In vitro and *In vivo* Immune Responses in Ethiopian Tuberculosis Patients with HIV Infection

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Dedication

This thesis is dedicated to my mother for her benevolence, support, and inspiration.

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List of abbreviations

AFB – Acid-fast bacillus

AIDS – Acquired Immunodeficiency Syndrome

ALERT – African Leprosy Educational Research and Training centre

APC – Antigen Presenting Cells

BCG – Bacille Calmette Gurein

BCIP – 5-bromo-4-chloro-3-indolyl-phosphate

BrdU- 5-Bromo-2-deoxyuridine

CDC – Centers for Disease Control

DMSO – Dimethyl Sulphoxide

DTH – Delayed type hypersensitivity

EASIA – Enzyme Amplified Sensitivity Immunoassay

EBSS – Earel`s balanced Salt Solution

ELISA- Enzyme Linked Immunosorbent assay

FACScan – Fluorescence activated cell sorter

HIV – Human Immunodeficiency Virus

HLA – Human leukocyte antigen

HRP – Horseradish Peroxidase

IFN- Interferon

Ig - Immunoglobulin

IL- Interleukin

IL-R –Interleukin receptor

IMDM – Iscove`s Modified Dulbecco`s Medium

iNOS – Inducible Nitrogen Oxygen Synthase

LAM - Lipoarabinomannan

LPS - Lipopolysaccharide

LTR – Long Terminal Repeat

MIP – Macrophage inflammatory protein

MOH – Ministry of health Organization

NASBA- Nucleic Acid Sequence Based Amplification

NBCS – New Born Calf Serum

NBT – Nitroblue tetrazolium

NF – Nuclear Factor

NTLP – National tuberculosis and Leprosy Program

OD – Optical Density

PBMC- Peripheral blood mononuclear cell

PBS – Phosphate buffered saline

PHA- Phytoheamagglutinin

PPD – Purified protein Derivative

RNIs – Reactive Nitrogen Intermediates

ROIs – Reactive Oxygen Intermediates

SIV – Simian Immunodeficiency Virus

ST-CF – Short Culture Filtrate

sTNF-R- Soluble tumour necrosis factor receptor

TB – Tuberculosis

TGF – Tumour growth factor

TMB – Tetramethylbenzidine

TNF- Tumour necrosis factor

WHO – World Health Organization

Abstract

Mycobacterium tuberculosis and HIV-1 are known to interact with each other with an impact on the immunological, virological/bacterial and clinical outcomes of both infections. We aimed to evaluate the *in vitro* and *in vivo* immunolgical alterations in TB and HIV-1 co-infection. The study population consisted of 101 subjects: HIV- and HIV+ individuals with TB (HIV-TB+, n=29; HIV+TB+, n=31), individuals with HIV only (HIV+TB-, n=19), and healthy controls (HIV-TB-, n=22). Lymphoproliferative responses were evaluated in mitogen- or antigen-stimulated PBMCs by BrdU ELISA. Cytokines (IFN-γ, IL-10, and IL-12) in cell culture supernatants of PBMCs, and plasma concentrations of TNF-α and TNF-RII were measured by sandwich ELISA. Plasma viral load was determined by nucleic acid sequence-based amplification; and CD4+ T-cells were enumerated by FACScan. Results showed that CD4+ cell counts (cells/mm^3) were significantly reduced in individuals with TB and/or HIV infections compared to the healthy controls. Patients co-infected with TB/HIV had a significantly elevated plasma viral load (log_{10} copies/ml) as compared to HIV+ without TB. Proliferative responses were reduced in TB+HIV- (1.36 and 0.58 to PHA and PPD, respectively), TB-HIV+ (1.17 and 0.40) as well as those with TB+HIV+ (1.13 and 0.47) compared to healthy controls (1.76 and 0.83). IFN-γ production was significantly depressed in TB+HIV- ($P<0.001$ to both PHA and PPD), TB-HIV+ ($P=0.04$, $P=0.03$ to PHA and PPD, respectively), and TB+HIV+ ($P=0.03$, $P=0.01$) when compared to healthy controls. Although we did not see statistically significant difference, there was a trend towards an increased IL-10 production in response to PPD from TB+HIV- < TB-HIV+ < TB+HIV+. In comparison to healthy controls, PPD-stimulated IL-12 levels were significantly reduced in the TB+HIV+ group ($P=0.02$). Plasma concentrations of TNF-α and sTNF-RII were progressively

increased from TB+HIV- (34.1 pg/ml and 7.8ng/ml, TNF-α and TNF-RII, respectively) < TB-HIV+ (52.1pg/ml, 9.9ng/ml) < TB+HIV+ (63.5pg/ml, 15.1ng/ml). There was a linear positive correlation between sTNF-RII and viral load in TB-HIV+ group (R=0.75, P=0.01). Immunosuppression appeared to be protracted after three months of anti-TB chemotherapy, and markers of immune activation remained elevated, irrespective of HIV status. The data suggest that TB and HIV infections display partly similar and partly distinct immune alterations, which may account for the exacerbation of disease outcomes associated with both infections. The pattern of non-specific immune activation during TB/HIV co-infection may be responsible for the enhanced replication of HIV-1 and accelerated disease progression.

The tubercle bacillus preferentially infects macrophages and is noteworthy for having evolved mechanisms that allow it to survive and multiply inside the phagocytic cell (Fine, 1994; Clemens, 1996). The cell envelope of the tubercle bacillus, exceptionally rich in the unusual lipids, glycolipids and polysaccharides, may account for many of the unusual characteristics of the bacillus, including ability to withstand the killing mechanisms of macrophages and to replicate intracellularly, resist chemical and physical stresses, and be impermeable to many antimicrobials and resist potent adjuvant activity, which may contribute to the immunopathological manifestations of the disease (Kaufmann, 1993; McKinney *et al.*, 1998).

1.2. Pathogenesis of tuberculosis

The primary route of infection of the tubercle bacillus is via the respiratory tract and the susceptible individual can acquire an infection by inhaling infectious droplet nuclei, each containing one to three bacilli with a diameter less than 5μm (Wiegeshaus *et al.*, 1989). The magnitude of the risk of acquiring *M. tuberculosis* infection is associated with the intensity of exposure, innate host defences (Rose, 1991; Stead, 1992), and the intrinsic virulence of the tubercle bacillus (Schlesinger, 1993; Strokes and Speert, 1995). On the other hand, the infectiousness potential of tuberculosis patients usually correlates with the number of organisms in the sputum, extent of pulmonary TB, and the frequency of coughing (Daniel, 1994).

Following arrival at the level of alveoli, *M. tuberculosis* bacilli are ingested by local macrophages and may promptly be killed before infection is established or grow to a limited extent within the host cell phagosome (Rich and Ellner, 1994). In individuals with intact immune system during the two to eight weeks after initial infection, a granulomatous

response is established. From then on the body's immune system maintains a standoff with the infection, sometimes for years. Most people undergo complete healing of their initial infection, and the tubercles calcify and lose their viability (Daniel, 1994).

In individuals who make either a poor or no granulomatous responses, the infection may progress to active disease. Moreover, in individuals in whom infection is controlled at the granulomatous state or earlier, any later imbalance of the host's immune system may promote reactivation of the disease (Dannenberg and Rook, 1994). Only a small proportion of the successfully infected people develop active disease some time in their lifetime, but this small group is the most important and direct source of transmission of new infections (Riley, 1995).

Active TB usually results from the spread of bacilli from the alveoli through the blood stream or lymphatic system to other sites, usually elsewhere in the lungs or local lymph nodes. In the minority of cases, the bacilli cause disease in other organs, such as the skin, kidneys, bones, or reproductive and urinary systems. At the new sites, the body's immune defenses kill many bacilli as well as immune cells and local tissues. The dead cells and tissue, along with live immune cells, form granulomas whose centres have the consistency of soft cheese, where the bacilli survive but do not flourish. The early symptoms of active tuberculosis can include cough, weight loss, fever, night sweats, and loss of appetite, or they may be vague and go unnoticed by the affected individual (Dannenberg, 1991; Rich and Ellner, 1994).

As the disease progresses, more lung tissues are destroyed; granulomas expand; and cavities in the lungs develop, which sometimes break into bronchi. This creates a rich medium in which the bacilli multiply rapidly and spread, creating further lesions and

characteristic chest pain, and, when a blood vessel is eroded, bloody sputum, and allows large numbers of bacilli to be ejected when patients cough (Has and DesPrez, 1994; Fenton and Vermeulen, 1996).

1.3.Immunity to tuberculosis

The successful containment of *M. tuberculosis* infection is primarily due to the development of acquired immunity, which predominantly is the cell-mediated type rather than humoral, involving the interactions of a variety of immunocompetent T cells and mononuclear phagocytes (Barnes and Rom, 1994). In addition, humans display native immunity to TB, with substantial individual variation. Epidemiologic data derived from twin studies clearly indicate that the susceptibility to TB has genetic determinants (Ellner, 1997). Several efforts have also been made to show an association between human leukocye antigen (HLA) phenotype and TB (Rich and Ellner, 1994). Recent interest has also focussed on the role of polymorphisms in cytokine promoters (e.g., tumour necrosis factor-alpha, TNF-α) and cytokine receptors (e.g., interferon-gamma receptor, IFN-γR) in susceptibility to mycobacterial disease (Ellner, 1997).

1.3.1. Role of mononuclear phagocytes

Mononuclear phagocytes are the natural effector cells involved both in innate immunity and acquired immunity to TB. They play multiple roles, including antigen processing and presentation in addition to their effector function. They are also the preferred host cells for *M. tuberculosis*, and hence play a dual role (Chan and Kaufmann, 1994).

The adherence of *M. tuberculosis* to macrophages may be mediated by several macrophage cell-surface molecules, including complement receptors, mannose receptors, the lipopolysaccharide (LPS) receptor (CD14), Fc receptors, the scavenger receptor, and possibly the toll-like receptor 2 (Hingley-Wilson *et al.*, 2000), and ingestion takes place via conventional phagocytosis. The precise route of *M. tuberculosis* entry is likely to determine the ultimate fate of bacilli within the macrophage (Fenton and Vermeulen, 1996).

There are many descriptions of possible mechanisms by which mononuclear phagocytes kill or inhibit *M. tuberculosis*. These include lysosomal enzymes that are delivered to the phagosome during phagosome-lysosome fusion, the generation of reactive oxygen intermediates (ROIs, such as H_2O_2 and O_2^-), reactive nitrogen intermediates (RNIs, such as NO and NO_2^-), and apoptosis (Hnigley-Wilson *et al.*, 2000). However, pathogenic mycobacteria have the capacity to evade killing, and various ways contributing to *M. tuberculosis* evasion have been described (Fenton and Vermeulen, 1996; Hingley-Wilson *et al.*, 2000).

In addition to their role as host cells that support the intracellular growth of mycobacteria and their potential ability to restrict the growth and kill the bacilli, mononuclear phagocytes produce and release inflammatory cytokines when exposed to *M. tuberculosis*. These cytokines have the potential to exert potent immunoregulatory effects and to mediate many of the clinical manifestations of TB. The cytokines produced by mononuclear phagocytes in response to *M. tuberculosis* include interleukin (IL)-1, IL-6, IL-10, tumour necrosis factor (TNF-α), transforming growth factor (TGB)-β, IL-12, and etc. (Table 1) (Barnes and Rom, 1994).

1.3.2. Role of T lymphocytes

Mononuclear phagocytes have a limited intrinsic capacity to reduce the growth of mycobacteria; therefore, additional acquired immune activation is provided by T lymphocytes to control the infection. Thus, after ingesting mycobacteria, macrophages sensitise T lymphocytes by secreting proinflammatory cytokines (IL-1, IL-6, TNF-α) and chemokines (e.g. macrophage inflammatory protein 1, interferon inducible protein 10) (Anderson, 1997) and presenting lymphocytes with processed mycobacterial antigens on their cell surfaces. Mycobacterial peptide antigens, expressed in association with human leukocyte antigen (HLA) class II or class I molecules, can be recognized by $CD4^+$ and $CD8^+$ αβ TCR^+ T cells respectively whereas non peptide mycobacterial antigens, such as LAM and mycolic acid are recognized by the double negative T cells ($CD4^-CD8^-$) in an MHC-independent but CD1-restricted manner (Fenton and Vermuelen, 1996).

The stimulated *M. tuberculosis*-specific precursor lymphocytes then proliferate and release an elaborate array of chemical signals, some of which produce inflammatory reactions, and others recruit and activate specialized cells to kill bacilli and wall-off infected macrophages in tubercles, constituting a granulomatous response in which mycobacteria are killed by repeated cycles of phagocytosis, cytolysis, and exposure to microbicidal products (Fig.1) (Rich and Ellner, 1994; Shafer, 1994).

Among the T lymphocyte subsets, αβ CD4+, αβ CD8+, and γδ T cells are believed to contribute to protective immunity against TB (Ellner, 1997). αβ CD4+ T lymphocytes are believed to be the primary subset involved in many aspects of the immune response to *M. tuberculosis*, including binding to the processed antigen, secreting cytokines, killing

mycobacteria-infected cells, and persisting as antigen-specific memory cells (Shafer, 1994). αβ CD8+ cells constitute the major cytolytic T cell population in defenses against many intracellular pathogens in animal models of infection. They also produce cytokines such as IFN-γ and TNF-α (Seder and Hill, 2000) and seem to be of importance for the normal generation of protective granulomas and the lack of this subset leads to diffuse and badly organized cellular infiltrates (Rich and Ellner, 1994). γδ T-cells may act as a first line of defence against *M. tuberculosis* (Janeway *et al.*, 1987). Functionally, γδ T cell clones are similar to αβ T cells, although respond optimally to distinct antigens of *M. tuberculosis*, in that they produce IFN-γ, IL-2, TNF-α, IL-4, IL-5, and IL-10 (Tsukaguchi *et al.*, 1995), and also have been shown to display cytotoxic activities (Patel *et al.*, 1989).

In the current model the CD4 cell subset is divided into Th1 and Th2, each producing a characteristic panel of cytokines. This division was originally based on data from murine T cell clones, but data on cytokine profiles in human patients with different clinical forms of parasitic disease indicate that at least a similar division of human CD4 cells exists (Abbas *et al.*, 1996). Subsets of CD4+ T cells designated as Th1 produce cytokines, such as IL-2, IFN-γ, lymphotoxin, and TNF-α. Th2, on the other hand, produce the cytokines IL-4, IL-5, IL-6, IL-10, and IL-13 (Mosmann and Sad, 1996).

A Th1 response has been postulated as conferring protection against *M. tuberculosis* whereas a Th2 response is associated with severe disease. Depressed Th1 responses have been reported for patients with active TB: specifically, production of Th1 cytokines by *M. tuberculosis*-stimulated peripheral blood mononuclear cells (PBMC) was lower for PMBC from patients with active TB than for PBMC from healthy tuberculin reactors (Surcel *et al.*, 1994; Zhang *et al.*, 1994; Zhang *et al.*, 1995a; Demissie *et al.*, 1999). Th1 cytokines are also

more prominent in tuberculous pleuritis, a manifestation of tuberculosis that often resolves without chemotherapy and patients with less radiologically advanced pulmonary TB have bronchoalveolar lavage lymphocytes that secrete IFN-γ and not IL-4 (Barnes *et al.*, 1993; Condos *et al.*, 1998).

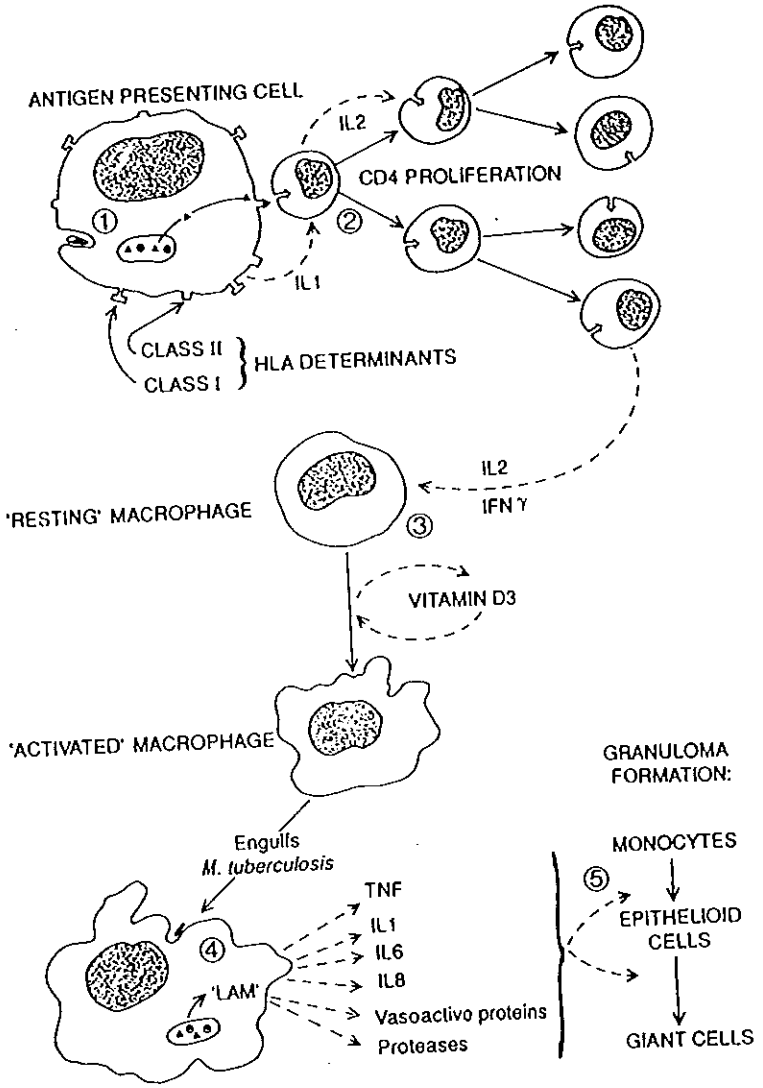

Figure 1. Major events in the cellular response to *M. tuberculosis* infection (*Adapted from* Fine, 1994).

1.3.3. Cytokines in tuberculosis

Various studies have demonstrated that cytokines produced in response to *M. tuberculosis* play an important role in the host defence, regulating the interactions of the immunocompetent cells and acting as effecter molecules in antimicrobial immunity. They may also mediate some of the clinical manifestations of TB.

Treatment of murine macrophages with IFN-γ and TNF-α induces to promote synthesis of inducible nitric oxide synthase (iNOS), production of RNI, and killing of ingested tubercle bacilli (McKinney *et al.*, 1998), and also causes B-cells to switch their Ig isotype to IgG1, which promotes phagocytosis by activating the classical pathway of complement and binding to Fc receptors on macrophages (Lynch *et al.*, 1995). Moreover, individuals with IFN-γ-receptor deficiency are particularly susceptible to mycobacterial diseases (Newport *et al.*, 1996).

TNF-α has the capacity to induce macrophage chemotaxis and enhances class II expression (Barnes *et al.* 1993), and is required for the generation of the granulomatous response to mycobacterial infection (Vanham *et al.*, 1997). However, excessive TNF-α production and/or increased sensitivity to TNF-α are involved in expression of many of the local and systemic toxicities evident in TB, including necrotizing reactions, which promote the replication and dissemination of the bacteria. It is also an important mediator of systemic inflammation, clinically manifested by fever and wasting (Beutler and Cerami, 1987).

Inhibition of antigen-specific T cell responses may be mediated by cytokines such as IL-10 and TGF-β, which probably alter antigen presentation by macrophages by the regulation of MHC class II expression, reduce IL-2 dependent T cell proliferation (Barnes and Rom,

1994), activation of macrophages mediated by IFN-γ (Silva *et al.*, 1991) and production of reactive oxygen intermediates (Bodgan *et al.*, 1991). Both TGF-β and IL-10, however, may also prevent excessive inflammation and tissue damage from an uncontrolled inflammatory response, regardless of their negative role in antimycobacterial responses (Barnes *et al.*, 1994).

Table 1. Cytokines produced by T cells and their antagonists or agonists (*Adapted from* Janeway *et al.*, 1999).

Cytokines	Cell source	Antagonist	Synergistic
IL-2	Th1, some CTL	Th2	Th1, NK
IFN-γ	Th1, CTL	Th2	Mo, NK
TNF-β	Th1, some CTL	Th2	Mo
IL-3	Th1, Th2, some CTL	-	-
IL-4	Th2	Th1, Mo	Th2, B, Ma
IL-5	Th2	-	Eo, B
IL-6	Th2	Th1	B
IL-10	Th2, Mo	Th1, Mo	Ma
IL-12	Mo, DC, B, Granulocyte	-	Th1
IL-13	Th2	Th1, Mo	Th2
IL-18	Mo, DC	-	Th1
TNF-α	Th1, some Th2, some CTL, Mo	-	Mo
TGF-β	T cells, Mo	Mo, B	Neu
GM-CSF	Th1, some Th2, some CTL	Mo	T & B cells

Abbreviations: B, B-lymphocyte; CTL, cytotoxic T lymphocyte; DC, dendritic cell; Eo, Eosinophil; Ma, mast cell; Mo, monocyte/macrophage; Neu, neutrophil; Th, T-helper.

Some cytokines may also be involved in response to TB through various mechanisms. In this regard, IL-12 may be important in favouring the development of the delayed type hypersensitivity (DTH) response by enhancing production of IFN-γ, facilitating the development of Th1 cells, and augmenting the cytotoxicity of antigen specific T-cells and natural killer cells (Clerici *et al.*, 1993). IL-12 favours development of Th1 responses in several experimental systems including that with mice infected with *M. tuberculosis* (Cooper *et al.*, 1995; Flynn *et al.*, 1995). In studying patients with tuberculous pleuritis, Zhang *et al.* (1994) noted that IL-12 was increased in pleural fluid from patients compared with the levels in peripheral blood.

In contrast, IL-4 can derive a naïve Th cell down a Th2 differentiation pathway (Abbas *et al.*, 1996). It also abrogates both the activation of monocytes and their anti-microbial activity mediated by IFN-γ (HO *et al.*, 1992), down-regulates CD14 expression on monocytes and production of IL-1 and TNF-α (Hart *et al.*, 1989), and can block macrophage nitric oxide generation (Liew and Cox, 1991).

1.4. Tuberculosis and HIV co-infection

1.4.1. Acquired Immunodeficiency Syndrome (AIDS) and the Virus

Acquired immunodeficiency syndrome (AIDS) is the clinical end stage of persistent HIV infection (CDC, 1993). HIV is a member of the lentivirus family of retrovirus (Chiu *et al.*, 1985). There are two subtypes of HIV, i.e. HIV-1 and HIV-2 with 40-50% of homology between them, and varying in geographical distribution and degree of virulence. HIV-1 is responsible for most cases of HIV infections, widely distributed throughout the world, and

more virulent than HIV-2 (Romieu *et al.*, 1990). Detailed analysis of molecular genetic data provide evidence that HIV-1 may have been introduced into human populations from the simian immunodeficiency virus (SIV_{cpz}) of the chimpanzees (Gao *et al.*, 1999), whereas HIV-2 from monkeys (sooty mangabeys and mandrills) (Hahn *et al.*, 2000).

Since the first recognition of AIDS in 1981 until the end of the year 2000, an estimated 36.1 million people worldwide (34.7 million adults and 1.4 million children younger than 15 years) were living with HIV/AIDS. Cumulative HIV/AIDS-associated deaths worldwide amounted to approximately 21.8 million (17.7 million adults and 4.1 million children younger than 15 years) (WHO, 2000). According to the same estimate, about 15000 people become infected every day despite progress in prevention and powerful drug combinations to treat HIV infection. Over 95% of new infections occur in developing countries for which the recent medical advances are not immediately available or affordable.

HIV can be transmitted horizontally by sexual contact, via contaminated blood or blood products, and vertically from infected mother to the foetus and child. The probability of HIV-1 transmission depends on the interplay of many different factors related to biological characteristics of HIV strains, infectiousness potential of HIV-positive individual, and susceptibility of the exposed individual (Buchaz *et al.*, 1998).

The principal targets for HIV-1 infection in the human host are the helper subset of T lymphocytes and monocyte/macrophages (Fauci, 1988). The entry of the virus into the host cell is mediated by interactions between the virus envelope glycoproteins, gp120 and gp41, and the surface molecule receptors of the CD4+ cells, CD4 and members of the chemokine receptor family mainly CCR5 and CXCR4 (Kristiansen *et al.*, 1998).

Primary infection with HIV-1 may be either asymptomatic or associated with an acute retroviral syndrome (ARS), which is characterized by flu and mononucleosis-like symptoms within a few weeks or months just before or at the time of seroconversion (Tindall and Cooper, 1991). The acute stage is associated with a burst of HIV viremia and often a concomitant abrupt decline of CD4+ T cells in the peripheral blood (De Wolf *et al.*, 1997). The acute stage is followed by an asymptomatic period with different rates of CD4+ T cell decline and increase in viral load, which eventually lead to severe immunodeficiency and clinical manifestations of AIDS, such as opportunistic infections, lymphadenopathy, neoplasms, and neurological disorders like dementia (Pantaleo *et al.*, 1993).

Immunological abnormalities, including disturbed T-, B- and antigen presenting cell (APC) functions, occur before and during the decline of CD4+ T cell in HIV infection (Miedema *et al.*, 1988). The loss in T helper cell function (such as cytokine production and proliferation) appears first as unresponsiveness to recall antigens, then to alloantigens, and finally to phytohemagglutinins (Clerici *et al.*, 1989). Several hypotheses contributory for T-cell dysfunction during disease progression have been postulated. These include the loss of memory cells and associated memory cell function, impaired T cell responses to co-stimulatory signals, programmed cell death of T cells, and changes of cytokine secretion (Pakker, 1998). In addition, certain functional macrophage defects such as abnormal cytokine secretion, decreased phagocytosis, decreased generation of reactive oxygen species, and decreased antigen presenting efficiency have also been observed (Shafer, 1994). These abnormalities may favour for the onset of opportunistic infections.

1.4.2. Interaction between tuberculosis and HIV infection

TB is one of the most common opportunistic infections in HIV-infected individuals (WHO, 1994). TB and HIV share a number of common features, i.e. both have similar geographic distribution and each exacerbates the other. In both diseases, the onset is slow; the pathogens are localized within immune cells, evade and suppress the host immune response; and the host may control both infections by multiple mechanisms. A Th1 response seems to be involved in attempt to control both pathogens (Bentwich *et al.*, 1999).

HIV-infected persons are at a markedly increased risk for primary or reactivation TB (Selwyn *et al.*, 1989) and for a second episode of TB from exogenous re-infection (Small *et al.*, 1993). The annual risk of developing active TB in individuals with HIV is about a 5-15% (Selwyn *et al.*, 1989). Unlike other AIDS-defining conditions, TB may appear at any stage throughout the spectrum of HIV-induced immunodeficiency (Shafer, 1994). The early onset of TB in HIV-infected individuals suggests a strict requirement for a fully competent cell-mediated immune response for protection against *M. tuberculosis* (Rich and Ellner, 1994).

The clinical presentation of HIV-associated TB is heavily influenced by the extent of the immunodeficiency. HIV-infected persons are more likely to have extrapulmonary and disseminated TB than HIV-negative individuals, and pulmonary TB is common in the earlier stage whereas extrapulmonary and disseminated forms in later stages. Diagnosis of TB in HIV-positive persons is rendered more difficult by increased frequency of false negative tuberculin skin tests, of sputum smear-negative, and of atypical radiological manifestations (DeCock, 1994).

HIV-infected TB patients have a shorter survival, more opportunistic infections, and a greater decrease in CD4+ T cell counts, compared to HIV patients without TB (Rich and Ellner, 1994). Furthermore, there is evidence that HIV-infected patients with TB have an accelerated decline in immune function compared with HIV-positive without TB (Whalen *et al.*, 1995). Similar findings have been reported from primate studies in which co-infection with simian immunodeficiency virus (SIV) and *M. tuberculosis* resulted in enhanced decline of peripheral CD4+ cell counts and faster progression to AIDS (Zhou *et al.*, 1999).

Immune activation has been considered as an important factor in the pathogenesis of HIV disease progression (Bentwich *et al.*, 1995). Accordingly, HIV-positive TB patients exhibit a more pronounced immune activation than HIV-negative TB patients or HIV-positive individuals without TB, as evidenced by increased expression of HLA-DR on both CD4 and CD8 T cells, enhanced levels of both FcγR-I and FcγR-III on monocytes, and elevated serum levels of TNF-α, β_2-microglobulin and neopterin (Vanham *et al.*, 1996).

The role of *M. tuberculosis*-induced immune activation in accelerating disease progression may be related to increased viral replication. In this regard, Goletti *et al.* (1996) reported a 5- to 160-fold increase in plasma viral load during the acute phase of TB as compared with those observed prior to the onset of TB and after successful anti-TB treatment. Pleural fluid from patients with TB increases HIV replication in activated lymphocytes (Garrait *et al.*, 1997). In addition, incubation with *M. tuberculosis* or its constituents resulted in activation of HIV-1 transcription and expression in T lymphoid and monocytoid cells (Zhang *et al.*, 1995b; Goletti *et al.*, 1996; Toosi *et al.*, 1997).

There are at least two mechanisms by which *M. tuberculosis* modulates HIV replication. First, macrophages could be co-infected, especially at sites of disease (Zhang *et al.*, 1995b) implying that *M. tuberculosis* may influence directly HIV gene expression and replication. The other mechanism is that infection with *M. tuberculosis* stimulates the release of cytokines from mononuclear phagocytes. This has been shown for IL-1, TNF-α, and IL-6 in animal models, in *in vitro* cell culture, and in humans with active TB (Barnes *et al.*, 1992; Zhang *et al.*, 1995b). These cytokines enhance replication of HIV-1 *in vitro* using several cell lines (Koyanagi *et al.*, 1988). The mechanism of this stimulation has been localized in the 5` long terminal repeat (LTR) region of HIV-1 and specifically in the nuclear factor (NF)-κB transcription enhancer site (Rosenberg and Fauci, 1991). Thus, systemic immune activation in patients with TB/HIV co-infection (Vanham *et al.*, 1996) may enhance viral replication at sites remote from the major site of TB infection. This, in particular, is relevant for countries like Ethiopia, whereby the predominantly circulating HIV-1 subtype C has more NF-κB binding motifs at the LTR sites compared to subtype B HIV-1 (Johansson *et al.*, 1996), making the former more susceptible to enhanced replication by TNF-α.

In addition to contributing for viral replication, *M. tuberculosis* may favour the productive infection of monocytes (Toossi *et al.*, 1993) as well as CD4+ T cells. This may be related to the increased production of TNF-α (Toossi *et al.*, 1997), or increased expression of requisite HIV-1 co-receptors (Wu *et al.*, 1997; Fraziano *et al.*, 1999). In addition, the expanded FcγR-III^{+} monocyte subset might be more permissive to HIV, although the role of FcγR-III as a possible co-receptor for HIV on monocytes remains highly controversial (Connor *et al.*, 1991).

1.4.3. The scope of the problem

The elimination of TB from parts of the industrialized world was once considered an achievable goal, and strategies for TB control had been designed in many developing nations (Shafer, 1994). However, a dramatic resurgence of TB has been noted in numerous countries, both industrialized and developing nations since the mid 1980`s. For instance, the annual incidence of TB in the United States as a whole rose by about 20%, with similar increases occurring in several Western European nations (Cantwell *et al.*, 1994). In Africa, notifications of TB approximately doubled between 1985 and 1990, rising to a level over 200 per 100,000 cases per year (Shafer, 1994).

About a third of the world's population (~2 billion) harbours *M. tuberculosis*, and is at risk of developing the disease (Anderson, 1997). In 1997, total number of new cases of TB was estimated to be 7.96 million, including 3.52 million cases of infectious pulmonary disease, and there were 16.2 million existing cases of disease. An estimated 1.87 million people died of TB and the global case fatality rate was 23% but exceeded 50% in some African countries with high HIV rates. (Dye *et al.*, 1999).

One of the main contributing factors for the dramatic resurgence of TB has been the HIV/AIDS epidemic. Globally, 4.6% of total TB cases were attributable to HIV infection in 1990 and the proportion was expected to increase to more than 14% in the year 2000 (Lienhardt and Rorigues, 1997). In addition, 3 million new TB cases attributed to HIV are expected to occur in four years time through 1999 to 2002 (Pilheu, 1998). Globally, about

10.7 million people had *M. tuberculosis*-HIV co-infection in the year 1997 (Dye *et al.*, 1999).

1.5. Burden of tuberculosis and HIV infection in Ethiopia

TB is a serious public health treat in Ethiopia. It was among the leading causes of outpatient morbidity, hospital admission, and hospital deaths in the country (MOH, 1997). In the urban areas of Ethiopia, the incidence of TB and the proportion of extra-pulmonary TB cases have already increased (Eyob *et al.*, 1999a; Eyob *et al.*, 1999b). According Dye *et al.* (1999) estimate, the annual incidence of TB cases is 260 per 100000 people, with a prevalence of 367 per 100000 people. This takes Ethiopia to be ranked among the top 22 highest-incidence countries. Various factors such as HIV epidemic, spread of virulent strains resistant to key anti-TB drugs, population explosion/crowding, famine, and war may contribute to the marked increase in TB burden.

Although the HIV/AIDS pandemic started relatively late in Ethiopia, the first HIV-1 positive sera being detected in 1984 and the first AIDS patients reported in 1986 (Tsega *et al.*, 1988), it has spread to the entire country to reach a prevalence ranging from 4.5% in the adult population (in rural areas) to 21% (in urban areas) with a mean of 7.4% (MOH, 1998). Various studies have indicated that HIV-1 subtype C is predominantly circulating in the country (Ayehunie *et al.*, 1990; Abebe *et al.*, 1997). The burden of TB/HIV co-infection has markedly increased through the years from 6.6% in 1990 to 20-25% in 1995, 44.4% in 1996 and 45.3% in 1998 (Kefene *et al.*, 1990; Gellete *et al.*, 1997; Mitikie *et al.*, 1997; Demissie *et al.*, 2000).

1.6.Control of Tuberculosis

There are several methods that are helpful to alleviate the problem of TB: improvements in socio-economic conditions, case finding and treatment, chemoprophylaxis and vaccination (Rodrigues and Smith, 1990). Nevertheless, control measures based on treatment of infectious cases and the use of a Bacillus Calmette Guerin (BCG) vaccination have limited success. There is increasing evidence that BCG may be less effective in the tropics than in the regions further from the equator (Fine and Rodrigues, 1990; KPTG, 1996). The reasons for this are not clear, but may relate to differences in the genetics of the population, nutritional status, or exposure to other organisms including viruses, environmental bacteria and helminths, which may modulate the immune response to BCG or to *M. tuberculosis* itself. Moreover, the emergence of multi-drug resistant strains of *M. tuberculosis*, a lengthy treatment with chemotherapeutic drugs, and associated side effects complicates the situation (Friend and Watson, 1998).

Therefore, there is an incessant need for developing new drugs and vaccines. In this regard, the potential role of cytokines for adjunctive immunomodaltities has been recognized in multidrug-resistance disease in attempt to improve on the prevailing 50% cure rates, and in drug-sensitive TB, to shorten the duration of treatment by bolstering the immune response, thereby accelerating the eradication of persisting organisms (Ellner, 1997).

Although it is postulated that a Th1 response as conferring protection against diseases caused by intracellular pathogens such as *M. tuberculosis*, and a Th2 response is associated with severe disease, cytokine profiles in TB are the subject of some debate. Factors, such as HIV co-infection (Leroy *et al.*, 1997), malnutrition (Mainali and McMurray, 1998), and

genetic factors (Newport *et al.*, 1996; Goldfeld *et al.*, 1998) may alter the balance between Th1 and Th2 cytokines; thus, enhancing susceptibility to TB and disease progression.

For instance, PBMCs from HIV-infected Ethiopian individuals stimulated *in vitro* with purified protein derivative (PPD) release lower levels of IFN-γ and show depressed blastogenesis, but was not associated with increased IL-4 production, which was similar in HIV-positive individuals and controls (Legesse, 2000). Moreover, Fan *et al.* (1993) have shown that, although IL-2 production seems to be impaired, expression of IL-4 in PBMC from HIV-seropositive and HIV-seronegative individuals was barely detected regardless of the stage of disease. In contrast, studies by Clerici and colleagues (1993) showed that there was an increased production of IL-4 together with a decreased production of IL-2 during progression to AIDS. This shows that cytokine profiles in HIV-infected individuals deserve some debate.

In vitro stimulation of lymphocytes by a recall antigen (such as PPD) leads mainly to cytokine production by memory T cell precursors that had been pre-committed to a Th1 or Th2 pattern years earlier (Murphy *et al.*, 1996). Such response can differ from that of new effector cells in subsequent infection (Strikiatkhachorn and Braciale, 1997). Studying patients with TB with HIV might circumvent these limitations. Such individuals are currently stimulated and most of the circulating antigen-specific lymphocytes represent effector cells that have been differentiated at the site of infection or draining lymph node and are in transit to and from lymphoid organs. The results of such a study should more accurately reflect the influence of the pattern of cytokine response. Therefore, it can be assumed that concurrent HIV infection in TB patients aggravate the depressed T cell responses and enhance immune activation.

Alterations of Th1 immune responses may be a consequence of sequestration of antigen-specific cells at the disease site (Dieli *et al.*, 1999), defects in antigen presenting cells (Pancholi *et al.*, 1993), death of antigen specific cells (Hirsch *et al.*, 1999a), reduced production of Th1-inducing cytokines, and actions of cross regulatory cytokines (Hirsch *et al.*, 1996). Therefore, there is a need to elucidate the precise mechanisms of depression of Th1 responses.

Markers of disease activity are needed to evaluate disease progression and to monitor response to therapy. A number of markers of immune activity in plasma have been studied in HIV infection. β_2-microglobulin, soluble CD27, soluble TNF-α receptor type II (sTNF-RII), IL-12 were elevated in HIV-positive Ethiopian individuals compared to negative controls (Messele *et al.*, 2001). Since the pattern of these markers could vary according to the pathogen, markers discriminating co-pathogens would be particularly useful for the monitoring infections with HIV and TB.

1.7. OBJECTIVES

General: To evaluate the *in vitro* and *in vitro* immunolgical profiles in tuberculosis and HIV infection.

Specific:

- To measure the *in vitro* T-cell proliferation and cytokine production responses to *Mycobacterium tuberculosis*-derived antigen.

- To determine the impact of TB and/or HIV infections on $CD4^+$ T-cells counts; and TB on HIV-1 plasma viral load.

- To assess the impact of short-term chemotherapy of tuberculosis on *in vivo* and *in vitro* immune responses.

- To evaluate the impact of concurrent TB/HIV infections on immune activation markers in plasma.

2. Materials and Methods

2.1. Study Population

This study included 101 individuals (42 females, 59 males), consisting of 22 healthy donors, 19 HIV-infected individuals without TB, and 60 TB patients, of whom 31 were seropositive for HIV. Newly diagnosed tuberculosis patients were recruited from African Leprosy Educational Research and Training Centre (ALERT) and Higher 23 Health Centre, and classified according to results of HIV serology, as tuberculous HIV-seronegative patients (TB+HIV-) or tuberculous HIV-seropositive patients (TB+HIV+). HIV-seropositive patients without clinical TB (TB-HIV+) and HIV-seronegative individuals without clinical TB (TB-HIV-) were recruited from Akaki and Wonji cohort study groups.

2.2. Diagnosis of tuberculosis

TB was diagnosed based on positive acid-fast bacillus (AFB) smear and/or culture, histology of biopsy/tissue aspirate material and/or radiographic evidences compatible with the diagnosis of pulmonary TB, in addition to clinical symptoms. All TB patients received directly-observed therapy (DOT) with isoniazid 5mg/kg body weight (300 mg maximum), rifampicin 10 mg/kg (600 mg maximum), pyrazinamide 15-30 mg/kg (2 gm maximum) and ethambutol 15-25 mg/kg, according to the protocols of the National TB and Leprosy control Program (NTLP).

2.3. HIV Serology

HIV serology was conducted as previously done by Legesse (2000) using HIVSPOT Test (Genelabs Diagnostics, Singapore Science Park) and by enzyme-linked immunosorbent assay (ELISA) (Vironostika HIV Uni-form II plus O kit, Organon Teknika, Boxtel, The Netherlands). Reactive samples or discrepant results were confirmed by Western Blot test (HIV BLOT 2.2; Genelabs Diagnostics, Singapore).

2.3.1. HIV-SPOT Test

After adding consecutively three drops of reconstituted buffer, phosphate buffered saline (PBS) containing Tween 20, bovine serum albumin, heat treated goat serum into the HIV-SPOT device and allowed to soak, a drop of plasma was added, which was then followed by the addition of two drops of reconstituted buffer, wash buffer, reconstituted conjugate (protein A-gold reagent), and finally three drops of wash buffer. Results were read within 10 minutes. Formation of one large distinct red spot in the centre of the membrane was interpreted to be positive for HIV antibodies whereas a clear membrane that lacks a red spot at the centre of the device was considered to be negative for HIV antibodies.

2.3.2. ELISA

100ul of specimen diluent (stabilizing protein) was pipetted into each well of microelisa strip plates coated with a mixture of HIV proteins, followed by the addition of 50ul of serum and controls into the respective wells and mixed by tapping the sides of the strip holder. After

incubation at 37^{0}C for an hour, the wells were then washed six times by soaking in phosphate buffer diluted in distilled water (1:25). Following this, 100ul of tetramethylbenzidine (TMB) substrate was added into all wells and incubated at room temperature for 30 minutes. Reaction was stopped by adding 100 µl of 1.8 M H_2SO_4 and the optical density (OD) was measured at 450nm using an ELISA reader (Reader 230, Organo Teknica, Belgium). In reference to the cutoff value (mean of the negative control results plus 0.1), a test sample was considered positive for HIV antibodies when the OD value was greater than or equal to the cutoff value, and negative when the OD value was less than the cutoff value.

2.3.3. Western Blot

Two ml of diluted wash buffer (Tris with Tween 20, 1:20) was added into each well and incubated with three controls (strong reactive inactivated human serum with high titered antibodies to HIV-1 and HIV-2, weak reactive inactivated human serum with low titered antibodies to HIV-1 only, and non-reactive inactivated human serum for HIV-1 and HIV-2 antibodies) for 5 minutes at room temperature on a platform shaker at 100rpm. After removing the wash buffer, 2 ml of blotting buffer (non-fat milk, 1:10) was added to each well followed by 20µl of inactivated sample serum and 20 ul of controls to appropriate wells. The tray was covered and incubated for 1 hour at room temperature on a platform shaker. After aspiration, strips were washed 3 times with 2ml diluted wash buffer and allowed for 5 minutes to soak on a platform shaker between each step. Two ml of working conjugate solution (Goat anti-Human IgG diluted 1:1000 in blotting buffer) was added into each well and incubated for 1-hour at room temperature on a platform shaker. Conjugate was aspirated from the wells, washed, and 2ml working substrate solution (5-bromo-4-chloro-3-indolyl-phosphate, BCIP, and nitroblue teterazolium, NBT) was added into each well. The

tray was then incubated for 15 minutes at room temperature on a platform shaker. After the substrate was aspirated, the strips were rinsed several times with distilled water to stop the reaction, removed onto paper towels, covered, and allowed to dry. The presence or absence of antibodies to HIV in the tested samples was determined by comparing the bands developed on each strip to the non-reactive, strong reactive and weak reactive nitrocellulose strips.

2.4. HIV virus load quantification

Viral load was determined by quantifying the amount of HIV-1 RNA using a nucleic acid sequence based amplification (NASBA) kit (Organon Teknika, Belgium) following the manufacturer's instructions. The NASBA methodology was previously shown to give quantitatively reliable results on HIV-1 subtype C plasma samples (Alaeus *et al.*, 1997).

In brief, the plasma sample was added to NucliSens lysis buffer containing guanidine isothiocyanate (GuSCN), Triton X-100, Tris/HCl, PH 6.4. Following centrifugation for 2 minutes at 1500g, 20µl-reconstituted calibrator solution (synthetic RNAs) and 50µl silica suspension consecutively were added to each lysis buffer tube. After leaving for about 10 minutes at RT, supernatants were removed from the lysis buffer tubes following spinning. Then, the silica pellets were washed twice with wash buffer, twice with 70% ethanol and once with acetone and dried in open test tubes at 56^0C for about 10 minutes. 50ul elution buffer was added to test tubes until the pellets were completely resuspended, and left for about 10 minutes at 56^0C. After centrifugation was done for two minutes at 10000g, about 5µl of the supernatant nucleic acid was transferred to fresh tubes, and 10µl of primer solution (primers, nucleotides, dithiothreitol, KCl, $MgCl_2$, Tris/HCl, dimethyl sulphooxide, DMSO)

was added and followed by 5 minutes incubation at 65^{0}C and subsequent cooling at 41^{0}C for minutes. 5µl enzyme solution (AMV-RT, Rnase H, T7-RNA polymerase and BSA, Tris/HCl) was also added and incubated further for 5 minutes at 41^{0}C. Finally, the quantity of amplified RNA was determined by using a NASBA reader (Micro SLT 510, Organon Teknika, Belgium). The lowest limit of detection of the assay was 80 RNA copies/ml of plasma.

2.5. Flow cytometry

Lymphocyte subsets (CD4+ and CD8+) were determined using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) as previously done by Messele *et al.* (2000). Briefly, after the addition of 10ul of monoclonal antibodies, 100µl whole blood on the bottom of the FACS tubes was pippetted, and incubated for 15minutes at room temperature in the dark. Two ml of diluted lysing solution (50% diethylene glycol and 15% formaldehyde, FACSlyse) was added to each tube. After incubation for a maximum of 10 minutes at room temperature in the dark, tubes were centrifuged at 300g for 5 minutes and the supernatants discarded. Two ml of isotone was then pippetted and centrifuged at 300g for 5 minutes. Following removal of supernatants, 0.5µl isoton was added, and mixed by vortexing. Cells were then analysed using the Cellquest/Multiset software of the FACScan.

2.6. PBMCs isolation

Blood samples were drawn into ethylene diamine tetra acetic acid (EDTA)-vacutainer tubes (Becton and Dickinson, USA) and transported to EHNRI-ENARP laboratory and rested overnight. On the following day, plasma was removed and stored at –80^{0}C until used. The

remaining sample was reconstituted with Earel's balanced salt solution (EBSS) supplemented with 5% new born calf serum (NBCS), 100U/ml penicillin, 100ug/ml streptomycin and 20U/ml heparin, and overlaid over 12.5ml of Ficoll-paque solution (Amersham Pharmacia Biotech AB, Sweden) and centrifuged at 1400rpm for 30 minutes. The cells at the interface were collected, washed two times in EBSS medium at 1500rpm for 10 minutes and resuspended in a final volume of 1ml culture medium consisting of Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% Fetal Calf Serum (FCS), 100 U/ml penicillin and 100µg/ml streptomycin. Number of cells/ml was then determined by Coulter counter (Coulter Electronics LTD, U.K.).

2.7. Culturing of cells and proliferation assay

PBMCs culture was done as described previously (Legesse, 2000). In brief, after PBMCs were resuspended at a concentration of $1x10^6$ cells/ml in culture medium, a total of $1x10^5$ cells/200µl was seeded in triplicates to flat-bottom, 96-well plates, and cultured at 37°C in 5% CO_2 and 95% humidified atmosphere. PBMCs were either unstimulated or were stimulated with: (1) the mitogen, phytohaemagglutinin (PHA 16 Murex Biothec Limited, Dartford, UK), a final concentration of 2µg/ml for 48-72 h; (2) Purified protein derivative (PPD-batch RT49, Statens Serum Institute, Denmark), a final concentration of 10µg/ml for 120-144 h. Br-DU labelling reagent (final concentration, 10µg/ml) was added to the cultures 18-24 h prior to termination. Before proliferation assay, the cells were centrifuged at 300xg for 10 minutes and placed in an incubator at 60°C for about 90 minutes following removal of the supernatants.

The proliferative responses of T cells were determined by using commercial ELISA kits (BIOTRAK cell proliferation ELISA system, version 2 Amersham Pharmacia Biotech, UK). In brief, 200µl of fixative (ethanol) was added to each well and incubated for 30 minutes at room temperature. A similar volume of blocking solution containing 1% (w/v) protein in 50mM Tris-HCl; 150mM NaCl, PH 7.4 was then added to each well and incubated for the same period of time. 100µl/well peroxidase-labelled anti-BrdU was added and incubated for 90 minutes. The plate was then emptied by inverting and tapping on blotting surface and the wells were washed three times with wash buffer (200-300µl/well) so as to remove the non-bound peroxidase-labelled anti-BrdU. As a substrate, 100µl TMB dissolved in 15% (v/v) (DMSO) was dispensed into each well and incubated for 10 min at room temperature. The reaction was stopped by adding 25µl of 1M sulphuric acid into each well. Then, 120µl of supernatants were collected from each well and transferred into a new 96-well flat bottom plate. OD was measured at 450 nm using an ELISA reader. Proliferative capacity of T cell was calculated as the difference between average of the three replicate stimulated wells and the average of the three un-stimulated control wells.

2.8. Cytokine induction and measurement

A total of $2x10^5$ cells/200µl was added, in triplicate, to 96-well flat-bottom plates, in medium alone or with medium supplemented with either 2µg/ml PHA or 10µg/ml PPD. The plates were then incubated at 37°C in 5% CO_2 in a humid atmosphere. The well contents were harvested at 24h (both PHA-stimulated and PPD-stimulated, IL-10 and IL-12), 72h (PHA-stimulated cultures, IFN-γ and IL-4), and 144h (PPD-stimulated cultures, IFN-γ and IL-4) of incubation and centrifuged for 5 min at 400g. The supernatants were collected and immediately stored at –80°C, until tested.

2.8.1. IL-10

IL-10 concentration in supernatants was determined by an Enzyme Amplified Sensitivity Immunoassay (EASIA) using commercial kits (BIOSOURCE IL-10 EASIA kit, BioSource Europe S.A., Belgium). Briefly, standards and 1:5 diluted samples were placed in monoclonal antibody coated microtiter plate wells and incubated for two hours on a horizontal shaker set at 700rpm ± 100rpm at room temperature. After the liquid was aspirated from each well, the plate was washed three times with wash solution. Anti-IL-10 horse-radish-peroxidase conjugate was then added to the wells (100µl/well) and incubated for 2 hours at room temperature on a horizontal shaker set at 700rpm ± 100rpm. After three washings in wash buffer, chromogenic solution (TMB, 200µl/well) was added and incubated for 30 minutes at room temperature on a horizontal shaker set at 700rpm ± 100rpm, avoiding direct sunlight. Reaction was then stopped with the addition of stop solution (1.8N H_2SO4, 50µl/well). Assay for each sample as well as for controls was done in duplicate, and results are expressed as the average of two readings, at 450nm, from an ELISA reader. A standard curve was plotted and IL-10 was quantified by interpolation from the standard curve. The results are expressed in pg/ml, and the lowest limit of detection was 0 pg/ml.

2.8.2. IL-12

A sandwich ELISA based Human IL-12 kit (The Biosource International, Inc. USA) was employed for the *in vitro* quantitative determination of IL-12 in cell culture supernatants. In brief, standards of known hIL-12 content and samples were pippetted into the wells (100µl/well) of the microtiter strips coated with an antibody specific for hIL-12, followed by

the addition of biotinylated anti-IL-12 (Biotin Conjugate) solution (50µl/well), and incubated for 2 hours at room temperature. After removal of excess, unbound biotinylated anti-IL-12 by four times washing in wash buffer, streptavidin-horseradish peroxidase working solution (100µl/well) was added, and incubated for 30 minutes. Again, the unbound enzyme was removed by four times washing in wash buffer. Then, a chromogen solution (TMB, 100µl) was added and incubated for 30 minutes, which was followed by the addition of stop solution (100µl/well). IL-12 assay was done in duplicate and results are expressed as the average of two readings, at 450nm, from an ELISA reader. A standard curve was plotted and IL-12 was quantified by interpolation from the standard curve. The results are expressed in pg/ml, and the lowest limit of detection was 0 pg/ml .

2.8.3. IFN-gamma

IFN-γ concentrations were determined by using commercially available ELISA kits (CLB, Pelikine compact human IFNγ ELISA kits, The Netherlands) as previously done by Legesse (2000). Briefly, 96-well plates (Nunc-Immuno plate, Denmark) were coated with anti-human IFN-γ Mabs and incubated overnight at room temperature. On the following day, after the supernatants were aspirated, the wells were washed with wash buffer (CLB, Pelikine Tool set, The Netherlands). Then, blocking solution (blocking reagent in PBS, 200µl/well) was pippetted and incubated at room temperature for 1 hour. After five washings, culture supernatants (1:10 for PPD-stimulated and 1:100 for PHA-stimulated) and serially diluted IFNγ standards in dilution buffer were pipetted in duplicate into the corresponding wells (100µl/well) and further incubated at room temperature for 1 hour. The supernatants were discarded, and the plates were washed five times. Biotinylated IFNγ antibody (1:100, 100µl/well) was added into the wells and incubated at room temperature for 1 hour.

Following five washings, streptavidin-horseradish peroxidase (HRP) conjugate (1:1000, 100µl/well) was added and incubated for 30 minutes at room temperature. The plates were washed five times, and 100ul of a substrate solution (TMB) was added into all wells and incubated for additional 30 minutes at RT in the dark. 100ul of 1.8 M H_2SO4 solution was used to stop the reaction. OD was measured at 450nm using an ELISA reader. A standard curve was plotted and IFNγ was quantified by interpolation from the standard curve. The results are expressed in pg/ml and the lowest limit of detection was 0 pg/ml.

2.9. Determination of plasma levels of TNF-alpha and TNF-R

2.9.1. TNF-alpha

Concentrations of TNF-α in the plasma of the study groups were measured using commercially available assays (Bender MedSystems, human TNF-α ELISA Version 2, Austria). In brief, microwell strips coated with monoclonal antibody (murine) to human TNF-α were washed twice with wash buffer (PBS with 1% Tween 20, 1: 20, PH 7.4). After adding 100µl sample diluent (protein matrix) in duplicate to standard wells, 100µl reconstituted TNF-α standard was pipetted into the first wells and serially titrated from 500 to 8pg/ml. Also, 50µl sample, in duplicate, was added to sample wells containing 50µl sample diluent. Then, 50µl of the diluted biotin-conjugate (1:100) was added to all wells and incubated at room temperature for two hours on a shaker set at 100rpm. After washing five times with wash buffer, diluted streptavidin-HRP solution (1:100) was pippetted to all wells. Following a one-hour incubation time at room temperature, TMB substrate solution was added to all wells. Then the microwell strips were incubated for about fifteen minutes at room temperature. The reaction was stopped by adding 100µl/well stop solution. And, the

O.D. values were determined using ELISA reader at 450nm and the results were expressed as the average of two readings. The levels of TNF-α were quantified with reference to standard curves generated using TNF-α standards. The results are expressed in pg/ml, and the lowest limit of detection was 0 pg/ml.

2.9.2. sTNF-R

Soluble TNF-R (80 kDa) was measured by ELISA using commercial kits (Bender MedSystems, sTNF-R (80 kDa), Austria), as previously done by Messele *et al.* (2001). Briefly, microwell strips coated with monoclonal antibody (murine) to human sTNF-R (80 kDa) were washed twice with wash buffer (PBS with 1% Tween 20, 1: 20, PH 7.4). 100µl assay buffer (PBS with 1% Tween 20, 1:20) in duplicate was added to all standard wells except the first wells. Then, 200µl (10ng/ml) sTNF-R (80 kDa) standard was pippetted into the first wells and standard dilutions ranging from 10ng/ml to 0.16ng/ml were created by serial dilution. To the sample wells containing 90µl assay buffer, 10µl sample in duplicate was added. Following this, 50µl of diluted HRP-conjugate (1:100) was added to all wells and incubated at room temperature for two hours on a shaker set at 100rpm. After washing three times with wash buffer, diluted TMB substrate solution (TMB in H_2O_2, 1:1) was added to all wells and incubated for about fifteen minutes at room temperature. Reaction was stopped by adding 100µl/well stop solution (1M phosphoric acid). The O.D. values were determined using ELISA reader at 450nm and the results were expressed as the average of two readings and quantified with reference to standard curves generated using standards. The results are expressed in ng/ml, and the lowest limit of detection was 0 ng/ml.

Statistical analysis

Data were analyzed using sigma plot version 4.01. Two-sample t test with equal variances and two-sample Wilcoxon rank-sum test were used to compare two different groups. Differences were considered significant when the P-value was less than 0.05. To evaluate an association between two parameters Spearman's rank correlation test were performed.

Ethical consideration

This study is performed as part of EHNRI-ENARP long-term cohort study on the progression of HIV infection, which was ethically approved by the National Ethical Committee (NEC) on 13/09/96 for Akaki and 21/01/98 for Wonji and a pilot study on HIV/TB infection at ALERT/Higher 23 Health centre approved by NEC to collect and use socio-demographic, behavioural, and medical information and to test for antibodies of HIV and other infectious diseases. Informed consent was obtained from each subject. Confidentiality of the data was preserved by using coded information.

3. Results

3.1. Study subjects' characteristics

Due to restricted selection of the study subjects (such as age-, sex-matched selection), the total number of individuals included in the study within the TB-HIV+ group as well as the healthy controls was less than to those of TB patients with/without HIV (Table 1). There was no significant difference in the mean age among the different groups, although the HIV-TB+ groups were relatively younger. The number of females and males in the healthy controls was the same, whereas there were more females in HIV-TB+ groups and more males in HIV-positive individuals with/without tuberculosis. Nevertheless, no statistically significant difference was noted with regard to the sex proportion among the different groups.

The median CD4+ count of the healthy controls (TB-HIV-) was found to be significantly higher than in the HIV-infected individuals with TB ($P< 0.001$) or without TB ($P=0.01$) (Fig. 2). According to Centre for Diseases Control (CDC) classification (MMWR, 1993), 33.3%, 50%, and 16.7% of HIV-infected individuals (HIV+) and 6%, 27%, and 67% of the TB/HIV dually infected patients were in class 1, 2, and 3 respectively. The TB/HIV co-infected individuals presented with more advanced stage of immunodeficiency as evidenced, in addition to clinical staging, by their mean CD4+ cell counts (121 cells/mm^3), which was also found to be significantly lower than either the TB+HIV- group or TB-HIV+ ($P<0.001$). Surprisingly, the TB+HIV- patients had also significantly lower CD4+ counts ($P=0.03$) than those reported apparently healthy controls. In addition, a considerable percentage of TB patients (TB+HIV-, 44%) had CD4+ counts below 500. But, 8% of the healthy controls had CD4 count below 500. In assessing the plasma viral load in HIV-infected individuals with/without TB, it was found that the dually infected patients had significantly higher

plasma viral load levels than those with HIV only (P=0.01). Viral loads ranged from 3.24 to 4.63 and 4.42 to 5.46 (log_{10} copies/ml) in HIV infected individuals without TB and with TB, respectively.

Table 2. Demographic and baseline characteristics of the study population

Characteristics	TB-HIV- (N=22)	TB+HIV- (N=29)	TB-HIV+ (N=19)	TB+HIV+ (N=31)
Mean Age (range)	39 (30-47)	32 (15-60)	39 (31-48)	36 (22-60)
Sex ratio, male: female	1:1	1:1.2	1.4:1	1.2:1
CD4 count (median cells/mm^3 , IQR)	692 (615-824)	573 (383-732)	429 (298-720)	121 (65-239)
Median Plasma viral load (log_{10}copies/ml)	NA	NA	4.04 (3.24-4.63)	4.76 (4.42-5.46)
TB type				
PTB (n, %)	NA	24	NA	24
EPTB (n, %)	NA	5	NA	7

Abbreviations: TB=tuberculosis; HIV= human immunodeficiency virus; IQR=interquartile range; NA= not applicable..

3.2. Proliferative responses

In each group, the magnitude of proliferative responses PBMCs was in the following order: medium alone < PPD-stimulated < PHA-stimulated. There was no difference in spontaneous proliferation when comparisons were made between any two groups. All healthy controls and TB patients without HIV, 95% of HIV-infected individuals without TB, and 83% of the TB+HIV+ responded to PPD, and it was found that the mean PPD-induced proliferative responses of PBMC from healthy controls (0.831) were higher in comparison to TB+HIV- (0.584), or HIV-infected individuals with TB (0.474), or without TB (0.402) (Fig. 3). Moreover, proliferative responses to PHA were significantly reduced in PBMCs from TB+HIV- group (P=0.013), TB-HIV+ group (P=0.002), and TB+HIV+ (P<0.001) as compared to the TB-HIV- group. Although proliferative responses to either PHA or PPD by PBMCs from HIV (with or without TB) tended to be lower than those with TB only, the differences were not statistically significant. Overall, it was noted that there was a trend towards progressive reduction of the proliferative responses to PHA in the following order TB-HIV- (1.762) > TB+HIV- (1.357) > TB-HIV+ (1.174) > TB+HIV+ (1.129). In any group, PPD-induced proliferative responses did not correlate with CD4+ counts.

3.3. IFN-gamma production

Evaluation of IFN-γ production revealed that 13.6% of TB-HIV-, 14.9% of TB+HIV-, 31.6% of TB-HIV+, and 23.3% of TB+HIV+ did not respond upon PPD stimulation. Intragroup comparative analysis of IFN-γ secretion response upon PHA, or PPD, or medium alone showed that the levels in culture supernatants from stimulated PBMC were increased in relation to the levels detected in supernatants from non-stimulated PBMC; and the highest

responses were for PHA-stimulated ones, regardless of the group. The mean IFN-γ level in culture supernatants in response to both PHA and PPD stimulation from TB+HIV- ($P < 0.001$, $P = 0.001$ to PHA and PPD, respectively), or TB-HIV+ (P=0.043, P=0.003), or TB+HIV+ (P=0.034, P=0.01) was significantly reduced when compared to TB-HIV- (Fig. 4). It was noted that no statistically significant difference in the level of PPD-driven IFN-γ between TB+HIV- and TB-HIV+ (P=0.132). Moreover, there was no significant difference in the levels of IFN-γ when comparisons were made between TB-HIV+ and TB+HIV+ (P=0.976, P=0.06 to PHA and PPD, respectively). Like the proliferative responses, no statistically significant correlation between CD4+ cell count and PPD-induced IFN-gamma production was noted, irrespective of the group.

3.4. IL-10 production

The number of study subjects examined for IL-10 production in each group was small (12 TB-HIV-, 9 TB+HIV-, 11 TB-HIV+, and 11 TB+HIV+) because of the reason mentioned in section 3.4. In supernatants collected from PHA-stimulated cultures, the level of IL-10 in the TB+HIV+ (30.63pg/ml) was significantly reduced as compared to the TB-HIV- (1055pg/ml; P<0.001), or TB+HIV- (549pg/ml; P= 0.009), but not with the TB- HIV+ group (167pg/ml; P=0.08) (Fig. 5). Furthermore, the healthy controls had a significantly elevated IL-10 level in comparison with the TB+HIV- (P=0.049), or TB-HIV+ (P<0.001). No statistically significant difference was noted in IL-10 level upon stimulation with PHA between TB-HIV+ and TB+HIV- groups (P=0.07). Moreover, PPD-induced IL-10 level in TB+HIV- group showed no statistically significant difference from either the TB-HIV+ group (P=0.279) or the TB+HIV+ group (P=0.14), but considerably reduced as compared with the healthy controls (P=0.026). It was noted that there was an increased trend in the mean level

of IL-10 in the following order: TB+HIV- < TB-HIV+ < TB+ HIV+. The HIV-infected individuals, irrespective of the TB status had no significant difference when compared to the healthy controls.

3.5. IL-12 production

Because of limitation as to the number of PBMCs available, we determined *in vitro* IL-12 production responses in supernatants of PBMCs only in 11 TB-HIV-, 7 TB+HIV-, 7 TB-HIV+, and 11 TB+HIV+ individuals. Following stimulation with PHA, the level of IL-12 in culture supernatants from TB-HIV- was significantly higher than from TB+HIV- group (P=0.01), but not with HIV infected individuals with TB (P=0.351), or without TB (P=0.191) (Fig. 6). Furthermore, no significant difference was noted when comparison was made between HIV-infected individuals and TB patients with HIV (P=0.510), or without HIV (P=0.065). However, the TB/HIV co-infected group had a significantly reduced response in comparison to TB+ HIV- (p=0.03). PPD stimulated cells from the TB+HIV+ secreted significantly reduced IL-12 in comparison with healthy controls (P=0.024), but not from the TB-HIV+ group (P=0.055), or from TB+HIV- (P=0.227). There was no statistical significant difference in PPD-induced IL-12 production between the TB-HIV+ group and TB+HIV- (P=0.06).

3.6. TNF-α levels in plasma

All patients and controls had detectable levels of TNF-α. The plasma level of the cytokine in TB+HIV+ (63.5pg/ml) was elevated, although not statistically significant, compared to the TB-HIV- group (29.76; P=0.081), or TB+HIV- (34.1; P=0.078) (Fig. 7). Furthermore, there

was no significant difference when comparisons were made between any two groups; TB+HIV+ *vs.* TB-HIV+ (P=0.601), or TB-HIV+ *vs.* TB+HIV- (P=0.710), or TB+HIV- *vs.* TB-HIV- (P=0.668), or TB-HIV- *vs.* TB-HIV+ (P=0.147). On the other hand, although not statistically significant, there was a positive correlation (but not linear) between the viral load (log_{10} copies/ml plasma) and the plasma TNF-alpha in TB+HIV+ group (r=0.27, P=0.16).

3.7. TNF-RII levels in plasma

In comparison with the TB-HIV- group, plasma concentrations of TNF-R were substantially increased in both HIV-infected groups (TB-HIV+, P=0.022 and TB+HIV+, P<0.001), but increased to a much smaller degree in those with TB only (P=0.087) (Fig. 7). Moreover, sTNF-R was significantly higher in the TB+HIV+ group compared with the TB+HIV-group (P<0.001), but not with the TB-HIV+ (P=0.185). There was also a higher sTNF-RII level in the TB-HIV+ group than the TB+HIV- group, although at the border of statistical significance (P=0.057). On the other hand, there was a linear positive correlation between viral load (log_{10}copies/ml plasma) and sTNF-R in the TB-HIV+ group (r=0.75, P=0.01), but not in the TB+HIV+ group (r=0.27, P=0.18).

3.8. Impact of three-months post anti-TB therapy

Assessment of the impact of three-months of anti-TB therapy by examining PPD-induced proliferative responses of PBMC from 12 TB patients (HIV-, 8 and HIV+, 4) showed a slight increase as compared to the baseline, which was not statistically significant, regardless of the HIV status (Fig. 9). The production of IFN-γ in response to PPD by PBMCs from TB

patients remained significantly reduced when compared to the healthy controls despite three-months of post anti-TB therapy. Moreover, no significant difference was noted in the levels of IFN-γ in TB patients before and after three-months of anti-TB therapy (Fig. 9). The elevated TNF-α and sTNF-R levels at the time of TB diagnosis appeared to be protracted (Fig. 10). On the other hand, we did not see any change in the CD4+ cell count as well as plasma viral load after three-months of anti TB therapy.

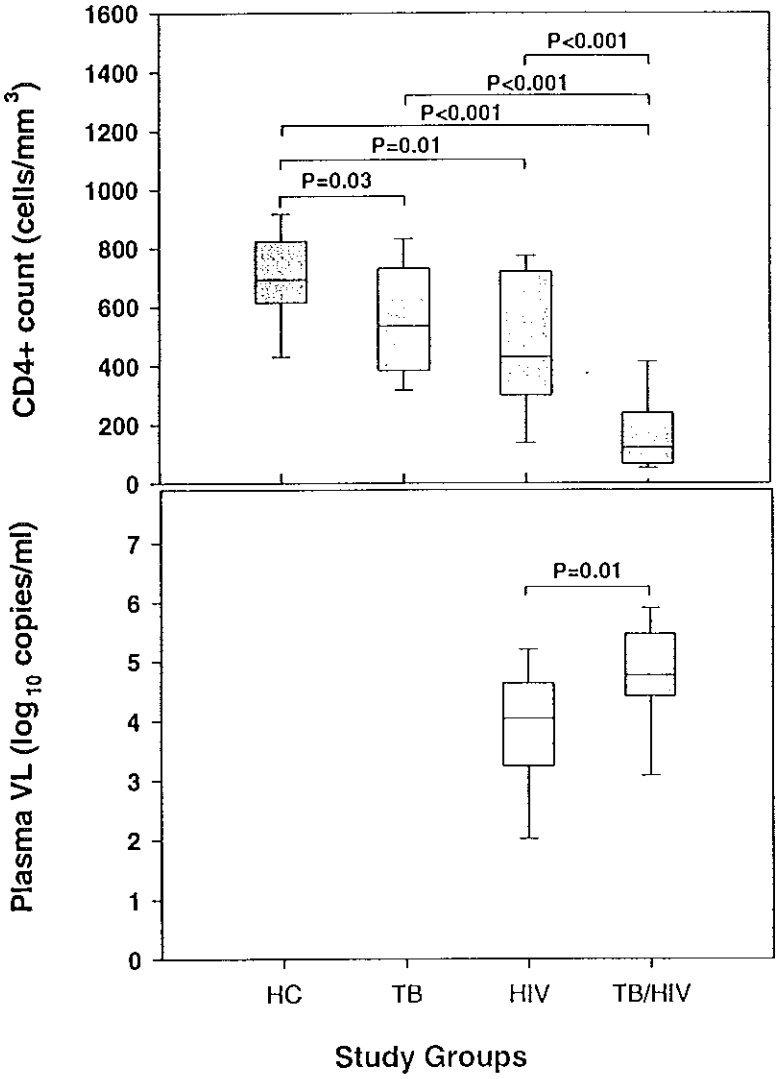

Figure 2. Absolute CD4+ counts (cells/mm^3) and plasma HIV-I load (log_{10} copies/ml). CD4+ counts were determined in 13 healthy controls (HC), 27 TB patients (TB), 18 HIV-infected individuals without TB (HIV), and 30 TB/HIV co-infected individuals (TB/HIV). Plasma viral load determination done in 17 dually infected individuals. Data are expressed as median values.

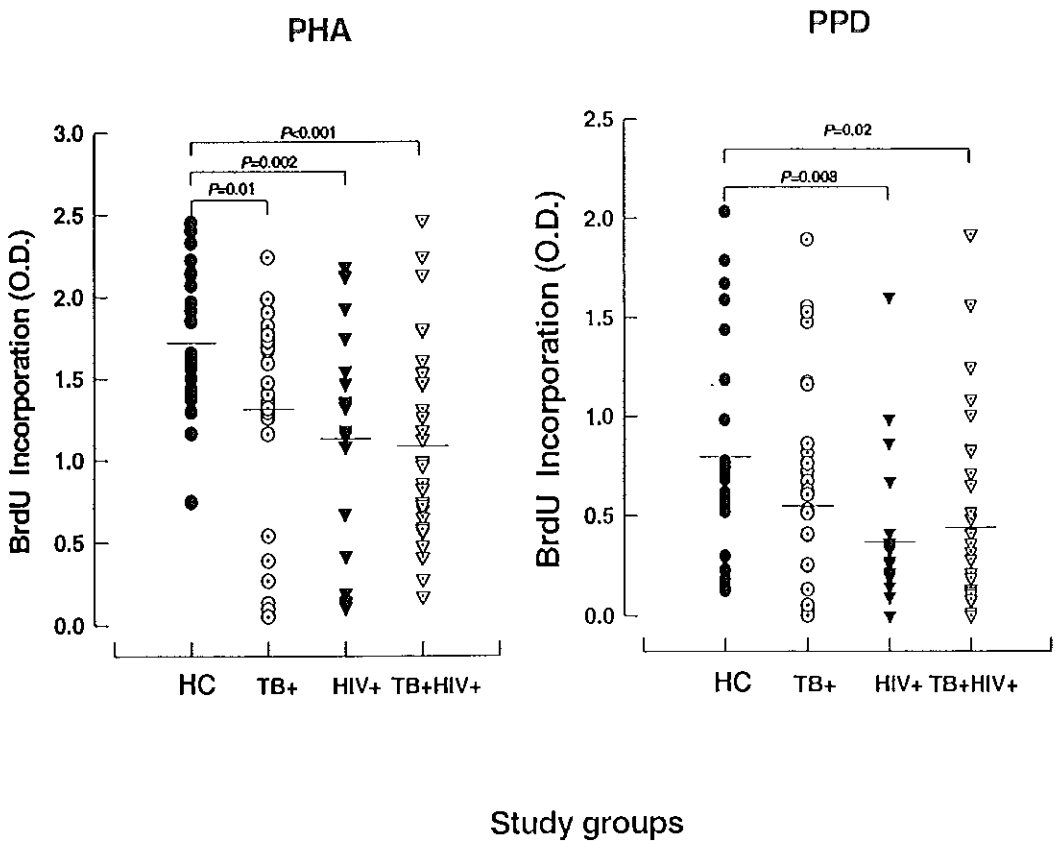

Figure 3. Proliferative responses of PBMCs to PHA and PPD in 22 healthy controls (HC), 29 TB patients (TB+), 19 HIV-infected individuals (HIV+), and 31 TB-HIV co-infected individuals (TB+HIV+). Data were normally distributed and presented as dot plots and horizontal lines are mean values.

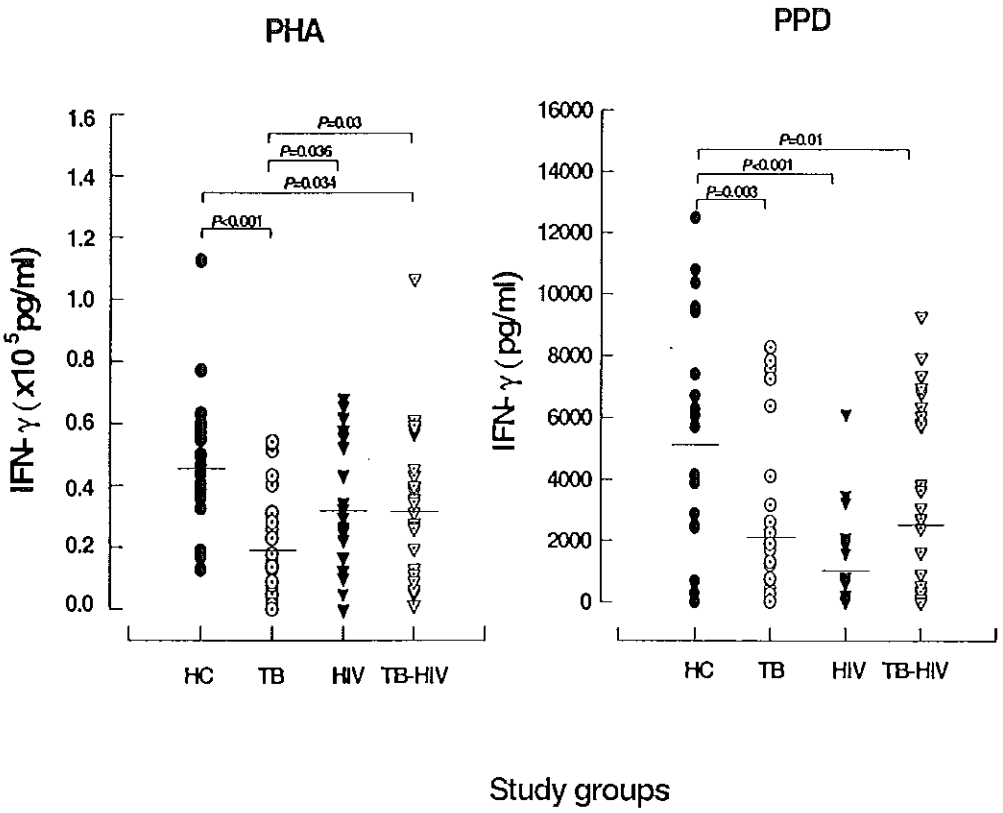

Figure 4. In vitro secretion of IFN-gamma (pg/ml) in response to PHA and PPD by PBMCs from 22 healthy controls (HC), 27 TB patients (TB+), 19 HIV-infected individuals (HIV+), and 31 TB-HIV co-infected individuals (TB+HIV+). (Data presentation are as in figure 3).

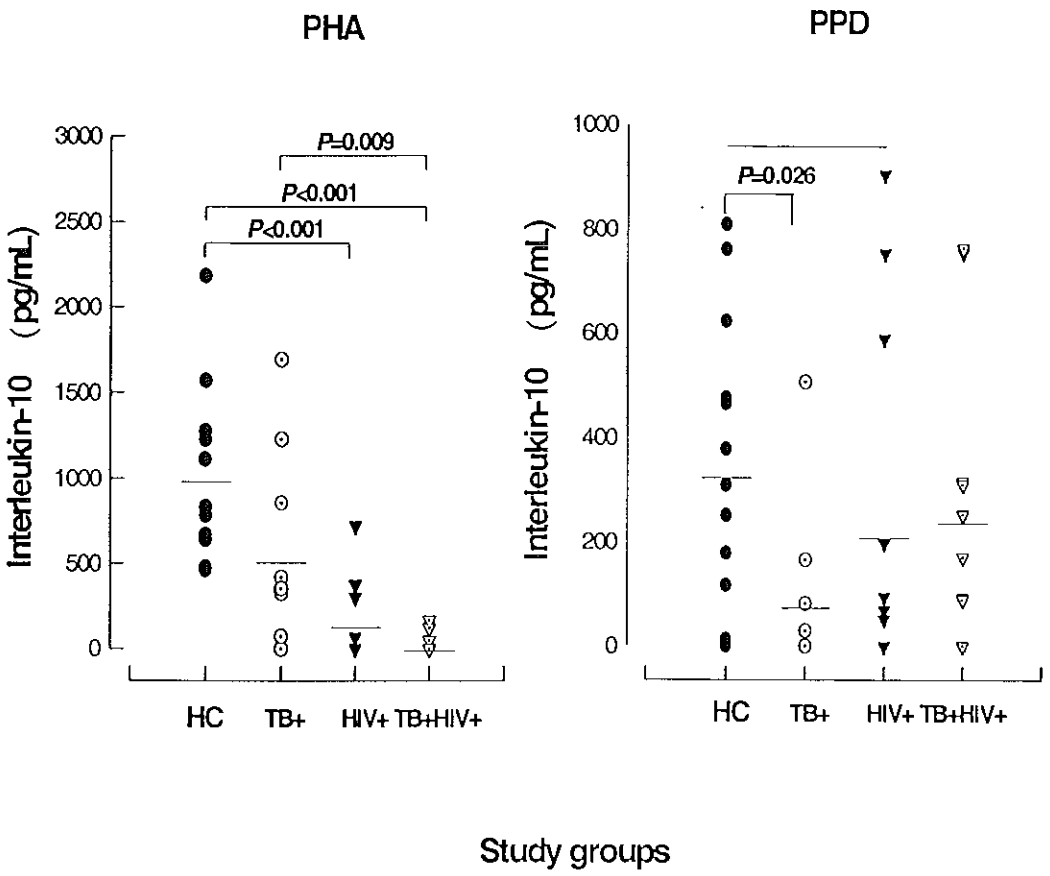

Figure 5. In vitro secretion of IL-10 (pg/ml) responses to PHA and PPD by PBMCs from 13 healthy controls (HC), 9 TB patients (TB+), 12 HIV-infected individuals (HIV+), and 11 TB-HIV co-infected individuals (TB+HIV+). (Data presentations are as in figure 3).

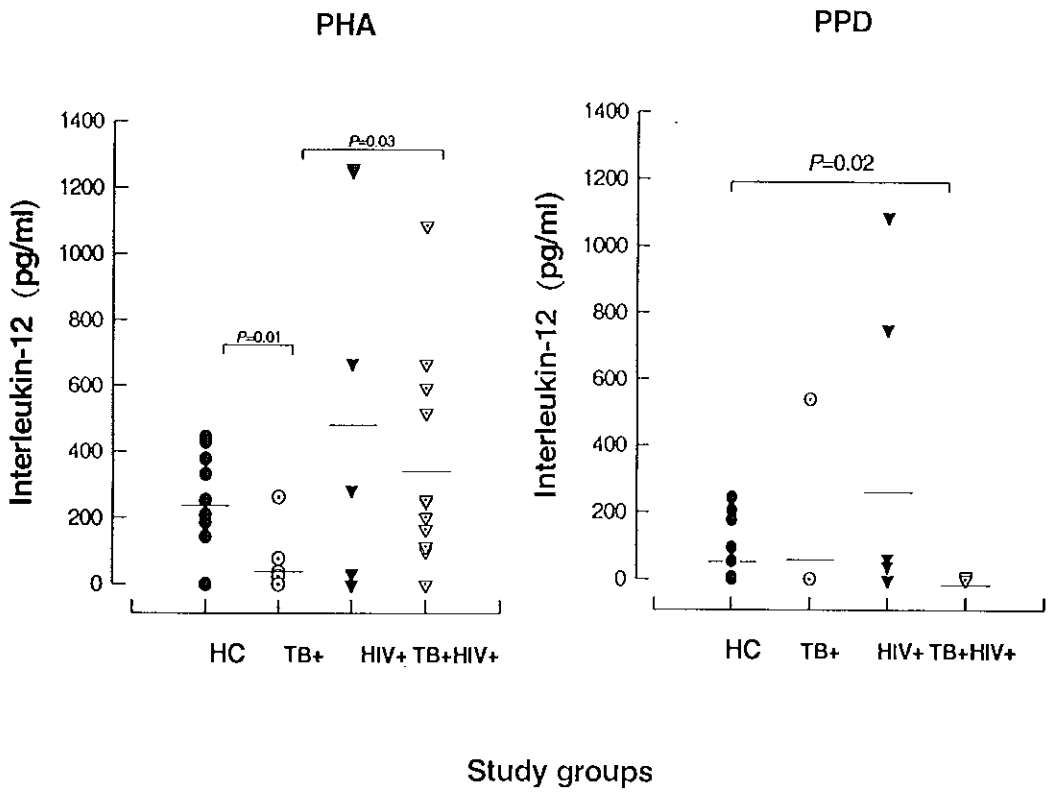

Figure 6. In vitro IL-12 secretion responses (pg/ml) to PHA and PPD by PBMCs from 11 healthy controls (HC), 7 TB patients (TB+), 7 HIV-infected individuals (HIV+), and 11 TB-HIVco-infected individuals (TB+HIV+). (Data presentations are as in figure 3).

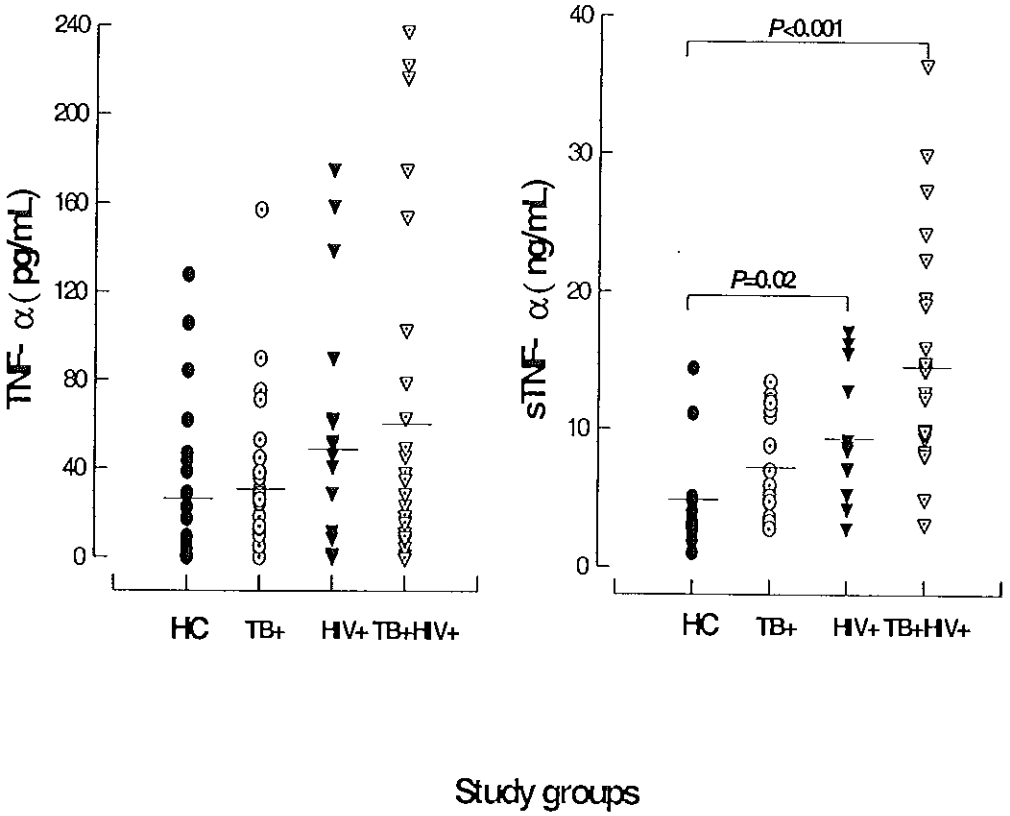

Figure 7. Plasma levels of TNF-alpha (pg/ml) and sTNF-RII (ng/ml) in 21 healthy controls (HC), 28 TB patients (TB+), 18 HIV-infected individuals (HIV+), and 29 TB-HIV co-infected individuals (TB+HIV+). (Data presentations are as in figure 3).

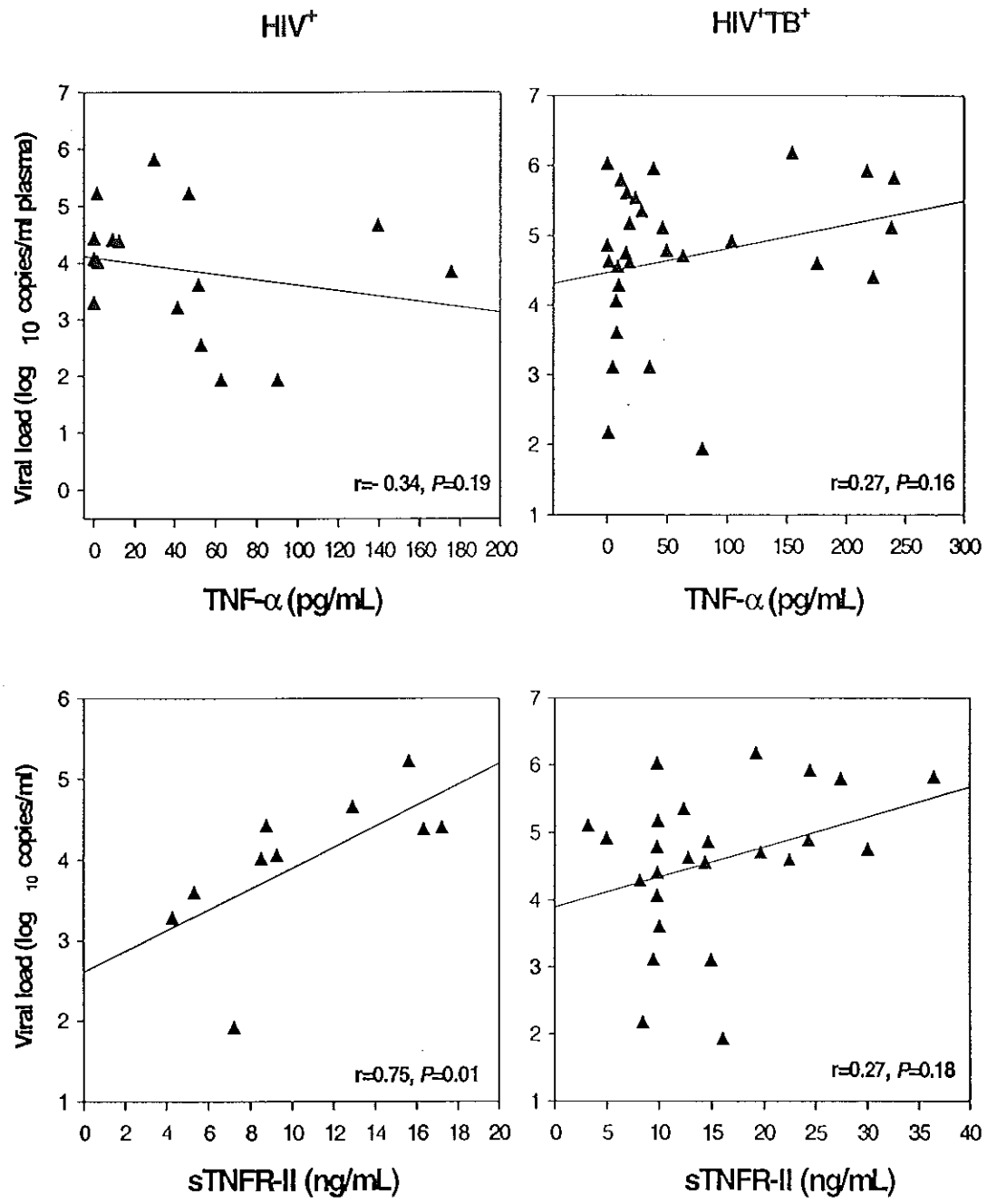

Figure 8. Correlation between plasma viral load (log10 copies/ml) and plasma levels of TNF-α (pg/ml) or sTNF-RII (ng/ml) in HIV-infected individuals with/without TB (HIV+ and TB+HIV+).

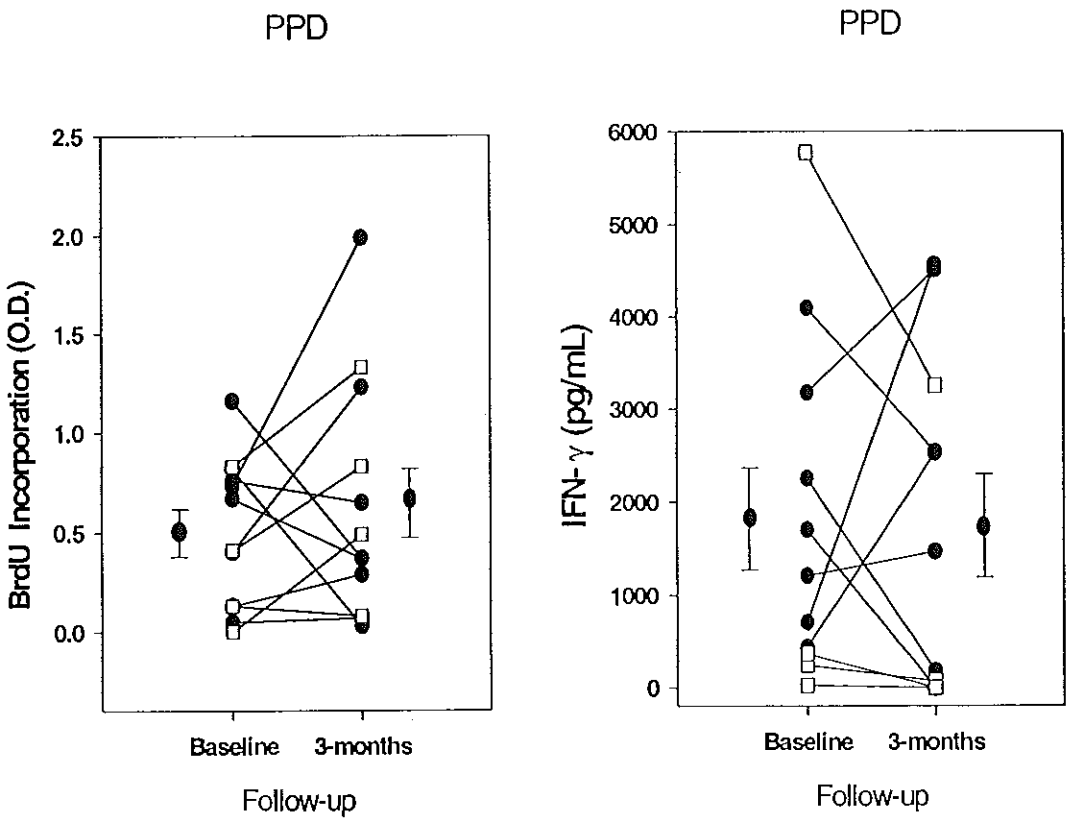

Figure 9. IFN-γ production and proliferative responses to PPD by PBMCs from 12 TB patients with HIV (□, n=4) and without HIV (•, n=8) before and after three-months of anti-TB therapy.

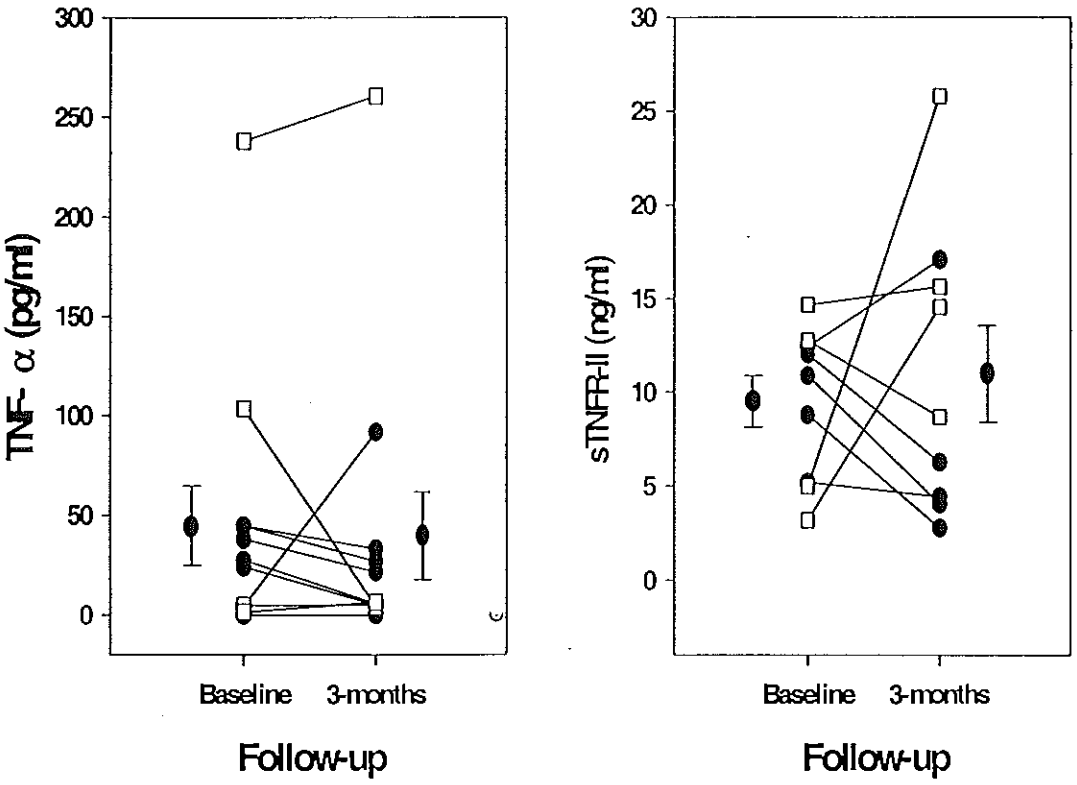

Figure 10. Plasma levels of TNF-α (pg/ml) and TNF-RII (ng/ml) in 12 TB patients with HIV (□, n = 4,) and without HIV (•, n = 8) before and after three-months of anti-TB therapy.

4. Discussion

With the advent of HIV/AIDS and multi-drug resistant strains of *M. tuberculosis*, TB has emerged as a disease of public health importance both in developed and developing countries (Narain *et al.*, 1992; Kochi *et al.*, 1993). The lack of availability of an effective protective vaccine has further aggravated the situation (Murray *et al.*, 1990). Elucidation of the immune response to TB may provide strategies for effective immunotherapeutic and prophylactic regimens (Ellner, 1997; Vanham *et al.*, 1997). Cell-mediated immunity, involving the interactions between *M. tuberculosis*-specific T cells and cells of the monocyte-macrophage lineage, is the major component of host defense against TB. Cytokines mediate the interactions between antigen-specific T cells and monocytes/macrophages (Barnes and Rom, 1994). The *in vitro* correlates of decreased cell-mediated immunity in active TB include lowered IFN-γ and IL-2 production and depressed blastogenesis by PBMCs after stimulation with *M. tuberculosis*, or with its products such as PPD (Barnes *et al.*, 1992; Zhang *et al.*, 1995a; Demissie *et al.*, 1999). Factors such as HIV infection (Silveira *et al.*, 1997; Legesse, 2000), helminths (Elias *et al.*, 2001), malnutrition (Mainali and McMurray, 1998), and genetic factors (Newport *et al.*, 1996; Goldfeld *et al.*, 1998) may alter the Th1 and Th2 cytokine balance in response to *M. tuberculosis*.

Taking into account the importance of evaluating *in vitro* T cell proliferation parameter as a measure of *in vitro* immune function, we employed BrdU-ELISA technique, which measures the incorporation of BrdU into newly synthesized DNA of proliferating T cells. It is a non-radioactive method previously shown to be applicable in the Ethiopian setting (Legesse, 2000; Messele *et al.*, 2000). The data provided additional evidence that active TB was associated with depressed *in vitro* PBMC proliferation upon stimulation with both PPD and PHA. Moreover, the response was lower in HIV-infected individuals than TB patients,

although not statistically significant. This, together with severely depressed proliferative response noted in TB/HIV co-infected patients, indicated that both TB and HIV could interfere proliferative responses, and highly pronounced when both TB and HIV occurred together. But, it seemed that HIV had a greater impact than TB in influencing blastogenesis responses of PBMCs. A decreased proliferative response to a mitogen might indicate disease progression in HIV infection (Clerici and Shearer, 1993) as well as TB (Dlugovitzky *et al.*, 1999). A number of possible mechanisms ensuing impairment of proliferative responses have been described. These include defects in *de novo* synthesis of nucleotides (Bofill *et al.*, 1999), production of cytokines such as IL-2 and expression of IL-2R (Hofmann *et al.*, 1989).

IFN-γ production was also profoundly affected in HIV-infected patients with/without TB, and there was no marked difference between TB patients and HIV-infected individuals, suggesting that alteration of IFN-γ production response is not specific for either HIV infection or TB. However, we noted a slight increase in IFN-γ production from TB/HIV co-infected individuals when compared with HIV patients without tuberculosis, in conformity with Heroghe and colleagues (2000). This might be ascribed to an activation of macrophages in attempt to control the *M. tuberculosis* infection, creating an increased expression of viral particles, which would exacerbate the IFN-γ response.

The present data also showed that the immuosuppression in TB was not only immediate but also long lasting. This was indicated by the finding that IFN-γ production as well as proliferative responses to PPD-stimulated culture supernatants from TB patients, regardless of the HIV status, remained depressed after three-months of anti-TB chemotherapy. In fact, Hirsch *et al.* (1999a) noted a similar observation even 1 year after successful completion of combination chemotherapy, despite significant improvement in patients' conditions.

However, TB patients treated with thalidomide had an improved proliferative response just after 42 days when compared to TB patients treated with anti TB drugs other than thalidomide (Bekker, *et al.*, 2000).

The severity of disease and low IL-2/IFN-γ production in TB patients appeared to correlate with CD4 lymphocytopenia (McDyer *et al.*, 1997). Moreover, PPD-induced proliferative responses or IFN-γ production was correlated with CD4+ cell count in HIV-positive individuals (Legesse, 2000). In the present study; however, there was a lack of correlation between CD4+ cell count and PPD-induced IFN-γ production or proliferative response in each group, suggesting that qualitative defects occur before quantitative change in CD4+ cells. On the other hand, the IFN-γ production was not correlated with proliferative responses, as observed by Surcel *et al.* (1994). This might suggest that cells producing IFN-γ do not necessarily proliferate.

The activities of down-regulatory cytokines such as IL-10 are expected to be involved in inhibiting IFN-γ production and proliferative responses. It has been shown that HIV-1-derived glycoproteins can induce the production of IL-10 by PBMCs *in vitro* (Schols and Clercq, 1996) and the serum level of IL-10 was found to be raised in these patients (Clerici *et al.*, 1994). On the other hand, Zhang *et al.* (1994) reported no enhancement of type 2 cytokines in HIV-infected patients even with concomitant TB.

In contrast, this study demonstrated that the level of IL-10 was reduced, although not statically significant, in supernatants of PBMC from HIV-infected individuals with/without concomitant TB. Moreover, TB patients showed a marked decrease in IL-10 production as compared to healthy controls. These observations, in general, suggested that TB as well as

HIV infection might be accounted to depletion of not only Th1 cells but also Th2 cells. This might be related to the generalized severe immunosuppression as evidenced by profoundly reduced CD4+ T cells in individuals with TB and/or HIV infection. In support of this, the level of IL-4 production was not affected by HIV infection (Legesse, 2000) or active TB (Zhang *et al.*, 1994). Moreover, Wolday *et al.* (1999) noted a depressed IL-10 level in HIV-infected individuals with visceral leishmania. Therefore, it seemed that other mechanisms, including other cross-regulatory cytokines might contribute to the depressed Th1 responses.

IL-12 has been reported to increase T cell proliferation, IFN-γ production, and increase natural cell activity and cytotoxic T lymphocyte (CTL) activity (Clerici *et al.*, 1993a). In this study, there was a markedly reduced IL-12 in TB/HIV co-infected individuals when compared to healthy controls, suggesting that the reduced proliferative response and IFN-γ production were probably associated with impaired IL-12 production. However, IL-12 level in the healthy controls was not significantly different from HIV-infected individuals or TB patients, but had depressed IFN-γ. Messele *et al.* (2001) also noted an elevated plasma level of IL-12 in HIV-infected individuals in comparison to healthy controls. Dysfunction of IL-12 might be attributed to defective expression of IL-12 receptors (Szabo *et al.*, 1997).

It has already been demonstrated that CD4+ cell count is an important independent predictor of disease progression in HIV infection (Schellekens *et al.*, 1995) and survival in HIV-related TB (Shafer *et al.*, 1996). In the present study, the CD4+ cell count was significantly reduced not only in HIV-infected individuals (with/without TB) but also in TB patients. In agreement with this, Beck *et al.* (1985) noted the occurrence of lymphocytopenia in the setting of non-HIV-related TB. In the present study, the two groups of HIV patients (with/without TB) had different immunological background, and the majority of persons in

each group are in different phases of HIV disease. The median CD4 cell count of the TB/HIV co-infected group was much lower than those in the other group. This is probably attributable to effects of both organisms: low CD4+ lymphocyte counts are associated with both increased plasma viral load and greater likelihood of systemic spread of mycobacteria (Gilkgs *et al.*, 1990). Although the precise mechanism of CD4 lymphocytopenia is unknown, a number of possible mechanisms have been described, such as apoptosis (Hirsch *et al.*, 1999a).

Recent research has significantly improved our understanding of apoptotic cell death and identified key molecules that induce and modulate this process. Overproduction of fasL and TNF-α provide direct apoptotic signals for activated T cells through interactions with their receptors, Fas and TNF-RI/II (Boise and Thompson, 1996; Fraser and Evan, 1996). It is possible that as part of the general immune activation during TB and/or HIV infection, in addition to up-regulation of expression of surface molecules such as HLA-DR (Vanham *et al.*, 1996), there is an up-regulation in expression of Fas, FasL, and TNF-RI/II. In support of this, plasma concentrations of TNF-α as well as its receptor, TNF-RII, were increased in TB and/or HIV-infected individuals. It has been demonstrated that TNF-α can induce apoptosis in activated murine T cells (Zheng *et al.*, 1995). Therefore, TNF-α might contribute to the depletion of CD4+ cells in TB as well as HIV-infected individuals via apoptosis.

Immune activation markers, including β_2-microglobulin, neopterin, soluble IL-2 receptor and sTNF-RII, have been shown to have prognostic significance (Messele *et al.*, 2001; Lawn *et al.*, 2000). The present study demonstrated that a markedly elevated sTNF-RII was seen in persons with HIV infection. In addition, TB patients had a higher sTNF-RII, although not significant, than the healthy controls. Both TB and HIV contributed to the greatly elevated

levels of sTNF-RII in the co-infected group. This fact, together with the finding of no significant correlation between plasma levels of TNF-RII and viral load in the TB/HIV co-infected group, suggested that TNF-RII showed no disease specificity. In support of this, the levels of sTNF-RII were elevated at the time of diagnosis of TB (Hirsch *et al.*, 1999b), and in AIDS patients when compared to HIV-positive individuals (Messele *et al.*, 2001). Moreover, Lawn *et al.* (2000) noted a significant elevation of sTNF-RII in TB and/or HIV infections.

Regardless of its lack of specificity, sTNF-RII might be a useful prognostic marker of HIV infection before the onset of opportunistic infections, as evidenced by its correlation with viral load only in HIV-infected individuals. In this regard, Lederman *et al.* (2000) noted that sTNF-RII was the marker of immune activation most independently correlated with HIV viral load. Moreover, sTNF-RII was found to be a useful indicator of progression to AIDS (Godfried *et al.*, 1994). On the other hand, the level of sTNF-RII in HIV-positive individuals was relatively higher than TB patients, which could be related to the role played by TNF-RII in the control of HIV replication and death of CD8 cells (Herbein and Gordon, 1997; Screaton and Xu, 2000).

It has been postulated that increased concentration of sTNF-RII could be related to its role in binding, transportation, stabilization and prolongation of the bioactivity of TNF-α. Thus, in the present data showed that there was an association between TNF-α and sTNF-RII in that both TNF-α and TNF-RII followed a similar pattern, highest in TB/HIV co-infected and lowest in healthy controls. However, there was no linear positive correlation between TNF-α and TNF-RII in any group.

TB was associated with enhanced HIV-1 replication with a significant increase in plasma viral load in TB/HIV co-infected individuals in comparison with HIV-infected individuals without TB, in agreement with observation made by Goletti *et al.* (1996). A higher viral load is associated with a more rapid progression of HIV-1 disease (Schellekens *et al.*, 1995). Thus, this study demonstrated that TB might act as a cofactor that accelerates the decline in immune function and shortens survival in HIV-infected persons. And, the impact of TB seemed to be protracted for there was no change in viral load after three months of anti-TB therapy.

One way by which *M. tuberculosis* modulates HIV replication is by stimulating the release of cytokines, such as IL-1, TNF-α, and IL-6 (Zhang *et al.*, 1995h). Garrait *et al.* (1997) also noted that the cytokine profile of the tuberculous microenvironment is conducive to HIV-1 replication. The present study revealed that the plasma levels of TNF-α in TB and/or HIV-infected individuals were higher than healthy controls; and the TB/HIV co-infected individuals had the highest TNF-α.

The increased TNF-α production might contribute to the immunopathogenesis associated with TB (Beutler and Cerami, 1987) and promoting the replication and dissemination of the bacilli (Imperiali *et al.*, 2001). On the other hand, although data from *in vitro* studies suggest that TNF-α plays an important role in the up regulation of HIV replication (Lederman *et al.*, 1991; Zhang *et al.*, 1995b), the role of TNF-α in HIV pathogenesis *in vitro* is not clear (Li *et al.*, 1997). But, there was no significant correlation between plasma viral load and TNF-α in this study. However, the failure of HIV plasma load to decrease was clearly associated with high, sustained systemic levels of TNF-α after three-months of anti-TB therapy. The fact that TNF-α has a very short half-life in the circulation (Beutler and Cerami, 1987) suggests that

there was ongoing systemic production in TB patients despite decrease in mycobacterial load. In part, this might be due to the fact that HIV infection itself induces elevated systemic levels of TNF-α as seen in HIV-infected individuals without TB. Although it seems that the increase in TNF-α levels in TB and/or HIV infection was attributed to TB and/or HIV infections, there may be a cytokine dysregulation in individuals with TB and/or HIV infection (Lawn *et al.*, 1999).

More recent data from ENARP laboratory (Wolday *et al*, Unpublished data) indicate that the cellular immune activation marker, HLA-DR and chemokine receptor expression of CCR5 and CXCR4 on CD4+ T-cells from TB/HIV co-infected individuals is significantly elevated than in those with TB only, and the levels of these coreceptors remains elevated during successful treatment of TB may suggest that both soluble and cellular immune activation coupled with chemokine co-receptors might be responsible for the observed sustained levels of plasma viremia.

In summary, the present study demonstrated that TB and HIV infection display partly similar and partly distinct immune alterations. And also, HIV and TB have additive effects in TB/ HIV co-infection. Both lead to immunosuppression, which are expressed in qualitative and quantitative changes, contribute to the synergistic interaction between them. In addition to immunosuppression, this study confirms previous findings that both TB and HIV infection are associated with immune activation as evidenced by the rise in plasma levels of TNF-α and sTNF-RII.

TB and/or HIV infections are associated with depletion of CD4 cells, which are not accompanied with qualitative defects. The depressed IL-10 level may provide evidence that

TB as well as HIV is associated with the depletion of not only Th1 cells but also Th2 cells from the peripheral blood. But, this needs further investigation.

On the other hand, association of IL-12 with Th1 responses was noted only in TB/HIV co-infected individuals. Therefore, future studies are needed to delineate mechanisms accompanied with reduced Th1 responses observed in TB patients or HIV infected individuals despite the production of comparable IL-12. In this respect, factors such as genetic defects in the expression of IL-12 receptor genes might need due consideration.

In Ethiopia, where CD4 count and viral load can often not be assessed, serological markers of immune activation could represent an affordable alternative to monitor disease progression and to assist in the management of patients. Therefore, future studies are required to examine other markers, which may serve a useful role in discriminating HIV infection or tuberculosis in co-infection.

Although previous data suggested that treatment of TB has a rapid beneficial effect in reducing HIV-1 plasma load (Goletti *et al.*, 1996), this is not clearly the case in the present study. Therefore, there is a need for improved TB prevention including the use of prophylactic drugs in HIV-infected subjects. Moreover, therapeutie strategies that enhance Th1 responses and inhibit the activity of TNF-α may be useful.

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