Decreased CD4$^+$ lymphocytes and innate immune responses in adults with previous extrapulmonary tuberculosis

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Background: CD4$^+$ lymphocytes control *Mycobacterium tuberculosis* infection through cytokine-mediated macrophage activation. Extrapulmonary tuberculosis is presumably a marker of immunodeficiency, but cytokine responses have not been well studied in such patients.
Objective: Assess immune defects in persons with previous extrapulmonary tuberculosis.
Methods: *In vitro* cytokine responses of PBMCs from HIV-seronegative adults with previous extrapulmonary tuberculosis (n = 10) were compared with responses from persons with previous pulmonary tuberculosis (n = 24) and latent *M tuberculosis* infection (n = 30) in a case-control study.
Results: Patients and controls did not differ according to age, sex, race, or monocytes. The median time between tuberculosis diagnosis and study entry was 72 and 122 weeks in extrapulmonary and pulmonary patients, respectively ($P = .2$). Median CD4$^+$ counts were 660, 814, and 974 lymphocytes/mm^3 in extrapulmonary, pulmonary, and latently infected patients, respectively ($P = .03$). At 48 hours, median unstimulated cytokine levels were uniformly lower in extrapulmonary patients than both sets of controls. These differences persisted after controlling for CD4$^+$ count by linear regression analysis. Despite lower unstimulated levels, median TNF-α response was higher in patients with extrapulmonary and pulmonary tuberculosis than latently infected persons after stimulation with PHA 1% ($P = .006$) and PHA+IL-12 (1 ng/mL; $P = .02$); IL-10 remained low in patients with extrapulmonary tuberculosis after the same stimuli ($P = .04$ and .06, respectively). There was no primary immunodeficiency in the IL-12/23–IFN-γ axis.
Conclusion: HIV-seronegative adults with previous extrapulmonary tuberculosis had lower CD4$^+$ lymphocytes and unstimulated cytokine production. This suggests a subtle abnormality in innate immune function.
Clinical implications: These characteristics could identify persons at risk for severe tuberculosis manifestations.
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Key words: Mycobacterium tuberculosis, *extrapulmonary tuberculosis, cytokines, innate immunity, CD4$^+$ lymphocytes*

Tuberculosis is a major health problem worldwide, with an estimated 8.8 million new cases in 2003 and approximately 2 million deaths each year.[1,2] Although 1/3 of the world's population is infected with *Mycobacterium tuberculosis*, the estimated lifetime risk of disease for a newly infected young child is only 10%.[3,4] Several underlying medical conditions are associated with an increased risk of progressing to tuberculosis disease (eg, HIV infection, diabetes mellitus, renal failure),[5] but tuberculosis can develop in persons who do not have these risk factors. A possible genetic predisposition to tuberculosis has been suggested in several studies,[6-12] but the functional immunologic correlate of the genetic polymorphisms identified is often unclear.

Extrapulmonary tuberculosis (EP-TB) appears to be a marker of an underlying immune defect. The risk of extrapulmonary disease is increased in HIV-infected persons[13-15]; it occurs in 10% to 20% of HIV-seronegative persons but in 40% to 80% of those infected with HIV.[16] The increased risk in HIV-infected persons has been associated with advanced immune suppression (ie, low CD4 count).[17] The risk of EP-TB is also increased in children, presumably because of an immature immune system.[13,14,18,19] Thus, if there is a predisposition to developing tuberculosis, the immunologic defects associated with this predisposition should be most readily identified among persons with extrapulmonary disease.

The importance of cell-mediated immunity in the protective response against *M tuberculosis* is well established. The production of cytokines such as IFN-γ and TNF-α are essential for macrophage activation, control of mycobacterial replication, and granuloma formation and maintenance

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Abbreviations used
BMI: Body mass index
EP-TB: Extrapulmonary tuberculosis
IFN-γR1: IFN-γ receptor 1
IQR: Interquartile range
MANOVA: Multivariate analysis of variance
MCP-1: Monocyte chemoattractant protein 1
PPD: Purified protein derivative
PPD^+: Latent *Mycobacterium tuberculosis* infection
P-TB: Pulmonary tuberculosis

in both mice and human beings.[20-22] The production of IFN-γ and TNF-α, as well as IL-1, IL-6, and IL-8, is important in the innate immune response.[23-26] Thus, an evaluation of the innate and acquired immune response to *M tuberculosis* must assess these and other proinflammatory and anti-inflammatory cytokines. Although the immune defect in patients with EP-TB is likely in cytokine and chemokine response pathways, these pathways have not been well studied in patients with EP-TB.

We previously noted defects in unstimulated IL-8 levels in HIV-seronegative adults with EP-TB compared with persons with latent *M tuberculosis* infection.[27] However, that study was limited by the lack of a control group with pulmonary tuberculosis, and the lack of stimuli that included mycobacterial cell wall antigens to assess cytokine responses. We therefore conducted a follow-up study in newly recruited patients to assess for defects in innate immune responses by measuring unstimulated *in vitro* cytokine responses from PBMC in persons with EP-TB (cases) and 2 sets of controls: persons with pulmonary tuberculosis and those with latent *M tuberculosis* infection. We also investigated cytokine responses after stimulation with mitogen (PHA), proinflammatory cytokines, and purified protein derivative (PPD). Because of the profound defects in cellular immunity that can occur because of HIV infection, we restricted our study to HIV-seronegative persons. Because of the effect of active disease on cytokine responses, we studied only patients who had received curative therapy and were not acutely ill with tuberculosis.

METHODS

Study population

Patients were identified through the Baltimore City Health Department Eastern Chest Clinic and Nashville Metropolitan Health Department Tuberculosis Clinic. Eligibility criteria for case patients included a history of treated culture-confirmed EP-TB, age ≥18 years, and HIV-seronegative status. Extrapulmonary disease was defined as any site outside of the pulmonary parenchyma. Patients with concomitant extrapulmonary and pulmonary disease were eligible. Exclusion criteria included serum creatinine >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 criteria for pulmonary tuberculosis control patients included HIV-seronegative adults ≥18 years old who had completed treatment for culture-confirmed pulmonary tuberculosis and had no evidence of EP-TB. Positive cultures of sputum, bronchoalveolar lavage, or pulmonary parenchyma were required. Controls with latent *M tuberculosis* infection were ≥18 years old, were HIV-seronegative, and had a positive tuberculin skin test (defined as ≥10 mm induration after intradermal placement of 5 tuberculin units of PPD) without evidence of active tuberculosis. Participants in this control group were US-born (and therefore not vaccinated with BCG) and were mostly close contacts of tuberculosis cases. Exclusion criteria for both control groups were the same as for the case group. Controls were drawn from the same 2 clinic populations as cases.

This study was approved by the institutional review boards of the Johns Hopkins Hospital, the Baltimore City Health Department, the National Institutes of Health, Vanderbilt University Medical Center, and the Nashville Metropolitan Health Department. All study participants provided written informed consent.

Laboratory methods

PBMCs were purified at the enrollment site (Baltimore or Nashville) within 24 hours of obtaining the specimens from study participants using density gradient separation from heparinized whole blood; 10^6 cells/mL were plated in 1 mL of complete RPMI (Gibco, Carlsbad, Calif).[27] Selected wells of the PBMCs were stimulated with PHA 1% (Life Technologies, Carlsbad, Calif); PHA plus IL-12p70 heterodimer (R&D Systems, Minneapolis, Minn), 1 ng/mL; *Escherichia coli*-derived LPS, 200 ng/mL (Sigma-Aldrich, St Louis, Mo); LPS plus IFN-γ, 1000 U/mL (Genentech, South San Francisco, Calif); and TNF-α, 10 ng/mL (R&D Systems). PBMCs were stimulated for 48 hours (except stimulation with TNF-α, which was performed for 8 hours, after 40 hours without stimulation) at 37°C in 5% CO_2; there was also a 48-hour unstimulated condition. Culture supernatants were obtained and frozen at –70°C for subsequent cytokine determinations. Cells not used for these experiments were immediately frozen and stored in vapor phase liquid nitrogen. For stimulation with PPD, frozen PBMCs were thawed, adjusted to 10^6 live cells/mL, and stimulated with PPD 10 μg/mL (Statens Serum Institut, Copenhagen, Denmark) for 96 hours.[28] A 96-hour unstimulated condition was also performed. Culture supernatants were obtained and frozen at –70°C as noted. Cell viability on thawing was verified by using the trypan blue exclusion method.

Cytokine detection

Cell culture supernatants were thawed once and examined for IL-1β, IL-4, IL-6, IL-10, IL-12p70, monocyte chemoattractant protein 1 (MCP-1), IFN-γ, and TNF-α concentrations in duplicate by multiplex cytokine array analysis performed by using the Bio-Plex protein multiarray system, which uses Luminex-based technology as specified by the manufacturer (Bio-Rad, Hercules, Calif). IL-8 levels were determined by a commercial ELISA (Pierce, Rockford, Ill) assay run in parallel. All cytokine determinations were performed with the same lots of reagents. Laboratory personnel were blind 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 cases and controls with 80% power and a 2-tailed α value of 0.05. Clinical and demographic characteristics were compared among the 3 groups (EP-TB, pulmonary tuberculosis [P-TB], and latent *M tuberculosis* infection [PPD^+]) using the Kruskal-Wallis test for continuous variables and the χ^2 and Fisher exact tests for categoric variables.

Given the possibility of increased type I error caused by multiple comparisons of cytokine responses, we conducted a global test for the combined effect of the 9 cytokine measures among the 3 patient groups for each stimulus condition using multivariate analysis