CONCISE COMMUNICATIONS

Depressed T-Cell Interferon-γ Responses in Pulmonary Tuberculosis: Analysis of Underlying Mechanisms and Modulation with Therapy

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Immunological and clinical profiles were evaluated in 2 groups: human immunodeficiency virus (HIV)–uninfected and HIV-infected patients, with newly diagnosed pulmonary tuberculosis (TB), and tuberculin-skin-test–reactive healthy control subjects. HIV-uninfected patients with TB were also followed up longitudinally during and after chemotherapy. At the time of diagnosis, purified protein derivative (PPD)–stimulated production of interferon (IFN)–γ by peripheral blood mononuclear cells from TB patients was depressed, compared with that of healthy control subjects, whereas levels of transforming growth factor (TGF)–β and interleukin (IL)–10 were increased. In longitudinal studies, PPD stimulated production of IL-10 and TGF-β returned to baseline by 3 months, whereas IFN-γ production remained depressed for at least 12 months. These data indicate that the immunosuppression of TB is not only immediate and apparently dependent (at least in part) on immunosuppressive cytokines early during the course of Mycobacterium TB infection but is also long lasting, presumably relating to a primary abnormality in T-cell function.

Existing studies of antituberculous immunity mostly rely on a comparison between patients with active tuberculosis (TB) and healthy tuberculin-reactive control subjects recruited from the community. These studies have demonstrated a relative depression of TH1-type cytokine responses and overproduction and/or enhanced effects of immunosuppressant molecules (transforming growth factor [TGF]–β, interleukin [IL]–10). Proinflammatory cytokines (tumor necrosis factor [TNF]–α, IL–6, and IL–1β) have also been shown to play a role in the immunopathogenesis of TB [1–5]. Furthermore, active TB has been associated with excessive immune activation, as evidenced by increased circulating levels of TNF–α, β2-microglobulin and neopterin [6] in the serum of patients and increased expression of HLA-DR on the surface of T cells and Fcγ-R1 and III on blood monocytes [7]. Recent evidence also suggests that IL-12, a cytokine that has been successfully applied to immunotherapy of individuals with malignant tumors [8], may have immunomodulatory properties during TB [9].

The current study was designed to simultaneously assess and correlate the whole spectrum of anti-Mycobacterium TB (MTB) immune responses in a single well-defined cohort of patients with active pulmonary TB and to follow up the cohort during and after completion of antituberculous chemotherapy.

Methods

Human subjects. We enrolled 42 Ugandans with newly diagnosed pulmonary TB (24 human immunodeficiency virus [HIV]–uninfected, 18 HIV-infected) and 18 HIV-negative healthy tuberculin-reactive control subjects. Screening included history and physical examination, complete blood count and chemistries, HIV testing (ELISA), a chest x-ray, and 3 sputum acid-fast bacilli smears. Sputum smear–positive individuals (aged 18–50 years) with symptoms and radiographs consistent with pulmonary TB, Karnofsky score >50%, and without prior TB treatment were enrolled. Patients receiving immunosuppressive therapy and those with serious medical illness were ineligible for study participation.

Assessment of radiographic extent of disease [10] showed that, of the patients with TB, 12 had moderately advanced disease, 30 had far-advanced TB, and none had minimal or miliary disease. Patients were treated with standard short-course therapy (daily isoniazid [INH], RMP, ethambutol, and pyrazinamide for 2
months, followed by daily INH and RMP for 4 months). Compliance with therapy was evaluated by urine INH metabolite testing.

Baseline immunological evaluation in TB patients was performed within 2 weeks of initiation of therapy. Follow-up studies were conducted at 3, 6, 12, and 18 months after enrollment. Healthy HIV-uninfected control subjects were recruited among laboratory personnel and nursing staff and were matched to patients by sex and age.

Reagents. Purified protein derivative (PPD) was obtained from Statens Serum Institute (Copenhagen, Denmark). Neutralizing antibodies to TGF-β and IL-10 and recombinant (r)-human IL-12 were obtained from R&D Systems (Minneapolis) and Pharmingen (San Diego). All reagents were used at concentrations found optimal in previous studies [2, 9]. Endotoxin content of these reagents was <0.01 ng/mg protein.

Preparation of cells and generation of cytokine-containing culture supernatants. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll Hypaque density gradient centrifugation [2]. PBMC suspended in Iscove’s Modified Dulbecco’s Medium (IMDM; BioWhittaker, Walkersville, MD) containing 2% pooled human serum (cells/mL) were incubated in 24-well microtiters plates for up to 72 h with or without PPD. Neutralizing antibodies to TGF-β, IL-10, and (r)-human IL-12 were added to cultures from patients and control subjects were cultured without antibody to TGF-β.

Immunoassays for cytokines, markers of immune activation, and immunoglobulins. Immunoreactivities of cytokines and markers of immune activation in culture supernatants (IFN-γ, TNF-α, TGF-β, IL-4, and IL-12) and serum (neopterin and TNF receptor type 2 [TNF-RII]) were assessed by commercially available ELISA kits. These assays are sensitive to 2 pg/mL (IFN-γ; Endogen, Boston), 8 pg/mL (TNF-α, TGF-β, TNF-RII, and IL-4; R&D), 0.5 EU/mL (neopterin; ICN, Costa Mesa, CA), and <0.5 pg/mL (IL-12; R&D). ELISA for IL-10 (using a pair of monoclonal antibodies to IL-10; Pharmingen) detects a minimum of 16 pg/mL of IL-10. Levels of IgG and IgM antibodies against culture filtrate of MTB were evaluated by ELISA [11].

Statistical analysis. Data were analyzed by Student’s t test, paired t test, and analysis of variance. P ≤ .05 was considered significant.

Results

Baseline immunological profiles in patients with newly diagnosed TB and in healthy control subjects. First, we assessed PPD-stimulated production of cytokines by PBMC of patients with TB and of control subjects in vitro. At the time of diagnosis, production of PPD-induced IFN-γ in PBMC of TB patients (both HIV-infected and -uninfected) was depressed, compared with that of healthy control subjects (table 1). By contrast, an excess of TGF-β, IL-10, and TNF-α was induced by PPD in PBMC from (HIV-positive and -negative) patients with TB, when compared with healthy PPD-positive control subjects (table 1). Immunoreactivities of IFN-γ, TNF-α, and IL-10 in PBMC supernatants from patients or control subjects cultured in the absence of PPD were below the detection limits indicated above (data not shown). In contrast (as shown elsewhere [2]), TGF-β immunoreactivities in unstimulated PBMC cultures from patients and control subjects were ~50% lower when compared with PPD-stimulated production of the cytokine in supernatants from either study group. A control antigen or mitogen was not included in this study, since numbers of cells available for study were limited.

To assess whether TH2-type cytokines play a role in depressed T-cell IFN-γ responses during active TB, we evaluated production of IL-4. No IL-4 immunoreactivity was detected in PBMC culture supernatants from either TB patients or control subjects (data not shown).

Next, we studied circulating markers of immune activation and IgG and IgM antibodies against culture filtrate of MTB in serum. Both neopterin and TNF-RII immunoreactivities were increased in samples from TB patients, compared with samples from healthy control subjects (table 1). Levels of IgG

### Table 1. PPD-stimulated production of cytokines and markers of immune activation in HIV-infected and -uninfected patients with TB and in PPD-positive control subjects and modulation of IFN-γ production by coculture with neutralizing antibodies to TGF-β and IL-10 or (r)-IL-12.

<table>
<thead>
<tr>
<th></th>
<th>TB patients</th>
<th>HIV/TB patients</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ (ng/mL)</td>
<td>1.53 ± 28³</td>
<td>1.03 ± 33⁴</td>
<td>8.00 ± 1.05</td>
</tr>
<tr>
<td>TNF-α (ng/mL)</td>
<td>3.91 ± 3⁷</td>
<td>4.00 ± 39⁵</td>
<td>2.78 ± 35</td>
</tr>
<tr>
<td>TGF-β (ng/mL)</td>
<td>5.56 ± 30⁶</td>
<td>4.20 ± 28⁷</td>
<td>3.20 ± 34</td>
</tr>
<tr>
<td>IL-10 (ng/mL)</td>
<td>30 ± 03 ⁸</td>
<td>53 ± 09⁹</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>TNF-RII (ng/mL)</td>
<td>4.20 ± 50 ¹</td>
<td>7.30 ± 60¹</td>
<td>2.40 ± 30</td>
</tr>
<tr>
<td>Neopterin (EU/mL)</td>
<td>10.4 ± 2.40</td>
<td>16.3 ± 2.20¹</td>
<td>4.50 ± 1.60</td>
</tr>
</tbody>
</table>

Modulation of PPD-stimulated IFN-γ production by coculture with neutralizing antibodies to TGF-β and IL-10 or (r)-IL-12.

<table>
<thead>
<tr>
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<th>TB patients</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PPD only</td>
<td>1.53 ± 28</td>
<td>1.03 ± 33</td>
<td>8.00 ± 1.05</td>
</tr>
<tr>
<td>PPD + α-TGF-β</td>
<td>5.02 ± 1.09</td>
<td>1.66 ± 20⁶ ¹</td>
<td>14.3 ± 2.35</td>
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<tr>
<td>PPD + α-IL-10</td>
<td>6.45 ± 1.31</td>
<td>2.43 ± 74⁶</td>
<td>15.04 ± 2.88</td>
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<tr>
<td>PPD + (r)-IL-12</td>
<td>11.39 ± 1.70</td>
<td>10.85 ± 2.13¹</td>
<td>28.83 ± 0.59</td>
</tr>
</tbody>
</table>

**NOTE.** Neopterin and TNF-RII levels were assessed in serum, and cytokine immunoreactivities were measured in PPD-stimulated culture supernatants. Results are presented as mean ± SE. PPD, purified protein derivative; HIV, human immunodeficiency virus; TB, tuberculosis; IFN, interferon; TGF, transforming growth factor; IL, interleukin; TNF, tumor necrosis factor; TNF-RII, TNF receptor type 2; PBMC, peripheral blood mononuclear cells.

³ P ≤ .001, when compared with levels of cytokines/markers of immune activation in supernatants/serum from control subjects.

⁴ P ≤ .02, when compared with TNF-α immunoreactivity in supernatants from control subjects.

⁵ P ≤ .01, when compared with TNF-α levels in supernatants from healthy control subjects.

⁶ P ≤ .03, when compared with cytokine immunoreactivity in culture supernatants from control subjects.

⁷ P ≤ .005, when compared with levels of markers of activation in serum from healthy control subjects.

⁸ P ≤ .004, when compared with IFN-γ production in supernatants of PBMC cultured without antibody to TGF-β.

⁹ P ≤ .003, when compared with IFN-γ production in supernatants of PBMC cultured without antibody to TGF-b.

¹ P ≤ .05, when compared with levels of IFN-γ in cultures without IL-10 antibody.

¹ P ≤ .01, when compared with IFN-γ levels in cultures containing PPD alone.

¹ P ≤ .01, when compared with IFN-γ immunoreactivity in cultures containing PPD alone.
and IgM antibodies against culture filtrate of MTB (assessed by ELISA and expressed as optical density) were increased by ~2-fold in sera from TB patients, compared with sera from healthy control subjects (data not shown).

Modulation of PPD-induced production of IFN-γ by neutralization of TGF-β and IL-10 and by coculture with rIL-12. Coculture with neutralizing antibody to TGF-β or IL-10 significantly increased PPD-stimulated production of IFN-γ by PBMC from patients with TB, irrespective of their HIV status (table 1), but not by PBMC from control subjects. However, neutralization of TGF-β and IL-10 was less effective in reconstituting IFN-γ production in patients with HIV/TB (table 1). In contrast, even though levels of IL-12 (p70) did not differ significantly between patients with TB and HIV/TB (data not shown), coculture with (r)-IL-12 was equally effective in boosting PPD-induced production of IFN-γ by PBMC from both HIV-uninfected and -infected patients. Results of flow-cytometric studies evaluating the source of the described augmented production of IFN-γ suggest that coculture with (r)-IL-12 results in increased frequencies of IFN-γ-producing CD4 and natural killer cells among PBMC from patients with TB but only in increased numbers of IFN-γ-producing CD4 cells in control subjects (C.S.H., unpublished data).

Longitudinal study of immunological parameters in HIV-uninfected patients with TB. Longitudinal studies (of HIV-uninfected individuals only) were performed to assess dynamic changes of molecules implicated in antituberculous immunity throughout TB treatment. Production of TGF-β, IL-10, and TNF-α in PPD-stimulated PBMC cultures from TB patients returned to baseline (levels found in culture supernatants from control subjects) by 3 months (figure 1). By contrast, IFN-γ immunoreactivity in patient supernatants remained significantly depressed for prolonged periods of time when compared with “control values.” In fact, whereas PPD-induced production of IFN-γ by PBMC from patients with TB increased throughout the follow-up period (1.8-fold by 3 months; 3-fold by 12 months [figure 1]), IFN-γ levels at 18 months follow-up were still 20% lower in culture supernatants from TB patients, compared with control values (but differences were no longer statistically significant).

Decreases in serum levels of TNF-RII and neopterin were most notable early in the course of antituberculous therapy (data not shown). Even though small decrements still occurred during and after completion of antituberculous therapy, neopterin levels at 3 months and TNF-RII immunoreactivities at 6 months follow-up were no longer statistically significantly different from levels found in sera from healthy control subjects.

Discussion

Data from studies evaluating MTB antigen-induced T-cell responses in patients with active pulmonary TB consistently indicate depressed blastogenesis and production of immunoprotective cytokines such as IFN-γ and IL-2 [1–4, 12]. These cytokines are important to the capacity of the host to contain MTB infection and in the formation of functional granulomas [13, 14]. By contrast, production of immunosuppressive/macrophage deactivating molecules, such as TGF-β, is up-regulated during active TB [2].

Findings presented here both confirm and extend our current understanding of the human immune response during active pulmonary TB. Neutralization of IL-10 and TGF-β was associated with enhancement of PPD-induced IFN-γ production, particularly when both neutralizing antibodies were present concomitantly for the duration of culture (data not shown); however, only coculture with rIL-12 resulted in complete reconstitution of the IFN-γ response. Finally, and most notably, the immunosuppression in TB was not only immediate, as well as related to overproduction of deactivating cytokines at the time of diagnosis of TB, but also long lasting. IFN-γ immunoreactivity in PPD-stimulated culture supernatants from HIV-uninfected patients remained depressed for at least 1 year after initiation of chemotherapy, a time when TGF-β and IL-10 immunoreactivities were indistinguishable from those encountered in culture supernatants from healthy control subjects. Similarly, effects of neutralization of TGF-β and IL-10 were most pro-
nounced at baseline and at 3-month evaluations. In contrast, coculture with (r)-IL-12 remained effective in boosting IFN-γ production for the whole duration of follow up (data not shown).

Little is known about the mechanisms underlying this IL-12–mediated increase in IFN-γ production. Our recent studies indicate that natural killer cells (in addition to CD4 cells) contribute to augmented IFN-γ production by PBMC from patients with TB in the presence of IL-12 (C.S.H., unpublished data). Presumably, this effect of IL-12 could bypass any defect in MTB-induced CD4 responses. It remains to be seen, however, whether the recruitment of IFN-γ–producing effector cells, other than (antigen-reactive) CD4 T cells, has any significant impact on the clinical course of MTB infection.

The persistence of low PPD-induced IFN-γ production beyond 6 months that dissociates from the production of immunosuppressive cytokines may indicate other mechanisms involved in the suppression of T-cell responses during MTB infection/disease. At 18 months one would expect in vitro IFN-γ production to be high, particularly in view of the recent challenge with large amounts of viable MTB. Therefore, one explanation for this persistently low IFN-γ production is a genetic defect in IFN-γ production/response in susceptible individuals that may also underlie their particular risk for reactivation of a latent MTB infectious focus.

Compartmentalization of antigen-responsive cells to sites of active MTB infection also may be involved in the observed peripheral blood hyporesponsiveness. However, since total numbers of CD4 cells are normal in the blood from HIV-uninfected patients with TB (C.S.H., unpublished data) and hyporesponsiveness persists even after completion of therapy, at a time when antigen-responsive T cells should have recirculated, sequestration of antigen-responsive cells to sites of active MTB infection is unlikely to be the sole factor responsible for T-cell hyporesponsiveness in the peripheral blood.

Another possibility is that there is an active and selective depletion of circulating MTB-responsive T-cells during TB, which accounts for persistently low PPD-induced IFN-γ production, even after completion of chemotherapy. Earlier studies indicate that during TB there is an activation of mononuclear cells [7]. Also, we found that levels of TNF-RII and neopterin, which are molecules associated with monocyte and T-cell activation, are elevated at the time of diagnosis of TB. Furthermore, it is well established that activated mononuclear cells are prone to programmed cell death. Therefore, it is of considerable interest that spontaneous and MTB-induced programmed cell death is increased among PBMC from patients with newly diagnosed TB, compared with that of healthy control subjects [15]. Whether accentuated programmed cell death of T cells and depletion of antigen-responsive T cells from the peripheral blood fully explains the observation of the persistent IFN-γ defect in patients with TB and whether or how this observation may relate to reactivation of the latent tuberculous focus needs to be elucidated.

Progress in understanding the immunopathophysiology of TB should provide the rationale for trials of immune-based therapies. These, in turn, hold the promise of shortening the course of treatment in drug-sensitive TB and promoting the efficacy of chemotherapy of multidrug-resistant TB.

Acknowledgments

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References

