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Abnormal Immune Responses in Persons with Previous Extrapulmonary Tuberculosis in an *In Vitro* Model That Simulates *In Vivo* Infection with *Mycobacterium tuberculosis*

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Persons with previous extrapulmonary tuberculosis have reduced peripheral blood mononuclear cell cytokine production and CD4$^+$ lymphocytes compared to persons with previous pulmonary tuberculosis or latent tuberculosis infection, but specific defects related to *Mycobacterium tuberculosis* infection of macrophages have not been characterized. The objective of this study was to further characterize the *in vitro* immune responses to *M. tuberculosis* infection in HIV-seronegative persons with previous extrapulmonary tuberculosis. Peripheral blood mononuclear cells were isolated from HIV-seronegative persons with previous extrapulmonary tuberculosis ($n = 11$), previous pulmonary tuberculosis ($n = 21$), latent *M. tuberculosis* infection ($n = 19$), and uninfected tuberculosis contacts ($n = 20$). Experimental conditions included *M. tuberculosis*-infected macrophages cultured with and without monocyte-depleted peripheral blood mononuclear cells. Concentrations of interleukin 1β (IL-1β), IL-4, IL-6, CXCL8 (IL-8), IL-10, IL-12p70, IL-17, CCL2 (monocyte chemoattractant protein 1), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) were measured by multiplex cytokine array. When *M. tuberculosis*-infected macrophages were cocultured with monocyte-depleted peripheral blood mononuclear cells, IFN-γ ($P = 0.01$), TNF-α ($P = 0.04$), IL-10 ($P < 0.001$), and IL-6 ($P = 0.03$) exhibited similar continua of responses, with uninfected persons producing the lowest levels, followed by extrapulmonary tuberculosis cases, pulmonary tuberculosis controls, and persons with latent *M. tuberculosis* infection. A similar pattern was observed with CXCL8 ($P = 0.04$), IL-10 ($P = 0.02$), and CCL2 ($P = 0.03$) when monocyte-depleted peripheral blood mononuclear cells from the four groups were cultured alone. Persons with previous extrapulmonary tuberculosis had decreased production of several cytokines, both at rest and after stimulation with *M. tuberculosis.* Our results suggest that persons who develop extrapulmonary tuberculosis have a subtle global immune defect that affects their response to *M. tuberculosis* infection.

Tuberculosis (TB) presents an interesting paradox: while one-third of the world's population is infected with *Mycobacterium tuberculosis*, only 5 to 10% of those infected will develop active disease (1, 23). This observation suggests that factors other than the mycobacterium play a role in the development of TB. Our understanding of protective immunity to *M. tuberculosis* infection is incomplete. The immune response to *M. tuberculosis* involves cells such as monocytes, macrophages, and T lymphocytes that produce cytokines such as CXCL8 (interleukin-8 [IL-8]), IL-12, gamma interferon (IFN-γ), and tumor necrosis alpha (TNF-α) (16). While hematogenous dissemination occurs in most persons soon after infection, subsequent extrapulmonary TB disease does not (25). Development of extrapulmonary disease could be a result of a breakdown in host immune surveillance, a change in the mycobacteria from a dormant to an active state, or a combination of the two. Discovery of factors that predispose to extrapulmonary disease will advance TB prevention efforts by identifying immune responses that could be boosted by TB vaccines. It will also help identify individuals at increased risk for progression from latent infection to clinical disease, so that more intensive observation and treatment of latent infection can be initiated.

Extrapulmonary TB is associated with underlying immune defects. For example, HIV-infected persons are at increased risk of extrapulmonary TB, and this risk increases as the CD4$^+$ T lymphocyte count declines (30). Young children also have an increased incidence of extrapulmonary TB, specifically TB meningitis, presumably due to an immature immune system (31). Our group has previously noted reduced peripheral blood mononuclear cell (PBMC) cytokine production and CD4$^+$ T lymphocytes in HIV-seronegative adults with previous extrapulmonary TB compared to persons with previous pulmonary TB or latent *M. tuberculosis* infection (2, 38). In a subset of patients from the present study, we also found that persons with previous extrapulmonary TB had increased regulatory T cell frequency and CD4$^+$ lymphocyte activation, indicating possible immune dysregulation (8). However, an evaluation of the sequential activation of innate and adaptive immune responses to *M. tuberculosis* infection in such patients has not been performed. We therefore assessed *in vitro* immune responses in persons with previous extrapulmonary TB under several experimental conditions, including one in which autologous monocyte-depleted PBMCs were cocultured with *M. tuberculosis*-infected host macrophages.

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MATERIALS AND METHODS

Subject recruitment. Cases were defined as persons with previously treated extrapulmonary TB. There were three sets of controls: (i) persons with previously treated pulmonary TB, (ii) persons with latent *M. tuberculosis* infection, and (iii) persons who had been exposed to culture-positive pulmonary TB but were not infected (i.e., tuberculin skin test [TST] negative). Inclusion criteria consisted of the following: age >18 years at the time of diagnosis of TB disease or infection; HIV seronegative; culture-confirmed disease and either near completion (within 1 month) or completion of therapy (for extrapulmonary cases and pulmonary controls); and TST induration of >10 mm (for latent *M. tuberculosis* infection controls). We did not require persons to complete therapy for latent infection in order to be enrolled. Only contacts of culture-positive pulmonary TB cases or known TST converters were included as controls. Contacts of culture-positive pulmonary TB cases were tested for latent infection at the beginning of the contact investigation and after 8 to 12 weeks (5). Exclusion criteria consisted of the following: serum creatinine of >2 mg/dl; use of corticosteroids or other immunosuppressive agents at the time of diagnosis or study entry; malignancy; and diabetes mellitus. Pleural TB represents an exaggerated (not diminished) cell-mediated immune response (3). Because persons with pleural TB may exhibit a unique immunopathogenesis compared with the other forms of extrapulmonary TB, we excluded these individuals from our study.

All cases and controls were enrolled in Tennessee. Extrapulmonary TB cases and pulmonary TB controls were identified by review of the Tennessee Department of Health TB registry. Ongoing contact investigations at local and regional TB clinics were reviewed to identify patients in the remaining control groups. Demographic and clinical characteristics were collected from the patient or the Tennessee TB registry.

The institutional review boards of Vanderbilt University, Nashville Davidson Metro Public Health Department, and the Tennessee Department of Health approved the study. Study participants provided written informed consent.

Sample processing. HIV serology and a complete blood count were performed for each subject. PBMCs were isolated within 24 h under sterile conditions by Ficoll-Paque (GE Healthcare Bio-Science) density centrifugation. Viability was estimated by trypan blue dye exclusion. PBMCs were immediately suspended at a concentration of 10^7/ml in complete RPMI (2).

$CD14^+$ monocytes were positively selected from PBMCs according to the manufacturer's instructions (Stemcell Technologies). Monocytes and monocyte-depleted PBMCs (PBMC-M) were stained with trypan blue to estimate viability. After isolation, aliquots of PBMCs, monocytes, and PBMC-M were stained for viability with the Live Dead Fixable Aqua Dead Cells stain kit (Invitrogen), followed by surface staining with anti-CD14 antibody (PE-Cy7; eBioscience) to assess purity. Flow cytometry results were obtained by gating on viable cells.

Isolated monocytes were plated into a 48-well plate at a concentration of 2.5×10^5/ml in complete RPMI. PBMC-M were frozen at a concentration of 10^7/ml in freezing media (10% dimethyl sulfoxide [DMSO], 90% fetal bovine serum [FBS]). Monocytes were incubated for 7 days at 37°C and 5% CO_2. Following incubation, wells were examined under magnification of ×40 for confirmation of morphological transformation of monocytes to confluent macrophages.

Infection of macrophages with *M. tuberculosis* H37Rv. *Mycobacterium tuberculosis* H37Rv (ATCC 25618) was cultivated as previously described (10). All stocks were quantified by serial dilution, and aliquots were frozen. On the day of infection, one vial of H37Rv was thawed, washed once with media (complete RPMI without penicillin or streptomycin), and suspended in media to a final concentration of 1.25×10^6 CFU/ml (multiplicity of infection, 5:1). One milliliter of H37Rv suspension was added to selected macrophage wells. After 24 h of infection, the same wells were washed twice with Dulbecco's phosphate-buffered saline 1× (DPBS×1) to eliminate extracellular mycobacteria, and 1 ml of antibiotic-free medium was replaced.

Twenty-four hours after the wash, autologous PBMC-M were thawed and stained with trypan blue to assess viability. PBMC-M were suspended in R-10 antibiotic-free medium at a concentration of 3×10^6/ml. In one infected macrophage well and one uninfected macrophage well, medium was removed and replaced with 1 ml of the PBMC-M suspension. Separate wells with PBMC-M alone and PBMC-M plus staphylococcal enterotoxin B (SEB) (10 ng/ml) were also plated as negative and positive controls, respectively.

Cytokine bead array. After 48 h of incubation following addition of PBMC-M, 150 μl of culture supernatant was withdrawn from each of six conditions and frozen at −80°C. Cell culture supernatant was thawed once and examined for IL-1β, IL-4, IL-6, CXCL8 (IL-8), IL-10, IL-12p70, IL-17, CCL2 (monocyte chemoattractant protein 1 [MCP-1]), TNF-α, and IFN-γ by multiplex cytokine array analysis using the Bio-Plex protein multiarray system (Bio-Rad, Hercules, CA). All cytokine determinations were performed with the same lot of reagents. Laboratory personnel performing the cytokine bead array were blind to the case-control status of the specimens.

Statistical analysis. The sample size was calculated based on log-transformed means of unstimulated cytokines (2). Twenty subjects in each group were required to detect an effect size of 1.7 standard deviation (SD) of the log-transformed mean, with 80% power and 2-sided 5% significance level using analysis of variance. Clinical and demographic characteristics were compared among the 4 patient groups using the Kruskal-Wallis test for continuous variables and the chi-square and Fisher exact tests for categorical variables. A P value of <0.05 was considered significant.

In order to minimize the risk of increased type I error caused by multiple comparisons of cytokine responses, we conducted a generalized estimating equation (GEE) as a global test to simultaneously compare differences in the 10 cytokine measurements among the four patient groups, using proportional odds logistic regression. A robust sandwich estimator was used to address heavily skewed cytokine responses. The global test was performed separately within each experimental condition and was controlled for white blood cell (WBC) count. We also controlled for the effect of race (black versus nonblack) because our previous epidemiologic work suggested that blacks were more likely to present with extrapulmonary TB (13). Also, persons of the black race have low numbers of WBCs and differences in monocyte function compared to persons of other races (6, 35). When the global test was rejected, showing a significant effect of patient groups, we further analyzed the effect of group on each cytokine through a similar multivariable regression model, again controlling for WBC count and race. This global test is not able to predict where the difference lies, and *post hoc* pairwise comparisons among the four patient groups were not performed to minimize the risk of type I error. To determine if a correlation existed between cytokine values and the time between TB therapy completion and study enrollment, a Spearman's rank correlation was performed with each cytokine only under experimental conditions that were found to be globally significant. If a correlation was found, a proportional odds regression model adjusting for the time variable was performed to determine if this correlation differed by group.

RESULTS

Clinical characteristics of the study population. Between 2008 and 2009, we enrolled 11 persons with previous extrapulmonary TB, 21 with previous pulmonary TB, 19 with latent *M. tuberculosis* infection, and 20 uninfected contacts of pulmonary TB cases. All of the persons with previous clinical TB had completed therapy. Two persons with latent infection declined preventive treatment, and three latently infected persons were enrolled during their final month of therapy. The clinical and demographic characteristics of

TABLE 1 Demographic characteristics of the study population[a]

Characteristic	TST negative ($n = 20$)	Latent TB infection ($n = 19$)	Pulmonary TB ($n = 21$)	Extrapulmonary TB ($n = 11$)	P value[b]
Age (yr) (IQR)[c]	43 (33–52)	45 (39–64)	49 (40–59)	41 (27–71)	0.27
Male	7 (35)	7 (37)	13 (62)	6 (55)	0.27
Black race[d]	7 (25)	2 (11)	9 (43)	6 (55)	0.02
Foreign born[e]	0 (0)	2 (11)	5 (24)	8 (73)	0.007
Homeless	0 (0)	1 (6)	5 (24)	0 (0)	0.03
Alcohol use	1 (5)	1 (5)	7 (33)	0 (0)	0.03
Tobacco use	6 (30)	4 (19)	11 (52)	1 (9)	0.05
Illicit drug use	0 (0)	0 (0)	2 (9)	0 (0)	0.18
No. of WBCs/mm^3 (IQR)[c]	8.2 (7.0–9.1)	7.2 (6.3–7.8)	5.9 (5.5–7.7)	5.4 (4.3–7.5)	0.01
No. of CD4$^+$ cells/mm^3 (IQR)[c]	1,078 (882–1,332)	1,175 (797–1,495)	1,058 (708–1,206)	917 (673–1,058)	0.15
No. of mos. from treatment completion to study entry[f]	NA[g]	18 (2–26)	5 (0.25–11)	21 (6–39)	0.09

[a] Data are presented as numbers (%) of individuals unless otherwise noted.
[b] Kruskal-Wallis test and Pearson's chi-square test.
[c] Values are median numbers, with IQRs (interquartile ranges) in parentheses.
[d] Other races that were represented in the study population were Caucasian, Asian, and Hawaiian/Pacific Islander.
[e] The classification of foreign-born individuals by race is as follows: Caucasians ($n = 4$), Black ($n = 5$), Asian ($n = 5$), Hawaiian/Pacific Islander ($n = 1$).
[f] Two persons with latent TB infection did not complete therapy.
[g] NA, not applicable.

the study population are listed in Table 1. Sites of disease among extrapulmonary cases included lymphatic sites ($n = 4$), areas near the spine ($n = 1$), vertebra ($n = 1$), miliary sites ($n = 1$), and the genitourinary tract ($n = 1$), peritoneum ($n = 1$), skin ($n = 1$), and breast ($n = 1$).

Cytokine bead array. All median cytokine results (pg/ml) measured at 48 h after the addition of PBMC-M are presented in Table 2, with the exception of IL-4, which was detectable only when PBMC-M were stimulated with SEB. Levels of IL-12p70 were low under each condition tested, perhaps due to the time point at which supernatants were collected (4). The two experimental conditions that were globally significant were (i) autologous PBMC-M cocultured with *M. tuberculosis*-infected macrophages and (ii) autologous PBMC-M cultured alone. Thus, differences between the patient groups for each cytokine could be evaluated statistically under these conditions.

Compared to infected macrophages alone, all cytokines tested increased when autologous PBMC-M were added to infected macrophages, but a pattern emerged in the latter condition when evaluating by patient group. After controlling for race and WBCs, median production of IL-6 ($P = 0.03$), IL-10 ($P < 0.001$), TNF-α ($P = 0.04$), and IFN-γ ($P = 0.01$) was lowest in TST-negative contacts, followed by persons with previous extrapulmonary TB, latently infected persons, and persons with previous pulmonary TB (Fig. 1). A similar trend was seen for IL-1β and CXCL8, but it was not statistically significant. Production of IL-17 was highest in persons with previous extrapulmonary TB ($P = 0.01$). Persons with previous pulmonary TB had the highest level of CCL2 ($P = 0.05$). There was no significant correlation between cytokine production and time between TB treatment completion and study entry (data not shown).

A similar pattern of cytokine production by patient group was noted when PBMC-M were cultured alone. TST-negative contacts produced the lowest median levels of CXCL8 ($P = 0.04$), IL-10 ($P = 0.02$), and CCL2 ($P = 0.03$), followed by persons with previous extrapulmonary TB, persons with previous pulmonary TB, and latently infected persons after controlling for race and WBCs (Fig. 2). A similar trend was seen for IL-1β, IL-6, TNF-α, and IFN-γ but was not statistically significant. A positive correlation between IL-1β and IL-10 production and time between TB treatment completion and study entry was noted (Spearman's coefficient, 0.21 [$P = 0.03$] and 0.23 [$P = 0.03$], respectively). A proportional odds logistic regression model adjusting for time as well as race and WBCs was performed, and the observed differences in these two cytokines between the four patient groups remained statistically significant (data not shown).

Similar to what was observed in our previous work, persons with previous extrapulmonary TB had lower levels of IL-6, IL-10, CCL2, TNF-α, and IFN-γ than did the other groups when uninfected macrophages were cocultured with autologous PBMC-M, but this trend was not statistically significant (see Table S1 in the supplemental material) (2). With the exception of CXCL8 and CCL2, cytokine levels increased in all study groups when autologous PBMC-M were cultured with infected macrophages compared to levels in PBMC-M cultured with uninfected macrophages. IFN-γ showed the greatest fold increase when all groups were compared under these two conditions, with increases ranging from 245 to 1,000-fold compared to the other cytokines.

Levels of IL-β, IL-6, CXCL8, IL-10, and CCL2 decreased when comparing uninfected to infected macrophages (see Table S1 in the supplemental material). This was most striking for CCL2, which decreased 200- to 600-fold when uninfected macrophages were compared to infected macrophages. The decrease in cytokine levels was not different between cases and controls.

DISCUSSION

Previous studies comparing the immune responses of persons with extrapulmonary TB to those with pulmonary TB and healthy controls have measured *in vivo* or *in vitro* cytokine production and have arrived at different conclusions. Some have found similar cytokine levels in sera from persons with various TB manifestations (40), while others such as Hasan et al. found that circulating levels of cytokines such as IFN-γ and CXCL9 differ based on the site of TB disease (19, 40). Other groups have stimulated whole

TABLE 2 Cytokine production of PBMCs 48 h after addition of PBMC-M[a]

Cytokine	Group	Median cytokine concn (IQR) or *P* value 48 h after addition of: Macrophage + *M. tuberculosis* H37Rv + PBMC-M	Median cytokine concn (IQR) or *P* value 48 h after addition of: PBMC-M
IL-1β	TST−	1,959 (754–3,502)	155 (69–252)
	EPTB	2,035 (906–3,813)	126 (60–188)
	PTB	2,253 (1,876–4,209)	236 (120–314)
	LTBI	3,722 (1,783–6,243)	225 (136–606)
	P value	0.15	0.11
IL-6	TST−	2,159 (710–4,206)	3,514 (950–5,490)
	EPTB	3,867 (1,375–10,212)	2,431 (1,311–5,880)
	PTB	4,266 (2,047–6,116)	4,835 (3,844–7,599)
	LTBI	4,827 (2,492–10,475)	5,387 (3,639–9,228)
	P value	**0.04**	0.07
CXCL8	TST−	16,777 (8,700–28,034)	27,790 (10,158–54387)
	EPTB	15,644 (8,465–64,617)	14,461 (94,301–44,438)
	PTB	21,495 (13,762–44,211)	36,949 (27,993–72,137)
	LTBI	27,499 (14,935–59,700)	46,454 (21,853–85,471)
	P value	0.12	**0.04**
IL-10	TST−	18 (11–37)	27 (12–44)
	EPTB	33 (23–103)	32 (16–49)
	PTB	69 (35–139)	44 (26–65)
	LTBI	64 (37–142)	51 (28–152)
	P value	**<0.001**	**0.02**
IL-12	TST−	1.3 (1.1–2.0)	0.53 (0–1.3)
	EPTB	1.7 (0–5.9)	0 (0–1.3)
	PTB	1.8 (1.4–2.5)	1.1 (0–1.6)
	LTBI	1.6 (1.1–2.4)	1.2 (1–1.7)
	P value	0.49	0.27
IL-17	TST−	5.6 (2.4–16)	2.0 (0.6–7.4)
	EPTB	22 (5.2–45)	1.7 (0–3.2)
	PTB	8.4 (5.6–53)	2.2 (1.1–4.7)
	LTBI	19 (12–79)	4.7 (2.0–15)
	P value	**0.01**	0.17
CCL2	TST−	146 (83–278)	988 (226–5,567)
	EPTB	358 (116–1,434)	623 (457–3,218)
	PTB	436 (167–655)	4,044 (1,565–6,484)
	LTBI	213 (121–1,686)	4,685 (1,678–6,381)
	P value	0.05	**0.03**
TNF-α	TST−	293 (141–694)	26 (12–44)
	EPTB	684 (223–1860)	20 (9.5–72)
	PTB	708 (416–2,000)	31 (17–45)
	LTBI	989 (268–2,107)	46 (21–79)
	P value	**0.04**	0.17
IFN-γ	TST−	1,301 (607–2,672)	46 (10–157)
	EPTB	2,599 (1,770–13,064)	19 (12–49)
	PTB	3,533 (1,931–7,743)	33 (9.7–91)
	LTBI	3,126 (1,974–9,709)	83 (17–177)
	P value	**0.01**	0.07

[a] Values are in pg/ml; *P* values of <0.05 are in bold. Abbreviations: EPTB, extrapulmonary TB; PTB, pulmonary TB; LTBI, latent TB infection; IQR, interquartile range.

blood or PBMCs from persons with various forms of TB and measured cytokine levels and T lymphocyte proliferation (20, 21, 36, 41, 42). These studies have focused more on the immune response to *M. tuberculosis* in persons with active TB, in some cases to identify diagnostic biomarkers. The present study differs from previous studies in the literature and builds on prior work in that we used a more precise and biologically relevant model of the sequential activation of innate and adaptive immunity following human infection with *M. tuberculosis*. We enrolled only persons who had completed TB therapy in order to evaluate for immune defects that may predispose to development of TB and to avoid possible confounding of our results with the tumultuous immune response that accompanies active TB (28). We observed a continuum of immune responses in which uninfected persons produced the lowest levels of several cytokines, followed by persons with previous extrapulmonary TB, persons with previous pulmonary TB, and persons with latent TB infection. Similar to our previous work (2, 38), our results suggest that people who develop extrapulmonary TB appear to have a subtle immune defect compared to persons with other TB manifestations.

Our findings could signify inadequate activation of memory T lymphocytes by antigen-presenting macrophages in persons with previous extrapulmonary TB compared to latently infected persons and persons with previous pulmonary TB. If so, it is not surprising that uninfected persons had the lowest cytokine responses to *in vitro* challenge with *M. tuberculosis*. A recent study found that IFN-γ production after stimulation with *M. tuberculosis* peptides in persons with pulmonary TB was mediated by effector memory T lymphocytes and this response was different based on severity of disease (17). Inadequate production or maintenance of memory T lymphocytes could potentially lead to the development of TB at sites distant from the site of primary infection after initial control of *M. tuberculosis* infection, and this may explain why persons with previous extrapulmonary TB produced low cytokine levels. There were differences between the groups in the time between completion of therapy and study enrollment, which could affect the memory immune response, but the differences in the immune response between study groups did not change when adjusting for this time variable.

We found that persons with previous extrapulmonary TB had the highest levels of IL-17. IL-17 is a proinflammatory cytokine that participates in the early defense against *M. tuberculosis* infection and has been shown in murine models to be essential for granuloma formation (33). In a separate study that included a subset of the current study's patient population, we observed that persons with previous extrapulmonary TB have increased frequencies of regulatory T cells, a category of T cells characterized by surface expression of CD4 and CD25, and intracellular expression of FoxP3 (8). In active TB, regulatory T cells suppress IFN-γ production but have less suppressive effects on IL-17 production (32). IFN-γ also regulates production of IL-17 (7). Our findings suggest a model whereby increased regulatory T cell frequency suppresses IFN-γ responses to *M. tuberculosis* infection, leading to elevated levels of IL-17. Future studies where production of IFN-γ and IL-17 is measured after regulatory T cell depletion could test this hypothesis.

We found that levels of CCL2 were elevated in persons with previous pulmonary TB, which is consistent with our previous work involving children (39). Flores-Villanueva et al. found that a polymorphism in the CCL2 promoter was associated with in-

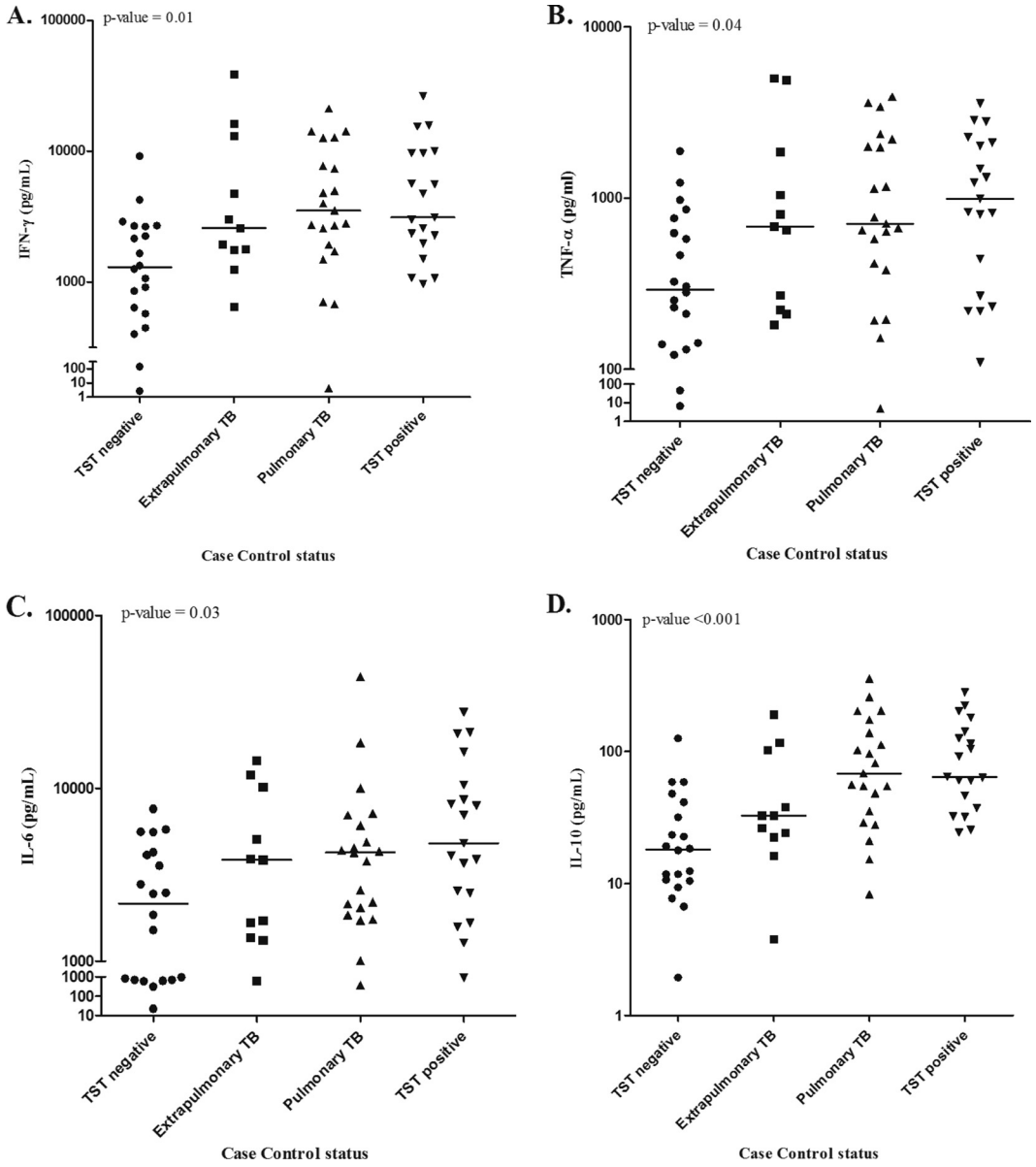

FIG 1 Levels of IFN-γ (A), TNF-α (B), IL-6 (C), and IL-10 (D) across study groups when autologous PBMCs are added to *M. tuberculosis*-infected macrophages. Macrophages from study participants were cultured for 7 days and then infected with *M. tuberculosis* H37Rv. Forty-eight hours later, autologous monocyte-depleted PBMCs were added to the macrophage culture. Supernatants were collected 48 h later and stored at −70°C until submission for cytokine bead array.

creased levels of CCL2 in Korean and Mexican adults with active pulmonary TB compared to healthy controls (15). The consistent finding of elevated CCL2 in various groups of patients in different settings suggests that it may have a direct effect on the pathogenesis of pulmonary TB.

Interestingly, we found that in addition to lower levels of inflammatory cytokines such as IL-6 and TNF-α, PBMCs from persons with previous extrapulmonary TB also had lower levels of IL-10, which is traditionally considered an anti-inflammatory cytokine that suppresses the immune response. We found a similar result in a previous study (2). These findings suggest a global defect in cytokine production rather than a defect in production of a specific cytokine or chemokine.

Several of the cytokines were noted to decrease significantly when infected macrophages were compared to uninfected macrophages. This was most striking with CCL2. All cytokines tested increased with the addition of autologous PBMC-M to infected macrophages. One possibility for this finding is that infected macrophages became "burned out" after several days of infection and required activating signals from PBMC-M to produce inflammatory cytokines. Also, certain cytokines, such as IL-6 and CXCL8, as well as IFN-γ and IL-17, were likely contributed by the added lymphocytes. Finally, it is possible that infected macrophages became nonviable after several days of incubation and that is why lower production of cytokines was observed. We did not assess for viability when obtaining supernatant from macrophage cultures.

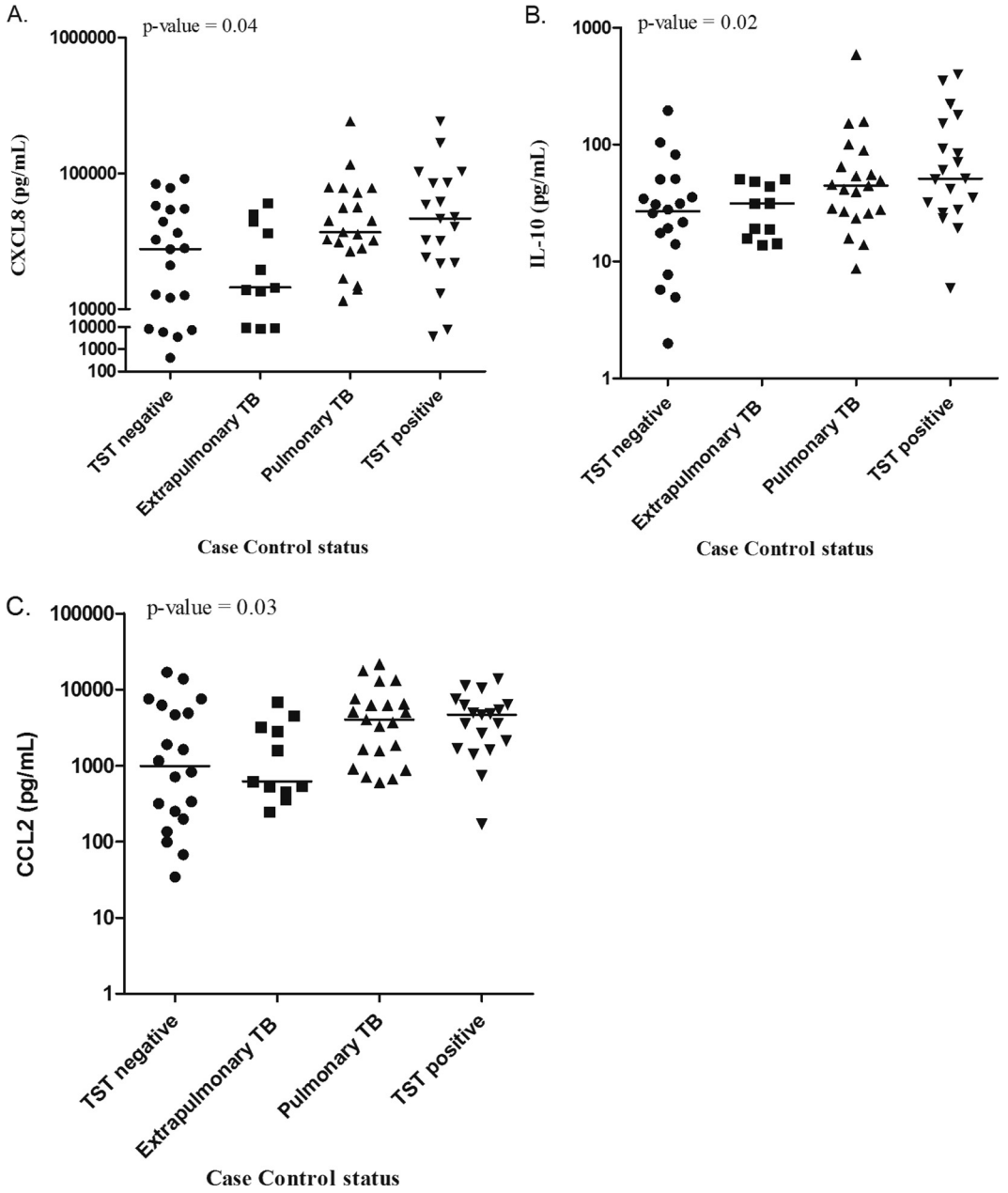

FIG 2 Levels of CXCL8 (A), IL-10 (B), and CCL2 (C) across study groups when autologous monocyte-depleted PBMCs were cultured alone. Supernatants were collected from monocyte-depleted PBMCs after 48 h of culture and stored at −70°C until submission for cytokine bead array.

Our study has several limitations. First, it is unclear whether cytokine production in response to *M. tuberculosis* after completion of anti-TB therapy is similar to cytokine production prior to developing TB disease. Previous studies have demonstrated that cytokine profiles differ between persons with active TB disease and uninfected controls (24) and before and after TB therapy (22, 29). Because the aim of our study was to identify predisposing immunologic factors leading to development of extrapulmonary TB, we chose to study persons who had completed TB therapy to avoid the fluctuations in cytokine production that have been observed in early-stage TB (28). Second, extrapulmonary TB is a complex disease, and different sites of disease manifestation may have different pathophysiology (9, 26, 34, 37). Previous studies have suggested that persons with different sites of TB disease have various immune responses (18). We included a wide spectrum of TB manifestations in the present study. The heterogeneous nature of the extrapulmonary group potentially makes our findings more broadly applicable to all persons with extrapulmonary TB, although it makes it impossible to draw conclusions about factors that may influence whether a person develops a limited or disseminated form of extrapulmonary TB. We are planning future studies to compare immune responses between persons with various forms of extrapulmonary TB. Also, immune activation in the periphery may differ from the immune response at the site of disease

(i.e., in the lung or lymph nodes). Finally, persons with previous pulmonary TB were more likely to smoke than persons in the other study groups. Smoking is strongly associated with pulmonary disease (11, 27), likely due to its local effect in the lung rather than an effect on systemic immune function. Alcohol use is often associated with tobacco smoking and has also been associated with pulmonary TB (14).

Taken together, our data suggest that persons with previous extrapulmonary TB may have a subtle immune defect that leads to an inability to control *M. tuberculosis* at the site of primary infection. This defect may lie in signaling pathways upstream of cytokine production. Our results expand our knowledge of the immune response to *M. tuberculosis* infection and help us to further understand the factors that may influence progression from infection to active TB disease.

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We report no potential conflicts of interest.

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