

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/mm³ 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. (J Allergy Clin Immunol 2006;117:916-23.)

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 $\frac{1}{3}$ 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 persons¹³⁻¹⁵; 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- γ R1:	IFN- γ receptor 1
IQR:	Interquartile range
MANOVA:	Multivariate analysis of variance
MCP-1:	Monocyte chemoattractant protein 1
PPD:	Purified protein derivative
PPD ⁺ :	Latent <i>Mycobacterium tuberculosis</i> 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% CO₂; 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 μ g/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-1 β , 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 categorical 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

TABLE I. Clinical and demographic characteristics of the study population

Characteristic	EP-TB (n = 10)	Pulmonary TB (n = 24)	PPD ⁺ (n = 30)	P value*
Age, y	46.0 (42.1-57.5)	43.6 (40.3-53.9)	44.6 (38.8-48.0)	.53
# Male sex (%)	7 (70)	14 (58)	18 (60)	.81
# Black race (%)	8 (80)	19 (79)	22 (73)	.42
Monocytes/mm ³	409 (362-580)	400 (283-522)	450 (330-510)	.57
BMI at time of diagnosis, kg/m ²	22.3 (20.1-27.2)	19.2 (17.7-22.3)	25.7 (23.2-29.7)	.0002
BMI at time of study, kg/m ²	27.0 (22.2-29.3)	20.6 (18.2-24.0)	26.4 (23.2-31.5)	.0002
CD4 count	660 (488-1125)	814 (594-938)	974 (703-1219)	.03

NS, Not significant.

Data are medians (IQRs) except as noted.

*Kruskal-Wallis test for continuous variables; χ^2 test for categorical variables.

of variance (MANOVA); MANOVA does not require that the 9 cytokine responses are all in the same direction for each patient group.²⁹ Because many variables were skewed, Box-Cox transformation was performed for all cytokine responses in the MANOVA analysis. The results of Kruskal-Wallis tests were presented for each stimulus condition-cytokine response only when the global test detected a significant effect among the 3 groups. Post hoc pairwise comparisons of the Kruskal-Wallis tests were not performed to minimize the risk of type I error.

Because median baseline CD4⁺ lymphocyte counts differed among the 3 groups, multiple linear regression analysis was performed to compare cytokine responses after adjusting for CD4⁺ lymphocyte count. When the Box-Cox transformation did not improve the normality of the residuals, we performed proportional odds (ordinal) logistic regression analysis with quintiles of each cytokine response as an outcome variable. The statistical packages STATA version 8.2 (Stata Corp, College Station, Tex) and SAS version 9.0 (SAS Institution, Cary, NC) were used for all analyses. A 2-sided significance level of .05 was used for statistical inference.

RESULTS

Clinical characteristics of study patients

There were 10 patients with EP-TB, 24 pulmonary tuberculosis controls, and 30 controls with latent *M tuberculosis* infection. The clinical and demographic characteristics of the study population are shown in Table I. There were no differences between cases and controls according to age, sex, or race. The sites of disease among extrapulmonary cases included lymphatic (n = 3), pleural (n = 3), meningeal (n = 1), disseminated (n = 1), bone/joint (n = 1), peritoneal (n = 1), and testicular (n = 1); 1 patient had both meningeal and disseminated disease. Four patients with EP-TB had concomitant pulmonary disease. None of the extrapulmonary or pulmonary patients had more than 1 episode of tuberculosis. The median time between tuberculosis diagnosis and study entry was 72 (interquartile range [IQR], 50-151) and 122 (IQR, 74-139) weeks in extrapulmonary and pulmonary tuberculosis patients, respectively ($P = .20$).

The median millimeters of induration of the tuberculin skin test was 16, 15, and 15.5 for EP-TB (n = 7), P-TB (n = 21), and PPD⁺ (n = 30) patients, respectively ($P = .98$). Body mass index (BMI) at both the time of diagnosis and time of study entry was high in controls with latent *M tuberculosis* infection and low in persons with previous

pulmonary tuberculosis (Table I). Median height did not differ among the 3 groups (data not shown).

Despite similar responses to tuberculin and monocyte levels in cases and controls, median CD4⁺ lymphocyte levels were lower in patients with EP-TB ($P = .03$; Table I).

Cytokine production of unstimulated and stimulated cells

Cytokine responses after 48 hours of incubation are provided in Table II. Median unstimulated cytokine levels were uniformly lower in patients with EP-TB; these differences were most pronounced for IL-4 and IL-1 β . After stimulation with PHA and PHA+IL-12, cytokine responses were generally robust among patients with EP-TB. Levels of TNF- α were significantly higher in persons with extrapulmonary and pulmonary tuberculosis compared with latent *M tuberculosis* infection (PHA, $P = .006$; PHA+IL-12, $P = .02$). The only cytokine that remained lower in patients with EP-TB after stimulation with PHA or PHA+IL-12 was IL-10 ($P = .04$ and .06, respectively).

Unstimulated cytokine production after 96 hours was notable for uniformly lower median levels in patients with extrapulmonary and pulmonary tuberculosis compared with controls with latent *M tuberculosis* infection; these differences were almost all statistically significant (Table III).

To assess the response to mycobacterial cell wall components and the acquired immune response, cytokine responses to PPD were measured. Frozen cells were available from 57 subjects. The percentage of viable cells was high in all 3 patient groups: mean $\pm$ SD for PPD⁺, P-TB, and EP-TB was 81.5% (± 10.6), 80.4% (± 11), and 81.3% (± 9.8), respectively. After 96 hours of stimulation with PPD, there were no statistically significant differences in cytokine production.

A summary of all statistically significant differences in cytokine responses is provided in Table IV. Cytokine responses that were correlated by factor analysis are provided in this article's Table E1 in the Online Repository at www.jacionline.org.

For both the 48-hour and 96-hour cytokine studies, similar differences in cytokine responses among the 3 patient groups were seen after controlling for baseline

TABLE II. Forty-eight-hour cytokine responses of study patients

Cytokine	Group	Condition					
		Unstimulated	PHA	PHA + IL-12	LPS	LPS+IFN- γ	TNF- α
IL-4	EP-TB	47 (14-413)	2.4 (1.5-3.3)	3.0 (1.4-3.6)	1.4 (1.0-2.1)	2.4 (1.8-3.3)	1.6 (1.5-2.3)
	P-TB	308 (76-726)	4.2 (3.1-6.5)	4.4 (3.1-6.0)	2.4 (1.4-3.4)	3.8 (2.3-5.0)	2.4 (1.9-3.8)
	PPD ⁺	320 (47-501)	2.6 (1.5-3.7)	3.2 (1.8-4.0)	2.0 (0.8-3.0)	3.7 (1.6-4.5)	2.4 (1.4-3.6)
	<i>P</i> value	.03	.001	.01	NS	NS	NS
IL-6	EP-TB	1536 (142-110,559)	733.8 (503-1396)	914.8 (527-1606)	714.7 (357-1008)	1434.0 (583-3239)	3.5 (2.0-192)
	P-TB	54,449 (785-97,700,000)	1018.1 (499-2193)	1167.3 (576-2057)	742.0 (492-1690)	1182.7 (566-4030)	109.5 (3.2-324)
	PPD ⁺	154,383 (1617-711,772)	931.7 (510-1284)	938.8 (637-1642)	718.1 (473-1077)	1749.7 (973-3619)	217.5 (20-306)
	<i>P</i> value	.10	.65	.77	NS	NS	NS
IL-10	EP-TB	12 (0-870)	3.1 (2.2-4.6)	3.5 (2.7-7.5)	2.0 (0.7-4.1)	1.6 (1.0-2.9)	0.1 (0-0.9)
	P-TB	297 (12-1558)	9.4 (4.9-15)	9.6 (4.6-15)	4.2 (2.4-6.2)	2.5 (1.4-3.6)	0.4 (0-2.5)
	PPD ⁺	574 (138-1957)	8.6 (4.4-13)	9.0 (3.7-15)	3.0 (1.4-4.9)	2.2 (0.8-3.4)	0.4 (0.2-1.4)
	<i>P</i> value	.17	.04	.06	NS	NS	NS
IL-12	EP-TB	1 (0-20)	0.1 (0-0.3)	4.1 (0.4-6.7)	0.06 (0-0.1)	0.4 (0.1-1.0)	0.02 (0-0.1)
	P-TB	16 (1-32)	0.2 (0.1-0.3)	1.3 (0.5-5.4)	0.09 (0-0.1)	0.8 (0.3-1.2)	0.07 (0-0.1)
	PPD ⁺	22 (5-28)	0.2 (0.1-0.2)	0.6 (0.4-0.9)	0.1 (0-0.1)	0.7 (0.2-1.4)	0.1 (0-0.1)
	<i>P</i> value	.12	.36	.07	NS	NS	NS
IFN- γ	EP-TB	43 (7-1663)	32.9 (24-56)	207.0 (74-345)	4.3 (2.5-7.7)	383.0 (224-810)	7.5 (6.3-9.2)
	P-TB	1047 (62-2824)	35.9 (20-83)	76.3 (40-180)	9.0 (5.1-11)	434.2 (268-799)	10.4 (8.6-15)
	PPD ⁺	1095 (88-1924)	23.2 (9.6-72)	53.5 (26-145)	6.3 (2.5-10)	170.5 (83-584)	9.8 (6.9-13)
	<i>P</i> value	.06	.42	.26	NS	NS	NS
TNF- α	EP-TB	12 (1-374)	16.6 (10-35)	23.8 (10-38)	1.3 (0.7-2.7)	14.7 (5.6-31)	—
	P-TB	350 (14-1719)	19.2 (0.9-28)	23.4 (10-32)	2.7 (1.5-3.9)	20.4 (8.1-35)	—
	PPD ⁺	332 (19-683)	7.1 (0.2-14)	11.9 (4.0-20)	1.7 (1.0-3.3)	17.5 (12-24)	—
	<i>P</i> value	.14	.006	.02	NS	NS	—
IL-1 β	EP-TB	1 (1-1004)	2.6 (1.8-3.4)	3.4 (2.2-4.1)	3.9 (1.1-8.7)	8.3 (4.0-13.8)	0.4 (0.3-1.2)
	P-TB	886 (6-3448)	4.6 (2.1-8.7)	4.5 (2.5-12.1)	6.6 (3.2-14.8)	12.7 (6.3-21.4)	1.4 (0.3-4.1)
	PPD ⁺	873 (173-1515)	2.9 (1.8-5.7)	4.2 (2.7-8.3)	5.1 (2.3-9.3)	13.5 (7.0-23.0)	1.1 (0.4-2.1)
	<i>P</i> value	.04	.20	.21	NS	NS	NS
MCP-1	EP-TB	24,770 (4319-109,961)	743.2 (162-1069)	649.7 (204-3622)	77.4 (37-160)	70.0 (43-164)	37.0 (5.3-73)
	P-TB	59,156 (16,101-17,700,000)	233.0 (87-1207)	174.0 (99-756)	63.3 (34-119)	39.0 (18-188)	51.0 (19-142)
	PPD ⁺	125,961 (3272-34,300,000)	403.5 (121-753)	291.0 (120-764)	85.5 (55-163)	69.3 (28-196)	136.8 (37-244)
	<i>P</i> value	.58	.58	.63	NS	NS	NS
IL-8	EP-TB	60,215 (36,927-427,892)	712.8 (577-797)	631.2 (588-806)	543.9 (417-669)	547.5 (365-789)	127.4 (87-554)
	P-TB	164,285 (57,298-483,245)	626.5 (537-847)	607.1 (519-810)	583.3 (495-776)	438.4 (342-690)	214.0 (102-508)
	PPD ⁺	284,291 (158,580-399,846)	563.8 (461-675)	612.6 (445-681)	554.0 (454-780)	524.1 (383-704)	403.2 (174-541)
	<i>P</i> value	.28	.17	.75	NS	NS	NS

Median cytokine production of PBMCs after 48 hours of incubation (except TNF- α , 8 hours). All *P* values are for the Kruskal-Wallis test. *P* values < .05 are in bold. IQRs are in parentheses. Concentrations are pg/mL for unstimulated conditions and ng/mL for stimulated conditions. Concentration of stimuli, all at final concentration: PHA, 1%; IL-12, 1 ng/mL; LPS, 200 ng/mL; TNF- α , 10 ng/mL. To account for multiple comparisons, a MANOVA test was performed for each stimulus condition to assess the combined effect of all 9 cytokine measures among the 3 patient groups. Kruskal-Wallis *P* values for individual conditions are presented only for conditions in which the MANOVA test detected a statistically significant difference among the 3 patient groups overall; otherwise, *P* values are presented as not significant (NS).

TABLE III. Ninety-six-hour cytokine responses of study patients

Cytokine	Group	Condition	
		Unstimulated	PPD
IL-4	EP-TB	116 (86-190)	1.4 (0.9-1.5)
	P-TB	90 (77-408)	1.2 (0.7-2.2)
	PPD ⁺	574 (369-806)	1.7 (1.2-2.0)
	<i>P</i> value	.02	NS
IL-6	EP-TB	5385 (1701-38,754)	421.0 (92-605)
	P-TB	2499 (1276-169,215)	456.8 (244-1143)
	PPD ⁺	1,176,435 (233,235-7,117,795)	608.5 (334-1529)
	<i>P</i> value	.02	NS
IL-10	EP-TB	17 (10-25)	0.4 (0.1-0.4)
	P-TB	12 (7-123)	0.4 (0.1-0.9)
	PPD ⁺	570 (85-1416)	0.5 (0.1-1.3)
	<i>P</i> value	.009	NS
IL-12	EP-TB	6 (3-13)	0.07 (0-0.06)
	P-TB	5 (0-14)	0.05 (0-0.08)
	PPD ⁺	18 (8-26)	0.05 (0-0.07)
	<i>P</i> value	.02	NS
IFN- γ	EP-TB	237 (120-539)	7.8 (3.1-10)
	P-TB	159 (98-1276)	5.1 (2.1-9.1)
	PPD ⁺	1911 (1199-2529)	6.5 (4.1-12)
	<i>P</i> value	.01	NS
TNF- α	EP-TB	46 (31-107)	2.4 (0.6-5.5)
	P-TB	21 (15-384)	1.2 (0.5-2.3)
	PPD ⁺	697 (225-1634)	1.7 (0.8-3.4)
	<i>P</i> value	.02	NS
IL-1 β	EP-TB	27 (6-165)	0.5 (0.2-0.6)
	P-TB	16 (9-2239)	1.3 (0.3-3.4)
	PPD ⁺	1815 (324-3300)	4.5 (1.4-6.9)
	<i>P</i> value	.07	NS
MCP-1	EP-TB	166,709 (112,494-34,300,000)	546.1 (105-881)
	P-TB	256,971 (44,275-924,724)	427.4 (300-1019)
	PPD ⁺	34,300,000 (171,974-34,300,000)	376.0 (195-914)
	<i>P</i> value	.13	NS
IL-8	EP-TB	73,932 (54,433-133,160)	363.3 (262-420)
	P-TB	83,461 (69,914-109,101)	385.5 (297-570)
	PPD ⁺	235,541 (93,192-356,017)	344.5 (283-463)
	<i>P</i> value	.004	NS

Median cytokine production of PBMCs after 96 hours of incubation. All *P* values are for the Kruskal-Wallis test. *P* values < .05 are in bold. IQRs are in parentheses. Concentrations are pg/mL for unstimulated conditions and ng/mL for stimulated conditions. Concentration of PPD: 10 μ g/mL (at final concentration). To account for multiple comparisons, a MANOVA test was performed separately for both conditions to assess the combined effect of all 9 cytokine measures among the 3 patient groups. Kruskal-Wallis *P* values for individual conditions are presented only for the condition in which the MANOVA test detected a statistically significant difference among the 3 patient groups overall; otherwise, *P* values are presented as not significant (NS).

CD4⁺ lymphocyte count via linear regression analysis (data not shown).

Assessment for complete functional immune defects

IFN- γ receptor 1 (IFN- γ R1) deficiency is characterized by severe and frequently disseminated mycobacterial infections.³⁰ To assess for evidence of defects in IFN- γ R1, we compared the ratio of TNF- α produced in response to LPS plus IFN- γ to TNF- α produced in response to LPS alone in cases and controls. The median ratios were 7.0, 6.5, and 9.7 in EP-TB, P-TB, and PPD⁺ patients, respectively (*P* = .20).

By using a similar approach, we assessed for evidence of IL-12 hyporesponsiveness. Because *in vitro* IL-12 levels can directly influence IFN- γ production, IL-12 hyporesponsiveness could predispose to disseminated mycobacterial infections. We compared the ratio of IFN- γ produced in response to PHA plus IL-12p70 to IFN- γ produced in response to PHA alone in cases and controls. The median ratios were 3.0, 2.2, and 2.5 in EP-TB, P-TB, and PPD⁺ patients, respectively (*P* = .30).

DISCUSSION

Persons in our study with previous EP-TB had significantly lower median CD4⁺ lymphocyte levels than persons with pulmonary tuberculosis or latent *M tuberculosis* infection. In both HIV-seronegative and HIV-seropositive persons with tuberculosis, acute disease is associated with decreased CD4⁺ lymphocyte levels. However, the CD4⁺ lymphocytopenia corrects to normal levels with antituberculosis therapy.³¹⁻³³ The patients in this study are different in that the CD4⁺ lymphocytopenia was detected long after therapy, and all had recovered from tuberculosis. Also of note, a gradient of median CD4⁺ lymphocyte counts was identified, decreasing from PPD⁺ to P-TB to patients with EP-TB. The finding that persons who have recovered from EP-TB have lower CD4⁺ lymphocyte levels is novel.

Unstimulated cytokine levels were uniformly low in patients with EP-TB at both 48 and 96 hours. This was not a result of decreased cell viability, because cytokine responses were normal after stimulation. These findings are consistent with our previous demonstration of low unstimulated IL-8 levels in persons with extrapulmonary tuberculosis compared with persons with latent infection.²⁷ These data indicate a subtle difference in immune function in persons with EP-TB that results in low constitutive levels of several soluble factors. The findings also suggest a global defect in cytokine production rather than a defect in the production of a particular cytokine or chemokine.

The rank order of unstimulated cytokine production differed between the 48-hour and 96-hour conditions. At 48 hours, patients with EP-TB had the lowest cytokine production, whereas at 96 hours, cytokine production was low among both extrapulmonary and pulmonary patients. However, the 48-hour condition was performed on fresh

TABLE IV. Summary of statistically significant cytokine responses

Cytokine	Group	Condition			
		Unstimulated (48 h)	Unstimulated (96 h)	PHA (48 h)	PHA+IL-12 (48 h)
IL-4	EP-TB	47 (14-413)	116 (86-190)	2.4 (1.5-3.3)	3.0 (1.4-3.6)
	P-TB	308 (76-726)	90 (77-408)	4.2 (3.1-6.5)	4.4 (3.1-6.0)
	PPD ⁺	320 (47-501)	574 (369-806)	2.6 (1.5-3.7)	3.2 (1.8-4.0)
	<i>P</i> value	.03	.02	.001	.01
IL-6	EP-TB		5385 (1701-38,754)		
	P-TB		2499 (1276-169,215)		
	PPD ⁺		1,176,435 (233,235-7,117,795)		
	<i>P</i> value		.02		
IL-10	EP-TB		17 (10-25)	3.1 (2.2-4.6)	
	P-TB		12 (7-123)	9.4 (4.9-15)	
	PPD ⁺		570 (85-1416)	8.6 (4.4-13)	
	<i>P</i> value		.009	.04	
IL-12	EP-TB		6 (3-13)		
	P-TB		5 (0-14)		
	PPD ⁺		18 (8-26)		
	<i>P</i> value		.02		
IFN- γ	EP-TB		237 (120-539)		
	P-TB		159 (98-1276)		
	PPD ⁺		1911 (1199-2529)		
	<i>P</i> value		.01		
TNF- α	EP-TB		46 (31-107)	16.6 (10-35)	23.8 (10-38)
	P-TB		21 (15-384)	19.2 (0.9-28)	23.4 (10-32)
	PPD ⁺		697 (225-1634)	7.1 (0.2-14)	11.9 (4.0-20)
	<i>P</i> value		.02	.006	.02
IL-1 β	EP-TB	1 (1-1004)			
	P-TB	886 (6-3448)			
	PPD ⁺	873 (173-1515)			
	<i>P</i> value	.04			
IL-8	EP-TB		73,932 (54,433-133,160)		
	P-TB		83,461 (69,914-109,101)		
	PPD ⁺		235,541 (93,192-356,017)		
	<i>P</i> value		.004		

Median cytokine production of PBMCs. All *P* values are for the Kruskal-Wallis test. *P* values < .05 are considered statistically significant and are in bold. IQRs are in parentheses. Concentrations are pg/mL for unstimulated conditions and ng/mL for stimulated conditions. Concentration of stimuli, all at final concentration: PHA, 1%; IL-12, 1 ng/mL.

cells and the 96-hour condition on cells that had been frozen and subsequently thawed. Such a difference in conditions makes comparisons between the 2 time points difficult. However, unstimulated cytokine levels in persons with extrapulmonary disease are clearly low at both time points.

The lower CD4⁺ lymphocyte and unstimulated cytokine levels in persons with previous EP-TB raise the possibility that these 2 findings may be related. However, multivariable regression analyses revealed that after controlling for CD4⁺ lymphocyte count, the differences in cytokine responses persisted. This suggests that the differences in cytokine responses among the 3 groups were independent of CD4⁺ count.

IL-10 production after stimulation with PHA and PHA+IL-12 was lower in patients with EP-TB. There was also essentially no difference in IL-10 response in any of the 3 patient groups after stimulation with PHA+IL-12

compared with PHA alone. Similar to a previous report, we also noted higher levels of IL-10 after stimulation with LPS in P-TB patients, although the difference was not statistically significant in our population.³⁴

Resting TNF- α levels were low in patients with EP-TB, but TNF- α responses after stimulation with PHA and PHA+IL-12 were higher in EP-TB and P-TB patients than in persons with latent *M tuberculosis* infection. TNF- α is important for controlling the extent of *M tuberculosis* infection and granuloma formation,^{21,35,36} and TNF- α blockers dis-inhibit latent *M tuberculosis* infection, resulting in active tuberculosis, particularly extrapulmonary disease.^{37,38} However, TNF- α does not play a strictly beneficial role in tuberculosis pathogenesis: increased plasma TNF- α levels have been associated with clinical deterioration early in the treatment of severe tuberculosis.³⁹ A possible unifying hypothesis on the role of TNF- α in tuberculosis pathogenesis is that low resting TNF- α levels

could predispose to the development of disseminated tuberculosis, and that TNF- α levels remain increased after the development of active disease.

Antigen stimulation with PPD did not result in significant differences in cytokine response among the 3 patient groups, suggesting that the differences in host immune response among the 3 groups are not antigen-driven.

The ratio of TNF- α produced in response to LPS plus IFN- γ /LPS alone did not differ among cases and controls, making it unlikely that defects in IFN- γ R1 predisposed to EP-TB in these study patients. IL-12 responsiveness, as assessed by the ratio of IFN- γ produced in response to PHA plus IL-12p70 / PHA alone, was also similar in all 3 patient groups. Therefore, we found no evidence of a primary immunodeficiency in the IL-12/23-IFN- γ axis.

There were statistically significant differences in BMI among the 3 patient groups. As one might expect, at the time of tuberculosis diagnosis, persons with either pulmonary or extrapulmonary disease had lower BMI than persons with latent *M tuberculosis* infection. At the time of this study, when PBMCs were obtained and all study patients had recovered from acute illness, the median BMI of persons with extrapulmonary disease was similar to that of persons with latent infection, but the median BMI of pulmonary TB patients remained lower. A previous study found that low BMI was an independent risk factor for tuberculosis, although a distinction was not made between pulmonary and extrapulmonary disease.⁴⁰ It is interesting to note that the median BMI of patients with pulmonary tuberculosis was within the normal range (18.5-24.9 m/kg²), whereas BMI > 25 is overweight (<http://nhlbisupport.com/bmi/bimcalc.htm>).

Given the several cytokines and conditions tested, the issue of multiple comparisons arises. To account for multiple comparisons, a MANOVA test assessed for the combined effect of all 9 cytokine measures per condition to identify those results that should be reported as statistically significant. Findings from each cytokine-condition pair was presented only when the global test detected the overall difference. Given the relatively small sample size and the acknowledgment that these global tests may be underpowered, further study with larger sample size may be warranted to confirm our findings for these measures.

Several limitations of this study should be noted. First, EP-TB may be a complex disorder in which the epidemiology and pathophysiology differ according to the site of disease.¹⁴ However, although there is enhanced, rather than diminished, immune response at the site of disease in pleural tuberculosis,^{41,42} we are unaware of differences in systemic immune response according to the site of EP-TB. Similarly, it is unclear whether patients with EP-TB in this study had disease because of reactivation of latent *M tuberculosis* infection or progressive primary disease, or whether PBMC cytokine responses are of similar importance for both mechanisms of disease. Second, patients with extrapulmonary and pulmonary tuberculosis were evaluated after having recovered from the disease. Although this avoided the bias caused by acute illness on CD4⁺ lymphocyte and cytokine levels, we do not

know whether levels after recovery are predictive of those before developing disease. Finally, it is possible that some of the controls with latent *M tuberculosis* infection may develop active tuberculosis in the future. However, all of our PPD⁺ controls received treatment for latent infection, making such an occurrence unlikely.

Collectively, these data demonstrate that HIV-seronegative persons with previous EP-TB have an innate immune response that differs significantly compared with persons with previous pulmonary tuberculosis and latent *M tuberculosis* infection. There was no evidence of a primary defect in the IL-12/23-IFN- γ axis. We speculate that a subtle immune defect leads to abnormal control of basal cytokine and chemokine production, and possibly CD4⁺ lymphocyte levels. This is associated with a decreased ability to contain *M tuberculosis* at the site of primary infection, permitting the development of EP-TB.

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Correction

With regard to the December 2005 article entitled "Innate immunity for biodefense: A strategy whose time has come" (2005;116:1334-42): Kyle S. Dunn, BS, was listed as an author. This inclusion was made in error and Mr. Dunn's name should not be affiliated with this article.