 (41.7)		**
TST ulceration	NA	NA	6 (4.8)	1 (2.1.)		

Values are *n (%)* of patients, except for age and TST **(**tuberculin skin test TST) induration are in mean and standard deviation (SD); BCG: Bacille Calmette-Guerin vaccination; NA**:** not available;

[a]**:** Comparison among all the study groups; * $p < 0.05$, ** $p < 0.001$

[b]**:** Scar noted

[c] :Significant difference in mean TST size of the HIV positives than the HIV negatives, $p < 0.05$

2. Laboratory parameters and response to the six month TB treatment

We also characterized the immunohematologic, virologic, BMI, and IFN-γ production to the *Mtb* specific antigen (ESAT-6/ CFP-10) of the five clinical groups at enrolment. Moreover, response of theses baseline parameters to the 6th month of TB treatment in pulmonary TB patients was investigated.

Overall, a total of 15 (9.7%) patients died within the first 6 months of follow-up of whom 8 (15.4%) were HIV+TB+, 4 (6.7%) were HIV-TB+ and 3 (7.0%) were HIV+TST+. Of the HIV+TB+ patients who died, 87.5% had CD4+ T cells below 200/µl, 66.7% were anemic and 37.5%, had malnutrition (data not shown).

We further investigated the risk factors for early mortality of the pulmonary TB patients using univariate logistic regression. As shown in Table 2, CD4+ cells <100/µl (OR: 6.3, 95% CI 1.7 - 23.5); CD4+ % < 14 (OR: 3.4, 95%CI 1.0 -12.1), TLC < 1200 cells/µl (OR: 7.6, 95% CI 1.0-63.7), and WHO-stages III plus IV (OR: 5.6 95% CI 1.1-28.3, *p = 0.03*), were significantly associated with early mortality.

Immunologic, virologic and hematologic values and response to TB treatment

The summary of the immunohematologic (CD4+ count, WBC, TLC, neutrophil, and Hgb), virologic (HIV RNA level), BMI, and IFN-γ production to the *Mtb* specific antigen (ESAT-6/ CFP-10) at diagnosis and after six months of TB treatment are shown in Table 3.

At diagnosis and relative to controls there was lower Hgb (g/dl) (*p < 0.0001),* CD4 cells/µl *(p < 0.000),* and TLC *(p = 0.02),* higher WBC and neutrophil counts (*p <0.001; p <0.0001)* during TB disease (HIV-TB+). We observed only lower Hgb *(p = 0.018)* but no difference in any of the leukocyte sub-populations in HIV-TST+ compared to controls.

In response to TB treatment, an increase in CD4+ cells (*p = 0.05),* CD4% *(p = 0.22),* Hgb (*p = 0.003*), and in TLC *(p = 0.0006),* but a decrease in total WBC *(p = 0.08)* and Neutrophil *(p < 0.001)* counts were observed in the HIV uninfected TB patients (Table 3).

Response to TB treatment in HIV infected TB patients however did not improve CD4+ cells, CD4%, Hgb, TLC, WBC and neutrophil values. Likewise, despite the lower CD4+ count *(p=0.003)* and elevated VL level (*p=0.02*) in HIV+TB+ than in HIV+TST+ patients at enrolment, there was no significant increase in either CD4+ count or VL level after TB treatment in the HIV+TB+ patients with no HAART (Table 3).

Moreover, there was a higher proportion of HIV+TB+ patients with abnormal laboratory values at diagnosis including CD4+ lymphopenia (24.9%), lymphopenia(73.7%), malnutrition (50%), anemia,(57.9%), Leucopenia(31.6%), and Neutropenia (21.1%)

BMI and response to TB treatment

As shown in Table 3, the BMI in HIV-TB+ patients was lower compared to that of controls (*p < 0.0001).* Moreover, although the mean BMI in HIV-TB+ patients at diagnosis was as low as of HIV+TB+ *(p = 0.82)*, BMI reached normal values in HIV-TB+ but not in HIV+TB+ patients after TB treatment.

Table 2. Risk factors associated with early deaths of active pulmonary patients (n=11) occurring within the first six months of follow-up.

Characteristics (variables)	Early deaths (%)	x^2 *(p-value)*	Crude OR (95% CI)	OR-*p Value*
Gender				
Male	7/80 (8.8)	0.5	1	
Female	4/72 (5.6)	(0.45)	2.0 (0.5-7.4)	0.28
Age (years				
15- 34	4/35 (11.4)	1.2	1	
35=-60	7/117 (6.0)	(0.27)	1.6 (0.5-5.8)	0.45
CD4+ count/µl				
≥100	4/80 (5.0)	9.2	1	
<100	7/30 (23.3)	(0.002)	6.3 (1.7 - 23.5)	0.002
CD4+ %				
≥14	5/82 (6.1)	4.0	1	
<14	6/33 (18.2)	(0.04)	3.4 (1.0 -12.1)	0.01
TLC				
≥1200	1/44 (2.3)	5.1	1	
< 1200	8/53 (15.1)	(0.02)	7.6 (0.9-63.7)	0.03
BMI				
≥18.5	6/75 (8.0)	0.12	1	
< 18.5	5/77 (6.5)	(0.72)	0.8 (0.2-2.7)	0.56
Hgb				
≥12	2/56 (3.6)	5.1 (0.03)	1	
<12	7/41 (17.1)		5.5 (1.1-28.3)	0.40
WHO staging				
III and IV	7/48 (14.6)	0.5	1	
I and II	0/3 (0)	(0.48)	5.6 (1.1-28.3)	0.03

OR: odds ratio; TLC: Total lymphocyte count (x1000 /µl of blood); BMI: Body Mass Index (kg/m^2); Hgb: Hemoglobin (g/dl); WHO staging: World Health Organization clinical stage

Table 3. BMI and Laboratory parameters of the latent TB infected populations (HIV+TST+ and HIV-TST+) and of the pulmonary TB patients (HIV+TB and HIV-TB+) with and without HIV coinfection at diagnosis and after six months of anti-TB treatment (after reatment)

			HIV+TB+		HIV-TB+		
	HIV+TST+ (n=35)	HIV-TST+ (n=22)	before treatment (n=38)	after treatment (n)	before treatment (n=54)	after treatment (n)	Controls (n=23)
BMI (kg/ m^3)	21.1 ±3.0	21.4 ±2.6	18.9 ±3.1[a]	21.8 ±1.8 (14)	18.9 ±2.9 [a]	20.1± 3.1[b] (58)	21.4 ±2.0
Hgb (g/dl)	12.6 ±2.7 [a]	16.4 ±1.8 [a]	11.8 ±3.0 [a]	13.8 ±1.5 (7)	13.4±2.5 [a]	16.0± 2.7[b] (19)	17.9 ±2.1
WBC	3971 ±1806	4686 ±1957	4854 ±1599 [a]	3838±883 (6)	6824±312 2 [a]	5320± 1595 [b] (21)	4547 ±1497
neutrophil	2117±1286	2331 ±1494	3265 ±1504 [a]	1860 ±772[b] (6)	4840 ±2825 [a]	2655± 1529 [b] (19)	2599 ±1170
TLC	1474 ±849	1975 ±1483	1137±798 [a]	1597 ± 581(7)	1477 ±887 [a]	2132± 908 [b] (21)	1667 ±559
CD% (n)	20.7±10.2[a] (n=38)	44.0±6.7 (22)	13.7 ±8.7 [a] (54)	19.0 ±9.1 (9)	37.1±9.4 [a] (61)	40.0± 6.0 (27)	42.7±6.5 (17)
CD4+cells/µl (n)	308±257 [a] (42)	787±274 (25)	173.7±181 [a] (56)	316.9 ±56)	473±254 [a] (61)	696.7 ±223. (30)	754±241 (23)
HIV RNA (log_{10}copies/ml) (n)	4.7± 5.0 (n=39)	NA	5.0± 5.2 [a] (44)	5.0 ±5.6 (n=8)	NA	NA	NA

n, number of subjects; NA, not applicable; values presented as mean ± standard deviation (SD); WBC, TLC and neutrophil counts are (x1000 /µl of blood)

[a] Significant difference ($P < 0.05$) from that of the controls (*t* test).

[b] Significant difference ($P < 0.05$) between values before and after treatment (paired *t* test).

IFN-γ production in response to ESAT-6/CFP-10 mycobacterium antigen

To study differences in immune reactivity against *Mtb* antigens and to determine whether TB treatment influences these responses, we measure IFN-γ production upon ESAT-6/CFP-10 stimulation. Compared to HIV-TST-, there was lower IFN-γ production in HIV-TB+ ($p = 0.0002$); and significantly lower IFN-γ production in HIV-TB+ compared to HIV-TST+ ($p = 0.003$). Moreover, the level of IFN-γ was severely lower during co-infection with HIV in the LTBI groups (HIV+TST+ vs. HIV-TST+; $p = 0.002$), as well as in the active TB cases (HIV+TB+ vs. HIV-TB+; ($p = 0.07$). (Figure 1A)

After six month of TB treatment, the level of IFN-γ in HIV-TB+ patients increased two fold relative to the values at diagnosis ($p = 0.02$), although it remained lower by two fold from that of LTBI groups. In the HIV+TB+ on TB treatment without HAART, however, there was no significant increase in IFN-γ compared to values at baseline ($p = 0.33$). (Figure 1B)

(A) **(B)**

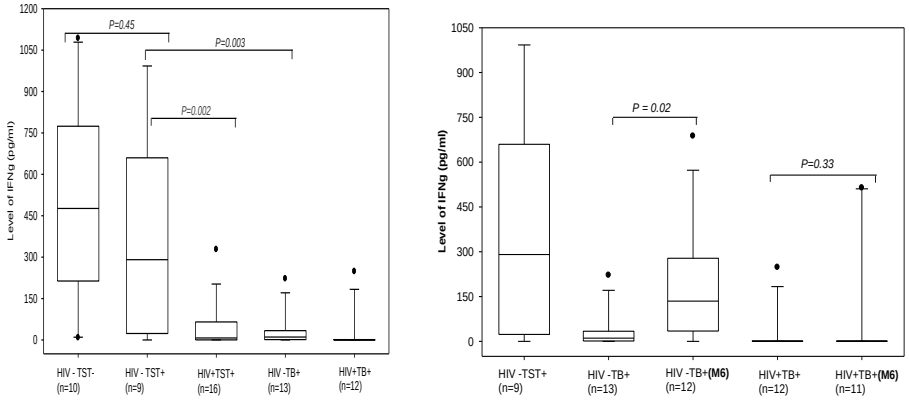

Figure -1. Level of IFN-γ in 7th day whole blood culture supernatants stimulated with ESAT-6/ CFP-10: **A)** in five clinical groups at diagnosis including controls (HIV-TST-), latent TB infected groups with and without HIV coinfection (HIV-TST+, HIV+TST+) and pulmonary TB patients with and without HIV coinfection (HIV-TB+, HIV+TB+); and **B)** Reconstitution of IFN-γ after six month of TB treatment in the pulmonary TB cases with (HIV+TB+ (M6)) and without (HIV-TB+ (M6)) HIV coinfection. Solid lines across the bars represent median values. P values showed significant difference in the values of IFN-γ between groups

Correlation between CD4+ T cell count recovery and IFN-γ production

We also assessed whether the dynamics in CD4+ cells in the HIV-TB+ patients before and after treatment is correlated with IFN-γ production upon ESAT6 stimulation (immune function specific to TB). We observed a strong correlation between the recovery of the absolute counts of CD4+ cells and IFN-γ production after 6 months of TB treatment *(r=0.76, p = 0.006, n=11)* (Fig 2), which might confirm the decrease in the level of IFN-γ observed in this study, could be associated with the depletion of the CD4+ cells from the periphery.

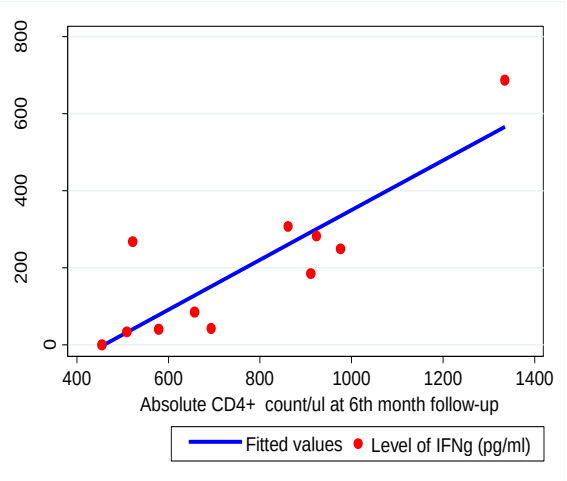

Figure-2. IFN-γ correlation with absolute CD4+ count in the HIV-TB+ patients after 6th month of TB treatment. A linear regression line is included: *r = 0.76, p = 0.006; n=11 (Spearman's correlation test).*

Discussion

Comprehensive management of TB patients becomes a challenge since the emergence of HIV/AIDS [10]. In this study, we characterized the immunohematological profiles, level of IFN-γ production specific to *Mtb* antigens and nutritional status in five clinical groups.

1. Clinical features

Similar to previous reports from elsewhere [24] and from Ethiopia [25], and cough was the most frequent symptom in HIV-TB+ patients in this study. However, we observed no difference in occurrence of TB symptoms between HIV infected and uninfected TB, which could be due to the inclusion criteria as all subjects were expected to be with symptoms of TB prior to enrolment as TB cases**.**

In agreement to Ong *et al* [26]**,** we observed strong overlap between the occurrence of the major symptoms of TB and imunosuppression in HIV+TB+ patients**.** This could be related to poor

immune function of the patients in whom the immuno-pathological mediated onset of symptoms are expressed at advanced disease stage [27]; or it could due to late presentation of the patients to the health facilities. As delay in TB diagnosis and initiation of treatment could result in higher death rate in TB/HIV patients [16]**,** our data emphasizes the need of early intervention for TB patients infected with HIV.

The 28.1% BCG scar prevalence in this study might indicate previous BCG immunization coverage [28]. The lower BCG scar prevalence in TB cases was comparable with a previous report from Ethiopia [17] and could be related to variations in BCG vaccination status, scar visibility, or scar failure among the groups [29]**.** The moderate decrease in BCG scar frequency in HIV positive compared to HIV negative groups could be due to waning of the scar due to diminished immunity associated with HIV infection. Collectively, our data emphasize the need of careful interpretation of BCG when applied as an indicator of previous BCG vaccination coverage in a population.

TST reactivity

In agreement with Chadha *et al* [30]**,** there was moderate variation in TST reactivity related to BCG scar, gender, age, and BMI. Our data re-confirms the need of careful interpretation of TST results during epidemiological studies or for treatment of LTBI.

2. Immunehematologic values

Hematologic and nutritional profiles

Similar to previous reports [7, 31] we observed alteration of hematologic (Hgb, TLC, Neutrophil, WBC) and BMI values during TB disease which were reversed to the level of healthy controls after six months of TB treatment. During co-infection with HIV (HIV+TB+), however, baseline immunohematological profiles were altered as compared to of HIV-TB+ patients. Even more, all the laboratory and BMI values were not normalized after TB treatment**.**

The distinctive nature of Hgb, TLC, neutrophil, and WBC during active TB before and after TB treatment, indicates they can serve as prognostic markers. Previous reports have shown the role of monocyte, neutrophil and total WBC counts to monitor TB treatment outcomes [31].

Immunologic and virologic profiles

Similar to previous reports from Ethiopia [23]**,** we observed lower CD4+ cell counts in the healthy control groups (HIV-TST-) compared to that of East African [32]**,** and Dutch [23] populations. The implication of lower baseline CD4+ count of healthy Ethiopians with respect to diseases progression during HIV infection has been discussed by others [33]. However, as the rate of CD4+ decline in HIV infected Ethiopians is slower despite the lower baseline values [33]**,**

and similar to that of other African populations [34], the lower CD4+ count of Ethiopians did not directly correlated with lower survival time during HIV infection [33].

Our observation of lower CD4+ cell counts in the HIV negative TB patients (HIV-TB+), which was normalized after TB treatment, was in agreement with others [6]. Thus, in areas where TB/HIV is endemic and monitoring of HIV/AIDS and response to ART is dependent on CD4+ counts, the effect of TB on the prognostic value of CD4+ cells should be accounted [6].

Despite the low CD4+ counts and elevated viral load in HIV+TB+ patients at diagnosis, there was no significant recovery in CD4+ counts or suppression in viral load after TB treatment. This could be related to persistent activation of the immune system despite TB treatment which would boost viral replication [35]. Similarly, higher level of plasma immune activation markers including neopterin, beta2M and sTNFalpha-RI were reported in TB/HIV patients despite effective clearance of *M tuberculosis* infection [35]. Our data and the report from others [35], strengthen the recent WHO recommendation of early initiation of HAART for TB/HIV patients despite CD4+ counts [36].

IFN-γ production to ESAT-6/CFP-10

As reported by others [37], ESAT-6/CFP-10 induced production of IFN-γ was lower in the HIV negative TB patients but higher in HIV negative LTBI groups. This might reflect the predominant role of IFN-γ in the immunity against *Mtb* infection [38]. However, although the level of IFN-γ was normalized in response to TB treatment in the HIV-TB+ patients, it remained lower than that of the LTBI by the six month of continuous TB treatment. We further investigated whether the dynamics of CD4+ count before and after TB treatment in HIV-TB+ cases is associated with immune functions specific to TB. The strong and positive correlation of the level of IFN-γ and the number of CD4+ counts following TB treatment (Fig. 2) could reflect the predictive value of higher CD4+ count for elevated IFN-γ levels, so as an improvement in immune function specific to TB.

Similar to other reports [39], we found the lowest level of IFN-γ production in response to *ESAT-6/CFP-10* stimulation in HIV+TB+ patients at diagnosis which is not reconstituted after TB treatment. This might reflect the degree of immunodeficiency (exhaustion of immune system) in TB+HIV+ patients at time of diagnosis, as well as persistent immune activation and persistent elevated plasma viral load despite TB treatment and clearance of the bacilli [35]. This further strengthens the recommendation of early initiation of HART in TB/HIV patients [36]

In conclusion, the distinctive profile of CD4+, TLC, WBC, neutrophil, Hgb, and BMI in TB patients before and after TB treatment indicates their prospective to serve as prognostic markers for patient monitoring and response to TB therapy. Likewise, the distinctive pattern of IFN-γ

during TB disease and LTBI reconfirms the central role of IFN-γ to control *M tuberculosis* infection as well as its potential to serve as a correlate of protective immunity. The higher early mortality and the impaired response in CD4+, VL and IFN-γ to TB treatment in TB/HIV patients, strengthen the recommendation of early initiation of HAART for TB/HIV patients [39]. Finally, studies aimed to assess whether the alteration of peripheral leukocyte subpopulations during TB and TB/HIV is associated with host immunologic deterioration or improvements are warranted.

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Chapter 4

Virologic and immunologic outcome of HAART in Human Immunodeficiency Virus (HIV)-1 infected patients with and without tuberculosis (TB) and latent TB infection (LTBI) in Addis Ababa, Ethiopia.

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Abstract

Background: HIV/TB coinfection remains a major challenge even after the initiation of HAART. Little is known about *Mycobacterium tuberculosis (Mtb)* specific immune restoration in relation to immunologic and virologic outcomes after long-term HAART during co-infections with latent and active TB.

Methods: A total of 232 adults, including 59 HIV patients with clinical TB (HIV+TB+), 125 HIV patients without clinical TB (HIV+TB-), 13 HIV negative active TB patients (HIV-TB+), and 10 HIV negative Tuberculin Skin TST positive (HIV-TST+), and 25 HIV-TST- individuals were recruited. HAART was initiated in 113 HIV+ patients (28 TB+ and 85 TB-), and anti-TB treatment for all TB cases. CD4+ T-cell count, HIV RNA load, and IFN-γ responses to ESAT-6/CFP-10 were measured at baseline, 6 months (M6), 18 months (M18) and 24 months (M24) after HAART initiation.

Results: The majority of HIV+TB- (70%, 81%, 84%) as well as HIV+TB+ patients (60%, 77%, 80%) had virologic success (HIV RNA< 50 copies/ml) by M6, M18 and M24, respectively. HAART also significantly increased CD4+ T-cell counts at 2 years in HIV+TB+ (from 110.3 to 289.9 cells/μl), HIV+TB- patients (197.8 to 332.3 cells/μl), HIV+TST- (199 to 347 cells/μl) and HIV+TST+ individuals (195 to 319 cells/μl). Overall, there was no significant difference in the percentage of patients that achieved virologic success and in total CD4+ counts increased between HIV patients with and without TB or LTBI. The *Mtb* specific IFN-γ response at baseline was significantly lower in HIV+TB+ (3.6 pg/ml) compared to HIV-TB+ patients (34.4 pg/ml) and HIV+TST+ (46.3 pg/ml) individuals; and in HIV-TB+ patients compared to HIV-TST+ individuals (491.2 pg/ml). By M18 on HAART, the IFN-γ response remained impaired in HIV+TB+ patients (18.1 pg/ml) while it normalized in HIV+TST+ individuals (from 46.3 to 414.2 pg/ml).

Conclusions: Our data show that clinical and latent TB infections do not influence virologic and immunologic outcomes of ART in HIV patients. Despite this, HAART was unable to restore optimal TB responsiveness as measured by *Mtb* specific IFN-γ response in HIV/TB patients. Improvement of *Mtb*-specific immune restoration should be the focus of future therapeutic strategies

Key words: HIV, tuberculosis, HAART

Background

Human immunodeficiency virus/Acquired immunodeficiency syndrome (HIV/AIDS) associated morbidity and mortality has reduced substantially since the introduction of Highly Active Antiretroviral Therapy (HAART) in the mid 90's [1, 2]. Access to Antiretroviral Therapy (ART) in low and middle income countries has been expanded following the launche of "3 by 5" global initiative [3], though only 54% of those eligible for ART were on treatment by 2011 [2]. In Ethiopia, where free ART was started in 2005, >250,000 (~79%) of the adults requiring ART were actually treated [4].

The primary goal of HAART is to suppress HIV-1 RNA lower than the detection level (LDL) of the assay within 3 to 6 months on treatment and restore immunologic function, to reduce morbidity and mortality, to reduce vertical transmission, and improve quality of life [5]. However, there are still un-resolved problems including early mortality [6], incomplete responses [7], variations in HAART outcomes [8], lack of universal consensus to define treatment failures and time to start ART [9], drug resistance [10] and lost to follow-ups [7].

While HIV RNA testing is the golden standard to monitor patients on ART [8], due to costs and technical demands of the HIV RNA test, CD4+ T cell measurements are recommended for resource poor settings [11]. Immunologic parameters, however, have lower performance to identify virologic failures which could lead to premature change or to continuous use of failed regimens [reviewed in. 12]. This leads to higher morbidity and mortality rates and more complex resistance in settings where virologic tests are not available [13]. Therefore, accurate diagnosis of treatment failure is necessary in settings where free ART service is accelerating and patient monitoring is exclusively dependent on clinical and CD4+ T cell measurements like in Ethiopia.

Furthermore, despite that HAART has significantly reduced morbidity and mortality in HIV/TB patients [14], studies showed defects of immune response in HIV/TB patients on HAART including suboptimal restoration of CD4+ T cells in number, phenotype and function [15,16], and incomplete TB specific immune restoration [17]. Higher TB incidence in individuals on continuous HAART as compared to the HIV negative local population, which could be due to incomplete immune restoration specific to TB, was also reported [18]. However, data regarding the magnitude of immune restoration specific to *Mtb* in relation to CD4+ and virologic responses to long-term HAART in patients with TB and LTBI is limited.

Overall, although extensive studies aiming to evaluate ART outcomes have been performed, the studies are predominantly from developed countries, and they are different in study design, ex-/inclusion criteria, ethnicity, ART experience, ARV regimen, duration, and definitions, which makes it difficult to generalize HAART outcomes in different countries [19]. Especially in

Ethiopia, like in many HIV/TB endemic settings, little data is available [20, 21] regarding HAART outcome in patients with and without active TB and LTBI.

In summary, these studies strongly support the need of recent data from well defined longitudinal cohort studies on HAART, which is crucial to provide answers and insights to the HAART related challenges and develop and update national ART guidelines [19, 22].

The aim of this observational cohort study which comprised three clinical groups including HIV+TB+, HIV+TB-, and HIV-TB+ patients, and two control groups including HIV-TST+, and HIV-TST- individuals , was to evaluate the long-term outcome of HAART by comprehensively measuring key parameters including mortality, virologic and immunologic responses, and *Mtb* specific immune restoration by measuring IFN-γ production in response to Early Secretory Antigenic Target-6/Culture Filtrate Protein-10 (ESAT-6/CFP-10).

Results

1. Characteristics of the study population at enrolment

Demographic, clinical and laboratory data of the study populations at baseline are shown in **Table 1.** A total of 232 participants were included. Thirteen HIV-TB+, 59 HIV+TB+, 125 HIV+TB- (of whom 43 were HIV+TST+, and 82 HIV+TST-), 10 HIV-TST+, and 25 Controls (HIV-TST-) were enrolled. HIV+TB+ patients had lower numbers of CD4+ T cells (p=0.003), total Lymphocyte count (TLC) (p=0.001), Hemoglobin (Hgb) (g/dl) (p=0.02), Body Mass Index (BMI) (Kg/m^2) (p=0.002), CD4% (p=0.006), but higher HIV RNA levels (p= 0.02) than HIV+TB- patients. There was no significant difference in CD4+, TLC, Hgb, BMI, CD4% and HIV RNA tests between HIV+TST- and HIV+TST+ individuals (data not shown). Of special interest, 10 (6.3%) of the ART naïve HIV patients had HIV RNA < 50 copies/ml (LDL) at enrolment. The mean CD4+ T cell count of these subjects was 476.1 (SD ± 260.7 cells/µl).

At enrolment, more than 50% of the HIV+TB+ patients had advanced diseases stages (anemic, malnourished, WHO stages 3 plus 4, and immunosuppressed) while only 15-36% of the HIV+TB- patients had these advanced disease stages. There was also a higher percentage of chronic hepatitis B virus coinfection in the HIV+TB+ patients, (19.3%), followed by the HIV+TB- ,(7.2%), and healthy controls (4.2%) (Table 1)**.**

Table 1. Baseline characteristics of the study populations (n=232)

	HIV+TB+ (n=59)	HIV+TB- (n=125)	HIV-TB+ (n=13)	HIV-TST+ (n=10)	HIV-TST- (n=25)
Demographic data					
Age, years	33.1± 8.7	33.2± 7.3	28.5±9.5 [b]	26.6± 7.4,	24.6 ± 6.6
Female, n (%)	28 (47.5)	83 (66.4)	6 (46.2)	6 (60)	16 (64)
Clinical data					
CHBV coinfection, n (%)	11/57 (19.3)	8/112 (7.2)	3 (15)	2 (20)	1 (4)
WHO stage, n (%)					
I+II	3/50 (6)	80 (64)			
III+IV	47/50 (94)	45 (36)	NA	NA	NA
BMI, kg/m2	18.9± 3.1[a]	21.3±3.5	18.9 ± 2.8	21.4 ± 2.5	21.4 ± 2.0
BMI < 18.50 kg/m^2, n (%)	29 (49.2)	18/124 (14.6)	6 (46)	8 (80)	24 (96)
Laboratory data					
Hgb, g/dl	12.2± 3.7 [a]	13.2± 2.5	13.5± 2.5 [b]	16.4 ±1.8 [c]	18.0 ± 2.1
Hgb < 12 g/dl (anemic), n (%)	23/43 (53.5)	29/107 (27.1)	2 (23)	0	0
CD4+ count/µl	173.7±180.8 [a]	279.2±212.4	478.1± 253 [b]	787.3± 274	754.8± 241
CD4+ category, n (%)					
< 100	27 (48.2)	26 (22.2)	1 (1.7)	0	0
100 –200	12 (21.4)	26 (22.2)	6 (10.0)	0	0
>=201	17 (30.3)	65 (55.6)	53 (88.3)	10 (100)	25 (100)
TLC	1175±754 [a]	1626±750	1485±895 [b]	1975±1483	1.668± 559
HIV RNA (log10copies/ml)	4.5 ± 0.8 [a]	4.1± 0.9	NA	NA	NA
HIV RNA category, n (%)	1 (2.2)	9 (8.0)			
LDL	14 (30.4)	97 (85.8)			
<= 100000	32 (69.6)	16 (14.2)	NA	NA	NA
>100000					

Data are means ± standard deviations (SD) unless stated; *n (%)*: Number of patients; **CHBV:** chronic Hepatitis B Virus, defined as the presence of hepatitis surface antigen (HBsAg) in the plasma; BMI: Body mass index; Hgb: Hemoglobin; **TLC**: total lymphocyte; **HIV RNA**: plasma viral load; LDL: lower than detection limit (HIV RNA < 50 copies/ml); **WHO**: World Health Organization; **NA**: not applicable

[a] Comparing HIV+TB+ and HIV+TB-; $P < 0.05$

[b] Comparing HIV+TB+ and HIV-TB+; $P < 0.05$

[c] Comparing HIV-TST+ and HIV-TST- ; $P < 0.05$

2. Outcome of HAART

2.1. ART initiation, mortality and follow-up status

A total of 113(61.4%) HIV patients including 28 HIV+TB+ and 85 HIV+TB- [of whom 31 were HIV+TST+ and 54 were HIV+TST-], initiated ART and were followed for a median of 23.9 (IQR 22.5-24.4) months. The majority (78%) of the HIV+TB- patients received D4T+3TC+NVP HAART regimens. At ART initiation, HIV+TB+ patients had lower mean CD4+ T cells (*p= 0.005)*, CD4 % (*p = 0.03)*, TLC (p=008), and BMI (*p<0.0001)* than HIV+TB- patients **(Table 2)**.

At 2 years, 18 (9.8%) patients died of which 63% within the first 24 weeks. The risk factors for early mortality in the HIV patients with and without TB were advanced disease stages including baseline CD4+ count <100 cells/µl (OR: 5.7, 95% CI 1.8 – 17.2, *p=0.03*), WHO-stages III plus IV (OR: 4.8 95% CI 1.3-17.5, *p = 0.01*), and BMI < 18.5 kg/m2 (OR: 1.2 95% CI 0.37-3.8, *p = 0.77*) (data not shown).

Overall, at M6, M18 and M24 of follow-up time, 21/59 (35.6%), 21/51 (41.2%) and 23/41 (56.1) of the HIV+TB+ patients; and 27/125 (21.6%), 36/120 (30%) and 43/115 (37.4%) of the HIV+TB- patients, respectively, were lost to follow-up (more than 60 days late for a scheduled date of clinic visit).

2.2. Virologic outcomes

We measured virologic outcomes by determining the HIV RNA level as well as the proportion of patients with virologic success. In both HIV+TB+ and HIV+TB- patients, the mean HIV RNA level significantly declined after HAART (p<0.05 and p<0.001, respectively) **(Fig 1A).** There was no significant difference in mean HIV RNA decline between patients with and without TB or LTBI **(Fig 1A & B)**.

Table 2: Therapeutic, clinical and immunovirologic characteristic of the study participants at ART initiation (n=113)

	HIV+TB+	HIV+TB-	*p value*
No of patients initiated ART	28	85	
Demographic			
Female, n (%)	14 (50)	56 (65.7)	
Age, years	34.1±8.3	34.4±7.7	
Treatment			
Mean follow-up days before ART initiation	78.7	37.7	
Mean delay in commencing HAART following TB treatment, days	100		
HAART regimen at ART initiation, n (%)	4 (15)	66 (78)	
D4T/3TC/NVP	11(41)	2 (2)	
D4T/3TC/EVZ	7(26)	12 (14)	
AZT/3TC/NVP	5 (16)	4 (5)	
AZT/3TC/EVZ		1 (1)	
Others			
Cortimoxzole treatment, n (%)	15 (83.3%)	58 (59.2%)	
BMI and Laboratory values at ART initiation			
BMI (kg/m^2)	18.1±2.5	21.3±3.7	0.0001
CD4+ (cells/µl)	110.3±71.3	197.8±153.2	0.005
CD4%	10.1±5.3	14.6±9.9	0.033
TLC (cells/µl)	1078±512	1521±692	0.007
Hgb (g/dl)	13.4± 3.4	13.0± 2.5	0.68
HIV RNA (log_{10}copies/ml)	4.3±0.9	4.3±0.9	0.73
Follow-up outcomes			
Deaths, n (%)	8/59 (13.6)	10/125 (8.0%)	

Data in mean± standard deviation (SD) unless stated; n: number of patients; D4T: Stavudine; 3TC: lamividine; NVP: Nevirapine; EVZ: Efavirenz; AZT: Zidovudin; BMI: Body mass index; Hgb, Hemoglobin; TLC, total lymphocyte count; M6, M18 and M24= 6, 18 and 24 months after HAART initiation

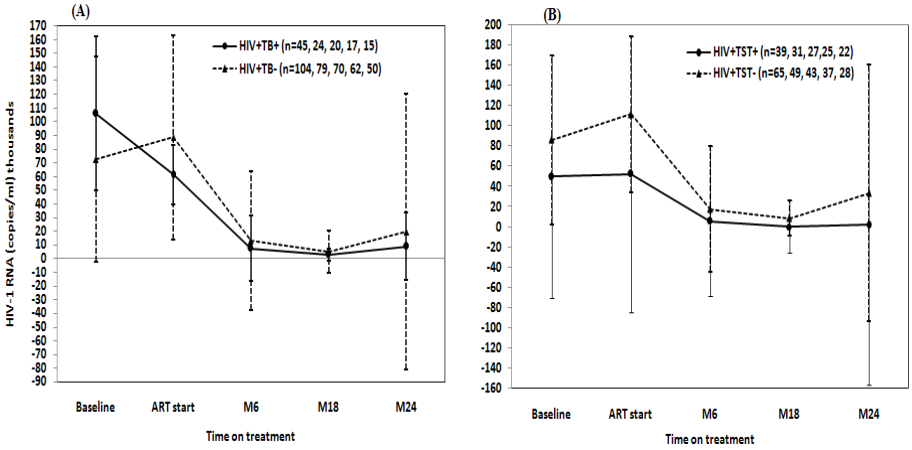

Figure 1. Changes in HIV RNA level (copies/ml) over time after the start of HAART. HIV RNA was measured at baseline, at ART start and at six month (M6), M18 and M24 of HAART in HIV patients with TB (HIV+TB+) (continuous line) and without TB (HIV+TB-) (dotted line) **(A)**, and HIV+TB- patients sub-grouped as those with TST positive (HIV+TST+) (continuous line) and TST negative (HIV+TST-) (dotted line) **(B)**. Values are shown as mean and standard deviation. n= number of participants per visit.

By M24 on HAART, the majority of the individuals (>80%) achieved virologic success (HIV RNA < 50 copies/ml). There was no significant difference in the proportion of patients achieving virologic success between individuals with and without TB or LTBI (**Fig 2A & B).** Moreover, whereas 28 (85%) of the patients on HAART maintained HIV RNA < 50 copies/ml at M6, M18 and M24 (**ever suppressed)**, 5 (15%) never reached HIV RNA < 50 copies/ml **(never suppressed)**.

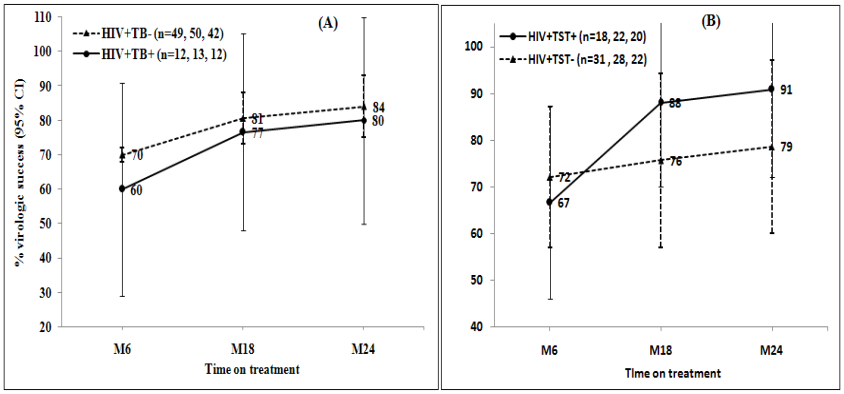

Figure 2. Percentages of patients with virologic success (plasma HIV RNA < 50 copies/ml) with 95% confidence interval (95% CI) overtime on HAART.

HIV RNA was measured at six month (M6), M18 and M24 of treatment in HIV patients with TB (HIV+TB+) (continuous line) and without TB (HIV+TB- (dotted line) **(A),** and HIV+TB- patients sub-grouped as those with TST positive (HIV+TST+) (continuous line) and TST negative (HIV+TST-) (dotted line) **(B)**. Percentage of patients with virologic success per follow up visit are shown on the line graphs; n= number of participants per visit.

At M6, 13 (19%) HIV+TB- patients had virologic failure (HIV RNA >5000 copies/ml), for which baseline BMI < 18.5 kg/m^2, CD4+ count < 100 cells/µl, and WHO stages 3 and 4 [OR: 3.8, 2.3, 4.9; p = 0.05, 0.32, 0.02, respectively] were risk factors. Other factors like sex (OR: 0.51) age (OR: 0.41), Hgb (OR: 0.19), and HIV RNA (OR: 0.89) were **not** associated with **the** risk of virologic failures **(Table 3).**

2.3. Immunologic responses

Quantitative restoration of CD4+ cells is one of the principal evidences for immune recovery during HAART. There was a significant increase in CD4+ T cell count at M6, M18 and M24 of HAART in both the HIV+TB- (P< 0.001 for all) as well as the HIV+TB+ patients (p=0.02, 0.001, 0.001, respectively); and in the HIV+TST+ (p=0.03, 003, 0.04, respectively) as well as in the HIV+TST- (P< 0.001 for all) **(Fig 3A & B).**

There was no significant difference in the total CD4+ T cells reached by M6, M18 and M24 in HIV+TB+ vs. HIV+TB- (p=0.37, 0.63 and 0.56, respectively) and in HIV+TST+ vs. HIV+TST- (*p=0.77, 0.62, and 0.74,* respectively) **(Fig 3A & B).** Interestingly, although the study population was small, HIV/TB patients coinfected with HBV (n=9) showed the least increase in CD4+ T cells **(Fig 3A)**.

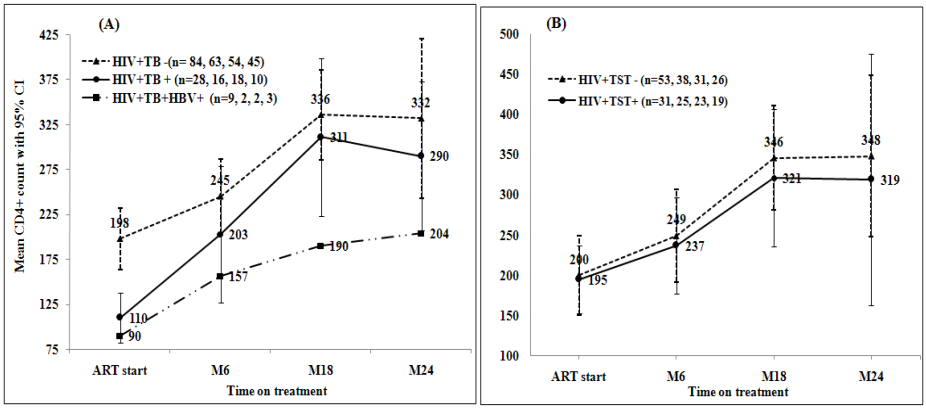

Figure 3. Mean CD4+ count increase (cells/µl) with 95% Confidence interval (95% CI) over time after the start of HAART.
CD4+ T cell count was measured at ART start, six month (M6), M18, and M24 of HAART in HIV patients with TB (HIV+TB+) (continuous line), with no TB (HIV+TB-) (dotted line), and HIV patients with TB and HBV (HIV+TB+HBV) (dash line) **(A)**, and HIV+TB- patients sub-grouped as those with TST positive (HIV+TST+) (continuous line) and those TST negative (HIV+TST-) (dash line) **(B)**. Number of CD4+ T cell counts per follow-up visit are shown on the line graphs; n= number of participants per visit.

There was an overall increase in CD4+ count over the two years with an average increase of 6.7 cells/µl per month in the HIV+TB- patients, and 5.9 cells/µl per month in the HIV+TB+. CD4+ increase was highest in the first six months. Despite lower CD4+ T cell count at ART initiation in HIV+TB+ patients (110 CD4+ cell/µl) than HIV+TB- patients (198 CD4+ cell/µl) (p=0.001), there was no significant difference in the net increase of CD4+ T cells per month as well as in the total CD4+ cells achieved at each time point between the patients with and without TB.

Overall, at two years on HAART, the total CD4+ cells in all clinical groups was still lower by more than two fold compared to healthy controls (HIV-TST-) (771 CD4+ cells/µl) **(Fig 3A and B)**. Moreover, the proportion of patients that attained the critical CD4+ count of ≥ 200 cells/µl by two years on ART was 19 (68%) for HIV+TB-, and 8 (73%) for the HIV+TB+. Six (21%) of the HIV+TB- but none of the HIV+TB+ patients had CD4+ count >500 cells/µl **(super responders)**.

At M6, M18 and M24 of HAART, 45%, 30%, 42% of the HIV+TB- and 54%, 43%, and 30% of the HIV+TB+ patients, respectively, were diagnosed with immunologic failure (an increase of less than 50 cells/ µl by M6, and less than 100 cells/ µl by M18 and M24 of HAART). The risk factors for immunologic failure at M6 of HAART for the HIV+TB- patients were WHO stages 3 + 4 [OR: 4.3, p = 0.01], and CD4+ < 100 cells/µl) [OR: 5.6, p = 0.008] at baseline (**Table 3).**

Table 3: Logistic regression analysis showing odds ratio for factors associated with the risk of immunologic and virologic failures of the HIV patients with no TB (HIV+TB-) at six months of ART

	Risk of **immunologic failure** (an increase of CD4+ < 50 cells/µl at 6 months (n=56)			Risk of **virologic failure** (HIV RNA> 5000 cells/µl) at 6 months (n=69)		
	OR	95% CI	P value	OR	95% CI	P value
Sex						
Male	1			1		
Female	0.62	1.0-1.9	0.40	0.51	0.12-2.1	0.93
Age (years)						
36-60	1			1		
16–35	0.65	0.21-2.0	0.44	0.41	0.10- 1.7	0.21
BMI						
<18.50	1			1		
>=18.50	0.90	0.25- 3.29	0.87	3.75	1.0-14.4	0.05
Hgb						
< 12	1			1		
>=12	0.38	0.08-1.7	0.21	0.19	0.02-1.6	0.13
CD4+ count/µl						
>=100	1			1		
<100	5.6	1.6-20.1	0.008	2.3	0.45-11.2	0.32
>=200	1			1		
>200	0.22	0.07-0.73	0.01	0.89	0.24-3.2	0.85
HIV RNA copies/ml						
>=100000	1			1		
<100000	1.2	0.3- 5.3	0.82	0.38	0.04- 3.3	0.4
WHO stages						
I + II	1			1		
III + IV	4.3	1.4-13.4	0.01	4.9	1.2-19.9	0.02
TB coinfection						
No	1			1		
Yes	1.4	0.4-1.8	0.5	0.23	0.03-1.9	0.17

OR: Odds ratio; 95% CI: 95% confidence Interval; n= total number of participants who have CD4+ count and plasma HIV RNA measurement at 6 month of HAART

As shown in **Figure 4,** for the HIV+TB- patients on ART, the CD4+ increase was steeper in women, in patients with older age, patients with baseline HIV RNA < 10000 copies/ml, and CD4+ count > 200 cells/µl with the difference becoming larger over time. More interestingly, the total CD4+ T cell count achieved in the HIV+TB- patients started ART at CD4+ > 200, was two fold higher than those started ART at < 200 cells/µl (495.0 vs. 243.2 cells/µl, respectively, (*P=0.007*). This indicated that advanced pre-treatment immunodeficiency is the most important factor for diminished restoration of CD4 cell counts after HAART.

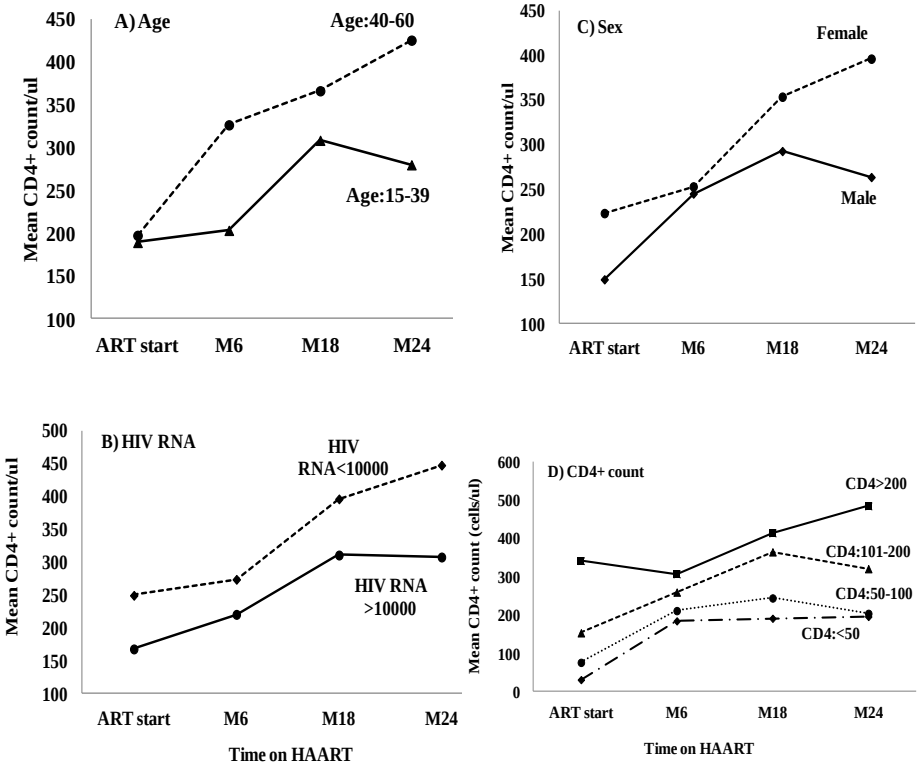

Figure 4. Mean CD4+ count increase (cells/µl) over time after the start of HAART in HIV patients with no TB (HIV+TB-) according to the baseline category of (A) Age, (B) HIV RNA (copies/ml), (C) Sex, and (D) CD4+ count (cells/µl).

Mtb antigen specific IFN-γ response before and after HAART

Although quantitative measurement of CD4+ counts and HIV RNA level provides a general insight in immune recovery, measuring qualitative restoration of TB specific immune responses will provide insight whether antigen specific immune responses are also restored. Therefore, we measured *Mtb* antigen (ESAT-6/CFP-10) specific IFN-γ responses during HAART **(Fig 5).** Compared to IFN-γ response in LTBI individuals (HIV-TST+) (491.2 pg/ml) at baseline, there was significantly lower IFN-γ production in HIV+TB+ (3.6 pg/ml) ($p = 0.004$), in HIV-TB+ (34.4 pg/ml) ($p = 0.004$), and in HIV+TST+ (46.3 pg/ml) patients ($p = 0.002$). Moreover, IFN-γ production at baseline was significantly lower in HIV+TB+ compared to HIV-TB+ ($p = 0.02$) and HIV+TST+ ($p = 0.04$) patients, and in HIV+TST+ compared to HIV-TB+ ($p = 0.004$).

In the HIV+TST+ individuals, following initiation of HAART, although the level of IFN-γ did not change by M6 (44.3 pg/ml), it increased sharply after that and normalized by M18 of treatment (414.2 pg/ml) ($p = 0.007$) and reached similar level as HIV-TST+ individuals (491.2 pg/ml) (p>0.05). In contrast, there was no significant increase in *Mtb* specific immunity for the HIV patients with TB (HIV+TB+) on TB treatment and on HAART by M6 (31.7 pg/ml) as well as by M18 (18.1 pg/ml) of treatment. For the HIV negative TB patients (HIV-TB+) on TB treatment, mean IFN-γ production significantly increased by M6 (181 pg/ml) (p=0.005)

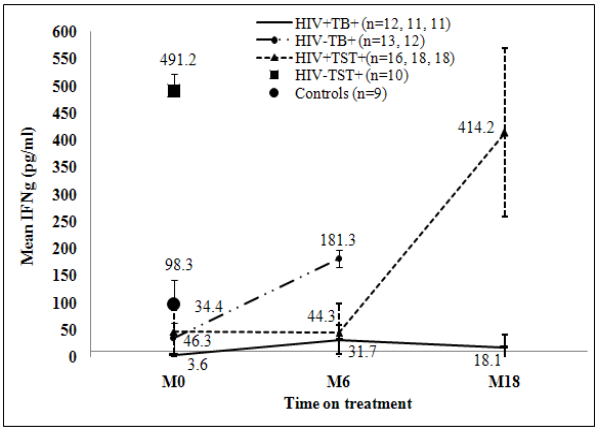

Figure 5. Restoration of *Mtb* specific IFN-γ (pg/ml) response overtime after the start of HAART and TB treatment. Level of IFN-γ (pg/ml) was measured at baseline (M0), and at six (M6) and M18 of treatment in 7th day culture supernatants of whole blood stimulated with *Mtb* specific (ESAT-6/CFP-10) antigen. **HIV+TB+:** HIV patients with TB (continuous line); **HIV-TB**: HIV negative TB patients (long-dash line); **HIV+TST+**: HIV positive tuberculin skin test (TST) positive patients (dot line); **HIV-TST+:** HIV negative TST positive individuals (square dot); and Controls (HIV-TST-) (circle dot). Mean of IFN-γ (pg/ml) with standard deviation per follow-up visit are shown on the line graph; n= number of participants per visit shown on the legend.

To see whether the poor *Mtb*-specific recovery is due to lower CD4 recovery, we correlated the increase in CD4+ cell count in the HIV+TB+ patients after HAART with IFN-γ production upon stimulation with ESAT-6/CFP-10 (immune function specific to TB). No significant correlation between CD4+ T cell count recovery and IFN-γ production was found *(r=0.19, p=0.40, n=22)*. In contrast, however, there was a strong positive correlation between the recovery of CD4+ cells and IFN-γ production in the HIV negative active TB patients (HIV-TB+)following TB treatment for six months *(r=0.71, p=0.0007, n=19)* (Fig 6).

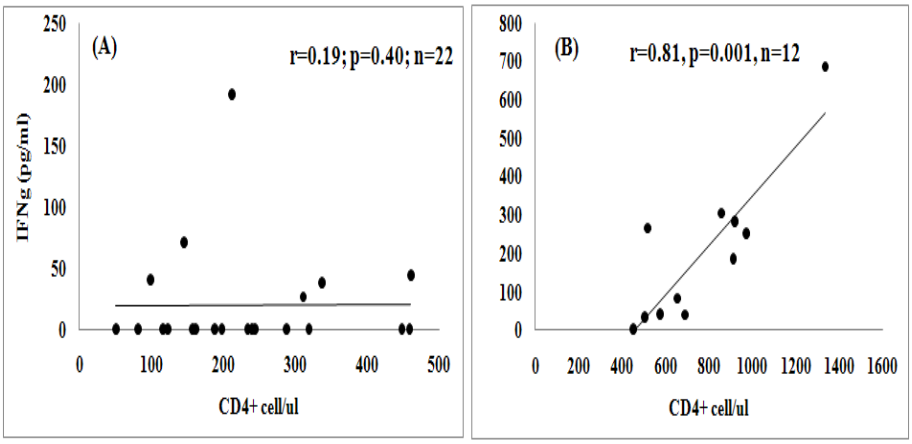

Figure 6. Spearman correlation between IFN-γ production and absolute CD4+ count recovery after the start of HAART and TB treatment. (A)HIV positive patients with active TB (HIV+TB+) at 6th and 18th months of HAART and TB treatment, and (B) HIV negative TB patients (HIV-TB+) at six months of TB treatment. IFN-γ secretion was measured in 7th day culture supernatants of whole blood stimulated with *Mtb* specific antigen (ESAT-6/CFP-10). r=correlation coefficient

Discussions

In this study, we determined the long-term outcome of HAART in HIV patients with and without TB and LTBI by comprehensively measuring HIV RNA suppression, CD4+ T-cell recovery, and immune reconstitution specific to *Mtb*.

The goal of ART is to suppress HIV-1 RNA below the detection limit of the assay within 12-24 weeks [5], or to less than 0·5-0·75 log copies/ml by 4 weeks [23]. In this study, 84% of the non-TB patients on HAART had HIV RNA <50 copies/ml at 24 months on ART. This is comparable to most studies from Africa [24, 25], Europe [15, 26], and United States [27].

Nevertheless, 19% of the non-TB patients in this study were virologic failures (HIV RNA>5000 copies/ml) at 6 months of ART. Reports from Cameroon [28] and Brazil [29] also showed virologic failure (HIV RNA>400 copies/ml) in 13% and 28% of patients at 6 months on ART. As reported by Tuboi SH *et al* [29], malnutrition and advanced WHO stages were risk factors for virologic failure in this study, which strongly indicate the need of earlier identification of eligible patients and earlier initiation of HAART for better treatment outcome.

Interestingly, comparable to other reports [30, 15], also 80% of the HIV patients with TB in this study had HIV RNA< 50 copies/ml at 24 months on HAART, which is similar to viral suppression in patients without TB (Fig 1 & 2) and has been reported recently in another study [31]. In contrast, others have reported a high hazard ratio for virologic failure in patients with TB on ART [32].

Since many patients in Sub-Saharan Africa present to the health facilities with advanced disease stages and low CD4 cell counts [24], they may have limited advantage for CD4+ recovery after ART [17]. However, despite the overall lower baseline CD4+ count of the healthy Ethiopians [33], and the lower CD4+ count at ART start (197 cells/ µl) in HIV patients without TB in this study, the increase in CD4+ cells after two years on ART (332 cells/µl) was comparable with a recent report from Ethiopia [34], and other reports from Africa [24], in low-income countries (Africa, Latin America and Asia) [35], and the United States [36, 27]. The mean CD4+ increase by 24 month on ART in this study (6.7 cells/µl/month) was also comparable to a report from South Africa [37].

However, 45% of the non-TB patients in this study had immunologic failure (an increase of <50 CD4+ cells/µl) at 6 months, while 32% failed to restore CD4+ T cell count to ≥ 200 cells/µl by 2 years on ART, which is similar to a study from Nigeria [38]. Similar to Lifson *et al.* [36], advance WHO stages and lower CD4+ count at baseline were risk factors for immunologic failure, which strongly suggests the need for earlier identification of eligible patients and initiation of HAART.

Furthermore, since more than 50% of the HIV/TB patients in Sub-Saharan Africa presented to the health facilities at advanced disease stages and start ART at CD4+ counts of 100–150 cells/μl [16, 24] the benefit of patients on ART could be limited [28, 39]. More than 50% of the HIV/TB patients in our cohort had advanced disease stage at enrolment and the CD4+ count at ART initiation was 110 cells/μl (Table 2). Interestingly, however, we observed no difference in the CD4+ cell increase over time on HAART in patients with and without TB as reported by Lawn SD *et al.* [40] and Dronda F *et al.* [31]. In contrast, others reported reduced CD4+ recovery after ART in patients with TB [41, 15, 42]. Interestingly, although the study sample was small, we did observe reduced CD4+ recovery in HIV/TB patients co-infected with HBV (Fig 3A) as reported by Pe´ rez-Molina JA *et al.* [43].

Overall, the total CD4+ count achieved by 2 years on HAART in patients with TB (290 cells/μl) and without TB (332 cells/μl) in this study was comparable to findings from African and other developed countries, although it was still lower compared to the healthy Ethiopian populations (754.8 cells/μl)**.** This poses the question whether immune function is restored after 2 year of HAART. Therefore, the *Mtb* specific immune restoration after 18 month on HAART was investigated.

It has been estimated that, if HAART is accessible to all patients with CD4+ < 200 cells/μl and would restore optimal immune responses specific to *Mtb*, the cumulative incidence of TB would decrease by 22% over 20 years [44]. However, if immune restoration to *Mtb* is incomplete, there would be a substantial number of patients on HAART which are continuously at high risk for TB. In this study we measured the level of IFN-γ, a cytokine which plays a key role in the control of *Mtb* infection [45] in response to *Mtb* specific antigen (ESAT-6/CFP-10) [46].

We observed lower IFN-γ secretion in HIV negative TB patients compared to LTBI individuals at baseline as reported by Hanna LE *et al.* [47]. Coinfection with HIV severely decreased the secretion of IFN-γ in both groups. Furthermore, in support to previous reports [48, 42, 41], IFN-γ production after 18 month of HAART was not restored in HIV patients with TB while it was normalized in those with LTBI. Among the possible factors contributing to the impaired IFN-γ response in the HIV/TB patients are exhaustion of immune system [47] defined as a reduced proliferation of immune cells and impaired cytokine production due to infection with HIV [49] and *Mtb* [50] , and depletion of *Mtb* specific CD4+ cells due to direct infection with HIV [15]. Our observation that there was no correlation between CD4+ count recovery after HAART and level of IFN-γ production in the HIV+TB+ patients, unlike to that of HIV-TB+ patients where there was a strong correlation between CD4+ T cell recovery and IFN-γ production following TB treatment (Fig 5), suggests that other factors may play a role in the impaired functional recovery of *Mtb* specific immune responses in the HIV+TB+ patients. Among the strategies proposed to

boost immune restoration specific to TB after HAART are early initiation of HAART and isoniazid prophylaxis, and adjunctives such as BCG vaccination or co-administration of IL-2 [48, 51].

Overall, addressing the long-term outcome of HAART by comprehensively measuring the key parameters of ART responses in a well defined cohort of patients with and without active TB and LTBI is the major strength of this study. The study addressed immune responses after HAART not only by measuring the absolute CD4+ cell recovery as a proxy for immune restoration but also by measuring IFN-γ response specific to *Mtb*. However, the fact that there is no golden standard definition of LTBI could be counted as limitations of the study

Conclusions

In this observational cohort study, we showed sustained outcomes of long-term HAART in HIV patients with and without TB and LTBI as evidenced by clinical, immunologic and virologic data. Advanced pre-ART disease stages were the risk factors for diminished CD4+ and virologic responses to HAART and high mortality, which strongly indicated the need of early identification of eligible patients and early access to care and treatment. *Mtb* specific immune reconstitution in HIV/TB patients remained impaired after 18 months on HAART, which suggested the need of strong prevention, earlier diagnosis, and treatment of TB, as well as earlier initiation of HAART. Factors contributing to impaired *Mtb* specific immune restoration in HIV/TB patients after HAART need to be investigated in order to develop intervention methods which could boost the immune response. In addition, we should do further study on the immunological mechanisms associated with HIV/TB coinfection.

Materials and methods

Study populations and settings

This observational cohort study was performed from April 2007-February 2011 at St Peter Specialized Referral TB Hospital, Akaki and Kality Health centers in Addis Ababa, Ethiopia. Adults of both sexes who were naïve to ART and TB treatment were enrolled after informed and written consent was sought.

Diagnosis of active tuberculosis (TB) was based on both clinical and bacteriological evidences. At least two sputum smears stained by the Ziehil-Neelsen direct method were required to be microscopy positive for Acid Fast Bacilli (AFB) **[52].** Except for TB patients, Mantoux Tuberculin Skin Testing (TST) for tuberculin was done for all participants. A diameter of skin induration with ≥ 10 mm in HIV un-infected, and ≥ 5 mm in HIV-infected individuals was graded as TST positive (TST+), and was considered as a LTBI [52].

The study participants were enrolled in 5 clinical groups: HIV patients with TB (HIV+TB+), and without TB (HIV+TB-) [sub-grouped further as Tuberculin Skin Test (TST) positive (HIV+TST+) and TST negative HIV+TST-], HIV negative TB cases (HIV-TB+), HIV-TST+, and controls (HIV-TST-). After enrolment, the HIV+TB+, HIV+TB-, and HIV-TB+ groups were scheduled for follow-up (FU) clinic visits at sixth month (M6), M18 and M24.

At enrolment and during FU visit, each participant was interviewed using a standard questionnaire and detailed clinical, anthropometric and demographic data were recorded by a clinician or a nurse. A total of 20 ml heparinized venous blood was collected and transported immediately to the National HIV Referral Laboratory (NHL), at EHNRI. HAART was prescribed for eligible HIV patients and anti-TB treatment for all the TB cases free of charge as per the national guidelines. Antibiotic prophylaxis was also prescribed by the physician at enrolment or during the clinic follow-up visits [52].

Laboratory tests

Laboratory examinations of blood were performed by automated machines following the manufacturer's protocol. Hematological values were determined using Cell Dyn (Abbott laboratories, Abott Par IC Jl 60064, USA); CD4+ T cell counts were determined using Becton Dickinson (BD) FACSCalibur (Becton Dickinson, San Jose, USA); and Plasma HIV-1 RNA load was measured using the NucliSens EasyQ NASBA diagnostic 2007/1 (Organon, Teknika) which has a detection range of 50-3,000,000 copies/ml. The level of IFN-γ (pg/ml) in the 7^{th} day whole blood culture supernatant stimulated with *Mtb* specific antigen (ESAT-6/CFP-10) was measured by xMAP multiplex technology (Luminex, Austin TX, USA), using Biosource reagents (Biosource, Camarillo, USA), and analyzed with the STarStation v2.0 software (Applied Cytometry Systems, United Kingdom) as described previously [53].

Definitions

Based on data from different studies [54, 55], the national [56] and international [11] guidelines, and considering the small sample size in this study, we choose the following cut-off values to define HAART outcomes. ***Body mass index (BMI)*** (kg/m^2) was categorized as malnutrition (BMI <18.50) and normal ($18.50 \geq BMI \leq 24.99$), and a**nemia** was defined as hemoglobin (Hgb) <12 g/dl [57]; v***irologic success*** was defined as achieving a viral suppression (HIV RNA < 50 copies/ml, or lower than the detection level (LDL) of the assay) after HAART, and ***virologic failure*** as a single HIV RNA >5000 copies/ml after a minimum of 6 months on HAART **[11]**. ***Immunologic success*** was defined as an increase of ≥ 50 CD4+ cells/μl at M6 and ≥100 cells/μl at M18 and M24 from that at ART initiation; ***immunologic failure*** as a failure to increase ≥ 50 cells/μl at M6, or ≥100 cells/μl at M18 and M24; and ***super-responders*** as patients able to achieve CD4+ count of >500 cells/μl after 2 years of HAART.

Statistical analysis

Data entered using Microsoft Access (DBse XI) was double-checked for discrepancies. All data analysis was done using Intercooled STATA version 11.0 (College Station, Texas, USA). Descriptive analyses including counts and frequencies for categorical variables, and mean (standard deviation, SD) or median (interquartile range, IQR) for continuous variables were computed. Results were compared using chi-square test and Fisher's exact test for categorical variables and non-parametric tests (Wilcoxon signed rank test and Mann-Whitney U test) for continuous variables. Fixed (sex, groups) and time-updated variables (age, CD4+ count, HIV RNA, BMI, Hgb, TLC, co-infections) were included in the logistic regression analysis to identify risk factors for failed immunologic and virologic responses to HAART. A p-value of < 0.05 was considered as statistical significant.

Ethical Approval

This study was ethically approved institutionally, by the Scientific and Ethics Review Office (SERO), EHNRI; and nationally by the National Health Research Ethics Review Committee (NHRERC), Ethiopian Ministry of Science and Technology Agency (ESTA).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DK was a lead author on planning, implementation of the study, data analysis, and writing of the draft, interim and final version of the manuscript; GG and YA participated in different laboratory tests; AA participated in counseling the study participants, filling study questionnaires and sample collection; DW, TE and DB – participated in providing advice and help during data analysis and also offered inputs and recommendations during the draft, interim and final version of the manuscript. All authors have seen and approved the final manuscript.

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PART-II

Immunologic responses to *M tuberculosis* antigens in latent and active TB individuals

Chapter 5

Analysis of immune responses against a wide range of Mycobacterium tuberculosis antigens in patients with active pulmonary tuberculosis.

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Abstract

Characterizing host immune responses to molecular targets of *Mycobacterium tuberculosis* (*Mtb*) is essential to develop effective immunodiagnostics and better vaccines. We investigated the immune response against a large series of *Mtb* antigens, including 5 classical and 64 non-classical (39 DosR regulon-encoded antigens, 4 Resuscitation Promoting Factor (RPF), 21 Reactivation associated) antigens in active pulmonary TB patients. Whole blood from TB patients (n=34) was stimulated *in vitro* with *Mtb* antigens. Interferon-γ (IFN-γ) was measured after seven days of stimulation using ELISA. The majority of the study participants responded to the classical *Mtb* antigens TB10.4 (84.8%), ESAT6/CFP-10 (70.6%), and PPD (55.9%). However, only 26.5% and 24.2% responded to HSP65 and Ag85A/B, respectively. From the 64 non-classical antigens, 23 (33.3%) were immunogenic (IFN-γ levels >62 pg/ml) and eight were strong inducers of IFN-γ (IFN-γ levels >=100 pg/ml). The RFP antigens were the most immunogenic. In addition, we observed distinct cytokine expression profiles in response to several *Mtb* antigens by Multiplex Immunoassay. TNF-α, IL-10, and IL-6 were commonly detected at high levels after stimulation with 4/15 latency antigens (Rv0081, Rv2006, Rv2629, Rv1733c) and especially found in supernatants of the three strong IFN-γ inducers (Rv2629, Rv1009, and Rv2389c). IL-8, IL-6, and IL-17 were exclusively detected after stimulation with Rv0574c, Rv2630, Rv1998, Rv054c, and Rv2028c.

In conclusion, in active pulmonary TB patients, we identified 23 new immunogenic TB antigens. The distinct expression level of IFN-γ, TNF-α, IL-6 and IL-10 in response to specific subsets of *Mtb* antigens may be promising for the development of immunodiagnostics.

Introduction

Mycobacterium tuberculosis (*Mtb*) infects one third of the world population (21) of whom 8-10 million developed active tuberculosis (TB) and 1.1 million died as a result of TB in the year 2010 (50). Due to social and economic factors (22), limitations in diagnostic tools, the lack of an effective vaccine for TB (3, 52 36), and the emergence of alarming multidrug resistance and extensive drug resistant *Mtb* (50), control of TB remains a major challenge. Effective diagnosis, drugs and vaccines are therefore urgently required. (22, 14).

To design new diagnostics or vaccines it is necessary to enlarge our knowledge on potential immunogenic *Mtb* antigens (41, 6). Ideally, antigens should represent the different stages of *Mtb* infection, and include *Mtb* antigens expressed during the early onset of the infection (growth stage), during the latent/dormancy stage and antigens expressed during resuscitation of the dormancy stage (19). Several immunogenic recombinant (32) and secreted (ESAT-6, Ag85B, MPT64, MPB70) (29) antigens have been identified using advanced molecular technologies (34). However, the absence of reliable methods able to predict which *Mtb* antigens may lead to protective immune responses warrants further screening of *Mtb* antigens (41).

Secreted antigens are produced early in the course of infection (5) and can elicit protective immunity (4), which rapidly stabilizes the bacterial load in the lung (5). These antigens have been recognized as potential vaccine components (Ag85 and ESAT-6) and specific immunodiagnostic reagents (ESAT-6, CFP-10) (33) for TB. *In vitro* (31) and *in vivo* (52) studies have shown the capacity of the resuscitation promoting factor (RPF) proteins to elicit both humoral and cellular immune responses which result in protection against TB infection (12, 13, 38).

Whereas latently TB infected populations have been used as human models for the screening of *Mtb* antigens (8), only few studies have been performed to assess the immunogenicity of *Mtb* antigens in active TB populations (2, 28). The fact that there is heterogeneity in T-cell repertoires between TB patients and LTBI subjects (22), and that immune recognition of *Mtb* antigens may vary in the course of TB infection and disease (43) reinforces the need to involve active TB patients in the screening of *Mtb* antigens.

This study aimed to reassess the immunogenicity of previously well established diagnostic TB antigens (classical antigens) (n=5) (TB10, ESAT-6, Ag85A/B, PPD, HSP65), as well as to analyze the immune response against a range of new *Mtb* antigens (n=64), including those for which the immunogenicity has not yet been assessed (non-classical antigens).

Since the strength of the host immune response against *Mtb* infection is directly proportional to the level of cellular (CD4+) production of IFN-γ (19), we employed a validated Whole Blood

Assay (49) to measure the level of *ex-vivo* IFN-γ induced by each antigen using Enzyme Linked Immunosorbant Assay (ELISA).

Although it is known that IFN- γ plays an important role against *Mtb* infection, the complex network of other cytokines such as TNF-α (19), interleukin(IL)-17 (44), IL-6 , IL-8 (48), IL-2 (9), and IL-10 (9) may play a role in the immunopathogenesis of *Mtb* infection. Therefore, we also assessed the level of TNF-α, IL-2, IL-6, IL-8, IL-17 and IL-10 cytokines using Luminex assay after stimulation with immunogenic antigens based on the ability to induce IFN-γ levels > =62 pg/ml as measured by ELISA.

Materials and Method

Study site and patients

This cross-sectional study was performed at St. Peter Specialized TB Hospital, Addis Ababa, Ethiopia, from November-December, 2006. A total of 37 pulmonary TB patients who were naïve for TB treatment were recruited consecutively after informed and written consent was sought. Mean (± standard deviation (SD)) age was 30.0 (±11.5) and 72.7% were men. Two (5.8%) HIV positive subjects and one (2.9%) subject with a BCG scar were excluded from analysis. Patients with clinical symptoms suggestive of TB were required to have at least two positive sputum smears for Acid Fast Bacilli (AFB) by direct microscopy to be diagnosed and confirmed with pulmonary TB. A total of 10 ml of whole blood (WB) was collected in a heparinized tube from each participant and was processed at the Ethiopian Health and Nutrition Research Institute (EHNRI). The study Nurse collected demographic data from the participants using a standardized questionnaire. Diagnosis and management of TB cases followed the national guidelines (18).

M tuberculosis antigens

As shown in Table 1, the 69 recombinant *Mtb* proteins evaluated in this study were grouped into two: 1) 5 classical antigens for which immunogenicity and specificity to *Mtb* is well defined (5, 33, 11) including TB10.4 [Rv0288], Early secreted antigenic target-6kDa (ESAT-6) [Rv3875]/Culture Filtrate Protein-10 (CFP-10) [Rv3874] (ESAT-6/CFP-10], Ag85A/B [Rv3804c/Rv1886v], 65K heat shock protein (HSP65) [Rv0440], and purified protein derivative (PPD)] and, 2) 64 non-classical antigens for which little or no data is available on immunogenicity, which included 39 dormancy (DosR)-regulon encoded or latency antigens (45), 21 reactivation (46), and 4 RPF (52) antigens. All the *Mtb* antigens were selected and produced at the department of Infectious Diseases, Leiden University Medical Center (20).

The 64 *Mtb* antigens were randomly grouped into 4 batches of 16, and each antigen batch was tested on whole blood from a group of on average 9 randomly divided TB patients. Thus, blood

samples from the TB patients in each study group were stimulated with a total of 21 antigens always including the 5 classical antigens plus 16 non-classical antigens. All *Mtb* antigens were reconstituted in sterile PBS to a concentration of 20 µg/ml, and PPD to 10µg/ml before stimulation.

Whole Blood Assay (WBA)

For each stimulus, coated plates were prepared. To this end 100ul of each stimulus, and Culture Medium (RPMI 1640) as negative control, was transferred in triplicate to 96 well round bottomed culture plates (Nunc; Cat No 163320), and stored at -80 ^{o}C until used for WBA. PHA (10µg/ml) was coated on the date of blood culture as a positive control. After dilution of the blood (1 in 5) with complete RPMI 1640 supplemented with 10% Fetal Calf Serum (FCS) and 1% Penicillin/Streptomycin (P/S), 100ul of blood/well was transferred to the thawed antigen coated plates to reach a final volume of 200 µl. The final concentration of all the recombinant antigens was 10µg/ml, while it was 5µg/ml for PPD and PHA. Culture plates were incubated in a 5% CO_2 incubator at 37°C for 7 days, and supernatants were collected and stored at -80 degree for further analysis.

IFN- γ measurement in supernatants using ELISA

The level of IFN- γ induced by each of the 69 *Mtb* antigens was quantified from the supernatants using an IFNg ELISA. In brief, Purified anti-human IFN-γ monoclonal antibody (mAb) [Pharmingen 551221, lot 72093] was diluted to a final concentration of 2µg/ml using coating buffer [0.1M $NaHCO_3$, pH 8.2] and 50µl of the mAb was transferred to each well (except well A1 – the blank) of a 96 well flat bottom ELISA plate (ThermoLab Immulon 4 HBX) and incubated overnight at 4 ^{0}C in a CO_2 incubator. After washing, 150µl blocking solution (PBS + 3% BSA) was added per well and incubated for 2 hours at room temperature. Finally, 50µl of the supernatant samples and positive control (PHA) was added to each well. Moreover, 100 µl/well of the standards were transferred to the wells following serial dilution (4000 down to 31 pg/ml) using standard diluent (RPMI+5%FCS). Plates were incubated overnight. After washing, 100µl of the detection antibody (biotin mouse anti-human IFN-γ mAb (Pharmingen 554550, lot 48629) with a final concentration of 1µg/ml was transferred to each well and incubated for 45 minutes at room temperature (RT). Then, 50 µl of the enzyme StrepABC complex was added per well and incubated at RT for one hour. Finally, 150 ul of the substrate ABTS was added to each well and plates were incubated at 37^{0}C for 15 minutes in the dark and read at 405 nm.

The IFN-γ response for individual antigens were considered positive or negative for each participant based on the cut-off value (IFN-γ=62.0 pg/ml) which was calculated as the mean plus 2 times the standard deviation of the negative control (7).

Measurement of additional cytokines using Multiplex Immunoassay

The level of Th1 (IL-2), Th2 (IL-10) and inflammatory (TNF-α, IL-6, IL-17, IL-8) cytokines (pg/ml) was measured from the same culture supernatant for a subset of 29 immunogenic antigens using multiplex immunoassay (MIA; LabMAP technology, Luminex Corporation Austin TX, USA) at the University Medical Center, Utrecht (UMCU), The Netherlands, as described previously (16). The lower limit of detection of the Luminex assay for IL-2, IL-4, IL-10, IL-17, TNFa, IL-8, and IL-6 were 0.25, 0.12, 0.50, 0.75, 0.25, 0.5 and 0.75 pg/ml, respectively.

Statistical analysis

The mean cytokine value (pg/ml) of the negative control (RPMI 1640 medium) was subtracted from the values induced by each *Mtb* antigens. The level of each cytokine induced was compared among the classical antigens. The expression level of cytokines induced by the newly evaluated *Mtb* antigens was compared to that of the classical antigens. Parametric and non-parametric test were used for statistical analysis. A P value <0.05 was considered significant. Data were analyzed using STATA software version 11.0 (Stata Corporation, College Station, Texas).

Ethical approval

This study was approved by the Research and Ethical Clearance Committee (RECC), EHNRI; and by the National Health Research Ethics Review Committee (NHRERC), Ethiopia.

Table 1: Description of the 5 classical and 64 non-classical *Mtb* recombinant antigens included latency (n=39), reactivation (n=21) and Resuscitation Promoting Factors (RPF) (n=4) which were screened for their immunogenicity based on the level of IFN-γ elicited.

Gene name [a]	**Size [aa]**	**Description**
Classical antigens (n=5)		
Rv3804c /RV1886c	338	Secreted Antigen 85-A (FBPA/85-B (FBPB)
Rv0440	540	Heat Shock Protein 65 (HSP65)
Rv3875/Rv3874	95/98	6-kDa early secretory antigenic target (ESXA; ESAT-6)/10 KDA Culture filtrate antigen ESXB (LHP) (CFP10)
Rv0288	96	Low molecular weight protein antigen (ESXH - TB10.4)
PPD		Purified protein Derivative
Non-classical (n=64)		
Latency antigens		
Rv0079	273	Hypothetical protein
Rv0081	114	Probable Transcriptional Regulatory Protein
Rv0570-C	354	Rv0570 C-term part (aa 1–354)
Rv0571c	443	Conserved Hypothetical protein
Rv0572c	113	Hypothetical protein
Rv0573c	463	Conserved Hypothetical protein
Rv0574c	380	Conserved Hypothetical protein
Rv1733c	210	Probable Conserved Transmembrane Protein
Rv1734c	80	Conserved Hypothetical protein
Rv1736c-C	380	Rv1736c C-term part
Rv1736c-N	308	Rv1736c N-term part
Rv1737c	395	Possible nitrate/nitrite transporter
Rv1738	94	Conserved hypothetical protein
Rv1812c	400	Probable Dehaydrogenase
Rv1813c	143	Conserved Hypothetical protein
Rv1997-C	430	Rv1997 C-term
Rv1997-N	504	Rv1997 N-term
Rv1998	258	Conserved Hypothetical protein
Rv2003c	285	Conserved Hypothetical protein
Rv2004c	498	Conserved Hypothetical protein
Rv2006	1327	Probable Trehalose-6-phosphate Phosphate
Rv2007c	114	Probable Ferredoxin
Rv2028c	279	Conserved Hypothetical protein
Rv2032	331	Conserved Hypothetical protein
Rv2624c	272	Conserved Hypothetical protein

Rv2625c	393	Probable Conserved Transmembrane Protein
Rv2626c	143	Conserved Hypothetical protein
Rv2627c	413	Conserved Hypothetical protein
Rv2628	120	Hypothetical Protein
Rv2629	374	Conserved Hypothetical protein
Rv2630	179	Hypothetical Protein
Rv2631	432	Conserved Hypothetical protein
Rv3126c	104	Hypothetical Protein
Rv3127	344	Conserved Hypothetical protein
Rv3128c	337	Conserved Hypothetical protein
Rv3131	332	Conserved Hypothetical protein
Table 1 continued		
Rv3132c	578	Two Component Sensor Histidine kinase
Rv3133c	217	Two Component Transcriptional Regulatory Protein
Rv3134c	268	Conserved Hypothetical protein
Reactivation antigens		
Rv0140	126	Conserved Hypothetical protein
Rv0246	436	Probable Conserved Integral membrane Protein
Rv0251c	159	Possible Heat Shock Protein
Rv0331	388	Putative Dehydrogenase
Rv0384c	848	Heat Shock Protein F84.1
Rv0753c	510	Methylmalmonate Semialdehyde Dehydrogenase
Rv1130	526	Conserved Hypothetical protein
Rv1131	393	Citrate Synthase 3
Rv1471	123	Thioredoxin Reductase
Rv1472	285	Enoyl-CoA Hydratase/Isomerase Super family
Rv1717	116	Conserved Hypothetical protein
Rv1874	228	Hypothetical protein
Rv1875	147	Conserved Hypothetical protein
Rv2465c	162	Phosphopentose Isomerase
Rv2466c	207	Conserved Hypothetical protein
Rv2662	90	Hypothetical Protein
Rv3054c	184	Conserved Hypothetical protein
Rv3223c	216	ECF subfamily Sigma Subuint
Rv3307	268	Probable Purine Nucleoside Phosphorylaseddeod
Rv3463	285	Conserved Hypothetical protein
Rv3862c	116	Possible Transcriptional Regulatory Protein WHIB6

RPF antigens		
Rv0867c	407	Possible Resuscitation Promoting Factor A
Rv1009	362	Possible Resuscitation Promoting Factor B
Rv1884c	176	Possible Resuscitation Promoting Factor C
Rv2389c	154	Possible Resuscitation Promoting Factor D

[a] , Annotations are from http://genolist.pasteur.fr/TubercuList/; [aa] , Amino acid; C-term, C-terminal; N-term, N-terminal

Results

1. Magnitude of the immune response (IFN-γ production) towards *Mtb* antigens in TB patients

To analyze the immunogenicity of the 5 classical and the 64 new *Mtb* antigens in active pulmonary TB patients, we assessed the level of IFN-γ (pg/ml) induced by each antigen after 7 days of whole blood culture. All the study participants (100%) responded to PHA (3389.9 pg/ml IFN-γ, SD ± 2999.2) (Table 2 and Fig. 1).

(i) IFN-γ response to the classical antigens

The highest level of IFN-γ production was induced after stimulation with TB10.4 (373.2 pg/ml), followed by PPD (242.0 pg/ml) and ESAT-6/CFP-10 (161.9 pg/ml) (*P<0.05*). In contrast, the level of IFN-γ induced by Ag85A/B and HSP65 was below the cut-off value (62 pg/ml) (P<0.001) (Table 2 and Fig. 1).

The proportion of study participants who responded to the *Mtb* antigens is essential for further prioritizing the antigens. TB10.4, ESAT6-CFP-10 and PPD were recognized by 81.8%, 67.7% and 58.8% of the TB patients, respectively, while the antigens HSP65and Ag85A/B were recognized only by 29.1% and 21.2% of the study group (Fig. 1).

Thus, of the classical antigens, TB10.4 followed by ESAT-6/CFP-10 were the most immunogenic antigens with respect to the level of IFN-γ induced and rate of recognition by the TB patients.

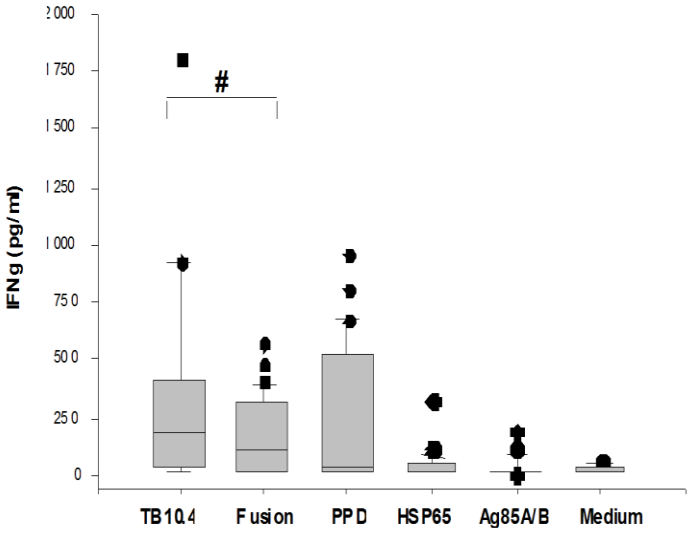

FIG 1 Production of IFN-γ by WB obtained from 34 active-pulmonary-TB patients. WB was stimulated *in vitro* with the classical *M. tuberculosis* antigens (TB10.4, PPD, ESAT-6/CFP-10 [fusion], HSP65, and Ag85A/B) and a negative control (RPMI medium). An IFN-γ ELISA analysis was done on a 7-day supernatant. The box plots show the 25th, 50th, and 75th percentiles, and the whiskers represent the minimum and maximum levels of IFN-γ (pg/ml) induced by each stimulus. The responses were compared using a Mann-Whitney U test. #, $P < 0.05$.

(ii) IFN-γ response to the non-classical antigens

To identify new immunogenic antigens, we tested 64 non-classical antigens for recognition by TB patients. Four of the 64 antigens (Rv2004, Rv3128, Rv0251, and Rv1717) were not recognized by any of the patients, whereas 23 antigens (4 RPF, 13 latency, 6 reactivation) were able to induce a positive response (elicit IFN- γ > 62 pg/ml). Of these identified immunogenic antigens 15 were intermediate inducers of IFN-γ (62>=IFN-γ<100 pg/ml), and eight (Rv1884c, Rv2389c, Rv1009, Rv0867c, Rv2629, Rv2627c, Rv2630, Rv3223c) were strong inducers of IFN-γ (IFN-γ >=100 pg/ml). Furthermore, 10 of these 23 immunogenic antigens (Rv1884c, Rv1009, Rv0867c, Rv2629, Rv1733, Rv1736-C, Rv2024, Rv3223c, Rv2662, and Rv0246) were recognized by 55.6-66.7% of the study participants. Results are summarized in Table 2.

None of the 23 immunogenic antigens induced higher IFN-γ than stimulation with TB10.4, ESAT-6/CFP-10 or PPD. However, all the RPF (Rv1009, Rv2389c, Rv1884c, Rv0867c), and 5 latency (Rv2627c, Rv2629, Rv2630, Rv1733c) antigens elicited higher IFN-γ levels than Ag85A/b and HSP65 ($P<0.05$ for all).

Taken together, from the families of antigens evaluated, 28.6%, 33.3% and 100% of the reactivation, latency and RPF antigens, respectively, were found to be immunogenic. The RPF antigens in particular were found to be the most immunogenic with respect to the level of IFN- γ induced and number of study participants that responded to these antigens (Table 2).

Table 2: IFN-γ (pg/ml) elicited by the negative control (RPMI medium), PHA, the 5 classical and the 23 identified immunogenic antigens including 7 reactivation, 12 latency and 4 Resuscitation Promoting Factors (RPF). The number of study participants, the proportion (%) of positive responders (induce IFN- γ > 62 pg/ml) and mean± SD of IFN-γ (pg/ml) are shown.

Stimuli/Antigen	Number of study participants	% of Positive [a] responders	Mean ± SD IFN- γ (pg/ml)
Negative control (RPMI medium)	34	5.9	26.2±19.9
PHA	33	100	3389.9± 2999.2
Classical			
PPD	34	55.9	242.0 ±297.5 †
Rv0288 (TB10.4)	34	84.8	373.2 ± 606.3 †
Rv3875/RV3874 (Fusion)	34	70.6	161.9±162.1 †
Rv0440 (HSP65)	34	26.5	38.2±57.9
Rv3804c/RV1886c (Ag85A/B)	34	24.2	32.2 ±42.5
Reactivation			
Rv0140	9	22.0	73.0±134.8
Rv0384c	9	22.2	90.5±190.1
Rv2662	9	44.4	65.7±70.3
Rv3223c[§]	9	22.2	125.4±213.2 †
Rv3307	9	22.2	76.9±151.1
Rv3862c	6	33.3	90.8±127.0
Latency			
Rv0079	9	11.1	76.3±175.7
Rv0081	6	33.3	61.3±84.3
Rv0574c	9	22.2	97.5 ±204.1
Rv1733c[*]	6	33.3	81.0±83.3
Rv1734c	9	33.3	57.3 ±84.8
Rv1998	9	22.2	67.4 ±101.7
Rv2006	6	16.7	72.0±127.5
Rv2007c	10	30.0	62.6±85.7
Rv2028c	10	20.0	83.5±189.1
Rv2627c[*]	6	33.3	100.9±147.5 †
Rv2629[*§]	6	50.0	121.9±121.0 †
Rv2630[*]	9	33.3	113.1±228.0 †

Rv3132c	9	22.2	97.5±191.4
RPF			
Rv0867c[*§]	6	66.7	223.6±234.6 [†]
Rv1009[*§]	6	50.0	154.0±152.1 [†]
Rv1884c[*§]	6	66.7	126.1±128.0 [†]
Rv2389c[*§]	6	33.3	182.4±307.8 [†]

Abbreviations: SD=standard deviation, CHP = Conserved Hypothetical Protein, HP = Hypothetical Protein, RPF=Resuscitation-Promoting Factor; PPD=Purified protein derivative, PHA= Phytohemagglutinin

[a] Proportion of positive respondents (able to elicit IFN- γ > 62 pg/ml) ;

[†] Strong IFN-γ inducers (IFN-γ >= 100 pg/ml

[*] Antigens induced significantly higher IFN-γ than that of Ag85A/B (*P<0.05)*

[§] Antigens induced significantly higher IFN-γ than that of HSP65 (*P<0.05)*

Measurement of pro-and anti-inflammatory cytokines after WB stimulation wit*h Mtb* antigens

To further characterize the immune response to the 26 immunogenic *Mtb* antigens, which included 3 classical (TB10.4, ESAT-6/CFP-10 and PPD) and 23 non-classical antigens, the level of pro- and anti-inflammatory cytokines (IL-7, IL-2, IL-8, IL-6, TNF-α, IL-10) were quantified using multiplex immunoassay from the same WB culture supernatants. Results are shown in Fig. 2.

Overall, the potent inflammatory cytokines (IL-6, and IL-17) were most commonly detected in WB culture supernatants (Fig. 2). IL-6 in particular was detected after stimulation of all 29 (100%) antigens and had the highest level of expression (mean value ranging from 12.9– 4127.1 pg/ml per antigen)**.** Likewise, IL-17 was detected in 26 (89.7%) of the antigen stimulations with a mean value ranging from 0.01–21.5 pg/ml per antigen. More interestingly, whereas most cytokines were expressed in response to Rv1737 and Rv1131, we observed distinct expression of cytokines (or a cytokine) towards a subset of antigens (or an antigen) within the different families of *Mtb* antigens as described below.

i) Immune responses to the latency associated antigens

As shown in Fig 2, IL-6, TNF-α, and IL-10 but not IL-17 and IL-8 were exclusively induced and at a higher level by 4 (Rv0081, Rv2629, Rv1733c, and Rv2006) of the 15 latency antigens evaluated ($p < 0.05$ for all). In contrast, only the potent inflammatory cytokines (IL-8, IL-6, IL-17), but not IL-2, TNF-α, and IL-10, were commonly detected after stimulation with the 6

antigens Rv0574, Rv2630, Rv1998, Rv1734c, Rv054, Rv2028c. ***ii) Immune responses to the Reactivation antigens***

After stimulation with the 7 immunogenic reactivation antigens (Rv1131, Rv3223c, Rv2662, Rv0140, Rv3307, Rv0384c, Rv3862c), neither IL-10 nor TNF-α were produced except for RV1131; while only IL-2, IL-6 and IL-17 were induced by Rv0140.

(iii) Immune responses to the RPF antigens

After stimulation with the 4 highly immunogenic RPF antigens (Rv1009, Rv2389c, Rv0867c, Rv1884c), only IL-2, IL-10, TNF-α and IL-6 were induced by Rv1009 and Rv2389c; but no detectable IL-8 and IL-17 was induced by any of the RPF antigens.

iv) Responses to the classical antigens (TB10.4, ESAT6/CFP10 fusion protein, PPD)

There was no detectable amount of TNF-α, IL-8, IL-17 and IL-10 after stimulation with PPD. However, the level of IL-2, TNF-α, IL-17 and IL-6 was elevated, whereas low levels of IL-8 and IL-10 were found in response to TB10.4 and ESAT6/CFP10 fusion protein.

In summary, our results revealed distinct expression of pro- and anti-inflammatory cytokines in response to a subset of *Mtb* antigens.

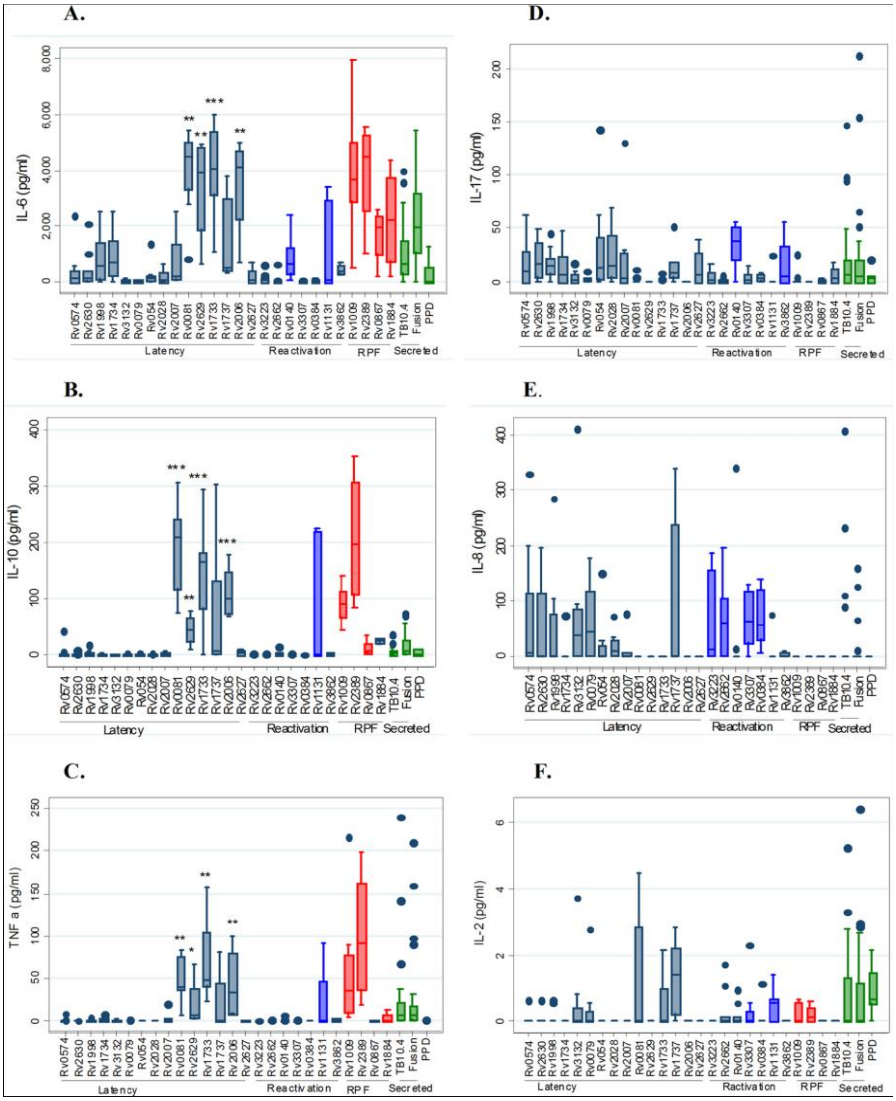

FIG 2: Concentration of L-6, IL-17, TNF-α, IL-8, IL-2, and IL-10 cytokines (pg/ml) in culture supernatants of whole blood stimulated with mycobacterial antigens. Whole blood obtained from active pulmonary TB patients (average n=9) were stimulation with wide range of mycobacterium antigens (n=29) included latency (n=15) (gray bar), reactivation (n=7) (blue bar), resuscitation promoting factor (RPF, n=4) (red bar), and secreted antigens (TB10.4, Fusion) and PPD (green bar). Concentration of the cytokines was measured from the 7th day supernatants using Luminex assay. Box and whisker plots showing minimum and maximum level and horizontal bars showing median concentrations of each cytokine. *p < 0.05*;* **p < 0.01*;* ***p < 0.001: comparison of the highly expressed IL-6, IL-10 and TNF-α cytokines induced by 4 latency antigens (RV0081, RV2629, RV1733c, RV1737, and RV2006) with that of the 11 latency antigens

Discussion

The challenges in TB diagnosis (33) and the absence of an effective vaccine against TB (52) support the need to identify immunogenic *Mtb* antigens. In this study we screened a large series of *Mtb* antigens for their ability to elicit Th1 cellular immune responses characterized by IFN-γ. Secreted *Mtb* antigens which are expressed early during the course of infection (5) have been widely reported as potential vaccine candidates (hsp60, Ag85A/Ag85B, and ESAT-6) and/or immunodiagnostic reagents (ESAT-6, CFP-10) for TB (33, 39). We included these antigens as classical controls. An additional 64 new *Mtb* antigens were tested for their immunogenicity relative to these antigens. Of the 64 non-classical antigens, 23 were immunogenic of which eight were strong inducers of IFN-γ.

Of further interest, IL-10, IL-6, TNF-α but no (or little) IL-17, IL-18 and IL-2 were detected in culture supernatants of WB assays stimulated with three (Rv2629, Rv1009, and Rv2389c) of the eight strong IFN-γ inducers.

IFN-γ response to the classical antigens

The strong immunogenicity of TB10.4 reported previously (1, 24, 7) is also supported by this study in which 84.4% of the donors recognize the antigen. Moreover, ESAT-6/CFP-10 was recognized by 67.2% of the donors in this study. In agreement to our results, other studies showed recognition of ESAT-6 in 80% of LTBI populations (41), and in 65% of TB patients in Ethiopia (37). Black *et al* (7) also showed recognition of ESAT-6/CFP-10 in >75 % of TB patients.

Although Ag85A/B is a promising candidate for subunit TB vaccines (39), in contrast to reports by Launois PR, *et al* (27), but similar to others (40, 7), we found low antigenicity of Ag85A/B compared to ESAT6-CFP10, TB10 and PPD. The lowest proportion of responding study participants and the lowest amount of IFN-γ produced was observed after stimulation with HSP65. This could be due to the nature of the antigen, which may be expressed to a lesser extent and thus lead to less immune responsiveness in the active TB patient population we studied (17).

Overall, results in the literature regarding IFN-γ responses to the classical antigens in active TB patients are inconsistent. Among the likely explanations for these inconsistencies are differences in host genetic makeup (25), in *Mtb* strains (47), in study methodologies (15), and the extent of TB disease progression, with diminished IFN-γ production during advanced disease (51). Results of our recent study performed in the same geographical location also point to diminished IFN-γ production in HIV negative TB patients compared to LTBI populations. (*D. Kassa submitted*)

IFN-γ response to the 64 non-classical Mtb antigens

The main objective of this study was to identify additional immunogenic *Mtb* antigens. We were able to prioritize 23 promising antigens, eight of which (Rv2627c, Rv2629, Rv3223c, Rv1884c, Rv2630, Rv2389c, Rv054, Rv0867c) induced high levels of IFN-γ (pg/ml>=100). Four of these (Rv1009, Rv0867c, Rv2389c, Rv1884c) were RPF antigens (Table 2).

An extensive study done by Zvi *et al* (53) identified 189 antigens from the complete *Mtb* genome, of these 45 were ranked as top-hits, and 20 as most immunodominant T-cell antigens. Of the 23 immunogenic antigens we identified, 16 were in the list of these 189 antigens, ten (Rv0079, Rv1733, Rv2028c, Rv2627c, Rv2629, Rv3132c, Rv1009, Rv0867c, Rv2389, Rv1884c) were in the list of 45 top-hit antigens, while two (Rv0867, Rv2627) were in the list of 20 most immunodominant antigens identified by Zvi and *et al* (53).

In agreement with our study which involved active TB patients, other studies from South Africa, The Gambia and Uganda which involved LTBI infected populations also showed that Rv0081, Rv1733c, and Rv2006 were the most frequently recognized antigens (7). Rv1733c, Rv0140, and Rv1009 in contrast, induced significantly higher IFN-γ responses in LTBI compared to TB patients (43). Interestingly, higher IFN-γ responses to Rv2031, Rv1733c and Rv2626c antigens were also reported in BALB/c mice persistently infected with *M. tuberculosis* compared to acutely infected mice (39)

It is also known that the majority of TB disease development occurs due to reactivation of LTBI, in which RPF antigens may be secreted and are involved in reactivation of the dormant bacterial (31). Interestingly, in this study we observed the highest immunogenicity for the RPF antigens compared to the latency and reactivation antigens, which may correlate with the characteristics of the study groups and the intrinsic nature of the antigens (52).

Whereas the recognition of the latency associated antigens by cells from active TB patients could reflect that most TB patients undergo a latent infection prior to TB disease (43); it might also indicate the involvement of latency antigens in the pathogenesis of TB. However, whether these antigens are differently recognized by cells from LTBI population warrants further study.

In summary, we identified additional immunogenic *Mtb* antigens composed of latency, reactivation and RPF families which can serve as additional antigens for further evaluation as supplementary diagnostic reagents and vaccine subunits.

Of special interest, five (Rv2662, Rv3223c, Rv3307, Rv3862c, Rv2630) of the immunogenic antigens we identified, were not in the list of the 189 antigens identified by Zvi *et al* (53) nor were they evaluated by other studies previously to our knowledge. Because they induced a very good immune response they may be potential candidates for future evaluations.

Pro-and anti-inflammatory cytokine response to a subset of immunogenic Mtb antigens

Although IFN-γ plays a pivotal role in controlling *Mtb* infection, the level of inflammatory vs. anti-inflammatory cytokines determines the clinical outcome of *Mtb* infection. It is also known, while antigens that evoke strong IFN-γ are candidates for TB vaccine development (19) that antigens that induce high level of TNF-α, IL-10, and inflammatory cytokines should be avoided from vaccine formulations for TB (34). On the other hand, combinations of antigen-specific cytokines with IFN-γ are known to strengthen the diagnostic potential of *Mtb* antigens (5, 33). We therefore also characterized the level of pro- and anti-inflammatory cytokines in the culture supernatants of the immunogenic *Mtb* antigen using Luminex assay.

The detection of IL-6 and IL-17 in 100% and 82.8% of supernatants (Fig. 2), may confirm unspecific inflammation during active TB (35). Likewise, the detection of only IL-6, IL-17, and IL-8 in supernatants of cultures with 5 latency antigens (Rv0574c, Rv2630, Rv1998, Rv054, and Rv2028c) might confirm the role of these antigens in inflammation and the pathogenesis of TB.

In agreement to Yeremeev VV. *et al* (52), we observed common secretion of TNF-α and IL-10 in only 5/15 latency, 1/6 reactivation, and 2/4 RPF antigens (Fig 2). This might indicate a role of these antigens in TB pathogenesis (42, 9); but could also support reports that a combination of TNF-α and IL-10 with specific *Mtb* antigens (5, 33), and IL-6, TNF-α and TGF-β (35) may be used as potential immunodiagnostics for active TB. Studies have also shown increased Th1 and Th2 cytokine production in patients with active TB, and that a configuration of IFN-γ, IL-2R, and IL-10 may predict (60-70% probability) susceptibility to acquire or not acquire acute TB (23). Others also reported the combination of IL-6, TNF-α and IFN-γ as effective markers to monitor TB treatment success (30).

The elevated level of IL-6, IL-17, and TNF-α in response to TB10.4 and ESAT-6/CFP-10 was in agreement with others (30). This might support previous reports that ESAT-6 and CFP-10 induced IFN-γ, TNF-α and IL-6 together with IgG are useful biomarkers of TB disease (30). On the contrary, the production of these inflammatory cytokines by TB10.4 and ESAT-6/CFP-10 might raise questions of utilizing these antigens for vaccine development (41).

Overall, the induction of multiple cytokines by a subset of antigens reflects the involvement of multifunctional subsets of T cell or leukocyte populations (15), and multiple cytokines (35) in TB pathogenesis. More importantly, our results might indicate a link between antigen specific T cells subsets and cytokine signatures during active TB, which might lead to new applications for the diagnosis of active TB and correlates of TB disease progression (51).

In conclusion, in this study we confirmed the immunogenicity of ESAT-6/CFP-10 and TB-10.4, but in addition also identified 23 immunogenic antigens which could serve as additional candidates for immunodiagnostics and vaccines for TB. Whereas the distinct expression level of

IFN-γ, TNF-α, IL-6 and IL-10 in response to specific subsets of *Mtb* antigens (Rv0081, Rv2629, Rv1733c, Rv2006) suggests a promising potential path for the development of immunodiagnostics, those antigens in favor of Th1 than that of Th2 or pro-inflammatory responses can be more relevant for new vaccine design against TB.

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Chapter 6

Analysis of host responses to secreted, latent and reactivation Mycobacterium tuberculosis antigens in a large multi-site study of subjects with different TB and HIV infection states in sub-Saharan Africa.

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Abstract

Background: (TB) remains a global health threat with 9 million new cases and 1.4 million deaths per year. In order to develop a protective vaccine, we need to define the antigens expressed by Mycobacterium tuberculosis (Mtb), which are relevant to protective immunity in high-endemic areas.

Methods: We analysed responses to 23 Mtb antigens in a total of 1247 subjects with different HIV and TB status across 5 geographically diverse sites in Africa (South Africa, The Gambia, Ethiopia, Malawi and Uganda). We used a 7-day whole blood assay followed by IFN-γ ELISA on the supernatants. Antigens included PPD, ESAT-6 and Ag85B (dominant antigens) together with novel resuscitation-promoting factors (rpf), reactivation proteins, latency (Mtb DosR regulon-encoded) antigens, starvation-induced antigens and secreted antigens.

Results: There was variation between sites in responses to the antigens, presumably due to underlying genetic and environmental differences. When results from all sites were combined, HIV- subjects with active TB showed significantly lower responses compared to both TST^- and TST^+ contacts to latency antigens (Rv0569, Rv1733, Rv1735, Rv1737) and the rpf Rv0867; whilst responses to ESAT-6/CFP-10 fusion protein (EC), PPD, Rv2029, TB10.3, and TB10.4 were significantly higher in TST^+ contacts (LTBI) compared to TB and TST^- contacts fewer differences were seen in subjects with HIV co-infection, with responses to the mitogen PHA significantly lower in subjects with active TB compared to those with LTBI and no difference with any antigen.

Conclusions: Our multi-site study design for testing novel Mtb antigens revealed promising antigens for future vaccine development. The IFN-γ ELISA is a cheap and useful tool for screening potential antigenicity in subjects with different ethnic backgrounds and across a spectrum of TB and HIV infection states. Analysis of cytokines other than IFN-γ is currently on-going to determine correlates of protection, which may be useful for vaccine efficacy trials.

Introduction

Mycobacterium tuberculosis (Mtb) complex is the causative agent of tuberculosis (TB). The scientific challenges in understanding immunity to Mtb arise from the observation that, although immune responses are generated after infection, eradication of the bacteria is rare [1]. Instead, host immunity causes Mtb to adopt a clinically silent, latent state of infection in which it is highly resistant to immune attack. Once immunity becomes dysregulated the bacteria can become reactivated [1]. Considering that over 2 billion people live with latent TB infection (LTBI) [2], this population provides an enormous reservoir for potentially new cases of active TB disease.

The Mtb life-cycle can be separated into three main stages: latent (dormant), reactivating and active TB. Each stage represents differences in Mtb gene expression and therefore determining the immune response to stage-specific antigens can inform the design of new vaccine candidates [3,4]. For instance, in LTBI, the Mtb DosR regulon is induced by conditions that inhibit aerobic respiration and prevent bacillary replication and is crucial for rapid resumption of growth by involving resuscitation-promoting factors (rpf) once Mtb exits the hypoxic/anaerobic or nitric oxide-induced non-respiring state [5]. Once reactivation has occurred, the induction of a strong immune response by the host may actually provide further benefit to the bacteria [1–3]: T cell responses to TB antigens have been shown to be significantly higher in active TB than LTBI [6,7] suggesting that increased immunity may promote lung pathology and subsequently transmission [1]. Indeed, immunogenicity does not necessarily equate to protection, as illustrated by the recent failure of a novel prime-boost vaccine, MVA85A, to protect children against TB [8], despite a proven antigen-specific T cell response [9]. Thus, more information is required to understand what constitutes protective immunity to TB and in turn to inform new vaccine design strategies.

Our consortium previously reported responses to 51 DosR antigens in latently infected HIV$^-$ subjects from Uganda, South Africa and The Gambia with Rv1733c being the most commonly recognised antigen [10]. However, whilst similarities between sites were observed, there were also significant differences between the populations. Another study of South African subjects showed that responses to rpf were significantly higher in TB cases compared to household contacts (HHC) but values were minute compared to responses to dominant antigens such as ESAT-6/CFP-10 fusion protein (EC) [11] and comprised a mixture of uninfected and latently infected HHC. Building on these preliminary findings, the present study analysed T cell responses to 23 Mtb antigens in a total of 1247 subjects with different HIV and TB status across five geographically diverse sites in Africa (South Africa, The Gambia, Ethiopia, Malawi and Uganda).

Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. Study protocols were approved by specific review boards at each institution (full details for each site are listed in the Table S1). All patients provided written informed consent for the collection of samples and subsequent analysis.

Subjects and Study sites

The study design for GC6-74 has been previously described [12]. For this sub-study, sites included The Gambia (Medical Research Council, MRC), Ethiopia (Armauer Hansen Research Institute, AHRI), Malawi (Karonga Prevention Study, KPS), Uganda (Makerere University, MAK) and South Africa (Stellenbosch University, SUN). Subjects were considered for inclusion if they were ≥18 years of age, had no concurrent infections and were willing to undergo an HIV test. Subjects without TB were recruited from households of TB patients (MRC, SUN, and MAK) or by random community selection and from HIV care clinics (KPS, AHRI) (termed household and community controls; HCC). All subjects underwent a clinical assessment, including a chest x-ray and a screen for malaria and inter-current illnesses. Tuberculin skin tests (TST; two tuberculin units [TU], PPD RT23, SSI, Denmark) were performed in order to detect latent infection status in the subjects without active disease. Subjects with induration ≥10mm for HIV^- or ≥5mm for HIV^+ subjects were classified as latently infected (TST^+). TB cases were confirmed by sputum culture (BACTEC™, Becton-Dickinson, USA). If BACTEC was not available, culture on Lowenstein-Jensen solid media was performed (KPS, AHRI). Following informed consent, heparinised whole blood was collected for the whole blood assay set-up.

Seven-day whole blood assay

200µl of 1:10 diluted whole blood was stimulated with each antigen in triplicate as previously described [10]. Each site used the same batch of quality controlled antigens, controls and reagents. The antigens used were generated by our consortium and immunogenicity determined previously [10,13] (Table S2). Following 7 days incubation at 37^oC, 5% CO2, supernatants were harvested and stored at -20^oC prior to analysis by IFN-γ ELISA. Antigens were evaluated at a final concentration of 10µg/ml except for the peptide pools, Rv2659c and Rv2660, which were used at 1µg/mL final concentration per peptide and Mtb PPD, which was used at 5µg/mL final concentration. Controls included unstimulated (negative control) and the polyclonal stimulator, phytohaemagglutinin (PHA 5µg/mL; positive control) (Sigma, USA). Antigens were produced at SSI (Denmark) or LUMC (The Netherlands).

IFN-γ ELISA

Supernatants were analysed by IFN-γ ELISA as previously described [10,14]. Briefly, plates were coated overnight with mouse anti-human IFN-γ monoclonal antibody (2μg/ml; Becton-Dickinson, USA) at 4^{o}C. Following washing with PBS-Tween 20, wells were blocked using PBS/FCS (Sigma, USA) for 2 hours. Samples, controls and standards were then added and the plate incubated overnight at 37^{o}C. After washing, biotinylated anti-rabbit detection antibody (1μg/mL; Becton-Dickinson, USA) was added, plates incubated for 45min. at RT and a final colorimetric step performed by addition of avidin-peroxidase followed by OPD Fast (Both from Sigma, USA). The reaction was stopped with 2M H2SO4 and the plates were read at 492nm with a four-parameter curve-fit. Each site used an aliquot of the same positive PHA-stimulated whole blood culture as a positive control. Additionally, the same batches of antibodies and standards were used in all the sites, using a standardised protocol.

Data analysis

We assumed non-parametric distribution of samples. All antigen-stimulated wells were adjusted for non-specific responses by background subtraction (media alone). IFN-γ levels below 16pg/mL were considered as non-responses and adjusted to '1'. A Kruskal-Wallis test followed by Dunn's post-test comparison was used for determining differences between TB cases and TST^{+} and TST^{-}HHC. A Mann–Whitney U-test was used for analysis of TB cases and TST^{+} controls at each site. Significance was defined as p-value ≤ 0.035 to adjust for false-discovery rates (FDR) with multiple comparison testing. For HIV+ subjects, analysis of CD4 counts within and between sites was performed using Kruskal-Wallis test and Dunn's post-test comparison. Due to differences between sites in regards to CD4 counts, HIV+ subjects were analysed both with unadjusted and adjusted CD4 counts using linear regression. Logistic regression and receiver-operator curve (ROC) analyses were performed to determine which parameters best discriminate between TB and TST^{+} controls. Analyses were performed using Graphpad Prism 6 (Software MacKiev, USA) and SPSSv20 (IBM, USA).

Results

Subject demographics

The majority of subjects in this study were HIV^{-} with a total of 262 $HIV^{-}TB^{+}$, 454 $HIV^{-}TST^{+}$ (HCC) and 204 $HIV^{-}TST^{-}$HCC from all sites combined (Table 1 depicts numbers from individual sites). No HIV^{+} subjects were analysed from MRC due to the low rate of HIV infection in The Gambia. For HIV-infected subjects, a total of 77 TB, 87 TST^{+} HCC and 163 TST^{-}HCC were

analysed (Table 1). There were significant differences between and within sites in regards to age and sex of the subjects so these were adjusted for accordingly in the statistical analyses where possible. A total of 71 TB index cases from MRC were also confirmed to be infected with either Mtb sensu stricto (n=39) or *M. africanum* (Maf; n=32); a strain only present in West Africa [15]. No significant difference in response to any of the antigens was seen in Mtb or Maf infected subjects (data not shown) and therefore this was not adjusted for in our analyses. HIV+ subjects had significant differences in CD4 counts within and between sites (Table 1). Whilst no difference was seen for TB cases or TST+ HCC, TST- HCC were significantly different between sites particularly for SUN ($p<0.05$ compared to KPS and $p<0.001$ compared to both AHRI and MAK). In addition, TB cases had significantly lower CD4 counts compared to TST+ contacts at AHRI, KPS and MAK ($p=0.01$, $p=0.001$ and $p=0.0001$ respectively) but not at SUN. As such, results are presented using unadjusted values, but also with adjustment for differences in CD4 counts.

Table 1. Participant information.

	HIV$^+$			HIV$^-$			
Site/TB status	**TB**	**TST$^+$**	**TST$^-$**	**TB**	**TST$^+$**	**TST$^-$**	**Total**
SUN	23	16	47	65	231	27	**409**
Age	34[28–40]	39[30–44]	39[31–47]	28[22–40]	26[16–37]	24[15–37]	
% males	57	56	43	40	40	37	
MRC	n/a	n/a	n/a	77	120	124	**321**
Age	n/a	n/a	n/a	28[23–39]	25[20–34]	24[18–31]	
% males	n/a	n/a	n/a	85	50	56	
AHRI	19	18	15	31	28	11	**122**
Age	35[27–45]	30[25–35]	32[24–36]	23[21–28]	31[20–45]	23[20–30]	
% males	41	14	20	65	36	27	
KPS	21	39	78	32	34	18	**222**
Age	32[27–38]	39[31–48]	39[31–45]	49[29–58]	39[32–50]	34[27–52]	
% males	52	33	36	56	51	44	
MAK	14	15	23	57	97	24	**230**
Age	28[25–39]	35[31–40]	30[27–39]	25[22–30]	26[19–35]	22[20–26]	
% males	43	13	30	49	43	38	
Total (n=)	**77**	**88**	**163**	**262**	**510**	**204**	**1304**

HIV$^+$ = human immunodeficiency virus-positive; HIV$^-$ = human immunodeficiency virus-negative; TB = active tuberculosis; TST$^+$ = tuberculin skin test-positive control; TST$^-$ = tuberculin skin test-negative control; SUN = Stellenbosch University, South Africa; MRC = Medical Research Council, The Gambia; AHRI = Armauer Hansen Research Institute, Ethiopia; KPS = Karonga Prevention Study, Malawi; MAK = Makerere University, Uganda; Age = median[Interquartile range]. Total = total subjects per site per TB/HIV group. CD4 counts indicated for HIV-positive subjects only.

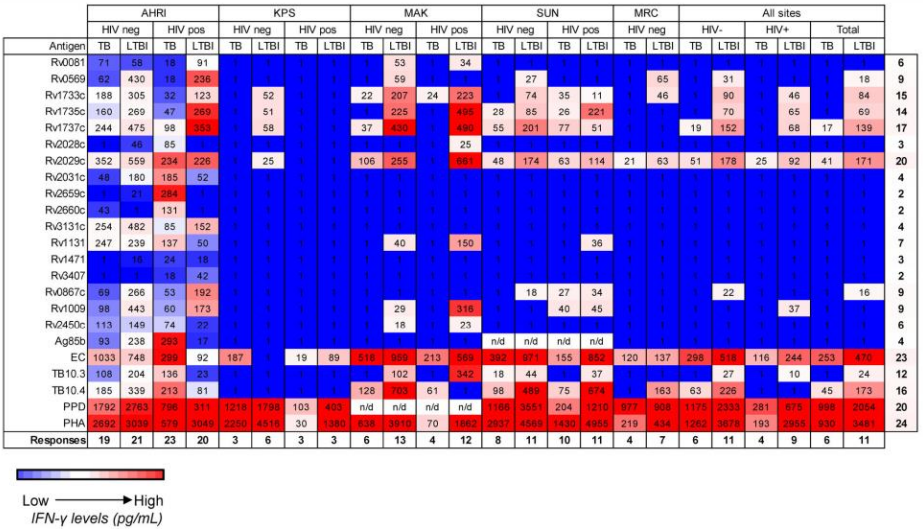

Figure 1. Heat map of IFN-γ responses to secreted, latent, and reactivation Mtb antigens stratified according to HIV status, TB status and location. . Median levels of IFN-γ are shown (pg/mL). Red indicates relatively high levels of IFN-γ and blue indicates relatively low levels. HIV^{+} = human immunodeficiency virus positive; HIV^{-} = human immunodeficiency virus negative; TB = active tuberculosis; LTBI = latently TB infected; n/d = not done; SUN = Stellenbosch University, South Africa; MRC = Medical Research Council, The Gambia; AHRI = Armauer Hansen Research Institute, Ethiopia; KPS = Karonga Prevention Study, Malawi; MAK = Makerere University, Uganda. The right column indicates the number of groups (out of a possible 24) who responded to a particular antigen. PPD was not used at MAK nor was Ag85b used at SUN, so the maximum number for these is 20.

IFN-γ responses to 23 antigens following a 7-day whole blood assay

Figure 1 shows a heat-map of responses from all sites with subjects separated based on TB and HIV status. The highest responses were to PHA, PPD, EC, TB10.4 and Rv2029c (Figure 1, red). The heat map illustrates the differences seen between sites, with subjects from AHRI showing responses (median) to the majority of antigens used regardless of HIV or TB status (Figure 1; AHRI); whilst KPS subjects responded to, at most, four antigens other than PPD and PHA (HIV^- LTBI: Rv1733, Rv1735, Rv1737 and Rv2029) and only ESAT-6/ CFP10 (EC) for the other 3 groups (Figure 1; KPS). The most consistent differences were seen to EC regardless of site, TB or HIV status. However the direction differed between sites with higher EC responses in TB patients compared to LTBI for both HIV^- and HIV^+ subjects from AHRI and HIV^- subjects from KPS but lower responses to LTBI from all other sites regardless of HIV status. After PHA, PPD and EC, the antigens that induced the greatest number of responses from each group were Rv2029c (20 out of a possible 24 HIV/TB groups) followed by Rv1737c (17/24) and TB10.4 (16/24) (Figure 1).

Control Stimulants

Control antigens used in this study included PPD and the mitogen PHA as a polyclonal stimulator. PPD cross-reacts with both environmental Mycobacteria and BCG vaccine and therefore is not specific for TB in the endemic countries analysed in this study. Indeed, few differences were seen in responses to PPD between TST^+ controls (LTBI) and active TB subjects (Figure 2). SUN was the only site to see a difference between the groups in response to PPD: both HIV^- ($p<0.01$) and HIV^+ ($p<0.01$) subjects with responses in active TB disease being significantly lower than in LTBI. Responses to the positive control PHA were significantly lower in subjects with active TB disease compared to LTBI for all sites studied regardless of HIV status. Intriguingly, these responses were often lower than for PPD (Figure 2). Whilst there was considerable variation in PHA responses within and between sites, we did not adjust for this since responses to PPD, which reflect TB relevant immune responses, were not significantly different.

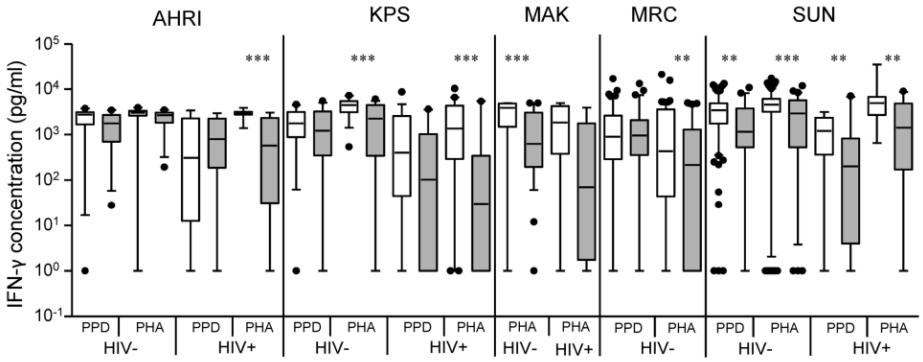

Figure 2. IFN-γ secretion in response to secreted, latent, and reactivation Mtb antigens in active TB and LTBI subjects, from five African sites. IFN-γ ELISA was performed on supernatants collected after 7-day antigen stimulation of diluted blood from TB cases (grey) and TST^{+} (LTBI) controls (white) from five sites in Africa. Line indicates median, whiskers indicate 5–95% range and dots indicate outliers. Data were analysed by Mann-Whitney U-test within sites for HIV^{-} and/or HIV^{+} subjects. Significant differences are indicated: *=p<0.05; **=p<0.01; ***=P<0.001. doi: 10.1371/journal.pone.0074080.g002

Secreted antigens

The secreted Mtb antigens used in this study were EC, Rv0288 (TB10.4), Rv1886 (Ag85b) and Rv3019 (TB10.3) (Table S2). There were no significant differences between TB and LTBI in response to these antigens with subjects from AHRI (HIV^{-} and HIV^{+}; Figure 3). HIV^{-} TB cases from KPS had a significantly higher response to EC than LTBI (p=0.0035; Figure 4A), but no difference in HIV+ subjects after adjusting for FDR (Figure 4B). LTBI subjects from Uganda (MAK) had significantly higher IFN-γ levels compared to TB following TB10.3 and TB10.4 stimulation (p<0.0001 for both) in HIV^{-} subjects (Figure 5A) and following EC (p=0.0342) and TB10.3 (0=0.0013) stimulation in HIV^{+} subjects (Figure 5B). However, when CD4 counts were adjusted, only TB10.3 responses were significantly different (p=0.026). Ag85b responses were not assessed in subjects from SUN, however, responses to EC and TB10.4 were both significantly higher in HIV^{-} LTBI compared to HIV^{-} TB (p=0.0109 and p=0.0001 respectively; Figure 6A). Similarly, HIV^{+} LTBI subjects from SUN had higher levels of IFN-γ in response to EC and TB10.4 than HIV^{+} TB after adjusting for CD4 counts (p=0.003 and p<0.0001 respectively; Figure 6B). Due to low levels of HIV infection in The Gambia, data from MRC is presented for HIV^{-} subjects only (Figure 7). Similar to AHRI and MAK, LTBI subjects from MRC had higher responses to secreted antigens than those with active TB with a significant

difference in IFN-γ production following stimulation with TB10.3 (p=0.0223) and TB10.4 (p=0.0001) and a higher (but not significant) response to EC (Figure 7).

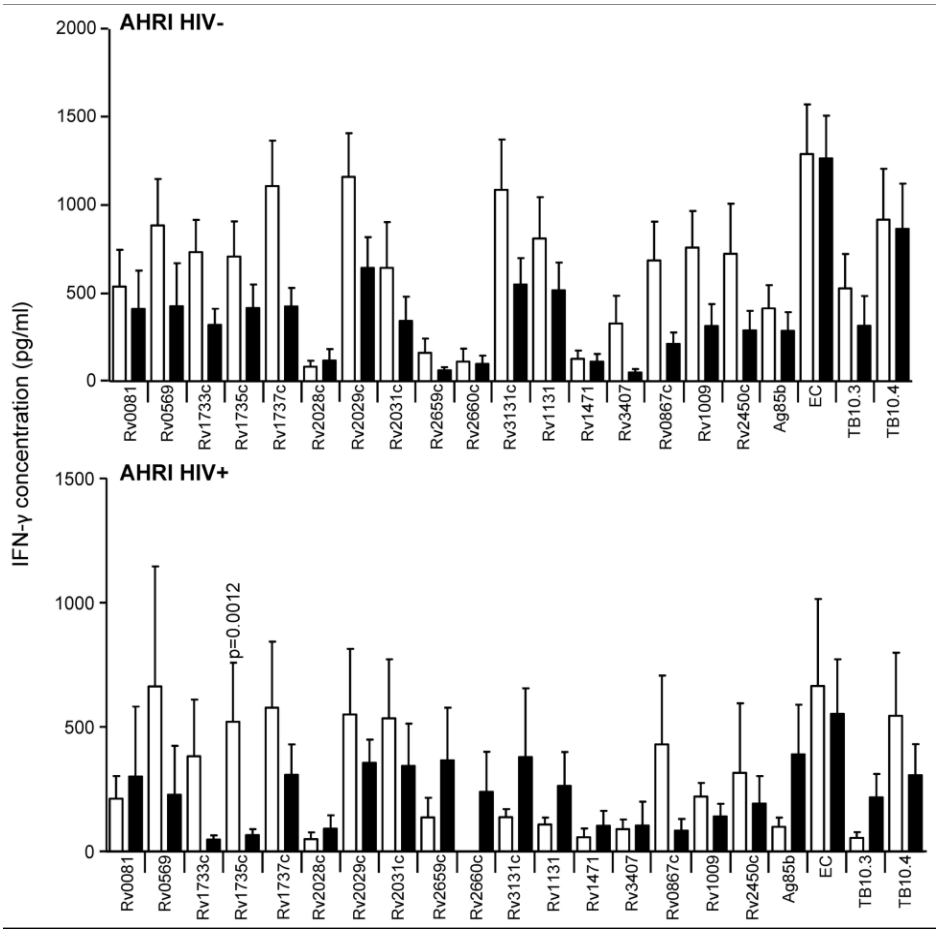

Figure 3. IFN-γ secretion in response to secreted, latent, and reactivation Mtb antigens in active TB and LTBI subjects, from Ethiopia. IFN-γ ELISA was performed on supernatants collected after 7 day antigen stimulation of diluted blood from TB cases (black) and TST^{+} (LTBI) controls (white) from AHRI (Ethiopia). Line indicates median, whiskers indicate 5–95% range and dots indicate outliers. Data were analysed by Mann-Whitney U-test for HIV^{-} and/or HIV^{+} subjects. Significant differences are indicated.

Reactivation antigens

The reactivation antigens used in this study were Rv1131, Rv1471, Rv3407, Rv0867c, Rv1009 and Rv2450c (Table S2); the latter three are so-called rpf proteins. Principally, responses to these antigens were lower than for the secreted antigens except for HIV^- subjects from AHRI but, despite higher levels in LTBI, there were no significant differences seen between the groups for any antigen in HIV^- or HIV^+ subjects from AHRI (Figure 3). HIV^- LTBI from KPS showed significantly higher responses than HIV-TB to Rv1131 and Rv0867c ($p=0.0073$ and $p=0.006$ respectively; Figure 4A). HIV^+ subjects from KPS showed low responses to all of the reactivation antigens except Rv1009, which was significantly higher in LTBI than TB ($p=0.0060$; Figure 4B). After adjusting for CD4 levels, Rv1131 was also significantly higher in LTBI compared to TB ($p<0.035$). HIV^- LTBI subjects from MAK revealed significantly higher esponses to all reactivation antigens compared to HIV^- TB from SUN generated low responses to the reactivation antigens except Rv1471 and Rv2450 (Figure 5A). HIV^+ LTBI subjects (Figure 6A), although these were significantly higher in HIV^- TB from MAK had significantly higher levels of IFN-γ following compared to LTBI in response to Rv0867c ($p=0.0020$; Figure stimulation with Rv1131 and Rv1009 compared to TB 6A). HIV^+ subjects from SUN did not respond to any ($p<0.0062$ and $p<0.0033$ respectively; Figure 5B) but only for reactivation antigen (Figure 6B) even after adjusting for CD4 Rv1009 after adjusting for CD4 counts ($p=0.013$). HIV^- subjects counts. While all other sites had higher levels of response to Rv0867c from LTBI compared to active TB, this was the reverse for HIV- subjects from MRC ($p=0.012$; Figure **7**). Responses to Rv2450c followed the same pattern as for the other sites with levels of IFN-γ significantly higher in LTBI than TB subjects from MRC ($p=0.0042$; Figure **7**).

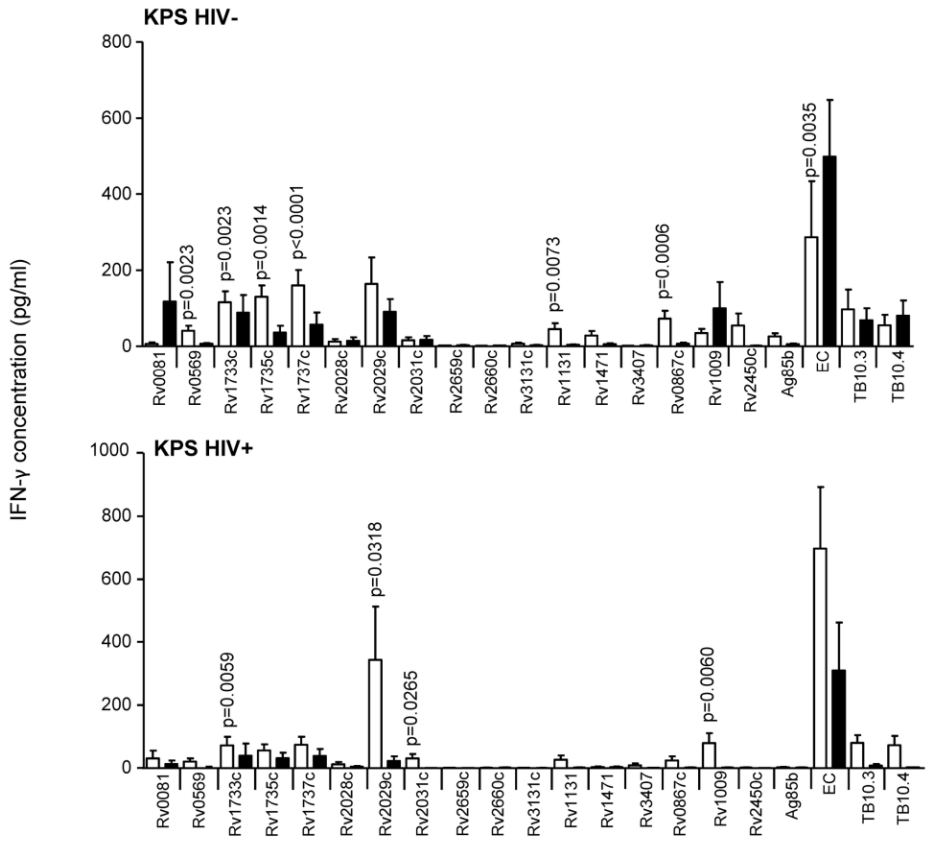

Figure 4. IFN-γ secretion in response to secreted, latent, and reactivation Mtb antigens in active TB and LTBI subjects, from Malawi. IFN-γ ELISA was performed on supernatants collected after 7-day antigen stimulation of diluted blood from TB cases (black) and TST+ (LTBI) controls (white) from KPS (Malawi). Line indicates median, whiskers indicate 5–95% range and dots indicate outliers. Data were analysed by Mann-Whitney U-test for HIV– and/or HIV+ subjects. Significant differences are indicated.

Latency antigens

Latency (i.e. Mtb DosR regulon-encoded) antigens included in this study were Rv0081, Rv0569, Rv1733c, Rv1735c, Rv1737c, Rv2028c, Rv2029c, Rv2031c, and Rv3131c, next to the two starvation-induced antigens Rv2659c, Rv2660c. Whilst HIV- subjects from AHRI showed no significant difference between LTBI and TB to any of these antigens (although LTBI were generally higher than TB), HIV+ LTBI had a significantly higher response to Rv1735c than HIV+ TB (p=0.0012; Figure **3B**); although this was lost after adjusting for CD4 counts. HIVLTBI from KPS generated significantly higher responses to Rv0569, Rv1733c, Rv1735c and

Rv1737c than HIV- TB (p=0.0023, p=0.0023, p=0.0014 and p<0.0001 respectively; Figure **4A**). Additionally, HIV+ LTBI from KPS revealed significantly higher responses to Rv1733 and Rv2029c and Rv2031c than HIV+ TB (p=0.0059, p=.0318 and p=0.0265 respectively; Figure **4B**); but again no difference after adjusting for CD4 counts. HIV- LTBI from MAK had significantly higher levels of IFN-γ than HIV- TB following stimulation with all the latency antigens used in this study (Figure **5A**). HIV+ LTBI also had higher responses than HIV+ TB to most of the latency antigens with significant differences found for Rv1733c, Rv1735c, Rv1737c and Rv2029c (p=0.0023, p=0.0018, p=0.0031 and p=0.0008 respectively; Figure **5B**).

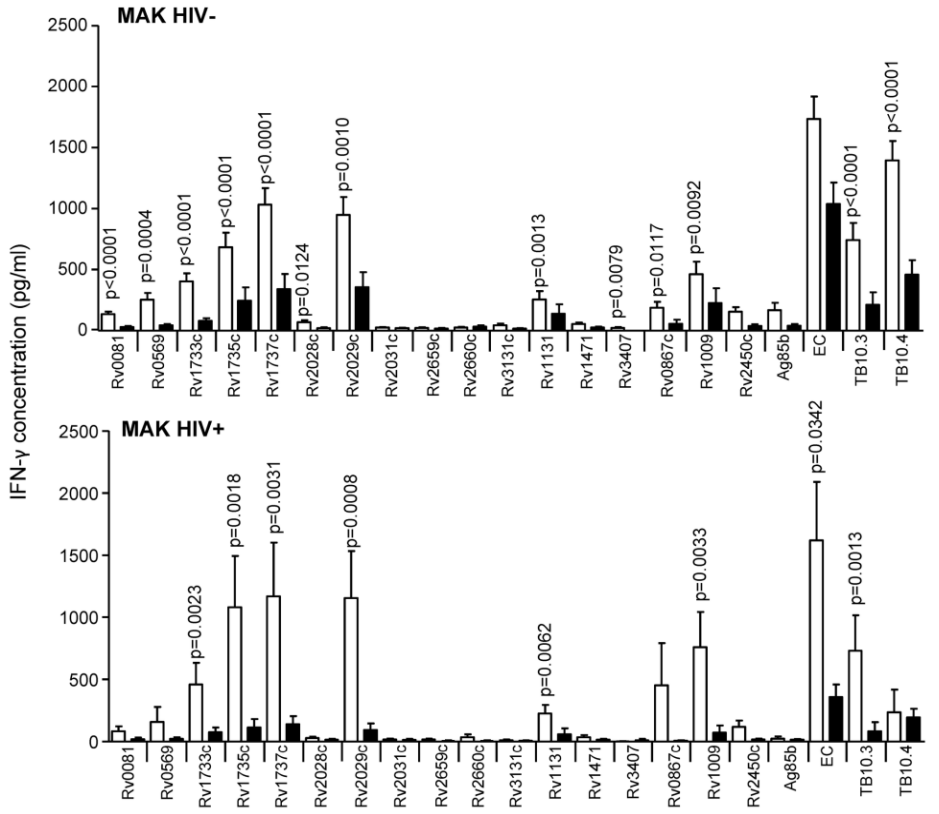

Figure 5. IFN-γ secretion in response to secreted, latent, and reactivation Mtb antigens in active TB and LTBI subjects, from Uganda. IFN-γ ELISA was performed on supernatants collected after 7-day antigen stimulation of diluted blood from TB cases (black) and TST+ (LTBI) controls (white) from MAK (Uganda). Line indicates median, whiskers indicate 5–95% range and dots indicate outliers. Data were analysed by Mann-Whitney U-test for HIV– and/or HIV+ subjects. Significant differences are indicated.

After adjusting for CD4 counts, Rv1737c and Rv2029c remained significantly different between LTBI and TB (p=0.011 and p=0.003 respectively). HIV- LTBI subjects from SUN had significantly higher levels of IFN-γ compared to HIV- TB subjects in response to Rv0569, Rv1733c, Rv1737c and Rv2029c (Figure **6A**). Again, HIV+ subjects from SUN showed no differences in response to any of the latency antigens between LTBI and TB (Figure **6B**). Whilst MRC subjects generated lower responses to most of the latency antigens than HIV- subjects from AHRI, MAK and SUN, they were significantly higher in LTBI compared to active TB subjects in response to Rv0569, Rv1733c, Rv1735c and Rv1737c.

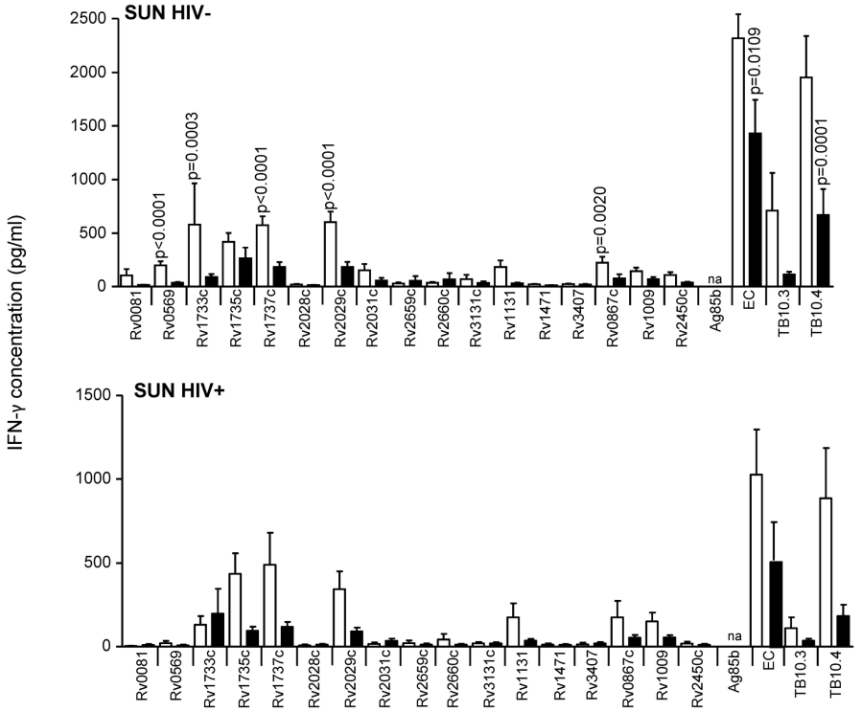

Figure 6. IFN-γ secretion in response to secreted, latent, and reactivation Mtb antigens in active TB and LTBI subjects, from South Africa. IFN-γ ELISA was performed on supernatants collected after 7-day antigen stimulation of diluted blood from TB cases (black) and TST+ (LTBI) controls (white) from SUN (South Africa). Line indicates median, whiskers indicate 5–95% range and dots indicate outliers. Data were analysed by Mann-Whitney U-test for HIV– and/or HIV+ subjects. Significant differences are indicated. n/d = antigen not analysed.

Comparison of TST- (Mtb-uninfected), TST+ (LTBI) and active TB groups

We analysed HIV- and HIV+ subjects from all sites based on their TB status (Mtb-uninfected (TST-), latent infection (TST+) or active disease (TB)). For HIV- subjects, active TB subjects had significantly lower levels of IFN-γ compared to both TST- and TST+ controls in response to

latency antigens Rv0569, Rv1733, Rv1735, Rv1737 and Rv0867 ($p<0.0001$ for all; Figure **8**). Responses to EC and PPD were significantly higher in HIVTST+ (LTBI) compared to HIV- TB subjects ($p=0.016$ and $p<0.0001$ respectively) who were in turn, significantly higher than HIV- TST- subjects ($p<0.0001$ for both; Figure **8**). Responses to Rv2029, TB10.3, TB10.4 and PHA were significantly higher in HIV- TST+ compared to both HIV- TB and HIV- TST- (who were comparable; $p<0.0001$ for all; Figure **8**). Fewer differences were seen with HIV+ subjects: the only significant difference between TB and TST+ subjects was in response to PHA with a median of 193 pg/mL for HIV+ TB+ compared to 2799 pg/mL for HIV+ TST+ ($p<0.0001$; Figure **8**). Similar to the HIV- subjects, we observed a hierarchical response to EC and PPD with LTBI higher than TB (but not significantly) and both significantly higher than HIV+ TST- ($p<0.0001$ for all; Figure **8**). When ROC analysis was performed, no single antigen response could discriminate between active TB disease and latent infection with >65% correct classification regardless of HIV status (data not shown).

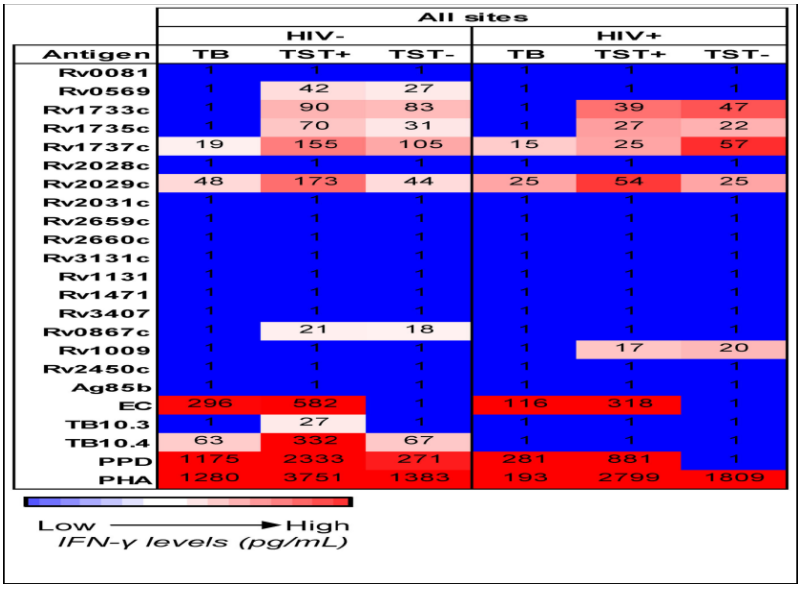

Figure 8. Heat map of combined responses from all sites for TST–, TST+ (LTBI) and active TB with or without HIV infection in response to secreted, latent, and reactivation Mtb antigens. . Median levels of IFN-γ are shown (pg/mL). Red indicates relatively high levels of IFN-γ and blue indicates relatively low levels. HIV+ = human immunodeficiency virus positive; HIV- = human immunodeficiency virus-negative; TB = active tuberculosis; LTBI = latently TB infected; SUN = Stellenbosch University, South Africa; MRC = Medical Research Council, The Gambia; AHRI = Armauer Hansen Research Institute, Ethiopia; KPS = Karonga Prevention Study, Malawi; MAK = Makerere University, Uganda.

Discussion

Discovery of new antigens that could provide protection against primary or reactivation TB disease is essential for development of next-generation vaccines. The fact that BCG remains the only licensed TB vaccine for over 90 years shows how difficult this accomplishment is. A recent trial using Mtb Antigen 85A as a boost to BCG in infants showed no increase in protection against development of TB, despite strong immunogenicity in Phase I/IIa trials [8]. Thus we also need to determine the exact correlates of protection to determine which antigens to incorporate in vaccine design strategies and for determining vaccine efficacy without the requirement for extremely large and expensive cohort studies. The Bill & Melinda Gates Foundation, Grand Challenges (GC) in Global Health, Biomarkers for TB: GC6-74 aims to determine biomarkers for protective immunity in the context of HIV/AIDS in Africa (http://www.biomarkers-for-tb.net/consortium). As one objective, we analysed responses from 1247 subjects to 23 Mtb antigens to elucidate the influence of TB status (latent infection, Mtb-uninfected, active TB disease), HIV status and geographical location on these responses.

The antigens analysed included PPD, ESAT-6 and Ag85B together with novel rpf, reactivation proteins, latency (Mtb DosR regulon-encoded) antigens, starvation-induced antigens and secreted antigens tentatively characterized for T-cell responses in HIV^- HHC in a previous pilot study of 86 antigens [10]. Antigens used in current diagnostic/vaccination strategies (TB10.4, PPD and ESAT-6/CFP-10) generated dominant responses from all sites but very few differences between active TB disease and LTBI. The next highest responses were seen to Rv2029c followed by Rv1733, Rv1735 and Rv1737. These are all dormancy-associated antigens, essential for the survival of Mtb during persistence *in vivo* and Rv1733, 1735 and 1737 were previously shown to induce dominant immunogenic responses in a small cohort of LTBI subjects from The Gambia, South Africa and Uganda [10]. Rv2029c is probable phosphofructokinase (pfkB) and is one of the most important enzymes of glycolysis [16]. A recent study suggests that glycolysis leads to accumulation of glucose-derived toxic metabolites that limits long-term survival of Mtb under hypoxic conditions [16] and is exacerbated when the glycolytic pathway is disrupted at the PKF step. Whilst HIV^+ and HIV^- subjects from Uganda and HIV^+ subjects from Malawi showed significant differences between LTBI and TB to Rv2029c, no differences were observed at the other three sites. This pattern was similar for the majority of antigens used in this study: whilst there were similarities between sites, there were also many differences. Notably, these included no differences between TB and LTBI in subjects from Ethiopia for any antigen, and much lower responses to all antigens in subjects from Malawi. Note that these differences between sites are unlikely due to differences in Mtb strain since Gambian subjects infected with Mtb *sensu stricto*

or *M. africanum* showed no differences in response to any of the antigens (data not shown). There was a large variation in CD4 counts for HIV+ subjects within and between sites, which will clearly affect levels of IFN-γ. However, whilst HIV+ subjects from KPS had the lowest responses (and CD4 counts), HIV negative subjects from KPS also had very low responses across the board. Additionally, subjects from SUN showed no difference in CD4 levels between HIV+LTBI and HIV+TB but were the only site to show a difference in responses to PPD with active cases significantly lower than LTBI regardless of HIV status. Even after adjusting for CD4 counts, there was still considerable variation between sites in regards to antigen reactivity suggesting that other confounding factors need to be considered such as ethnicity (host genetics), nutritional status and microbial environment.

Although accuracy of combined analyses from all sites were impeded by considerable variations in responses, the main purpose of this study was the search for antigens which distinguish between TST^-, TST^+ (LTBI) subjects and TB patients regardless of geographical location (and hence of ethnicity, nutritional status, microbial environment, etc.). When results from all sites were combined, HIV^- patients with active TB showed significantly lower responses compared to both TST^- and TST^+ (LTBI) contacts to latency antigens (Rv0569, Rv1733, Rv1735, Rv1737) and the rpf Rv0867; whilst responses to EC, PPD, Rv2029, TB10.3, TB10.4 and PHA were significantly higher in TST^+ HCC (LTBI) compared to active TB and TST^- HCC. Fewer differences were seen in subjects with HIV co-infection, with response to PHA being significantly lower in subjects with active TB compared to those with LTBI and no difference with respect to any other antigen. Interestingly, PHA-induced polyclonal responses were often lower than PPD-induced responses to Mtb. This may relate to the highly activated immune system of TB cases making their T cells more susceptible to activation-induced cell death [17] with PHA-induced IFN-γ inversely correlating with disease severity [18]. Interestingly, the heat-shock protein, Rv2031c, which was recently used as a post-exposure sub-unit vaccine enteric to *M. avium* infection in cattle [19], induced low responses in our study with the only difference seen for HIV^+ subjects from KPS (LTBI higher than TB). However, protection induced by Rv2031c in cattle was shown to be mediated by antibodies rather than T cells [19], emphasizing the importance for multi-parametric analysis of responses to novel vaccine candidates.

Since the inception of this study, dominant Mtb antigens were reported to be evolutionary hyperconserved [1,20] leading to the suggestion that immunogenicity to these antigens could benefit survival of Mtb. Disparities in responses between sites in our study despite a large cohort, and the relatively few differences seen between TB and LTBI, regardless of HIV status, suggests that new Mtb epitope discovery is required for determining the optimal candidates for

development of novel vaccines [21,22]. Furthermore, underlying genetic influences are clearly playing a role since responses differed considerably between sites despite the use of the same batch of reagents for all assays and adjusting for age, sex and other confounders. The fact we saw few differences between TB and LTBI in this study may be explained partly by the use of a long-term culture assay, which will detect both effector and central memory cells. This may mean we are analysing responses to antigens which were expressed during the asymptomatic stage but which could not prevent progression to active disease in the TB cases. Additionally, we only measured IFN-γ in our study, which is required but not sufficient to protect against TB disease [23]. As such, we are currently assessing levels of cytokines other than IFN-γ in each of the culture supernatants to determine correlates of protection, which may be used for future vaccine efficacy trials.

In conclusion, we have shown the utility of performing large, multi-site studies for TB research. The IFN-γ ELISA is a cheap and useful tool for screening potential antigenicity in subjects across the spectrum of TB and HIV infection. These studies allow critical evaluation of responses to TB antigens in a large cohort of subjects with different TB and HIV status and genetic backgrounds: essential for finally elucidating what constitutes protective immunity to TB.

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Chapter 7

The effect of HIV coinfection, HAART and tuberculosis (TB) treatment on cytokine/chemokine responses to TB antigens in active TB patients and latent TB infected (LTBI) individuals.

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Summary

Identification of *Mtb* specific induced cytokine/chemokine host biomarkers could assist in developing novel diagnostic, prognostic and therapeutic tools for TB.

Levels of IFN-γ, IL-2, IL-17, IL-10, IP-10 and MIP-1α were measured in supernatants of whole blood stimulated with *Mtb* specific fusion protein ESAT-6/CFP-10 using xMAP technology. The study groups were HIV positive TB patients (HIV^+TB^+), HIV negative TB patients (HIV^-TB^+), HIV positive tuberculin skin test positive (TST+) (HIV^+TST^+), HIV negative TST+ (HIV^-TST^+), and HIV^-TST^- individuals.

Compared to HIV^-TST^-, latent TB infection led to increased levels of IP-10, IFN-γ and IL-17, while levels of IL-2 and IP-10 were increased with active TB. Levels of IFN-γ, IL-17, MIP-1α, and IL-10 were increased in HIV^-TST^+ individuals compared to HIV^-TB^+ patients. HIV coinfection decreased the level of IFN-γ, IL-17, IP-10 and IL-2. After six months (M6) of anti-TB treatment (ATT) in HIV^-TB^+ patients, IFN-γ, IL-10, and MIP-1α levels normalized. After M6 and M18 of ATT plus HAART in HIV^+TB^+ patients, levels of MIP-1α and IL-10 normalized, while this was not the case for IFN-γ, IL-2, IL-17, and IP-10 levels. In HIV^+TST^+ patients on HAART, levels of IFN-γ, IL-17, IL-10 and MIP-1α normalized, while no change in the levels of IL-2 and IP-10 were observed.

In conclusion, the simultaneous measurement of IFN-γ, IL-17 and IP-10 may assist in diagnosing LTBI; IL-2 and IP-10 may assist in diagnosing active TB; while IFN-γ, IL-17, MIP-1α, and IL-10 levels could help to discriminate LTBI and active TB. In addition, IL-10 and MIP-1α levels could help to monitor responses to TB treatment and HAART.

1. Introduction

Despite that nearly 20 million lifes have been saved from tuberculosis (TB) associated deaths in the past 17 years, and mortality from TB has decreased by 41% since 1990, TB remains a major health problem particularly in developing countries where 90% of TB incidence and death occurs. In 2011, there were 8.8 million incident TB cases (13% co-infected with HIV) and 1.4 million deaths from TB worldwide [1].

Due to the lack of an effective vaccine, control of TB largely depends on the diagnosis and treatment of active TB [2]. However, Acid Fast Bacilli (AFB) smear sputum microscopy, the gold standard for active TB diagnosis especially in resource limited settings where TB is endemic, has low sensitivity (50 -70%) [3] especially in individuals coinfected with HIV (~ 35%) [4] and children (<50%) [5]. In addition, *Mtb* sputum culture has limitations including the relatively higher costs per test, long time to get results (4-8 weeks) and requirement of higher expertise and laboratory infrastructure [3]. Moreover, the current recommended regimen for treatment of active TB is requiring a minimum of 6 months to complete and is often hampered by non-adherence and drug-related toxicity.

Nonetheless, treatment of asymptomatic latent TB infection (LTBI), which is recommended when the risk of reactivation is high, is a critical strategy to control TB [6]. The role of the century old Tuberculin Skin Test (TST) and the recently emerged IFN-γ release assay (IGRA) is important to diagnose LTBI [7]. However, TST and IGRA have low sensitivity and specificity especially in malnutritioned and HIV infected individuals [8]. The fact that TST and IGRAs are dependent on the detection of a single biomarker, IFN-γ, [9] is the main reason for the lower performance of both TST as well as IGRA [10].

Based on the notion that *Mtb* infection is associated with a spectrum of overlapping clinical conditions which can be poorly separated, there is a need to identify additional biomarkers that correlate with the clinical stages of *Mtb* infection. This could accelerate the development of novel diagnostic and therapeutic tools for both latent and active TB. Furthermore, for better utilization of TB biomarkers in clinical practice, the effect of HIV infection and therapy on the biomarker profile also needs to be investigated [11, 12, 13].

The clinical outcome of *Mtb* infection is determined by a complex interplay of various cytokines (intercellular signaling molecules that regulate the differentiation, proliferation, and activation of immune cells [14]; and chemokines (8–10 kDa cytokines that direct cell migration) [15]. The main cytokines shown to be important in the occurrence, progression and control of TB infection are the pro-inflammatory (INF-γ, TNF-α, IL-12, IL-2), inflammatory (IL-6, IL-17), and immunoregulatory cytokines (IL-10, TGF-β) [16]. A combination of IP-10, IL-2 and TNF-α

[17], and IP-10 and IFN- γ , [18] could be promising biomarkers for active TB diagnosis. IP-10 and MCP-2 were reported to be able to discriminate TB disease from latent infection [19].

Besides that measuring of multiple pro- and anti-inflammatory cytokine/chemokines specific to TB will give insight into the pathogenesis mechanism of *Mtb* infection, it may also provide the opportunity to identify candidate immunologic biomarkers for TB disease and infection. Therefore, we measured the secretion of six cytokines [T helper cell (Th) type 1 (Th1) (INF-γ, IL-12, IL-17), IL-10, and two chemokines (IP-10, MIP-1α)] after 7 day culture of whole blood stimulated with Early Secreted Antigenic Target-6/Culture Filtrate Protein-10 (ESAT-6/CFP-10) antigen. Five clinical groups namely $HIV^{+}TB^{+}$, $HIV^{-}TB^{+}$, $HIV^{+}TST^{+}$, $HIV^{+}TST^{+}$ and $HIV^{-}TST^{-}$ were included in this study.

2. Materials and methods

2.1. Study population and samples

This observational cohort study was performed at St Peter Specialized Referral TB Hospital, Akaki and Kality Health centers, in Addis Ababa, Ethiopia from April 2007-February 2011. Adults (age 18-69 years) of both sexes who were naïve to antiretroviral therapy (ART) and TB treatment were enrolled after informed and written consent was sought.

Diagnosis of active TB was based on both clinical and bacteriological evidence. At least two sputum smears were required to be microscopy positive for Acid Fast Bacilli (AFB) by Ziehl-Neelsen method [20]. Except for TB patients, TST test was performed for all participants by intradermally injecting 2 TU (tuberculinunit) of PPD RT23 (Staten Serum Institut, Copenhagen, Denmark) by Mantoux method. A diameter of skin induration was measured by a trained nurse after 48-72 hours. The cut-off for TST positivity was ≥ 10 mm in HIV un-infected, and ≥ 5 mm in HIV-infected individuals [20].

A total of 79 participants in five clinical groups were included in this study: 1) HIV positive TB patients ($HIV^{+}TB^{+}$, n=26) of whom 14 were on anti-TB treatment (ATT) and were followed-over a six month (M6) period, and 12 were on ATT plus HAART and were followed for 6 and 18 months; 2) HIV negative TB patients ($HIV^{-}TB^{+}$, n=14), all were on ATT and followed for 6 month; 3) HIV positive TST positive individuals ($HIV^{+}TST^{+}$, n=19), all were on HAART and followed for 6 and 18 months; 4) HIV negative TST positive individuals ($HIV^{-}TST^{+}$, n=10), and 5) Healthy Controls without HIV infection, *Mtb* infection and without signs of active TB ($HIV^{-}TST^{-}$, n=10). HAART and ATT were provided to the patients free of charge and according to the national guidelines [20].

At enrolment and during the follow-up visit each participant was interviewed using a standard questionnaire and detailed clinical, anthropometric and demographic data were recorded by a clinician. A total of 20 ml heparinized venous blood was collected and transported to the

Ethiopian Health and Nutrition Research Institute (EHNRI) for laboratory tests. All laboratory tests were performed by automated machines by adhering to the manufacturer's manual. Quantification of absolute counts of **CD4+ T cells** was performed using a FACScan (Becton Dickinson, San Jose, USA). **Plasma HIV RNA** load was determined using the NucliSens EasyQ NASBA diagnostic 2007/1 assay (Organon, Teknika) which quantifies HIV-1 with a linear dynamic range from 50-3,000,000 copies /ml.

2.2. Whole blood stimulation *in vitro*

We adopted a previously established whole blood assay (WBA) and determined cytokines in the supernatants [21]. Whole blood obtained from each participant before and after treatment was diluted 1 in 5 with complete Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, cat R0883) supplemented with 10% Fetal Calf Serum (FCS) (Invitrogen, cat 10106169), and 1% Penicillin/Streptomycin (P/S). Then 100ul of blood/well was transferred in triplicate in round-bottom microtiter plates (Nunclon Surface; NUNC; Roskilde, Denmark) which has been coated with ESAT-6/CFP-10, and Culture Medium (RPMI 1640) as negative Control. The final concentration of ESAT-6/CFP-10 was 10μg/ml. Culture plates were incubated in a 5% CO_2 incubator at 37°C for 7 days, and supernatants were collected and stored at -80 degrees for further analysis. The ESAT-6/CFP-10 fusion protein was produced at the department of Infectious Diseases, Leiden University Medical Center [22].

2.3. Measurement of cytokine/chemokine biomarkers

The level of 4 cytokines (IFN-γ, IL-2, IL-17, IL-10) and two chemokines (IP-10,MIP-1α) was measured in the 7^{th} day whole blood culture supernatant using xMAP technology (Luminex, Austin TX, USA) using a biorad FlexMap3D system (biorad USA). Acquisition was performed using xPonent 4.2 software (Luminex) and data analysis was performed using BioPlex Manager 6.1.1 (biorad)

at the multiplex core facility of the University Medical Center, Utrecht (UMCU), The Netherlands, as described previously [23].

2.4. Data analysis

Initially, the value of each cytokine/chemokine measured after no stimulation (RPMI medium) was subtracted from that of ESAT-6/CFP-10 induced cytokine/chemokines. Nonparametric statistical methods (Wilcoxon signed rank test for paired data, Mann-Whitney u tests for unpaired data, and Kruskal-Wallis tests for data from different groups) were performed to compare differences within and between groups. Spearman's rank correlation analysis was performed between cytokines/chemokines within each patient group. All data analysis was done using Intercooled STATA version 11.0 (College Station, Texas, USA). A p-value of <0.05 was considered significant.

3. Results

3.1. Study population characteristics at baseline

The demographic, clinical and laboratory characteristics of the study populations are shown in **Table 1**. A total of 79 participants were included among which 26 $HIV^{+}TB^{+}$, 14 $HIV^{-}TB^{+}$, 19 $HIV^{+}TST^{+}$, 10 $HIV^{-}TST^{+}$ and 10 Controls ($HIV^{-}TST^{-}$). Mean age of the HIV infected groups ($HIV^{+}TB^{+}$ and $HIV^{+}TST^{+}$) was significantly higher than the HIV negative groups ($HIV^{-}TB^{+}$, $HIV^{-}TST^{+}$, $HIV^{-}TST^{-}$) ($p<0.05$ for all).

At baseline, HIV+TB+ patients had higher HIV RNA (copies/ml) than the $HIV^{+}TST^{+}$ patients; and lower body mass index (BMI) (Kg/m^2) and CD4+ T-cell counts (cells/µl) than $HIV^{+}TST^{+}$, $HIV^{-}TST^{+}$ and Controls, ($p<0.05$ for all). BMI and hemoglobin (Hgb) were lower in $HIV^{+}TB^{+}$ and $HIV^{-}TB^{+}$ patients compared to Controls ($p<0.05$). The majority of $HIV^{+}TST^{+}$ patients (68%) were on D4T/3TC/NVP antiviral regimen and the majority of $HIV^{+}TB^{+}$ patients (57%) were on D4T/3TC/EVZ.

Table 1. Baseline demographic and clinical characteristics of the 5 clinical groups (n=79).

	HIV^+TB^+ (n=26)	**HIV^-TB^+ (n=14)**	**HIV^+TST^+ (n=19)**	**HIV^-TST^+ (n=10)**	**HIV^-TST^- (n=10)**
Age, years	32.7± 7.2	25.3± 8.1[a]	34.6±8.0[b]	26.6± 7.9[a,c]	27.5 ± 8.2
Male, n (%)	14 (50.0)	8 (57.14)	8 (42.1)	6 (60)	4 (40)
BCG scar frequency, n (%), 27/76 (35.5%)	4/23 (17.4%)	5/14 (35.7%)	7/19 (36.9%)	5/10 (50%)	6/10 (60%)
WHO stage, n (%) I+II III+IV	1 (4.2) 23 (95.8)	NA	7 (38.9) 11 (61.1)	NA	NA
BMI (kg/m2)	18.9± 3.3	17.7±2.8	21.6± 3.8[ab]	21.0± 2.5 [ab]	21.0± 2.2 [ab]
Hgb (g/dl)	12.4± 3.7	14.2± 2.3	12.7± 2.3	16.5±2.0 [abc]	17.7± 1.0[ab]
CD4+ count/µl	163±110.7	445±236.3[a]	166.5± 95.5 [b]	901.7± 316.7[ab]	727.8±315.3[ab]
HIV RNA (copies/ml)	93015.0± 172824.4 [a]	67592.3± 140772.5	NA	NA	NA

Data are means ± standard deviations (SD); *n (%)*: Number of patients; BMI: Body mass index; Hgb: Hemoglobin; WHO: World Health Organization; BCG, Bacillus Calmette–Gue´rin; NA: not applicable
a: Comparison with HIV^+TB^+, $p < 0.05$
b: Comparison with HIV^-TB^+, $p < 0.05$
C: Comparison with HIV^-TST^+, $p < 0.05$

3.2. Comparison of cytokine/chemokine responses to *Mtb* antigen at baseline

Comparison of the concentration of each cytokine in response to ESAT-6/CFP-10 stimulation among the five clinical groups is shown in **Fig 1 and Table S2**. Compared to HIV^-TST^- controls, HIV^-TST^+ individuals had elevated IFN-γ (p=0.04), IL-17 (p=0.0007) and IP-10 (p=0.03) levels, while HIV^-TB^+ patients had elevated IL-2 (p=0.03) and IP-10 (p=0.02), but lower IL-10 (p=0.0001) levels. Interestingly, the chemoattractants showed similar expression during TB disease and infection, where IP-10 levels were elevated and MIP-1α level were decreased in both HIV^-TB^+ and HIV^-TST^+ compared to HIV^-TST^- ($p<0.05$ for all) **(Fig 1 & 2)**. We found significantly elevated IFN-γ (p=0.005), IL-17 (p=0.0001), MIP-1α (p=0.009), and IL-10 (p=0.0005) in HIV^-TST^+ individuals compared to HIV^-TB^+ patients **(Fig 1 & 2)**.

3.3. The effect of HIV coinfection on Mtb specific immune response.

We next studied the effect of HIV coinfection on the cytokine/chemokine response to ESAT-6/CFP-10 (**Fig 1 & 3**). Interestingly, HIV infection lead to lower secretion of Th1-derived cytokines (IFN-γ, IL-17, IP-10 and IL-2) in active TB patients (HIV^+TB^+ vs. HIV^-TB^+, p=0.02, 0.02, 0.0001, 0.003, respectively) as well as in LTBI individuals (HIV^+TST^+ vs. HIV^-TST^+; p=0.002, 0.0002, 0.07, 0.01, respectively). The secretion of all cytokines (IFN-γ, IL-17, IL-10, IL-2, IP-10 and MIP-1α) was significantly lower in HIV^+TB^+ patients compared to HIV^+TST^+ patients ($p< 0.05$ for all)

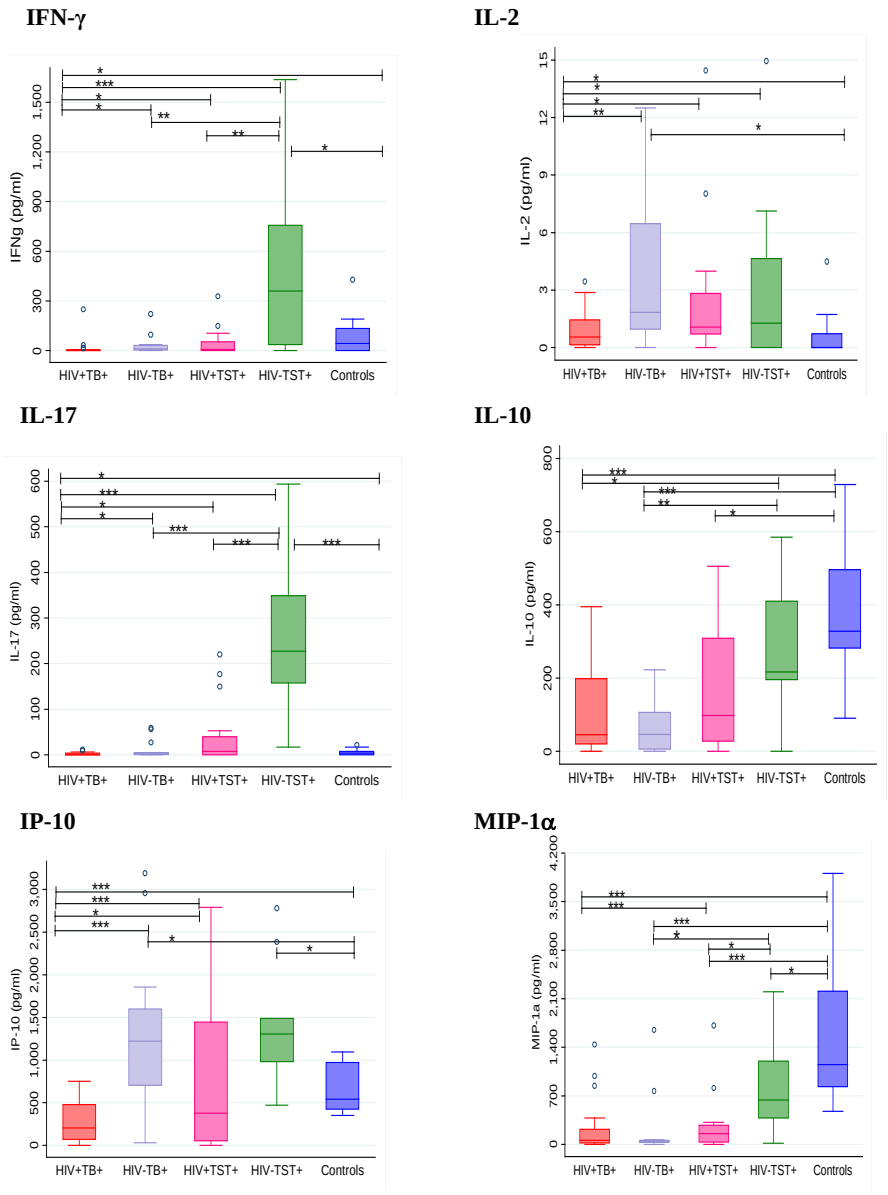

Figure 1. Comparison of cytokine/chemokine concentration among five clinical groups at baseline: HIV+TB+, (n=24); HIV-TB+ (n=14); HIV+TST+ (n=17), n=; HIV-TST+ (n=10), and Controls (HIV-TST-, n=10). Y-axis represents cytokine/chemokine concentrations and X-axis represents the study groups. The concentration of Th1 (IL-2, IFN-γ), Th17 (IL-17), Th2 (IL-10) cytokines, and chemokines (MIP-1α, IP-10) (pg/ml) was measured by Luminex assay from culture supernatants of whole blood stimulated with ESAT-6/CFP-10. In the figure, horizontal lines in the boxes show median values, boxes boundaries representing 25th and 75th percentiles and whiskers represent the highest and lowest values. The responses were compared using a Mann-Whitney U test. P-values of those with significant difference are shown on the horizontal lines. *: $p<0.05$; **: $p<0.01$, ***: $p<0.001$

3.4. Comparison of cytokine/chemokine levels using heat map analysis

Hierarchal cluster analysis of all of the HIV negative participants (TST^+HIV^-, TB^+HIV^-, TST^-HIV^-) displays a distinct pattern of cytokine/chemokine production in each group in response towards ESAT-6/CFP-10. Increased levels of IFN-γ, IL-17, IL-2 and IP-10 are observed in TST^+HIV^- participants, whereas IL-2 and IP10 is increased in TB^+HIV^- participants; and IL-10 and MIP-a in healthy controls (TST^-HIV^-) **(Fig 2A).**

Furthermore, cluster analysis of all of the HIV positive participants (TB^+HIV^+ and HIV^+TST^-), revealed decreased levels of IFN-γ, IL-17, and IL-2 in TB^+HIV^+group **(Fig 2B).**

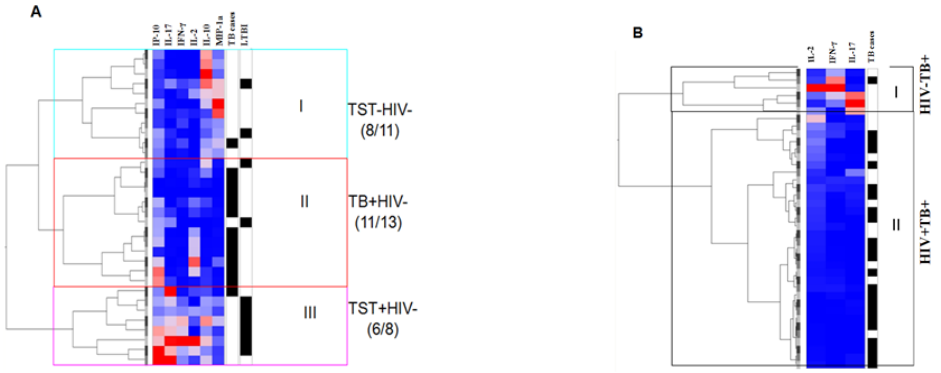

Figure 2: Heat map analysis comparing cytokine levels after stimulating with ESAT-6/CFP-10. A) HIV negative participants: Black box under "TB cases" column = Active TB patients (TB^+HIV^-); Black box under "LTBI" column=tuberculin skin test (TST) positive individuals (HIV^-TST^+); white box under both "TB cases" and "LTBI"=controls (TST^-HIV^-). **B) HIV positive participants:** Black box under "TB cases" column = HIV positive TB patients (TB^+HIV^+), and white box= HIV positive non-TB individuals (TB^-HIV^+).

The individual values are represented as colors. Data was normalized for each parameter individually displaying, highest level in red and lowest level in blue. Symbols I, II and III, indicates clustering of each study participant in response to individual cytokine levels.

3.5. The effect of anti-TB treatment (ATT) and HAART on the cytokine/chemokine responses

Analysis of the cellular immune responses after therapy provides novel opportunities to understand the complex pathogenesis mechanism of *Mtb* infection as well as identify biomarkers

for treatment effect. Therefore, we measured the dynamics of the cytokine response to ESAT-6/CFP-10 in patients who were on ATT and/or HAART (Fig 3 & 4).

In the $HIV^{-}TB^{+}$ patients by M6 of ATT, there was a significant increase in IFN-γ (p=0.005), IL-10 (p=0.003), and MIP-1α (p=0.05), but a decrease in IL-2 (p=0.003) and IP-10 (p=0.02) relative to baseline values, and all reached normal control values (p> 0.45 for all) (Fig 3). However, in the $HIV^{+}TB^{+}$ patients by M6 of ATT without HAART, we found no significant change in the level of Th1 derived cytokine (IFN-γ, IL-2, IL-17and IP-10). However, the level of both IL-10 and MIP-1α increased and reached normal control values (Fig 3).

In $HIV^{+}TB^{+}$ patients by M6 and M18 on combined ATT and HAART, the levels of Th1 derived cytokines (IFN-γ, IL-17, IL-2 and IP-10) remained impaired, while there was an increase in MIP-1α (p=0.05, 0.02) and IL-10 (p=0.05, 0.003) which reached normal values by M18 (Fig 4).

In $HIV^{+}TST^{+}$ patients by M6 and M18 of HAART, there was an increase in IL-10 (p=0.02, 0.008) and MIP-1α (p=0.06, 0.0007) levels, and a decrease in IL-2 (p=0.03, 0.32) and IL-17 (p=0.03, 0.01) levels and all reached normal values by M18. Although there was no significant change in IFN-γ by M6, it sharply increased by M18 (p=0.006).

Of special interest, this study showed a distinctive expression pattern of the chemoattractants (MIP-1α and IP-10) and IL-10 in response to therapy (Fig 3 & 4). Whereas the concentration of both IL-10 and MIP-1α normalized in all the patients by M6 of ATT, ATT plus HAART, and HAART, respectively; there was no significant change in the level of IP-10 in $HIV^{+}TB^{+}$ and $HIV^{+}TST^{+}$ patients on standard therapy, while it reached normal values in the $HIV^{-}TB^{+}$ patients by M6 of ATT.

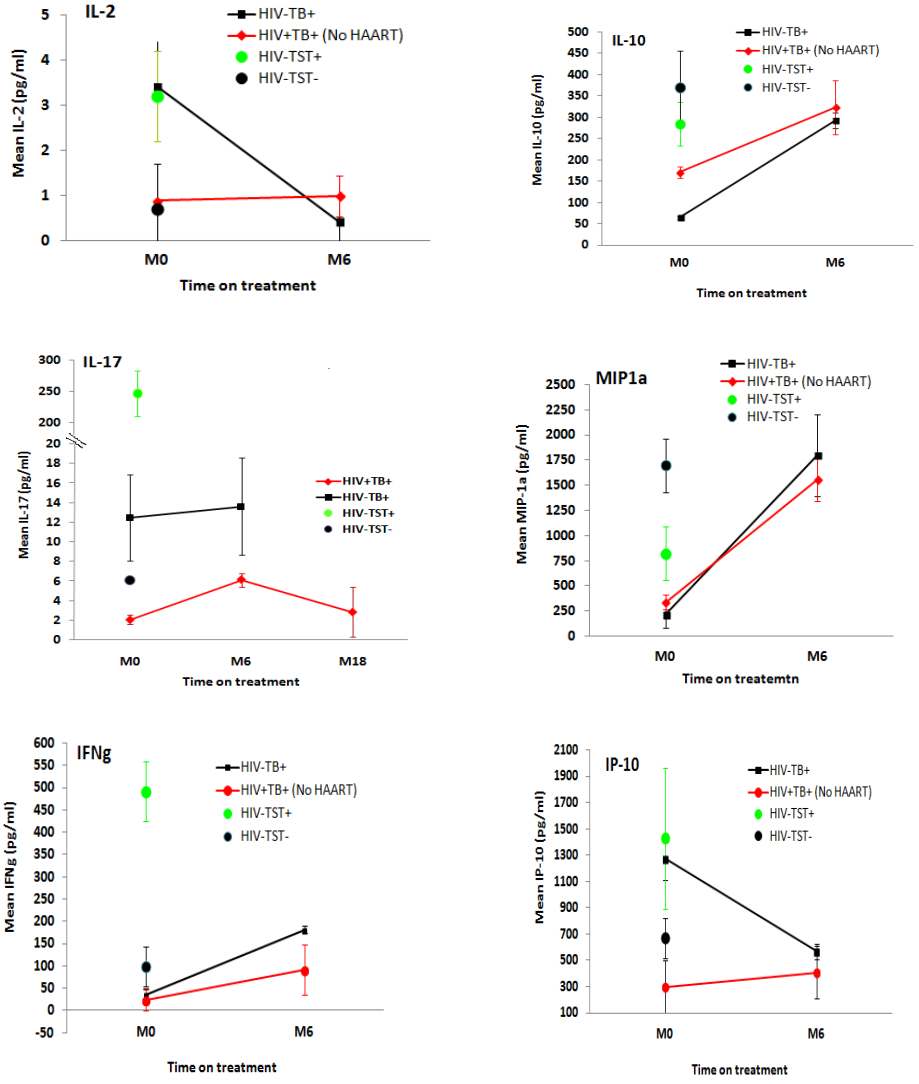

Figure 3. Change in cytokine/chemokine concentrations during TB treatment. HIV negative TB patients on TB treatment (HIV-TB+, n=14) (black line); HIV positive TB patients on TB treatment with no HAART (HIV+TB+, n=12) (red line), HIV-TST+ individuals (n=10) (blue triangle), and HIV-TST- controls ((n=10) (black dot). The level of cytokine/chemokines measured in 7th day culture supernatants of whole blood stimulated with ESAT-6/CFP-10 at baseline (M0), and by six month (M6) and M18 of HAART and/or anti-TB treatment. Months at which cytokines were measured are shown on X-axis, and the cytokine values expressed in mean and standard deviation are shown on Y axis.

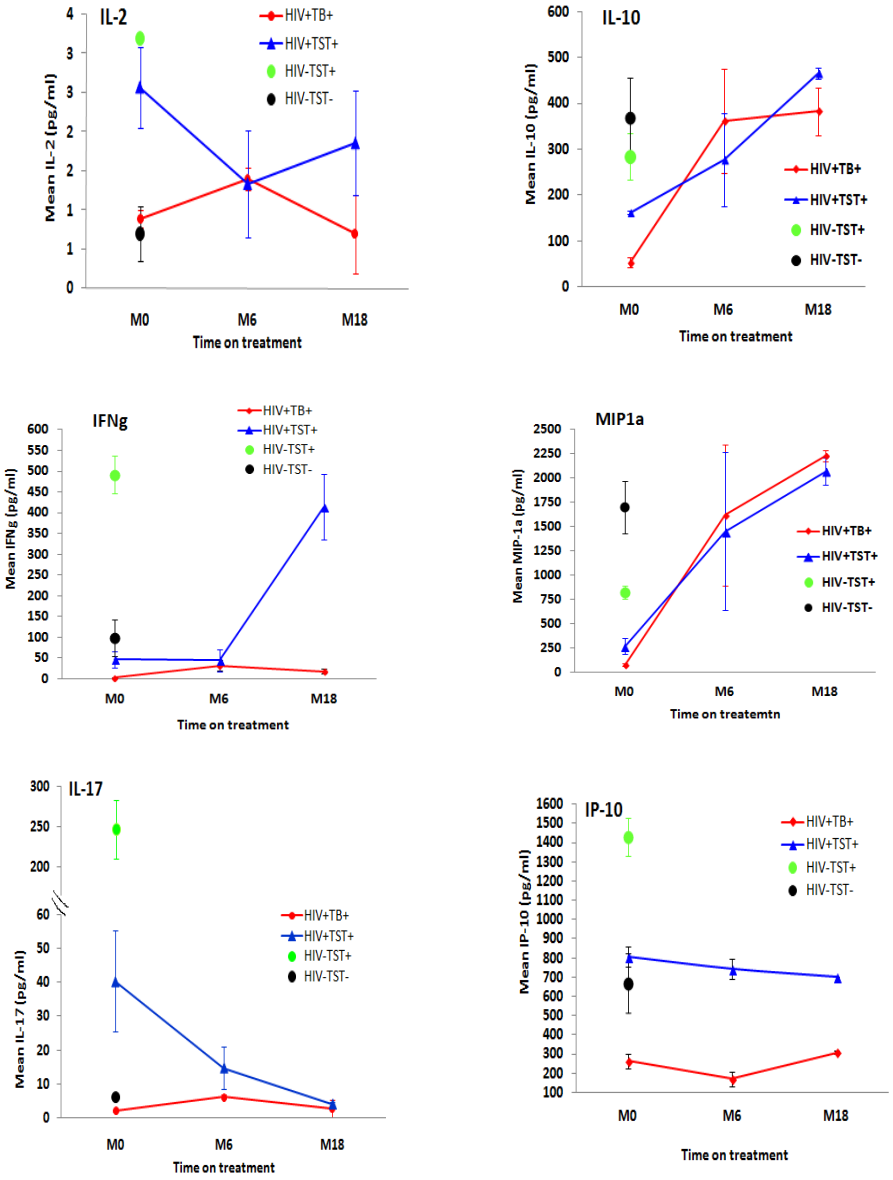

Figure 4. Change in cytokine/chemokine concentrations during HAART and/or TB treatment. HIV positive TB patients on HAART and TB treatment (HIV+TB+, n=12) (red line); HIV infected TST positive groups on HAART (HIV+TST+, n=17) (blue line); HIV-TST+ individuals (n=10) (blue triangle) and HIV-TST- controls (n=10) (black dot). The level of cytokine/chemokines was measured in 7th day culture supernatants of whole blood stimulated with ESAT-6/CFP-10 at baseline (M0), and by six-month (M6) and M18 of HAART and/or anti-TB treatment. Months at which cytokines were measured are shown on X-axis, and the cytokine values expressed in mean and standard deviation are shown on Y-axis.

3.6. The effect of anti-TB treatment (ATT) and HAART on Th1/Th2 ratios overtime

We analyzed the dynamics of Th1/Th2 ratio over time in response to ATT and/or HAART. Interestingly, we found a completely different Th1/Th2 expression in the $HIV^{+}TB^{+}$ versus $HIV^{-}TB^{+}$ patients by M6 of ATT alone. While there was a sharp increase in IFN-γ /IL-10 but a decrease in IL-17/IL-10 and IL-2/IL-10 ratio in $HIV^{-}TB^{+}$ patients (Fig 5D), the reverse was observed in the $HIV^{+}TB^{+}$ patients (Fig 5C).

However, there was an increase in the Th1/Th2 ratio (IFN-γ /IL-10, IL-17/IL-10 and IL-2/IL-10) in the $HIV^{+}TB^{+}$ patients by M6 of ATT and HAART (Fig 5A), as also found in $HIV^{+}TB^{+}$ patients by M6 of ATT without HAART (Fig 5C), which could be associated with the occurrence of Immune Reconstitution Inflammatory Syndrome (IRIS).

In the HIV+TST+ patients on HAART (Fig 5B), we found a decrease in IL-17/IL-10 and IL-2/IL-10 by M6 and M18 (a shift to Th2), but a sharp increase in IFN-γ /IL-10 by M18 (a shift to Th1).

The results suggested that IL-7/IL-10 and IL-2/IL-10 ratios can be used to monitor TB treatment and HAART responses, except in HIV+TB+ patients. IFN-γ /IL-10 ratio behaves differently relative to the IL-17/IL-10 and IL-2/IL-10 ratio in all the clinical groups, which indicates less value of IFN-γ /IL-10 to monitor therapy responses

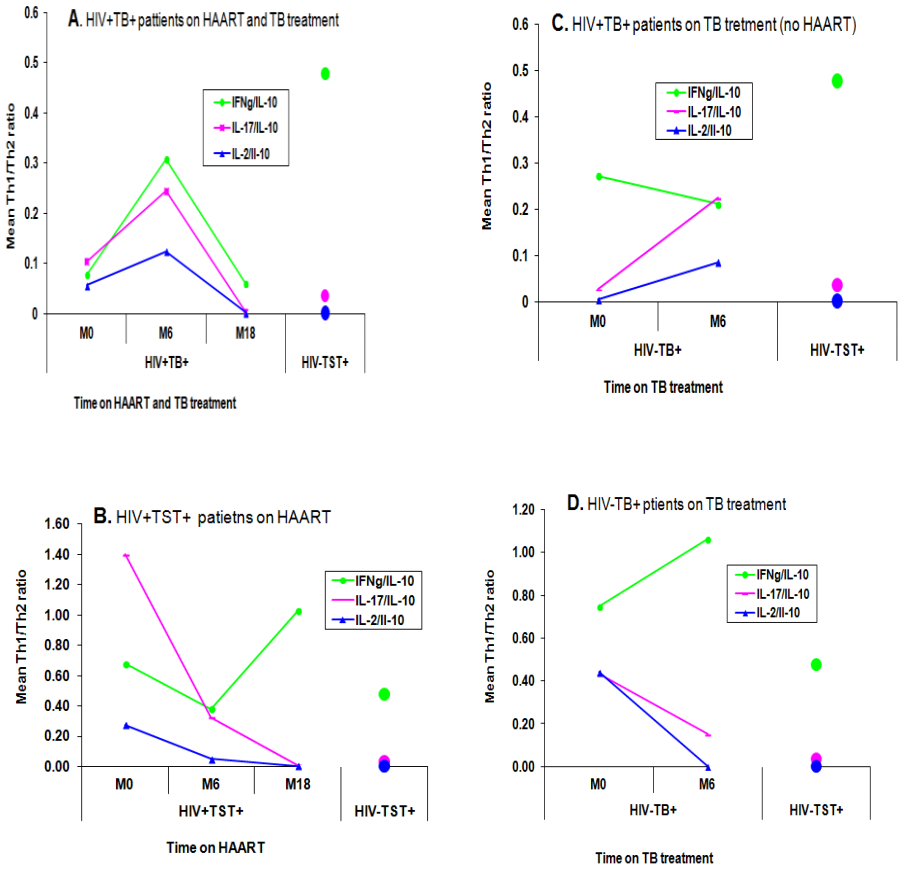

Figure 5. Dynamic changes in the Th1/Th2 ratio during treatment. A. HIV+TB+ (n=11) on HAART and TB treatment; B. HIV+TST+ (n=18) on HAART; C. HIV+TB+ (n=10) on TB treatment with no HAART; and HIV-TB+ (n= 10) on TB treatment. The Th1/Th2 ratio was derived by dividing the concentrations (pg/ml) of Th1 cytokines (IFN-γ, IL-17 and IL) by that of Th2 (IL-10) for each donor and then calculating the mean level in each group. Colored lines represent different Th1/Th2 ratios: IFN-γ /IL-10 ratio, (black line); IL-17/IL-10 ratio, (pink line); IL-2/IL-10 ratio, (Blue line). Y-axis represents mean values of the Th1/Th2 ratios in 7th day culture supernatants of whole blood stimulated with ESAT-6/CFP-10 at baseline (M0), and by six-month (M6) and M18 of HAART and/or TB treatment.

4. Discussion

In this study, the levels of four cytokines (INF-γ, IL-12, IL-17) and two chemokines (MIP-1α, IP-10) with ESAT-6/CFP-10 antigen stimulation were compared in five clinical groups (HIV^+TB^+, HIV^-TB^+, HIV^+TST^+, HIV^-TST^+ and controls (HIV^-TST^+) before and after treatment. As a result, candidate cytokine/chemokine biomarkers that may serve as diagnostic and prognostic markers for TB were identified.

Distinct expression of cytokines in LTBI individuals

The increased expression of Th1derived cytokines (IFN-γ, IP-10 and IL-17) in LTBI individuals (HIV^-TST^+) relative to healthy controls **(Fig 1 & 2)**, indicates that immune cells producing these cytokines are abundantly present in most LTBI individuals [7]. Our results suggest that simultaneous measurement of these cytokine/chemokines could increase the accuracy to predict and diagnose LTBI. To support this, previous studies indicated the value of IP-10, IFN-γ and IL-2 [24], and IP-10 and MCP-2 [19], to diagnose LTBI. Even more, since IP-10 is produced by a variety of cells (neutrophils, monocytes, endothelial cells and fibroblasts) which are less affected by HIV infection [25],detection of IP-10 could improve the sensitivity to diagnose LTBI in populations with HIV infection **[**26,27**]**.

Distinct expression of cytokines in active TB patients

In agreement with other reports [27] active TB patients (HIV^-TB^+) in this study showed elevated expression of IL-2 and IP-10 **(Fig 1 & 2)**, which suggest that combined assessment of these cytokines may assist in the prediction and diagnosis of active TB. Indeed, others showed that combined detection of IP-10 , IFN-γ and MIP-1β 27]; IFN-γ, IP-10 with MIG [27]; IP-10, IL-2 with TNF-α [17], and IP-10 with IFN- γ [18], in response to *Mtb* specific antigens, improved diagnostic performance for active pulmonary TB. However, the secretion of IFN- γ, IL-2 and IP-10 at baseline was suppressed in active TB patients coinfected with HIV **(Fig 1 & 3)**, which could be due to a reduction in the number and functionality of *Mtb* and HIV specific T cells and macrophages due to infection with *Mtb*, HIV or TB/HIV [28]**.** Thus, the value of IFN- γ, IL-2 and IP-10 to diagnose active TB could be compromised during HIV co-infection.

Distinct expression of cytokines between LTBI individuals and active TB patients

Identification of stage specific cellular biomarkers which can accurately discriminate LTBI from active TB could play a significant role in control of TB [8]. In this study, HIV^-TST^+ individuals showed elevated IFN-γ, IL-17, IL-10 and MIP-1α response to ESAT-6/CFP-10 antigen stimulation compared to HIV^-TB^+ patients, which suggests that simultaneous analysis of these cytokines can discriminate LTBI from active TB, which is in agreement to other studies [29, 30].

However, IP-10 appears to be less relevant to distinguish LTBI from active TB in this study as reported by others **[**31].

The distinctive expression of cytokine/chemokines in LTBI and active TB groups in the present study also indicates the role of these cytokine in the pathogenesis of *Mtb* infection. Whereas increased secretion of Th1 cytokines (IFN-γ, IL-17, IP-10) in $HIV^{-}TST^{+}$ individuals may indicate the protective role of the cellular immune response against *Mtb* infection; the increased production of IL-10 in $HIV^{-}TST^{+}$ may indicate the positive effect of IL-10 to counter-act immunopathology [16]. Moreover, whereas increased production of IP-10 in $HIV^{-}TB^{+}$ patients may show ongoing pro-inflammatory response during active TB, the lower production of IFN-γ and IL-17 in $HIV^{-}TB^{+}$ patients may confirm the defective Th1 response accompanying active TB disease [32].

The effect of ATT on cytokine responses in HIV-TB+ patients

IL-10, IFN-γ, IL-2, MIP-1α, and IP-10 all increased to normal values in the $HIV^{-}TB^{+}$ patients by six month of ATT **(Fig. 4)**. Similarly a recent study done in Ethiopia showed that anti TB treatment significantly improves the plasma level of Th1 cytokines and level of chemokines in HIV negative TB patients [33].Thus, our results suggests a role for IL-10, IFN-γ, IL-2, MIP-1α, and IP-10 in the pathogenesis of TB on hand, and their value to monitor TB treatment. Others also showed the value of plasma IP-10 [34] and IFN-γ **(De**, [35] and IP-10 and IFN-γ in response to ESAT-6 and CFP-10 stimulation [36] to monitor TB treatment in HIV negative TB patients.

The effect of ATT and /or HAART on cytokine responses in HIV+TB+ patients

In the $HIV^{+}TB^{+}$ patients by six month of ATT without HAART, except that MIP-1α and IL-10 were normalized, there was no significant change in the restoration of Th1 derived cytokines (IFN-γ, IL-2, IL-17 and IP-10) **(Figs 4)**. Similarly a recent study done in Ethiopia, showed no restoration of the plasma level of Th1 cytokines in HIV positive individuals after anti TB treatment [33]**.** Our results support the scenario of early HAART initiation in TB/HIV patients [1] which could boost optimal *Mtb* specific immune restoration. Nonetheless, we found persistently lower and weaker Th1 derived cytokines in the HIV+TB+ patients on ATT and HAART**,** which is similar to other reports [37]. This could be due to inefficient therapy, lack of treatment compliance**,** [34] and exhaustion of the immune system to produce these Th1 derived cytokines [9].

However, there was a sharp increase in Th1/Th2 ratio in the $HIV^{+}TB^{+}$ patients by six month of ATT and HAART **(Fig 5)**, which is similar to previous reports [37]**.** This might reflect the occurrence of IRIS which can developed within a month to even years after commencing HAART [38]**.** Our results suggest, therefore, the need for diagnosis and management of IRIS in

TB/HIV patients initiated HAART in settings like in Ethiopia where TB and HIV are endemic and access to ART is expanding [39].

The effect of HAART on cytokine/chemokine responses in HIV+TST+ patients

Chronic immune activation, inflammation, and immune dysfunction which cause non-AIDS pathologies including lymphoid fibrosis, cardiovascular diseases, lipoatrophy, and osteoporosis persist despite potent ART. Understanding the dynamics of antigen-specific immune responses in HIV patients on HAART, may assist to identify biomarkers for ART monitoring, as well as to develop effective therapeutic strategies for the non-AIDS comorbdites and for HIV cure [40, 41]. The progressive reduction in the pro-inflammatory Th1 cytokines (IL-2, IL-17, IL-2/IL-10 and IL-17/IL-10), but progressive increase in the anti-inflammatory cytokine (IL-10), and the chemoattractant (MIP-1α) in HIV+TST+ patients **(Fig 5)**, indicates the value of these cytokines to predict HIV disease progression, and also to monitor HAART outcomes in HIV patients. It seems that the Th1 and Th2 response in HIV patients on HAART behave in opposite direction. Others also showed a progressive increase in IL-10 [42]; IL-12 and IFN- γ [43]; but a decrease in IP-10 44] in HIV patients on HAART. Our data indicate that HAART benefits HIV patients not only by inhibiting virus replication but also by adjusting pro-and anti-inflammatory cytokine production. The dynamics of cytokine/chemokines in response to therapy might have long-term implication for progression or regression of the immunological health of HIV patients [43], which strongly suggests the need to improve our understanding of when, where and how modulation of immune activation will be beneficial for HIV patients on HAART.

Of special interest, the level of IL-10 and MIP-1α normalized in HIV+TB+, HIV-TB+ and HIV+TST+ groups on standard therapy **(Fig 6)**, which could indicate the value of both IL-10 and MIP-1α to predict HIV and TB disease progression and to monitor ATT and/or HAART outcomes.

In summary, we showed distinct cytokine/chemokine production in response to ESAT-6/CFP-10 in LTBI and active TB groups. Simultaneous measurement of IFN-γ, IL-17 and IP-10 may assist to diagnose LTBI, while IL-2 and IP-10 may assist to diagnose active TB. Likewise, combined measurement of IFN-γ, IL-17, MIP-1α, and IL-10 may assist to discriminate LTBI from active TB. HAART plus ATT did not restore IFN-γ, IL-17, IP-10 and IL-2 response in $HIV^{+}TB^{+}$ patients. However, HAART adjusts pro-and anti inflammatory cytokine/chemokine production in $HIV^{+}TST^{+}$ patients except IP-10. Combined measurement of IL-2, IFN-γ and IP-10 for $HIV^{-}TB^{+}$ patients; and IL-10 and MIP-1α for $HIV^{-}TB^{+}$, $HIV^{+}TB^{+}$ and $HIV^{+}TST^{+}$ patients, can be useful surrogate biomarkers to monitor therapeutic responses and disease progression. However, further studies should be performed to validate the diagnostic and prognostic value of these

cytokine/chemokine biomarkers by including latent *Mtb* antigens as stimulants; and larger groups of LTBI individuals, extra pulmonary TB patents and children where the need of better diagnostic methods is enormous.

Ethical approval

This study was ethically approved institutionally, by the Scientific and Ethics Review Office (SERO), EPHI; and nationally by the National Health Research Ethics Review Committee (NHRERC), Ethiopian Ministry of Science and Technology Agency (ESTA).

Conflict of interest

The authors declare that they have no financial or personal conflict of interests in this study.

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PART-III

Whole blood host gene expression profiles in latent and active TB individuals

Chapter 8

Differential gene expression of activating Fcγ receptor classifies active TB regardless of HIV status or ethnicity.

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Abstract

New diagnostics and vaccines for tuberculosis (TB) are urgently needed, but require an understanding of the requirements for protection from/susceptibility to TB. Previous studies have used unbiased approaches to determine gene signatures in single-site populations. The present study utilized a targeted approach, reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA), to validate these genes in a multisite study. We analysed ex vivo whole blood RNA from a total of 523 participants across four sub-Saharan countries (Ethiopia, Malawi, South Africa, and The Gambia) with differences in TB and human immunodeficiency virus (HIV) status. We found a number of genes that were expressed at significantly lower levels in participants with active disease than in those with latent TB infection (LTBI), with restoration following successful TB treatment. The most consistent classifier of active disease was FCGR1A (high-affinity IgG Fc receptor 1 (CD64)), which was the only marker expressed at significantly higher levels in participants with active TB than in those with LTBI before treatment regardless of HIV status or genetic background. This is the first study to identify a biomarker for TB that is not affected by HIV status or geo-genetic differences. These data provide valuable clues for understanding TB pathogenesis, and also provide a proof-of-concept for the use of RT-MLPA in rapid and inexpensive validation of unbiased gene expression findings.

Introduction

Mycobacterium tuberculosis (Mtb) complex is the causative agent of tuberculosis (TB). The scientific challenges in understanding immunity to Mtb arise from the observation that, although immune responses are generated after infection, eradication of the bacteria is rare [1]. Instead, host immunity causes Mtb to adopt a clinically silent, latent state of infection in which it is highly resistant to immune attack. Once immunity becomes dysregulated the bacteria can become reactivated [1]. Considering that over 2 billion people live with latent TB infection (LTBI) [2], this population provides an enormous reservoir for potentially new cases of active TB disease. The Mtb life-cycle can be separated into three main stages: latent (dormant), reactivating and active TB. Each stage represents differences in Mtb gene expression and therefore determining the immune response to stage-specific antigens can inform the design of new vaccine candidates [3,4]. For instance, in LTBI, the Mtb DosR regulon is induced by conditions that inhibit aerobic respiration and prevent bacillary eplication and is crucial for rapid resumption of growth by involving resuscitation-promoting factors (rpf) once Mtb exits the hypoxic/anaerobic or nitric oxide-induced non-respiring state [5]. Once reactivation has occurred, the induction of a strong immune response by the host may actually provide further benefit to the bacteria [1–3]: T cell responses to TB antigens have been shown to be significantly higher in active

TB than LTBI [6,7] suggesting that increased immunity may promote lung pathology and subsequently transmission [1]. Indeed, immunogenicity does not necessarily equate to protection, as illustrated by the recent failure of a novel primeboost vaccine, MVA85A, to protect children against TB [8], despite a proven antigen-specific T cell response [9]. Thus, more information is required to understand what constitutes protective immunity to TB and in turn to inform new vaccine design strategies. Our consortium previously reported responses to 51 DosR antigens in latently infected HIV-subjects from Uganda, South Africa and The Gambia with Rv1733c being the most commonly recognised antigen [10]. However, whilst similarities between sites were observed, there were also significant differences between the populations. Another study of South African subjects showed that responses to rpf were significantly higher in TB cases compared to household contacts (HHC) but values were minute compared to responses to dominant antigens such as ESAT-6/CFP-10 fusion protein (EC) [11] and comprised a mixture of uninfected and latently infected HHC. Building on these preliminary findings, the present study analysed T cell responses to 23 Mtb antigens in a total of 1247 subjects with different HIV and TB status across five geographically diverse sites in Africa (South Africa, The Gambia, Ethiopia, Malawi and Uganda).

Methods

Participants and study sites

The study design for GC6-74 has been previously described [4]. Sites included: The Gambia (Medical Research Council Unit; MRC), Ethiopia (Ethiopian Health and Nutrition Research Institute; EHNRI), Malawi (Karonga Prevention Study; KPS) and South Africa (Stellenbosch University; SUN). Participants were considered for inclusion if they were >18 years of age, had no concurrent infections and were willing to undergo an HIV test. Participants without TB were recruited from households of TB patients (MRC, SUN) or by random community selection and from HIV care clinics (KPS, EHNRI). All participants underwent a clinical assessment, including a screen for malaria (except SUN) and inter-current illness and chest x-ray. Tuberculin skin tests (TST; 2 tuberculin units; TU, PPD RT23, SSI, Denmark) were performed in order to further classify the HIV^- participants without active disease into TST^+ and TST^-. Due to the influence of prior BCG vaccination and environmental mycobacteria in TB-endemic settings, HIV^- participants with skin test induration of ≥10mm diameter were categorized as TST^+. TB cases were confirmed by sputum culture (BACTEC™, Becton-Dickinson, USA). If BACTEC was not available, culture on Lowenstein-Jensen solid media was performed (KPS, EHNRI). TB treatment consisted of a 2- month intensive phase with rifampicin, pyrazinamid, isoniazid and ethambutol followed by a 4-month continuation phase with only isoniazid and rifampicin. Following informed consent, PAXgene blood RNA tubes (Qiagen, USA) were collected for subsequent RNA isolation. Follow-up assessment at 6 months from treatment initiation was performed for all participants. Ethical approval was obtained from local ethics committees and also from the London School of Hygiene and Tropical Medicine (LSHTM) for KPS participants.

Reverse transcription MLPA

RNA was isolated from PAXgene tubes using extraction kits according to manufacturer's instructions (Qiagen, USA). Dual color RT-MLPA was performed [22, 23] with several major modifications, including probe-primer design for 45 genes of interest [21]. Briefly, 100–150 ng RNA was reverse transcribed using gene-specific RT primers and MMLV reverse transcriptase. This was denatured and hybridized overnight at 60°C with a SALSA probe mix (MRC Holland, The Netherlands). After treating the samples with ligase-65 (MRC-Holland, The Netherlands) for 15 min at 54°C, PCR amplification was performed with specific SALSA FAM- or HEX-labeled MAPH primers (2 μM each, forward primer 5'-GGCCGCGGGAATTCGATT-3' and reverse primer 5'-GCCGCGAATTCACTAGTG-3'), 13.75 μL H20 and 0.25 μL SALSA polymerase [21]. PCR conditions were 33 cycles of 30s at 95°C, 30s at 58°C and 60s at 72°C, followed by 1 cycle of 20 min at 72°C. PCR products were then diluted 1:10 in HiDi formamide

containing 400 HD ROX size standards and analysed on an ABI PRISM 3730 capillary sequencer (Applied Biosystems, UK). Data were analysed using GeneMapper software (Applied Biosystems, UK) and peak areas were exported to a Microsoft Excel file. Sample- and peak-related differences in PCR and electrophoresis efficiency were corrected by adjusting to GAPDH housekeeping gene. Signals below the threshold value for noise cut-off (peak area ≤ 200) were adjusted accordingly. A positive control that encompassed the combined target-specific sequences of the left- and right-hand half-probes was used for all runs. Detailed descriptions of the genes analysed are shown in **Supplementary Table 1**.

Statistical Analysis

Due to differences in the sequencing machines used, it was necessary to normalize and standardize the data from all sites so participants could be grouped together for final analyses. This involved normalization over GAPDH and discarding any samples where the number of genes was below the required amount for accurate analysis. This differed per site and was determined based on the average value GAPDH at each site. Mann-Whitney U-test was used for analysis of participants at each site and values adjusted using Bonferroni correction for multiple comparisons. Kruskal-Wallis followed by Dunn's post-test comparison was used for determining differences between TB cases and TST^+ and TST^- contacts. Logistic regression and receiver-operator curve (ROC) analyses were performed to determine which parameters best discriminate between TB cases and controls. Analyses were performed using Graphpad Prism 5.04 (Software MacKiev, USA) and SPSSv19 (IBM, USA) and adjusted for age and sex.

Results

Analysis of gene expression relative to TB and HIV status

Gene expression levels were analysed for individual sites, and also collated for analysis of total HIV-negative and HIV-positive participants. Some genes showed baseline expression in all participants, and were excluded from the final analyses. These were generally cytokine-related genes, including IL4, IL4D, IFNG, TNFA, and IL2. Detailed descriptions of the final 33 genes analysed are given in Table S2. Many genes were expressed at low levels (Figs 1 and 2), including CXCL10 and the lactotrans-ferrin gene (LTF), but their expression tended to be higher in Gambian participants than in South African or Malawian HIV-negative participants. For HIV-positive participants, low expression of CD163, CCL19 and RAB13 was seen at all sites, for all participants (Fig. 2). High expression of SEC14L1, FPR1, TGFBR2 and TNFRSF1A was seen for HIV-negative participants at all sites, regardless of TB status (Fig. 2). For HIV-positive participants, all sites showed high expression of SEC14L1, TIMP2, CD3E, and TNFRSF1A (Fig. 2). There were also some notable differences between sites, with participants from Malawi and South Africa, but not from The Gambia, showing significantly different expression of several

genes in HIV-neg-ative TB cases and vice versa. These included CD8, SEC14L1, TGFB1, TGFBR2, and CASP8; all had significantly lower expres-sion in participants with active TB than in contacts from South Africa and Malawi, but no difference was seen between the groups in The Gambia (Fig. 1). Interestingly, median CCR7 expression levels were significantly lower in TB cases (3483) than in contacts (6494) from South Africa, but were signifi-cantly higher in cases (11091) than in contacts (200) from Malawi (again, the difference was not significant for The Gambia; Fig. 1). Conversely, LTF expression was significantly higher and RAB13 expression was significantly lower in cases than in contacts in The Gambia, but no differences were seen for either in South Africa or Malawi (Fig. 1).

Gene	SUN TB^+	SUN TB^-	p-value	KPS TB^+	KPS TB^-	p-value	MRC TB^+	MRC TB^-	p-value
CD8A	1729	2624	0.0022	3721	7780	0.0061	1494	1515	ns
CXCL10	200	200	ns	200	200	ns	249	217	ns
CD4	5742	6933	ns	4765	6918	<0.0001	4623	5680	0.0004
BLR1	1340	3218	<0.0001	200	200	0.0002	911	1774	<0.0001
SEC14L1	15191	21633	0.0023	11112	17952	<0.0001	19224	19931	ns
TIMP2	20583	23673	ns	18239	19358	ns	18340	16476	ns
CCL19	200	200	ns	200	200	ns	566.5	532	ns
FPR1	17627	13547	ns	9553	8536	ns	22881	17075	ns
NCAM1	200	200	ns	200	200	ns	985.5	1087	ns
FOXP3	200	1808	ns	2290	200	ns	2598	3428	ns
TGFBR2	4127	5622	0.0049	3087	4743	<0.001	5400	6085	ns
IL7R	12932	25678	<0.0001	6897	24426	<0.0001	10695	17518	<0.0001
BCL2	3905	6560	<0.0001	2230	5196	<0.0001	2011	2961	0.0032
CASP8	18164	22558	0.0088	13124	17343	<0.0001	11675	13217	ns
BPI	200	200	ns	200	200	ns	1134	948	ns
CCR7	3483	6494	<0.0001	11091	200	<0.0001	13922	16220	ns
CCL22	200	200	ns	200	200	ns	477	400	ns
MMP9	1477	200	ns	200	200	ns	859	414	ns
RAB24	200	200	ns	2022	200	ns	3238	3088	ns
LTF	200	200	ns	200	200	ns	404	200	0.0008
CD163	200	200	ns	200	200	ns	1003	1168	ns
TGFB1	3599	4197	0.0153	5486	6733	<0.0001	7017	7261	ns
TNFRSF1B	200	200	ns	2184	3039	ns	4116	4434	ns
FCGR1A	4880	200	<0.0001	3551	200	<0.0001	2501	947	<0.0001
TNFRSF1A	22275	22756	ns	18264	17239	ns	15798	15195	ns
RAB13	200	200	ns	200	200	ns	527	1063	0.0004
CD3E	15550	31909	<0.0001	13052	31409	<0.0001	13552	20815	<0.0001

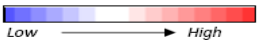

FIG. 1. Differential gene expression values in human immunodeficiency virus (HIV)-negative participants from South Africa (SUN), Stellenbosch University; Malawi (KPS), Karonga Prevention Study; The Gambia (MRC), Medical Research Council Unit. RT-MLPA was performed on ex vivo RNA extracted from tuberculosis cases (TB^+) and household or community controls (TB^-). Median values are shown. Blue indicates relatively low expression and red indicates relatively high expression. Data were analysed with Mann–Whitney U-test for each site; p-values are indicated; NS, not significant.

Differential BLR1, FCGR1A, CD3E and IL7R expression levels were seen between TB cases and controls, regardless of HIV status (Fig. 2 (all sites) and Fig. 3). There were also some genes that only showed differences in HIV-negative participants, including apoptotic marker genes (BCL2 and CASP8), CD4, regulatory T-cell marker genes (FOXP3, TGFB1, and TGFBR2) and intracellular trafficking marker genes (RAB13, RAB24, and SEC14L1). Similarly, some differences were only associated with HIV-positive participants, including the chemokine marker genes CXCL10 and CCL22, and the innate immunity marker genes LTF

and MMP9 (Fig. 2). Owing to significant differences between sites with regard to CD4 counts at the time of recruitment of HIV-positive participants, we adjusted for CD4 counts by using linear regression, and found the expression of all genes to be significantly different between HIV-positive cases and controls, both with site-specific analysis and with grouped analysis (data not shown). The only marker whose expression was consistently higher in active disease than in controls was FCGR1A, which was significantly different regardless of HIV status or site, even with unadjusted CD4 counts (Figs 1–3).

Gene	SUN TB^+	SUN TB^-	p-value	KPS TB^+	KPS TB^-	p-value	EHNRI TB^+	EHNRI TB^-	p-value	HIV^- All sites	HIV^+ All sites
CD8A	3257	4581	0.0067	4390	200	0.0177	4513	2634	0.0129	ns	ns
CXCL10	2225	200	0.0022	1794	200	ns	2094	2889	0.0295	ns	<0.0001
CD4	5066	4658	ns	7357	7221	ns	967	536	ns	<0.0001	ns
BLR1	1470	1733	ns	200	200	ns	1031	836	ns	<0.0001	0.0065
SEC14L1	19341	14555	ns	9864	12949	ns	13551	12516	ns	<0.0001	ns
TIMP2	34046	18727	0.0012	19323	19437	ns	7395	14718	0.0005	ns	ns
CCL19	200	200	ns	200	200	ns	200	370	ns	ns	ns
FPR1	18341	12425	0.0061	11287	7984	ns	8708	13960	0.0064	ns	0.0002
NCAM1	200	200	ns	200	200	ns	2765	2501	ns	ns	ns
FOXP3	200	1810	0.0013	200	200	ns	200	200	ns	0.0061	ns
TGFBR2	5078	3962	ns	2997	4015	ns	1522	1459	ns	<0.0001	ns
IL7R	6868	9420	ns	4107	9049	0.0009	2477	1224	ns	<0.0001	0.0003
BCL2	4520	4035	ns	1932	3063	0.0031	483	382.5	ns	<0.0001	ns
CASP8	20041	20605	ns	12960	14632	ns	5091	5281	ns	<0.0001	ns
BPI	4033	200	ns	200	200	ns	200	200	ns	ns	ns
CCR7	2126	4522	ns	200	200	ns	5727	4923	ns	ns	ns
CCL22	200	200	ns	200	200	ns	757	1891	<0.0001	ns	<0.0001
MMP9	2415	200	0.0013	200	200	ns	200	491	0.0025	ns	<0.0001
RAB24	3025	200	<0.0001	3505	3017	ns	200	200	ns	<0.0001	ns
LTF	2053	200	ns	200	200	ns	200	200	ns	ns	0.0084
CD163	200	200	ns	200	200	ns	200	200	ns	ns	0.0143
TGFB1	4439	4068	ns	5209	5518	ns	2719	3056	ns	<0.0001	ns
TNFRSF1B	2564	200	ns	1964	3442	<0.0001	1645	2558	ns	ns	ns
FCGR1A	5127	200	<0.0001	2589	200	0.0028	200	1751	<0.0001	<0.0001	<0.0001
TNFRSF1A	24487	19283	ns	13639	11979	0.0014	5052	8198	0.0006	ns	ns
RAB13	200	200	ns	200	200	ns	200	200	ns	<0.0001	ns
CD3E	17660	30658	0.0003	16199	22950	0.0043	6028	3996	ns	<0.0001	<0.0001

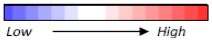

FIG. 2. Differential gene expression values in human immunodeficiency virus (HIV)-positive participants per site and total HIV-negative and HIV-positive participants (all sites grouped). RT-MLPA was performed on ex vivo RNA extracted from tuberculosis cases (TB^+) and household or community controls (TB^-). Median values are shown. Blue indicates relatively low expression and red indicates relatively high expression. Data were analysed with Mann–Whitney U-test for each site and for total HIV-negative and HIV-positive participants (all sites combined); p-values are indicated; EHNRI, Ethiopian Health and Nutrition Research Institute; SUN, Stellenbosch University; KPS, Karonga Prevention Study; NS, not significant.

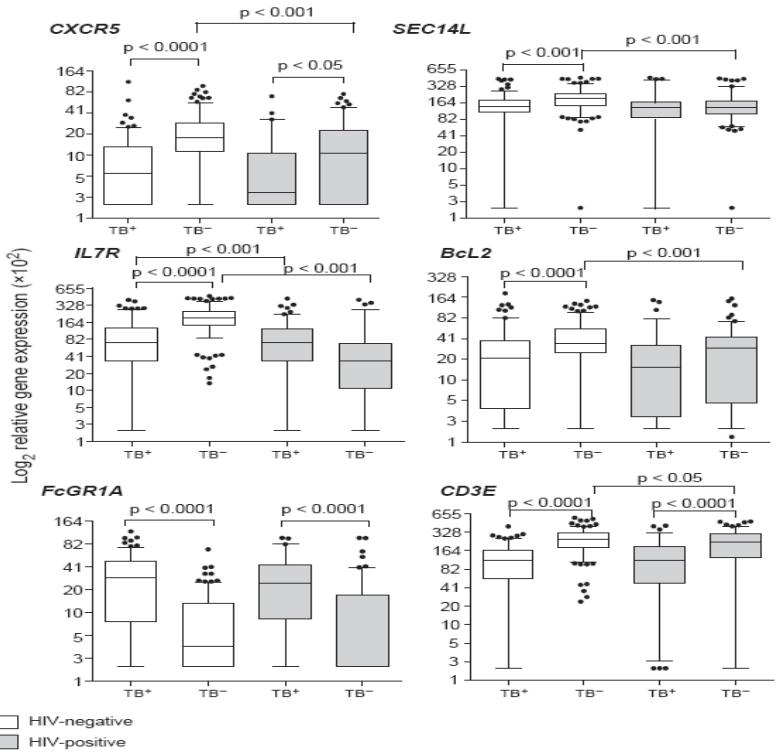

FIG. 3. Reverse transcriptase multiplex ligation-dependent probe amplification was performed on ex vivo RNA extracted from tuberculosis cases (TB$^+$) and household or community controls (TB$^-$) at four sites in Africa. Data ara presented from a total of 141 human immunodeficiency virus (HIV)-negative TB-positive, 195 HIV-negative TB-positive, 74 HIV-positive TB-positive and 120 HIV-positive TB-negative participants. Line indicates median, whiskers indicate 5–95% range, and dots indicate outliers. Data were analysed with the Kruskal–Wallis test followed by Dunn's post-test comparison.

Gene expression differences between participants with active TB disease, participants with LTBI (TST-positive), and non-infected (TST-negative) healthy controls

When TST-positive (LTBI) and TST-negative healthy controls were separated and compared with participants with active disease (data available only for HIV-negative participants), a similar pattern emerged (Table 1). TB cases had significantly lower expression of BCL2, CASP8, CXCR5, CD3, CD4, CD8, IL7R, TGFB1, TNFRSF1B, TGFBR2 and SEC14L1 but significantly higher expressionof FCGR1A thanparticipantswithLTBI(TST-positive) (Table 1). Participants with LTBI had significantly higher expression of BCL2 and CASP8 than TST-negative participants. However,expressionlevelsof CCL22, CD163, NCAM1 and CD19 were all significantly lower in TST-positive participants than in TST-negative contacts (Table 1). The majority of markers had significantly lower expression in TB cases than in TST-positive

participants, except for FCGR1A, which had a median expression level of 4200 in TB cases as compared with 200 in TST-positive and TST-negative contacts (p <0.001; Table 1). The expression levels of all markers were restored to LTBI levels following successful TB treatment (data not shown).

TABLE 1. Analysis of gene expression variation with different tuberculosis infection and disease states for human immunode-ficiency virus-negative participants (median (interquartile range) 3 10^2)

Gene	TB $n = 99$	TST$^+$ HHC $n = 91$	TST HHC $n = 49$	TB vs. TST p-value	TB vs. TST$^+$ p-value	TST vs. TST$^+$ p-value
BCL2	29 (19–42)	5 (30–71)	33 (26–46)	NS	<0.001	<0.01
CASP8	145 (106–186)	180 (141–224)	145 (120–179)	NS	<0.001	<0.01
TNFRSF1B	22 (2–39)	31 (2–39)	36 (30–48)	<0.001	NS	NS
CXCR5	2 (2–15)	24 (12–41)	19 (2–27)	<0.001	<0.001	NS
CCL22	2 (2–2)	2 (2–2)	2 (2–5)	NS	NS	<0.01
CD163	2 (2–11)	2 (2–10)	9 (2–14)	NS	NS	<0.01
NCAM1	2 (2–8)	2 (2–9)	9 (2–17)	<0.001	NS	<0.01
CD19	2 (2–2)	2 (2–5)	6 (2–11)	<0.001	NS	<0.001
FCGR1A	42 (23–61)	2 (2–13)	2 (2–7)	<0.001	<0.001	NS
CD3	140 (105–197)	294 (220–351)	249 (190–308)	<0.001	<0.001	NS
CD4	53 (31–67)	65 (52–83)	65 (53–81)	<0.01	<0.001	NS
CD8A	18 (2–36)	26 (13–74)	19 (2–41)	NS	<0.01	NS
IL7R	102 (62–139)	227 (172–278)	191 (143–256)	<0.001	<0.001	NS
FOXP3	21 (2–26)	26 (2–43)	3 (2–26)	NS	NS	NS
TGFB1	49 (40–60)	64 (45–73)	65 (56–74)	<0.001	<0.001	NS
TGFBR2	39 (21–54)	55 (44–65)	54 (45–64)	<0.001	<0.001	NS
RAB13	2 (2–2)	2 (2–7)	7 (2–12)	<0.001	NS	NS
SEC14L1	147 (109–207)	197 (154–244)	212 (156–239)	<0.001	<0.001	NS

NS, not significant; TB, participants with active tuberculosis disease; TST$^+$ HHC, tuberculin skin test-positive household contacts; TST HHC, tuberculin skin test-negative household contacts.
Data are shown for the most differentially expressed genes.
Data were analysed with a Kruskal–Wallis test followed by Dunn's post-test comparison.

Classification of active TB

We performed receiver operator curve analysis to determine which marker resulted in the best specificity and sensitivity for classification of active TB. For HIV-positive participants, the best classifier was FCG1RA (area under the curve (AUC) = 0.75), followed by CXCL10 (AUC = 0.73) and CD3E (AUC = 0.70), and this did not change when we adjusted for CD4 counts (Fig. 4, left panel); however, controls were a mixture of TST-positive and TST-negative. For HIV-negative participants, classification into active TB disease or LTBI (only TST-positive participants) was much better: CD3E correctly classified 89% of participants (AUC = 0.89), followed by IL7R (AUC = 0.88) and FCGR1A (AUC = 0.87) (Fig. 4, right panel). Multiple logistic regression analysis revealed that a combination of FCGR1A, BCL2 and IL7R could correctly classify 91% of HIV-negative participants into active TB disease or LTBI. The success rate was much lower for HIV-positive participants, with FCGR1A, CD3 and CXCL10 resulting in a 75% classification of TB with all sites combined, with or without adjustment for CD4 counts.

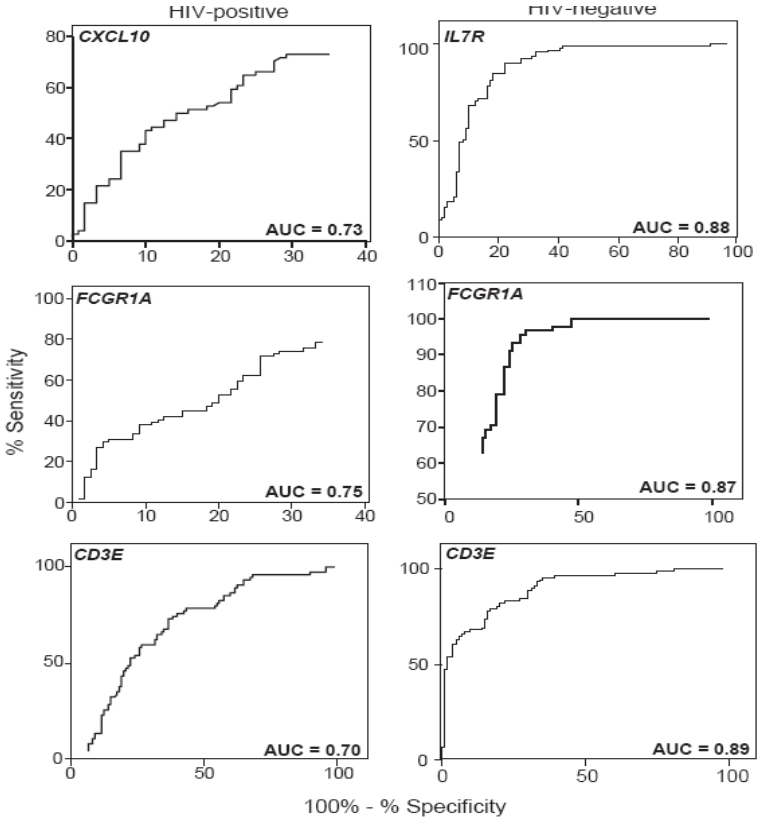

FIG. 4. Reverse transcriptase multiplex ligation-dependent probe amplification was performed on ex vivo RNA extracted from tuberculosis (TB) cases abd tuberculin skin test (TST)-positive household or community controls (HCCs) from four sites in Africa. Data are presented from a total of 99 TB cases and 91 TST-positive HHCs (all HIV-negative). Data were analyzed using reviever operator curves, and senditivity and specificity are shown fot IL7R, FCGR1a and CD3e. AUC: area under the curve

Discussion

The ability to determine natural protective immunity to TB requires multisite studies, owing to the relatively low rate of progression from latent infection to active TB, and the influence of HIV co-infection and genetics on the outcome of the immune response. The Bill & Melinda Gates Foundation Grand Challenge 6 was designed with this in mind: it was a large, multisite project that incorporated all infectious states of TB (TST-negative, TST-positive and active disease, pretreat-ment and post-treatment) in HIV-positive and HIV-negative participants from seven sites in Africa (four included in this study), providing a unique opportunity to identify

biomarkers for protection against TB that could be targeted for vaccine efficacy, diagnostics and treatment outcome studies.

In this study, we used RT-MLPA to analyse ex vivo whole blood RNA with a specific set of gene targets that are known to be involved in or affected by TB pathogenesis. We show that this approach is feasible for identifying differential gene expres-sion across different geographical sites, across HIV and TB infection phenotypes, and in larger participant groups than are currently practicable, through unbiased transcriptomic analysis. This is important, because the majority of previous studies have been based on HIV-negative participants from one site only.

There were a number of genes in HIV-negative participants that showed distinct differences between the index cases and their household contacts regardless of the study site (and therefore underlying genetic make-up), particularly genes encoding T-cell markers (CD3 and IL7R), BCL2 (apoptotic regulator), CXCR5 (chemokine), FCGR1A (CD64) (high-affinity IgG receptor), and SEC14L (Golgi marker). There were also some notable differences between sites, with participants from Malawi and South Africa showing significant reductions in expression levels of genes in TB cases that were not observed in The Gambia and vice versa. For instance, the expression of CD8, which encodes the characteristic marker of the T-cell population with cytolytic functions, was signif-icantly lower in TB cases from South Africa and Malawi but not The Gambia, whereas RAB13 and LTF expression levels were different between cases and controls in The Gambia but not in South Africa or Malawi. LTF regulates immune responses to a number of infections by modulating functions of antigen-presenting cells [24], and has recently been shown to boost bacille Calmette–Gu_erin vaccine responses when used as an adjuvant [25]. The differences between sites may be attributable to the selection criteria for the control group, but is more probably attributable to underlying genetics, as participants from The Gambia appeared to diverge more than participants from South Africa and Malawi. Pathogen strain and lineage differences may also be relevant, particularly as Mycobacterium africanum, which appears to be less virulent than M. tuberculosis [26], is prevalent only in West Africa.

Fewer genes showed consistent results across sites in HIV-positive participants, with CD14, FCGR1A and CD8A being the three main markers that differed between TB cases and controlsforallsites.When results were combined from all sites, 14 genes showed a difference between cases and controls in HIV-negative participants and ten in HIV-positive participants. These were quite distinct, with HIV-negative participants being more likely to show differences in secretory genes and regulatory T-cellgenes(i.e. FOXP3 and CTLA4),andHIV-positiveparticipants showing most differences in chemokine markers and innate immune markers. Four genes showed significant differences in both HIV-negative and HIV-positive (all sites combined) partic-ipants, including BLR1, IL7R, FCGR1A, and CD3E. Importantly, when

TB cases vs. TST-positive HHCs (LTBI) were analysed, a similar pattern of markers emerged, with the addition of CASP8 (apoptotic regulator), CD4, CD8, TGFB1, and SEC14L1.

Interestingly, whereas the majority of markers had signifi-cantly lower expression in TB cases than in controls, FCGR1A was the only marker whose expression was consistently and significantly increased in TB cases, regardless of HIV status, showing that identification of universal biomarkers is achievable. KEGG pathway analysis has revealed that FCGR1A is functionally associated with immunoregulatory genes, particularly with apoptotic regulators and proinflammatory regulators involved in the JAK–STAT pathway [20]. Our data are in line with previous findings in South African participants, showing a significant reduction in FCGR1A expression following treatment for TB [27]. FCGR1A is mainly expressed by monocytes and macrophages, is induced by interferon-c, and plays a central role in antibody-dependent cytotoxicity and the clearance of immune complexes. It also helps to control Mtb via stimulation of the respiratory burst in intracellular mononuclear phagocytes [28]. These findings require further validation, but our results indicate the likely importance of FCGR1A in TB pathogenesis regardless of the immune status of the host.

In conclusion, the findings from this study incorporated within the GC6-74 consortium have provided valuable clues for understanding TB pathogenesis, and also provides a proof-of-concept for the use of RT-MLPA in rapid and inexpen-sive validation of unbiased gene expression findings.

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Chapter 9

Discriminative expression of whole blood genes in HIV patients with latent and active TB in Ethiopia

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Abstract

Background: Transcriptomic host biomarkers could assist to develop effective diagnostics and therapeutics for TB. However, different biomarkers may be discriminatory in different populations depending on the host and bacilli genetics, and host immune reactivity due to HIV infection.

Methods: the expression levels of 45 mRNA genes were analyzed using Reverse Transcriptase Multiplex Ligation Probe Amplification (RT-MLPA) assay in whole blood of 106 HIV positive individuals who were either active TB patients (TB^+HIV^+, n=29), tuberculin skin test positive(TST+) (TST^+HIV^+, n=26), or tuberculin skin test negative (TST^+) (TST^-HIV^+ (n=51) individuals.

Results: Although there was no gene that show significant difference in expression level between HIV infected individuals with and without latent TB infection (TST^+HIV^+ vs. TST^-HIV^+), 16 genes (CCL22, CCL19, CXCL10, TNFRSF1A, IL2RA, FCGR1A, TIMP-2, MMP9, FPR1, LTF, CD14, IL7R, SEC14L1, CD4, CD8A and CD3E) showed distinct expression between TB^+HIV^+ and TST^-HIV^+ participants ($p<0.05$ for all). Furthermore, 9 genes were differentially expressed between TB^+HIV^+ and TST^+HIV^+ participants , of which 7 genes (CCL22, TNFRSF1A, FCGR1A, TIMP-2, MMP-9, LTF and CD14) had a higher expression in the TB^+HIV^+ patients, while the levels of IL-7R and CD8A were higher in the TST^+HIV^+ participants ($p<0.05$ for all). More importantly, hierarchical cluster analysis of the data from all the 106 study participants revealed distinct expression of FCR1a, TIMP-2, IL-7R and CD8A in TB^+HIV^+ patients compared to both TST^+HIV^+ and TST^-HIV^+ participants.

Conclusions: We showed that four genes (FCGR1A, TIMP-2, IL-7R and CD8A) have the potential to discriminate active TB in HIV patients in Ethiopia. Further elucidating of these genes could help to develop better diagnostic tools for active TB in HIV patients, and also to understand the pathogenesis of TB/HIV coinfection.

Introduction

Tuberculosis (TB) remains a major global health threat next to HIV/AIDS **(WHO, 2010)**. Factors which impair the success of TB control include lack of effective TB diagnostics (1); lack of an effective vaccine (2); the emergence of multi- and extensively drug-resistant TB (MDR- and XDR-TB), HIV infection (2); limitations in anti-TB drugs and therapeutic regime (3); social and economic factors (3); and the nature of the spread of *Mtb* infection and the complex host immune response (4).

TB biomarkers which can accurately predict reactivation of latent TB infection (LTBI), TB cure, eradication of latent TB, efficiency of vaccine and drug therapy are important for development of effective diagnostic and therapeutic tools for TB. The discovery of specific and universal biomarkers for TB, therefore, is a priority of TB research **(**5, 6**)**. Biomarkers can be host and pathogen specific and can be determined at cellular, protein or transcript level (7).

Several transcriptome studies have identified host gene signatures that are able to classify active from latent TB, including gene markers expressed by mononuclear cells (8), regulatory T cells (9), B and T cells, and innate immunity pathways **(**10**).** However, there are no universal biomarkers that can be utilized in the TB eradication program. The existence of at least six *Mtb* lineages that have co-evolved with and adapted to human populations **(**11), which may result in host resistance in specific human populations **(**12**)**, suggests that different biomarkers may be discriminatory in different populations depending on the underlying *Mtb* epidemiology. Likewise, population-based differences in immune reactivity due to immune deviation by coinfections such as HIV and malaria may impact biomarker signatures in the local TB setting. Therefore, biomarker signatures for TB need to be investigated and validated across geographically and ethnically diverse populations (13, 14, 15).

In line with this, in a previous study, the expression pattern of 45 host genes implicated in TB pathogenesis was investigated in four sub-Saharan countries including Ethiopia, Malawi, South Africa, and The Gambia **(**16**)**. The study revealed differential expression of BLR1, FCGR1A, CD3E and IL7R between TB cases and community controls in all sites regardless of HIV infection. More interestingly, FCGR1A was the most significant biomarker to classify active TB (16). Furthermore, the study identified 11 genes (SEC14L1, Caspase8, CXCR5, FCGR1A, CD3, CD4, CD8A, IL7R, TGFB1, TGFBR2, BCL2) which were differently expressed between HIV negative active TB patients (**$TB^{+}HIV^{-}$)** and LTBI individuals (**$HIV^{-}TST^{+}$**) (16). Here we extend this previous study by including HIV infected individuals with latent and active TB, and aim to identify host gene markers that have discriminative potential for active TB infection in HIV infected individuals.

2. Materials and methods

Study population

This cross-sectional study was performed from April 2007-January 2009 in Addis Ababa, Ethiopia. The study participants were adults (age 15-60 years) of both sexes and included HIV positive active TB patients (TB^+HIV^+), HIV positive tuberculin skin test positive (TST+) (TST^+HIV^+), and TST^-HIV^+ individuals. The participants were naïve for Highly Active Anti-Retroviral Treatment (HAART) and anti-tuberculosis treatment. Cutaneous test for tuberculin (PPD RT23, SSI, Copenhagen, Denmark) for the non TB cases, and TB diagnosis were performed at the study site according to the national protocol (17).

Laboratory Testing

Twenty ml heparinized venous blood was collected from all subjects at diagnosis and processed at the National HIV Referral Laboratory of the Ethiopian public health institute (EPHI), Addis Ababa. Laboratory examinations including blood CD4+ T cell counts and Plasma HIV RNA load were determined using BD FACSCalibur (Becton Dickinson, San Jose, USA), and NucliSens EasyQ NASBA diagnostic 2007/1 (Organon, Teknika), respectively, according to manufactures instruction.

Reverse transcriptase Multiplex ligation-dependent probe amplification (RT-MLP)

RNA was extracted from 2.5 ml whole blood using PAXgene tubes and extraction kits according to the manufacturer's instructions (Qiagen). The expression level of 45 genes of interest that are associated with the host immune response components against *Mtb* infection including innate immunity, T-cell immunity and production of cytokines/chemokines (18,19) was determined using RT-MLPA as previously described (10, 16). Detailed characteristics of the panel of host mRNA genes included in this study was described previously (16).

Statistical data analysis

A descriptive analysis of the level of expression of each gene was conducted. Results were compared using Mann-Whitney U test. The level of significance was at $P<0.05$ and all P values were two –tailed. All the analyses were performed using Intercooled STATA version 10.0 (College Station, Texas, USA).

3. Results

3.1. Demographic and clinical characteristics of the study participants

A total of 106 participants (29 TB^+HIV^+, 26 TST^+HIV^+, and 51 TST^-HIV^+) were included in this study. Mean (standard deviation, SD) age of the study participants was 31.6 (8.6) years, and 59 (39.6%) were males. At diagnosis, the median CD4+ T cell count was lower in the TB^+HIV^+ patients compared to TST^+HIV^+ and TST^-HIV^+ groups ($p<0.001$ for all). HIV plasma RNA level was higher in TB^+HIV^+ patients compared to TST^-HIV^+ ($p=0.02$) and TST^+HIV^+ individuals ($p=0.01$). There was no significance difference in HIV plasma RNA load between TST^-HIV^+ and TST^+HIV^+ patients (data not shown).

3.2. Comparison of gene expression among the clinical groups

Of the 45 host genes analyzed, six genes including MARCO, IL22RA1, BPI, CCL13, TNFRSF18, IL-4, were not expressed at all in the study groups and were excluded from further analysis.

Between HIV infected individuals with and without active TB (**TB^+HIV^+ vs. TST^-HIV^+**),16 genes were differently expressed, of which 11 genes (CCL22, CCL19, CXCL10, TNFRSF1A, IL2RA, FCGR1A,TIMP-2, MMP9, FPR1, LTF, and CD14) were expressed at a significantly higher level in the **TB^+HIV^+** participants and 5 genes (IL7R, SEC14L1, CD4, CD8A and CD3E) were expressed at a higher level in the **TST^-HIV^+** participants ($p<0.05$ for all) **(Table 1)**.

Furthermore, between HIV+ individuals with active TB and LTBI **(TB^+HIV^+ vs. TST^+HIV^+)**, 9 genes were differently expressed, of which 7 genes (CCL22, TNFRSF1A, FCGR1A, TIMP-2, MMP-9, LTF and CD14) were expressed at a higher level in the TB^+HIV^+ patients, while the expression level of two genes (IL-7R and CD8A) was higher in the TST^+HIV^+ participants ($p<0.05$ for all) **(Table 1)**.

Interestingly, between HIV^+ individuals with and without LTBI **(TST^+HIV^+ vs. TST^-HIV^+)**, there were no genes differentially expressed**(Table 1)**.

We next analyzed the gene expression pattern among all the three clinical groups **(TB^+HIV^+, TST^+HIV^+ and TST^-HIV^+)** using Kriskal-Walls test. Interestingly, levels of 6 genes (CCL22, CXCL10, TNFRSF1A, FCGR1A, TIMP-2, and LTF) were elevated in **TB^+HIV^+** patients , and levels of 3 genes (CD3E, CD8A and IL-7R) were elevated in **TST^-HIV^+** individuals (**Table 1 and Fig 1).**

Table 1. Analysis of gene expression in HIV infected individuals with latent and active TB

Gene	TB^+HIV^+	TST^+HIV^+	TST^-HIV^+	TB^+HIV^+ vs. TST^-HIV^+ p-value	TB^+HIV^+ vs. TST^+HIV^+ p-value	TST^+HIV^+ vs. TST^-HIV^+ p-value
CCL22	693.5±297.1	396.4 ±329.94	341.3± 318.1	**<0.0001**	**0.001**	NS
CCL19	220 ± 201.2	131.8±186.2	133.6.6± 163.8	0.02	NS	NS
CXCL10	3656.9±3954.0	2456.1±2967.2	2037.1±2278.6	0.02	NS	NS
TNFRSF1A	3316.2 ± 1319.4	2131.3 ± 1113.9	2352.5±1405.2	**0.001**	**0.0009**	NS
IL7R	1330.4±1117.6	2281.4±2003.4	2582.9±1953.4	**0.001**	**0.03**	NS
IL2RA	265.1± 275.3	218.9±259.8	137.7± 226.5	0.02	NS	NS
SEC14L1	8148.5±2343.7	9139.6± 2358.0	9512.4± 3207.3	0.04	NS	NS
FCGR1A	707.3 ± 355.3	376.8 ± 510.7	221.6± 312.0	**<0.0001**	**0.006**	NS
TIMP-2	9869.0 ± 4374.4	6650.6 ± 3651.8	6929.7±4149.0	**0.003**	**0.004**	NS
MMP9	418.8 ± 576.6	176.1 ± 463.9	149.6±317.6	**0.01**	**0.007**	NS
CD4	494.1±473.4	745.8±665.3	833.0±844.6	0.04	NS	NS
CD3E	2241.7±1799.5	3209.2± 2788.3	3345.9±2424.2	0.02	NS	NS
CD8A	1994.3 ± 1043.2	2901.9 ± 1604.1	3998.5±2854.6	**<0.0001**	**0.01**	**0.07**
FPR1	10666.9±5048.6	8891.8± 5209.4	8784.8±6222.2	**0.03**	NS	NS
LTF	182.3± 355.5	13.4± 67.2	67.5± 187.5	**0.02**	**0.001**	NS
CD14	2173.8±1124.5	1682.8± 1694.7	1501.5±1281.5	**0.003**	**0.01**	NS

Data is shown only for genes that show significant difference in expression level among the three clinical groups (TB^+HIV^+, TST^+HIV^+ and TST^-HIV^+). Data was tested with Kriskal-Walls test followed by Mann-Whitney U test; data is shown as mean±standard deviation; NS: Not significant.

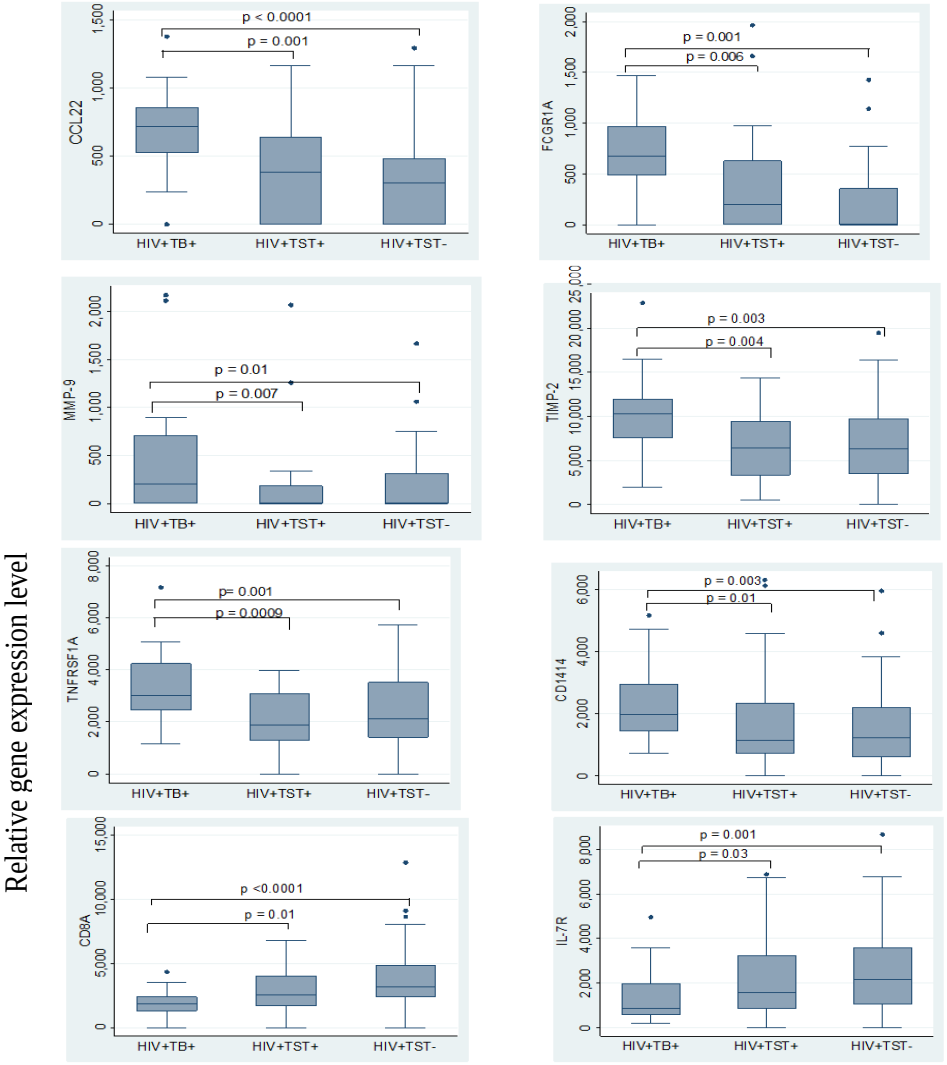

Figure 1. Host mRNA genes which showed distinct expression among HIV infected individuals with latent and active TB and with no TB. **$TB^{+}HIV^{+}$**, HIV positive active TB patients; **$TST^{+}HIV^{+}$**, HIV positive tuberculin skin test positive (TST+) participants; **$TST^{-}HIV^{+}$**, HIV positive TST negative participants**.** RT-MLPA was done on RNA extracted from whole blood from each study participant. Bar plots indicate mean expression values of the host mRNA genes. Horizontal lines in the boxes show median values, boxes boundaries representing 25th and 75th percentiles and whiskers represent the highest and lowest values. The responses were compared using a Mann-Whitney U test. P-values are shown on the horizontal lines.

3.3. Analysis of gene expression using heat map analysis

Hierarchical cluster analysis of the data from HIV positive participants with and without active TB (TB^+HIV^+ vs. TST^-HIV^+) displayed a distinct expression of 13 genes between the groups **(Fig 2A)**. However, using the most stringent power of analysis (t test pooled variance), the expression of four genes (FCR1a, CCL22, TIMP-2 and TNFRSF1A) was significantly increased in the TB^+HIV^+ patients , while levels of IL-7R and CD8A were increased in TST^-HIV^+ patients **(Fig 2B).**

Likewise, cluster analysis of the data from TB^+HIV^+ and TST^+HIV^+ participants, showed increased expression of FCR1a and TIMP-2, but decreased levels of IL-7R and CD8A in the TB^+HIV^+ patients**(Fig 2C).**

Since no difference in gene expression was revealed in the cluster analysis of HIV infected participants with and without LTBI (TST^+HIV^+ vs. TST^-HIV^+), we merged the TST^+HIV^+ and TST^-HIV^+ data and compared these with the data from the TB^+HIV^+ patients. Again, the result showed increased expression of FCR1a and TIMP-2, but decreased levels of IL-7R and CD8A in the TB^+HIV^+ patients **(Fig 2C).** Together, the results indicated the potential of these four genes (FCR1a, TIMP-2, IL-7R and CD8A) to discriminate active TB during HIV coinfection.

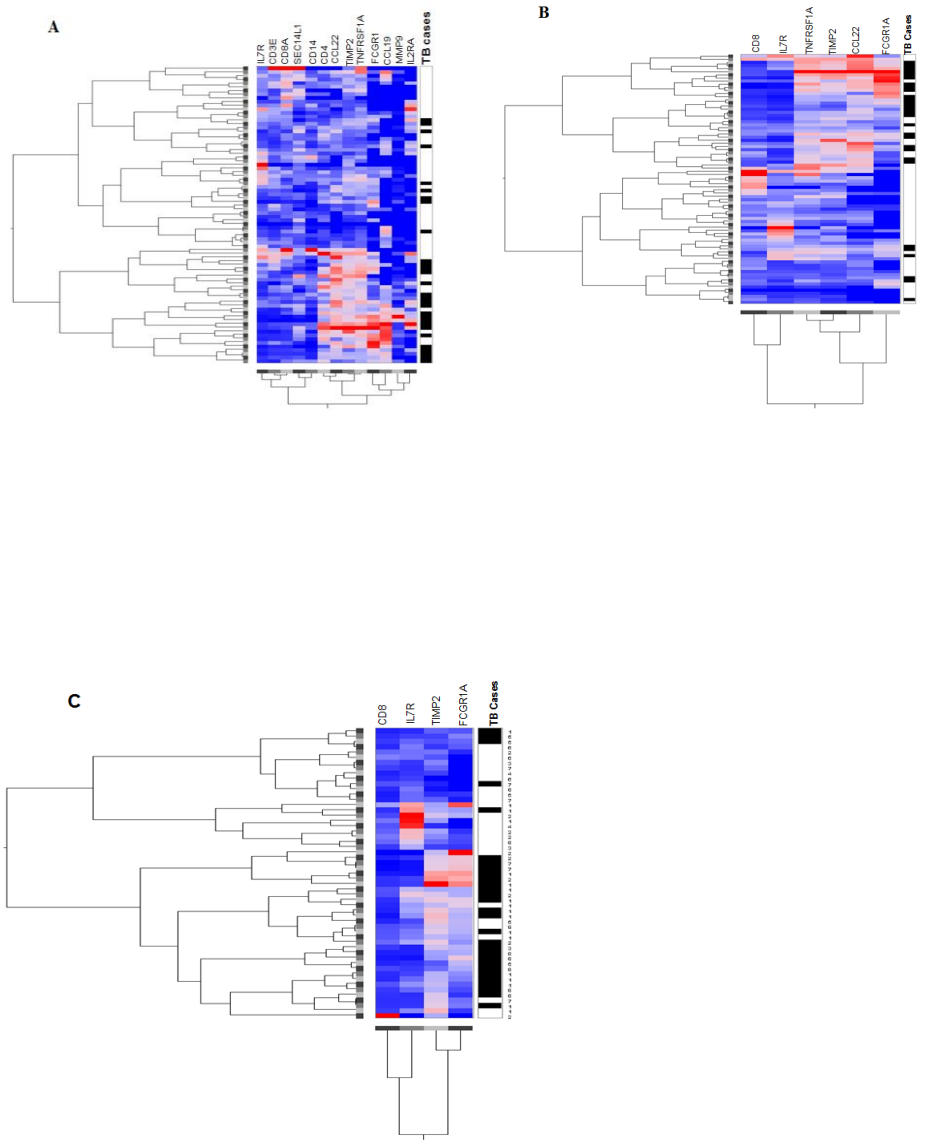

Figure 2: Heat map analysis of gene expression between HIV positive active TB cases (TB^+HIV^+), HIV positive tuberculin skin test positive (TST+) (TST^+HIV^+), and TST^-HIV^+ participants. A) Comparison between TB^+HIV^+ (black box), and TST^-HIV^+ (white box), using Mann-Whitney U-tests; **B)** Comparison between TB^+HIV^+ (black box), and TST^-HIV^+ (white box), using a stringent testing (t test pooled variance); **C)** Comparison between TB^+HIV^+ (black box), and TST^+HIV^+ (white box) participants, using Mann-Whitney U-tests

3.4. Correlation of gene expression levels with CD4 cell count and plasma HIV RNA level

As shown in **Fig 3** , of all the 39 host genes analyzed, 4 genes including MMP9,TIMP2, and LTF showed a negative correlation with absolute CD4+ T cell counts, but a positive correlation with plasma HIV RNA level (copies/ml) in the $TB^{+}HIV^{+}$ patients. On the other hand, only SEC14L1 gene expression negative correlated with CD4+ T cell counts (r= -0.30; p=0.03) while it positively correlated with plasma viral load (r= - 0.41; p=0.004) in the HIV patients with no TB (TST+HIV+ and TST-HIV+) (Data not shown).

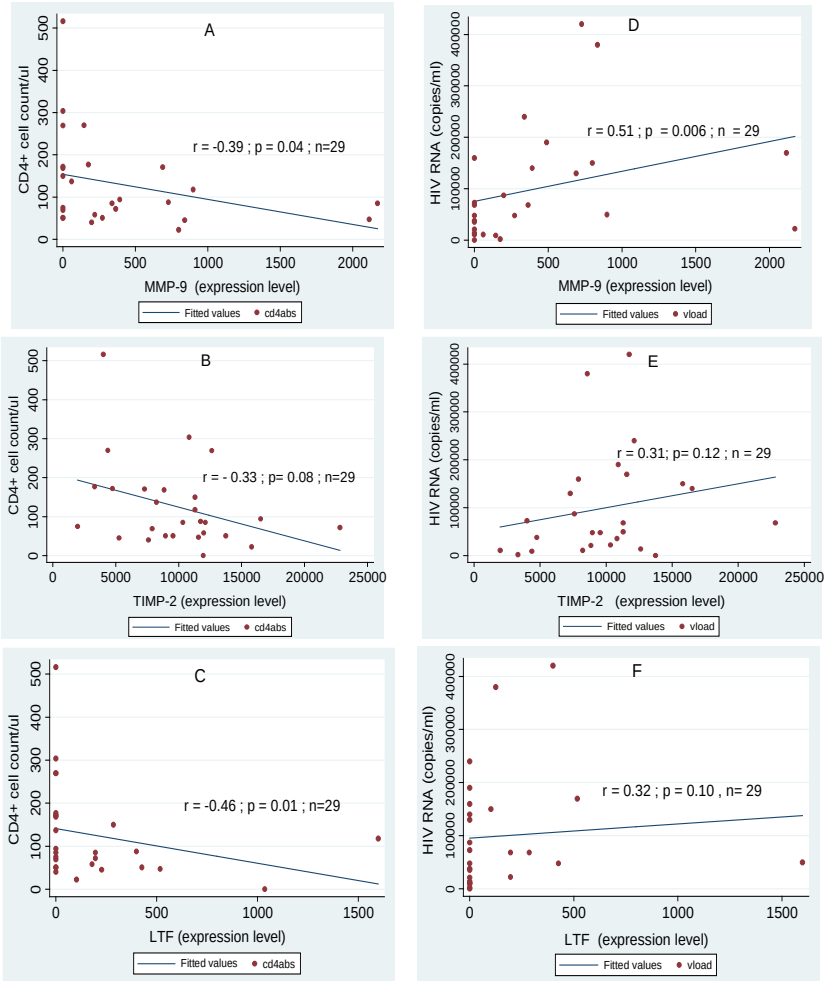

Figure-3. Correlation analysis between the expression level of the genes, MMP-9 (A), TIMP2 (B) and LTF (C), and absolute CD4+ counts (cells/ul) (A,B,C) and plasma HIV RNA level (copies/ml) (D,EF) in HIV+TB+ patients. A linear regression line is included: *r and p values (Spearman's correlation test) are shown.*

4. Discussion

Understanding host gene expression pattern in HIV patients with latent and active TB could help to identify candidate gene biomarkers for TB, as well as to understand the complex pathogenesis of TB and TB/HIV coinfection (20**,**10**)**.

We identified 16 genes that showed distinct expression between HIV patients with and without active TB (TB^+HIV^+ vs. TST^-HIV^+), of which the levels of 11 genes (CCL22, CCL19, CXCL10, NFRSF1A, IL2RA, FCGR1A,TIMP-2, MMP9, FPR1, LTF, and CD14) was significantly elevated in TB^+HIV^+ patients. A previous study also has shown differential expression of FCGR1a, IL7R, BLR1 and CD3E between HIV infected TB cases and community controls (16).

More importantly, in depth comparison of gene expression using heat map analysis, showed differential expression of four genes (FCGR1A,TIMP-2, IL-7R, and CD8A) in TB^+HIV^+ patients compared to the other HIV infected groups (TST^-HIV^+ and TST^+HIV^+), which strongly indicates the value of these genes to discriminate active TB in HIV infected individuals. A study performed in four Sub Saharan African countries (Ethiopia, Malawi, South Africa, and The Gambia) also showed that the potential of FcγRs/ FCGR1A to discriminate active TB patients from latently infected individuals irrespective of HIV infection and ethnicity (16). Others also reported the ability of FCGR1B, FCGR1A/CD64, and LTF (9**)** and FCGR1A, LTF, RAB13 (10) to discriminate active from latent TB**.**

Furthermore, the findings of our study may give insight into the host-bacilli-viral interrelation and TB/HIV pathogenesis. Several studies have shown association between expression level of host immune genes and pulmonary TB in Africa **(**21, 22**)**.

The higher expression patterns of genes in HIV patients with active TB **(Table 1)**, may indicate the influence of actively dividing bacilli (bacilli antigen load) to trigger host gene expression. In support of this, a rapid change in blood gene expression profile which correlates with the clearance of the actively dividing bacilli in TB patients on therapy was reported (23).

Furthermore, the higher expression level of specific host mRNA genes in HIV patients with active TB, may indicate the activation of cellular immune responses in an attempt to control the *Mtb* infection (24 Oh, *et al*., 2004), or the mechanism by which *Mtb* infection increases the progression of asymptomatic HIV infection to AIDS (25). The distinct expression of four immune genes (FCGR1A, TIMP-2, IL-7R, and CD8A) in particular in HIV patients with active TB may indicate, the role of these genes in the pathogenesis of TB during coinfection with HIV.

Lower expression of the genes associated with T cells (CD3E, CD8, and CD4) in TB^+HIV^+ patients could be associated with the depletion of T cells from the periphery blood due to HIV infection and may indicate the mechanisms by which HIV infection aggravates TB disease progression.

Humoral immune response is one of the host defense mechanisms against *Mtb* infection (26). In the present study the levels of the B cell associated gene, the high-affinity Fc receptors (FcRs) for immunoglobulin (Igs) (FCGR1A/CD64), and the Matrix metalloproteinases (MMPs) members (TIMP2, MMP9), were elevated in $TB^{+}HIV^{+}$ patients **(Table 1and Fig 1)**.

FcγRs, which are expressed solely by innate cells, binds the Fc portion of Igs and modulates immunity (27 ,28). Studies showed that FcγRs regulate immune activation and susceptibility during *Mtb* infection by divergently affecting cytokine productions (27). On the other hand, MMPs, which are secreted by monocytes, macrophages and activated lymphocytes, are known to degrade extracellular matrix such as collagen and proteoglycans (29). It has been shown that infection of monocytes and macrophages with *Mtb in vitro* and *in vivo* induces MMP9 secretion (30). The involvement of large amount of MMP-9 and TIMP-2 in the pathogenesis and pathological changes of PTB is also reported **(**31,32**)**. In support to this, we observed a negative correlation of both MMP-9 and TIMP-2 expression with CD4+ T cell counts, but a positive correlation with plasma HIV RNA level in $TB^{+}HIV^{+}$ patients **(Fig 5)**. Together, our findings suggest further elucidating of the FCGR1A, MMP-9 and TIMP-2 in TB/HIV patients may provide new insights to the pathogenesis of TB, and to develop effective therapeutic and diagnostic tools for TB in HIV patients.

T regulatory cells (Tregs) play a crucial role in the maintenance of self-tolerance and prevention of excessive responses against infection (33). In this study, IL-7R and IL-2RA were suppressed in HIV patients with active TB compared to those infected with latent TB, as reported by others (34,16). However, whether this could be beneficial for the host or for the pathogen needs further investigation.

Apoptosis of macrophages is an effective host strategy to control intracellular pathogen mycobacteria (35). TNFR1 and TNFR2 are receptors for tumor necrosis factor-α (TNF-α) which is known to modulate apoptosis of infected macrophages (35). Association of TNFR1 and TNFR2 with Pulmonary TB in Africa (36) has been reported. In agreement to others (37), we found higher expression of TNFRSF1A in $TB^{+}HIV^{+}$ patients **(Table 1)**. Although this needs further investigation, it may be beneficial to the host as it would be associated with diminished pathogen viability (38).

Chemokines play a protective role against *Mtb* infection by assisting cell migration and subsequently granuloma formation (19). CCL22 is a chemotactic factor for Th2 cells (33), and for Tregs into the pleural space of patients with tuberculous pleurisy (39). Thus, CCL22 could negatively modulate Th1 polarization and aggravates TB disease progression (40). CCL9 which is the major beta-chemokine is known to facilitate the transport of *Mtb* from the lung to the lymph node via DCs; the accumulation of antigen-specific IFN-γ producing T cells in the lung;

and the development of the granuloma and control of mycobacteria (41). Therefore, the higher expression level of CL22 and CCL9 in $TB^{+}HIV^{+}$ patients in the present study, strongly indicate the role of CL22 and CCL9 in the pathogenesis of TB during HIV infection.

In summary, we identified several genes from different families of host mRNA genes that are differentially expressed in HIV patients with active and latent TB. More importantly, this study showed distinctive and elevated expression of four mRNA genes (FCGR1A, TIMP-2, IL-7R and CD8A) in HIV patients with active TB, which strongly indicates the prospect of these genes to discriminate active TB in HIV patients.

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Chapter 10

Summarizing discussion

TB remains a major health threat worldwide, which is mainly caused by lack of effective diagnostic and therapeutic tools. Identification of universal and specific biomarkers could accelerate the development of novel diagnostics and therapeutics for TB (1). Although there are a number of biomarkers identified, there is no biomarker that is used for TB control programs (2, 3). The focus of this thesis is to identify immunologic host biomarkers for latent and active TB in the context of HIV/AIDS and therapy in Ethiopia (Chapter 1).

Outcomes of HAART in HIV patients with latent and active TB

In Chapter 2, the success and challenges in the era of rapid expansion of access to ART in resource limited settings where TB/HIV is endemic are discussed. Despite the fact that HAART reduces morbidity and mortality at a global level, there are challenges, including early mortality, incomplete responses to therapy, lack of consensus to define treatment failures and time to start ART. These HAART associated challenges may be more pronounced in resource-limited settings where the long-term effect of the rapid roll-out of ART is not well investigated. Overall, the need for data from well defined longitudinal cohort studies of patients on HAART is crucial to provide insights to HAART related challenges. Another health challenge since the emergence of HIV infection, even in the era of ART, is the comprehensive management of TB/HIV coinfection (4, 6). To address some of these challenges, HAART and TB treatment outcomes in HIV patients with latent and active TB is addressed in **Chapters 3 and 4**.

In **Chapter 3,** detailed description of the baseline characteristic of the different clinical groups enrolled to investigate candidate biomarkers for TB is provided. The study groups were HIV+TB+, HIV-TB+, HIV+TST+, and HIV+TST- patients, and individuals with (HIV-TST+) and without (HIV-TST-) latent TB infection. In addition, the effect TB of treatment on immuno-hematologic and virologic values and IFN-γ response to ESAT-6/CFP-10 simulation is presented.

In the HIV-TB+ patients, levels of CD4+ T cells, total lymphocytes (TL), hemoglobin level (Hgb), body mass index (BMI), white blood cell count (WBC), neutrophiles, and the IFN-γ response to ESAT-6/CFP-10 stimulation, all normalized after TB treatment. Others also reported normalization of TLC and neutrophil values in un-infected TB patients after TB treatment (7). This suggests that these immuno-hematological parameters can serve as prognostic markers for HIV-TB+ patients, as reported by others (8). In contrast, in the HV+TB+ patients, immuno-hematologic values, BMI, plasma HIV RNA, and IFN-γ response did not normalize after TB treatment. This suggests that early initiation of HAART is prompted in TB/HIV patients irrespective of CD4+ T cell counts.

In **Chapter 4,** we extended the study in **Chapter-3**, to add knowledge regarding HAART outcome in HIV patients with latent and active TB. We followed HIV+TST+, HIV+TST- and

HIV+TB+ patients for 6 months (M6), 18 months (M18) and 24 months (M24) after initiation of HAART. At M24, the majority of patients (both non-TB patients (84%) and TB patients (80%) had achieved virologic success (HIV RNA <50 copies/ml). A study done in Italy showed also 74% of the non TB patients had viral supression (<50 copies/ml) at 15 months of ART (9), while another report from US has shown 81% of the patients attained LDL (< 400 copies/ml) at 24 months on HAART (10).

Overall, there was no significant difference in the percentage of patients that achieved virologic success and in total CD4+ T cell count increase between HIV patients with and without TB or LTBI, in agreement to other reports (11). Despite this, HAART was unable to restore IFN-γ responses to ESAT-6/CFP-10 stimulation in the HIV+TB+ patients, which could be associated with exhaustion of the immune system (15, 9). Other studies also showed suboptimal restoration of CD4+ cells in terms of phenotypic and functions in TB/HIV patients on ART (13,14). The results suggest the need to develop intervention methods, which could boost the immune response in TB/HIV patients on HAART.

***Mtb* antigen specific immunologic markers in latent and active TB**

Characterizing host immune responses to *Mtb* antigens expressed at the different stages of the infection is essential to develop effective diagnostics and therapeutics for TB (16,17). Furthermore, although the interplay of several cytokines determine the clinical outcome of *Mtb* infection, the main cytokines shown to be important in the pathogenesis of *Mtb* infection are IFN-γ, TNF-α, IL-12, IL-2, IL-6, IL-17, IL-18, IL-10, and TGF-β (18, 19, 20). In **Chapters 5, 6, and 7,** immune response of IFN-γ, TNF-α, IL-2, IL-6, IL-17, IL-18, IP-10 and MIP-1a to a wide range of *Mtb* antigens in the context of HIV infection and ethnicity are investigated

Immune responses to Mtb antigens in HIV negative active TB patients

Only a few studies have been performed to assess the immunogenicity of *Mtb* antigens in active-TB patients. (21). **In Chapter 5,** we investigated the immune response (IFN-γ, TNF-α, IL-2, IL-6, IL-8, IL-17, and IL-10) against a large series of *Mtb* antigens including TB10, ESAT-6, Ag85A/B, PPD, and HSP65, and 64 *Mtb* antigens for which little or no data is available on immunogenicity (39 latency, 4 RPF, and 21 reactivation) in HIV negative active TB patients.

We identified 23 (4 RPF, 13 latency, and 6 reactivation) immunogenic antigens (IFN-γ ≥ 60 pg/ml), that could serve as additional candidates for immunodiagnostics and vaccines for TB. Interestingly, these identified antigens are all among the list of 189 immunogenic antigens which are identified from the *M. tuberculosis* genome-scale dataset that is constructed by analyzing published data of global gene expression, cellular and humoral immunity, and vaccine potential studies (22). More importantly, we observed induction of multiple cytokines by a subset of antigens, which indicate a link between antigen-specific T-cell subsets and cytokine signatures

during active TB (23). Our data suggest that combined analysis of IFN-γ, TNF-α, IL-6, and IL-10 in response to a subsets of *Mtb* antigens including Rv0081, Rv2629, Rv1733c, and Rv2006 could be promising for the development of immunodiagnostics.

Immune responses to Mtb antigens in geographical diverse sites

Consecutively, in **Chapter 6,** the influence of TB infection status, HIV infection and ethnicity on the immune responses to *Mtb* antigens was elucidated by measuring IFN-γ response to 23 *Mtb* antigens (reactivation, latency, starvation-induced and secreted) of which eight antigens were also included in the previous study (Chapter 5). The study population were latent and active TB groups with and without HIV coinfection recruited from five sites in Africa (South Africa, The Gambia, Ethiopia, Malawi and Uganda). The results showed that next to TB10.4, PPD and ESAT-6/CFP-10, the highest responses were seen to Rv2029c followed by Rv1733, Rv1735 and Rv1737 in all sites irrespective of HIV infection and TB status. Rv1733, Rv1735 and Rv1737 were previously shown to induce dominant immunogenic responses in LTBI subjects from The Gambia, South Africa and Uganda in all sites irrespective of HIV infection and TB status. (24). Our results in Chapter 5 showed also strong immunogenic responses to Rv1733 and Rv1737in HIV negative active TB patients in Ethiopia.

The study revealed also disparities in responses to antigens between sites presumably due to host genetic, nutritional status and environmental differences. This suggests that additional epitope discovery is required to determine the optimal candidates for novel vaccines, as also reported by others (25, 26).

Immune responses to Mtb specific antigens during HIV infection, HAART and TB treatment

For effective clinical utilization of TB biomarkers, the effect of HIV infection and therapy on the dynamics of the biomarkers need to be investigated (2). In **Chapter 7,** therefore, the effect of therapy on the modulation of TB specific biomarkers was investigated, by measuring the levels of IFN-γ, IL-2, IL-17, IL-10, IP-10 and MIP-1α (pg/ml) in response to ESAT-6/CFP-10 in HIV^+TB^+, HIV^-TB^+, HIV^+TST^+ patients after 6 months, 18 months and 24 months of HAART and/or TB treatment.

At baseline, HIV^-TST^+ individuals showed distinct expression of Th1derived cytokines (IFN-γ, IP-10 and IL-17) compared to controls, while HIV^-TB^+ patients showed increased IL-2 and IP-10 levels. Others also showed higher *Mt* antigen induced IP-10 and IL-2 in household contacts of TB patients than controls;[27, 28] and in active TB patients than in Controls (29, 30. Moreover, HIV^-TST^+ individuals showed higher levels of IFN-γ, IL-17, IL-10 and MIP-1α compared to HIV^-TB^+ patients, in agreement to others (31, 32). In conclusion, our results indicated simultaneous measurement of IFN-γ, IL-17 and IP-10 may assist in diagnosing LTBI that IL-2

and IP-10 may assist in diagnosing active TB; while IFN-γ, IL-17, MIP-1α, and IL-10 may assist to discriminate latent and active TB.

Moreover, our observation that the level of IL-10, IFN-γ, IL-2, MIP-1α, and IP-10 was normalized by six months of TB treatment in the HIV-TB+ patients, suggest that these cytokines can be used to monitor TB treatment. Others also showed ESAT-6 and CFP-10 induced IP-10 and IFN-γ response as biomarkers for TB treatment response in HIV negative patients (33).

However, HAART was not able to restore levels of IFN-γ, IL-2, IL-17 and IP-10 in the HIV+TB+ patients, as reported by others (34). This may be due to inefficient therapy, lack of treatment compliance, and exhaustion of the immune system to produce the cytokines (30 ,15). Therapeutic interventions such as co-administration of IL-2 that could improve *Mtb*-specific immune restoration in the HIV/TB patients should be the focus of treatment strategies in the future.

Transcriptomic whole blood biomarkers in latent and active TB patients

Transcriptomic analysis is one of the platforms to identify candidate gene markers for TB (35). **In Chapter 8,** the expression pattern of 45 whole blood genes was investigated in 4 sub-Saharan countries (Ethiopia, Malawi, South Africa, The Gambia) in participants with TB (TB^+HIV^+ and TB^+HIV^-), and without TB (TB^-HIV^+,TST^+HIV^-, TST^-HIV^-).

The best biomarkers for classification of TB^+HIV^+ patients were FCG1RA followed by CXCL10 and CD3E; while for the classification of TB^+HIV^- patients the best markers were CD3E, followed by IL7R and FCGR1A. Multiple logistic regression analysis revealed that a combination of FCGR1A, BCL2 and IL7R could correctly classify 91% of HIV-TB+ participants into active TB disease or LTBI. Interestingly, FCGR1A was the most significant promising biomarker to classify active TB irrespective of HIV infection and ethnicity. Other studies showed that decreased expression of FcγR1A is associated with an increased progression from LTBI to active TB (36, 37), suggesting that progression to active TB may switch the balance from inhibitory to activating FcγR on antigen presenting cells. **In Chapters 9,** we extended the study i**n Chapters 8** by including HIV infected individuals with latent TB (TST^+HIV^+, TB^+HIV^+, TST^-HIV^+). The findings showed distinctive and elevated expression of four genes (FCGR1A, TIMP-2, IL-7R and CD8A) in TB^+HIV^+ patients, which suggests that these genes can be used to classify active TB in HIV patients.

Together, the conclusive finding in Chapter 8 which showed FCGR1A to be the most promising biomarker to classify active TB irrespective of HIV infection and ethnicity, also reflected in Chapter 9, that FCGR1A can classify active TB in HIV patients.

Chapter 11

Conclusion and future perspectives

If biomarkers that could accurately differentiate latent and active TB could be identified , it will be possible to develop effective and simple point-of-care devices such as immunochromatography that would improve the diagnosis of TB disease and infection. In the studies included in this thesis, several candidate biomarkers for latent and active TB have been identified.

Our study design for testing novel *Mtb* antigens, revealed combined measurement of specific cytokines (IFN-γ, TNF-α, IL-6 and IL-10) in response to a subset of antigens (Rv0081, Rv2629, Rv1733c, Rv2006) could be a promising path to develop immunodiagnostics.

We also presented data, which suggest combined measurement of IFN-γ IFN F γ-17, and IP-10 in response to ESAT-6/CFP-10 could be useful immune markers for the diagnosis LTBI; IL-2 and IP-10 for the diagnosis of active TB; and IFN-γ, IL-17, MIP-1α, and IL-10 to discriminate latent and active TB. In addition, FCGR1A, TIMP-2, IL-7R and CD8A whole blood genes could be useful to classify active TB in HIV patients

We showed that IL-2, IFN-γ and IP-10 in response ESAT-6/CFP-10; and the leukocyte subsets (TLC, WBC, neutrophil) to be useful markers to monitor treatment in HIV negative TB patients. Similarly, IL-10 and MIP-1α in response to ESAT-6/CFP-10 may assist to monitor TB treatment and HAART.

In summary, whereas we feel that these identified biomarkers will have significant contribution towards TB control in the future, the effects of other potential confounding factors including endemic infections such as malaria and helminthes, granulomatous disease, host genetics, and *Mtb* lineages need to be addressed before these new biomarkers are moving to clinical practices.

Thus, we propose context-dependent validation studies by comparing findings from prospective cohorts from different epidemiological settings and populations with samples and data collected in a standardised way. The ideal approach for this will be the GC-6-74 TB consortium repository samples which consists of five African countries including Ethiopia, Uganda, South Africa, The Gambia and Malawi.

We suggest further biomarker research by including extrapulumonary TB patients and children where the need of better TB diagnostics is enormous; and from other easy to access specimens including urine, saliva and sputum. To increase the efficiency of biomarker discovery and consecutively to deliver novel clinical tests, there is a need of better understanding of the TB biomarker research process (from study design to validation). Nonetheless, research aimed to understand host-pathogen interactions, the interplay between innate and adaptive immune responses, and the complex cytokine interplay need to be continued.

Chapter 12

Samenvatting in het Nederlands

Tuberculose(tbc) is een ernstige, soms besmettelijke bacteriëleinfectieziekte, die vooral de longen aantast, veelal veroorzaakt door *Mycobacterium tuberculosis(Mtb).* Besmetting met Mtb vindt plaats door het inademen van kleine tuberculosebacteriën bevattende druppeltjes die door tuberculosepatiënten worden verspreiddmv hoesten. Bij besmetting komt de bacterie binnen via de longen, waar de bacterie zich vermenigvuldigt. Vervolgens wordt de bacterie via de lymfe naar de dichtsbijzijnde lymfeklier getransporteerd, waar de afweerreactie begint. Hierna verspreidt de tuberculosebacterie zich door het hele lichaam. Na ongeveer 6 weken heeft het lichaam genoeg afweer ontwikkeld om de infectie tegen te gaan. Wel blijven overal in het lichaam nog latente ('slapende') bacteriën achter.

Ongeveer 1/3 van de wereldbevolking is besmet met Mtbtbc-bacteriën, zonder ziek te zijn. Deze mensen hebben een zogenaamde "latente tuberculose infectie" (LTBI). Bij circa 1% van de mensen zal direct aansluitend aan de besmetting ‘actieve’ tuberculose ontstaan. Bij 10% zal de 'slapende' tuberculosebacterie op een later moment weer actief worden en de ziekte tbc veroorzaken. Meestal treedt ziekte binnen een jaar of 2 na de besmetting op. Bij mensen met een verlaagde afweer of jonge kinderen is de kans groter op het ontwikkelen van tuberculose. Ook infectie met het Humane immunodeficiëntievirus (HIV) bespoedigt het ontstaan en ontwikkelen van tuberculose. In landen waar HIV veel voorkomt is daarom vaak ook veel tuberculose. In Afrika ten zuiden van de Sahara zijn de meeste tbc-patiënten ook HIV-positief.

Tuberculoseis een grote bedreiging voor de gezondheid wereldwijd. Dit wordtmet nameveroorzaakt door het ontbreken van effectieve diagnostische en therapeutische mogelijkheden. Identificatie van universele en specifieke biomarkers kunnen de ontwikkeling van nieuwe diagnostica en therapeutica voor TB versnellen. Hoewel er een aantal biomarkers geïdentificeerd zijn, is er geen biomarker die wordt gebruikt voor tuberculose. De focus van dit proefschrift is om immunologische en gastheer-specifiekebiomarkerste identificeren voor latente en actieve tuberculose in het kader van HIV/AIDS en therapie in Ethiopië (hoofdstuk 1).

Uitkomsten van ART bij HIV-patiënten met latente en actieve tuberculose

In hoofdstuk 2, wordt het succes en de uitdagingen in het tijdperk van de snelle uitbreiding van de toegang tot antiretrovirale therapie (ART) in gebieden met beperkte middelen waar tbc enHIV-infectie endemisch is besproken. Ondanks het feit dat ART de morbiditeit en mortaliteit op mondiaal niveau vermindert, zijn er uitdagingen, waaronder vroegtijdige sterfte, onvolledige reacties op de therapie, gebrek aan consensus mbt identificatie van falen van behandeling en het moment waarop behandeling met ART moet beginnen. Deze uitdagingen kunnen groter zijn in gebieden met beperkte middelen, waar het lange termijn effect van de snelle uitrol van ART nog niet goed is onderzocht. Kortom, de behoefte aan gegevens uit goed gedefinieerde longitudinale

cohort studies van patiënten die ART krijgen is cruciaal om inzicht te krijgen in ART gerelateerde uitdagingen.

Om een aantal van deze uitdagingen aan te pakken, worden HAART en TB behandelingsresultaten bij HIV-patiënten met latente en actieve tuberculose beschreven in de hoofdstukken 3 en 4.

In hoofdstuk 3, geven we een gedetailleerde beschrijving van de baseline kenmerken van de verschillende klinische groepen die deelnamen aan de onderzoeken beschreven in dit proefschrift. De studiegroepen waren HIV + TB +, HIV-TB + (beiden actieve tbc patiëntengroepen), HIV + TST +, en HIV + TST- patiënten (HIV geinfecteerden met of zonder latente tuberculose-infectie gemeten met een Mantouxtest) en gezonde personen met (HIV-TST +) en zonder (HIV-TST-) latente tuberculose-infectie. Bovendien wordt het effect van tbc behandeling op immuno-hematologische en virologische waarden en IFN-γresponsen op ESAT-6 / CFP-10 stimulatie (Mtb specifieke eiwitten) gepresenteerd.

In de HIV-TB +-patiëntenzijn niveaus van CD4 + T-cellen, de totale lymfocyten (TL), hemoglobinegehalte (Hb), body mass index (BMI), witte bloedcellen (WBC), neutrofielen, en de IFN-γ respons op ESAT -6 / CFP-10 stimulatie al genormaliseerd na de behandeling van tbc. Dit suggereert dat deze immuno-hematologische parameters als prognostische markers kunnen dienen voor HIV-TB +-patiënten. In tegenstelling, in de HIV+/ TB+-patiëntennormaliseren immuno-hematologische waarden, BMI, plasma HIV-RNA, en IFN-γ respons niet na de behandeling van tbc. Dit suggereert dat een vroege start van ART noodzakelijk is in HIV+TB-patiënten, onafhankelijk van het aantal CD4 + T-cellen.

In hoofdstuk 4, hebben we de studie in Hoofdstuk3 uitgebreid, om kennis te vergaren met betrekking tot ART uitkomst bij HIV-patiënten met latente en actieve tuberculose. We volgden HIV + TST +, HIV + TST- en HIV + TB +-patiënten gedurende 6 maanden (M6), 18 maanden (M18) en 24 maanden (M24) na het starten van ART. Op M24, waren de meeste van de patiënten (zowel niet-TB-patiënten (84%) en TB-patiënten (80%) virologische succesvol (HIV RNA <50 kopieën / ml). Over het algemeen was er geen significant verschil in het percentage patiënten met virologische succes en de stijging in totale CD4 + T cel aantallen tussen HIV patiënten met en zonder tuberculose en LTBI. Desondanks was ART niet in staat om de IFN-γ responsenop ESAT-6 / CFP-10 stimulatie te herstellen in de HIV + TB + patiënten, wat veroorzaakt zou kunnen worden door uitputting van het immuunsysteem. Deze resultaten suggereren dat de ontwikkeling van methoden waarmee we de immuunrespons kunnen boosten nodig zijn in HIV / TB patiënten die ART krijgen.

Mtb antigeen-specifieke immunologische markers in latente en actieve tuberculose

Het karakteriseren van gastheer immuunresponsen op antigenen die in de verschillende stadia van de Mtbinfectie tot expressie komen, is essentieel voor de ontwikkeling van doeltreffende diagnostica en therapeutica voor tbc. Ofschoon de interactietussen de verschillende cytokines de klinische uitkomst van Mtb-infectie kunnen bepalen, zijn de belangrijkste cytokines voor de pathogenese van Mtb infectie: IFN-γ, TNF-α, IL-12, IL-2, IL-6, IL-17, IL-18, IL-10 en TGF-β. In hoofdstuk 5, 6 en 7, worden deimmuunrespons (productie van IFN-γ, TNF-α, IL-2, IL-6, IL-17, IL-18, IP-10 en MIP-1a)onderzocht tegen een groot aantal Mtb antigenen in de context van HIV-infectie en etniciteit.

Immuunrespons op Mtb-antigenen in HIV-negatieve actieve tbc-patiënten

In hoofdstuk 5 onderzochten we de immuunrespons (IFN-γ, TNF-α, IL-2, IL-6, IL-8, IL-17 en IL-10) tegen een groot aantal Mtbantigenen waaronder TB10, ESAT -6, Ag85A/B, PPD, en HSP65 en 64 Mtb antigenen waarvoor weinig of geen gegevens beschikbaar zijn over de immunogeniciteit (39 latency, 4 RPF, en 21 reactiverings antigenen) bij HIV-negatieve actieve tbc-patiënten. We identificeerden 23 (4 RPF, 13 latentie, en 6 reactiverings) immunogene antigenen (IFN-γ≥60 pg / ml), die als extra kandidaten voor immunodiagnostiek en vaccins voor tuberculose zouden kunnen dienen. Een subset van antigenen is zelfs in staat om meerdere cytokinente induceren. Dit geeft aan dat er een verband is tussen antigeen-specifieke T-cel subsets en cytokine signaturen tijdens actieve tbc. Onze gegevens suggereren dat gecombineerde analyse van IFN-γ, TNF-α, IL-6 en IL-10 in reactie op een subset van Mtb-antigenen waaronder Rv0081, Rv2629, Rv1733c, Rv2006 veelbelovend zou kunnen zijn voor de ontwikkeling van (nieuwe) immunodiagnostiek.

Immuunrespons op Mtb-antigenen in geografische diverse locaties

Vervolgens hebben we in hoofdstuk 6 de invloed van TB-infectie status, HIV infectie en etniciteit op de immuunrespons op Mtb-specifieke antigenen opgehelderd door het meten van IFN-γ responsenastimulatie met 23 verschillende Mtb-antigenen (reactivatie, latentie, honger-geinduceerd en uitgescheiden antigenen), waarvan acht antigenen ook werden geanalyseerd in de vorige studie (hoofdstuk 5). De onderzoekspopulatie bestond uit latente en actieve tuberculose groepen met en zonder hiv coinfectie afkomstig uit vijf locaties in Afrika (Zuid-Afrika, Gambia, Ethiopië, Malawi en Oeganda). De resultaten toonden dat naast TB10.4, PPD en ESAT-6 / CFP-10, de hoogste immuun responsen werden gedetecteerdtegen Rv2029c gevolgd door Rv1733, Rv1735 en Rv1737, ongeacht HIV infectie enTB status. Rv1733, Rv1735 en Rv1737 waren al

eerder als dominante immunogene antigenen gevonden in latent geïnfecteerdenuit Gambia, Zuid-Afrika en Uganda, ongeacht de HIV-infectie en de TB status. Onze resultaten in hoofdstuk 5 laten ook sterkeresponsen zien na stimulatie met Rv1733 en Rv1737 antigenen in HIV-negatieve actieve tbc-patiënten in Ethiopia. De studie laat ook verschillen zien in respons op antigenen tussen locaties vermoedelijk als gevolg van genetische, voedingstoestand en milieu-verschillen van de gastheer. Dit suggereert dat additionele epitoopidentificatievereist is om optimale kandidaten voor nieuwe vaccins te bepalen.

Immuunrespons op specifieke Mtbantigenen tijdens HIV-infectie, HAART en de behandeling van tuberculose

Voor een effectief klinisch gebruik van TB biomarkers, moet het effect van HIV-infectie en therapie op de dynamiek van de biomarkers onderzocht worden. In hoofdstuk 7, hebben we derhalve het effect van behandeling op TB biomarkers onderzocht door het meten van IFN-γ, IL-2, IL-17, IL-10, IP-10 en MIP-1α niveau's (pg / ml) in reactie op stimulatie met ESAT-6 / CFP-10 bij HIV + TB +, HIV-TB +, HIV + TST + patiënten na 6 maanden, 18 maanden en 24 maanden HAART en / of behandeling van tuberculose. Bij aanvang toonden HIV-TST + personen duidelijke expressie van zgnTh1 cytokines (IFN-γ, IP-10 en IL-17) vergeleken met controles, terwijl HIV-TB + patiënten verhoogde IL-2 en IP-10 niveaus hadden. Bovendien vertoonden HIV-TST + individuen hogere niveaus van IFN-γ, IL-17, IL-10 en MIP-1α ten opzichte van HIV-TB + patiënten. Deze resultaten geven aan dat gelijktijdige meting van IFN-γ, IL-17 en IP-10 kan bijdragen aan de diagnose van LTBI en dat meting van IL-2 en IP-10 kan bijdragen aan de diagnose van actieve TB; terwijl analyse van IFN-γ, IL-17, MIP-1α en IL-10 kan assisteren in de discriminatie van latente en actieve tuberculose. Bovendien suggereert onze waarneming dat het niveau van IL-10, IFN-γ, IL-2, MIP-1α en IP-10 normaliseertna zes maanden behandeling van tuberculose in de HIV-TB + patiënten, dat deze cytokines kunnen worden gebruikt om TB behandeling te monitoren. Echter, HAART kon de niveaus van IFN-γ, IL-2, IL-17 en IP-10 niet herstellenin de HIV+/ TB+-patiënten. Dit kan het gevold zijn van inefficiënte behandeling, gebrek aan therapietrouw en uitputting van het immuunsysteem om de cytokinen produceren. Therapeutische interventies zoals gelijktijdige toediening van IL-2, zou de Mtb-specifieke immuunrespons kunnen verbeteren en herstel daarvan moet de focus van behandelingsstrategieën zijn in de HIV / TB-patiëntenin de toekomst.

Analyse van volbloed biomarkersop transcriptoomniveau in latente en actieve tbc-patiënten

Transcriptoom analyse is een van de platforms om kandidaat-gen expressie markers voor TB te identificeren. In hoofdstuk 8werd het expressiepatroon van 45 volbloed genen onderzocht in 4

sub-Sahara landen (Ethiopië, Malawi, Zuid-Afrika, Gambia) in deelnemers met tbc (TB + HIV + en TB + HIV), en zonder tbc (TB-HIV+, TST + HIV, TST-HIV). De beste biomarkers voor het classificeren van TB + HIV + patiënten waren FCG1RA gevolgd door CXCL10 en CD3e; terwijl voor de classificering van TB + HIV-patiënten de beste markers CD3e, gevolgd door IL7R en FCGR1A waren. Multipele logistische regressie-analyse toonde aan dat een combinatie van FCGR1A, BCL2 en IL7R 91% van de HIV-TB + deelnemers met actieve tcb of LTBI correct kon classificeren. FCGR1A was de belangrijkste veelbelovende biomarker om actieve tuberculose te classificeren, ongeacht HIV-infectie en etniciteit. In hoofdstuk 9, hebben we de studie in hoofdstuk 8 uitgebreid met inclusie van HIV-geïnfecteerde personen met latente tuberculose (TST + HIV +, TB + HIV +, TSTHIV +). We vonden een verhoogde expressie van vier genen (FCGR1A, TIMP-2, IL-7Ren CD8a)in TB + HIV + patiënten, wat suggereert dat deze genen kunnen worden gebruikt om actieve TB te classificeren bij HIV-patiënten. De overtuigende bevinding in hoofdstuk 8 dat FCGR1A de meest veelbelovende biomarker is om actieve tuberculose te classificeren, onafhankelijk van HIV-infectie en etniciteit, is ook terug te vinden in hoofdstuk 9 waarbij de expressie van FCGR1A actieve tuberculose kan classificeren in HIV-patiënten.

Conclusie en toekomstperspectieven

Als biomarkerskunnen worden geïdentificeerddie latente en actieve tuberculose nauwkeurig kunnen differentiëren, is het mogelijk effectieve en simpele methoden zoals immunochromatografiete ontwikkelen om de diagnose van tuberculose ziekte en besmetting te verbeteren. In de studies die in dit proefschrift zijn gedaan, zijn verschillende kandidaat-biomarkers voor latente en actieve tuberculose geïdentificeerd. Ons studie-design voor het testen van nieuwe Mtb antigenen, onthulde dat gecombineerde analyse van specifieke cytokines (IFN-γ, TNF-α, IL-6 en IL-10) in response op een subset van antigenen (Rv0081, Rv2629, Rv1733c, Rv2006) veelbelovend kan zijn om te ontwikkelen als nieuwe immunodiagnostiek.Tevens presenteerdenwe gegevens die suggereren dat gecombineerde analysevan IFN-γ IFN γ F-17 en IP-10 in respons op ESAT-6 / CFP-10 stimulatie nuttig kan zijn als immuun markersvoor de diagnose van LTBI; IL-2 en IP-10 voor de diagnose van actieve tuberculose; en IFN-γ, IL-17, MIP-1α en IL-10 om latente en actieve tuberculose te onderscheiden.

Daarnaast kan het gebruik van de expressie van FCGR1A, TIMP-2, IL-7R en CD8a in volbloed nuttig zijn om actieve tuberculose te classificeren bij HIV-patiënten. Ook toonden we aan dat IL-2, IFN-γ en IP-10 in reactie op ESAT-6 / CFP-10 stimulatie; en de leukocyten subsets (TLC, WBC, neutrofielen) bruikbare markers zijn om de behandeling in HIV-negatieve tbc-patiënten te

monitoren. IL-10 en MIP-1α productie in reactie op ESAT-6 / CFP-10 stimulatie kunnen bijdragen aan het monitoren van de behandeling van tuberculose en HAART.

Terwijl wij van mening dat de door ons geïdentificeerde biomarkerseen belangrijke bijdrage aan de tbc-bestrijding in de toekomst zullen hebben, moeten de effecten van andere mogelijke verstorende factoren, waaronder endemische infecties zoals malaria en helmints, granulomateuze ziekte, gastheer genetica, en Mtbstammen worden bestudeerd voordat deze nieuwe biomarkersgebruikt kunnen worden in de klinische praktijk. Daarom stellen we voor dat meer contextafhankelijke validatie studies uitgevoerd worden door het vergelijken van de bevindingen uit prospectieve cohorten uit verschillende epidemiologische settings en populaties met samples en data die op een gestandaardiseerde manier verzameld zijn. De ideale aanpak hiervoor is het gebruiken van de binnen het GC-6-74 TB consortium opgeslagen samples die bestaat uit materiaal uit vijf Afrikaanse landen, waaronder Ethiopië, Oeganda, Zuid-Afrika, Gambia en Malawi. We raden aanaanvullendbiomarker onderzoek te doen in patiënten met extrapulmonaire tbcen kinderen, waar de noodzaak van een betere TB diagnostiek groot is; en in anderemakkelijktoegangkelijke materialen zoals urine, speeksel en sputum. Om de efficiëntie van biomarkeridentificatie te vergroten en vervolgens nieuwe klinische test te leveren, is er een tevens behoefte aan beter begrip van het TB biomarker onderzoeksproces (van studieontwerp tot validatie). Hoe dan ook moet onderzoek gericht op het begrijpen van gastheer-pathogeen interacties, de wisselwerking tussen aangeboren en adaptieve immuunrespons, en het complexe cytokine samenspel worden voortgezet.

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Curriculum vitae

Desta Kassa was born on 26 December 1968 at Egela, Tigray, Ethiopia. After he completed secondary school in 1986 at Ethiopia Tikdem Secondary School, Addis Ababa, Ethiopia; he joined Asmara University and graduated in Marine Biology (BSc degree) in 1992. From 1992-2000, Desta has been working as a Fishery expert and researcher in the Ministry of Agriculture, Ethiopia.

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Desta Kassa lives with his wife ,Abeba Gebresadic, and his daugher, Mahlet Desta.

List of publications

1. **Desta Kassa**, Wilco de Jager , Gebremedhin Gebremichael, Yodit Alemayehu, Leonie Ran, Justin Fransen, Dawit Wolday, Tsehaynesh Messele, Belete Tegbaru, Tom H. M. Ottenhoff, and Debbie van Baarle.The effect of HIV coinfection, HAART and tuberculosis (TB) treatment on cytokine/chemokine responses to TB antigens in active TB patients and latent TB infected (LTBI) individuals. *Tuberculosis (Submitted)*

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