Cytokine responses induced by *Mycobacterium tuberculosis* in patients with HIV-1 infection and tuberculosis

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Summary

Objective: Tuberculosis (TB) is an important opportunistic infection in HIV patients. Immune responses to *Mycobacterium tuberculosis* in HIV/TB patients were evaluated.

Methods: Fifteen patients with HIV/TB, ten with HIV, four with TB, and five controls were enrolled. Peripheral blood mononuclear cells were isolated and stimulated with mycobacterial antigen (PPD). Interferon (IFN)-\textgamma and TNF-\alpha in culture supernatants were measured by ELISA.

Results: IFN-\gamma and TNF-\alpha production after PPD stimulation was markedly decreased in HIV patients, but not in HIV/TB patients. In HIV patients with a CD4 cell count of less than 200/mm\textsuperscript{3}, IFN-\gamma and TNF-\alpha production after PPD stimulation was higher in HIV/TB patients than in HIV patients. Cytokine responses to *M. tuberculosis* reconstituted after highly active antiretroviral therapy (HAART) and were prominent in HIV/TB patients.

Conclusions: Cytokine responses to *M. tuberculosis* were retained in HIV-infected patients with tuberculosis, even in patients with a CD4 cell count of less than 200/mm\textsuperscript{3}, and reconstituted after HAART.

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**Introduction**

Tuberculosis (TB) is one of the most common opportunistic infections in human immunodeficiency virus type 1 (HIV-1)-infected patients.1,2 The production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α appears to be crucial in the control of TB.3,4 Moreover, the impaired production of IFN-γ correlates with the progression of immunodeficiency.3 Understanding the biology of IFN-γ production and responsiveness among HIV-1 infected patients with TB (HIV/TB) is important because this cytokine may have a predominant protective role in TB infection, and it is emerging as a possible immunotherapeutic agent for patients with hard-to-treat TB.3,6 In addition, the cellular production of TNF-α may be particularly important, as this cytokine is produced in response to mycobacterial products, is synthesized at elevated levels in active tuberculosis, and is important in granuloma formation.4

Host immune responses to TB during its development in HIV patients, especially during highly active antiretroviral treatment (HAART), are not fully understood. Therefore, cellular immune responses against *Mycobacterium tuberculosis* in HIV patients with tuberculosis were evaluated.

**Materials and methods**

**Subjects**

Thirty-four individuals (31 males, three females) were enrolled in this study. The study population was divided into four groups: HIV-1 infected patients with TB (HIV/TB), HIV-1 infected patients (HIV), TB patients (TB), and normal subjects.

**Definition**

TB patients were classified as definite, probable and possible cases. Definite cases were defined as cases from which *M. tuberculosis* had been isolated. Probable cases were defined as acid-fast bacilli smear positive cases with symptoms and radiographic findings consistent with tuberculosis. Cases with pathologic findings compatible with tuberculosis, such as granuloma and caseation necrosis were also defined as probable cases. Possible cases were defined as cases with symptoms and radiological findings compatible with tuberculosis, and whose symptoms and radiological findings improved after anti-TB medication. HIV infection was diagnosed by ELISA and confirmed by Western blot. The control group was composed of BCG-vaccinated, healthy individuals who had no previous history of TB, and who were also seronegative for HIV. All HIV-infected patients were also BCG-vaccinated in their childhood as part of the routine vaccination schedule.

**Cell culture**

Peripheral blood mononuclear cells (PBMCs) were isolated from 15 ml of whole blood by centrifugation through a Ficoll-Paque separation (Amersham Pharmacia Biotech, Sweden). PBMCs were resuspended (5 × 10^6 cells/ml) in RPMI 1640 with glutamate (2 μM), penicillin and streptomycin (1 U/ml and 100 μg/ml, respectively), 20 mM HEPES, and 10% (vol/vol) heat-inactivated human albumin. Assays were performed with 2.5 × 10^5 cells/well in a 96-well plate. Cells were stimulated with a purified protein derivative (PPD; Statens Seruminstitut, Denmark). Phytohemagglutinin (PHA; Sigma, USA) was used as a positive control. Cell cultures were maintained at 37 °C in 5% CO₂, and culture supernatants were harvested after 72 h of PHA stimulation, and after 120 h of PPD stimulation. Supernatants were stored at −70 °C for IFN-γ and TNF-α measurement.

**Measurement of IFN-γ and TNF-α**

IFN-γ and TNF-α levels in culture supernatants were determined using commercial ELISA kits (Quantikine, R&D Systems, Inc. USA), in accordance with the manufacturers’ instructions.

**Statistical analysis**

Nonparametric methods, namely, the Mann-Whitney U test or the Wilcoxon signed rank test were used to compare continuous variables. All P values were two-tailed, and P < 0.05 was considered to indicate statistical significance. SPSS (version 10.0) software was used for the analysis.

**Results**

**Demographic characteristics**

Thirty-four subjects were enrolled in this study between January 2000 and April 2002. Of these, 15 patients had HIV/TB infection, ten had HIV infection, four had TB, and five were healthy HIV-seronegative subjects. Of the 15 HIV/TB patients, three had proven TB, eight had probable TB, and four had possible TB. Of the four TB patients, three were proven cases and one was a probable case. Clinical characteristics of patient groups are shown in Table 1. Median CD4 cell count (per microliter)
was 160 in HIV/TB patients and 145 in HIV patients, which was not significantly different ($P = 0.781$, Mann-Whitney $U$ test).

**IFN-γ and TNF-α production by PHA or PPD**

IFN-γ and TNF-α levels after PHA stimulation were not different in the four groups (Table 2). The median IFN-γ level after PPD stimulation was significantly higher in HIV/TB patients (1555 pg/ml; 347 and 3059 pg/ml for the 25th and 75th quartiles, respectively) than in HIV patients (0 pg/ml; 0 and 204 pg/ml for the 25th and 75th quartiles, respectively; $P = 0.001$, Mann-Whitney $U$ test). Also, the median TNF-α level after PPD stimulation was higher in HIV/TB patients (562 pg/ml; 190 and 1620 pg/ml for the 25th and 75th quartiles, respectively) than in HIV patients (132 pg/ml; 55 and 240 pg/ml for the 25th and 75th quartiles, respectively; $P = 0.013$, Mann-Whitney $U$ test).

Furthermore, the median IFN-γ level after PPD stimulation was significantly lower in HIV patients (0 pg/ml; 0 and 204 pg/ml for the 25th and 75th quartiles, respectively) than that in normal control (1519 pg/ml; 821 and 1752 pg/ml for the 25th and 75th quartiles, respectively; $P < 0.001$, Mann-Whitney $U$ test) (Table 1).

**Changes of IFN-γ and TNF-α production during HAART**

Five HIV patients and six HIV/TB patients on highly active antiretroviral treatment (HAART) were prospectively monitored for IFN-γ and TNF-α measurement. The HIV/TB patients also received anti-TB medication together with HAART. During a six-month period of HAART, all patients except two showed increased CD4 cell counts. In HIV patients, initial mean CD4 cell count increased significantly after six months ($72 ± 61$ cells/mm$^3$ vs. $174 ± 104$ cells/mm$^3$, $P = 0.043$, Wilcoxon signed rank test). But in HIV/TB patients, the observed increase of mean CD4 cell counts was not significant ($174 ± 115$ cells/mm$^3$, $242 ± 176$ cells/mm$^3$, $P = 0.345$, Wilcoxon signed rank test).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV/TB (n = 15)</th>
<th>HIV (n = 10)</th>
<th>TB (n = 4)</th>
<th>Control (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 cell count, no. (%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;200 CD4 cells/μL</td>
<td>8 (53.3)</td>
<td>6 (60)</td>
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<td>200–500 CD4 cells/μL</td>
<td>7 (46.7)</td>
<td>4 (40)</td>
<td>0</td>
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<tr>
<td>&gt;500 CD4 cells/μL</td>
<td>0</td>
<td>0</td>
<td>4 (100)</td>
<td></td>
</tr>
<tr>
<td>Age, median years (range)</td>
<td>39 (25–60)</td>
<td>39 (30–65)</td>
<td>52 (41–64)</td>
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<tr>
<td>Male sex, no. (%)</td>
<td>14 (93.3)</td>
<td>9 (90)</td>
<td>3 (75)</td>
<td></td>
</tr>
</tbody>
</table>

Values not otherwise identified are no. (%) of patients.
Although IFN-\(\gamma\) and TNF-\(\alpha\) levels after PHA stimulation increased in HIV patients after HAART in parallel with increasing CD4 cell count, the recovery of cytokine responses by PPD stimulation was not prominent, except in one patient whose CD4 cell count recovered to 320/mm\(^3\) (Figure 2, panel I). In HIV patients, the increased median IFN-\(\gamma\) level after PPD stimulation was not significant (0 pg/ml; 0 and 707 pg/ml for the 25th and 75th quartiles, respectively; and 987 pg/ml; 615 and 1294 pg/ml for the 25th and 75th quartiles, respectively; \(P = 0.08\), Wilcoxon signed rank test). The increased median TNF-\(\alpha\) level after PPD stimulation was also not significant (0 pg/ml; 0 and 84 pg/ml for the 25th and 75th quartiles, respectively, and 91 pg/ml; 10 and 774 pg/ml for the 25th and 75th quartiles, respectively; \(P = 0.068\), Wilcoxon signed rank test).

In HIV/TB patients, the median IFN-\(\gamma\) level after PPD stimulation significantly increased (755 pg/ml; 352 and 2145 pg/ml for the 25th and 75th quartiles, respectively, and 1998 pg/ml; 818 and 3801 pg/ml for the 25th and 75th quartiles, respectively; \(P = 0.028\), Wilcoxon signed rank test). However, increased median TNF-\(\alpha\) level after PPD stimulation was not significant (378 pg/ml; 129 and 1217 pg/ml for the 25th and 75th quartiles, respectively, and 552 pg/ml; 353 and 880 pg/ml for the 25 and 75 quartiles, respectively; \(P = 0.345\), Wilcoxon signed rank test). IFN-\(\gamma\) and TNF-\(\alpha\) responses to PPD stimulation were retained in HIV/TB patients, even in patients with CD4 cell count of less than 200/mm\(^3\), and these response were sustained during the HAART period (Figure 2, panel II).

Discussion

In the present study, cytokine responses were compared against mycobacterial antigen in four groups, namely, HIV, HIV/TB, TB, and a normal subject group. Proliferative responses to mycobacterial antigen in HIV patients were less vigorous than in normal subjects, even when the CD4 cell count was greater than 200/mm\(^3\) in HIV patients. These results suggest that HIV patients are susceptible to \(M.\) \(tuberculosis\) infection, even when the CD4 cell count was greater than 200/mm\(^3\) in HIV patients. This result is consistent with a previous study showing that 18% (8/44) of HIV patients with tuberculosis had CD4 cell counts greater than 200/mm\(^3\). Indeed, a previous study showed that 18% (8/44) of HIV patients with tuberculosis had CD4 cell counts greater than 200/mm\(^3\). In contrast, IFN-\(\gamma\) and TNF-\(\alpha\) production induced by mycobacterial antigens was retained in HIV patients with TB, even when their CD4 cell counts were less than 200/mm\(^3\). Sodhi et al. also reported that production of TNF-\(\alpha\) was three to ten times greater in HIV patients with TB than in controls.

The increased IFN-\(\gamma\) and TNF-\(\alpha\) production in HIV patients with TB, compared with HIV patients without TB, might be due to the activation of cellular immune responses in an attempt to control the \(M.\) \(tuberculosis\) infection. Mayanja-Kizza et al. showed that there was a marked variability in \(M.\) \(tuberculosis\)-stimulated IFN-\(\gamma\) production in HIV patients with tuberculosis and that this was related to the degree of immunodeficiency. Several studies have reported a low level of IFN-\(\gamma\) production in HIV patients with tuberculosis, but the majority of these studies examined the dually infected patients as a group, regardless of individual CD4 cell status, or the patients enrolled had severe tuberculosis, with frequent extrapulmonary dissemination. Sodhi et al. reported that the radiographic extent of disease and the site of disease
were the only independent predictors of IFN-\(\gamma\) production in HIV-negative and in HIV-infected patients, suggesting that reduced IFN-\(\gamma\) production by PBMCs is a marker of severe tuberculosis regardless of HIV seropositivity.\(^{12}\) Some HIV/TB patients with CD4 cell counts of more than 200 cells/mm\(^3\) retained the ability to produce IFN-\(\gamma\) in response to mycobacterial antigens.\(^{12}\) In the present study, it is noted that cytokine responses against \(M.\) \(tuberculosis\) were retained in HIV-infected patients with tuberculosis, even in patients with CD4 cell counts of less than 200/mm\(^3\). However, the difference between this report and others with respect to the preservation of PPD-induced IFN-\(\gamma\) responses in HIV/TB cases may reflect differences in CD4 count.

**Figure 2**  Effect of HAART on the expression of IFN-\(\gamma\) and TNF-\(\alpha\) by PHA and PPD stimulation in HIV-infected patients without tuberculosis (I) and with tuberculosis (II). Panel I. Initial mean CD4 cell count increased significantly after six months of HAART (72 \(\pm\) 61 cells/mm\(^3\), 174 \(\pm\) 104 cells/mm\(^3\), \(P = 0.043\), Wilcoxon signed rank test). Although IFN-\(\gamma\) and TNF-\(\alpha\) levels after PHA stimulation increased in parallel with the CD4 cell count, the recovery of cytokine responses to PPD stimulation was not prominent except in patient 5 whose CD4 cell count had recovered to 320/mm\(^3\), Panel II. The increased mean CD4 cell count was not significant (174 \(\pm\) 115 cells/mm\(^3\), 242 \(\pm\) 176 cells/mm\(^3\), \(P = 0.345\), Wilcoxon signed rank test). However, the expression of IFN-\(\gamma\) and TNF-\(\alpha\) after PPD stimulation was retained, and in particular IFN-\(\gamma\) levels were markedly increased after six months. A. IFN-\(\gamma\) response to PHA stimulation; B. IFN-\(\gamma\) response to PPD stimulation; C. Change of CD4 cell count during the HAART period; D. TNF-\(\alpha\) response to PHA stimulation; E. TNF-\(\alpha\) response to PPD stimulation.
Although only four TB patients without HIV were available, this study shows that low levels of mycobacterial antigen-induced IFN-\(\gamma\) and TNF-\(\alpha\) have not been found in TB patients. These results are consistent with those of Surcel et al. in this regard. These workers showed that the number of IFN-\(\gamma\) secreting cells was not depressed in active TB patients.\(^{13}\) Silveira et al. also suggested that there was no down-regulation of IFN-\(\gamma\) production in active pulmonary TB patients in Portugal.\(^{14}\) However, another study demonstrated that IL-2 and IFN-\(\gamma\) expression was depressed when PBMCs were stimulated with mycobacterial antigens in tuberculosis patients.\(^{15}\) Possible explanations for these discrepancies among studies include the difference in the prevalence of the latent infection with tuberculosis or the BCG vaccination rate among the study population. Since South Korea is an endemic area for tuberculosis, the majority of the population receive the BCG vaccination routinely.\(^{16,17}\) A recent nationwide tuberculosis prevalence survey in South Korea demonstrated that observed tuberculin positivity (\(\geq 10\) mm in diameter) in subjects aged under 30 was 15.5%, that the prevalence of active pulmonary tuberculosis was approximately 1%, and that the BCG scar prevalence of subjects aged under 30 was 91.8%.\(^{17}\) The high prevalence of latent infection or exposure to \(M.\) tuberculosis prior to HIV infection may contribute to the preserved immune response to \(M.\) tuberculosis in the patients in the present study.

\(Mycobacterium\) \(tuberculosis\) infection results in the activation of T cells and macrophages, which may harbor latent HIV. This activation is beneficial to the host immunity to mycobacterial disease, but it may result in a reactivation of latent HIV. There is a report which found that the production of TNF-\(\alpha\) and the mean \(\beta 2\)-microglobulin level were greater in HIV-1 seropositive patients with pulmonary tuberculosis.\(^{8}\) These observations suggest that HIV-1-associated tuberculosis is accompanied by immune activation that may result in increased HIV replication and accelerated progression to AIDS.\(^{8}\)

The restoration of lymphocytes responses to mycobacterial antigens by HAART was first reported by Autran et al.\(^{18}\) It was demonstrated that cell-mediated immune responses to \(Mycobacterium\) \(avium\) reconstituted rapidly after HAART, and that this was sustained even with partial viral suppression.\(^{19}\) In HIV-infected patients receiving HAART, primary and secondary prophylaxis against \(Pneumocystis\) \(carinii\) pneumonia can be safely discontinued after the CD4 cell count has increased to more than 200/mm\(^3\) for more than three months.\(^{20}\) HAART also restored immune responses to \(M.\) \(tuberculosis\), although this restoration was found to be delayed and did not reach the levels seen in healthy, HIV-negative control subjects.\(^{21}\) Indeed, one cohort study found that HAART reduced the incidence of HIV-associated tuberculosis by more than 80% in an area endemic with tuberculosis and HIV.\(^{22}\)

The results of this study suggest that the cellular immune responses of HIV-infected patients with/without tuberculosis recovered as the CD4 cell count increased during the HAART period. In HIV-infected patients without tuberculosis, the IFN-\(\gamma\) and TNF-\(\alpha\) production by PPD was not as prominent as that induced by PHA stimulation. In HIV-infected patients with tuberculosis, IFN-\(\gamma\) and TNF-\(\alpha\) production after PPD stimulation were retained and sustained during the HAART period. The relative lack of reconstitution of PPD-induced IFN-\(\gamma\) responses in HIV patients might be due to relative lack of increment of CD4 cell count. Indeed, in a patient with an improved CD4 cell count of 320/mm\(^3\) after HAART, IFN-\(\gamma\) and TNF-\(\alpha\) levels after PPD stimulation were found to be similar to those after PHA stimulation.

The differences in recovery of immune responses between HIV only and dually infected subjects might also reflect a different level of exposure to the antigen. Therefore it would have been informative to see whether there is a similar or a different trend in the recovery of responses to non-TB antigens, to which both populations had a similar exposure.

In conclusion, cytokine responses against \(M.\) \(tuberculosis\) were retained in HIV-infected patients with tuberculosis, even in patients with a CD4 cell count of less than 200/mm\(^3\). Cytokine responses to \(M.\) \(tuberculosis\) reconstituted after HAART, and were prominent in HIV/TB patients.

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Conflict of interest: No conflict of interest to declare.

References


