Immunological Consequences of M*ycobacterium tuberculosis* and Human Immunodeficiency Virus Coinfection in Ethiopia

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Immunological Consequences of M*ycobacterium tuberculosis* and Human Immunodeficiency Virus Coinfection in Ethiopia

Immunologische consequenties van Mycobacterium tuberculosis en Humaan Immunodefientie Virus Coinfectie in Ethiopië

(met een samenvatting in het Nederlands)

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This thesis is dedicated to:

WODAJE, NEGUSSIE AND GONE TEGBARU

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Introduction

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Chapter 1

1.1. *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (MTB) is an aerobic intracellular microorganism which has a preference for the lung tissue rich in oxygen supply. Infection occurs via the respiratory route. There are four stages of pulmonary tuberculosis disease. The first stage begins with the inhalation of the tubercle bacilli and the phagocytosis of MTB by the alveolar macrophages in the lung **(1, 2).** However, if mycobacterial growth inhibition/killing fails, within 2 to 6 weeks of time the cell mediated immunity (CMI) develops, which results in an influx of lymphocytes and activated macrophages to the site of infection to form granuloma that contain the bacilli in the caseous center, which is the second phase of the infection. In macrophages, anti-mycobacterial effector mechanisms such as generation of reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) are required to control the expansion of the bacilli. In the acquired phase of immunity, the role of humoral immunity is minor compared to the role of CMI. The presentation of MTB through Major Histocompatibility Complex Class-II (MHC-class II) molecules, which activates CD4+ T cells to produce interferon gamma (IFNγ) and activate macrophages to control the bacilli, is the major event. However, CD8+ T cells and CD1 molecules are also found to be participating in the immune response towards MTB **(3, 4).** Cytokines, such as IL-12 (produced by macrophages and Dendritic cells following phagocytosis), IFNγ (produced by both CD4+ and CD8+ T cells), tumor necrosis factor- alpha (TNF-α , produced by macrophages, Dendritic cells and T cells), Interleukin-1 (IL-1), IL-2, IL-4, IL-6, IL-10 and transforming growth factor-beta (TGF-β) are the major cytokines playing roles in host-pathogen interactions. Furthermore, chemokines such as IL8, monocyte chemoattractant protein-1 (MCP-1), RANTES, macrophage inflammatory protein-1alpha (MIP-1α), MIP-1β, MIP2, MCP3 and MCP-5 also play roles in controlling the pathogen by the host. In this phase of the infection (third phase of the infection), the early logarithmic bacillary growth stops (which prevents the bacilli from spreading) and a latent phase of infection is entered. Finally, in the last phase of the infection, the immune system may fail to control the bacilli in which case the disease may progress, and hematogenous dissemination may take place **(1)**.

1.2. Screening for MTB infection and laboratory diagnosis of the disease in the era of HIV

Exposure of individuals to tuberculosis infection can be screened by skin tests by challenging individuals to MTB proteins such as purified protein derivative (PPD). In such cases delayed type of hypersensitivity (DTH) response rates will be measured depending on the inflammation that is caused by MTB antigens by measuring the indurations developed on the arms of the individuals after 48 to 72 hours of exposure. However, skin tests have limitations due to BCG vaccination and reaction to non tuberculosis environmental bacteria, which lead to loss of specificity **(5-8).** Impaired immunity, especially in HIV infected individuals also affects the response rate in skin tests towards these antigens. Indeed, in culture positive TB cases these tests are often negative among HIV positive cases **(9,10).** Due to major inherent problems of the skin tests such as the use of PPD, there are efforts to increase the detection of both latent and active forms of TB infection using different antigens of MTB. Therefore, tests based on early secreted antigen target-6 (ESAT-6) **(11-18)** and culture filtrate protein-10 (CFP-10) are now getting attention due to the fact that these tests can differentiate between latent and active TB infections **(19,20)**.

Once the disease has developed, diagnosis is based on the identification of bacilli from sputum by conventional methods: Acid Fast Bacilli (AFB) smear microscopy and culture. The methods are based on the availability of the bacilli in the sputum. Factors such as bacilli concentration and the status of the immune system of the individuals could affect the outcomes of these tests. Moreover, currently the higher frequency of smear negative

individuals, especially in HIV infected persons, and the limited number of laboratory facilities to perform culture complicates the diagnosis of tuberculosis disease in the developing world **(21)**.

Generally, the diagnosis of smear positive TB cases in the community, be it by active or passive surveillance methods, is important in controlling tuberculosis infection since diagnosing of active smear positive cases would help to reduce the transmission by treating the active cases, which is one of the recommended ways to control TB **(21)**.

1.3. Epidemilogy of tuberculosis infection

Tuberculosis (TB) remains one of the major health threats of mankind as one-third of the world's population harbor inactive (latent tuberculosis) TB infection that represents an infection reservoir and (future) source of transmission. The majority of the affected people are living in the developing world especially in Sub-Saharan Africa. TB accounts for nearly 6% of all-cause deaths worldwide by killing one fourth of the eight to nine million people who developed the disease **(21,22,23)** and it is the foremost cause of death from a single infectious agent in adults **(24).** It is also reported that nearly 90 million new cases occurred between 1990 and 1999 **(25).** Despite the use of standard anti-TB treatment worldwide, the occurrence of TB as new infection (incidence) or reactivation is increasing. In 2004 alone 9 million people were infected (140 per 100,000 populations) with TB and 2 million of them died of TB disease **(26)**. In 2004, globally, the coverage of treatment schemes (Directly Observed Short course chemotherapy, DOTS) was expected to detect more than 60% of new TB cases. In Ethiopia, like other Sub-Saharan African countries, the incidence of TB is higher; 370 per 100,000 populations with a DOTS coverage of 39% in 1995 and 70% in 2004 **(26)**. However, the detection and treatment success rates were lower than 50% by the end of 2005 **(26)**.

1.4. HIV-TB Coinfection

At the end of 2005 about 39 million people were HIV infected worldwide and more than 70% of them were in sub-Saharan African countries. In Ethiopia alone (with a population of more than 70 million), there were an estimated 1.5 million people living with HIV (a prevalence rate of 3.5%) **(27,28)**.

In 2005 nearly 11.5 million of HIV-infected people were coinfected with MTB in the world. Of these 70% live in Sub-Saharan Africa, 20% in South-East Asia and 4% in Latin America and the Caribbean **(26).** In Ethiopia, 15% of TB patients in rural **(29)** and 33 to 45% in the urban areas were reported to be coinfected with HIV **(29,30,31).** The reported lower absolute CD4+ T cell counts, and especially naïve CD4+ T cells, in the normal population of several African countries including Ethiopia **(32,33)** and other environmental factors such as parasitic infections and malnutrition may result in an increased incidence or reactivation of latent MTB infections. The occurrence of TB as early as the first year of HIV infection **(34),** and the mechanism by which it aggravates the progression towards AIDS and/or vice-versa **(35,36),** could be the major reasons why these two diseases are widespread, particularly in Sub Saharan Africa. In HIV-infected persons, early progression of newly acquired tuberculosis infection may occur in almost 40% of persons within four months, compared with 2-5% of non-infected controls in the first 2 years. However, among individuals infected with tuberculosis, co-infection with HIV leads to the development of active tuberculosis at the annual rate of 7-10%, compared with a lifetime risk of reactivation of 5-10% among HIV-negative controls **(24).**

HIV-TB coinfection has long been associated with generalized immune activation involving both lymphocytes and macrophages **(37)**. It is known that HIV preferentially infects activated

CD4+ T lymphocytes and monocytes and efficient induction of its replication is initiated through immune activation. Immune activation caused by HIV is believed to mediate the increased proliferation of CD4+ T cells, which in turn makes these cells more vulnerable to infection and destruction by HIV, accelerating the progressive decline of the naïve CD4+T cell pool **(38,39)**. Furthermore, increased proliferation of CD4+ T cells leads to increased consumption of naïve T cells, which cannot be compensated by new production from the thymus, and thereby a reduction in CD4+ and especially naïve T cell numbers. In addition, some studies suggest that a decrease in T regulatory cells ($CD4^+$, CD25+) was associated with HIV disease progression; suggesting that loss of regulatory T cells (Tregs) could contribute to increased T cell hyperactivation, through the reduction in active Treg cell mediated suppression of conventional T cells **(40).**

The higher risk of HIV infected individuals to develop TB disease includes rapid progression of recently acquired TB as well as reactivation of latent infection, making it the most common opportunistic infection in HIV infected individuals. Studies conducted in both developing and developed countries have demonstrated that occurrence of TB in HIV-infected subjects is significantly associated with progressive disease and mortality. Co-infected patients have a 4- to 8-fold higher likelihood of death as compared to HIV negative TB patients **(41)**. Furthermore, among HIV/TB co-infected patients with CD4+ counts below 200 cells/µl, mortality at 6 months to 1 year is 2 to 3 times higher than the death rate with higher CD4+ counts. Poor survival correlates with the anatomical site of MTB, and is higher in HIV infected patients with extra-pulmonary TB foci (meninges, blood and bone marrow) than in those with only pulmonary TB. Tuberculosis meningitis accounts for 10% of TB disease in patients that are HIV infected, and the risk of death is 4 times higher than pulmonary TB disease.

1.5. Effects of HIV on MTB infection

It is suggested that HIV-infected individuals coinfected with TB have an ineffective immune responses against MTB as shown by total lymphocyte count and reduced CD4+ T cell count and decreased IFNγ expression **(35)**. In addition, impairment of many of the macrophage effector functions in HIV infected persons has been documented **(42).** For example, receptor mediated phagocytosis and oxidative burst are impaired. Moreover, HIV causes CD4+ T cell depletion and functional abnormalities in both CD4+ and CD8+ T cells **(43)**. In addition, studies have shown that interactions of T cells with antigen presenting cells (APCs) in the context of MHC class II are impaired, which may also result in hypo-responsiveness to soluble tubercle antigens. Also the fact that HIV impairs mainly the T-helper type-1 (TH1) response **(24,44),** patients with HIV infection are reported to have suppressed IFNγ and IL2 responses which paves the way for susceptibility to intracellular infections such as TB.

1.6. Effects of MTB on HIV infection

Several findings indicate that active TB accelerates the progression of HIV infection to AIDS. Indeed, clinical observations show that the response of HIV-infected TB patients to anti-tuberculous drugs is similar to HIV-uninfected TB patients, indicating that the increased morbidity and mortality in co-infected patients is attributable to the worsening of HIV disease **(45)**. Moreover, the course of HIV-1 infection may be accelerated after TB diagnosis. It has been shown that HIV-infected patients with TB have reduced survival, more opportunistic infections, and a greater decrease in CD4+ counts compared to HIV-1-infected CD4+ matched control subjects **(46,47)**.

In HIV-infected patients, MTB seems to augment HIV replication as measured in the peripheral circulation **(46**), lung **(48,49)**, and lymphoid tissues, through mechanisms involving

immune activation **(50)**. Although the lung could be a preferential organ for HIV replication during active TB, available data indicate that an increase as high as 5- to 160-fold in plasma HIV viremia may occur during the acute phase of MTB disease **(36,46,51).** There is also some evidence that indicate that the increase in HIV load is particularly significant in HIV-infected TB patients with higher CD4+ counts **(48)**.

Opportunistic infections such as MTB and also CMV are potent activators of the immune system, which in turn lead to profound changes in the cytokine network, providing favorable environment for viral replication **(52)**. Tuberculosis infection may induce HIV through TNF-α production **(35, 53, 54,55)**. In addition, it is also interesting to note that increased expression of CCR5 and/or CXCR4, major chemokine HIV co receptors, as well as their chemokine ligands, has been demonstrated in human monocyte derived macrophages, alveolar macrophages, and CD4+ T cells in the course of *in vivo* and *in vitro* MTB infection **(56,57,58).** The expression of these coreceptors seems to depend on the activation state of these cells which then further amplifies viral replication by enhancing entry in to uninfected cells. Therefore, the rapid decline of CD4+ T cell number, faster disease progression and increased mortality observed in HIV infected TB patients could be due to MTB-induced hyper-activation of the immune system.

Interestingly, individuals from sub-Saharan African countries, whose baseline immune profile is characterized by chronic immune activation, have been shown to have increased susceptibility to HIV-infection **(59),** indicating that immune activation plays a major role in HIV disease progression and pathogenesis.

1.7. Treatment of tuberculosis disease and TB/HIV- coinfection

The most cost effective public health measures to control TB are the identification and cure of the infectious cases, i.e. patients with smear-positive pulmonary TB. However, the efforts to control TB have so far failed to reduce the number of TB cases worldwide **(21)**. Despite all this, treatment of TB is based on two major drug regimens, i.e. long course anti-TB chemotherapy (given for 12 months) and directly observed chemotherapy, short course (DOTS, for 6 to 8 months). DOTS is currently the most effective anti-TB treatment scheme and mainly used in the developing world, where the health care worker takes care of the adherence of the patients to the therapy. In spite of this, the case fatality of HIV/TB patients 1 year after starting TB treatment is reported to be about 20% and is greater than in HIV-negative TB patients**.** However, the response rate to DOTS in both HIV infected and non-infected individuals is reported to be similar **(60)**.

The treatment of active TB disease is supposed to decrease the HIV RNA level in HIV coinfected individuals **(46,61).** Nevertheless reports from Africa showed that treatment of TB in HIV/TB coinfected individuals has a minimal effect on the levels of plasma viral RNA level **(19, 56,62, 63, 64).** This could be due to higher levels of immune activation **(31)**. In contrast, treatment with antiretroviral drugs (ART) results in reduction of plasma viral RNA levels and in the incidence of tuberculosis in HIV infected individuals. However, treatment of TB together with ART may cause adverse drug-drug interactions. For Example Refampicin influences the activity of protease and non-nucleoside reverse transcriptase inhibitors (NNRTIs) **(65).** Furthermore, some reports showed reactivation/ new infection of TB occurring as immune reconstitution syndrome (IRS) in ART treated patients **(66**) This could be a big challenge for national TB control programs as the coinfected individuals could be a source of infection in the general population.

1.8. Scope of the thesis

Because HIV-TB coinfection remains a major problem in developing countries, especially in sub-Saharan Africa, in this thesis we investigate the immunological interaction of MTB and HIV and their effect on disease progression in HIV infection.

There are three parts in this thesis.
In the first part of the thesis, we try to understand the rate of HIV infection in TB patients (Chapter-2). The burden of HIV infection and its influence on the conventional diagnostic methods of TB disease are critical for planning of the rational use of anti-TB and antiretroviral drugs in particular for implementation of treatment schemes. Following to this, the thesis describes the use and limitations of skin tests in the screening of MTB infection in the era of HIV (Chapter-3). The information generated in this section is helpful to address the limitations of the screening tests, especially in HIV infected individuals, as the awareness of the presence of latent TB infection may help to prevent reactivation by the administration of prophylaxis.

The second part of the thesis focuses on the role of TB and HIV on immune activation (Chapter-4). The dynamics of CD4+ T cell depletion, plasma viral RNA level and immune activation (Chapter-5), the number of regulatory T cells (Chapter-6) and expression of HIV coreceptors (Chapter- 7) in the presence and absence of anti-TB chemotherapy among HIV/TB coinfected individuals are studied. This part of the thesis tries to define and explain why HIV-disease progression rate remains aggressive despite the administration of standard anti-TB chemotherapy in HIV/TB coinfected patients.

The third part of the thesis describes the disease progression rate in HIV infected individuals (Chapter-8) and the clinical outcomes of HIV/TB coinfection in pre- and post ART era (Chapter-9). The first section of this part investigates why the disease progression rate among HIV infected Ethiopians is comparable to Caucasians despite Ethiopians having a higher baseline immune activation level and lower CD4+ T cell number. The second section addresses the clinical outcomes (mortality) of HIV/TB coinfected patients pre and post-ART.

Finally, the last chapters of the thesis (Chapter–10 and Chapter-11) provide a general discussion and summary of the studies included in this thesis towards the role of effective diagnostic , treatment and controlling aspects of TB, HIV and HIV/TB coinfection.

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PART-I

THE BURDEN OF HIV INFECTION IN TUBERCULOSIS PATIENTS AND ITS IMPACT ON SCREENING AND DIAGNOSTIC METHODS OF MTB INFECTION

Clinical outcomes and laboratory results of tuberculosis patients with or without HIV infection in two health institutions in Addis Ababa, Ethiopia

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Chapter 2

Abstract

Background**:** Diagnosis of tuberculosis (TB) in the developing world is based on acid-fast bacilli (AFB) smear microscopy and chest x-ray, if available, with limited mycobacterium culture facilities.

Objectives: To determine the burden of HIV infection in TB patients and to investigate the clinical and laboratory outcomes of TB patients with or without HIV infection after standard anti-TB chemotherapy

Patients and settings: 251 suspected TB patients from two health institutions in Addis Ababa, Ethiopia were prospectively enrolled to evaluate both the clinical and laboratory outcomes.

Methods: Clinical examination including chest X-ray, AFB smear microscopy and culture test were performed to diagnosis TB. The study subjects were classified as confirmed TB cases (concordant smear and culture positives) or non-TB cases (concordant smear and culture negatives).

Results: Fifty-one percent of the individuals were HIV-infected (54.8% and 44.6% in smear negatives and positives, respectively). Chest X-ray results were indicating TB to be very likely in 86.2% of the smear positives and 89.0% of the confirmed (smear and culture positive) TB cases. Twenty-four deaths occurred [mortality: 7.0/100 (PYO)]. Mortality was higher in HIV infected compared to non infected suspected TB patients (13.2% versus 5.8%, p=0.045). Interestingly, more deaths occurred in non-TB cases (smear and culture negatives) [mortality: 19.4/100 PYO] compared to confirmed TB cases [mortality: 3.7/100 PYO, p=0.02] of whom the majority (6/8) were HIV infected. There was a 51.8% cure rate in smear positive TB cases after standard anti-TB treatment, which was not different by HIV status.

Conclusion: Higher HIV infection rate in TB patients and higher rate of smear negativity was observed in HIV infected TB patients. The increased death rate in HIV infected non-TB cases suggest that HIV infected TB patients may be missed both clinically and in the laboratory. This may contribute to the higher transmission of TB and increase TB related deaths in the community.

Introduction

Tuberculosis (TB) is still one of the leading causes of mortality and morbidity in Sub-Saharan African Countries. Ethiopia is one of the highly affected countries with TB and stands 7th in the world rank. The incidence of TB (all cases) was reported to be 370/100,000 population/year with a mortality rate of 79-deaths/100,000 population/year (1). The detection and treatment success rate of smear positive TB patients using directly observed short course anti-tuberculosis chemotherapy (DOTS) in Ethiopia is 33% and 76%, respectively. Therefore, immediate and accurate diagnosis is required to provide proper treatment. In this respect, sputum smear Acid Fast Bacilli (AFB) is a critical element in the TB control program and it is still the simplest and most rapid procedure to detect acid-fast bacilli in all the laboratories in the country. Although, the sensitivity of smear microscopy is low, its specificity is reported to be higher and can be improved by examining multiple sputum samples (2,3).

To diagnose TB for subsequent treatment at different levels of health institutions smear positivity in two or more sputum samples is required. However, it has been reported that nearly 50% of the TB cases are smear negative. In one study, out of 352 suspected TB patients, 56.3% of the cases were smear negative (4). Despite the magnitude of the problem of TB in HIV infected persons, with nearly 75-100% having pulmonary diseases, smear negativity poses a challenge on the diagnosis and efficiency of treatment of HIV infected TB patients at an early stage before the immune system deteriorates. The absence of diagnostic methods other than smear microscopy and limited number of culture facilities in most of health facilities in developing world may also influence the control program to detect the undiagnosed potential transmitters in the community, especially among HIV infected individuals. Therefore, in this study we investigate the clinical and laboratory outcomes of suspected TB patients in both HIV infected and non-infected individuals

Methods

In two health institutions (Higher 23 Health Centre and All African Leprosy Rehabilitation and Training Centre (ALERT) Hospital both in Addis Ababa, Ethiopia) suspected TB patients were enrolled in this study. The suspected TB patients were clinically examined and were analyzed for Acid-Fast-Bacilli (AFB) smear microscopy and *mycobacterium tuberculosis* culture test. Besides to the clinical and laboratory investigations, chest X-rays were taken and read by three radiologists at the center to support the diagnosis and interpreted in three levels: Unlikely, probably and very likely to be TB (see definitions below). At least two agreements were required to take the results as indicated. Based on the results of AFB smear microscopy and culture confirmation (see below), the study subjects were classified into two groups: confirmed TB cases (concordant smear AFB and culture positive) and non-TB cases (concordant smear AFB and culture negative). The socio demographic characteristics of the suspected TB patients are depicted in Table-1.

Diagnosis of *Mycobacterium tuberculosis*

Diagnosis of *M. tuberculosis* in the laboratory was done using sputum Acid Fast Bacilli staining for 3 consecutive days from each suspected patient at the sites. A patient is positive, at least, if two acid-fast bacilli stained on two sputum samples for the bacilli were identified by smear microscopy. Moreover, *M. tuberculosis* culture (on LJ medium) was performed at the Ethiopian Health and Nutrition Research Institute (EHNRI) TB laboratory. The diagnosis was also supported by chest X-ray and if EPTB (TB lymphadenitis) was suspected, fine needle aspiration was done. The diagnostic methods and treatment protocols followed in this study were according to the guidelines of Ministry of Health, Ethiopia **(5).**

Definitions of parameters:

Confirmed TB cases**:** Individuals suspected to be TB patients based on clinical grounds with concordant smear AFB and culture positive results.

Confirmed non-TB cases: Individuals suspected to be TB patients based on clinical grounds with concordant smear AFB and culture negative results.

Cured: A smear positive and/or confirmed TB patient who is smear negative after taking the standard anti-TB chemotherapy for 2, 5 and 7 months of follow-up according to the guideline of the Ministry of Health, Ethiopia **[5].**

Unlikely chest X-ray result: the probability of 0-29% to be a TB patient

Probable chest X-ray result: the probability of 30-79% to be TB patient and treatment can start after having additional information such as smear result

Very likely chest X-ray result: the probability of 80-100% to be TB patient and treatment can start without any additional information

Determination of Hematological parameters, HIV testing, Plasma viral RNA level and T cell subsets.

Total white cell count, total lymphocyte counts and Hemoglobin were done using Coulter Counter (T450, BD, CA). CD4+ and CD8+ T cell numbers were determined using a standard three-color flow cytometry (FACScan, BD, CA). HIV testing was done using Determine HIV rapid test (Abbott, Japan) and followed by Vironostika Uniform II PLUS O (Organon Teknika). All positive and discrepant results were confirmed with Western Blot (HIV Blot, Version 2.2, Singapore). Plasma viral RNA levels were determined using nucleic acid-sequence based amplification assay (Organon Teknika, The Netherlands) with a minimum detection limit of <80 copies/ml.

Data analysis

Data analysis was carried out using STATA (STATA version-7, College Texas, USA). Median values were compared using Wilcoxon rank-sum (Mann-Whitney) test before and after treatment. p values <0.05 were indicative of level of significance. Mortality rates were determined assuming a Poisson distribution and compared using log-rank sum test.

Ethical clearance

The National Ethical Clearance Committee approved the study protocol and informed consent was obtained from all study participants.

Results

Study subjects

From March 1999 to April 2001, 251 suspected TB patients were enrolled and followed in two health institutions (Figure 1). Of these suspected patients, 174 had smear AFB results, 203 had culture results and 186 had chest X-ray results. Of the 251 subjects, 169 had both AFB smear microscopy and culture results. Of these, 83 of them were concordant positive cases (we will refer to them as TB cases here after) and 42 of them were concordant negatives (we will refer to them as non-TB cases). There were 16 AFB smear negative cases

and culture positive cases and there were 26 AFB smear positive and culture negative cases. Of the 251 suspected TB patients 208(82.9%) had TB related disease/illness (Table 2).

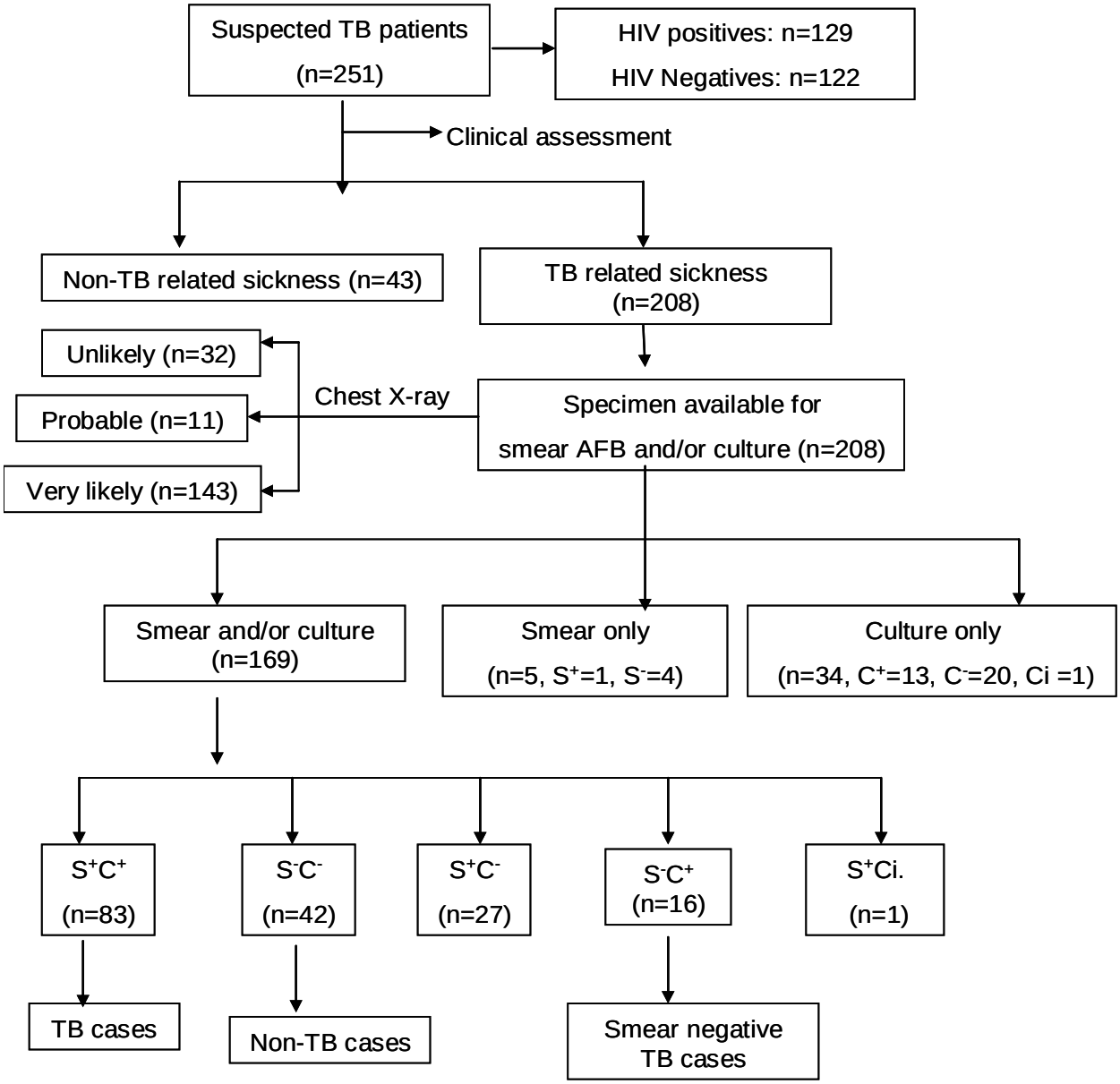

Figure-1. Distribution of study subjects (C^+: Culture positive, C^-: Culture negative, S^+: AFB Smear positive and S^- : AFB Smear negative, Ci: culture inconclusive (contaminated case))

Table-1. Socio-demographic characteristics of the study participants at enrolment

Characteristics	n(%)
Total number of suspected TB cases enrolled	251
Gender (M/F)	135/116
Age (in years)	
Total (Years, IQR)	29[23-38]
Female	27[22-35]*
Male	30[24-42]
Occupation	
Housewife	33(13.1)
Daily laborer	52(20.7)
Unemployed	47(18.7)
Students	24(9.6)
Government employee	22(8.7)
Others	73(29.1)
Education (year of schooling)	
1-6years	127(50.6)
≥7 years	120(47.8)
Unknown	4(1.6)
Average number of household living together	5[3-7]
Marital status	
Married	97(38.6)
Single, never married	100(39.8)
Divorced	27(10.8)
Widowed	14(5.6)
Others	13(5.2)
How long you reside in the area you are now (years)?	9[5-20]
Definite diagnosed TB in the past	47(18.7)
Contact tracing:	
Ever contacted with TB patient (yes)	45(17.9)
Was the person a TB patient (yes)	37(82.2)
Is the person in contact alive?	
Yes	24(53.3)
No	18(40.0)

*-p=0.004, IQR: 25th and 75th interquartile ranges

Table-2. Clinical diagnosis and outcomes of suspected TB patients

Clinical Symptoms and diagnosis	n(%)
Total number of patients examined for TB related sicknesses	208(80.9)
Dry cough	105(50.5)
Cough with sputum	121(58.2)
Chest pain	133(63.9)
Blood in sputum	35(16.8)
Short breath	95(45.7)
Fever	135(64.9)
Night sweats	168(80.7)
Tiredness	166(79.8)
Weight loss	144(69.2)
Loss of appetite	131(62.9)
Swelling around the neck, Inguinal region	47(22.6)
Diarrhea	41(19.7)
Skin rash	23(11.1)
Bone/joint tuberculosis	3(1.5)
X-ray findings (n=186)	
Unlikely	32(17.2)
Probable	11(5.9)
Very likely	143(76.9)
Tuberculosis type at diagnosis (n=156)	
Pulmonary tuberculosis (PTB)	100(64.1)
Extra-pulmonary tuberculosis (EPTB)	39(25.0)
Both mixed infections (PTB +EPTB)	17(10.9)
HIV positives	
Of the total (n=251)	129(51.4)
Of the smear negatives (n=62)	34(54.8)*
Of the smear Positives (n=112)	50(44.6)
Of the confirmed TB cases (n=83)	36(43.4)
Of the non-TB cases (n=42)	25(59.5)
Cure rate after 8 months of follow up (median)	
In smear positives (n=112)	58(51.8)$^{\varphi}$
In confirmed TB cases (n=83)	46(55.4)
Death	
Of the total (n=251)	24(9.6)
Of the smear negatives (n=62)	9(14.5)**
Of the smear positives (n=103)	9(8.0)

*-NS compared to smear AFB positives, φ-NS compared to smear AFB negative cases,**-NS compared to smear AFB positives

HIV prevalence in suspected and confirmed TB patients

Of the 251 suspected TB patients, 129(51.4%) were HIV infected. HIV positive individuals had lower CD4+ T cell number than HIV negative individuals (130.5 cells/μl; IQR: 68.5-216 cells/μl versus 504 cells/μl; IQR: 388-679 cells/μl) at enrolment ($p<0.001$) and in the last visit ($p<0.001$). Of the confirmed TB cases (n=83), 36(43.4%) were HIV-infected and of the non-TB cases (n=42), 25(59.5%) were HIV infected.

Comparison of Acid Fast Bacilli (AFB) Smear Microscopy and *Mycobacterium tuberculosis* (MTB) culture tests

Of the TB suspected individuals with AFB smear microscopy results (n=174), 112(64.4%) were smear positive (Table-2). There was no difference in smear positivity in HIV infected (59.5%) and non-infected individuals (68.9%, p=0.20). Of smear positives (n=112), 50(44.6%) and 62(55.4%) were HIV positive and negative individuals, respectively. However, among smear negatives (n=62), 34(54.8%) were HIV infected. There was no difference in CD4+ T cell number between smear positive and smear negative HIV infected suspected TB patients (124 cells/μl; IQR: 63-198 cells/μl versus 116 cells/μl, IQR; 56-193 cells/μl, p=0.62).

Among those individuals with MTB culture test results (n=203), 113(55.7%) had positive, 88(43.4%) negative and 2(0.98%) had inconclusive results (Figure-1). There were 51(52.0%) and 62(60.2%) culture positive suspected TB cases in HIV infected and non-infected individuals, respectively. Compared to the clinical diagnosis, 53(73.6%) of the PTB, 1(25%) EPTB and 1(33.3%) of the mixed infections were confirmed TB cases (concordant smear and culture positives, p=0.046). Among HIV positive subjects 33% of EPTB was observed.

Of the total study population (n=251), 208(82.5%) had smear and/or culture results. 81.0% (n=169)) had both culture and smear results, 5 (2.4%) had only smear results (1 positive and 4 negatives) and 34 (16.3%) had only culture results **(Figure-1)**. Overall, only 16(27.6%, CI: [16.6-40.8]) were smear negative TB cases (smear negatives but culture positives) resulting in 83.8% sensitivity of AFB smear microscopy. 27(24.5%) were smear positive and culture negative cases leading to a specificity of 60.9% for AFB smear microscopy. In HIV positive individuals, smear negative TB cases were observed more often in individuals with low CD4+ T cell numbers (<200 cells/μl: 5/6(83.3%)) compared to high CD4+ T cell numbers (≥200 cells/μl: 1/6(16.7%)).

Comparison of Chest X-ray results with laboratory findings

Three experienced physicians read 186 chest X-ray results and classified them in to three levels. There were 32(17.2%) unlikely, 11(5.9%) probable and 143(76.9%) very likely TB cases **(Figure-1 and Table-2)**. Of these 186 individuals, 112 had laboratory confirmed results (73 (65.2%) of them were confirmed TB and 39(34.8%) were non-TB cases). Of the confirmed TB cases (n=73), 65(89.0%) were classified as very likely to be TB patients in their chest X-ray results. However, of those with unlikely chest X-ray findings and with laboratory results (n=15), 4(26.6%) were found to be confirmed TB cases of which 3(75.0%) of them were HIV infected **(Table-3)**.

Table-3. Chest X-ray results among confirmed TB and non-TB cases by HIV status

	Total (n=112)		HIV positives (n=59)		HIV negatives (n=53)	
	TB cases (n,%)	Non-TB cases (n,%)	TB cases (n,%)	Non -TB cases (n,%)	TB cases (n,%)	Non-TB cases (n,%)
Unlikely	4(5.5)	11(28.2)	3(8.8)	9(36.0)	1(2.6)	2(14.3)
Very likely	65(89.0)	25(64.10	29(85.3)	13(52.0)	36(92.3)	12(85.7)
Probable	4(5.5)	3(7.7)	2(5.9)	3(12.0)	2(5.1)	0(0.0)

In Ethiopia, in hospitals where X-ray is available, physicians use a combination of smear AFB microscopy and chest X-ray results to diagnose active TB disease for routine diagnosis purposes. To investigate this alternative way of diagnosing TB, we combined smear AFB and chest X-ray results (AFB positives and very likely in chest X-ray result) and correlate it to the

confirmed laboratory results (smear and culture concordant). We found 65/81 (80.2%) of suspected TB patients (with smear positive and very likely chest X-ray results) were confirmed TB cases. Finally taking concordant smear and culture test results (concordant positive and negative results) as a reference and excluding probable chest X-ray results for TB disease, chest X-ray was found to have 65/69(94.2%) sensitivity, 11/36(30.5%) specificity and 65/90(72.2%) positive predictive value (PPV) to detect TB disease (Table-3).

Comparison of clinical symptoms and laboratory findings for TB diagnosis

To compare clinical diagnosis and laboratory findings, we correlate the symptoms diagnosed clinically in the suspected TB patients and laboratory results. Of those suspected patients with dry cough and laboratory results (n=62), cough with sputum (n=73) and those with blood stained sputum (n=18); 39(62.9%), 50(68.5%) and 13(72.2%) of them were confirmed to be TB patients, respectively. Moreover, from those patients with fever (n=71), 43(60.6%) of them were confirmed TB patients. Among those who reported having a contact with a TB patient (n=45), 21 of them had a laboratory results and 18(85.7%) were found to be confirmed TB cases. In individuals with past history of tuberculosis (n=47), 26 had laboratory results and 15(57.7%) of them were confirmed to have TB of which 9 of them were HIV infected.

Immunological outcomes of suspected TB patients in the follow-up period

Of the enrolled study subjects, 141(58.2%) of the suspected TB cases had CD4+ T cell count data after taking 2 months of intensive phase of anti-TB chemotherapy. However, as indicated in Table-4, there was no difference in CD4+ T cell number before and after 2 months of anti-TB therapy (p=0.20). Similarly, no difference was observed in HIV infected patients (130.5 cells/μl versus 144 cells/μl, p=0.50) after therapy.

Table-4. Immunological parameters at baseline and after two months of intensive phase of anti-TB treatment of the confirmed TB cases

Parameter	Baseline	After Treatment	P-value
Absolute lymphocyte count (cells/μl , IQR)	1236[880-1647]	1265[1075-1680]	0.14
Absolute CD4+ T cells (cells/μl, IQR)	309[125-519]	373[146-554]	0.22
Absolute CD8+ T cells (cells/μl, IQR)	528[342-810]	565[399-917]	0.25
Plasma HIV viral RNA level (HIV+, log_{10}coopies/ml, IQR)	4.9[4.1-5.4]	4.9[4.0-5.3]	0.64

* p<0.001 compared to HIV negative subjects.

Clinical outcomes of the suspected TB patients after anti-TB chemotherapy in the follow up period

(A) Cure and defaulter rates

As showed in table-2, of the treated AFB smear positive cases (n=112), 58(51.8%) were cured (became AFB smear negatives) after 8 months [7.8-8.5] of follow up. Cure rate was not different by HIV status (54.8% versus 48.0% in HIV negative and positive smear positive TB patients, respectively p=0.50). There were about 15(5.9%) defaulters of whom 11 were smear negatives, of these 6 of them were culture confirmed.

(B) Mortality rates

In the follow-up period (25.6 months), 24 (9.5%) deaths were reported giving a mortality rate of 7.0 per 100 persons years observation (PYO). It was 17(13.2%) [mortality rate: 7.8 per 100 PYO] in HIV infected compared to 5.7 per 100 PYO in HIV non -infected TB patients. The survival estimates of the study subjects by HIV status is given in figure-3. However, when deaths were classified between confirmed TB and non-TB cases, more deaths occurred in non-TB cases 8/42(19.0%) [mortality: 19.4 per 100PYO; CI: 8.8-34.9] compared to TB cases 7/83(8.4%) [mortality: 3.7 per 100 PYO; CI: 1.5-7.4], p=0.02. Of the deaths occurring in non-TB cases (n=8), 6/8(75%) occurred in HIV infected suspected TB patients.

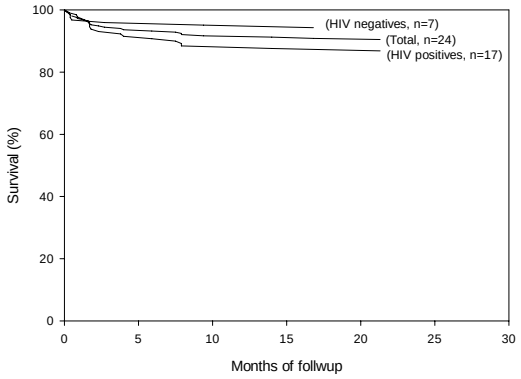

Figure-3. Kaplan-Meier survival estimates for survival rate by HIV status. Y-axis shows the percent of survival rate and X-axis shows follow up period in months

Discussion

In this study we investigated the clinical outcomes and laboratory results in a longitudinal follow-up of suspected TB patients who presented themselves in two clinics to be treated for their TB related sicknesses.

The overall HIV prevalence in the suspected TB patients was higher (51%). The rate of infection was 45% in smear positives, which confirms other studies in urban areas of Ethiopia **(7,8,9)** and other parts of Africa **(1).** Moreover, the rate of HIV infection was higher **(**57%) in smear negatives which might suggest that the majority of HIV positive patients are smear negative and may have a paramount importance on influencing the diagnosis of tuberculosis.

Although, in the general population the case detection rate for TB disease is very low (below 50%), in this study, we found over 60% of smear positivity, which was higher compared to other studies **(4, 8).** The difference might be due the nature of subjects that we had (with TB related sicknesses) or might be due to an increase in TB incidence over the past 10 years due to increase in HIV prevalence in the population **(10)**.

Unlike to the report **(8)** with a significant difference of smear positivity by HIV status, we observed no difference in smear positivity in HIV infected and non-infected individuals. Similarly, other studies also reported the absence of difference in smear positivity by HIV status **(11,12,13).** However, out of the suspected TB patients with the negative AFB smear microscopy results, more than 50% of the cases were HIV infected. This might be explained by the fact that a small number of bacilli can cause already disease in the immunocompromised individual, which can not be diagnosed using the conventional tools due to small number of bacilli in the sputum **(1)**.

The better diagnostic capacity of smear AFB microscopy for the diagnosis of PTB than ETPB **(14**) and the presence of higher number of EPTB suspected patients in HIV infected individuals (33%) than non-infected patients confirms other reports **(1).** The presence of increased level of EPTB might aggravate the problem related to diagnosis and treatment of EPTB, especially in HIV infected patients **(14)**.

In this study, using chest X-ray as a diagnostic tool, we found that 89% of the confirmed TB cases were classified as very likely to be TB patients (sensitivity > 90%), which was similar to a reported value **(8)**. Moreover, nearly 70% of the TB patients can be diagnosed using chest X-ray (positive predictive value) as reported elsewhere **(4)**. In the majority of the hospitals in Ethiopia, combinations of smear AFB microscopy and chest X-ray are being used to diagnose active TB disease. To check whether this combination is sensitive enough to detect TB patients, we compared the combined tools with the rate of TB confirmation by AFB smear and culture test and observed that the combination was able to diagnose at least 80% of the TB cases. Therefore, applying a combination of chest X-ray and smear AFB methods could help to maximize the detection rate in the diagnosis of TB disease.

The mortality rate we observed in this study was higher compared to the reported value for TB deaths for the whole population estimates by UNAIDS, 79 /100,000 population/year **(1).** This could be due to the nature of the study subjects since all of them were suspected TB patients with proximate clinical diagnosis and TB related sickness. Moreover, the low health seeking behavior of the patients (to the health institution for treatment), the health worker and the health delivery system delay to diagnose and treat TB disease immediately might contribute and add an effect on the high mortality rate that we found, as reported **(7,15)**. However, the higher death rate in non-TB cases with majority of the deaths occurring in HIV infected individuals with CD4+ T cell numbers less than 200 cells/μl indicates the effect of HIV on diagnosis of tuberculosis (more smear and culture negative results). In HIV infected suspected TB patients, chest-X ray results might have some abnormalities, which might add difficulties in the interpretation **(16, 17)**. Therefore, the diagnosis of TB in HIV infected suspected TB patient might be difficult and some patients may not be diagnosed which might add more deaths in non-TB HIV infected cases as reported **(18, 19)**.The results indicate the need for strict AFB smear microscopy diagnosis and integration of HIV and TB diagnosis services **(20)** so that either in undiagnosed TB cases or due to HIV disease progression deaths could be averted using standard anti-TB and antiretroviral therapies.
One major finding that we observed in this study was the presence of higher level of smear negative TB cases in the study subjects (27.6%), which were culture positive. This indicates AFB smear microscopy, which is being used at lower health facility levels in third world countries, leaves high numbers of undiagnosed TB cases in the community that can potentially transmit TB. This needs special attention either to improve the technique it self as indicated **(2,21)** or make available culture facilities and other molecular techniques at referral levels in the country to increase the case detection rate, which is currently below 50% **(1).**

The cure (smear conversion to negative) rate we found among smear positives is lower compared to the ultimate goal of the Ministry of Health in Ethiopia, and to those reported **(16,22,23)**. This can't be explained by the presence of multidrug resistance tuberculosis (MDR-TB) in the study subjects since the same study in the same area showed only 5.3% of the isolates were isolated with MDR-TB and this was not associated with HIV status **(24)**. Thus the treatment success rate in Ethiopia needs special attention. By increasing the detection and cure rate the development of drug resistance TB could be minimized. The absence of restoration of CD4+ T cell number and reduction of plasma viral RNA level confirm other reports in Africa, especially in HIV infected patients **(25,26,27,28).** Thus, in HIV coinfected TB patients, other factors may have influence disease progression rate there by may increase mortality.

In conclusion in this study we found higher percentage of HIV in the suspected TB patients and more deaths were occurred in non-TB cases than those confirmed TB cases. The presence of more smear negative TB patients may impose diagnostic challenges on TB diagnosis and therefore, applying strict diagnosis methods, if possible smear AFB, chest X-ray and/or culture combinations could maximize detection and treatment success rates in the TB control program.

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Tuberculin skin test conversion and reactivity rates among adults with and without Human Immunodeficiency Virus in Urban settings, Ethiopia

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Chapter 3

Abstract

To investigate whether low CD4 + T cell counts in healthy and human immunodeficiency virus (HIV)-infected Ethiopians influence TB immunological memory, tuberculin skin test (TST) conversion and reactivity rates were investigated among adults with and without HIV infection in urban settings in Ethiopia. Reaction to the TST was analyzed with Purified Protein Derivative by Mantoux technique. A total of 1,286 individuals with TST results of ≥ 5mm (n=851) and ≤4mm (n=435) indurations diameters were included. Individuals with ≤4 mm indurations sizes were followed up for 21.4 ± 9.5 months (mean ± standard deviation) to observe skin test conversion. The overall TST reactivity (≥5mm indurations diameter) was 66.2% (n=851). Reactivity was significantly lower among HIV positive persons (40.5%) than among negative persons (68.7%) (P<0.001). Of the above persons, 32 incident TB patients were checked for their TST status 13.05 ± 11.1 months before diagnosis and reactivity was found among 22(68.7%) of them. Of the TST-negative persons with 0-4mm indurations who were followed up 3 years, the conversion rate to positivity was 17.9/100 Person-Years-Observation (PYO) [14.4/100 PYO and 18.3/100 PYO in HIV positive and negative persons, respectively]. Despite lower absolute CD4+ T cell numbers in Ethiopians, higher TST conversion and reactivity rates show the presence of a higher rate of latent TB infection and/or transmission. The lower TST positivity rate before the diagnosis of TB disease showed the lower sensitivity of the test. This indicates the need for other sensitive and specific diagnostic and screening methods to detect TB infection, particularly among HIV positive persons, so that they can be given prophylactic isoniazid therapy.

Introduction

Tuberculosis (TB) is still one of the major health threats in the world. One-third of the world's population is estimated to be infected with *Mycobacterium tuberculosis*. The majority of the affected people are living in the developing parts of the world, especially in sub-Saharan Africa. TB accounts nearly for 6% of all deaths worldwide by killing every fourth person of the eight to nine million people who develop the disease **(1)** and is the foremost cause of death from a single infectious agent in adults **(2).** Infection with human immunodeficiency virus (HIV) substantially increases the risk of developing clinically apparent TB **(3)**.

The tuberculin skin test (TST) done with purified protein derivative (PPD) by the Mantoux method, which is highly sensitive but less specific than other cellular based methods, is useful for the screening of TB infection *in vivo* **(4,5,6)**. It is reported to be less sensitive among immunocompromised individuals such as those infected with HIV **(7, 8, 9).** Its prevalence of reactivity is reported to be positively correlated with absolute counts of CD4+ and total lymphocytes **(10).** Despite its poor specificity, TST is still thought to be useful to diagnose TB infection in non- HIV- infected individuals **(11, 12).**

M. tuberculosis (the causative agent of TB) and HIV are the most common infectious agents in Ethiopia as 50% of the TB infected individuals are HIV infected **(13,14,15,16).** Therefore, it is expected that a high proportion of Ethiopians could be exposed to TB and have an immunological memory for TB antigens. In this country, the national bacilli Calmette-Guerin (BCG) vaccination coverage at birth was reported to be 67% in 1997 and 72% in 2004 **(17).** However, few national surveys have been done on the tuberculin response rate in Ethiopia **(18, 19).** In addition, a lower absolute CD4+ T-cell count among healthy Ethiopian individuals compared to other population in the world, including African countries, was observed **(15, 20).** However, whether this lower absolute CD4+ T cell count among apparently healthy, HIV negative individuals influences the TST conversion, reactivity or positivity rate is unknown. Furthermore, to our knowledge, no study was conducted on the tuberculin test conversion rate and its relationship to absolute CD4+ T cell counts in HIV infected individuals in Ethiopia. Therefore, in this study, we assessed the rates of TST conversion, reactivity, and positivity and their correlation with CD4+ T cell counts among HIV-infected and non-HIV infected adult Ethiopians enrolled in a longitudinal cohort study using TST.

Materials and methods

Study subjects

To investigate TST reactivity and the rate of conversion positivity among adult Ethiopians, we analyzed longitudinal data from the HIV/AIDS natural history cohort study of ENARP at the Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia. Out of 1,701 enrolled cohort participants (since 1995), a total of 1,286 individuals with complete TST results at enrolment were included in this study. The study subjects were grouped into three categories: **(i)** individuals with TST reactive (≥5 mm diameter of skin test result, n=851) at enrollment; **(ii)** those with TST results of ≤ 4mm indurations diameter (n=435) at enrolment and followed up from 1997 to 2000 for 21.4 ± 9.3 months (mean ± standard deviation [SD]) to observe TST conversion, and **(iii)-**those clinically diagnosed incident TB cases (n=32). The third group includes those who were not TB patients at enrollment but were diagnosed as TB patients who developed the disease in the follow-up period (**Fig. 1)**. Each participant in the cohort was followed up every 6 months at the clinics of the cohort sites by a full clinical examination. Study participants were factory workers who were living in urban settings, a suburb of Addis Ababa (25 kilometers from the capital city) and 114 km southeast of Addis Ababa. The details of the study sites and study conditions have been described elsewhere

(21). All HIV-infected patients were antiretroviral agent naïve. Characteristics and selection of the study subjects are depicted in **Table-1 & Fig.1.**

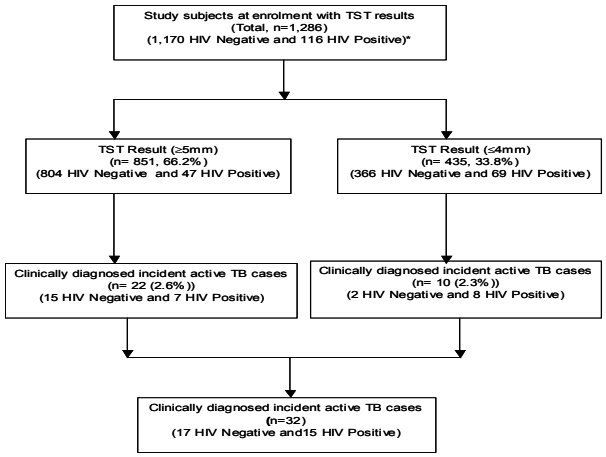

Figure-1. Flowchart for the selection and composition of the study subjects, *- This is a longitudinal HIV/AIDS natural history study conducted by Ethio-Netherlands AIDS Research project (ENARP) at the Ethiopian Health and nutrition Research Institute (EHNRI), in the period 1995 to 2003, Addis Ababa, Ethiopia.

TST

The TST was done by the Mantoux method. At the forearm, 0.1 ml of PPD (RT-23 SSI 2TE, Staten Serum institute, Copenhagen, Denmark) was intradermally injected and results (indurations diameters) were read by an experienced physician after 48 to 72 h. Two perpendicular diameters of the skin indurations were measured, and the average was taken for interpretation. The interpretation was based on four cut off values: anergy or no response (0mm indurations diameter), negativity (>0mm to ≤4mm indurations diameter), reactivity (≥5mm indurations diameter), and positivity (≥10mm indurations diameter). However, TST results for HIV positive persons were considered positive if indurations sizes were ≥5mm according to the recommendation given by the National Tuberculosis and Leprosy Control and Prevention Program, Ministry of Health, Ethiopia **(8, 13)**. TST conversion to positive was indicated by an increase in indurations diameter of 10mm or more over a previously negative TST result (< 5mm indurations diameter) over a period of 2 years **(8, 22, 23, 24).** Conversion rates are expressed in persons-years of observations (PYO).

Laboratory methods

HIV testing and T-cell subset determination

Blood samples collected from each participant during each visit were tested for HIV using HIVSPOT (Genelab Diagnostics, Singapore) and/or Determine (Abbott Laboratories) and enzyme-linked immunosorbent assay (Vironostika Uniform II PLUS O; Organon Teknika, Boxtel, The Netherlands)). Western Blotting (HIV Blot 2.2, Genelab Diagnostics, Singapore) was used to confirm reactive samples. Lymphocyte subsets were analyzed by standard three-color flow cytometry (FACScan, Becton Dickinson, San Jose, CA). Plasma HIV plasma RNA levels were analyzed by a nucleic acid sequence-based amplification assay (Organon Teknika, Boxtel, The Netherlands).

Diagnosis of *Mycobacterium tuberculosis*

Diagnosis of *M. tuberculosis* was done by sputum Acid Fast Bacilli staining for 3 consecutive days for each suspected patient. A patient is positive, at least, if two acid-fast bacilli stained sputum samples for the bacilli were identified by smear microscopy. Furthermore, culture was performed if requested by the physicians. The diagnosis of TB was also supported by chest X-ray and pathology. Treatment was provided according to the guidelines of Ministry of Health, Ethiopia **(13).**

Ethical consideration

This study is a part of a longitudinal study of the natural history of HIV/AIDS in Ethiopia, which was ethically cleared by the National Ethical Committee. Each study participant gave consent to participate in the study.

Statistical analysis

Analysis was performed with STATA (Intercooled STATA, version 7, Stata Corporation, College Station, TX). TST reactivity differences (medians) were compared by the nonparametric Mann-Whitney U test. A p value of less than 0.05 was considered indicative of statistical significance. The incidence of TST conversion was computed by assuming a Poisson distribution of events. Kaplan-Meier analysis was performed to compare conversion rates by HIV status. Incidence curves were compared by log-rank test.

Results

Characteristics of the study subjects

A total of 1,286 study subjects had complete TST results. The percentage of males was higher (77.6%). Age was not significantly different between males (34 years; Inter Quartile Range [IQR], 29 to 40 years] and females (35 years; IQR, 30 to 39 years, p=0.284) or between those with and without HIV (p=0.78). Of the total study subjects, 116(9.0%) were HIV positive. HIV negative persons had higher absolute CD4+ T cell counts (693 cells/μl; IQR, 544 to 853) than HIV positive persons (326 cells/μl; IQR, 197 to 483;, p<0.001) **(Table-1).**

able-1. Characteristics of the study population (total n= 1,286), those with TST result ≥5mm (reactive cases, n=851), with ≤4mm (follow-up cases**,** n=435) and active TB incident cases (n=32).

Characteristics	**Total (n=1,286)**	TST ≥5mm (**n=851)**	TST ≤4mm **(n=435)**	active incident TB cases **(n=32)**
Sex (male, n, %)	998(78.1)	665(78.1)	333(76.5)	26(81.2)
Age (Years, IQR)				
HIV Positives	33[30-39]	32[30-39]	34[30-39]	35[30-38]
HIV negatives	35[29-40]	35[29-40]	35[29-40]	39[32-42]
BCG scar (yes, n, %)	535(41.6)	385(45.2)	150(34.5)	9(28.1)
HIV antibodies (n, %)	116(9.0)	47(5.5)	69(15.9)	15(46.9)
CD4 + T cells (Cells/μl, IQR)	326[197-483]*	363[252-483] *	306[167-462] *	237[140-375]
HIV positives	693[544-853]	713[576-865]	660[509-819]	NA
HIV negatives				
CD4+ T cell category				
<200 cells/μl (n, %)	29(6.2)	7(24.1)**	22 (11.8)	NA
≥200 cells/μl (n, %)	441(93.8)	277(62.8)	164(88.2)	
HIV Plasma viral RNA (log_{10} copies/ml)	3.9[3.2-4.7]	3.9[3.3-4.6]	3.8[3.3-4.8]	4.3[3.4-4.9]

n- number of cases, %-percentage, IQR: 25th and 75th Inter Quartile Ranges, *- p<0.001 compared to HIV negatives using Mann Whitney U test, **-p<0.001 compared to ≥200 cells/μl CD4+ T cell count (χ2 test) , NA- complete data was not available.

Tuberculin Skin Test (TST) reactivity

In 1,286 participants with TST results at enrollment, the overall reactivity rate (≥5mm indurations diameter) was 851(66.2%) and was not different by year (1997 to 1999). The reactivity rate of HIV negative persons (68.7%) was higher than that of HIV positive persons (40.5%), p<0.001. TST positivity (≥10mm indurations diameter) among HIV negative persons was 56.1% (**Fig. 2)**. Skin test indurations sizes were higher in HIV negative persons (median, 10mm) than in HIV positive persons (median, 4 mm; p<0.001) (data not shown). However, the presence of anergy (0 mm indurations diameter or no response) was higher (45.7%) in HIV positive persons compared to negative subjects (24.4%; p<0.001 **(Fig. 2).** Of 1,284 subjects with BCG results, 535(41.7%) had a scar.

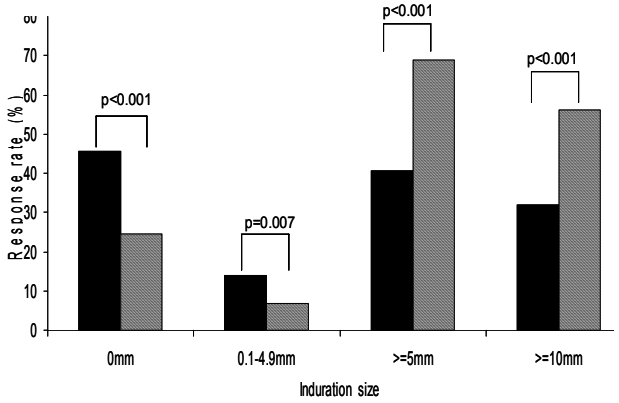

Figure-2. TST response rates among the total subjects (n=1,286) at different TST indurations for HIV negative (gray bars) and HIV positive (black bars) individuals (including the incident TB cases in both groups).

TST reactivity was higher in those individuals with a BCG scar than in those without a scar (71.9% versus 62.1%, p<0.001), which might be the effect of BCG. Furthermore, among those without a BCG scar, reactivity was higher in HIV negative persons (64.1%) than HIV positive persons (43.1%; p<0.001 (data not shown).

Tuberculin Skin Test (TST) response in relation to absolute CD4+T-cell count

To investigate whether TST reactivity is influenced by the degree of HIV-infection **(5)**, we analyzed CD4+ T cell numbers in relation to reactivity. In TST reactive cases, HIV negative persons had higher absolute CD4+ T cell counts (713cells/μl; IQR, 576 to 865) than HIV positive persons (363cells/μl; IQR, 252 to 483), (p<0.001). TST reactive persons (≥ 5mm indurations diameter) had higher absolute CD4+ T-cell counts (673.5 cells/μl; IQR, 511 to 822.5 cells/μl) than anergic (no response) persons (538 cells/μl; IQR, 333 to 761; data not shown) (p<0.001). When the absolute CD4+ T cell counts were classified into two categories (<200 and ≥200 cells/μl), TST reactivity was higher in those with absolute CD4+ counts ≥200 cells/μl (24.1% versus 62.8 %, p<0.001), whereas anergy (no response) was higher in those with <200 cells/μl (72.4% versus 30.8%, p<0.001) **(Table-1)**. The same was true of HIV positive individuals. Logistic regression analysis showed an increase in TST reactivity with a BCG scar (odds ratio [OR], 1.5, confidence interval [CI], 1.1 to 1.9; p<0.01), and a decrease with HIV infection (OR, 0.39; CI, 0.26 to 0.57; p< 0.001). Reactivity increased with the absolute CD4+ T cell number in the study subjects (OR, 1.00; CI, 1.00 to 1.00; p<0.001).

Tuberculin Skin Test (TST) conversion rate

To analyze the TST conversion rate, 435 individuals (0 to 4 mm indurations diameters at enrolment) were followed up for a mean follow up time of 21.4 months (± 9.3 months [SD]). With an indurations diameter increase of ≥10 mm from the previous negative result as the cutoff, the conversion rate was 14.3/100 PYO (CI, 7.9 to 25.9/100PYO) and 18.3/100 PYO (14.8 to 22.6/100PYO) in HIV positive and negative persons, respectively (p=0.453). Even after using an indurations diameter cut-off value for conversion in HIV positive persons of ≥5mm (since reactivity for HIV positive persons was defined as an indurations diameter of ≥5mm), the conversion rate was 16.2/100 PYO (CI, 9.2 to 28.6), which was not statistically significantly different from the ≥10mm indurations increase. Among HIV-negative persons, an increasing trend in TST conversion was observed in the years 1997(14.9/ 100 PYO), 1998(21.5/100 PYO), and 1999(20.6/100 PYO). Moreover, a higher rate of conversion (30.2/100 PYO; CI, 22.3 to 40.9) was observed among those HIV-negative persons with a BCG scar compared to those without a scar (13.2/100 PYO; CI, 9.8 to 17.8/100PYO; p=0.0001 (**Table-2).** HIV-positive persons with absolute CD4+ T cell counts <200 cells/μl had a lower TST conversion rate (4.9/100 PYO; CI, 0.7 to34.7/100PYO) than those with absolute CD4+ T cell counts ≥ 200 cells/μl (19.9/100 PYO; CI, 10.7 to 37.1/100PYO). Almost 50% of the study subjects converted within 2.5 years a different between HIV-positive and HIV-negative persons (**Fig. 3)**.

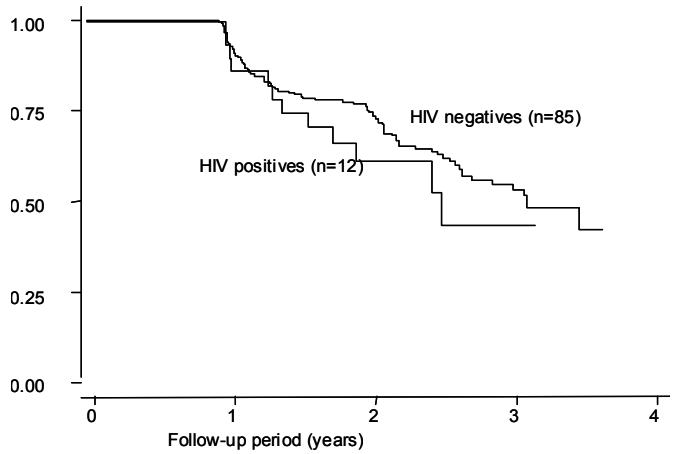

Figure-3. Kaplan-Meier survival estimates for the rate of TST conversion by HIV status. Y-axis shows the percent of conversion rate and X-axis shows follow up period in years

Table-2. Incidence of TST conversion rates among follow up cases (≤ 4mm indurations, n=435) in the period 1997 to 2000.

	Incidence of Conversion rate			
	n/PYO	Rate per 100 PYO	95% CI	p-value
Overall TST conversion (≥10mm increase) *	99/552.6	17.9	14.7-21.8	
HIV: Positives (≥5mm increase)	12/73.9	16.2	9.2-28.6	
Positives (≥10mm increase)	11/76.4	14.4	7.9-25.9	
Negatives (≥10mm increase)	85/464.6	18.3	14.8-22.6	ns
BCG Scar (HIV negatives):				
Yes	42/139.1	30.2	22.3-40.8	
No	43/325.6	13.2	9.8-17.8	0.0001
Gender (HIV negatives):				
Female	11/87.1	12.6	6.9-22.7	
Male	53/230.2	23.0	17.6-30.1	0.06
Year (HIV negatives):				
1997	32/214.3	14.9	10.5-21.1	
1998	33/153.4	21.5	15.2-30.2	
1999	20/96.9	20.6	13.3-31.9	ns
CD4 count (cells/mm^3, HIV positives)				
<200	1/20.4	4.9	0.7-34.7	
≥200	10/50.1	19.9	10.7-37.1	ns

95% CI – 95% confidence intervals, n-number of converted cases, PYO-Person-year-Observation in the years 1997-2000, p-values- Mantel-Haenszel**,** ns- statistically not significant, *-the overall inicidence rate of conversion was calculated based on an increase ≥10 mm skin test induration size, since 88.5% of the converters were from HIV negatives

Tuberculin Skin Test (TST) results among clinically diagnosed incident TB cases

As indicated in Materials and Methods, in this group, 32 incident TB cases (22 with ≥ 5 mm and 10 with ≤4mm indurations sizes at enrolment) were included to examine TST reactivity and positivity; 17 of them were HIV-negative and 15 HIV- positive. As shown in Fig. 1, the occurrence of TB in the total study group (n=1,286) was higher in HIV positive persons (12.9%) than HIV negative persons (1.4%), as reported earlier **(15)**. Among individuals with HIV infection, the median absolute CD4+ count and viral RNA level were 237 cells/μl and 4.3 $\log_{10}$copies/ml of plasma, respectively (**Table-1**).

On average at 13.05±11.1 months (mean ± SD) before the diagnosis of clinical TB, 68.7% of the 32 persons had reactive (≥ 5mm indurations diameter) TST results. Reactivity was higher in HIV-negative persons (88.2%) than HIV-positive persons (46.7%), p<0.001. However, a negative response (anergy or a 0mm indurations diameter) was higher among HIV positive persons (46.7%) than negative persons (5.9%) (p=0.008), which clearly indicates the effect of HIV on the test (data not shown).

A second TST result from 21 (65.6%) of TB incident cases at an average of 14.7±10.7 months (mean ± SD) after the diagnosis and treatment of TB showed that TST reactivity (≥5mm indurations diameter) was increased to 17/21(80.9%).

Discussion

TST reactivity and conversion are considered to be indicative of infection of individuals with *M. tuberculosis.* In addition, a higher risk of developing active TB within two years following conversion is well documented **(22, 25, 26).** In this study, we investigated the rates of TST conversion and reactivity in adults with and without HIV infection in urban settings. A TST conversion rate higher than in other reports **(23,26,27)** was found and it was comparable to the observations in Mexico **(28).** The higher conversion and reactivity rates among HIV negatives despite lower absolute CD4+ T cell numbers might be attributable to latent TB infection and a higher incidence of TB or transmission of TB in the population **(13,14).**

Despite the effect of HIV on TST conversion, reactivity, and anergy **(8, 29),** the observed reactivity among HIV positive persons (40.5%) was still lower than those in United States (52.3%), Haiti (65%), and Uganda (73.8%) **(28, 29,30).** This could partially be explained by the presence of lower absolute CD4+ T cell counts in Ethiopians **(20)**. However, the role of other factors such as nutrition and concomitant infection among HIV positives persons (parasitic infections) could not be ruled out.

The finding of higher absolute CD4+ T-cell counts in TST reactive persons than anergic persons is in agreement with other studies **(11)** and could be due to an increase in the TST response rate when the immune system is intact **(10).** An increase in the TST reactivity rate among those clinically diagnosed incident TB cases after TB treatment was found. this suggests that TB affects the immune system negatively and that treatment reverses this. Other studies also showed that the *in vitro* PPD response rate was increased after treatment of helminthic infections among Ethiopians **(31).**

The lower sensitivity of TST to detect clinically diagnosed TB cases before their clinical presentation of TB, the presence of higher incidence and prevalence of TB and HIV coinfection in the country **(14, 16),** and the presence of a higher prevalence of anergy among HIV positive persons compared to other reports **(10,30)** show the need for other sensitive and specific methods to help the detection of *M. tuberculosis* infection among HIV infected

individuals. This is due to the increased risk that anergic persons will develop TB **(11, 32, 33).**

The occurrence of equal percentage of TST conversion among HIV positive and negative individuals was most probably due to the presence of a higher TB incidence, which might indicate both groups had equal chances *M. tuberculosis* exposure and/or infection. However, HIV positive individuals are at higher risk of developing TB than HIV-negative persons **(15).** The occurrence of a higher conversion rate among those individuals with a BCG scar might indicate true conversion due to recent infection than boosting effect of BCG since these individuals were TST negative at enrolment.

In conclusion, in this study, with the TST as readout, the higher percentage of reactivity and conversion is probably a reflection of a higher rate of latent TB infection and transmission in the country. However, the lower sensitivity of TST among the incident TB cases, negative effect of HIV on the test, the absence of sufficient numbers of supportive TB culture facilities, and the lower ability of the smear microscopy diagnostic method (~20%-50%) to detect TB infection, will all have a negative effect on the treatment of persons with undiagnosed cases in the population (who are the potentials transmitters of TB). Thus, the data presented here will contribute to the efforts made to control and preventing TB infection by increasing the awareness of latent TB infection, conversion rates and the problems related to TST, particularly among HIV positive persons. Therefore, introducing alternative sensitive, specific and affordable diagnostic and screening methods for TB into HIV testing schemes could probably help to reduce the spread of TB by allowing cases to be treated earlier and latently infected HIV infected individuals to benefit from Isoniazid prophylaxis.

Acknowledgements

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PART-II

THE ROLE OF MTB AND HIV IN IMMUNE ACTIVATION AND DISEASE PROGRESSION

Increased CD4+ T cell proliferation level in tuberculosis patients coinfected with Human Immunodeficiency Virus before and after the treatment of active tuberculosis

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Chapter 4

Abstract

Objective: To investigate the role of *M. tuberculosis* and anti-TB chemotherapy on immune activation in HIV/TB coinfection

Methods: Measurement of intracellular expression of Ki-67 nuclear antigen as a marker for immune activation within naïve and memory T cells in tuberculosis (TB) patients with and without HIV before and after anti-TB chemotherapy.

Results: Increased Ki-67-expressing CD4+ T-cells in $HIV^{+}TB^{+}$ coinfected (13.2%) compared to asymptomatic HIV-infected individuals (7.1%), TB patients without HIV-infection (3.6%) and healthy controls (1.9%) was observed. In contrast, absolute number and percentages of naïve CD4+ T cells were lower in coinfected individuals compared to other groups. Compared to asymptomatic HIV infected individuals, T-cell proliferation rates were higher in coinfected individuals with ≥200 CD4+ T cells/μl but not with <200 CD4+ T cells/μl. The percentage of Ki-67+CD4+ T cells was positively correlated with plasma viral RNA level and negatively with absolute CD4+ T-cell counts. The latter was lost after controlling for plasma viral RNA level, which suggests HIV-induced immune activation. Strikingly, T-cell proliferation rates decreased after anti-TB chemotherapy in TB patients without HIV infection but not in $HIV^{+}TB^{+}$ coinfected individuals. ART decrease the rate of proliferation in coinfected individuals.

Conclusions: Increased levels of CD4+Ki-67+T cells in HIV+TB+ coinfected patients which paralleled higher plasma HIV viral RNA level suggests that TB-related immune activation in coinfected individuals sustained replication of HIV which could lead to faster disease progression due to naïve CD4+ T-cell depletion. The absence of reduction of plasma viral RNA level and immune activation after anti-TB chemotherapy indicate disease progression rate may not be reversed despite anti-TB therapy. The presence of higher proliferation rates in $HIV^{+}TB^{+}$ individuals with higher CD4+ T cell count provide evidence for TB as a cause for immune activation due to a new infection, whereas TB occurrence in individuals with lower CD4+ T cells suggests that it occurs as an opportunistic infection after reactivation of latent TB.

Introduction

In sub-Saharan African countries, *Mycobacterium tuberculosis* (MTB) and human immunodeficiency virus (HIV) infections remain to be the major health problems as 50-70% of tuberculosis (TB) infected individuals are HIV infected [1]. It is well documented that HIV increases the risk of TB infection either by predisposing to new infections or by reactivating latent infections [2, 3, 4]. On the other hand TB has been shown to enhance HIV viral replication especially at higher CD4+ T-cell numbers [5-9]. The effect could be minimized after treatment of TB disease, which leads to a decrease in plasma viral RNA levels in Caucasians [10, 11]. However, an absence of reduction of plasma HIV viral RNA level despite the resolution of TB disease was observed among treated individuals in African countries [12-20].

HIV infection has been associated with persistent immune activation. Even more, the presence of high immune activation levels (especially higher levels of Ki-67 expression) on T cells was shown to be the best predictor for HIV-disease progression [21,22]. Persistent immune activation has been suggested to play a major role in CD4+ T-cell depletion and may have a more detrimental effect on the naïve population. Not only HIV infection but also other chronic antigen stimulation has been shown to induce persistent high immune activation. In healthy Ethiopians lower numbers of CD4+ T cells (especially naive CD4+ T cells) and high levels of T-cell activation as measured by CD38 and HLA-DR expression were described [23]. The high level of immune activation is probably caused by exposure to environmental pathogens such as parasites [24]. Tuberculosis, as a common pathogen, may therefore also contribute to chronic/persistent immune activation. Furthermore, HIV-infected individuals with TB disease may have further enhanced immune activation levels, leading to faster disease progression.

To investigate the rate of immune activation, the expression of the proliferation marker Ki-67, a nuclear antigen expressed in late G1, S, G2 and M phase but not in G0 state of the cell cycle, is reported to be an accurate marker [25,26,27]. In this study, we investigated the effect of TB infection on immune activation and its role in disease progression. In addition, the effect of anti-TB chemotherapy on immune activation in the presence or absence of HIV coinfection was studied. To this end, we measured the expression of Ki-67 nuclear antigen intracellularly within CD4+ and CD8+ T cell subsets (including naïve and memory phenotypes) in TB patients without HIV infection and HIV^+TB^+ coinfected individuals before and after anti-TB chemotherapy. Furthermore, to study the consequences of higher immune activation on the levels of CD4+ T cells in HIV-TB coinfection, we analyzed the number of naïve T-cells within the CD4+ T cells.

Patients and methods

Study population

In this study we conducted a two-step investigation of T cell division/proliferation rate in HIV^+TB^+ coinfected individuals.

First, to investigate the effect of TB on T cell division/ proliferation rates with and without HIV coinfection, we performed a comprehensive cross-sectional investigation in four different groups of study subjects: TB patients infected with HIV (HIV^+TB^+, n=18), asymptomatic HIV infected persons (HIV^+TB^-, n=27), TB patients without HIV infection (HIV^-TB^+, n=26) and healthy controls (HIV^-/TB^-, n=20). All TB and HIV or coinfected groups were studied before both anti-TB or antiretroviral therapy (ART). The characteristics of the groups are depicted in Table-1. There was no age difference among the groups. In all groups the percentage of males was higher.

Table-1. Characteristics of studied subjects in the absence of anti-TB chemotherapy in TB patients coinfected with HIV (HIV^+TB^+), asymptomatic HIV infected individuals (HIV^+TB^-), TB patients without HIV infection (HIV^-TB^+) and Healthy controls.

Parameters	HIV^+TB^+ (n=18)	HIV^+TB^- (n=27)	HIV^-TB^+ (n=26)	Controls (n=20)
Gender (F/M)	6/12	7/20	14/12	7/13
Age (Years, IQR)	30[25-40]	34[30-39]	26[20.37]	34[31-39.5]
Absolute CD4+ T cell count (cells/μl)	133[76-326]*	252[186-491]*	627[458-751]*	811.[690.5-950]*
Absolute CD8+ T cell count (cells/μl)	647.5[306-1294]	1188[889-1815]	517[418-622]	554.[387.5-714.5]
Plasma HIV viral RNA level (Log_{10}copies/ml)	4.9[4.5-5.6]**	4.4[3.9-5.1]	NA	NA
Antiretroviral therapy	Naïve	Naïve	NA	NA

Numbers in brackets are IQR: 25th and 75th inter quartile range, *-p<0.001 in all comparisons in the groups with each other, **-p<0.001 when compared to HIV^+TB^- subjects

Second, to investigate the effect of anti-TB chemotherapy on T cell division/proliferation rate in HIV^+TB^+ coinfection; we studied two groups of coinfected individuals. TB patients coinfected with HIV after 3.6 ±1.9 months (mean ± standard deviation [SD]) of anti-TB chemotherapy (group-I, n=15) and HIV^+TB^+ coinfected individuals before and after 2 months of ART (n=9), (group-II). Furthermore, TB patients without HIV infection (n=16) after anti-TB chemotherapy (follow-up of the cross-sectional TB group) were included to investigate the effect of anti-TB chemotherapy on T cell division rate without HIV infection. The characteristics of the groups are given in Table-2.

Table-2. Characteristics of studied subjects after anti-TB chemotherapy: TB patients coinfected with HIV (HIV^+TB^+, naive to ART), TB patients coinfected with HIV (HIV^+TB, before and after ART) and TB patients without HIV infection (HIV^-TB^+).

	HIV^+TB^+ (n=15)*	HIV^+TB^+ ART Cases (n=9)		HIV^-TB^+ subjects (n=16)**
		Before (n=9)	After (n=9)	
Gender (F/M)	5/10	2/7	2/7	8/8
Age (Years, IQR)	37[30-43]	38[36-48]	-	25[18.5-40.5]
Absolute CD4+count (cells/μl)	147[58-295]	64[45-78]	191[135-238]$^{\phi}$	517[397-773]
CD8+absolute count (cells/μl)	1372[867-1678]	870[540-1465]	853[617-1113]	512[338-658]
Plasma HIV viral RNA level (Log_{10}copies/ml)	5.5[4.9-5.9]	5.9[5.6-6.3]	3.1[2.7-3.2]	NA
Antiretroviral therapy	Naive	Naive	Yes	NA

Number in brackets are IQR: 25th and 75th inter quartile ranges, *-Cross sectional data after a mean of 3.6 ± 1.9 months anti-TB chemotherapy, **-longitudinal data after a median of 2.1± 1.0 months anti-TB chemotherapy, NA-not applicable, $^{\phi}$p<0.001 compared to the baseline CD4+ T cell count.

Patients were prospectively recruited and followed from the following clinical Centers. (i) The All African Leprosy Research and Training Centre (ALERT) and Higher 23 Health Centre,

both in Addis Ababa, Ethiopia (ii) from two cohort sites of the Ethio-Netherlands AIDS Project (25 and 112 kilometers from Addis Ababa) and (iii)- from St. Paul Hospital, Addis Ababa, Ethiopia. Patients were enrolled after thorough clinical evaluation by primary clinicians at the respective health centers. Informed consent was obtained from all study participants and the study protocol has been reviewed and approved by Institutional and National Ethical Clearance Committee's.

Diagnosis and treatment of tuberculosis disease

Diagnosis of MTB infection was done by sputum acid-fast bacillus staining for 3 consecutive days for each suspected patient. A patient was considered positive, at least, if two acid-fast bacillus-stained sputum samples positive for the bacilli were identified by smear microscopy. Furthermore, culture was performed if requested by the physicians. The diagnosis of TB was also supported by chest X-ray and pathology. Treatment was provided according to the guidelines of the Ministry of Health, Ethiopia [28].

Flow Cytometry

To measure Ki-67 nuclear antigen intracellularly, CD4- and CD8- PerCP (Peridinin chlorophyll Protein), CD45RO-PE (Phycoerythrin) monoclonal Antibodies (mAbs) (all from Becton Dickinson, San Jose, CA), Flourescein Isothiocyante (FITC) labeled Ki-67 mAb (Monosan, The Netherlands), Biotinylated-CD27 (Sanquin, The Netherlands) and Streptavidin- Allophycocyanine (Strep-APC)(Molecular Probes) were used. Naïve and memory T cell phenotypes were defined as CD45RO-CD27+ and CD45RO+CD27+, respectively [29, 30].

Peripheral Blood Mononuclear Cells (PBMCs) were thawed with RPMI (20% Fetal Calf Serum (FCS), with L-glutamine, 1% Streptomycin-Penicillin) and centrifuged at 1400 RPM for 5 minutes. The cells were then washed with RPMI (10% FCS), PBA (Phosphate Buffer Saline with 0.05% Bovine Albumin) and stained with CD4+, CD8+, $CD45RO^{+}$ and Biotinylated-CD27 mAbs for 20 minutes in dark. After additional washing step, the cells were stained with Strep-APC for 20 minutes. The stained cells were then washed (PBA), lysed and permeabilized with lysing and permeabilizing solution, respectively (Becton Dickinson) for 10 minutes (each) and stained intracellularly with mAb of Ki-67 for 20 minutes in dark. After fixing (Cell fix solution, BD) the cells were analyzed using a four-color FACSCalibuir with Cellquest software.

Plasma HIV viral RNA level

Plasma HIV viral RNA levels were measured using nucleic acid-based amplification assay (Organon Teknika, The Netherlands). HIV RNA concentrations below the detection limit of the NASBA assay (<80 copies/ml of plasma) were considered at 80 copies/ml.

Statistical analysis

Median CD4 counts and plasma HIV levels were compared using the nonparametric Mann-Whitney *U* test. The Spearman's rank test was used to assess correlation unless indicated. A *p* value of less than 0.05 was considered indicative of statistical significance. The statistical analysis was performed using STATA (Intercooled STATA, Version-7, and College Station, Texas).

Results

Higher levels of Ki-67 expression within CD4+ and CD8+ T cells in HIV^+TB^+ coinfected individuals

To study the effect of TB on T cell division rates in individuals with and without HIV co-infection, we performed a cross-sectional investigation of Ki-67 expression in CD4+ and CD8+ T cell subsets of TB patients infected with HIV (HIV^+TB^+), asymptomatic HIV infected persons (HIV^+TB^-), TB patients without HIV infection (HIV^-TB^+) and healthy controls (HIV^-/TB^-). In HIV^+TB^+ coinfected patients (HIV^+TB^+), absolute numbers of CD4+ T cells were lower (median: 133.5 cells/μl) compared to asymptomatic HIV-infected persons (HIV^+TB^-; 252 cells/μl), TB patients without HIV-infection (HIV^-TB^+; 627 cells/μl) and healthy controls (811.5 cells/μl) (test for trend, $p<0.001$). Plasma HIV viral RNA level was higher in HIV^+TB^+ coinfected patients (median: 4.9 $\log_{10}$ copies/ml) than HIV^+TB^- groups (4.4 $\log_{10}$ copies/ml, $p<0.001$), (Table-1). The difference in viral load was only significant in individuals with CD4+ T cell counts ≥200/μl ($p=0.04$).

The proportion of Ki-67 expressing CD4+ T cells was significantly higher in HIV^+ TB^+ coinfected patients (13.2%) than HIV^+TB^- persons (7.1%, $p=0.02$), HIV^-TB^+ patients (3.6%, $p<0.001$) and healthy controls (1.9%, $p<0.001$, Fig. 1A). A 4 and 2 fold increase due to HIV and TB alone in Ki-67 expression within the CD4+ T cells was observed, respectively. In the CD4+ memory T cells Ki-67 expression was higher in the HIV^+TB^+ group (15.8%) than HIV^+TB^- (8.8%, $p=0.05$), HIV^-TB^+ (3.9%, $p<0.001$) and controls (1.8%, $p<0.001$). A similar pattern was observed in the naïve compartment; there was 4.1% Ki-67 expression in HIV^+TB^+, 2.9% in HIV^+TB^-, 1.8% in HIV^-TB^+ and 1.2% in healthy controls and it was higher in HIV^+TB^+ coinfected groups compared to other groups ($p<0.02$) except HIV^+TB^-groups ($p=0.85$). We observed a 4-fold increase due to HIV or a 2.2 fold increase due to TB alone on Ki-67 expression in the memory CD4+ T cells. A 2.4 fold increase due to HIV or a 1.5 fold increase due to TB alone in proliferation rate was observed in the naïve CD4+ T cell population (Fig. 1A-C). These data indicate that the increased Ki67 expression in CD4+ T cells is not merely due to a shift in naïve and memory ratio.

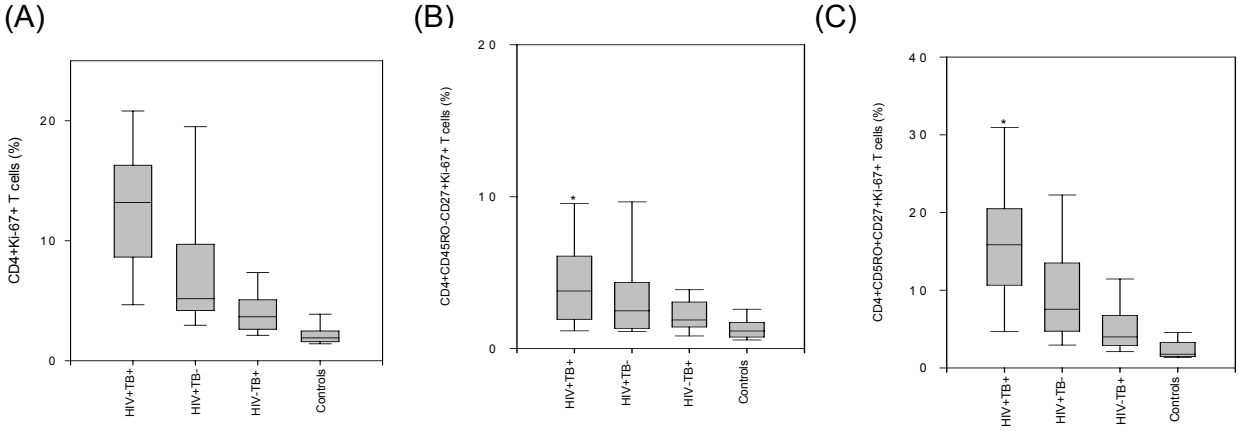


Figure-1. Expression of $Ki67^+$ T cells within **(A)-** total CD4+ T cells **(B)**- within naïve and **(C)** within memory CD4+ T cells (Boxes: 5th and 75th ranges, confidence interval are 5th and 95th percentiles and bar lines are medians, *-indicates the absence of significant difference between HIV+TB+ and HIV+TB- groups, **- p=0.07, all other comparisons were statistically significant)

The expression of Ki-67 in HIV^+TB^+ individuals within CD8+ T cells was higher than TB patients without HIV infection (6.5% versus 2.8%, $p<0.001$) and healthy controls 1.9%, $p<0.001$). Interestingly, unlike for CD4+ T cells, Ki-67 expression was not higher in HIV^+TB^+ compared to HIV^+TB^- groups (6.5 % versus 6.0%, $p=0.71$). HIV alone induces a 3-fold increase while TB does not induce a relevant increase in Ki-67 expression within the CD8+ T cell population (Fig. 1B). Memory CD8+ T cells in HIV^+TB^+ coinfected individuals expressed higher levels of Ki-67

(9.3%) than TB patients without HIV (5.8%, p<0.001) and healthy controls (3.2%, p<0.001) but not higher than HIV^+TB^- individuals (9.2% p=0.7). Similarly in naïve CD8+ T cells, HIV^+TB^+ coinfected individuals expressed more Ki-67 than TB patients without HIV infection (3.4% versus 1.6%, p<0.001) and healthy controls (1.2%, p<0.001) but not higher than HIV^+TB^- individuals (3.7%, p>0.05). There was a 3 and 2 fold increase in Ki-67 expression due to HIV and TB alone, respectively (Fig. 2A-C).

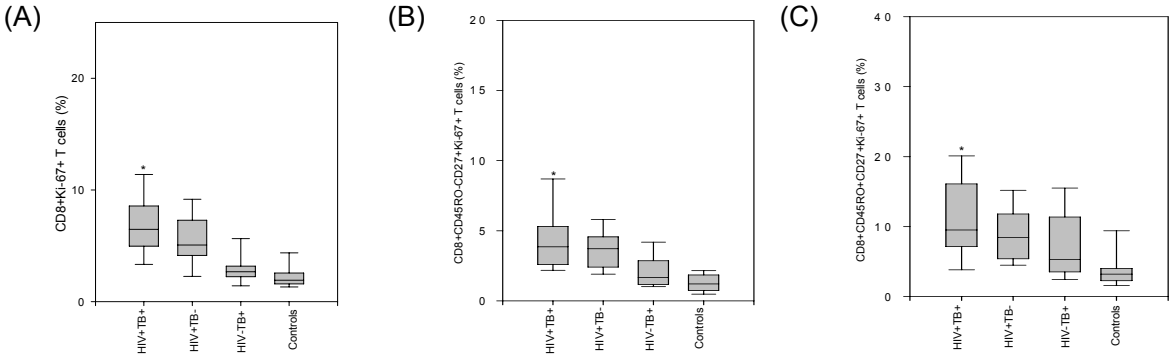


Fig. 2.- Expression of $Ki67^+$ T cells **(A)** within the total CD8+ T cells **(B)** within the naïve CD8 + T cells **(C)** within the memory CD8+ T cells (Boxes: 5th and 75th ranges, confidence interval are 5th and 95th percentiles and bar lines are medians, *-indicates the absence of significant difference between HIV+TB+ and HIV+TB- groups, **- p=0.07, all other comparisons were statistically significant)

Low numbers of naïve CD4+ T cell in HIV^+TB^+ coinfected individuals

As high levels of immune activation may lead to lower numbers of CD4+ T cells, and naïve T cells in particular, we investigated the number of naïve CD4+ T cells as defined by expression of CD27 and lack of CD45ROexpression. Indeed, the absolute number of naïve CD4+ T cells was lower in HIV^+TB^+ coinfected individuals before anti-TB chemotherapy (28.5 cell/μl) than TB patients without HIV infection (144.2 cells/μl, p<0.001) and healthy controls (205.1 cells/μl, p<0.001) but not different from asymptomatic HIV^+TB^- individuals (65.3 cellsμl, p=0.09) (Fig.3A & 3B). Interestingly, the level of naïve CD4+ T cells was observed to be inversely related (r= -0.70, p<0.001) to the level of immune activation in all the groups

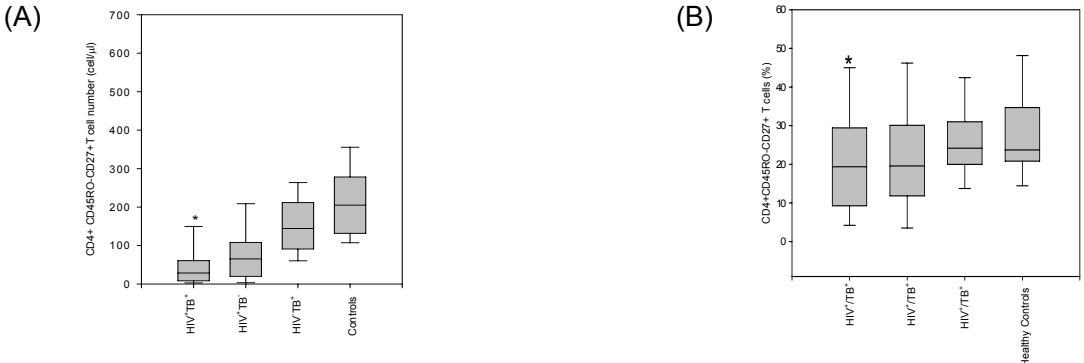


Fig.3. (A) Number of naïve CD4+ T cells (CD4+CD45RO-CD27+ T cells, cells/μl) (B) the percentages naïve CD4+ T cells (CD4+CD45RO-CD27+T cells, %), Boxes: 5th and 75th ranges, confidence interval are 5th and 95th percentiles and bar lines are medians, *- indicates the absence of significant difference between HIV+TB+ and HIV+TB- groups.

Effect of CD4+ T cell and plasma viral RNA level on T cell division

To investigate the relationship between absolute CD4+ T cell number and plasma viral RNA level and T cell division, we compared HIV^+TB^+ and HIV^+TB^- groups with CD4+ T cell numbers below or above 200. Despite higher levels of CD4+Ki-67+T cells and plasma viral RNA level in HIV^+TB^+ patients with CD4+ T cell numbers <200-cells/μl (15.1%, 4.9 log_{10}copies/ml) compared to HIV^+TB^- subjects (11.1%, 4.7 log_{10} copies/ml), the difference was not statistically significant (p=0.52 and 0.77). However, in subjects with ≥ 200 CD4+ T cells/μl, HIV^+TB^+ coinfected individuals had significantly higher level of Ki-67 expression in CD4+ T cells (9.6% versus 5.2%, p=0.011) and plasma viral RNA level (5.0log_{10}copies/ml versus 4.2log_{10}copies/ml, p=0.04) than HIV^+TB^- persons (Fig. 4).The difference in Ki67 expression in CD8+ T cells was not significant in the two CD4+ T cell categories (<200 cells/μl (p=0.3) and ≥200 cells/μl (p=0.22).

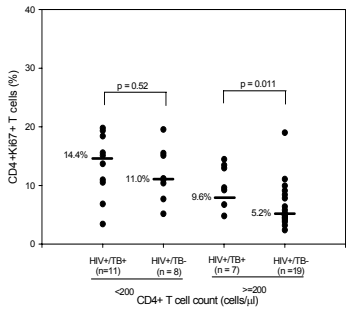


Figure-4. Expression of $Ki67^+$ T cells within the total CD4+ T cells in HIV^+TB^+ and HIV^+TB^- individuals at two different CD4+ T cell categories (<200 and ≥200 cells/μl) (bar lines are medians)

To further investigate the relationship between absolute CD4+ T cells count, plasma viral RNA level with expression of Ki67 within CD4+T cells and CD8+T cells, correlations were computed using the Ki67 expression data before anti-TB chemotherapy including asymptomatic HIV persons and healthy controls.

A negative correlation between CD4+Ki-67+T cells (r=-0.70, p<0.001) and CD8+Ki-67+T cells (r= -0.58, p<0.001) with absolute CD4+ T cell counts was observed (Fig. 5A). However, after correcting for the effect of plasma vial RNA level (by computing the partial correlation), the significant correlation was lost (r=-0.26, p=0.09 and r= -0.15, p=0.35 for CD4+Ki-67+T cells and CD8+Ki-67+T cells expression, respectively). In contrast there was a positive correlation between expression of CD4+Ki-67+T cells and plasma viral RNA level (in HIV^+TB^+ and HIV^+TB^- subjects, r= 0.50, p<0.001) but not with CD8+Ki-67+T cells(r= 0.22, p=0.16). After controlling for the effect of CD4+ T cell count the correlation remains significant (r= 0.48, p= 0.002). Moreover, a positive correlation between expression of Ki-67 within total CD4+ T cells and CD8+ T cells (r=0.81, p<0.001), which remains significant after correcting for the effect of CD4+ T cell number (r=0.57, p=0.004), plasma viral RNA level (r=0.45, p=0.001) or both (r=0.43, p=0.005) was observed (Fig. 5B).

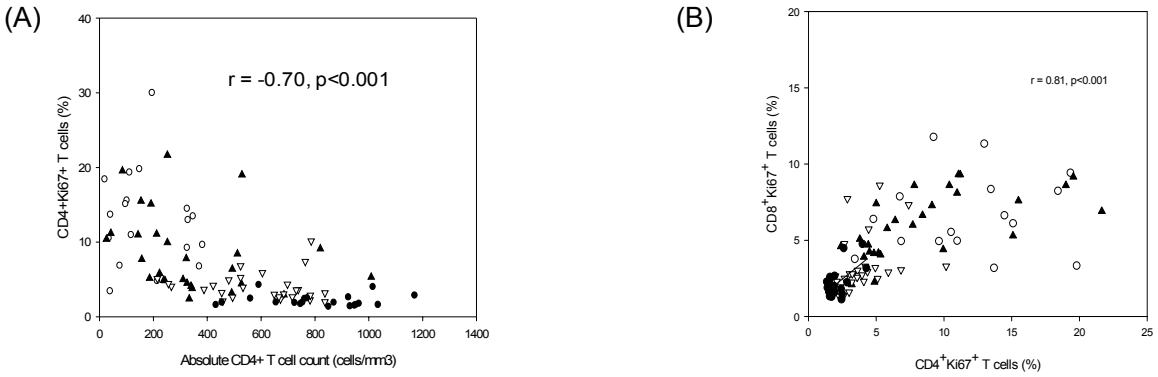
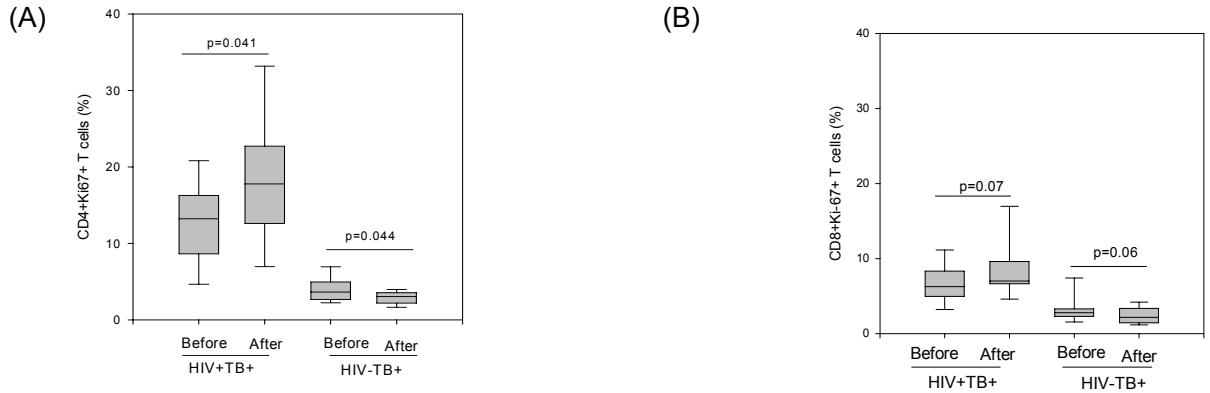


Fig. 5. **(A)-** Correlation between expression of Ki67+ T cells within CD4+ T cells and absolute CD4+ T cell count **(B)**- Correlation between expression of Ki67+ T cells within CD4+ T cells and within CD8+ T cells. HIV^+TB^+ (open circles), HIV^+TB^- (black triangles), HIV^-TB^+ (open triangles) and Healthy controls (black circles).

Effect of anti-TB chemotherapy and ART on T cell proliferation

To investigate the effect of anti-TB chemotherapy on immune activation, we studied HIV^+TB^+ coinfected patients after the treatment of clinical TB disease (3.6 months of anti-TB chemotherapy). Compared to HIV^+TB^+ coinfected patients before treatment (cross-sectional), there was no difference in absolute CD4+ T cell numbers. Plasma viral RNA level, however, was significantly higher in HIV+TB+ after treatment (5.5 $\log_{10}$ copies/ml) than before treatment (4.9 $\log_{10}$ copies/ml, p<0.001). The expression of CD4+Ki-67+T cells was significantly decreased after 2 months of anti-TB chemotherapy in TB patients without HIV infection than those before therapy (3.6% versus 3.0%, p=0.04, Fig. 6A). In contrast, the percentage of Ki-67 expressing cells within the CD4+ T cells was higher after the treatment of TB disease (13.2% versus 17.8%, p=0.04) (Fig. 6A). Similar observations were made with respect to Ki-67-expression within naive and memory CD4+ T cells (Fig. 6C & 6D). For CD8+ T cells there was a trend to higher levels of Ki-67 upon anti-TB treatment (p=0.07).



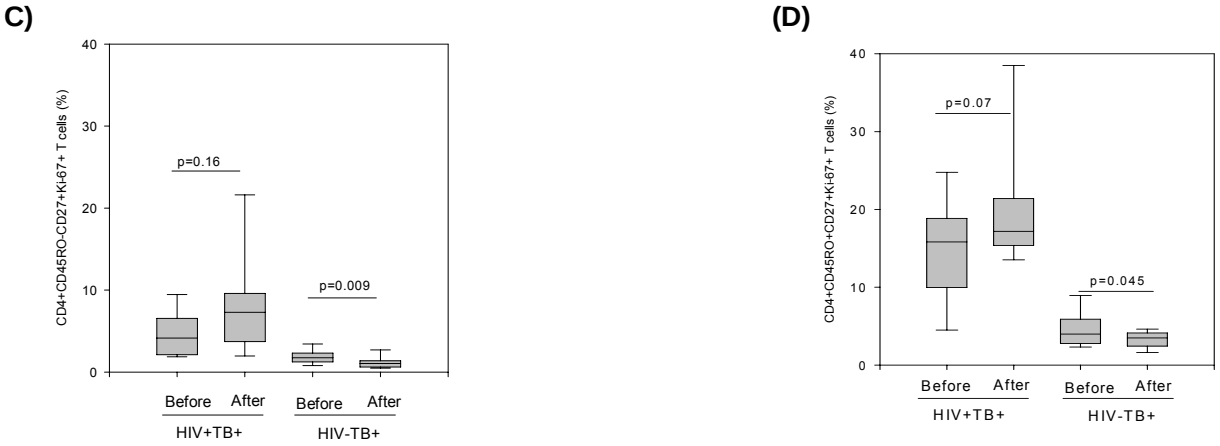


Figure-6. (A)- Expression of Ki67$^+$ T cells within the total CD4+,**(B)** CD8+ T cells before and after anti-TB chemotherapy **(C)**- Expression of Ki67$^+$ T cells within the naïve and **(D)** within the memory CD4+ T cells before and after anti-TB chemotherapy (Boxes: 5th and 75th ranges, confidence interval are 5th and 95th percentiles and bar lines are medians).

In addition, to investigate the effect of ART on immune activation, we studied HIV$^+$TB$^+$ coinfected patients before and after two months of ART. This group of patients had completed anti-TB chemotherapy and commenced ART thereafter**.** The absolute CD4+ T cell number increased from 64 cells/μl to 191 cells/μl (p=0.02). The percentage of CD4+Ki-67+T cells decreased from 22.7% to 15.3% (p=0.02, Fig. 7A) in parallel with plasma viral RNA levels (5.86 $\log_{10}$ copies/ml to 3.1$\log_{10}$copies/ml, p=0.0005). However, the expression of Ki-67 within CD8+ T cells did not decrease significantly (p=0.31, Fig. 7B). The reduction of Ki-67 expression was significant in memory CD4+ T cells (p=0.04), but not in naïve CD4+ T cells (p=0.23, Fig. 7C & 7D).

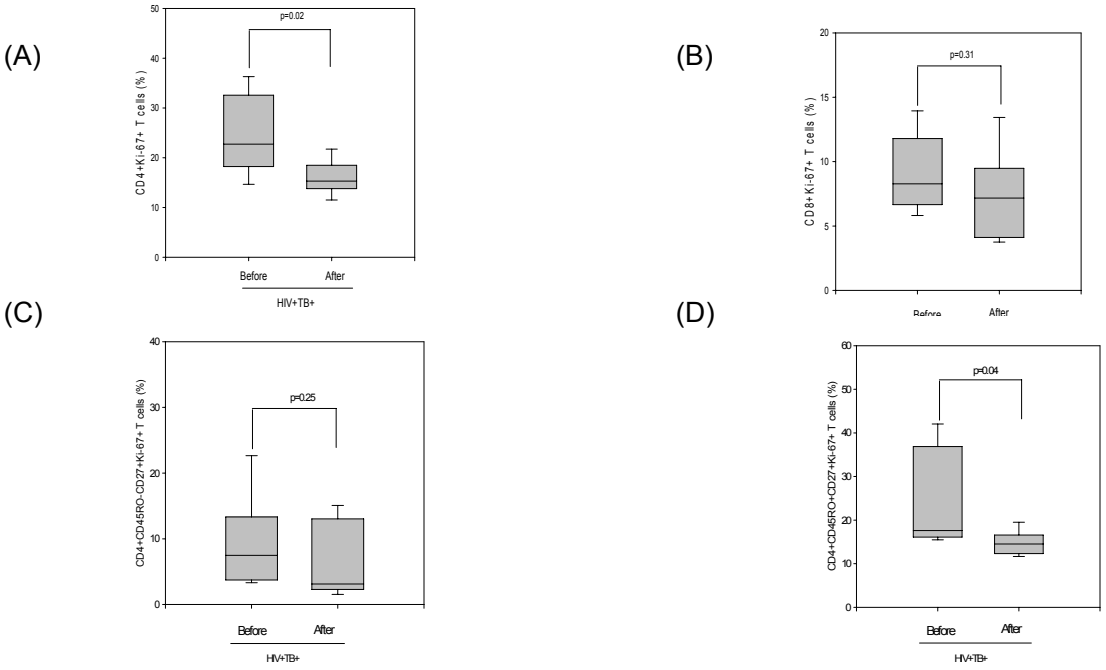


Figure 7. (A)-Expression of Ki67$^+$ T cells within the total CD4+, **(B**) CD8+ T cells before and after ART **(C)** Expression of Ki67$^+$ T cells within the naïve, **(D**)- and memory CD4+ T cells before and after ART chemotherapy (bar lines are medians).

No change in naïve CD4+ T cells despite anti-TB and ART chemotherapy

To investigate the level of naïve CD4+ T cells in the HIV^+TB^+ coinfected patients, naive T cells were enumerated within the CD4+ T cells. As shown in figure 8, in HIV^+TB^+ coinfected individuals, the naive CD4+ T cell population was not different both in percentage (19.4% versus 7.9%, p=0.42) and absolute number (28.5 cells/μl versus 5.5 cells/μl, p=0.07) before and after anti-TB chemotherapy, respectively. Despite the decrease in immune activation after anti-TB chemotherapy in TB patients without HIV infection, percentage and absolute numbers of naïve CD4+ T cells remained similar (24.2% and 144.5 cells/μl) before and (23.3% and 129.9 cells/μl) after treatment. Furthermore, in HIV^+TB^+ patients naïve CD4+ T cells did not change after two months of ART both in percentages and absolute numbers (7.9%, 5.7 cells/μl and 9.7%, 12.9 cells/μl, p>0.05).

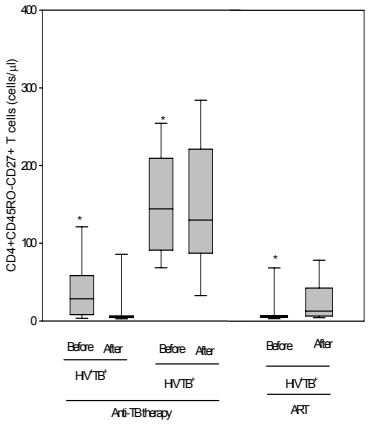


Figure. 8. Proportion of naïve CD4+ T cells before and after anti-TB chemotherapy and ART chemotherapy (*-shows the absence of significant difference before and after treatment, bar lines are medians).

Discussion

In this study we investigated the role of TB and effect of anti-TB chemotherapy in immune activation/T-cell proliferation in TB patients with and without HIV infection. A comprehensive analysis of Ki-67 within CD4+ and CD8+ T cells (including the naïve and memory phenotypes) in four different groups of study subjects that includes TB patients with and without HIV infection, asymptomatic HIV infected persons and healthy controls were performed.

We found a higher proliferation rate of CD4+ T cells in HIV^+TB^+ coinfected individuals compared to asymptomatic HIV, TB patients without HIV infection and healthy controls. The T-cell proliferation rate obtained in apparently healthy individuals, asymptomatic HIV infected persons and TB patients without HIV infection confirm other reports [31,32,33,34].

Our findings show a role for TB in immune activation by increasing the proliferation rate and by causing an additive effect on HIV-induced proliferation by two fold in total, naïve and memory CD4+ T cells. However, HIV on its own still induced the highest level of immune activation by increasing T cell proliferation four-fold. Interestingly, the absence of any effect of TB on immune activation in coinfected individuals with CD4+ T cells number <200 cells/μl may indicate that TB could not be the primary cause of immune activation rather it could be a consequence since TB specific immunity could be impaired at this stage. However, in individuals with CD4+ T cells ≥200-cells/μl TB seems to cause increased immune activation, which might be explained by new TB infections. This supports the presence of higher plasma

viral RNA level in this category of HIV^+TB^+ co infected individuals since the effect of TB on viral replication was significantly higher when the CD4+ T cell count is preserved as reported elsewhere [31,35]. Alternatively, the HIV+TB+ patients presenting themselves at CD4+ T cell numbers <200 cells /μl may be the result of TB reactivation and therefore TB acts merely an opportunistic infection not influencing immune activation parameters.

We observed an effect of antigen load on immune activation levels in HIV infected individuals. There was a negative correlation between CD4+Ki-67+T cells; CD8+Ki-67+T cells and absolute CD4+ T cell count as reported elsewhere [32,33]. However, when controlled for the effect of plasma HIV viral RNA levels, the correlations were lost. This indicates a major role of HIV (plasma viral RNA level) in induction of immune activation. Indeed, we also found a positive correlation between CD4+Ki-67+T cells with plasma viral RNA level, which was still significant after controlling for the effect of CD4+ T cell counts. The presence of a positive correlation between CD4+Ki-67+T cells and CD8+Ki-67+T cells, which remains significant after controlling for CD4+ T cell count or controlling for plasma viral RNA level (the effect of HIV antigen) and/or both, interestingly indicates the presence of an additional common factor for immune activation [21, 32, 35, 36, 37, 38], presumably TB infection in our cases.

In this study we observed reduced naïve CD4+ T cell populations and considerable proportions of proliferating naïve T cells in HIV^+TB^+ coinfected individuals. In HIV infection increased immune activation (proliferation) rates were shown to lead to the depletion of CD4+ T cells [21, 22, 34]. The presence of significant level of proliferation in the naïve T cell compartment, which leads to lower naïve CD4+ T cell numbers, may contribute to faster disease progression among these individuals. Surprisingly, in recent report Mekonnen *et al*. [39] showed disease progression rate in HIV infected Ethiopians was not different compared to other populations such as Dutch. However, their report did not address the rate of disease progression after the development of opportunistic infections such as TB.

By studying the effect of anti-TB therapy, we found that in TB patients without HIV infection, although there was a significant reduction of CD4+Ki-67+T cells after anti-TB chemotherapy, it did not reach levels of healthy controls. This could be explained by the residual effects of TB [14, 31], which we believe will be resolved after completion of the therapy.

The absence of plasma HIV viral RNA level reduction after anti-TB chemotherapy in our HIV^+TB^+ coinfected study subjects confirms other reports for 2 months [4,15], 3 months [13], 6 months [12] and 12 months [14] anti-TB chemotherapy. Of note is the higher rate of proliferation found after the completion of anti-TB chemotherapy in the coinfected individuals, which are in contrast to TB patients without HIV infection that showed a marked reduction. This might indicate that immune activation in HIV+TB+ may not be reversed by anti-TB chemotherapy alone, as HIV is the major source of immune activation. Interestingly, the level of immune activation after the completion of anti-TB chemotherapy was higher in coinfected individuals with CD4+ T cells <200-cells/μl compared to ≥200 cells/μl, with higher plasma viral RNA level, which suggests these individuals are at a further more advanced stage of their HIV-infection.

We used Ki-67 as a marker for immune activation in this study. Indeed we observed a positive correlation between other previously used activation markers (CCR5 and HLA-DR [15] with CD4+Ki-67+T cells in TB and HIV^+TB^+ coinfected individuals (data not shown). The elevated expression of these surface markers among HIV^+TB^+ coinfected individuals as reported recently [15] might cause the CD4+ T cells to be susceptible for HIV infection and increase viral replication [16-19, 26, 33, 35].

In strengthening our findings, the reduction of proliferation rate and plasma HIV viral RNA level after two months of ART in $HIV^{+}TB^{+}$ coinfected patients showed that immune activation caused by HIV antigen was suppressed [40]. However, since these individuals had completed anti-TB chemotherapy, they might benefit from dual treatment. In fact during ART there was a significant increase in absolute CD4+ T cell number, especially in the memory compartment as reported earlier [41]. Despite all these, neither the CD4+Ki-67+T cells nor plasma HIV viral RNA levels reached the level of healthy individuals or became undetectable, respectively. This may indicate that replication of the virus was not completely suppressed and may need longer treatment period.

In conclusion, in this study, we showed a role of TB in immune activation in HIV-negative TB patients and the absence of reduction in plasma HIV viral RNA level and immune activation in $HIV^{+}TB^{+}$ coinfected individuals after anti-TB chemotherapy. The presence of higher proliferation rates in $HIV^{+}TB^{+}$ individuals with higher CD4+ T cell count provide evidence for TB as a cause for immune activation, which may have impact on viral replication to sustain or increase the level of the plasma viral RNA level [8,10,42].

Acknowledgements

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Persistent immune activation and sustained plasma viral RNA level in Human Immunodeficiency Virus-infected individuals developing tuberculosis despite anti-TB treatment

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Chapter 5

Abstract

Immune activation plays an important role in HIV-disease progression and may also affect TB disease progression in HIV-infected individuals. In this study we investigated the relation between the occurrences of TB; level of plasma viral RNA, the dynamics of CD4+ T cells and trend of immune activation on T cells in HIV infected individuals. We observed significantly higher plasma viral RNA levels and T-cell immune activation levels after completing standard anti-TB treatment in a cross-sectional analysis of HIV/TB coinfected individuals. This was especially observed in individuals <200 CD4+ T cells/µl. In addition, we longitudinally analyzed HIV-infected individuals who developed TB during follow-up (n=9, followed for a mean of 44 months) before and after the diagnosis and treatment of clinical TB. These individuals showed a marked decline in CD4+ T cells and increase in plasma viral RNA level already before TB diagnosis. This trend was continued even after completing their standard anti-TB treatment. Parallel to this, the level of immune activation (as indicated by the percentage of Ki67+ CD4+ T cells) also continued to increase even after anti-TB therapy. The marked reduction in CD4+ T cell numbers, elevated plasma viral RNA levels and persistent immune activation in these individuals suggests the need to start ART and prophylaxis for latent tuberculosis infection at an earlier stage of immunodeficiency to slow HIV disease progression.

Introduction

The risk for the development of tuberculosis disease (TB) is 10 times higher in HIV infected than HIV-uninfected individuals **(1-3).** The risk of TB is reported to be higher during the first two years of HIV infection. However, TB disease remains dormant for a prolonged period (4-9 years) and become manifest following impairment of the immune system **(4-9).**

In developing countries the cause for TB reactivation includes different factors such as involvement/influence of other infections(such as parasitic infections), malnutrition, lower CD4+ T cell number and higher immune activation levels as has been previously reported in HIV-uninfected Ethiopians **(10).** The low baseline CD4+ T cell count, coupled with further decline in CD4+ T cells usually precedes the development of clinical TB in HIV-infected individuals **(2)**.

It has been suggested that immune activation plays an important role in CD4+ T cell depletion at the periphery. Immune activation of T cells was shown to result in higher levels of dividing cells as determined by Ki67 nuclear antigen expression, which may lead to more rapid turnover of T cells in HIV infection **(11)**. The presence of other opportunistic infections such as tuberculosis may increase immune activation further leading to an enhanced reduction in CD4+ T cell numbers. To elucidate the role of immune activation in disease progression in HIV/TB coinfected individuals, we investigated the dynamics of CD4+ T cell numbers, the trend in plasma viral RNA levels and immune activation (Ki67 expression) in ART naive HIV infected individuals with incident TB before and after diagnosis/treatment of TB longitudinally.

Patients and methods

In this study we included 39 HIV/TB coinfected individuals, 24 before TB treatment and 15 patients after 3.6 ±2.0 months (mean ± standard deviation [SD]) of TB treatment for cross-sectional analysis. The characteristics of the study subjects are depicted in **Table- 1 & Chapter-4.**

Table-1. Characteristics of HIV/TB coinfected patients before and after anti-TB Chemotherapy

	CD4 category	Before anti-TB	After anti-TB	p-value*
Subjects (n)	<200 cells/μl	16	8	
	≥200 cells/μl	8	7	
Sex (M/F)		15/9	12/3	
Age(years, IQR)		30[25-39.5]	37[30-43]	0.13
CD4+ T cells (cells/μl, IQR)	<200 cells/μl	78.5[30.5-115]	61[48-89.5]	0.90
	≥200 cells/μl	338.5[326-377]	295[207-352]	0.11
Plasma viral RNA level(log_{10}copies/ml, IQR)	<200 cells/μl	4.9[4.5-5.3]	5.9[5.5-6.1]	0.02
	≥200 cells/μl	5.0[4.3-5.4]	5.3[4.9-5.5]	0.60
p-value**		0.9	0.03	
Hemoglobin (gm/dl, IQR)	<200 cells/μl	15.1[10.5-19.3]	21.2[15.8-29.6]	0.03
	≥200 cells/μl	9.6[6.7-13.5]	14.9[9.3-17.8]	0.15
p-value**		0.08	0.03	

Numbers in brackets are 25th and 75th interquartile ranges (IQR), *-p values for comparison of before and after treatment, **-p values of the comparison between CD4+ T cell counts <200 cell/μl and ≥200 cells/μl categories

Secondly, from a cohort study for the natural history of HIV/AIDS (12), 9 HIV infected individuals who developed TB during follow-up were followed for an average of 14.7 ± 9.6 months (mean ± standard deviation) before and 22.3 ± 16.4 months after the diagnosis and treatment of tuberculosis disease. The characteristics of the study subjects are depicted in Table- 2. Informed consent was obtained from all study participants and the study protocol was reviewed and approved by both Institutional (EHNRI) and National Ethical Clearance Committees.

Table-2. Characteristics of HIV infected incident tuberculosis patients at enrolment

Parameters	$HIV^{+}TB^{+}$ (n=9)
Gender (F/M)	2/7
Age (Years, IQR) at enrolment	35[32-38]
Absolute CD4+ T cell count (cells/μl) at enrolment	382[252-379]
Absolute CD8+ T cell count (cells/μl) at enrolment	1376[1068-1535]
Plasma HIV viral RNA level (Log_{10}copies/ml) at enrolment	4.0[3.3-4.7]
Average follow up period before the diagnosis of active clinical TB disease (mean ± standard deviation)	14.7±9.6 months
Average follow-up period after the diagnosis and treatment of active clinical TB disease (mean ± standard deviation)	22.3±16.4 months
Antiretroviral therapy	Naïve
Anti-TB chemotherapy	No/Yes*

Numbers in brackets are 25th and 75th interquartile ranges (IQR), *- there was no anti-TB chemotherapy before the diagnosis of active clinical TB disease; however, the patients received standard anti-TB chemotherapy after being diagnosed.

Determination of plasma viral HIV RNA level and T cell subsets and intracellular Ki67 staining

Plasma HIV viral RNA level was measured using nucleic acid sequence based amplification assay (Organon Teknika, The Netherlands, detection limit of <80 copies/ml). Surface staining for CD4- and CD8- PerCP (Peridinin chlorophyll Protein) was performed using standard flow cytometry procedures. Intracellular staining for Ki67 nuclear antigen was done using Fluorescein Isothiocyante (FITC) labeled Ki67 monoclonal antibody (Monosan, The Netherlands) after lysing and permeabilizing the cell and nuclear membrane, respectively (lysing and permeabilizing solutions, Becton Dickinson). Analysis was done using a four-color FACSCalibuir (Cellquest software, BD, San Jose, CA).

Diagnosis of *M. tuberculosis* infection

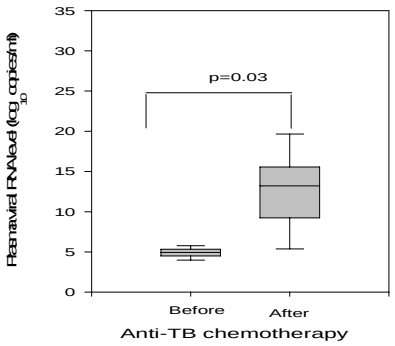
Diagnosis of *M. tuberculosis* infection was done by sputum analysis for acid-fast bacillus. A patient was considered positive, if at least two acid-fast bacillus-stained sputum samples positive for the bacilli were identified by smear microscopy. The diagnosis of TB was also supported by culture, chest X-ray and pathology. Treatment was provided according to the guidelines of the Ministry of Health, Ethiopia (**13)**.

Results

Plasma viral RNA and immune activation level are increased after anti-TB chemotherapy

In a cross sectional analysis, 39 HIV/TB coinfected individuals (24 before and 15 after anti-TB chemotherapy) were included. Of these, 24 patients had CD4+ T cell numbers <200-cells/μl (16 before and 8 after anti-TB treatment) and 15 individuals (8 before and 7 after anti-TB treatment) had ≥200 cells/ μl (Table-1). As discussed in Chapter-4, there was no difference in CD4+ T cell number before and after anti-TB chemotherapy. However, there was a higher median plasma viral RNA level (5.5log10copes/ml compared to 4.9log10copies/ml before therapy) and higher percentage of Ki67+ CD4+ T cells (17.8% compared to 13.2% before therapy) after treatment of TB (p=0.03 and 0.04, respectively, Table-1 & for comparision purposes see **figure-1**). Interstingly, the % of Ki-67 within CD4+ T cells was higher in individuals with less than 200 CD4+ T cells/μl compared to individuals with ≥200 CD4+ T cells/μl (p=0.03) and paralleled with plasma viral RNA level before and after anti-TB chemotherapy **(**p=0.02**, Fig. 2)**.

(A)



(B)

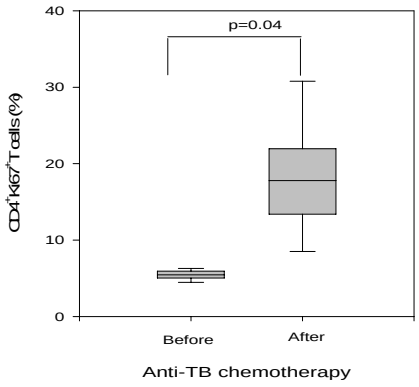
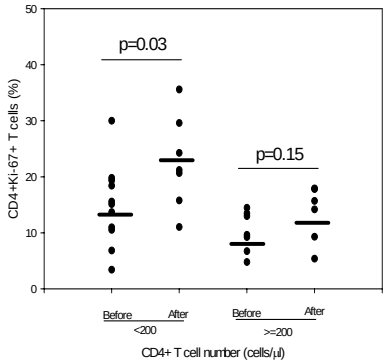


Figure-1. (A) Levels of plasma viral RNA (log_{10}copies/ml) (B) Levels of expression of CD4+ Ki67+ T cells (%) before and after anti-TB chemotherapy (boxes are 25th and 75th interquartile ranges, limits are 5th and 95th confidence intervals and bar is median value)

(A)



(B)

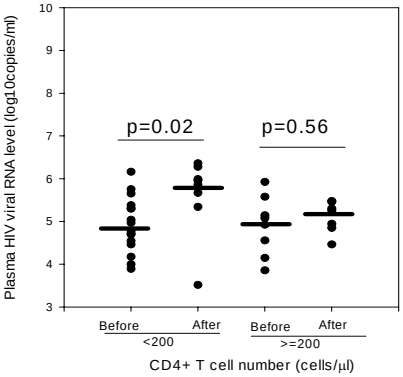


Figure-2. (A)-The expression of CD4+Ki67+ T cells (B)-level of plasma viral RNA level in two CD4+ T cell categories

Persistent plasma viral RNA and immune activation level with more advanced HIV disease progression continues after occurrence of TB in the presence of anti-TB therapy

To investigate the relation between immune activation on T cells and plasma viral RNA level in HIV disease progression with anti-TB treatment, we determined the expression of Ki67 within CD4+T cells and CD8+ T cells in HIV positive incident TB cases (n=9). In this study group, the median absolute CD4+ T cell at enrollment was 382 cells/μl. Eight subjects had ≥200 cells/μl at baseline. Upon follow up the absolute CD4+ T cell number decreased significantly to below 200 cells/μl ($p<0.001$, test for trend) in all individuals. Based on the CD4+ T cell number at the diagnosis of clinical TB disease, the development of TB was higher among those with higher CD4+ T cell counts (≥200 cells/μl, n= 6/9) compared to <200 cells/μl (3/9) **(Fig. 3A).** At enrolment, the level of viral RNA level was 4.0 $\log_{10}$copies/ml and was increased during follow-up despite the resolution of TB **(Fig. 3B)**.

(A) (B)

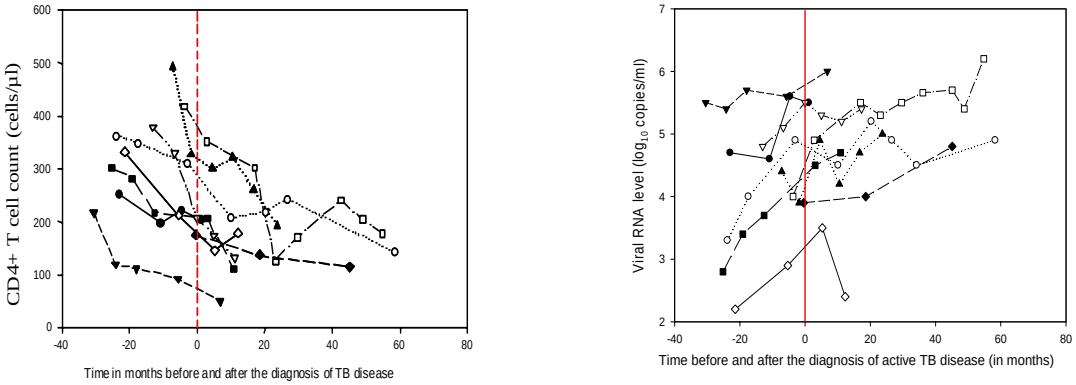


Figure-3. **(A)** The dynamics of CD4+ T cell depletion **(B)** plasma viral RNA level among the coinfected individuals before and after the diagnosis and treatment of TB disease (vertical line-time of TB diagnosis)

In contrast to the CD4+ T cell counts and parallel with the plasma viral RNA level, immune activation (Ki67 expression within CD4+ T cells but not within the CD8+ T cells) increased in the follow-up period **(Fig. 4A & 4B)**.

(A) (B)

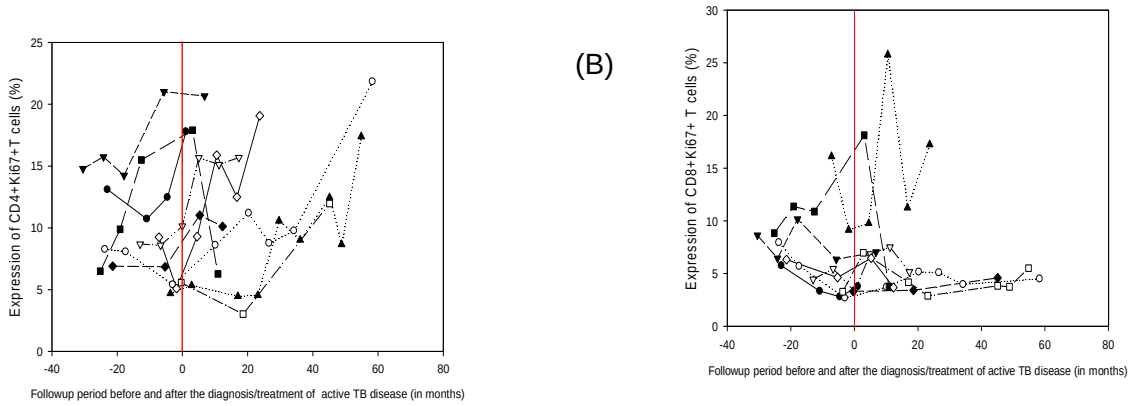


Figure-4. (A) Expression of CD4+Ki67+ T cells (%) **(B)** Expression of CD8+Ki67+ T cells (%) in HIV infected individuals with incident TB disease before and after the diagnosis/treatment of clinical active TB disease at different follow-up periods(dotted vertical lines are time of TB diagnosis).

In these individuals, unlike to the TB patients without HIV infection (data not shown), the level of hemoglobin, as a means of monitoring TB disease for anti-TB treatment **(22)** was not restored (Fig. 5):

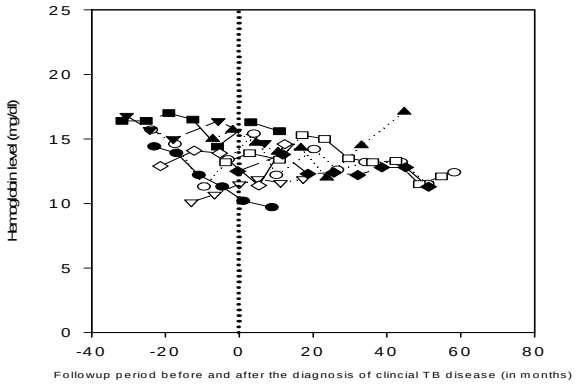


Figure-5. Level of hemoglobin (mg/dl) in HIV infected individuals with incident TB disease before and after the diagnosis/treatment of clinical active TB disease at different follow-up periods (dotted vertical lines are time of TB diagnosis)

Discussion

In this study we investigated the relation between the occurrences of TB; level of plasma viral RNA, trend of immune activation and the dynamics of CD4+ T cells in HIV infected individuals before and after the diagnosis and treatment of clinical TB longitudinally.

We confirmed the occurrence of TB at different level of immunodeficiency (defined by the level of CD4+ T cells). Interestingly, we observed especially occurrence of TB individuals with ≥200-cells/μl CD4+ T cell level, suggesting new infections. Treatment of clinical TB had no effect on plasma viral RNA and immune activation levels. Rather CD4+ T cell number progressively decreased despite anti-TB therapy. Moreover, the level of hemoglobin was not restored after anti-TB chemotherapy, which indicates that the resolution of TB disease did not succeed to normalize patients' hemoglobin level that lacks indication for the improvement of the patients from the disease.

Interestingly, the increasing level of immune activation (as indicated by the presence of sustained Ki67+ CD4+ T cells) was not averted after successful anti-TB chemotherapy. As evidenced from different reports **(3,8,9)**, the occurrence of a higher level of immune activation is expected to sustain the level of plasma viral RNA level, which is also supported by the findings of our study. However, in contrast to TB patients coinfected with HIV, there was a marked reduction in the level of immune activation in TB patients without HIV infection following chemotherapy for TB (data not shown). This indicates that, in HIV/TB coinfected individuals, the effect of HIV overshadowed the effect of anti-TB treatment on the effect of TB on the level of immune activation.

In spite of effectiveness of the anti-TB chemotherapy, there was an increase in the level of Ki67+ CD4+ T cells (immune activation), which paralleled the increase in plasma viral RNA levels. This might be due to either the residual effect of tuberculosis (which can persist for a long period) or alternatively might be caused by HIV itself. The latter seems more obvious as both viral load and immune activation levels were already increasing before the development (and treatment) of TB. This suggests that HIV-induced disease progression carries on even after implementation of TB-treatment.

Because we observed the occurrence of active TB disease at low level of immunodeficiency (CD4 T cells $\geq$ 200 cell/μl) and an absence of reduction in plasma viral RNA level despite effective anti-TB treatment suggests the advantage of initiating ART earlier, either before the development of TB or at the moment of TB diagnosis in parallel with anti-TB treatment. In addition, this suggests the need for anti-TB prophylaxis in HIV infected individuals. Thus, this study indicates the need to examine earlier HIV treatment schemes in coinfected individuals so that they could benefit from initiating ART at a lower level of immunodeficiency despite problems related to drug supply and logistics, which pose major challenges in the developing world. ART has been shown to decrease plasma viral RNA level and immune activation markers and thereby restoring the CD4+ T cell number, potentially leading to a better prognosis of TB.

Unlike other reports **(14,15)**, this report confirmed studies from Africa **(16-20)** that showed the absence of reduction in plasma viral RNA level following anti-TB therapy. This might be due to higher or sustained levels of immune activation that could not be resolved using standard anti-TB chemotherapy alone.

In conclusion, sustained plasma viral RNA levels and persistent immune activation by HIV in HIV/TB coinfected individuals leads to progressive loss in CD4+ T cell numbers even after successful treatment for TB, which could be due to HIV disease progression. As reported elsewhere continuous increase in plasma viral RNA level, immune activation and decrease in CD4+ T cells is normal HIV-progression. Moreover, our finding suggests that this trend of disease progression in HIV/TB coinfected individuals may not be influenced by both the occurrence of new TB infections (as TB increases immune activation) as well as TB treatment. This affects both tuberculosis and HIV treatment schemes since restoration of TB-specific immunity could be gradual even after ART **(21)**. This strongly supports the notion to start ART earlier at the moment of TB diagnosis or maybe already at an earlier time point. Monitoring viral load and CD4 T cells (and immune activation levels) frequently, can be helpful to decide when to initiate ART to prevent TB and improve immunity.

Acknowledgements

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Increased percentage of regulatory T cells is associated with advanced HIV disease and HIV-TB coinfection: a reflection of enhanced immune activation?

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Chapter 6

Abstract

CD4+ T cells characterized by up-regulated expression of IL-2 receptor (CD25), especially with high-density expression of CD25 (CD4+CD25^{high}), and the transcription factor protein FOXP3 (Tregs) are known for their regulatory functions as they have been shown to suppress T cell responses. In this study, we investigated the levels of Tregs in tuberculosis patients with and without HIV coinfection. Compared to healthy controls (8.1%), a higher-level of CD25-expression on T cells was observed in HIV/TB coinfected individuals (12.9%), asymptomatic HIV infected persons (11.5%) and TB patients without HIV infection (11.0%). There was a 4, 2.5 and 2 fold increase in percentages of CD25highCD4+ T cells in HIV/TB coinfected, asymptomatic HIV infected and TB patients without HIV infection respectively compared to healthy controls. Moreover, a 3 fold increased level in the expression of FOXP3 within the CD4+ T cells was observed in coinfected patients compared to healthy controls. Individuals with <200 CD4+ T cells/μl had higher levels of FOXP3+ and CD25high CD4+ T cells than individuals with ≥200 CD4+ T cells/μl. Absolute CD4+ T cell number was negatively correlated with the level of Tregs. Interestingly, T cell immune activation as determined by the expression of KI67 on T cells was positively correlated with the level of Tregs. The percentage of Tregs did not change after anti-TB and/or antiretroviral treatment. In conclusion, Tregs increase with disease severity in parallel to immune activation levels, suggesting that their level is related to the burden of infection. The absence of reduction of Tregs after anti-TB and antiretroviral treatment may indicate the longevity of these T cells.

Introduction

Tuberculosis (TB) patients coinfected with human immunodeficiency virus (HIV) have a worse disease outcome (mortality), suggesting that their immune system does not operate optimal any longer. Lower TB specific immunity could be due to the capacity of HIV to down regulate antigen presentation using MHC class II, inhibiting CD4+ T cell responses to be initiated. In addition, HIV leads to a depletion of $CD4^+$ T cell numbers, which are necessary to fight MTB **(1)**. On the other hand, the level of regulatory T cells, a subpopulation of CD4+ T cells, may influence this response.

Regulatory T cells are a specialized subpopulation of T cells that act to suppress immune responses to foreign and self-antigens **(2).** They are characterized by a decrease in both proliferation and IL2 secretion in response to T-cell receptor (TCR) stimulation and up regulated expression of interleukin-2 alpha chain receptor (CD25). Regulatory T cells comprise about 5-10% of $CD4^+$ T cells in humans and are known to express the forkhead family transcription factor FOXP3 (forkhead box p3) **(3,4,5),** which is required for the development and control of the genetic program to specify regulatory T cell fate **(6)**. Within the $CD4^+CD25^+$ T cells, high-density $CD4^+CD25^+$ T cells ($CD4^+CD25^{high}$) are reported to be the cells with regulatory functions (Tregs). They express the highest level of FOXP3 (~90%). Recently, it has been shown that regulatory T cells can regulate virus-specific or memory CD8+ T cell responses by diminishing the magnitude of the immune response **(7, 8, 9).**

Despite conflicting results, increased percentage of regulatory T cells is reported in HIV infection especially among those with low percentage of $CD4^+$T cells and higher level of immune activation **(9, 10)**. However, the susceptibility of these cells for HIV infection and apoptosis is suggested to contribute to their lower absolute count especially in advanced stage of HIV disease **(8)**.

The presence of higher levels of Tregs may lead to reduced immune responses and favor pathogen over the host. Particularly in HIV and tuberculosis coinfection, if Tregs are present in a greater amount, the immunodeficiency is expected to be aggravated **(11, 12, 13, 14).**

To date, to our knowledge, there are no reports on the level of Tregs in HIV+TB+ coinfected and in TB patients without HIV infection before and after the treatment of active tuberculosis disease. Therefore, in this study we investigated the level of Tregs in TB patients with or without HIV coinfection in relation to immune activation, antigen burden and the effect of standard anti-TB and antiretroviral treatment (ART) on the level of Tregs.

Materials and methods

Study subjects

In this study four groups of study subjects were included. TB patients coinfected with HIV (HIV^+TB^+, n=18), asymptomatic HIV infected individuals (HIV^+TB^-, n=22), TB patients without HIV infection (HIV^-TB^+, 21 before and 17 after anti-TB chemotherapy) and healthy controls (HIV^-TB^-, n=20). The HIV^+TB^+ group was further classified in to two groups: HIV^+TB^+ patients before anti-TB treatment (non chronic patients who came to the clinic for anti-TB treatment, $NCHIV^+TB$, n=19) and HIV^+TB^+ patients who are chronically ill after completing their anti-TB therapy and presented themselves for ART (CHIV+TB+; 9 before and 9 after 2 months of ART).

Patients were prospectively recruited and followed from the following clinical Centers: (i) The All African Leprosy Research and Training Center (ALERT) and Higher 23 Health Center,

both in Addis Ababa, Ethiopia (ii) from two cohort sites of the Ethio Netherlands AIDS Project (25 and 125 kilometers from Addis Ababa) and (iii)- from St. Paul's Hospital in Addis Ababa, Ethiopia, which currently provides ART service for HIV infected patients. Patients were enrolled after thorough clinical evaluation by primary clinicians at the respective health centers. This study was part of a longitudinal study on the Natural History of HIV/AIDS in Ethiopia, which was ethically cleared by the National Ethical Committee. Each study participant gave consent to participate in the study. Characteristics of the study population are depicted in **Table-1**.

Table-1. Characteristics of study subjects

Parameters	ncHIV+TB+	cHIV+TB+		HIV+TB-	HIV-TB+		Controls
		Before ART	After ART		Before anti-TB	After anti-TB	
Total number (n)	19	9	9	22	21	17	20
Age (Years)	37[33-46]	32[25-40]	-	33[31-39]	28[20-36]	-	34[29-40]
CD4+ count (cells/μl)	118[41-326]	69[42-91]	191[135-278]**	317[174-509]	666[458-731]	528[400-718]	811[725-948]
Plasma HIV viral RNA level (Log_{10}copies/ml)	4.9[4.2-5.3]*	5.9[5.4-6.3]	3.1[2.7-3.2]**	4.3[3.8-4.5]*	NA	NA	NA
Antiretroviral therapy	Before	Before	Yes	Before	NA	NA	NA
Anti-TB chemotherapy	Before	After	After	NA	Before	After	NA

nc-non-chronic, c- chronic, ART- antiretroviral therapy, NA-not applicable, numbers in brackets are 25th and 75th inter quartile ranges, *-p<0.001 compared to cHIV+TB+(baseline), **-p<0.001 compared to the baseline(before ART)

Diagnosis of *M. tuberculosis* infection

Diagnosis of *M. tuberculosis* infection was done by sputum acid-fast bacillus. A patient was considered positive, at least, if two acid-fast bacillus-stained sputum samples positive for the bacilli were identified by smear microscopy. The diagnosis of TB was also supported by culture, chest X-ray and pathology. Treatment was provided according to the guidelines of the Ministry of Health, Ethiopia **(15)**.

Plasma HIV viral RNA level and CD4 count determination

Plasma HIV RNA levels were analyzed by a nucleic acid sequence-based amplification assay (EasyQ, Organon Teknika, The Netherlands) with a detection limit of <50 copies/ml. T cell subsets were determined using standard three color flow cytometry (Becton Dickson, San Jose, CA).

Flow Cytometry for the determination of regulatory T cells

Surface markers staining ($CD4^+$ and $CD8^+$ and $CD25^+$ T cells) was performed using mAbs for CD4 [APC, Allophycocyanine]; CD8 [PercP, Peridinin chlorophyll Protein] and CD25 [FITC, Flourescein Isothiocyante] (all from Becton Dickinson, San Jose, CA). In detail, peripheral mononuclear cells (PBMCs) were thawed (RPMI with 10% Fetal calf Serum, FCS), washed with PBA (Phosphate Buffer Saline with 0.05% Bovine Serum Albumin) and stained for CD4, CD8 and CD25 mAbs in dark for 20 minutes. After a second washing step with PBS, the cells were re-suspended with freshly prepared Fix/Perm (1:3) solution (eBioscience, San Diego, USA) and incubated for another 30 minutes in dark at 4 degrees. The stained cells were then washed and re-suspended with permeabilization solution followed by washing for two times using permeabilization solution. Finally cells were stained intracellularly for FOXP3 with anti-human FOXP3 mAb–PE (Phycoerythrin, PHA101 clone, eBiosciences, San Diego, USA) and incubated in the dark for 30 minutes. After a final washing step with permeabilization buffer, analysis was performed using four color FACSCalibur (Cellquest Software, BD).

The ex-vivo immunospot (ELISPOT) assay

To analyze T-cell responses of TB patients with or without HIV coinfection, ELISPOT was performed using two known *M. tuberculosis* antigens (recombinant early secreted antigen target –6; rESAT-6, and culture filtrate protein –10; CFP-10). The cells (10^5 cells/ well) were stimulated with ESAT-6 and CFP-10 antigen (final concentration of 10μg/ml) for 24 hours after coating the nitrocellulose plates (Multiscreen IP clear acrylic PVDF 96-well plates, 0.45 um non sterile) with IFNγ monoclonal antibody (mAb 1-D1K, Mabtech, 5μg/ml, 50 μl/well). PHA-stimulated (10μg/ml, Sigma-Aldrich) and non-stimulated (no antigen) PBMCs were used as positive and negative controls, respectively. Plates were incubated overnight at 37°C in 5% CO_2 and 95% humidity. After washing with PBS/0.05% Tween-20, biotinylated anti-IFNγ mAb (7-B6-1 biotin; Mabtech, 1:1000 dilution, 1μg/ml) was added. Subsequently Streptavidin-conjugated Poly-Horse Reddish Peroxidase (Strep-Poly-HRP, CLB, Amsterdam, 1:6000 diluted) was added. After washing the plates, Tetra-Methyl-Benzilidine (TMB, 50μ/well, CLB, Amsterdam) substrate was added. Finally after stopping the reaction with water, the plates were analyzed using ELISCAN reader (AELVIS). Wells with ≥100 SFC responses after subtracting the mean SFC of the negative control well were considered as positive.

Statistical analysis

Analysis was performed using STATA (Intercooled Stata Version-7, Stata Corporation, College Station, TX). Medians were compared using the nonparametric Mann-Whitney U test. A p value of less than 0.05 was considered as indicative for statistical significance. Correlations were given with Spearman correlation coefficients unless indicated.

Results

At baseline there was no age difference among the groups of study subjects (Kruskal -Wallis test; p=0.14). The absolute CD4+ T cell counts were lower in chronic HIV^+TB^+ (CHIV+TB+, 69 cells//μl) and non-chronic HIV^+TB^+ coinfected patients (NCHIV+TB+, 118 cells/μl) than asymptomatic HIV infected persons (HIV^+TB^-, 317cells/μl), TB patients without HIV infection (HIV^-TB^+, 666 cells/μl) and healthy controls (811.5/cells/μl) (Kruskal -Wallis test; p<0.001). There was a higher plasma viral RNA level in CHIV+TB+ individuals (5.9 $\log_{10}$ copies/ml) and in NCHIV+TB+ (4.9$\log_{10}$ copies/ml) than asymptomatic HIV+TB- persons (4.3$\log_{10}$copies/ml), p<0.01 in both cases **(Table-1)**.

In TB patients without HIV infection and treated for TB, the absolute $CD4^+$T cell number was not different before and after treatment of TB disease (non-parametric t-test, p=0.90). In $CHIV^+TB^+$ coinfected patients after two months of ART, plasma HIV viral RNA level significantly decreased from 5.9 log_{10}copies/ml to 3.1 log_{10}copies/ml (p=0.0008). Moreover, CD4+ T cell number significantly increased from 69-cells/μl to 191-cells/μl (p=0.014), especially in the memory T-cell compartment (from 29.5 cells/μl to 93.8 cells/μl, p=0.011) **(Table-1)**.

Proportion of $CD4^+CD25^+$ T cells in HIV/TB coinfection

Since there are reports that showed up-regulated expression of CD25 within the CD4+ T cells in HIV infection **(10)** as a marker of regulatory T cells, in this study we initially measured the level of CD4+CD25+ T cells in TB patients with and without HIV infection. Compared to healthy controls (median % CD4+CD25+ T cells: 8.1), the percentage of CD25+ $CD4^+$T cells was higher in NCHIV+TB+ groups (12.9%, p=0.009), HIV^+TB^- persons (11.5%, p= 0.008) and HIV-TB+ patients (11.0%, p= 0.04**)**. However, CHIV+TB+ patients had lower percentages of CD4+CD25+ T cells (4.0%) than NCHIV+TB+ patients (12.9%, p=0.08, Fig. 1A). Interestingly we found a strong correlation between memory CD4+ T cell numbers (CD4+CD45RO+CD27+ T cells) and CD4+CD25+ T cells (r=0.80, p<0.001), which indicate these cells are highly expressed in memory CD4+ T cell phenotypes **(16)** (**Fig.1B).**

(A) **(B)**

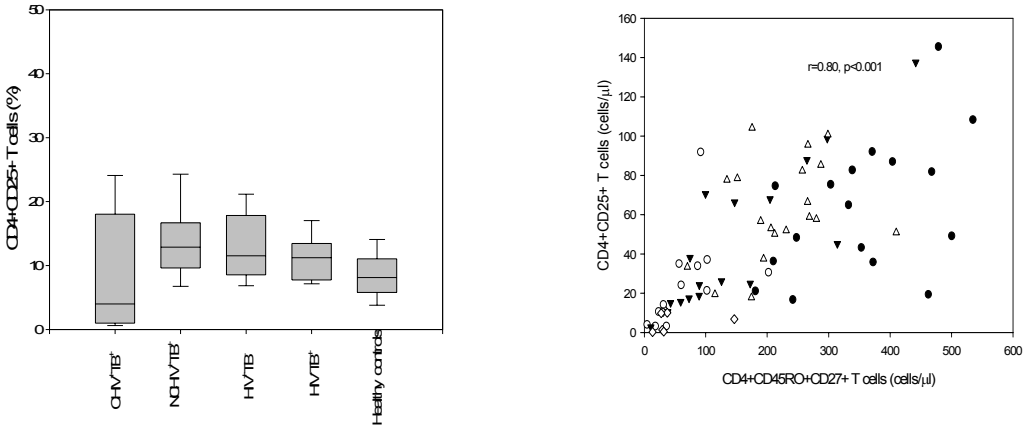

Figure-1. (A)- Expression of CD4+CD25+ T cells in different groups of study subjects **(B)-** Correlation of expression of CD4+CD25+ T cells with memory CD4+ T cell phenotype. Open Diamonds (CHIV/TB), Open Circles (NCHIV/TB), Black Downward Triangles (asymptomatic HIV persons), Open Upward Triangles (HIV negative TB patients), Open Circles (Healthy controls)

$CD4^+CD25^{high}$ T cells in HIV+TB+ coinfection

CD4+ T cells expressing high-density CD25+ cells ($CD4+CD25^{high}$) are reported to have regulatory functions within the CD25+CD4+ T cell subset. Although generally there was lower (<1%) level of expression of $CD25^{high}$ $CD4^+$ T cells across all the groups, at baseline, it was higher in NCHIV+TB+ (median: 0.9% confidence interval; CI: 0.3-1.2, p<0.001) and CHIV+TB+ (0.7%, CI: 0.5-1.8, p<0.001) followed by HIV^+TB^- (0.5%, CI: 0.3-0.7, p<0.001) and HIV^-TB^+ patients (0.40%, CI: 0.2-0.7, p=0.002) compared to healthy controls (0.20%, CI: 0.1-0.3) (**Fig. 2A)**. The level of $CD25^{high}$ $CD4^+$ T cells was higher in individuals with <200 than ≥200-CD4+ T cells/μl (p=0.02) **(Figure-2B).**

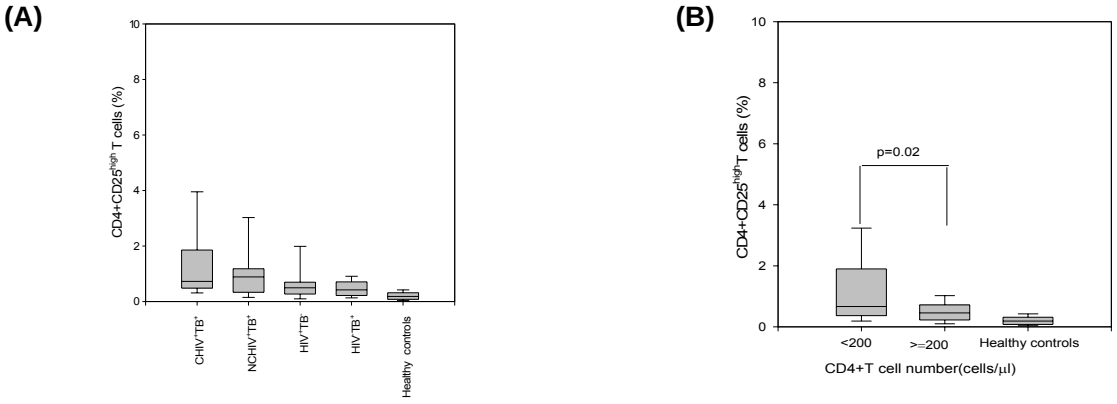

Figure-2. (A)- Expression of CD4+CD25^{high} T cells in different groups of study subjects **(B)**- Expression of CD4+CD25^{high} T cells in two different categories of CD4+ T cell numbers

Analysis of all groups at baseline showed a trend for $CD4^{+}CD25^{high}$ T cells to be negatively correlated with absolute CD4 + T cell counts (r=-0.43, p <0.001, **Fig. 3**) and a positive correlation with immune activation (expression of Ki67 within the CD4+ T cells; r = 0.44, p<0.001).

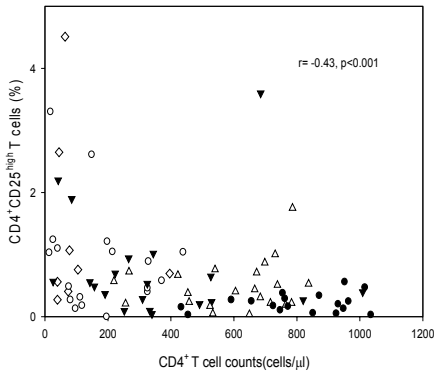

Figure-3. Correlation of CD4+CD25^{high} T cells with CD4+ T cell number. Open Diamonds (CHIV/TB), Open Circles (NCHIV/TB), Black Downward Triangles (asymptomatic HIV persons), Open Upward Triangles (HIV negative TB patients), Open Circles (Healthy controls):

Expression of FOXP3 within CD4+ T cells in HIV/TB coinfection

The level of FOXP3 expression in T cells (especially in CD4+ T cells) has recently been shown to indicate the level of regulatory/suppressive T cells within the CD25+T cell subpopulation **(6)**. Therefore, next we determined the expression of FOXP3 within the CD4+ T cells. The median expression of FOXP3 within the CD4+ T cells was 19.4 %: (median) in CHIV+TB+ before ART, 8.1% in NCHIV+TB+ patients, 7.5% in asymptomatic HIV infected persons ($HIV^{+}TB^{-}$), 3.9% in TB patients without HIV infection ($HIV^{-}TB^{+}$) and 2.8% in healthy controls. Compared to TB patients without HIV infection (HIV-TB+) and healthy controls; all HIV+ groups (CHIV+TB+, NCHIV+TB+ and $HIV^{+}TB^{-}$) had higher expression of CD4+FOXP3+ T cells (p<0.001 in all cases). However, there was no difference between TB patients without HIV infection and healthy controls (p=0.12, **Fig. 4A**). The level of expression was higher in those with <200-cells/μl than ≥200 cells/μl CD4+ T cell counts (p<0.001) **(Fig. 4B).** The level of CD4+FOXP3+T cells (%) positively correlated with plasma viral RNA levels (r = 0.53,

p=0.002), immune activation (Ki67 expression) (r=0.72, p<0.001**, Fig. 4C**) and CD4+CD25^{high} T cells (r= 0.60, p<0.001) (data not shown). However, it was negatively correlated with absolute CD4^{+}T cell numbers (r = -0.61, p<0.001, **Fig. 4D**). In addition, higher level of FOXP3 expression within CD4+CD25^{high} T cells was observed in all the groups and was not different among the groups (median: 73%, CI: 68-82).

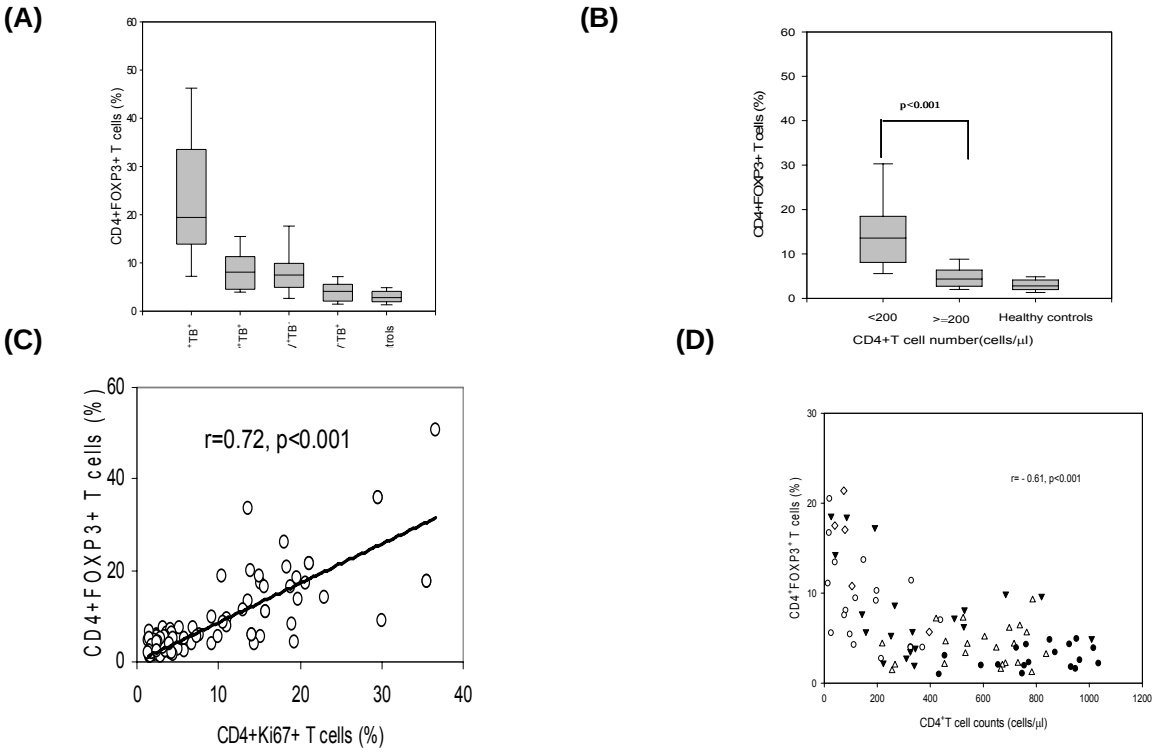

Figure-4. (A) Expression of CD4+FOXP3+ T cells in different groups of study subjects **(B)**-Expression of CD4+ FOXP3+ T cells at two different categories of CD4+ T cell numbers **(C)**- Correlation of the expression of CD4+FOXP3+ T cells with absolute CD4+ T cell number (**D) C**orrelation Ki67 and FOXP3 expression on CD4+ T cells. Open Diamonds (CHIV/TB), Open Circles (NCHIV/TB), Black Downward Triangles (asymptomatic HIV persons), Open Upward Triangles (HIV negative TB patients), Open Circles (Healthy controls).

Effect of anti-TB and antiretroviral chemotherapy on the level of regulatory T cells.

Since the expression level of CD4+CD25+ T cells was augmented in the HIV/TB coinfection compared to the healthy controls, we investigated whether the level could be affected by standard anti-TB and antiretroviral treatments. To this end, we included TB patients without HIV infection and HIV/TB coinfected (CHIV+TB+ group) after two months of anti-TB and antiretroviral chemotherapy, respectively. However, there was no change in the expression level of CD4+CD25+ T cells, CD4^{+}FOXP3^{+}T cells and CD4^{+}CD25^{high} T cells in both groups before and after therapy (**Fig. 5A & 5B).**

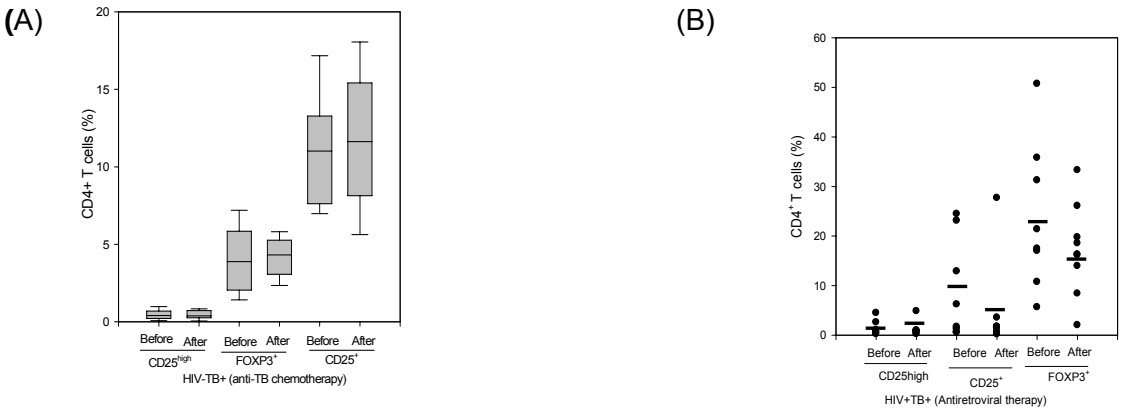

Figure-5. (A) Expression of CD4+CD25+, CD4+FOXP3+ and CD4+CD25^{high} T cells before and after anti-TB chemotherapy (B)- Expression of CD4+CD25+, CD4+FOXP3+ and CD4+CD25 ^{high} T cells before and after antiretroviral therapy

Discussion

To date, the absence of true marker to characterize Tregs in humans at the periphery imposed a limitation to investigate the functions and levels of these T cells in different infectious diseases. However, recently the availability of FOXP3 monoclonal antibody provided the opportunity to characterize regulatory T cells. In this study we investigated the levels of T regulatory (Tregs) in tuberculosis (TB) patients with and without HIV coinfection using CD25 and FOXP3 as markers.

The level of CD4$^+$CD25$^+$ T cells that we observed in this study in healthy individuals (8.1%) is in agreement with reported values **(2)**. We found an increased level of expression of CD25$^+$ T cells within the CD4$^+$ T cells in HIV$^+$TB$^+$ coinfected patients (non chronic) followed by asymptomatic HIV infected, TB patients without HIV infection and healthy controls. As CD25 is up-regulated on activated T cells and is expressed at a higher level in memory CD4+ T cells, the trend of increase in CD4+CD25+ T cells from healthy controls to NC HIV$^+$TB is due to more immune activation as a result of antigenic stimulation. Indeed we recently observed that also immune activation levels in HIV+TB+ coinfected patients were higher compared to other groups as evidenced by HLA-DR and CD38 expression, CCR5 expression and Ki-67 expression on CD4 and CD8+ T cells **(17 and Chapter-4)**. However, we also found lower percentage of CD4+CD25+ T cells in chronic HIV/TB coinfected patients, which might be due to the disruption of these cells with higher HIV disease progression **(8).** Moreover, reports showed also the level and activity of regulatory T cells could be higher in the early stage of HIV infection and decreases as the immunodeficiency increased at advanced level of the disease **(18)**.
Within the CD4$^+$CD25$^+$ T cells, CD4$^+$CD25^{high} T cells were reported to have a crucial role in regulatory functions **(19,20)**. In our study we found below 1% of the CD25$^+$ T cells express the CD25 ^{high} T cells in the healthy controls (0.2%), which is lower compared to other reports **(18)**. However, the 2.2 fold increased expression in TB patients without HIV infection than healthy controls, is in agreement to the recently reported data in TB patients **(12).** This increase was 2.6 fold in asymptomatic HIV infected individuals and significantly increased by 4 fold in chronic and non-chronic HIV+TB+ coinfected individuals (CHIV+TB+ & NCHIV+TB+), which indicates the presence of more Tregs in HIV-TB coinfected patients.

Also FOXP3 expression was higher within the CD4$^+$ T cells in coinfected patients. In addition, FOXP3 had a higher expression in individuals with <200 CD4+ T cells/μl, very

similar to what has been observed for Ki67-exprerssion, a marker for proliferation and associated with high immune activation. Furthermore, the positive correlation between FOXP3 expression and immune activation further corroborates that immune activation and FOXP3 expression are linked. The positive correlation of FOXP3 with HIV plasma viral RNA level suggests that the expression of this transcription factor can be up regulated due to the presence of antigenic burden (both plasma viral load and TB antigens in coinfected patients). This would also fit with the observed relationship between the level of regulatory gene FOXP3 (and thereby regulatory T cells) and disease progression **(10, 21)**.

Since the presence of higher levels of regulatory T cells is reported to have a down regulatory effect on immune responses, we performed ELISPOT assays in all subjects (except chronic HIV^+TB^+ persons) to analyze MTB-specific T cell responses (against the MTB antigens ESAT-6 and CFP10). Interestingly, although not significantly different, the number of IFN-γ producing T cells towards the CFP-10 TB antigen shows a trend of reduction in immune response in HIV^+TB^+ coinfected individuals with higher level of regulatory T cells and immune activation (data not shown). However, since we were not able to deplete CD4+CD25+ T cells from the PBMCs, we could not prove that the CD25+ cells were causing the reduction in immune response towards TB antigens.

We found no differences in levels of regulatory T cells after anti-TB chemotherapy and antiretroviral therapy in TB infected patients without HIV infection and chronic HIV^+TB^+ coinfected individuals, respectively. This might be due to short treatment periods in both cases (TB and HIV). However, as anti-TB treatment also does not lead to a reduction in immune activation levels on T cells (**Chapter-4**), the treatment is also likely not able to change Tregs levels. However, we have observed significant reduction in plasma viral RNA and immune activation levels in this short period **(Chapter-4)**. Alternatively, the established Treg population could be a population with a different fate that cannot be reverted by ART, which results in a stable Treg population (at a higher level) after induction of these T cells. The Treg population has indeed been shown to be very stable (without interference) and they may be able to replenish themselves by proliferation **(22)**.

In conclusion, in this study we showed high percentages of regulatory T cells as defined by $CD25^{high}$ expression and the proportion of FOXP3 expression within CD4+ T cells in HIV/TB coinfection. The positive and negative correlations of regulatory T cells with plasma viral RNA level, immune activation and CD4+ T cell number, respectively suggest that their induction could be antigen driven. The absence of reduction and restoration with anti-TB and antiretroviral treatments indicate the need for longer treatment periods and/or may indicate the longevity of these T cell subsets despite anti-TB and ART.

Acknowledgements

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Expression of chemokine receptors CCR5 and CXCR4 on CD4+ T cells and plasma chemokine levels during treatment of active tuberculosis in HIV-1 co-Infected patients

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Chapter 7

Abstract

The pathogenesis of persistently elevated plasma HIV viremia in patients co-infected with tuberculosis (TB) during anti-TB treatment in Africans remains unknown. We examined the expression of chemokine receptors CCR5 and CXCR4 on CD4+ T-cells and plasma chemokine levels of macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal T expressed and secreted (RANTES) and stromal cell-derived factor (SDF)-1α among TB patients with HIV co-infection during the first 2 months of anti-TB treatment. During treatment of TB, both the plasma HIV-1 load and CD4+ T cell count remained unchanged. Levels of CCR5 and CXCR4 expression on CD4+ T cells as well as plasma levels of chemokines remained persistently elevated during anti-TB treatment. Persistently elevated plasma HIV viremia paralleled also with persistently elevated expressions of activated CCR5+ or CXCR4+ CD4+ T-cells. These results suggest that increased expression of CCR5 and CXCR4 on activated CD4+ T-cell population coupled with persistently elevated chemokines may provide a suitable condition for continuous replication of HIV associated with TB co-infection. This, in turn, may contribute, at least in part, to the observed persistently elevated plasma HIV viremia in co-infected patients despite anti-TB treatment.

Introduction

HIV-infected patients are at increased risk of developing active *Mycobacterium tuberculosis* (MTB) disease, from reactivated latent infection or exogenous re-infection, and active MTB in HIV-infected patients has been shown to accelerate HIV disease progression (1-3). The immune background profile of individuals living in sub-Saharan Africa is characterized by chronic activation (4-7). In this respect, increased susceptibility to *de novo* infection with HIV and/or accelerated HIV disease progression in individuals from sub-Saharan Africa might be attributable to the chronic and persistent immune activation by ongoing immune responses to various endemic infections that prevail in the continent (7). It is worth noting that in patients with TB, there is evidence for immune activation (8-11). In addition, the level of immune activation of peripheral monocytes from patients with active pulmonary TB is sufficient to enhance susceptibility to productive infection with HIV *in vitro* (12), and MTB (and its antigens thereof) can up-regulate HIV replication *in vitro* (10, 13-17). In HIV-infected patients, mycobacteria appear also to augment HIV replication as measured in the peripheral circulation (10), the lung (15, 17-19), and lymphoid tissues (20, 21), through mechanisms involving immune activation (10, 21). It is interesting to note that increased expression of CCR5 and/or CXCR4, major chemokine HIV co-receptors, as well as their chemokine ligands, have been demonstrated in human monocyte-derived macrophages, alveolar macrophages and CD4+ T-cells in the course of *in vivo* and *in vitro* MTB infection (16, 19, 21-23), and the expression of these co-receptors appears to depend on the state of activation of the cells (24-26). Although the lung could be a preferential organ for HIV replication during active TB (15, 17-19), it is worth noting that a significant increase (as high as 3- to 160-fold) in plasma HIV viremia has been observed during the acute phase of MTB disease (10, 27), possibly related to mycobacteremia which is frequently observed in HIV co-infected patients (28). Indeed, CD4+ T cells, the principal target for HIV (29, 30), contribute to more than 95% of the total virus production (31). In contrast to what has been observed in MTB disease in the Western world where plasma HIV viremia often declines after successful TB treatment (10), few studies showed that HIV viral load remains unchanged both during and after completion of successful anti-TB treatment in Africans (11, 27, 32, 33). The pathogenesis of the persistently elevated plasma HIV viremia in patients co-infected with TB in Africans remains undefined, although several studies suggested the role of persistently activated immune system (11, 33).

In the present study, therefore, we studied the expression of chemokine receptors CCR5 and CXCR4 on CD4+ T-cells and plasma chemokine levels of macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal T expressed and secreted (RANTES) and stromal cell-derived factor (SDF)-1α among TB patients with or without HIV co-infection during the first 2 months of anti-TB treatment. Our findings indicate that persistently increased expression of chemokine receptors and chemokines may provide a potential mechanism of increased replication of HIV, and in part, may contribute to the persistence of HIV viremia observed in Africans despite anti-tuberculosis treatment.

Patients and methods

Patient population

HIV-1-infected patients with TB (*n* = 21; CD4+ T cell count range, 20 to 439 median 149 cells/μl; plasma viremia range 2,200-840,000, median 120,000 HIV-1 RNA copies/ml) were prospectively recruited and followed at the two Clinical Centers [the All African Leprosy Rehabilitation and Training Center (ALERT) and Higher 23 Health Center, both in Addis Ababa, Ethiopia]. HIV-negative persons with TB only (*n* = 15; CD4+ T cell count range, 215 to 837, median 464 cells/μl) were also included as controls. Patients were enrolled after

thorough clinical evaluation by primary clinicians at the respective health centers. The diagnosis of TB was made after demonstration of acid-fast bacilli in sputum samples confirmed by positive culture for MTB and radiological or histological evidence compatible with TB. Blood was obtained from each patient before or less than seven days of initiation of anti-TB chemotherapy and during follow-up visits two months after enrollment. After TB diagnosis, all patients received directly observed therapy with isoniazid 5 mg/kg body weight (300 mg maximum), rifampicin 10 mg/kg (600 mg maximum), pyrazineamide 15-30 mg/kg (2 gm maximum) and ethambutol 15-25 mg/kg. All HIV-infected patients were antiretroviral naïve, as the drugs are not available in Ethiopia at the time of the study. Informed consent was obtained from all study participants and the study protocol has been reviewed and approved by Institutional and National Ethical Clearance Committee's.

Table-1. Comparison of patients with active tuberculosis (TB) with or without HIV co-infection in terms of CD4 counts and/or plasma HIV-1 viral load at baseline and during the first 2 months of anti-TB treatment

Patient category and laboratory data	Baseline	After 2 months	$P^{\dagger}$
*TB patients (*n = *8)* [*]			
CD4 count			
Percentage	49.0 (34.0-51.5)	44.5 (32.5-49.5)	0.195
Absolute count (cell/mm^3)	461 (284-596)	563 (444-737)	0.008
*TB/HIV co-infected (*n = *21)*			
CD4 count			
Percentage	11.0 (8.8-13.8) [‡]	10.0 (8.0-15.3)	0.174
Absolute count (cell/mm^3)	149 (81-194)[‡]	146 (89-200)	0.931
Plasma HIV-1load Copies/ml	120,000 (49,000-227,500)	105,000 (48,250-297,500)	0.465
Log_{10} copies/ml	5.08 (4.69-5.35)	5.08 (4.67-5.47)	0.677

Data are expressed as median (inter quartile ranges), [*] Only 8/15 TB patients had CD4 counts both at baseline and follow-up, [†]Mann-Whitney *U*-test, [‡]$P < 0.001$ when compared to TB/HIV co-infected group, Wilcoxon Signed Rank Test.

FACS analysis

Cells were stained with the following Ab combinations: CD3 (FITC) or CD8 (FITC) and CD4 (PE) and CD45 (PerCP), HLA-DR (FITC) and 2D7 or 12G5 (PE) and CD4 (PerCP). PE-conjugated CCR5 (2D7) and CXCR4 (12G5) mAbs were obtained from Pharmingen (La Jolla, CA). mAbs (FITC-, PE-, PerCP-conjugated) to CD3, CD4, CD8, CD45, and HLA-DR were obtained from Becton Dickinson (San Jose, CA). For each stain, thawed peripheral blood cells were incubated with appropriate mAbs for 30 min, after which stained cells were analyzed using a three-color FACScan with Cellquest software (Becton Dickinson). Expression of HLA-DR, CCR5 and CXCR4 on CD4+ T cell subset was analyzed by a combination of side scatter and setting of a

gate around the CD4-PerCP stained cells. At least, a total of 2500 live cells were acquired before analysis was performed.

Plasma HIV-1 viremia

Plasma HIV viremia was quantitated by using nucleic acid-based amplification assay (NASBA, Organon Teknika, The Netherlands). HIV RNA concentrations below the detection limit of the NASBA assay (<80 copies/ml of plasma) were considered at 80 copies/ml.

ELISA for chemokines

The chemokines MIP-1α, MIP-1β, RANTES and SDF-1α were measured by commercial ELISA kits (R&D Systems) with limits of detection 10, 11, 8 and 18 pg/ml, respectively.

Statistical analysis

Means were compared using the paired Student's *t*-test. Median CD4 counts and plasma HIV levels were compared using the nonparametric Mann-Whitney *U* test. The individual changes in CD4 counts and plasma HIV load from baseline were compared using the nonparametric Wilcoxon Signed Rank test. Correlation was assessed by using the Spearman's rank test. A *P* value of less than 0.05 was considered indicative of statistical significance. The statistical analysis was performed using the SPSS statistical package (SPSS Inc., Chicago, IL).

Results

Levels of immune activation, expression of CCR5 and CXCR4 on CD4+ T cell population and plasma chemokines

Since there is evidence for immune activation in HIV-infected patients with tuberculosis (8-11), we evaluated the state of activation of the CD4+ T-cells as reflected by the expression of the cellular marker HLA-DR. As shown in Fig. 1, the proportion of circulating CD4+ T cells expressing HLA-DR was significantly higher in TB/HIV co-infected patients when compared with those with TB only (median [IQR], 47.1% [32.6-57.9] *vs.* 13.6% [11.7-19.3], respectively; *P*<0.001).

In addition, CCR5 expression within CD4+ T cell subpopulation from TB/HIV co-infected patients was increased significantly than in those with TB only (35.0% [22.4-45.8] *vs.* 3.3% [2.5-7.5], respectively; *P*<0.001). Overall, a 10-fold increase in CCR5 expression was observed on CD4+ T cell subsets from individuals co-infected with TB/HIV than those with TB only. Similarly, CXCR4 expressing CD4+ T cells were significantly increased in TB/HIV patients than in those with TB only (96.9% [94.4-98.7] *vs.* 71.7% [60.8-76.4], respectively; *P*<0.001). The level of CD4+ T-cells also expressing CXCR4 was significantly greater than those expressing CCR5, regardless of the HIV status of the patients (Fig. 1; *P*<0.001).

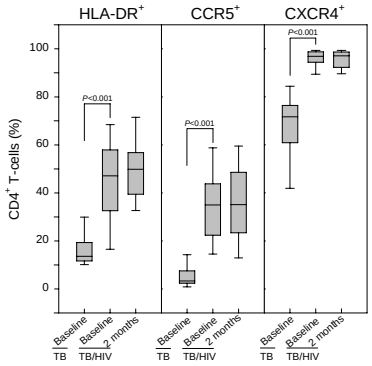

Figure-1. Expression of HLA-DR, CCR5 and CXCR4 on CD4+ T-cells from patients with tuberculosis (TB) without or with HIV co-infection (at baseline and/or after two months of anti-TB treatment).

Since the expression of chemokine receptors appears to depend on the state of activation of the cells (23-25), we further analyzed whether CCR5 and CXCR4 expression on CD4+ T cells correlated with cellular activation. As shown in Fig. 2, the proportion of CCR5+CD4+ T-cells also expressing HLA-DR was significantly higher in cells from TB/HIV patients than in TB patients without HIV (20.6% [11.4-35.3] *vs.* 2.0% [1.3-3.8], respectively; *P*<0.001). Similarly, the proportion of CXCR4+CD4+ T-cells also expressing HLA-DR was significantly higher in TB/HIV patients than in those with TB only (48.4% [29.6-54.9] *vs.* 9.1% [6.7-10.8], respectively; *P*<0.001).

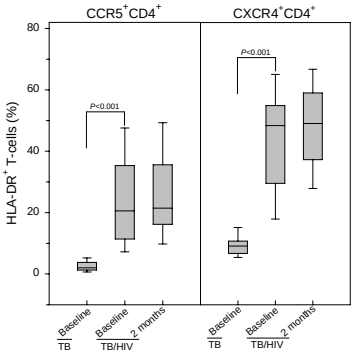

Figure- 2. Expression of HLA-DR of CCR5+ and CXCR4+ CD4+ T-cells from patients with TB without or with HIV co-infection at baseline and/or after two months of anti-TB treatment.

The proportion of activated CXCR4+CD4+ T-cells was higher than activated CCR5+CD4+ T-cells in both patient groups. In addition, the proportion of CD4+ T-cells expressing CCR5 or CXCR5 positively correlated with the activation state of the cells, with Spearman correlation coefficients of 0.73 (*P*<0.0001) for CD4+ T-cells expressing CCR5 and 0.53 (*P*=0.001) for CD4+ T-cells expressing CXCR4 (Fig. 3). Moreover, the plasma levels of chemokines in TB/HIV co-infected patients were not significantly different from those with TB only (Fig. 4)

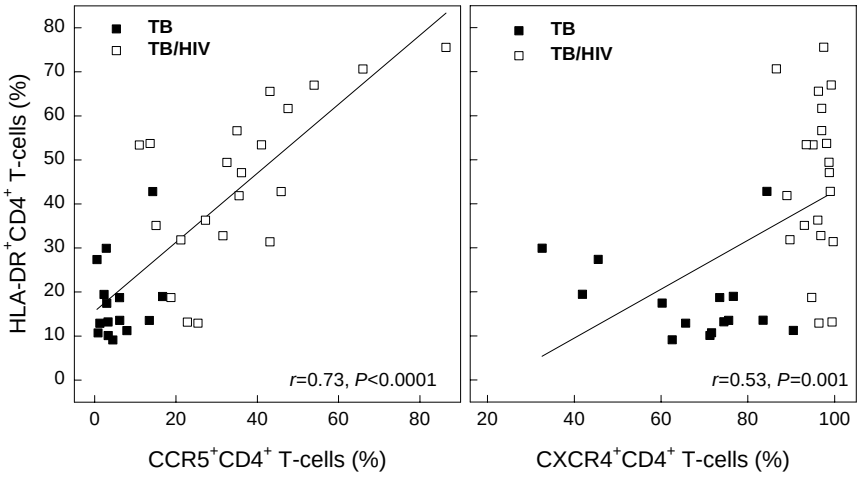

Figure-3. Correlation between stage of immunodeficiency and chemokine receptor expression (left panel) and the state of activation of CCR5+- or CXCR4+-expressing CD4+ T-cells (right panel) among TB patients with or without HIV co-infection.

Levels of cellular activation, chemokine expression and plasma chemokines during anti-TB treatment in HIV co-infected patients

Since efficient induction of HIV replication is initiated through immune activation (34), we evaluated changes in the level of expression of activation marker HLA-DR, chemokine receptor expression on CD4+ T cells and plasma chemokine levels. Patients with MTB disease were treated with anti-TB chemotherapy and we evaluated the effect of anti-TB treatment during the initial 2 months on the virological and immunological parameters.

Treatment of TB was associated with a significant increase in the absolute CD4 count only in those who were not co-infected with HIV (Table 1). However, there was no significant change in CD4+ T-cell percentage or absolute cell count in the TB/HIV group. In addition, plasma HIV load remained persistently elevated during anti-TB treatment. Although (24%) patients had a significant decrease in plasma HIV-1 viremia, the majority (48%) exhibited a significant increase (> 0.5 $\log_{10}$) and 20% had no significant change during anti-TB treatment. Lack of significant change in CD4 cell count and plasma HIV viremia paralleled also with lack of significant change in the proportion of activated cells, chemokine receptor expression on CD4+ T cells as well as plasma chemokine levels during anti-TB treatment. Thus, compared to the baseline levels, the percentage of CD4+ T-cells also expressing HLA-DR remained sustained after 2 months of successful treatment of TB (Fig. 1, 47.1% [32.6-57.9] *vs.* 49.9% [39.5-56.8], respectively; *P*=0.281). Also, no significant change was noted in both the levels of CCR5- and CXCR4-expressing CD4+ T-cell population (Fig. 1). The percentage of CCR5+CD4+ T-cells expressing the activation marker HLA-DR remained persistently elevated after 2 months of anti-TB treatment (Fig. 2). Likewise, we observed no significant change with regard to plasma chemokine levels measured at baseline compared to 2 months following anti-TB treatment (Fig. 4).

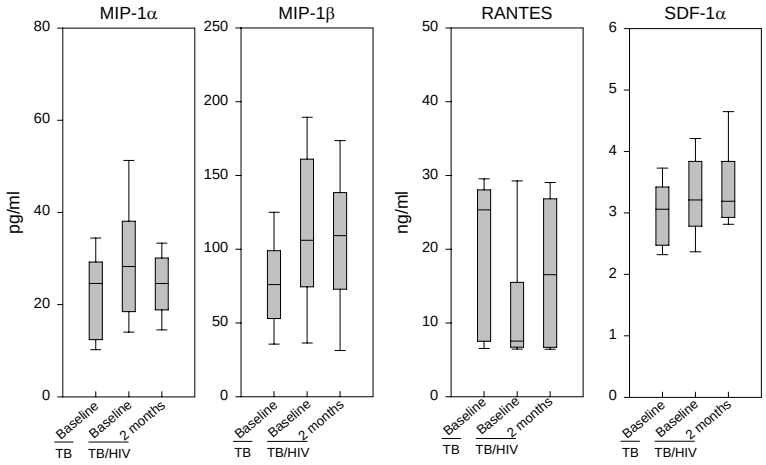

Figure- 4. Plasma levels of chemokines in patients with TB without or with HIV co-infection at baseline and/or after two months of anti-TB treatment.

Discussion

In this study, we noted that during anti-TB treatment, there was no significant increase in CD4 cell count among the TB/HIV co-infected patients when compared in those who were not co-infected with HIV. The findings are similar with that reported by Morris *et al* (33). Poor CD4 T cell restoration during TB treatment may the consequence of uncontrolled HIV-1 replication. In addition to the CD4 molecule (29, 30), the chemokine receptors CCR5 and CXCR4 have been identified as the major requisite HIV-1 entry into CD4+ T cells and macrophages (35-38). The β-chemokine receptor CCR5 is the most important coreceptor used by macrophage (M)-tropic viruses, whereas the α-chemokine receptor CXCR4 is the dominant co-receptor for T cell line (T)-tropic viruses (35-39). The expression pattern of the chemokine receptors on various cells is believed to have an influence on susceptibility, viral tropism and HIV disease progression (39, 40). It is interesting to note that there is ample evidence for immune activation in the course of active MTB disease (8-11). In addition, the expression of these receptors has been shown to be dependent on the state of activation of the target cells (24-26).

Previous studies have demonstrated that there was an increase in CCR5 expression on mononuclear cells in the lung (16, 19) and lymphoid tissue (21) from HIV patients co-infected with mycobacteria. CD4+ T-cell latently-infected with HIV is a major source of circulating viremia (31). Since mycobacteremia is also frequent in HIV-infected patients (28) and MTB-derived antigens have been shown to induce chemokine receptor expression on immune cells *in vitro* (16, 22, 23) and chemokines (16), we undertook the present study in order to evaluate the impact of anti-TB treatment in TB/HIV co-infected persons. This study demonstrates that both CCR5- and CXCR4-expressing CD4+ T cells were elevated in TB patients also co-infected with HIV. The findings from the present study concur with those reported previously (16, 23). In the present study, in addition we have shown that activated CD4+ T cells express high levels of CCR5 and CXCR4, and the expression of the co-receptors and cellular activation paralleled with each other. Susceptibility to infection with HIV is associated with levels of CCR5 expression (25, 41), and MTB can activate CD4+ T cells to induce HIV replication (10). Thus, increased expression of chemokine receptors within the CD4+ T subpopulation, may at least in part, explain as a mechanistic pathway of increased HIV replication observed during co-infection with TB. In addition, dysregulation in the chemokines might play important role in this process.

We have previously demonstrated that acute MTB infection is associated with significant increases in plasma HIV viremia (32). However, the plasma HIV load remained persistently elevated during anti-TB treatment. The data are consistent with prior studies from Africa of plasma HIV viremia during MTB disease (11, 27, 33), but is in contradiction with an earlier report (10), that successful treatment of MTB in Western subjects is associated with a significant reduction in HIV-1 RNA levels. Lawn *et al* (11) demonstrated that sustained plasma HIV viremia is associated with persistently elevated concentrations TNF-α during anti-TB treatment. Morris *et al* (33) also recently showed that sustained plasma HIV-1 load during anti-TB treatment was associated with persistently elevated HLA-DR and CD38 in CD8+ T-cell populations. In the present study, we observed that the levels of CCR5 and CXCR4 expressed on CD4+ T-cells remained persistently elevated and paralleled with the level of cellular activation as well as chemokines during anti-TB treatment. The fact that plasma viremia declines after successful treatment of infections other than TB (42-44), but remains persistently elevated during co-infection with TB indicates that TB-induced immune activation remains protracted, and suggest that TB infection might modulate HIV-1-specific immune responses, which are not restored once TB is successfully treated. Similar observation has been noted in treated schistosomiasis patients co-infected with HIV in Uganda (45)

In Ethiopia, the predominant circulating HIV virus is subtype C (46-48). Although CXCR4 usage by HIV-1 isolates often evolves during disease progression associated with the emergence of syncytium-inducing variants (49), this is not the case from our studies in Ethiopia (48). Indeed, in advanced HIV disease, CCR5 usage by non- syncytium-inducing isolates was the predominant phenomenon observed, and the emergence of syncytium-inducing viruses that use CXCR4 was found to be very rare for subtype C viruses (48, 50). Moreover, Morris et al showed (51) that R5 HIV-1 subtype C variants are preferentially recovered from patients with active TB. Taken together, the data suggest that increased expression of particularly CCR5 on activated CD4+ T-cell population may provide the availability of target cells for increased replication of HIV associated with TB co-infection.

The above studies suggest that expression of chemokine receptors in the course of TB along with other components of the immune activation process may provide a background for a more efficient availability of target cells for enhanced replication of HIV, and hence accelerated HIV disease progression, in the co-infected individual, and increased susceptibility of TB+HIV-negative patients following exposure with HIV-1. Although with the introduction of highly active antiretroviral therapy (HAART) may alter the sequela of increased HIV replication in the course of MTB disease, in as much of the developing world, where both HIV and TB prevail, the findings underscore the need for continued prophylaxis and prompt chemotherapy of TB among co-infected persons.

Acknowledgements

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PART-III

DISEASE PROGRESSION AND OUTCOMES OF HIV/TB COINFECTION

Slower CD4 T cell decline in Ethiopian versus Dutch HIV-1 infected individuals is due to lower T cell proliferation rates

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Chapter 8

Abstract

It has recently been shown that HIV-infected Ethiopians have a slower rate of $CD4^+$ T cell decline than Dutch HIV-infected individuals. This was unexpected, because healthy Ethiopians have a higher basal level of immune activation than healthy Dutch individuals and high immune activation is one of the best predictors for HIV disease progression. Here we found that when Dutch and Ethiopian HIV-infected patients were matched for $CD4^+$ count, the percentage proliferating, $Ki67^+$, cells within the $CD4^+$ and $CD8^+$ T cell subsets were lower in Ethiopians. Thus, the slower $CD4^+$ T cell decline in HIV^+ Ethiopians might be explained by lower immune activation.

Introduction

It has previously been found that T-cell activation as determined by HLA-DR [1] and proliferation as measured by Ki67 expression [2] are increased in healthy Ethiopians compared to Caucasians. This high level of immune activation in healthy Ethiopians is most likely caused by exposure to environmental pathogens and is accompanied by low numbers of total and naïve $CD4^+$ T cells [1-4]. Similarly, persistent immune activation has been proposed to cause $CD4^+$ T cell depletion in HIV-infection [5]. Indeed, the presence of high T-cell immune activation and proliferation levels was found to be the best predictor for HIV-disease progression [6-9].

Because of the basal persistent immune activation in healthy Ethiopians, we hypothesized that upon HIV-infection Ethiopians would have higher levels of immune activation than Dutch HIV^+ individuals, and concomitantly show a faster rate of $CD4^+$ T cell decline and progression to AIDS. Surprisingly, Mekonnen et al [10] found that the rate of $CD4^+$ T cell decline was in fact significantly lower in HIV^+ Ethiopian compared to HIV^+ Dutch patients. The rate of $CD4^+$ T cell decline was strongly dependent on the $CD4^+$ T cell count in both Dutch and Ethiopian HIV-infected individuals, and since HIV^+ Ethiopians have lower baseline $CD4^+$ T cell counts, this could explain their lower loss rates. However, even when the data were stratified according to $CD4^+$ T cell counts, the loss rate of $CD4^+$ T cells remained lower in HIV^+ Ethiopians [10]. Differences in viral load or the lack of syncytium-inducing (SI), CXCR4 tropic virus variants amongst Ethiopian HIV-infected individuals [11], also did not explain the faster $CD4^+$ T cell decline in Dutch HIV-infected individuals [10], suggesting other factors play a role.

On one hand, the pre-existing higher immune activation levels in healthy Ethiopians imply that, upon HIV infection, Ethiopians would similarly have higher levels of immune activation compared to HIV^+ Dutch individuals. On the other hand, if immune activation is considered as the driving force for disease progression, the slower rate of $CD4^+$ T cell decline in HIV-infected Ethiopians suggests that lower levels of immune activation are induced upon HIV-infection in Ethiopians compared to HIV^+ Dutch individuals. To discern these hypotheses, we determined the fraction of proliferating ($Ki67^+$) T cells in both Ethiopian and Dutch HIV-infected therapy-naïve individuals matched for $CD4^+$ T cell count.

Material and methods

Study Population

Ethiopian HIV-infected individuals (n=19) were recruited from (i) The All African Leprosy Rehabilitation and Training Centre (ALERT) and Higher 23 Health Centre (ii) from two cohort sites of the Ethio-Netherlands AIDS Research Project. Dutch HIV-infected individuals (n=19) were included from the Amsterdam Cohort Studies on HIV infection and AIDS and were matched to the HIV^+ Ethiopians according to $CD4^+$ T cell count. All studied HIV-infected individuals were naïve to antiretroviral therapy and had $CD4^+$ T cell counts above 200 cells per µl blood. The study protocol was approved by ethical clearance committees and informed consent was obtained from all study participants.

Flow cytometry

As a measure for immune activation we determined the percentage proliferating $Ki67^+$ T cells (measured as previously described in **Chapter-4**) in total, naïve ($CD45RO^-CD27^+$), memory ($CD45RO^+CD27^+$), effector/memory ($CD45RO^+CD27^-$) and ($CD45RO^-CD27^-$) effector $CD4^+$ and $CD8^+$ T cells by FACS analysis.

Statistical analysis

Differences between Dutch and Ethiopian HIV-infected individuals were analyzed by the non-parametric Mann-Whitney test using the software program SPSS 12.0.1 (SPSS Inc., Chicago, Illinois, USA). Of note, if we were unable to measure Ki67 expression in a specific cell subset, the Ki67 expression of that particular T cell subset in the individual with matched $CD4^+$ T cell count was also omitted for analysis.

Results

Since the rate of $CD4^+$ T cell decline is dependent on $CD4^+$ T cell numbers[10] and because the fraction of proliferating ($Ki67^+$) T cells increases during disease progression[6], every HIV^+ Ethiopian individual was matched with a Dutch HIV^+ individual according to $CD4^+$ T cell count. Given that SI virus variants are rare amongst Ethiopian HIV-infected individuals, we only included Dutch HIV-infected individuals who exclusively harbored NSI (CCR5-using) virus variants at the time of analysis. The characteristics of the HIV^+ Ethiopian and HIV^+ Dutch groups are depicted in Table 1. Age and viral RNA load in plasma were not significantly different between Ethiopian and Dutch HIV-infected individuals. However, only in 10 out of 19 Dutch HIV^+ individuals viral load was determined at the time-point at which PBMC were analyzed. If we included the mean of surrounding plasma viral loads for the other 8 patients (for one patient plasma viral load was not available) the median load of the Dutch HIV-infected individuals and statistical significance were unaffected. As reported before [12], $CD8^+$ T cell counts were significantly higher in the HIV-infected Ethiopian compared to HIV-infected Dutch individuals (p=0.002).

Table 1. Characteristics of Ethiopian and Dutch HIV^+ individuals

	Ethiopian (n=19)	Dutch (n=19)
Gender (F/M)	8/11	0/19
TB co-infected	7	0
Age (median, range)	31 (22-50)	31 (24-50)
$CD4^+$ T cell count in cells/µl (median, range)	326 (212-493)	330 (210-490)
$CD8^+$ T cell count in cells/µl (median, range)*	1128 (315-3231)	800 (400-1430)
Viral RNA load in plasma in copies/ml (median, range)	46,000 (5,100-840,000) n=18	39,000 (1,000-260,000) n=10

* significantly different between the two groups (p=0.002)

In all measured $CD4^+$ and $CD8^+$ T cell subsets a similar trend of increased $Ki67^+$ fractions in Dutch compared to Ethiopian HIV-infected individuals could be observed. However, probably due to the small sample size, statistically significant differences were not reached for every T cell subset. The median percentage $Ki67^+$ within total $CD4^+$ T cells was 9.79% in HIV^+ Dutch and 6.11% in HIV^+ Ethiopian individuals (p=0.145). Median percentages of $Ki67^+$ were 4.01% versus 1.96% within naïve (p=0.017), 14.93% versus 9.03% in memory (p=0.019) and

10.64% versus 5.71% in effector/memory (p=0.139) $CD4^+$ T cells in HIV-infected Dutch and Ethiopian individuals, respectively (Figure -1).

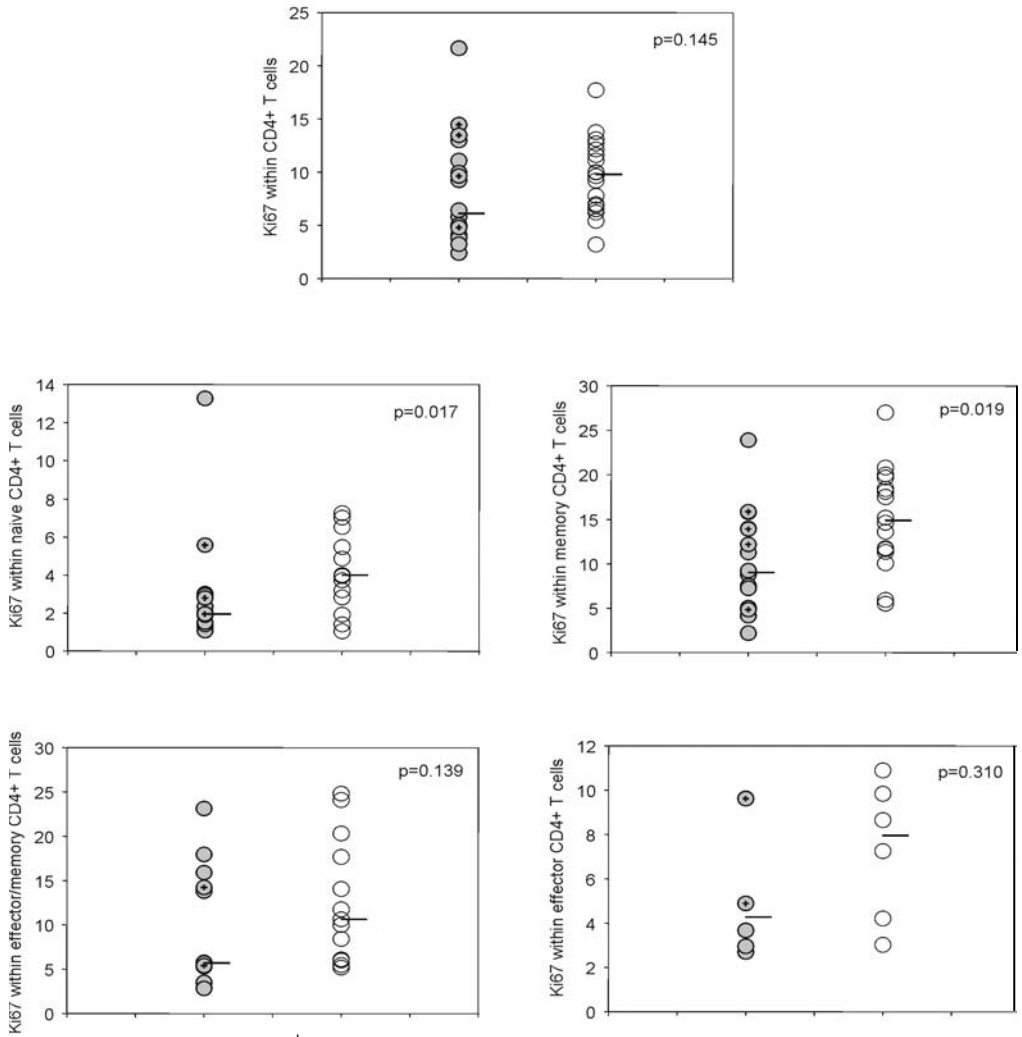

Figure 1. Percentage $Ki67^+$ T cells within total, naïve, memory, effector/memory and effector $CD4^+$ T cells of HIV^+ Dutch and Ethiopian individuals. Grey circles denote HIV^+ Ethiopians

Although the $CD4^+$ effector population is usually very small in healthy individuals, this population of $CD4^+$ T cells tends to be enlarged during chronic immune activation [12]. We could measure Ki67 expression in the $CD4^+$ effector population in 6 Dutch and Ethiopian HIV-infected individuals and $Ki67^+$ fractions were 7.95% versus 4.28% (p=0.310), correspondingly. The median percentage $Ki67^+$ within total $CD8^+$ T cells was 8.77% in Dutch versus 5.78% in Ethiopian HIV-infected individuals (p=0.030). Differences in median $Ki67^+$ fractions between HIV^+ Dutch and Ethiopian individuals in $CD8^+$ T cells subsets were 4.46% versus 3.59% within naïve (p=0.239), 13.99% versus 8.44% in memory (p=0.050), 10.62% versus 5.55% in effector/memory (p=0.034) and 5.08% versus 3.47% in effector (p=0.034) $CD8^+$ T cells, respectively (Figure 2).

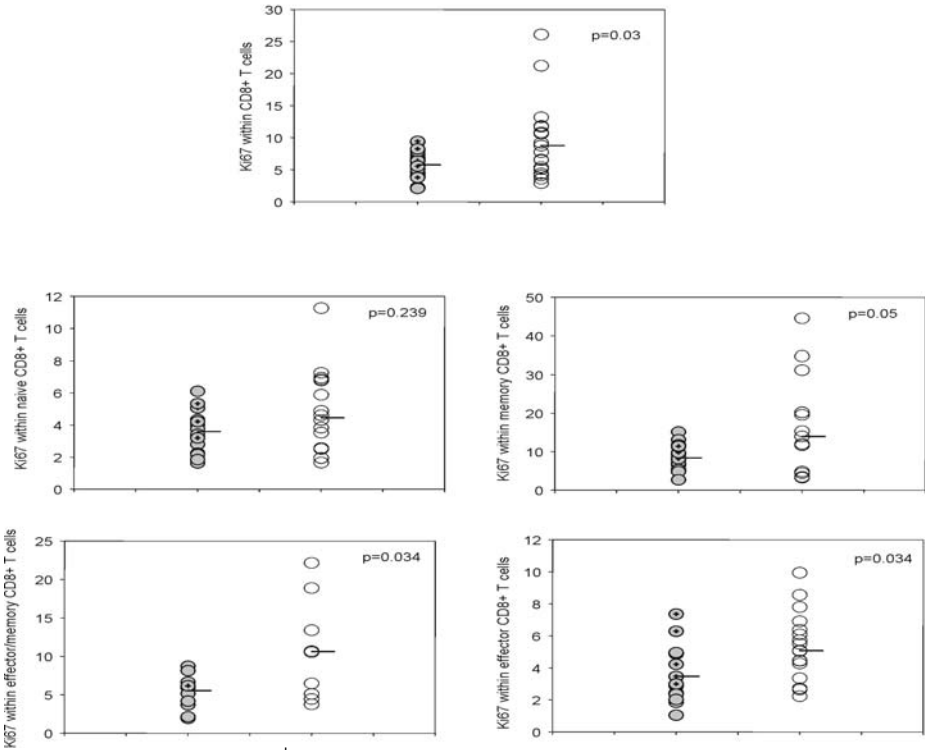

Figure -2. Percentage Ki67$^+$ T cells within total, naïve, memory, effector/memory and effector CD8$^+$ T cells of HIV$^+$ Dutch and Ethiopian individuals. Grey circles denote HIV$^+$ Ethiopians.

Interestingly, seven out of nineteen HIV$^+$ Ethiopians suffered from *Mycobacterium tuberculosis* (TB) (Figure 1, +-marked grey circles), which we previously showed to result in increased T cell proliferation (**Chapter-4**). Thus in spite of TB co-infection in 7 HIV$^+$ Ethiopians, Dutch HIV$^+$ individuals appeared to have significantly higher fractions of Ki67$^+$ T cells.

Discussion

Immune activation has been proposed as the driving force for HIV disease progression [5]. Therefore, the slow rate of CD4$^+$ T cell decline in HIV$^+$ Ethiopian compared to Dutch individuals [10] suggests immune activation levels to be lower in HIV$^+$ Ethiopians. On the other hand, this would be counterintuitive in view of the higher basal level of immune activation found in healthy Ethiopians. In line with the slower rate of CD4$^+$ T cell decline in HIV$^+$ Ethiopian compared to Dutch individuals [10], although not statistically significant in all subsets, the level of CD4$^+$ and CD8$^+$ T-cell proliferation was found to be lower in HIV$^+$ Ethiopian compared to HIV$^+$ Dutch individuals. The fact that Dutch HIV-infected individuals tended to have higher Ki67$^+$ fractions, even though 7 out of 19 HIV$^+$ Ethiopians were co-infected with TB, which we have shown to increase Ki67$^+$ T cell fractions in Ethiopian HIV-infected individuals (**Chapter-4**), reinforces our conclusion.

To exclude transient high fractions of Ki67$^+$ T cells due to opportunistic infections we studied HIV-infected individuals who had CD4$^+$ T cell counts above 200 cells per µl blood and all HIV$^+$ Dutch individuals had not progressed further than CDC group III. Higher levels of T cell proliferation in Dutch HIV$^+$ individuals could not be explained by syncytium inducing, CXCR4 using HIV-1 variants because we only included HIV$^+$ Dutch individuals that exclusively harbored NSI virus variants.

Generally viral load is correlated with immune activation and both have been shown to be related to progression to AIDS [6-9]. HIV viral load is typically lower in Ethiopians early after HIV infection [13]. This is mainly due to Ethiopians infected with HIV-1 subtype C, who have a lower viral load than Ethiopians with the predominant subtype C' early in infection [13]. During HIV infection, however, viral load in Ethiopians with subtype C is known to increase, and 2 years after seroconversion the viral load of Ethiopians was found to be similar to that of Dutch HIV-infected individuals [13]. As mentioned above, there was no evidence for lower viral load in Ethiopians compared to Dutch HIV-infected individuals in our study group (Table 1) that could explain the lower $Ki67^+$ fractions in HIV^+ Ethiopians. Our results could also not be explained by including 8 (out of 19) women in the HIV^+ Ethiopian group, who are known to have a lower viral load than men [14], since $Ki67^+$ fractions were not significantly lower in HIV-infected women compared to men in any of the T cell subsets.

HIV infection has been reported to increase the fractions of proliferating $Ki67^+$ T cells 3 to 4-fold in Ethiopians. In addition, TB infection increased the fractions of $Ki67^+$ T cells in both healthy and HIV-infected Ethiopians, HIV^-TB^+ Ethiopians having intermediate fractions of $Ki67^+$ T cells (**Chapter-4**). Thus although HIV infection increases the fraction of proliferating cells in both Ethiopian and Dutch HIV-infected individuals, apparently HIV infection induces less proliferation in Ethiopians. It has been proposed previously that Ethiopians might have been evolutionary selected for having lower immune activation, to minimize accelerated aging of the immune system due to continuous high level exposure to pathogens [10]. Alternatively, parasitic helminthes in Ethiopians might create an anti-inflammatory environment [15]. It remains to be determined whether the lower $Ki67^+$ fractions within T cell subsets of Ethiopians are indeed determined by host factors or are related to HIV-1 subtype C.

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Mortality in *Mycobacterium tuberculosis* patients coinfected with human immunodeficiency virus before and in the era of antiretroviral therapy

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Chapter 9

Abstract

Mortality was investigated in HIV/TB coinfected individuals before and after initiation of antiretroviral treatment (ART) in two-cohorts of 70 and 64 TB cases followed for 43.9 and 12 months, respectively. Pre-ART a higher mortality in HIV-TB coinfected individuals (24.2/100 PYO) than in HIV negative TB patients (1.1/100 PYO, $p<0.001$) was observed. Mortality in the ART group was 10.6/100 PYO, which was higher than in HIV-uninfected TB cases ($p<0.001$). The majority of deaths (62.5%) in the ART group occurred within six months. There were 30.4% and 12.5% deaths in pre- and post ART groups within one year of follow-up, respectively. Although mortality is higher in ART treated individuals than in HIV uninfected TB patients, survival increased due to ART. Detailed follow-up of patients may avert mortality and helpful to maximize the effect of ART among coinfected patients.

Introduction

The risk of development of tuberculosis (TB) in HIV infected individuals is 10 times higher than HIV uninfected individuals and can occur at different levels of immunodeficiency [1], leading to a higher mortality rate. Among TB patients coinfected with HIV, in developed countries, treatment of TB was reported to decrease plasma viral RNA level and increase the CD4+ T cell counts [2]. This restoration of immunity might lead to an increase in survival rate. However, in Africa absence of a reduction in viral load and recovery of CD4+ T cells was observed after TB treatment [3]. Moreover, mortality in coinfected individuals before the advent of antiretroviral treatment (ART) was reported to be higher than in HIV uninfected TB patients [4], suggesting that lack of immune recovery may lead to higher mortality in HIV-coinfected patients.

Recent findings showed ART could increase the survival rates of HIV infected patients by reducing the risk of TB. However, the occurrence of TB in HIV+ individuals despite the commencement of ART is reported to be higher, which challenges the current strategies for controlling TB especially in underdeveloped countries where both infections are prevalent [5].

In the developing world, especially in Sub-Saharan Africa, infections including latent tuberculosis are prevalent in the face of high levels of immune activation, malnutrition, low body- mass-index and low CD4+ T cells, which could exacerbate disease progression in TB patients coinfected with HIV. In Ethiopia, low CD4+ T cell numbers (especially naïve T cells) and high immune activation levels already in HIV uninfected individuals, are common. Surprisingly, despite these lower CD4+ T cell counts and higher immune activation background, HIV disease progression rate in HIV infected Ethiopians was not different from Caucasians [6]. However, still the adult mortality rate among HIV infected individuals remains higher. Whether this mortality rate reported due to HIV [7] could be increased due to high TB infection rate in the presence of anti-TB chemotherapy in HIV infected TB cases before and post ART is not known. Therefore, in this study we investigated mortality in HIV/TB coinfected individuals before and after the implementation of antiretroviral drugs in Ethiopia.

Methods

Study subjects

This study was a part of a cohort study on the natural history of HIV/AIDS by the Ethio-Netherlands AIDS Research Project in the years 1995 to 2005, Addis Ababa, Ethiopia. Moreover, a cohort of HIV/TB patients presenting themselves for ART were followed in St. Paul Hospital, Addis Ababa, Ethiopia from February 2005 to June 2006 for 12 months after initiating ART. In this study we included two groups of study subjects. Baseline characteristics of the study subjects are depicted in **Table-1**.

Table-1. Characteristics of study subjects

Characteristics	TB cases pre- ART		TB cases on ART (n=64)
	TB cases with TB at enrolment or with past history of TB (n=40)	Incident TB cases (n=30)	
Gender (F/M)	13/27	7/23	28/26
Age in years (IQR)	35[31-40.5]	35[30-40]	3[28-38]
HIV status (HIV+, n)	13	15	64
Absolute CD4+ T cells counts (cells/μl, baseline)	264[124-538]	349[252-492]	79.5[56.5-152]
Absolute CD4+ T cell counts (cells/μl, last visit)	113[51-225]	143[111-178]	209.5[151-287]
Plasma viral RNA level (log10copies/ml, baseline)	4.8[4.0-5.1]	4.1[3.9-4.8]	5.4[5.0-5.9]
Plasma viral RNA level (log10copies/ml, last visit)	5.0[4.5-5.3]	5.2[4.3-5.7]	LDL
Average follow-up period (months, mean ±SD)	43.6±21.3	44.4±15.4	-
Average follow-up period from the development (diagnosis) of TB to death (months)	24.2[15.2-52.3]	16.1[7.9-46.2]	3.2[1.5-8.1]*
Anti-TB chemotherapy	Yes	Yes	Yes
Antiretroviral treatment	Naïve	Naive	Yes
TB relapse	NA	NA	4(4.7%)
Mortality	24.2 per 100 PYO		10.6 per 100 PYO
Deaths within one year of follow-up among HIV positives (%, n)	45.5(2/11)	167(2/12)	12.5(8/64)
	Total: 30.4 (7/23)		

* - follow-up period from enrolment to death, numbers in brackets are 25th and 75th interquartile ranges, LDL- below detection limit , NA- not available, PYO- Persons-Years-Observation

Study subjects (Pre-ART)

The first group of the study subjects was from a cohort study that includes 1,870 cohort participants of which a total of 70 TB infected persons were followed for a mean of 43.9 ± 18.4 months. Sixty-three of them had two or more visits, while the remaining 7 had only one visit.

Of the 70 followed TB patients, 40 were clinically diagnosed either at enrolment or had a known date of TB diagnosis before enrolment in the cohort and were followed for a mean of 43.6 ± 21.3 months. Thirteen patients were HIV infected. The other 30 cases were diagnosed upon follow-up (incident TB cases; 15 with HIV infection). All HIV infected persons were ART naïves.

Study subjects after initiating ART

This group of study subjects consisted of TB patients infected with HIV who initiated ART (n=64). They were followed for a maximum of 18.9 months (2 months, 6 months, 1 year and 1.5 years). Cohort participants gave informed consent to participate in the study and the protocol has been reviewed and approved by both Institutional and National Ethical Clearance Committee's. ART and Diagnosis and treatment of *M. tuberculosis* infection were done according to the guidelines of the Ministry of Health, Ethiopia [8].

Table-2. Changes in CD4+ T cell counts and plasma viral RNA level at different periods of follow-up after the implementation of antiretroviral therapy

	Baseline	2 months	6 months	12 months	p-values
CD4+ T cell count (cells/μl, median)	79.5[56.5-152]	191[145-285]	204[149-281]	234[171-425]	<0.001
Plasma viral RNA level (log_{10}copies/ml, median)	5.4[5.0-5.9]	2.5[1.7-3.1]	LDL	LDL	
Numbers with detectable plasma RNA level	64/64	7/13	5/37	0/14	
CD4+ T cell count for those with undetectable plasma viral RNA level (cells/μl)	-	201[169-396]	211[162-285]	234[171-425]	
Plasma viral RNA level for those with detectable plasma viral RNA level (log_{10}copies/ml)	-	3.1[2.9-3.3]	4.1[2.9-4.6]	-	

Determination of plasma viral RNA level and T cell subsets

Lymphocyte subsets were analyzed by standard three-color flow cytometry (FACScan; Becton Dickinson, San Jose, CA). Plasma HIV RNA levels were analyzed by a nucleic acid sequence-based amplification assay (EasyQ, Organon Teknika, The Netherlands) with a detection limit of <50 copies/ml.

Statistical analysis

Analysis was performed using STATA (Intercooled Stata Version-7, Stata Corporation, College Station, TX). Median differences were compared by the nonparametric Mann-Whitney U test. A p value of less than 0.05 was considered indicative for statistical significance. Survival estimates were computed by assuming a Poisson distribution of events and the Kaplan-Meier analysis was performed to compare survival rate by HIV status.

Results

CD4+ T cell count and plasma viral RNA level in HIV/TB coinfected individuals Pre-ART and after initiating ART

Of the 70 Pre-ART TB cases with available CD4+ T cell count at baseline (n=34), the median CD4+ T cell count was different by HIV status(325.5-cells/μl and 261.5 cells/μl, respectively, p<0.001). Plasma viral RNA level in HIV-infected cases (n=27) was 4.6log_{10}copies/ml at enrolment and increased by 0.4log during follow-up (p=0.04). The median absolute CD4+T cell count decreased during the follow-up from 325.5 cells/μl to 137.5 cells/μl (p<0.001), particularly in HIV positive persons (from 261.5 cells/μl to 119 cells/μl, p=0.001). The CD4+ T cells and plasma viral RNA level in the follow-up period is given in figure-1. There were 2 patients diagnosed with extra pulmonary tuberculosis

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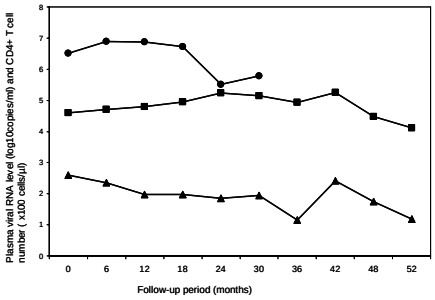

Figure-1. CD4+ T cell number in HIV positives (filled upward triangles), plasma viral RNA level (filled rectangle) and CD4+ T cell number in HIV negative idividuals (filled circles) of TB cases pre-ART.

Of the 64 HIV/TB coinfected individuals presenting themselves for ART, 48 had pulmonary TB (PTB), 11 extra pulmonary TB (EPTB) and 5 had both mixed infections. The median plasma viral RNA level decreased significantly from 5.4$\log_{10}$copies/ml at baseline (which was higher than the pre-ART groups, p<0.001) to 2.7$\log_{10}$copies/ml at 2 months (n=13, p<0.001) and reached below the detection limit at 6 (n=39) and 12 (n=14) months **(Table-1).** Median CD4+ T cell count increased from 79.5 cell/µl at enrolment, which was lower than pre-ART HIV/TB coinfected patients (p<0.001), to 191 cells/µl at 2 months (n=13, p<0.001), 204 cells/µl at 6 months (n=37, p<0.001) and 234 cells/µl at one year (n=13, p<0.001).

Mortality in Pre-ART HIV/TB coinfected individuals

Out of 1,870 individuals followed, a total of 96 (5.1%) deaths occurred. More deaths occurred among HIV infected persons (36.7%, 66/180) than among HIV uninfected persons (1.8%, 30/1682, p<0.001). Of the total deaths reported, 11.4% (11/96) were diagnosed as TB-related.

Among the Pre-ART 70 TB cases, 26 (37.1%) deaths occurred (mortality rate: 26 deaths in 373.45 followup peirod = 6.96 per 100 person year observations (PYO) during the study period. Of these 3 (7.1%) (mortality rate 1.1 per 100 PYO) of the HIV negative and 23 (82.1%) (mortality rate 24.2 per 100 PYO; CI: 16.0-34.1) of the HIV positive individuals died (p<0.001, Table-1). Twenty-seven percent (7/26) deaths occurred within one-year (30.4% (7/23) in HIV positive persons (survival rate of 75%). Individuals with <200 cells/µl CD4+ T cell counts at baseline had higher death rates (9/9) than those with ≥200 cells/µl CD4+T cell counts (13/25, p=0.01). The median time from diagnosis of TB to death (n=26) was 22.1 months and this was longer in HIV negative TB cases (52.9 versus 21.3 months). HIV negative individuals had higher survival rate than HIV positive TB cases **(Figure-2)**.

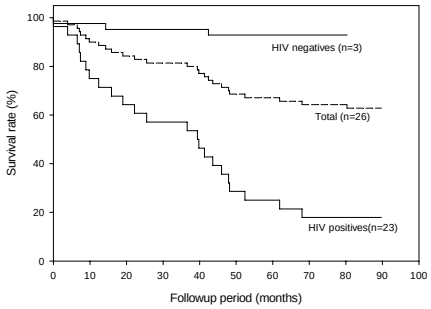

Figure-2. Kaplan-Meier survival estimates for survival rate by HIV status. Y-axis shows the percent of survival rate and X-axis shows follow up period in months

Mortality in ART treated HIV/TB coinfected individuals

From HIV/TB coinfected patients upon ART, 8(12.5%) deaths occurred (mortality rate 10.6 per 100 PYO; CI: 4.6-19.7, Table-1] in the follow-up period. Mortality decreased by 50% compared to Pre-ART patients within one-year follow-up (30.4% versus 12.5%) and was higher than HIV negative TB patients (12.5% versus 7.1%, p<0.001). Twenty-five% of the deaths (3/12) occurred among individuals with <50 cells/μl of CD4+ T cell counts compared to 9.6% (5/52) in individuals with ≥50 cells/μl CD4+ T cell count. The majority of deaths, 62.5% (5/8), occurred within six months. Moreover, there were 4.7% (3/64) TB relapsed cases, of which 1(33.3%) died.

Mortality was affected by nadir CD4+ T cell count. In fact, 8 individuals that died had a nadir CD4+ T cell count of 55-cells/μl (median), which was significantly different from survivors in the follow-up period (89.5 cells/μl, p=0.05). Patients with plasma viral RNA level below the detection limit at six months follow-up had higher nadir CD4+ T cell counts than those above the detection limit (89.5 cells/μl vs. 57 cells/μl,p=0.06). The survival estimate of these individuals within the follow-up period is given in Figure-3.

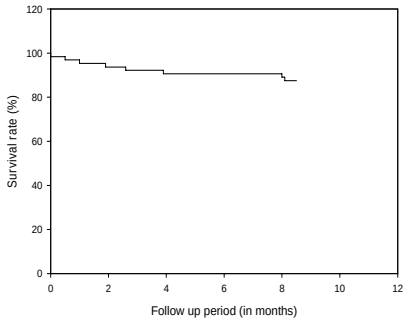

Figure-3. Kaplan-Meier survival estimates for survival rate after initiating ART. Y-axis shows the percent of survival rate and X-axis shows follow up period in months

Discussion

Previously we reported failure of reduction of plasma viral RNA level and restoration of CD4+ T cell count despite anti-TB chemotherapy in HIV/TB coinfected patients [9]. This might contribute to the presence of higher mortality in these individuals due to HIV disease progression. In this study, we found a four fold increase in mortality in HIV/TB coinfected individuals compared to HIV uninfected TB patients before the introduction of ART. Other reports also showed higher level of mortality among Pre-ART coinfected individuals in Ethiopia [3], Senegal [10] and Kenya [11]. The mortality reported in this study is higher compared to the adult mortality rate due to HIV for Ethiopia reported in 2003 (4.04 per 1,000) [7], which might be partially explained by the presence of faster disease progression in HIV/TB coinfected individuals. As expected, higher mortality was observed in coinfected individuals with CD4 counts below 200-cells/μl, which might be due to the severely impaired immune system and strengthens the need for ART in this category.

Despite the presence of standard anti-TB chemotherapy among the individuals, the mortality we found was higher compared to HIV uninfected TB patients. Similar reports from Kenya showed higher mortality in spite of the administration of similar drug regimens. The survival rate that we obtained in the Pre-ART group within one-year follow-up in the presence of anti-TB treatment (75%) was comparable to a report from Kenya (66%) [12], which might indicate the higher mortality may not be due to the failure in anti-TB chemotherapy. This could be explained by the presence of lower rate of multi-drug resistance pattern. In fact, the multi-drug resistance pattern observed in HIV/TB coinfected individuals in a similar cohort in Ethiopia was indeed reported to be lower [3]. However, recently we observed, despite successful anti-TB chemotherapy the plasma viral RNA level was not reduced.

In this study, mortality among ART treated HIV/TB coinfected individuals remained higher (12.5%) than TB patients without HIV infection (7.1%). The majority of the deaths occurred within six moths of follow-up. Such higher mortality was also reported from South Africa [13]. The relatively higher mortality observed in this study might be due to the presence of active TB cases after the commencement of ART as nearly 10% of the post ART patients are reported to have active TB disease [5,14]. Indeed we also observed 3(4.7%) patients with relapsed TB out of which one of them died.

Although mortality was higher among individuals upon ART, it is still by far lower than those without ART. Interestingly, a 50% reduction in mortality was found comparing coinfected patients within one year of follow-up pre- and post-ART (30.4% vs. 12.5%).This could be explained by the presence of suppression of plasma viral RNA level and restoration of CD4+ T cells in agreement with previous reports [15]. In addition, we observed that the median level of plasma viral RNA level did not reach undetectable levels within the first six months of treatment in some patients, which might contribute to the presence of higher mortality.

In conclusion, in this study, we showed the presence of higher mortality in coinfected individuals in the absence of ART. Although significantly reduced, it remains higher among ART treated HIV/TB patients within one year of follow-up and higher than HIV negative TB patients. This calls attention to closely monitor drug adherence, drug-drug interaction and handling of other opportunistic infections including TB, which can occur as immune reconstitution diseases and may affect survival rates, especially within the first six months.

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Discussion

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Chapter 10

The higher incidence and prevalence of tuberculosis infection in the developing world, especially in sub-Saharan African countries, remains to be one of the major health threats and a big burden for the economy of these countries. Moreover, the situation is exacerbated due to high prevalence of HIV-infection. The higher risk of HIV infected individuals for reactivation of latent TB infection or new infections, increases the challenges to control both infections in these developing countries **(1-7)**. The presence of HIV/TB coinfection before the introdcution of antiretroviral therapy had an increased effect on the rate of mortality. Moreover, TB disease also occurs even after initiating ART, which needs to be investigated to clearly define how the immune system copes with synergistic effect of these two diseases. Therefore, in this thesis we investigated the immunological consequences of human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* coinfection in Ethiopia by examining the level of immune activation due to either HIV or TB or both. Moreover, the effects of anti-TB and ART on the level of immune activation and disease progression were studied.

Screening of MTB infection and the diagnosis of TB disease

Screening and diagnosis of TB infection in a community with high HIV prevalence helps to minimize the occurrence of incident cases to identify active TB cases at an early stage and to provide timely treatment to prevent transmission in the community. To address the issue of burden of HIV infection and its effect on the diagnostic and screening methods we investigated the level of smear and culture positivity and rate of HIV infection in the suspected TB cases in Ethiopia. Moreover, the probability of detection by using both smear AFB and chest X-ray was investigated (Chapter 2). We found that nearly 80% of the cases were detected by the combination of the two methods, which are frequently used in majority of the health institutions in Ethiopia. Thus, the use of a combination of these methods will increase the TB detection rate in the country which is currently very low (36%) **(1)**.

The clinical outcomes of the patients were studied to investigate how the HIV/TB coinfection influenced the treatment scheme of TB chemotherapy. This was determined by indicating cure rate (conversion to negative after standard anti-TB chemotherapy) and mortality in the follow up period. The rate of smear conversion to negative (cure rate) after 8 months of anti-TB chemotherapy and follow-up was lower (51%) compared to the ultimate goal (85%) of the Ministry of Health in Ethiopia **(1)**. Similarly, the treatment success rate (cure rate plus treatment completion) registered and reported in the country is low (70%) and lower compared to other parts of Africa **(1),** which calls special attention to control tuberculosis especially in the era of HIV/AIDS.

To support our finding and to investigate whether the reactivity and conversion rate of skin tests (using PPD as a recall antigen) could be affected by the presence of lower CD4+ T cell counts among HIV positive and negative individuals in Ethiopia (Chapter-3), we also evaluated PPD tuberculin test (screening test) as a screening method for TB infection. We observed higher TST reactivity and conversion rate despite lower absolute CD4+ T cell numbers, which might be attributable to the presence of higher level of latent TB infection, higher incidence of TB and/or transmission of MTB in the population. However, HIV infection seems to affect both test positivity and reactivity but not the conversion rate as evidenced by the fact that HIV infected and non-infected individuals had equal chances of exposure despite HIV infected individuals having a higher chance to develop active TB disease than HIV uninfected persons **(8)**. This was also further supported by the absence of a difference in smear positivity in HIV infected and non-infected individuals. To decrease the higher proportions of smear negatives in HIV infected individuals and thereby mortality in smear negative TB cases, alternative screening and accurate diagnostic methods require special attention. Therefore, introduction of sensitive, specific and affordable tests remains to be the target to optimize screening for TB infection **(9, 10)**. To circumvent these problems, tests

based on early secreted antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are now getting attention due to the fact that the tests can differentiate latent and active TB infections **(11,12)**. The response rates, based on IFN-γ production by T cells in PBMC cultures can be determined by Enzyme Linked Immunosorbent Assay (ELISA, IFN-γ concentration from the supernatants of the culture after stimulating with antigen, mostly 5-6 days) or Ex vivo immunospot Assay (ELISPOT, T-cell response at a single cell level). These tests are not affected by age, BCG vaccination or environmental bacteria, HIV infection and malnutrition **(13).** This is vital to the developing world as these conditions, especially malnutrition, exacerbate other infectious diseases. Another additional advantage of these tests could be the use of these antigens to monitor TB therapy as reported **(14, 15, 16, 17),** which may support the intervention activities of the disease.

Immune activation and CD4+ T cell depletion in HIV/TB coinfection

Patients infected with HIV have been reported to have higher immune activation levels **(18, 19)**, which could be aggravated by opportunistic infections such as tuberculosis **(20, 21, 22)**. Therefore, we studied the contribution of TB to immune activation and subsequent depletion of CD4+ T cells (in HIV infection) **(23, 24).** In addition we investigated the role of anti-TB chemotherapy in immune activation and T cell proliferation in TB patients with or without HIV infection. Higher immune activation, depletion of naive CD4+ T cells and increased plasma viral RNA levels indicate that disease progression is faster in these individuals (Chapter-4). Unlike in Caucasians **(25,26)**, in Africans including Ethiopians, despite successful anti-TB chemotherapy, no reduction in plasma viral RNA level and no effect on the decline of CD4+ T cell numbers **(27,28,29,30,31)** was reported**.** In support of this we found progressive loss of CD4+ T cells, sustained/increased level of plasma viral RNA levels parallel to increased/sustained levels of immune activation in HIV infected individuals who developed active TB during follow up (Chapter-5). This trend was continued despite anti-TB chemotherapy in these individuals. Therefore, sustained immune activation may contribute to the absence of immune recovery, increased disease progression rate and increased mortality among HIV/TB coinfected patients (**30, 32)**. This calls for alternatives to reduce plasma viral RNA levels and immune activation. Indeed, antiretroviral therapy showed a significant reduction in plasma viral RNA level and immune activation (Chapter-4). In addition, among HIV/TB coinfected individuals with higher CD4+ T cell counts (≥200 cells/µl), higher levels of immune activation were observed compared to those in HIV infected individuals without TB disease. This suggests TB itself may cause this immune activation. On the other hand, the lack of higher immune activation in individuals with <200 cells/µl T cell numbers suggests that TB occurs due to lack of TB-specific immune responses as a consequence of severe T cell depletion. The sustained immune activation despite anti-TB treatment indicates that immune activation is caused mainly by progressive HIV-infection, as evidenced by high plasma viral RNA and decreased CD4 numbers after the treatment of tuberculosis**.**

From the studies that we conducted and to indicate the interaction and the consequences of HIV and TB coinfection, we developed a model that shows how these infections influence one another (Figure-1).

The presence of HIV infection leads to activation of the immune system and progressively depletes the CD4+ T cell pool. This may lead to either reactivation of latent TB infection or may increase the risk for new TB infections leading to HIV/TB coinfection. HIV infection therefore increases the risk of developing TB disease with progressively increased/sustained immune activation and thereby increased plasma viral RNA level (PVL).

Administration of standard anti-TB chemotherapy will have a minimal effect on the immune activation level as shown, because TB is not the main cause for the observed high immune activation levels. This can be explained by the dominant effect of HIV on immune activation. Thus, the effect of TB treatment on the reduction of immune activation could be masked by the effect of HIV. In contrast, the presence of antiretroviral therapy significantly reduced plasma viral RNA level and concomitantly the level of immune activation, thereby reducing the risk of mortality in the coinfected individuals. This data suggests that early initiation of ART in HIV/TB coinfected individuals is beneficial and necessary since early initiation of ART before the immune system deteriorates lead to better recovery of the immune response and potentially leads to a higher survival rate **(33,34 and Tegbaru B, Unpublished data).**

In contrast, in TB patients without HIV infection, the immune activation caused by TB decreased significantly due to the presence of standard anti-TB chemotherapy, thereby resulting in reduced mortality. However, due to the residual effects of tuberculosis, the level of immune activation may not reach to the level of healthy individuals, which may require more time to clear the antigen and/ or contain the infection in latent form.

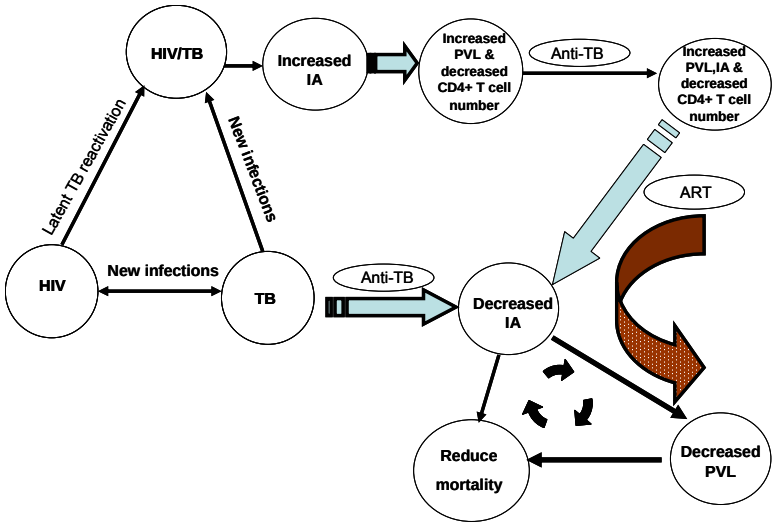

Figure-1. Schematic representation indicating the correlation of HIV, TB and HIV/TB infections with immune activation, HIV plasma viral RNA level and mortality (IA-immune activation and PVL-plasma Viral Load).

TB-specific immunity and regulatory T cells

Regulatory T cells (Tregs), which express high levels of CD25, are CD4+ T cells are known for their effect on the immunopathological responses in diseases such as arthritis **(35)** and reducing immune responses such as HIV. They have been implicated to affect immune responses in chronic infections such as cytomegalovirus and HIV **(36,37)**. However, they are also reported to be infected with HIV **(38)**. Moreover, the proportion of regulatory T cells was found to be higher in individuals with lower CD4+ T cell numbers, which might explain how these T cells could aggravate immune responses in this category of patients.

The effect of HIV/TB coinfection in disease progression may be related to higher numbers of regulatory T cells, which may lead to suppression of specific immune responses **(39)**. Increased numbers of Tregs were reported in TB patients **(40)** and their frequency was not decreased significantly after anti-TB chemotherapy **(41).** Since there are no reports about

regulatory T cells in HIV/TB coinfection before and after anti-TB chemotherapy, we investigated this. We observed HIV/TB coinfected individuals and especially those who were chronically ill showed higher levels of FOXP3 expression within the total CD4+ T cells. Increased levels of FOXP3 expression might contribute to lower immune responses in these patients compared to non-chronic HIV/TB coinfected individuals. Moreover, their higher level in HIV/TB coinfection compared to TB patients without HIV infection underscores the role of HIV infection in increasing the proportion of these cells (Chapter-6). The positive correlation that we found between the percentage of regulatory T cells and the immune activation level suggests that these T cells may be expanded due to a higher burden of antigen, which supports the notion that they are antigen driven **(42).** Furthermore**,** the positive correlation of these T cells with memory CD4+ T cells shows that these cells are antigen driven as reported **(37,38)**. However, administration of standard anti-TB chemotherapy did not affect their level. Similarly administration of ART (although for a short period), had no effect on their level, which might indicate these cells are stable and/or replenish themselves by proliferation **(40)**.

In support of the above findings, other additional markers of immune activation, chemokine receptors (CCR5 and CXCR4, which are also the major entry points of HIV to the host cell in HIV infection), are reported to be increased in different infections such as tuberculosis **(28,43)**. In this study we found increased levels of CCR5 and CXCR4 expression in HIV/TB coinfected individuals compared to HIV uninfected TB patients **(28)** (Chapter-7).

Disease progression and mortality in HIV/TB coinfected patients

High levels of immune activation (especially among HIV non-infected individuals) could lead to faster disease progression once HIV infection is initiated **(44,45)**. In Ethiopians due to the presence of lower CD4+ T cell numbers among HIV negative individuals **(46,47,48),** increased basal immune activation **(49)** and various infections **(50,51,52,53)** the disease progression rate in the course of HIV infection was hypothesized to be fast compared to their CD4+ T cell matched Caucasians. However, a recent report **(54)** showed the absence of faster disease progression in Ethiopians. Even more the reduction in CD4+ T cell per annum is smaller in Ethiopians compared to Caucasians. We hypothesized that, despite higher basal immune activation level in HIV non-infected Ethiopians, upon HIV infection, the rate of immune activation increase could be minimal (and thus lower) compared to their CD4+ T cell number matched Caucasians (Chapter-8). Indeed, despite equal number of CD4+ T cells, plasma viral RNA level and other socio-demographic data, there was lower immune activation in Ethiopians than in Dutch HIV infected individuals. Therefore, the reduced decline in CD4+ T cell number and absence of faster disease progression in Ethiopians could be related to the presence of lower immune activation of T cells in the presence of HIV compared to their Caucasian counterparts (Chapter-8)**.** However, the rate of disease progression after developing opportunistic infection may be faster in Ethiopians, since this phase of disease progression is dependent on many other factors such as standard health care and nutrition**.** Thus, to see the outcomes of the effects of this disease progression rate especially after developing opportunistic infections such as tuberculosis, studying the mortality rate might give indications**.** The presence of higher mortality in HIV/TB coinfected individuals is widely reported before the introduction of antiretroviral treatment **(55,56)** and is reported to be related to the presence of higher plasma viral RNA level**.** The sustained or higher level of plasma viral RNA and immune activation despite anti-TB chemotherapy in 2 **(28)**, 6 **(31)** and 12 months **(30)** of anti-TB chemotherapy is expected to increase the mortality among these individuals **(30).**

Higher immune activation, lower CD4+ T cell counts especially in naive T cells, increased plasma viral RNA level and higher level of regulatory T cells in HIV/TB coinfected individuals

may lead to faster disease progression which results in a higher mortality rate. The increased mortality rate could be reduced significantly by ART by minimizing the risk of new opportunistic infections such as tuberculosis **(57,58)**. In support of these findings, the mortality rate we observed in HIV/TB coinfected individuals (30.4%) was reduced by 50% when compared to ART cases within one year of follow-up (Chapter-9). However, still these rates were more than four-fold increased compared to HIV uninfected TB patients and more deaths occurred within the first six months of ART, which might add challenges for HIV and TB control programs. This especially requires close follow-up of patients and treating opportunistic infections effectively, which may avert the mortality that we observed among those on ART.

In conclusion, the presence of higher and sustained immune activation, higher plasma viral RNA level, depleted naive CD4+ T cells counts and higher level of regulatory T cells in HIV/TB coinfected individuals showed faster disease progression once these individuals develop opportunistic infection such as tuberculosis. However, unlike ART, the presence of anti-TB chemotherapy could not reverse the situation. The presence of persistent immune activation, residual effect of *M. tuberculosis* and HIV disease progression after the treatment of active TB disease may contribute to the occurrence of active TB disease even after the administration of ART **(59,60)**. This might be the reason why high numbers of active TB cases and higher mortality were observed in the HIV/TB coinfected individuals. Lower pre-treatment CD4+ T cell counts and higher plasma viral RNA levels extend to increased deaths in the coinfected individuals. Therefore, early initiation of ART in HIV/TB coinfected individuals before the immune system deteriorates in the course of the disease will help to increase the immune response and thereby increase survival rates.

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Summary

Chapter 11

In this study we investigated the interaction and the immunological consequences of human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* coinfection. This thesis contains eight parts.

chapter II demonstrates a high rate of HIV infection among suspected TB patients. There was no significant restoration of CD4+ T cell numbers or a reduction of plasma viral RNA levels among HIV infected TB cases despite successful anti-TB chemotherapy. The presence of high percentages of smear negative TB patients among HIV infected persons showed the effect of HIV on the diagnostic tests. The combination of AFB smear microscopy and chest X-ray detected nearly 80% of the TB cases. High mortality in the non-TB cases, especially in the HIV infected individuals indicates that some patients who were coinfected might be missed and were not diagnosed by the current diagnostic tests. These missed diagnoses increase the risk of transmission of TB in the community.

In Chapter III we characterized the *in vivo* immune response of HIV infected and uninfected individuals towards the recall antigen Purified Protein Derivative (PPD) that might indicate latent TB and possible transmission of tuberculosis in the community. Although a high prevalence of reactivity was observed, it was lower in HIV infected individuals and was affected by the presence of lower CD4+ T cell count. Moreover, conversion to a PPD positive test in HIV infected individuals was not different from HIV uninfected individuals, probably due to equal chance of exposure to *M. tuberculosis*, since tuberculosis infection is common in Ethiopia. Furthermore, the test (as a screening) was not able to identify incident TB cases within a certain period before the diagnosis of clinical active TB disease. Therefore, sensitive, specific and affordable techniques to screen TB infection within the community, especially among HIV positives, are required so that HIV infected persons can benefit from Isonaizid prophylaxis.

Chapter IV aimed to characterize immune activation of HIV/TB coinfected patients. T cell division was measured using intercellular staining of nuclear antigen Ki67. The fraction of CD4+ Ki67+ T cells was higher in HIV/TB coinfected individuals compared to asymptomatic HIV infected, TB patients without HIV and healthy controls. There was a significant percentage of Ki67+ in naïve T cells. Immune activation was not affected by anti-TB chemotherapy in HIV/TB coinfected individuals unlike to TB patients without HIV infection. Plasma viral RNA was also not reduced due to anti-TB chemotherapy. The effect of TB on immune activation was significant in HIV/TB coinfected individuals with CD4+T cell counts $\geq$200 cell/μl (TB as a cause for immune activation) compared to those with <200-cells/μl (TB as a consequence of immune activation). Similarly, there was a higher plasma viral RNA level in those with $\geq$200-cell/μl CD4+ T cell count and was higher after anti-TB chemotherapy. Unlike anti-TB chemotherapy alone, however, the level of immune activation and plasma viral RNA level was significantly reduced within two months of antiretroviral therapy (ART).

Chapter V aimed to discuss the kinetics of CD4+ T cell number, immune activation and plasma viral RNA level in HIV/TB coinfected individuals before and after the diagnosis and treatment of TB disease. Among patients followed for about 4 years, the plasma viral RNA did not show any decline despite treatment of active clinical TB disease. The level of immune activation, as seen by the expression of Ki67, was not reduced significantly in the follow-up period. The expression of Ki67 within the CD4+ T cell remained elevated in parallel to the level of plasma viral RNA in these individuals. However, CD4+ T cell counts showed a gradual decline. Therefore, the lack of reduced immune activation in HIV/TB coinfected patients might be explained by the absence of a reduction in plasma viral RNA level. Moreover, persistent immune activation or disease progression could be due to residual effects of TB after successful completion of anti-TB chemotherapy.

Chapter VI addressed the role of regulatory T cells in HIV/TB coinfection. We found higher levels of regulatory T cells in HIV/TB coinfection. The proportion of regulatory T cells was increased in chronically sick HIV/TB coinfected individuals compared to those non-chronic HIV/TB coinfected individuals. Upon treatment (both anti-TB and antiretroviral), the level did not change. This might indicate these T cell population is either stable or replenish themselves with proliferation at the periphery. The positive correlation that we observed between immune activation and level of regulatory T cells and the presence of higher levels of regulatory T cells in higher immunodeficiency stage indicates these cells, like immune activation, are antigen dependent.

Chapter VII addressed the expression of HIV co- receptors and immune activation markers in HIV/TB coinfected individuals before and after two months of anti-TB chemotherapy. Among these individuals, the expression of CCR5 and CXCR4 was higher than in TB patients without HIV coinfection. The level was positively correlated with plasma viral RNA level and negatively with CD4+ T cell count. In this study, parallel with plasma viral RNA level, we also found no decrease in expression of immune activation markers, coreceptors and β-chemokines after 2 months of anti-TB chemotherapy.

Chapter VIII concentrated on proving why HIV-infected Ethiopians had a slower progression rate than their Caucasian counterparts, despite higher baseline immune activation in HIV negative Ethiopians. Interestingly, there was lower immune activation level in HIV infected Ethiopians than their CD4+ T cell number matched Dutch counterparts. The prevalence of NSI and SI virus phenotypes in Ethiopian and Dutch HIV infected people was not involved since all of the Dutch and the majority of the Ethiopian (>90%) HIV-infected individuals harbored only NSI strains. Therefore, the absence of faster disease progression in HIV infected Ethiopians, despite their lower baseline CD4+ T cell numbers and higher immune activation might be due to the presence of lower CD4+ T cell proliferation rates in response to HIV.

Chapter IX addressed mortality of HIV/TB coinfected individuals before and in the era of antiretroviral therapy. Before the introduction of antiretroviral drugs in Ethiopia, among TB cases coinfected with HIV and followed for one year, there was a higher death rate (30.4%) compared to coinfected individuals after one year of ART (12.5%). However, mortality was still higher among those ART cases compared to those TB cases without HIV infection in the follow-up period (7.1%). Mortality decreased by 50% compared to those before the era of ART. Moreover, mortality and detectable plasma viral RNA level after six months of ART were associated with low nadir CD4+ T cell count at baseline. Majority of the deaths occurred within six months of ART with 4.5% tuberculosis disease relapse rate. Therefore, close follow-up and careful management of opportunistic infections, including TB, needs to be done to minimize the mortality within six months of ART.

Taking the data together, the thesis showed high rate of HIV infection in TB patients. Immune activation was contributing to the presence of higher level of plasma viral RNA level, which was not decreased significantly due to anti-TB chemotherapy. Ethiopians did not progress to AIDS faster, despite lower baseline CD4+ T cell number and higher baseline immune activation level, which was due to slower decline of CD4+ T cell number per year and lower immune activation after HIV infection. After the introduction of ART, a high mortality rate occurred within the first six months of follow-up in HIV/TB coinfected individuals that reduced by 50% within one year of follow-up. Close follow-up of patients to reduce mortality and introduction of sensitive, specific and affordable screening and diagnostic tests and methods will have a benefit, especially in HIV infected individuals, to be diagnosed and treated early and benefit from INH prophylaxis.

Samenvatting

Chapter 12

Coinfectie met Mycobacterium Tuberculose tijdens HIV-infectie is een groot probleem in de derde wereld, daar dit leidt tot een grotere sterfte. (**hoofdstuk I**) In de studies beschreven in dit proefschrift wilden we onderzoeken hoe coinfectie met tuberculose het verloop van HIV-infectie beïnvloedt en of daar een immunologische basis voor is.

Hoofdstuk II laat zien dat HIV-infectie veel voorkomt onder patiënten die verdacht worden Tuberculose te hebben. Daarnaast lieten de HIV-geïnfecteerde TB patiënten geen herstel van CD4+ T cel aantallen zien of een verlaging van de virale RNA load ondanks succesvolle anti-TB chemotherapie. Het hoge aantal HIV+ patiënten met een negatieve 'smear-test' suggereert dat HIV een effect heeft op deze diagnostische test. De combinatie van 'smear-test' en röntgen diagnostiek detecteerde bijna 80% van de TB patiënten. De hoge mortaliteit onder niet-TB patiënten, speciaal in de HIV-geïnfecteerden laat zien dat sommige co-geïnfecteerde patiënten niet gediagnosticeerd worden voor TB mbv de huidige testen. Deze gemiste diagnoses verhogen het risico op transmissie van MTB in the gemeenschap.

Om te bestuderen wat de impact van HIV-infectie is op een klinische uitlees methode van MTB infectie, hebben we in **hoofstuk III** de *in vivo* immuunrespons tegen het 'recall' antigeen 'Purified Protein Derivative' (PPD) gekarakteriseerd (de zgn tuberculine huidtest oftewel Mantoux-test). Alhoewel een hoge prevalentie van PPD-reactiviteit werd aangetoond, was de reactiviteit lager in HIV-geïnfecteerden, met name bij lage CD4 aantallen. Conversie naar een PPD-positieve test was daarentegen niet verschillend tussen HIV-geïnfecteerden en niet-geïnfecteerde personen. Dit kan waarschijnlijk verklaard worden door de gelijke expositie aan MTB, die hoog is in Ethiopie aangezien MTB veel voorkomt. De mantoux test was echter niet in staat om nieuwe TB gevallen te identificeren binnen een bepaalde periode voor de diagnose van klinisch actieve TB. Daarom zijn gevoelige, specifieke en betaalbare technieken vereist om te kunnen screenen voor TB infectie, met name onder HIV-positieven zodat ook zij gebruik kunnen maken van TB profylactische therapie met Isonaizid.

Het is beschreven dat een hogere mate van activatie van het immuun systeem leidt tot snellere progressie naar AIDS. In **Hoofstuk IV** hebben we immuun activatie van de T-cellen gekarakteriseerd in HIV/TB co-geïnfecteerden, door T-cel deling te meten door middel van intracellulaire kleuring van het nucleaire antigen Ki67. Het percentage Ki67+CD4+ T cellen was hoger in HIV/TB co-geïnfecteerden vergeleken met niet symptomatische HIV-geïnfecteerden, TB patiënten zonder HIV en gezonde controles. Een significant percentage van de Ki67+ cellen bevond zich in de naïeve T cel populatie. Het immuun activatie niveau in HIV/TB co-geïnfecteerden veranderde niet na anti-TB chemotherapie. Dit in tegenstelling tot TB patiënten zonder HIV-infectie waar de immuun activatie daalde. Ook de HIV virale RNA load daalde niet na anti-TB chemotherapie. Het effect van TB op immuun activatie was significant in HIV/TB co-geïnfecteerden met CD4+T cel aantallen boven de 200 cellen/μl (suggererend dat TB een veroorzaker is van de immuun activatie) vergeleken met HIV/TB co-geïnfecteerden met CD4+ T cel aantallen onder de 200 cellen/μl (suggererend dat TB een consequentie is van verhoogde immuun activatie). De HIV virale RNA load in het plasma was ook hoger in diegene met hogere CD4+ T-cel aantallen. In tegenstelling tot anti-TB chemotherapie, was behandeling met antiretrovirale therapie wel in staat om zowel de hoeveelheid immuun activatie als de virale RNA load te reduceren.

In Hoofstuk V hebben we gekeken naar de kinetiek van CD4+ T cel aantallen, immuun activatie markers en HIV RNA load in HIV/TB co-geïnfecteerden die 4 jaar vervolgd werden in het ENARP cohort en vervolgens actieve Tuberculose ontwikkelden. De HIV virale RNA load bleef toenemen na diagnose van klinisch actieve TB ondanks behandeling. Ook immuun activatie binnen de CD4+ T cellen, gemeten door naar de expressie van de celdelingsmarker Ki67 te kijken, bleef verhoogd. Tevens bleven de CD4 T-cel aantallen dalen. Het lijkt daardoor dat de HIV-ziekte progressie gewoon doorzet na de diagnose van

klinische TB. Het gebrek aan verlaging van de immuun activatie kan verklaard worden door een gebrek aan verlaging van de HIV load. Persisterende immuun activatie of ziekte progressie na TB therapie zouden veroorzaakt kunnen worden door restant effecten van TB, ofwel een reflectie zijn van doorgaande HIV ziekte progressie. Dus in tegenstelling tot Caucasiërs leidt het aanwezig blijven van immuun activatie in Afrikaanse HIV/TB co-geïnfecteerden tot afwezigheid van immuun herstel en reductie in virale load na therapie.

In Chapter VI is de mogelijke rol van regulatoire T cellen bestudeerd in HIV/TB coinfectie. Deze zgn Tregs kunnen (specifieke) immuun responsen downreguleren en worden gekarakteriseerd door de aanwezigheid van de IL-2 alpha receptor (CD25) op de celmembraan en intracellulaire expressie van de transcriptie factor Foxp3 die verantwoordelijk is voor de suppressieve functies. Wij vonden hogere percentages Tregs in HIV/TB co-geïnfecteerden. Het percentage Tregs was verhoogd in chronisch zieke HIV/TB patiënten vergeleken met niet-chronische HIV/TB co-geïnfecteerden. Na behandeling (zowel met anti-TB chemotherapie als met antiviral therapie) veranderde het niveau van Tregs niet. Dit kan suggereren dat deze Treg T cel populatie of stabiel is of dat ze regelmatig vervangen wordt door proliferatie van deze cellen in de periferie. De positieve correlatie tussen immuun activatie en Treg aantallen en de aanwezigheid van hogere aantallen Tregs in individuen met een later stadium van immuundeficiëntie (die dus hogere immuun activatie hebben), suggereert dat Tregs, net zoals geactiveerde T cellen, antigen afhankelijk zijn.

Een immunologische parameter die van invloed kan zijn op gevoeligheid voor HIV-infectie en ziektebeloop, is expressie van de chemokine receptoren CCR5 and CXCR4 op CD4+ T cellen, die opgereguleerd worden na activatie, en chemokines in plasma. Daartoe werden deze parameters onderzocht in HIV-TB co-geïnfecteerden voor en na anti-TB behandeling in **Hoofstuk VII**. De expressie van CCR5 and CXCR4 op T cellen was hoger in HIV/TB co-geïnfecteerden dan in TB patiënten zonder HIV-infectie. Het percentage CCR5/CXCR4+ T cellen was positief gecorreleerd met HIV virale load en negatief met CD4+ T-cel aantallen. Expressie van HIV coreceptoren en andere immuun activatie markers en de aanwezigheid van β-chemokines veranderde niet na anti-TB behandeling. De verhoogde expressie van CCR5 en CXCR4 op geactiveerde CD4+ T cellen samen met verhoogde chemokine niveaus vormt een basis voor continue HIV-replicatie geassocieerd met TB co-infectie.

Recent hebben we laten zien dat in het ENARP cohort, ondanks hogere initiële immuun activatie en lagere initiële CD4 aantallen (al aanwezig in gezonde Ethiopiërs), HIV ziekte progressie niet sneller en zelfs langzamer verloopt dan Caucasiers (in dit geval Nederlanders) verloopt. In **hoofdstuk VIII** hebben we een mogelijke verklaring hiervoor onderzocht door immuun activatie (CD4 proliferatie mbv Ki67 expressie) tussen HIV-geïnfecteerde Ethiopiërs te vergelijken met nedrlanders met eenzelfde CD4 aantal. (gematched) We vonden lagere immuun activatie in HIV-geïnfecteerde Ethiopiërs dan Nederlanders, wat niet veroorzaakt was door een verschil in prevalentie van de verschillende HIV NSI and SI virus fenotypes omdat alle Nederlanders en de meerderheid van de Ethiopiërs (>90%) in deze studie enkel NSI virus varianten bij zich droegen. We concludeerden daarom dat de afwezigheid van een snellere ziekteprogressie in HIV-geïnfecteerde Ethiopiërs, ondanks hogere immuun activatie en lagere initiële CD4 aantallen, veroorzaakt wordt door lagere CD4 proliferatie in response op de HIV-infectie.

Tenslotte lieten we in Hoofdstuk IX overleving analyses in HIV/TB co-geïnfecteerden zien voor en na implementatie van antiretrovirale therapie (ART). Voor de introductie van ART was er onder de TB patiënten met HIV coinfectie die gevolgd zijn voor een jaar een lagere overleving (69,6%) vergeleken met co-geïnfecteerden na 1 jaar ART (87.5%). Maar de overleving onder ART behandelden was nog steeds lager vergeleken met TB patiënten zonder HIV-infectie (92.9%). Mortaliteit en detecteerbare HIV virale load na 6 maanden ART

waren geassocieerd met lage nadir CD4+ T cel aantallen. De meeste doden vielen in de eerste 6 maanden na start ART. Dus, alhoewel de overleving toeneemt na therapie, is deze nog steeds lager in HIV-geïnfecteerden vergeleken met HIV-negatieve TB patiënten. Het nauwkeurig vervolgen van HIV-geïnfecteerden en opportunistische infecties zoals TB is vereist om de mortaliteit binnen 6 maanden na ART te minimaliseren.

In conclusie bespreken we in hoofdstuk X van dit proefschrift alle data in relatie tot de huidige literatuur en elkaar. Dit proefschrift laat een hoog percentage van HIV-infectie zien in TB patiënten. TB veroorzaakte alleen meer immuun activatie in de afwezigheid van HIV, maar leek geen actieve bijdrage te leveren aan immuun activatie onder HIV/TB co-geïnfecteerden. Zowel HIV virale load als de immuun activatie veranderde niet na anti-TB chemotherapie. Het feit dat Ethiopiërs geen snellere progressie naar AIDS hebben, ondanks hoge initiële immuun activatie en lagere CD4 aantallen, kon verklaard worden door lagere immuun activatie na HIV-infectie. ART verhoogde de overleving van HIV/TB co- geïnfecteerden. Nauwkeurige vervolging van patiënten om de mortaliteit te reduceren en de introductie van methoden om gevoelig, specifiek en betaalbaar individuen te screenen op aanwezigheid van MTB en klinische TB te diagnosticeren verdient de aandacht. Met name in HIV-geïnfecteerden is het van belang om snel te diagnosticeren en vroegtijdig profylactische behandeling te initiëren.

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20. Persistent immune activation and sustained plasma viral RNA level in human immunodeficiency virus infected Individuals developing tuberculosis despite anti-TB treatment. *Manuscript in preparation*
21. Increased percentage of regulatory T cells is associated with advanced HIV disease and HIV-TB coinfection: a reflection of enhanced Immune activation? *Submitted for publication*
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