Human Immunodeficiency Virus–Seronegative Adults with Extrapulmonary Tuberculosis Have Abnormal Innate Immune Responses

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Extrapulmonary tuberculosis is presumably a marker of underlying immunodeficiency, but cytokine response pathways in these patients have not been well studied. Cytokine responses of peripheral blood mononuclear cells from human immunodeficiency virus–seronegative adults with prior culture-confirmed extrapulmonary tuberculosis were compared with those of persons with latent Mycobacterium tuberculosis infection. Mitogen-stimulated interferon (IFN)-γ production, interleukin (IL)-12 production, and IFN-γ receptor– and IL-12 receptor–mediated cytokine production did not differ between case patients and control patients. However, median resting IL-8 production was significantly lower in case patients than control patients (8051 pg/mL vs. 19,290 pg/mL; \( P < .009 \)). In addition, the median tumor necrosis factor (TNF)–α response was lower in case patients than control patients after stimulation with lipopolysaccharide (833 pg/mL vs. 1149 pg/mL; \( P = .06 \)) and lipopolysaccharide plus IFN-γ (3301 pg/mL vs. 4411 pg/mL; \( P = .04 \)). These abnormalities in resting IL-8 and lipopolysaccharide-induced TNF-α production were not associated with IFN-γ or IL-12 abnormalities and were detected up to several years after cure of disease, suggesting an abnormality in innate immunity.

Although about one-third of the world’s population is infected with Mycobacterium tuberculosis [1], the vast majority of infected persons never develop active tuberculosis, presumably because of an effective host immune response. IFN-γ and TNF-α play critically important roles in the immune response to M. tuberculosis infection [2, 3]. IFN-γ activates macrophages, resulting in inhibition of intracellular mycobacterial growth [4, 5]. IFN-γ production by peripheral blood mononuclear cells (PBMC) after stimulation with mycobacterial antigens is higher in persons with latent M. tuberculosis infection than in persons with active pulmonary tuberculosis [6, 7]. Studies in mice demonstrate that TNF-α is important in controlling the extent of mycobacterial infection [3, 8] and granuloma formation [9]. There have been reports of disseminated tuberculosis in humans treated with the TNF-α blockers etanercept or infliximab [10]. The production of TNF-α by macrophages is stimulated by lipopolysaccharide (LPS) and is enhanced by IFN-γ [11]. LPS stimulation of PBMC results in lower TNF-α production in patients with chronic refractory pulmonary tuberculosis than in persons with latent M. tuberculosis infection [12]. However, the TNF-α response to stimulation with LPS has not been studied in persons with extrapulmonary tuberculosis nor in persons who have recovered from...
The cytokine response of PBMC to mycobacterial antigens varies according to the stage and severity of tuberculosis disease, possibly because of selective anergy, recruitment of antigen-specific T cells to sites of infection, or other mechanisms [6, 7, 13]. In contrast, mitogen-mediated responses, such as cell proliferation or IFN-γ production of PBMC, are relatively independent of M. tuberculosis disease stage [14]. The agents needed to probe the innate immune system differ from those required to examine acquired responses. One example is LPS, which is highly conserved among bacteria and signals through the Toll-like receptor pathway in conjunction with other cell surface markers such as CD14. The pathways mediating the LPS response have been well characterized at both the functional and molecular levels [15].

The incidence of extrapulmonary and disseminated tuberculosis is increased among persons with compromised immune function, particularly HIV-infected persons [16]; in such patients the risk of extrapulmonary tuberculosis is associated with low CD4+ T lymphocyte levels [17] and decreased antigen-stimulated IFN-γ production [18]. The risk of extrapulmonary tuberculosis is also increased among young HIV-seronegative children, presumably because of an immature immune system [19]. In addition, in HIV-seronegative persons with fundamental defects in the immune response (e.g., involving the IFN-γ receptors, IL-12 p40, or IL-12 receptor β-1) who develop nontuberculous mycobacterial infection, mycobacterial disease is almost always disseminated [20]. Therefore, extrapulmonary tuberculosis appears to be a clinical marker for an underlying host immune defect.

We hypothesized that persons who develop extrapulmonary tuberculosis have abnormalities in the host immune response. We also hypothesized that any host defense defect in such persons would be most readily identified by comparing their immune responses with those of persons with well-controlled latent M. tuberculosis infection. Because of the profound defects in cellular immunity that are known to occur with HIV infection, investigations into specific immunologic risk factors for extrapulmonary tuberculosis are best carried out among HIV-seronegative persons. To assess for host defense defects distinct from the transient antigen-specific responses reported elsewhere [13, 14, 18], we assessed PBMC responses after stimulation with mitogen and LPS. To avoid confounding of cytokine responses by acute illness, we studied only patients who were not acutely ill with tuberculosis.

METHODS

Patient population. Case patients and control patients were identified through the Baltimore City Health Department Eastern Chest Clinic. Inclusion criteria for case patients included a history of culture-confirmed extrapulmonary tuberculosis, age ≥18 years, and being HIV-seronegative. Exclusion criteria included serum creatinine level >2 mg/dL, use of corticosteroids or other immunosuppressive agents at the time of diagnosis or time of study entry, malignancy, or diabetes mellitus. The eligibility criteria for control patients included evidence of latent M. tuberculosis infection (≥10-mm induration on tuberculin skin test, consisting of intradermal placement of 5 tuberculin units of purified protein derivative) without evidence of active tuberculosis, age ≥18 years, and being HIV-seronegative. Exclusion criteria for control patients were the same as for case patients. The date of tuberculosis diagnosis was defined as the date that antituberculosis therapy was initiated. The date of study entry was the date that blood was drawn from participants.

Laboratory methods. PBMC were separated from whole blood within 24 h of obtaining blood from the study participants. PBMC were prepared by density gradient separation from heparinized whole blood, and 10^6 cells/mL were plated in 1 mL of complete RPMI [21]. Selected wells were stimulated with phytohemagglutinin, 1:100 (PHA; Life Technologies); Escherichia coli–derived LPS, 200 ng/mL (Sigma); LPS plus IFN-γ, 1000 U/mL (Genentech); PHA plus IL-12 p70 heterodimer, 1 ng/mL (R&D Systems). PBMC were stimulated for 48 h at 37°C in 5% CO₂; culture supernatants were frozen at −20°C for cytokine determinations. Samples were thawed once and examined for IFN-γ, TNF-α, IL-12, IL-8, and IL-1β concentrations in duplicate by ELISA (R&D Systems) as specified by the manufacturer. All cytokine determinations were done with the same lots of reagents. Laboratory personnel were blinded to the case-control status of the specimens.

Statistical analysis. The sample size was determined to detect a 2-fold difference in median cytokine production between case patients and control patients with 80% power and a 2-tailed α of .05. Cytokine responses between case patients and control patients were compared with the Mann-Whitney U test. χ² and Fisher’s exact tests were used to compare categorical variables. The statistical package STATA, version 6, was used for all analyses.

RESULTS

The demographic and clinical characteristics of all study participants are included in table 1. There were 15 case patients and 28 control patients. Although the sex and age distribution of case patients and control patients were similar, control patients were more likely to be African American and have a history of injection drug use than were case patients. The CD4+ T lymphocyte count and body mass index at the time of cytokine analysis did not differ between case patients and control patients. The site of disease, tuberculin skin test status, and...
time between tuberculosis diagnosis and study entry of each case patient are listed in table 2. Only 1 of the 15 case patients had had concomitant pulmonary tuberculosis. There were 11 cases of reactivation tuberculosis, 2 cases of primary disease, and 2 in which a distinction could not be made. The median time between diagnosis of tuberculosis and cytokine analysis among case patients was 26 months (range, 3–64 months). All of the patients had recovered from symptoms of active tuberculosis by the time of study entry.

Because the innate immune response is activated by bacterial cell wall products, we examined the ability of PBMC to respond to LPS. The median TNF-α response after stimulation with LPS alone was lower in case patients than control patients (833 pg/mL vs. 1149 pg/mL; \( P = .06 \); figure 1). Stimulation of PBMC with LPS plus IFN-γ at 1000 U/mL also elicited less TNF-α production in case patients than control patients (median, 3301 pg/mL vs. 4411 pg/mL; \( P = .04 \)). However, the ratio of TNF-α production in response to LPS plus IFN-γ to TNF-α production in response to LPS alone was no different in case patients (3.72) than in control patients (3.55; \( P = .58 \)), indicating that the IFN-γ receptors in case patients were able to respond normally to IFN-γ.

Having demonstrated normal IFN-γ receptor function, we next looked at production of IFN-γ. The median IFN-γ production by PBMC after stimulation with 1% PHA was lower in case patients than control patients, but the difference was not statistically significant (7888 pg/mL vs. 9517 pg/mL; \( P = .31 \); figure 1). There was no difference in median IFN-γ production between case patients and control patients after stimulation with PHA plus IL-12 (15,983 vs. 16,683 pg/mL; \( P = .22 \)). There was also no difference in median IL-12 production following stimulation with LPS (below detectable levels in both case patients and control patients) or LPS plus IFN-γ (357 pg/mL in case patients vs. 275 pg/mL in control patients; \( P = .68 \)). The ratio of IFN-γ production in response to PHA plus IL-12 to IFN-γ production in response to PHA alone was no different in case patients (1.55) than control patients (1.65; \( P = .65 \)). This ratio is a measure of IL-12 responsiveness mediated by the IL-12 receptor and associated signaling pathways. Therefore, the low levels of LPS- and LPS plus IFN-γ-stimulated TNF-α production among case patients were not due to functional abnormalities of the IFN-γ receptor or the

### Table 1. Characteristics of non–HIV-infected patients with extrapulmonary tuberculosis (case patients) and patients with evidence of latent *Mycobacterium tuberculosis* infection but not active tuberculosis (control patients).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case patients (n = 15)</th>
<th>Control patients (n = 28)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median years</td>
<td>52</td>
<td>47</td>
<td>.26</td>
</tr>
<tr>
<td>Male sex</td>
<td>10 (67)</td>
<td>18 (64)</td>
<td>.88</td>
</tr>
<tr>
<td>Black race</td>
<td>10 (67)</td>
<td>28 (100)</td>
<td>.005</td>
</tr>
<tr>
<td>No alcohol use</td>
<td>12 (80)</td>
<td>21 (75)</td>
<td>.64</td>
</tr>
<tr>
<td>History of injection drug abuse</td>
<td>0</td>
<td>13 (46)</td>
<td>.002</td>
</tr>
<tr>
<td>Body mass index, median kg/m²</td>
<td>24.05</td>
<td>25.08</td>
<td>.9</td>
</tr>
<tr>
<td>CD4 lymphocytes/mm³, median</td>
<td>772</td>
<td>839</td>
<td>.58</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of patients, unless otherwise indicated.

### Table 2. Characteristics of culture-confirmed extrapulmonary tuberculosis cases according to site of disease.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Site of disease</th>
<th>Tuberculin skin test result</th>
<th>Time between diagnosis and study entry (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bone/joint</td>
<td>Positive</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>Bone/joint</td>
<td>Positive</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Bone/joint, soft tissue</td>
<td>Positive</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>Pericardial</td>
<td>Unknown</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>Pericardial</td>
<td>Unknown</td>
<td>47</td>
</tr>
<tr>
<td>6</td>
<td>Genitourinary</td>
<td>0-mm induration</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>Gastrointestinal, pulmonary</td>
<td>Positive</td>
<td>47</td>
</tr>
<tr>
<td>8</td>
<td>Laryngeal</td>
<td>Positive</td>
<td>64</td>
</tr>
<tr>
<td>9</td>
<td>Pleural</td>
<td>Positive</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>Lymphatic–cervical</td>
<td>Positive</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>Lymphatic–axillary</td>
<td>Positive</td>
<td>11</td>
</tr>
<tr>
<td>12</td>
<td>Lymphatic–cervical</td>
<td>Unknown</td>
<td>57</td>
</tr>
<tr>
<td>13</td>
<td>Lymphatic–cervical</td>
<td>Positive</td>
<td>18</td>
</tr>
<tr>
<td>14</td>
<td>Lymphatic–cervical</td>
<td>Positive</td>
<td>37</td>
</tr>
<tr>
<td>15</td>
<td>Lymphatic–mediastinal</td>
<td>Positive</td>
<td>8</td>
</tr>
</tbody>
</table>
IL-12 receptor, nor were they due to decreased production of IFN-γ or IL-12.

Having demonstrated that the IFN-γ synthesis and response pathways that have been implicated in disseminated mycobacterial infection were intact in these patients with prior extrapulmonary tuberculosis, we turned to other cytokines that might be relevant to innate immunity and production of TNF-α. IL-1β is a cytokine critically involved in the generation of fever and cellular activation. Median IL-1β levels did not differ among case patients and control patients after stimulation with LPS (656 pg/mL in case patients vs. 710 pg/mL in control patients; P = .63) or LPS plus IFN-γ (1740 pg/mL in case patients vs. 1635 pg/mL in control patients; P = .90). IL-8 is a chemokine that is involved in leukocyte recruitment to sites of inflammation and has been implicated in the development of severe inflammation in the lung. Median resting levels of

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Figure 1. Cytokine production of peripheral blood mononuclear cells from non–HIV-infected patients with extrapulmonary tuberculosis (cases) and patients with evidence of latent Mycobacterium tuberculosis infection but without active tuberculosis (controls). Left to right: A, TNF-α production without stimulation, after stimulation with lipopolysaccharide (LPS), and after stimulation with LPS plus IFN-γ at 1000 U/mL; B, IFN-γ production without stimulation, after stimulation with phytohemagglutinin (PHA), and after stimulation with PHA plus IL-12; C, IL-8 production without stimulation, after stimulation with LPS, and after stimulation with LPS plus IFN-γ at 1000 U/mL. Units of measurement along Y-axes are pg/mL; scale varies according to values in each boxplot. Line within box represents median, top and bottom of box represent interquartile range, and circles represent outliers. P values for differences between case patients and control patients were not statistically significant, except as noted.
IL-8 were significantly lower in case patients than control patients (8051 pg/mL vs. 19,290 pg/mL; P = .009; figure 1). There were 3 outliers among case patients in terms of IL-8 production, but they were not more likely to have been recently diagnosed with tuberculosis than were others (8, 47, and 57 months between diagnosis and study entry). Interestingly, IL-8 production in case patients and control patients did not differ after stimulation with LPS or LPS plus IFN-γ (figure 1), confining this robust difference to the resting state.

There was no statistically significant difference in cytokine production between case patients with lymphatic versus non-lymphatic extrapulmonary tuberculosis. Among control patients, there were no statistically significant differences in cytokine production between drug users and non–drug users in any of the parameters tested.

**DISCUSSION**

All of the genetic defects predisposing to mycobacterial infection identified to date have involved aspects of the IFN-γ synthesis or response pathways. These include abnormalities in IL-12 production and defects in the IFN-γ receptor 1, IFN-γ receptor 2, IL-12 receptor β1, and IL-12 p40 [20]. All of these defects share the common feature in vitro of impaired mitogen-stimulated IFN-γ production, as would be predicted by the interdependence of IL-12 and IFN-γ production [22–25]. In addition, all patients with IFN-γ receptor 1 or IFN-γ receptor 2 defects have demonstrated in vitro defects in IFN-γ–dependent TNF-α production [26]. Therefore, in this study of HIV-seronegative adults with prior extrapulmonary tuberculosis, we initially sought defects in mitogen-stimulated IFN-γ production and IFN-γ–stimulated TNF-α production. To our surprise, none were found. Although this could be due in part to the small sample size, in each of the functional characterizations done, the IFN-γ synthesis and response pathways did not differ between case patients and control patients. The consistency of these results suggests that there were no significant defects in the IFN-γ pathway among the persons assessed in this study. Therefore, we then assessed other aspects of the immune response relevant to mycobacterial control.

IL-8 production by resting PBMC was significantly lower in case patients than control patients, but this difference did not persist after stimulation with LPS or LPS plus IFN-γ. This suggests that the factors regulating the resting IL-8 “set point” are significantly different in persons with prior extrapulmonary tuberculosis than in persons with latent *M. tuberculosis* infection. The reasons for this difference are unclear but warrant further investigation. IL-8, a CXC chemokine, is chemotactic for T lymphocytes, including those stimulated with *M. tuberculosis* purified protein derivative [27, 28]. IL-8 is required for granuloma formation in rabbits challenged with purified protein derivative [29] and may play a similar role in humans [30]. *M. tuberculosis* and the mycobacterial cell wall component lipoarabinomannan (LAM) stimulate the synthesis and release of IL-8 in vitro from human alveolar macrophages [31]. In pleural fluid from patients with tuberculosis, anti–IL-8 antibodies have been shown to completely block lymphocyte chemotaxis, indicating that IL-8 may be an important mediator of lymphocyte trafficking in tuberculosis [32]. Dysregulation of IL-8 pathways may therefore have important implications for host defense against *M. tuberculosis*. IL-8 pathways have not been previously evaluated in HIV-seronegative patients who have recovered from extrapulmonary tuberculosis, and intrinsic defects in IL-8 production have not previously been associated with tuberculosis.

Although resting TNF-α production did not differ between case patients and control patients, LPS-stimulated TNF-α production was lower in case patients. This difference in TNF-α production persisted when PBMC were stimulated with both LPS and IFN-γ. Recently, Toll-like receptors have been identified as critical components of the signaling pathways of both LPS [33, 34] and LAM [35–37]. Because LPS stimulation has been successfully used in previous studies of cytokine defects in patients with severe nontuberculous mycobacterial infections, we chose to use LPS instead of LAM to explore the LPS-dependent pathways. Although it is possible that stimulation with LAM might produce results different from those obtained with LPS, the demonstration of an abnormality in LPS-mediated cytokine production in these patients is novel and potentially important. Persons with extrapulmonary tuberculosis may have defects in Toll-like receptors and/or elsewhere in the LPS signaling pathway. The defects in TNF-α production and resting IL-8 levels seen in these patients point to abnormalities in the innate immune system, distinct from defects in the IFN-γ synthesis or response pathways seen in patients with severe nontuberculous mycobacterial disease. However, the mechanism underlying these defects is unclear.

There are several limitations of this study. First, the sample size was small, precluding an analysis of potential confounding factors. Second, persons with a history of injection drug use accounted for 46% of control patients but none of the case patients. However, there was no difference in cytokine production among control patients with and without a history of injection drug use. Third, control patients were more likely than case patients to be African American. This could be important if there are racial differences in cytokine production, although this has not been shown to date. Malnutrition [38] and decreased CD4+ lymphocyte level [39] may be associated with an increased risk of tuberculosis and are therefore potential confounders when assessing for risk factors for extrapulmonary tuberculosis. In this study, however, neither nutritional status...
(as determined by body mass index) nor CD4+ lymphocyte count differed among case patients and control patients.

Studies among larger patient populations are necessary to confirm these results. In addition, cytokine responses should be assessed among persons with prior pulmonary tuberculosis and after stimulation with mycobacterial LAM. However, these data demonstrate that in HIV-seronegative patients with prior extrapulmonary tuberculosis, aspects of the innate immune response, but not the IFN-γ and IL-12 receptor pathways, differ significantly in comparison to patients with latent *M. tuberculosis* infection.

**Acknowledgments**

We thank Steven Goodman for assistance with study design, Bernethia Williams for assistance with phlebotomy, and all of the patients who participated in this study.

**References**