

HIV Alters Plasma and *M. tuberculosis*-induced Cytokine Production in Patients with Tuberculosis

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ABSTRACT

To test the hypothesis that HIV infection brings about an alteration in the immune response to tuberculosis (TB), mycobacterial antigen-induced production and plasma levels of the inflammatory cytokine interferon- γ (IFN- γ) and its regulatory cytokines interleukin-12 (IL-12), IL-18, and IL-10 were determined in patients infected dually with HIV and TB and compared with individuals with either disease and with healthy controls. Peripheral blood mononuclear cells (PBMCs) of TB patients with HIV infection produced lesser amounts of IFN- γ and IL-12 compared with TB patients without HIV infection after *in vitro* stimulation with mycobacterial antigens. There was no difference in antigen-induced IL-18 production in TB patients with or without HIV infection. The *in vivo* cytokine pattern did not correlate with that seen *in vitro*. Higher levels of IFN- γ , IL-12, and IL-18 were detected in the plasma of TB patients infected with HIV compared with TB patients without HIV infection. The presence of significantly higher plasma levels of proinflammatory cytokines suggests a greater degree of immune activation in individuals with HIV and TB, particularly those with low CD4 counts. *In vitro* IL-10 production by HIV-positive TB patients was similar to that of the HIV-negative TB group and higher than in HIV-positive individuals without TB, but the plasma levels were similar. HIV infection downregulates the *in vitro* Th1 cytokine response to TB and simultaneously increases systemic levels of these cytokines.

INTRODUCTION

TUBERCULOSIS (TB) CONTINUES TO BE the most prevalent cause of death from an infectious agent globally, and its interaction with HIV is having devastating effects.⁽¹⁾ HIV infection has emerged as by far the most important of all the predisposing factors for the development of TB⁽²⁾ (primary or reactivation),^(3,4) and TB is often the sentinel illness of HIV infection.⁽⁵⁾ TB causes worsening of HIV disease status, increases viral replication, decreases immunity, and hastens the onset of AIDS.⁽⁶⁾

The pattern of cytokines produced by T lymphocytes plays a central role in the susceptibility to TB. Activation of Th1 lymphocytes, interferon- γ (IFN- γ) production, and macrophage activation are crucial in the defense against mycobacteria.⁽⁷⁾ On the other hand, Th2 cell activation and increased interleukin-4 (IL-4) production have been associated with poor clinical outcome after treatment.⁽⁸⁾ HIV infection is associated with a profound deregulation of the immune system and alterations in the cytokine profile.⁽⁹⁾ The present study

was carried out to test the hypothesis that HIV infection brings about an alteration in the host immune response to TB. To this end, *in vivo* levels of the proinflammatory cytokine IFN- γ and its regulatory cytokines (IL-12, IL-18, and IL-10) in plasma, as well as mycobacterial antigen-induced production of these cytokines by peripheral blood mononuclear cells (PBMCs) *in vitro*, were investigated in TB patients with and without HIV infection.

MATERIALS AND METHODS

Study population

Patients suspected of having HIV infection or TB were investigated at the Tuberculosis Research Centre, Chennai. The diagnosis of TB was based on sputum smear examination for acid-fast bacilli and confirmed by culture for *Mycobacterium tuberculosis*. HIV infection was diagnosed using two rapid tests (HIV Tridot, J. Mitra, New Delhi, India, and CombAids, Span

Diagnostics, Surat, India), and a positive result was confirmed by an ELISA test (Enzaids, Span Diagnostics).

The study population comprised four groups of individuals: (1) 29 HIV-seropositive patients with active TB (22 males and 7 females, age range 21–52 years, mean age 35 years), (2) 21 HIV-seronegative patients with TB (15 males and 6 females, age range 18–70 years, mean age 42 years), (3) 26 HIV-seropositive patients without active TB (19 males and 7 females, age range 19–50 years, mean age 33 years), and (4) 22 healthy controls (11 males and 11 females, age range 21–48 years, mean age 32 years) who were laboratory workers in the Tuberculosis Research Centre.

All HIV-positive patients tested positive for HIV-1, and 3 also had HIV-2 co-infection. Among the TB patients, 1 had pleural effusion, and all the rest had sputum-positive pulmonary TB. The group comprising HIV patients without TB was highly diverse. The majority of the individuals in this group had other opportunistic infections, such as pneumonia, diarrhea, or central nervous system (CNS) infections, and were being treated as inpatients in the Government Hospital for Thoracic Medicine, Tambaram, Chennai at the time of the study. One person had hepatitis B co-infection, 1 was being treated for sexually transmitted disease, and 4 were asymptomatic. The most common risk factor for HIV infection in the study population was heterosexual promiscuity. None of the patients were receiving antituberculosis or antiretroviral therapy at the time of intake into the study.

Cytokine assay

PBMCs were obtained by density gradient centrifugation on Ficoll diatrizoate. The cells were washed with RPMI 1640 medium (Bio-Whittaker, Walkersville, MD), and their viability count was ascertained by the trypan blue dye exclusion method. PBMCs were cultured in 24-well tissue culture plates (Falcon, Lincoln Park, NJ) at a concentration of 2×10^6 cells/well in a volume of 1 ml RPMI 1640 medium supplemented with 1 mM L-glutamine, 80 $\mu\text{g/ml}$ gentamicin, 25 mM HEPES, and 10% fetal bovine serum (FBS). The cells were stimulated *in vitro* with two mycobacterial antigens and a mitogen. The antigens used were purified protein derivative (PPD) (Conaught, Willowdale, Ontario, Canada) and heat-killed *M. tuberculosis* H37Rv (Hk-M.tb.) (prepared in our laboratory). The mitogen used was phorbol myristate acetate (PMA) (Calbiochem, San Diego, CA) in combination with ionomycin (Calbiochem). PPD was used at a concentration of 10 $\mu\text{g/ml}$, Hk-M.tb. at 10 $\mu\text{g/ml}$, PMA at 50 ng/ml, and ionomycin at 1 $\mu\text{g/ml}$. The cell culture supernatants were collected after 24 h for estimation of IL-10, IL-12, and IL-18 and after 72 h for estimation of IFN- γ . Concentrations of the antigens and mitogen used and the duration of culture were found previously to give optimal cytokine responses in our laboratory. The cell culture supernatants were stored at -70°C until use. Cytokines were measured by capture ELISA using kits purchased from Immunotech (Coulter, Hialeah, FL). To rule out technical error, about 10% of all the samples were run in duplicate, and the paired results were compared for concurrence.

Immunophenotyping

The monoclonal antibodies (mAbs) used for immunophenotyping were anti-CD14, anti-CD45, anti-CD3, anti-CD4, and

anti-CD8. Irrelevant isotope-matched antibodies (mouse IgG1 and mouse IgG2a) were included as controls for nonspecific immunofluorescence. All the mAbs were directly conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and were purchased from Becton Dickinson Immunocytometry (Mountain View, CA).

Lymphocyte subpopulations were analyzed in whole blood by dual-color flow cytometry using the standard staining protocol. The cells were analyzed on a FACSort flow cytometer (Becton Dickinson) using CellQuest software. Data were collected per 10,000 cells for each tube and analyzed as dot-plots.

Statistical analysis

All results are expressed as mean \pm SE. Wherever necessary, log transformation was used to stabilize variance. Comparison of the means between any two groups was performed using unpaired *t*-test. The four groups were compared simultaneously using analysis of variance (ANOVA). Tukey's multiple comparison test was also used to classify the groups. The significance was fixed at the 5% level.

RESULTS

Routine hematologic and biochemical tests as well CD4⁺ cell counts were performed for all study subjects. HIV-infected patients had significantly lower CD4⁺ cell counts than HIV-negative patients (284 ± 56 cells/ μl vs. 758 ± 53 cells/ μl , $p < 0.001$) (Table 1). Based on the CD4 counts, both groups of HIV patients (categories 1 and 3) were further divided into two subgroups, those having CD4 counts >200 cells/ μl and those with CD4 counts <200 cells/ μl . Each of these subgroups had more or less equal numbers of patients. Thus, there were six groups (I, II, III, IV, V, VI).

IFN- γ in plasma and in supernatants of mycobacterial antigen-stimulated PBMCs

Antigen-induced IFN- γ production was measured in the supernatants of PBMCs cultured with PPD or Hk-M.tb. for 72 h. Mean IFN- γ levels in mycobacterial antigen-stimulated cell cultures for TB patients were 2-fold higher than the corresponding values for healthy controls (Table 1). A trend toward a decline in IFN- γ production was observed in patients co-infected with HIV. Very small amounts of IFN- γ were measured in culture supernatants of HIV patients without active TB. Although plasma IFN- γ levels were higher in patients with either TB or HIV than in healthy controls, this increase did not reach statistical significance. Among HIV patients with advanced disease, low CD4 counts (<200 cells/ μl), and active TB, there was a significant increase in the amount of circulating IFN- γ ($p < 0.05$). On the other hand, in HIV patients with moderate CD4 counts (200–500 cells/ μl) and active TB, circulating IFN- γ levels were not elevated to the same extent.

IL-12 in plasma and in supernatants of *in vitro* stimulated PBMCs

PBMCs of TB patients produced significantly higher amounts of IL-12 in response to *in vitro* stimulation with PPD

TABLE 1. IFN- γ LEVELS IN PLASMA AND CULTURE SUPERNATANTS OF *IN VITRO* STIMULATED PBMCs

Group	Subjects	CD4 counts (cells/ μ l)	PPD-stimulated PBMC (pg/ml)	Hk-M.tb.- stimulated PBMC (pg/ml)	Plasma (pg/ml)
I (n = 14)	HIV + TB + (CD4 > 200)	284 \pm 20 ^a	280.8 \pm 124.0	333.2 \pm 128.8	31.0 \pm 10.1
II (n = 15)	HIV + TB + (CD4 < 200)	89 \pm 16	409.4 \pm 275.0	170.5 \pm 87.3	91.6 \pm 25.1*
III (n = 21)	TB +	609 \pm 56	730.2 \pm 184.5	799.1 \pm 438.5	23.6 \pm 5.1
IV (n = 14)	HIV + TB - (CD4 > 200)	432 \pm 76	1.8 \pm 0.4	15.2 \pm 6.8	36.0 \pm 32.2
V (n = 12)	HIV + TB - (CD4 < 200)	63 \pm 10	4.1 \pm 2.0	5.4 \pm 2.4	39.7 \pm 8.2
VI (n = 22)	Healthy controls	758 \pm 53	323.3 \pm 130.4	279.5 \pm 135.7	7.5 \pm 1.5

^aMean \pm SE.

* p < 0.05 compared with all other groups.

compared with healthy controls (p < 0.005) (Table 2). HIV co-infection in TB patients resulted in a significant decrease in IL-12 production in response to PPD (p < 0.005). Though Hk-M.tb.-induced IL-12 production was also lower in HIV-positive TB patients compared with TB patients without HIV infection, the difference between the groups was not statistically significant. PBMCs of HIV-positive individuals without TB produced very low amounts of IL-12 in response to mycobacterial antigens *in vitro*. Plasma IL-12p70 levels did not differ significantly in any of the study groups. However, a trend toward an increase in circulating IL-12 levels was observed in HIV-infected TB patients, particularly the group with low CD4 counts, compared with those with TB alone.

IL-18 in plasma and in supernatants of *in vitro* stimulated PBMCs

No significant difference was observed in IL-18 production in PPD and Hk-M.tb.-stimulated cultures of PBMCs from

healthy controls and from patients (Table 3). Mean IL-18 concentrations in cell culture supernatants of TB patients were similar irrespective of the presence or absence of HIV infection. PBMCs of HIV-positive individuals without TB produced very low amounts of IL-18. Plasma IL-18 levels were significantly higher in TB patients than in healthy controls (p < 0.001). Patients with both HIV and TB (irrespective of CD4 counts) had significantly higher IL-18 levels than the other four groups (p < 0.01).

IL-10 in plasma and in supernatants of *in vitro* stimulated PBMCs

PPD-induced IL-10 production was highly variable and not significantly different in any of the study groups. However, Hk-M.tb. induced significantly higher production of IL-10 in individuals with TB compared with healthy controls (p < 0.001) (Table 4). Both among HIV patients with moderate CD4 counts (200–500 cells/ μ l) and those with low CD4 counts (<200

TABLE 2. IL-2 LEVELS IN PLASMA AND CULTURE SUPERNATANTS OF *IN VITRO* STIMULATED PBMCs

Group	Subjects	PPD-stimulated PBMC (pg/ml)	Hk-M.tb.- stimulated PBMC (pg/ml)	Plasma (pg/ml)
I	HIV + TB + (CD4 > 200)	9.1 \pm 2.9 ^{a,*}	17.0 \pm 5.3	15.4 \pm 4.2
II	HIV + TB + (CD4 < 200)	4.7 \pm 1.4*	13.6 \pm 4.4	41.1 \pm 14.5
III	TB +	16.9 \pm 3.0**	39.2 \pm 15.0	9.2 \pm 2.1
IV	HIV + TB - (CD4 > 200)	5.9 \pm 2.2	17.6 \pm 1.5	5.2 \pm 1.9
V	HIV + TB - (CD4 < 200)	13.6 \pm 1.2	3.5 \pm 0.1	17.8 \pm 3.6
VI	Healthy controls	3.6 \pm 0.7	30.3 \pm 13.0	8.0 \pm 1.8

^aMean \pm SE.

* p < 0.005 compared with group III.

** p < 0.005 compared with group VI.

TABLE 3. IL-18 LEVELS IN PLASMA AND CULTURE SUPERNATANTS OF *IN VITRO* STIMULATED PBMCs

Group	Subjects	PPD-stimulated PBMC (pg/ml)	Hk-M.tb.- stimulated PBMC (pg/ml)	Plasma (pg/ml)
I	HIV + TB + (CD4 > 200)	138.3 ± 92.7 ^a	204.4 ± 124.5	1191.6 ± 139.1*
II	HIV + TB + (CD4 < 200)	78.3 ± 55.5	106.4 ± 20.9	1167.8 ± 150.5*
III	TB +	87.5 ± 26.2	236.6 ± 49.3	767.8 ± 240.0*****
IV	HIV + TB – (CD4 > 200)	5.5 ± 5.1	5.5 ± 2.9	635.5 ± 79.7*****
V	HIV + TB – (CD4 < 200)	11.2 ± 0.1	12.5 ± 4.7	790.6 ± 64.5*****
VI	Healthy controls	92.2 ± 25.2	99.1 ± 31.3	333.7 ± 63.5*****

^aMean ± SE.

**p* < 0.01 compared with group III.

***p* < 0.05 compared with group I.

****p* < 0.05 compared with group II.

cells/μl), patients with TB produced significantly higher amounts of IL-10 in response to Hk-M.tb. compared with those without active TB (*p* < 0.05). No statistically significant differences were observed in plasma IL-10 levels in any of the study groups.

DISCUSSION

To our knowledge, this is the first study to simultaneously evaluate both mycobacterial antigen-induced cytokine production by PBMCs *in vitro* and their plasma concentrations in patients with both HIV and TB. Many investigators have evaluated cytokine levels either in plasma or in culture supernatants of PBMCs obtained from patients with HIV or TB,^(10–12) but only a few have measured IFN-γ levels in patients with dual infection.^(13,14) We found that in TB patients with HIV infec-

tion, *in vitro* IFN-γ production in response to mycobacterial antigens was variable but tended to be lower than in TB patients without HIV infection. Elliott *et al.*⁽¹⁴⁾ also reported profoundly impaired IFN-γ responses to mycobacterial antigens in HIV patients with TB. Depression of *in vitro* immune responses in patients with dual infection could be the result of either the loss of antigen-specific cells or their reduced response to stimulation with mycobacterial antigens. In contrast, plasma levels of IFN-γ were significantly higher in HIV-infected TB patients with low CD4 counts (<200 cells/μl) than in HIV-negative TB patients as well as in HIV-positive TB patients with higher CD4⁺ cell counts, indicating that immune activation is maximal in patients with advanced HIV infection and TB.

These observations indicate that plasma cytokine levels do not reflect *M. tuberculosis*-induced cytokine production by PBMCs, and *vice versa*. A similar finding has been reported recently in TB patients by Vankayalapati *et al.*,⁽¹⁵⁾ suggesting that

TABLE 4. IL-10 LEVELS IN PLASMA AND CULTURE SUPERNATANTS OF *IN VITRO* STIMULATED PBMCs

Group	Subjects	PPD-stimulated PBMC (pg/ml)	Hk-M.tb.- stimulated PBMC (pg/ml)	Plasma (pg/ml)
I	HIV + TB + (CD4 > 200)	446.7 ± 112.7 ^a	4525.2 ± 482.9*	6.3 ± 2.0
II	HIV + TB + (CD4 < 200)	378.1 ± 281.4	3028.9 ± 500.6**	17.8 ± 3.9
III	TB +	393.7 ± 165.2	3792.1 ± 319.3***	7.8 ± 3.3
IV	HIV + TB – (CD4 > 200)	140.0 ± 50.6	1258.0 ± 513.6	24.0 ± 5.2
V	HIV + TB – (CD4 < 200)	53.3 ± 42.8	741.7 ± 234.6	16.7 ± 5.3
VI	Healthy controls	225.8 ± 84.1	1683.1 ± 351.3	24.8 ± 2.9

^aMean ± SE.

**p* < 0.05 compared with group IV.

***p* < 0.05 compared with group V.

****p* < 0.05 compared with group VI.

serum cytokine levels do not parallel *M. tuberculosis*-induced cytokine production by PBMCs. It has been demonstrated that T cells and macrophages at the site of disease in TB have the capacity to produce IFN- γ , and increased local vascular permeability is likely to favor diffusion of cytokines into the bloodstream.⁽¹⁵⁾ It is, therefore, speculated that elevated plasma levels of IFN- γ and its regulatory cytokines in patients with HIV and TB are caused by their leakage from tissues into the circulation.

Production of IFN- γ by T cells depends on the synergistic effects of the monokines IL-12 and IL-18, both of which are believed to be necessary for full expression of Th1 responses.^(16–18) IL-12 is produced by various cell types, such as phagocytic cells, dendritic cells (DC), and B lymphocytes, and induces the production of IFN- γ from T cells and natural killer (NK) cells after binding to its own receptor.⁽¹⁹⁾ Like IL-12, IL-18 is synthesized by various cell types and synergizes with IL-12 in the enhancement of NK cell cytotoxicity and of T cell proliferation, as well as the induction of IFN- γ production by T cells, particularly CD4⁺ cells.⁽¹⁹⁾ In our study, lower amounts of IL-12 were produced by PBMCs of patients with HIV and TB compared with those with TB alone in response to both the mycobacterial antigens used. The difference was found to be statistically significant, however, only with PPD and not with Hk-M.tb., probably because of the purified and specific nature of PPD compared with the crude Hk-M.tb. antigen. There was no significant difference in plasma levels of IL-12 between TB patients with and without HIV co-infection, although there was a trend toward an increase in IL-12 levels in patients with severe immunodeficiency. In contrast, although IL-18 production by PBMCs was not significantly different between the HIV-positive and HIV-negative TB patients, plasma IL-18 levels were higher in individuals with dual infection compared with those with TB alone. Mayanja-Kizza et al.⁽²⁰⁾ studied *in vivo* levels of IL-12 and IL-18 in HIV-infected and uninfected patients with TB and reported findings similar to ours.

IL-10, a cytokine produced by macrophages, is a potent immunosuppressive factor. Some intracellular pathogens specifically target macrophages for infection and use IL-10 to dampen the host immune response and stall their elimination from the host.⁽²¹⁾ We found that although *in vitro* production of IL-10 in response to PPD was not significantly different in any of the study groups, Hk-M.tb. induced significantly higher production of IL-10 in HIV patients with TB than in HIV patients without TB. Patients with HIV infection have been shown to have higher IL-10 production in response to *Mycobacterium avium* antigen.⁽²²⁾ Plasma IL-10 levels in the different study groups varied considerably and did not show statistical significance, although the lowest levels were seen in HIV-uninfected TB patients. In the literature, increased levels of plasma IL-10 have been associated with adult HIV disease progression, with the highest levels in patients with the most advanced disease.⁽²³⁾ Surprisingly, high levels of plasma IL-10 were detected in the healthy controls included in our study. A probable explanation for this phenomenon could be the high prevalence of and consequently high exposure to parasitic, helminthic, and other tropical diseases. Lukwiya et al.⁽²⁴⁾ reported an increased frequency of IL-10-producing cells in both HIV-infected Africans and uninfected Africans compared with Italian controls. Yet another study described high levels of plasma IL-10 in healthy contacts

of TB patients.⁽²⁵⁾ Because the healthy controls included in our study were laboratory workers from a TB hospital who had prolonged exposure to TB, their immune response could be similar to that of contacts of TB patients. However, this finding needs to be confirmed in larger groups of individuals with and without contact with TB. In addition, the role of IL-10 in modifying disease progression in HIV-infected patients in India needs further study.

In summary, we have demonstrated significant immune activation, as seen by high plasma IFN- γ , IL-12, and IL-18, in patients dually infected with TB and HIV compared with those with either infection alone. The Th1 arm of the immune system appears to be maximally stimulated, particularly in individuals with advanced HIV disease, and this is likely to be a double-edged sword. It is, therefore, unlikely that further stimulation of T lymphocytes will be beneficial, whereas the effect of antagonizing these cytokines is still unknown. Although the findings of our study reveal an active inflammatory response *in vivo*, a downregulation of the *in vitro* immune response was observed in patients with TB and HIV.

The present study has a few limitations. These include a relatively small sample size in each study group, heterogeneity of the HIV-positive TB-negative group, and lack of information on the PPD status of the study subjects. Although it is not possible to draw definitive conclusions at this stage on the significance of circulating cytokines as a predictor of activation state, our study provides preliminary evidence on the high levels of circulating Th1 cytokines in patients with advanced HIV disease and TB. Further research is needed into the mechanisms of cytokine dysregulation in HIV-infected patients and also the impact of treatment of both HIV and TB on cytokine production. Plasma levels of IFN- γ and IL-18 or their surrogate markers need to be studied longitudinally in order to correlate them with disease outcome.

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