

Increased Frequency of Regulatory T Cells and T Lymphocyte Activation in Persons with Previously Treated Extrapulmonary Tuberculosis

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Extrapulmonary tuberculosis may be due to underlying immune compromise. Immunosuppressive regulatory T cells (Treg cells), and CD4⁺ T lymphocytes in general, are important in the host immune response to *Mycobacterium tuberculosis*. We evaluated T lymphocytes from patients after recovery from extrapulmonary tuberculosis, which may reflect conditions before *M. tuberculosis* infection. A case-control study was conducted among HIV-uninfected adults with previously treated extrapulmonary tuberculosis and 3 sets of controls: (i) subjects with previously treated pulmonary tuberculosis, (ii) close tuberculosis contacts with *M. tuberculosis* infection, and (iii) close tuberculosis contacts with no infection. Monocyte-depleted peripheral blood mononuclear cells (PBMC-M) were stained for CD4⁺ CD25^{hi} CD127^{low} FoxP3⁺ cell (Treg cell) and T lymphocyte activation. Both characteristics were compared as continuous variables between groups with the Kruskal-Wallis test. There were 7 extrapulmonary tuberculosis cases, 18 pulmonary tuberculosis controls, 17 controls with *M. tuberculosis* infection, and 18 controls without *M. tuberculosis* infection. The median Treg cell proportion was highest among persons with previous extrapulmonary tuberculosis (1.23%) compared to subjects with pulmonary tuberculosis (0.56%), latent *M. tuberculosis* infection (0.14%), or no *M. tuberculosis* infection (0.20%) ($P = 0.001$). The median proportion of CD4⁺ T lymphocytes that expressed the activation markers HLA-DR and CD38 was highest for CD4⁺ T lymphocytes from persons with previous extrapulmonary tuberculosis (0.79%) compared to subjects with pulmonary tuberculosis (0.44%), latent *M. tuberculosis* infection (0.14%), or no *M. tuberculosis* infection (0.32%) ($P = 0.005$). Compared with controls, persons with previously treated extrapulmonary tuberculosis had the highest Treg cell frequency, but also the highest levels of CD4⁺ T lymphocyte activation. Immune dysregulation may be a feature of individuals at risk for extrapulmonary tuberculosis.

Of the estimated 2 billion people infected with *Mycobacterium tuberculosis*, only 5 to 10% will develop tuberculosis (TB) (14, 28). Although most persons with TB have pulmonary disease, approximately 20% have extrapulmonary involvement (8). Extrapulmonary tuberculosis may be a marker of underlying immune compromise, as illustrated by the increased risk of such disease among HIV-infected persons and young children (31, 35). We previously demonstrated decreased CD4⁺ T lymphocyte numbers and a decreased ability of unstimulated peripheral blood mononuclear cells (PBMC) to produce cytokines among HIV-uninfected adults who have recovered from extrapulmonary tuberculosis compared to controls with pulmonary tuberculosis or latent *M. tuberculosis* infection (1, 47). The factors that predispose individuals to extrapulmonary disease may provide insights into the risk factors for progression to all forms of active tuberculosis after *M. tuberculosis* infection.

The increased incidence of tuberculosis, specifically extrapulmonary tuberculosis, among individuals with HIV infection (31) or individuals receiving tumor necrosis factor alpha (TNF- α) inhibitors (32) demonstrates the importance of cell-mediated immune responses for the containment of *M. tuberculosis* infection. Activated effector T lymphocytes migrate to granulomas and presumably control infection through the release of cytokines and through cytolytic function (34). These immune responses appear to be modulated through the recruitment of regulatory T lymphocytes (Treg cells) to the sites of active infection (22). This suggests that Treg cells may play a significant role in the host immune

response to *M. tuberculosis* infection, specifically a role in determining the site of tuberculosis disease (22, 43).

Treg cells are a subset of CD4⁺ T lymphocytes and constitute 1 to 5% of all circulating CD4⁺ cells (40). Their main function is to prevent autoimmunity and maintain self-tolerance (18, 55). Treg cells also play a role in the immune response to infections, where they minimize excessive tissue destruction from adaptive immune responses via cell-cell contact and secretion of cytokines such as transforming growth factor beta (TGF- β) (6, 42, 51). However, by limiting the adaptive immune response, Treg cells may allow persistence and establishment of chronic infections. Depletion of Treg cells has been shown to increase *in vitro* immune responses to pathogens that cause chronic infections, such as *Helicobacter pylori* (38), HIV (33), hepatitis C virus (HCV) (7, 48), and *M. tuberculosis* (22, 43). The role of Treg cells in the pathogenesis of *M. tuberculosis* is not known. Treg cells could possibly be a response

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to the generalized immune activation that occurs in chronic infections such as HIV infection and tuberculosis (11, 44, 50), and they may dampen the immune response directed against *M. tuberculosis* (43); however, the relationship of Treg cells and immune activation to the site of tuberculosis disease is not clear. To date, studies of Treg cells and immune activation have been performed in persons with active tuberculosis disease (10). However, active tuberculosis is characterized by aberrations in the host immune system (5, 26) and may not be an accurate depiction of the immune dysregulation that leads to active tuberculosis.

To determine the immune response characteristics that may predispose individuals to extrapulmonary tuberculosis, we measured the frequency of Treg cells and the extent of CD4⁺ and CD8⁺ T lymphocyte activation in peripheral blood among HIV-seronegative adults who completed treatment for either extrapulmonary or pulmonary tuberculosis or latent *M. tuberculosis* infection. The optimal surface and intracellular markers to identify Treg cells continue to evolve. Based on previous studies that have found Treg cells to have high-level expression of CD25 (2, 3) and low-level expression of CD127 (36) and to be regulated by FoxP3 (17, 27, 57), we defined Treg cells as CD4⁺ CD25^{hi} CD127^{low} FoxP3⁺ cells in the current study. These measurements were performed for a subset of persons that were recruited for a study evaluating the *in vitro* immune response of monocyte-derived macrophages to infection with *M. tuberculosis* in persons with different manifestations of tuberculosis (16).

MATERIALS AND METHODS

Subjects. Case subjects 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 of >18 years at the time of diagnosis of TB disease or infection; HIV-seronegative status; culture-confirmed disease, with therapy either near completion (within 1 month) or completed (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 to be enrolled. Only contacts of persons with culture-positive pulmonary TB or known TST converters were included as controls. Contacts of persons with culture-positive pulmonary TB were tested for latent infection at the beginning of the contact investigation and after 8 to 12 weeks (9). Exclusion criteria consisted of the following: serum creatinine level of >2 mg/dl, use of corticosteroids or other immunosuppressive agents at the time of diagnosis or study entry, and malignancy. All laboratory procedures were performed blinded to case-control status.

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, the Nashville Davidson Metro Public Health Department, and the Tennessee Department of Health approved the study. Study participants provided written informed consent.

Sample preparation. Sixty-milliliter blood samples were collected in EDTA tubes, and plasmas were separated and frozen. PBMC were isolated under sterile conditions by Ficoll-Paque (GE Healthcare Bio-Science) density gradient centrifugation. Cells were washed twice in 1 × Dulbecco's phosphate-buffered saline (DPBS) (Cellgro-Mediatech Inc.), and viability was estimated by trypan blue dye exclusion. PBMC were then cryopre-

served in fetal bovine serum (FBS) (GemCell-Gemini Bioproducts). Blood samples were submitted to a commercial laboratory for HIV serology and complete blood counts. To determine CD4⁺ T cell numbers, the total lymphocyte counts were measured with an automated hematology analyzer and multiplied by the percentage of CD4⁺ T lymphocytes determined by flow cytometry.

Flow cytometry. Aliquots of cryopreserved PBMC were thawed, washed in DPBS (Cellgro-Mediatech) twice, and stained for viability at room temperature with a Live/Dead fixable aqua dead cell staining kit (Invitrogen). Cells were surface stained with the following directly conjugated human antibodies: phycoerythrin (PE)–Cy5.5–anti-CD127 (Beckman Coulter), PE–Cy7–anti-CD38 (eBioscience), PE–Texas Red–anti-CD4 (Invitrogen Corp.), AF-700–anti-CD3, Pacific Blue (PB)–anti-CD8, fluorescein isothiocyanate (FITC)–anti-HLA-DR, and PE–anti-CD25 (Becton Dickinson). Cells were washed, fixed, and permeabilized for intracellular staining with anti-FoxP3–allophycocyanin (APC) (eBioscience) and were analyzed on a FACSaria flow cytometer. Fluorescence-minus-one controls for anti-CD38 and anti-CD127 were used to optimize compensation. Treg cells were defined as CD4⁺ CD25^{hi} CD127^{low} FoxP3⁺ cells. The absolute Treg cell frequency was determined by multiplying the percentage of CD4⁺ Treg cells by the absolute CD4⁺ T lymphocyte number.

Statistical analysis. The primary study outcome was the difference in the frequency of Treg cells between subject groups. Based on a previous study, we determined that 10 subjects in each study group would provide 80% power to detect a difference in Treg cell frequency of 7% at a two-sided 5% significance level, using the two-sample *t* test (43). Comparisons of continuous variables between groups were performed using the Kruskal-Wallis test. Categorical variables were compared using the chi-square test. The correlation between Treg cell frequency and CD4⁺ immune activation was calculated using Spearman's rank correlation. Differences were considered statistically significant if the *P* value was <0.05. Statistical analyses were performed using GraphPad Prism software, version 5.0, for Windows and STATA, version 10.0 (College Station, TX).

RESULTS

There were 60 individuals enrolled in the study: 7 with previously treated extrapulmonary tuberculosis, 18 with previously treated pulmonary tuberculosis, 17 with latent *M. tuberculosis* infection (TST positive), and 18 without *M. tuberculosis* infection (TST negative). Demographic and clinical characteristics and laboratory data are summarized in Table 1. Five persons had received *M. bovis* BCG vaccination in the past (2 extrapulmonary cases, 2 pulmonary controls, and 1 latent infection control). There was no difference in median age or sex between groups. Persons with previous extrapulmonary or pulmonary tuberculosis were more likely to be black than persons with or without *M. tuberculosis* infection (*P* = 0.01). Pulmonary tuberculosis controls were more likely to have a history of smoking than the other groups, but the difference was not statistically significant. Persons with previous pulmonary tuberculosis were enrolled sooner after completion of therapy than the other groups. Persons with previous extrapulmonary tuberculosis had the lowest median white blood cell (WBC) count. They also had the lowest median number of CD4⁺ T lymphocytes and the highest median number of CD8⁺ T lymphocytes, but these differences were not statistically significant. There was no difference in the CD4⁺/CD8⁺ T lymphocyte ratio among groups (data not shown). Persons with previous extrapulmonary tuberculosis had a significantly lower median absolute number of neutrophils than the other groups. There were no differences between the groups in the median number of monocytes, eosinophils, or basophils (data not shown).

TABLE 1 Demographic, clinical, and laboratory characteristics of the study population

Characteristic	Value				P value ^a
	Extrapulmonary TB (n = 7)	Pulmonary TB (n = 18)	Latent <i>M. tuberculosis</i> infection (n = 17)	No latent <i>M. tuberculosis</i> infection (n = 18)	
Age (yr) ^b	53 (26–70)	47 (40–59)	43 (36–48)	42 (32–50)	0.44
No. (%) of male subjects	3 (43)	11 (61)	6 (35)	8 (44)	0.48
No. (%) of Hispanic subjects	1 (14)	2 (11)	0 (0)	1 (5)	0.48
No. (%) of black subjects	3 (43)	8 (44)	2 (12)	3 (17)	0.01
No. (%) of subjects who smoked	1 (14.3)	10 (55)	5 (29)	5 (28)	0.12
Time from end of treatment to blood draw (mo) ^b	12 (6–25)	1 (0–8)	15 (1.5–26)	NA	0.02
Cell count (per mm ³) ^b					
WBC	5,900 (4,900–7,500)	6,000 (5,300–7,700)	7,200 (6,400–7,800)	8,400 (7,300–9,200)	0.008
Monocytes	0.4 (0.3–0.5)	0.4 (0.3–0.5)	0.5 (0.4–0.6)	0.4 (0.3–0.6)	0.38
CD4 ⁺ lymphocytes	975 (962–1,078)	1,060 (708–1,206)	1,200 (863–1,495)	1,136 (933–1,326)	0.36
CD8 ⁺ lymphocytes	643 (340–945)	443 (368–556)	507 (440–673)	452 (335–783)	0.45
Neutrophils	3,100 (2,400–4,600)	3,700 (2,700–4,700)	3,900 (3,600–4,200)	5,300 (4,700–6,200)	0.013

^a By the Kruskal-Wallis test and Pearson's chi-square test.

^b Data are medians (interquartile ranges). NA, not applicable.

Figure 1A and B show the flow cytometry gating for Treg cells in the study population. Persons with previous extrapulmonary tuberculosis had the largest proportion of Treg cells (1.23%) compared to subjects with previous pulmonary tuberculosis (0.56%), TST-positive individuals (0.14%), and TST-negative individuals

(0.20%) ($P = 0.001$) (Fig. 1C) and the largest absolute number of Treg cells among CD4⁺ T lymphocytes compared to controls ($P = 0.002$) (Fig. 1D).

We measured the degree of generalized immune activation in study subjects by measuring expression of the activation markers

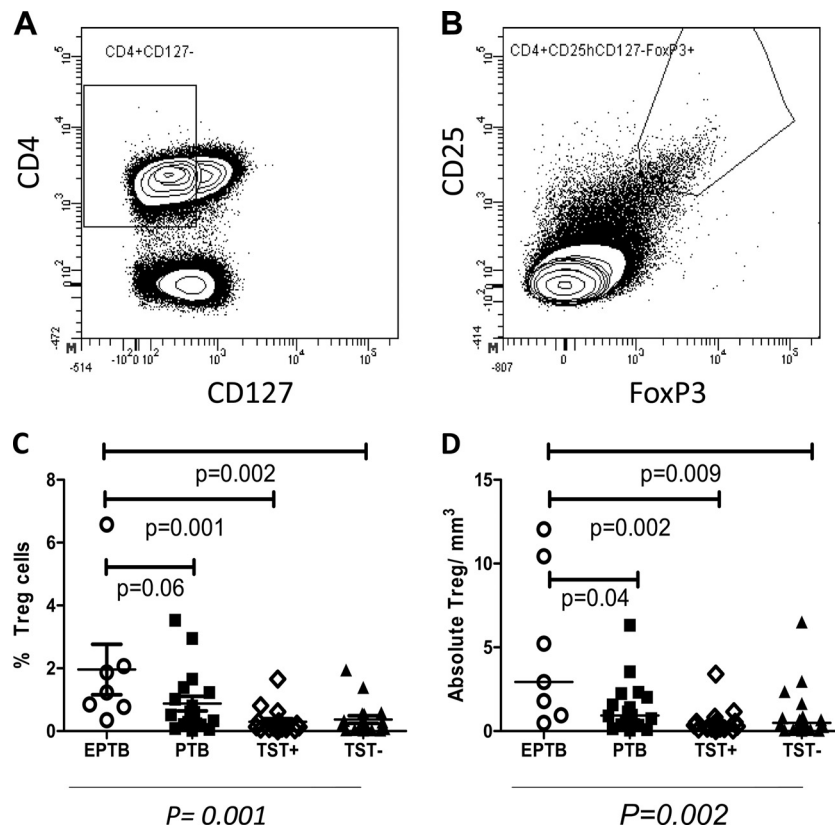


FIG 1 Frequencies of regulatory T cells among study groups. (A and B) Gating strategy for Treg cells. Treg cells were defined as CD4⁺ CD25^{high} CD127^{low} FoxP3⁺ cells. (C) Frequencies of Treg cells as percentages of CD4⁺ T cells among four study groups. (D) Absolute numbers of Treg cells/mm³ of blood among four study groups. Statistical comparisons of global differences between the study groups were performed with the Kruskal-Wallis test. Individual comparisons between the extrapulmonary group and each control group were calculated using the Wilcoxon rank sum test and were not adjusted for multiple comparisons. Horizontal bars represent the median values. EPTB, extrapulmonary TB; PTB, pulmonary TB; TST+, latent *M. tuberculosis* infection; TST-, no latent *M. tuberculosis* infection.

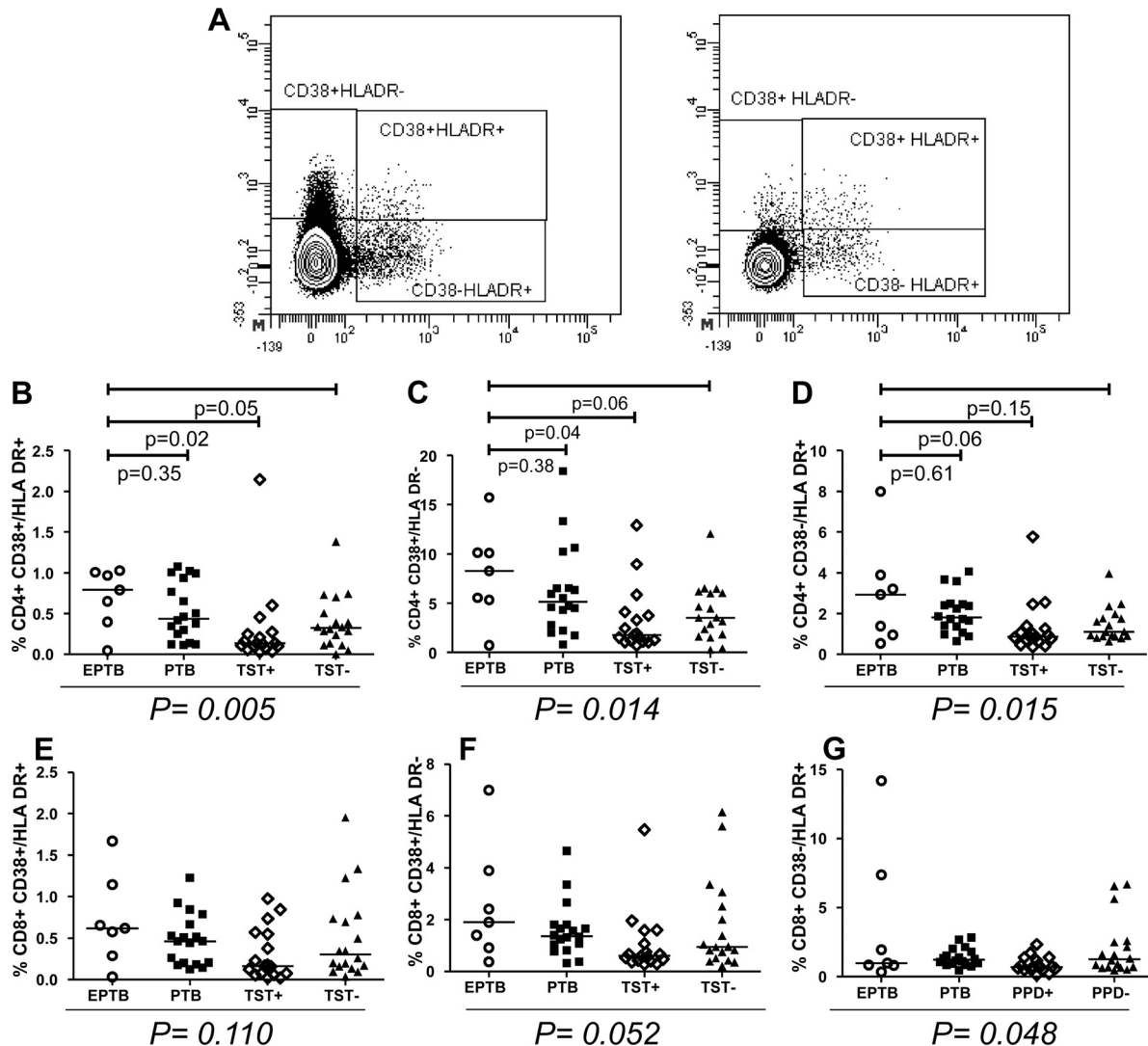


FIG 2 Proportions of cells with activation markers on CD4⁺ and CD8⁺ T lymphocytes among study groups. (A) Gating strategy for measuring HLA-DR and CD38 expression on CD4⁺ and CD8⁺ T lymphocytes. (B to D) Distributions of activation markers on CD4⁺ T lymphocytes among study groups. (E to G) Distributions of activation markers on CD8⁺ T lymphocytes among study groups. Statistical comparisons were performed with the Kruskal-Wallis test. Individual comparisons between the extrapulmonary group and each control group were calculated using the Wilcoxon rank sum test and were not adjusted for multiple comparisons. Horizontal bars represent the median values. EPTB, extrapulmonary TB; PTB, pulmonary TB; TST+, latent *M. tuberculosis* infection; TST-, no latent *M. tuberculosis* infection.

CD38 and HLA-DR on CD4⁺ and CD8⁺ T lymphocytes (Fig. 2A). Treg cells express HLA-DR and therefore were excluded from the activation marker analysis (12). The frequency of CD4⁺ T lymphocytes that expressed the activation markers CD38 and HLA-DR was highest in persons with previous extrapulmonary tuberculosis (0.79%) compared to subjects with previous pulmonary tuberculosis (0.44%), TST-positive individuals (0.14%), and TST-negative individuals (0.32%) ($P = 0.005$) (Fig. 2B). Likewise, the proportions of CD4⁺ CD38⁺ HLA-DR⁻ ($P = 0.014$) and CD4⁺ CD38⁻ HLA-DR⁺ ($P = 0.015$) cells were highest in subjects with resolved extrapulmonary tuberculosis, as shown in Fig. 2C and D, respectively. We observed a trend of increased activated CD8⁺ T lymphocytes in persons with prior extrapulmonary tuberculosis. This trend was of borderline significance with either CD38 or HLA-DR (CD8⁺ CD38⁺ HLA-DR⁻ cells [$P = 0.052$]

[Fig. 2F] or CD8⁺ CD38⁻ HLA-DR⁺ cells [$P = 0.048$] [Fig. 2G]) but was not significant when both activation markers on CD8⁺ T lymphocytes were compared between the four groups (CD8⁺ CD38⁺ HLA-DR⁺ cells [$P = 0.11$]) (Fig. 2E).

We found strong correlations between Treg cell frequency and CD4⁺ immune activation in TST-negative subjects ($r = 0.77$; $P = 0.0002$) and subjects with prior pulmonary tuberculosis ($r = 0.74$; $P = 0.0005$). We found a similar trend among subjects with prior extrapulmonary tuberculosis ($r = 0.70$; $P = 0.08$). We saw no association between Treg cell frequency and immune activation in TST-positive individuals ($r = 0.35$; $P = 0.16$) (Fig. 3).

DISCUSSION

Extrapulmonary tuberculosis is likely a marker of underlying immune suppression (31, 35) and has been associated with decreased

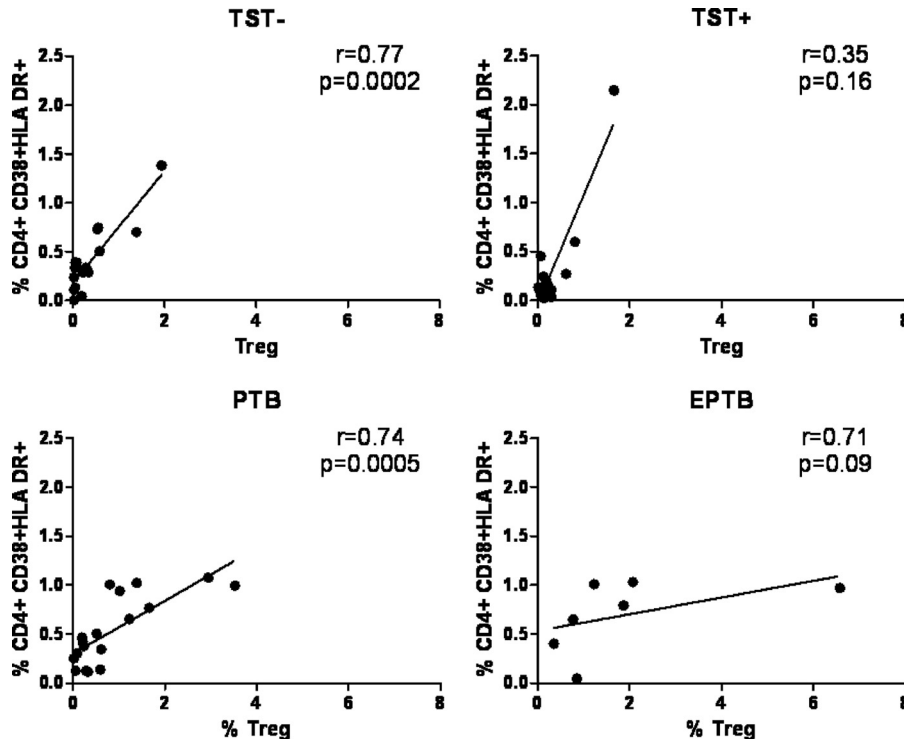


FIG 3 Correlation between CD4⁺ T cell activation and Treg cell frequency among study groups. Statistical calculations of correlation were performed with Pearson's correlation test. EPTB, extrapulmonary TB; PTB, pulmonary TB; TST+, latent *M. tuberculosis* infection; TST-, no latent *M. tuberculosis* infection.

in vitro secretion of gamma interferon (IFN- γ) in response to mycobacterial antigens compared to that of *M. tuberculosis*-infected controls (30). We found that subjects with prior extrapulmonary tuberculosis had both the highest frequency of Treg cells and the highest frequency of activated CD4⁺ T lymphocytes compared to those with prior pulmonary tuberculosis, individuals with latent *M. tuberculosis* infection, and uninfected controls. These findings suggest that immune dysregulation may be a feature of persons who develop extrapulmonary tuberculosis.

Previous studies have demonstrated an increase in Treg cell frequency and FoxP3⁺ mRNA both peripherally and at the site of disease in persons with active extrapulmonary TB (10, 22, 23, 46). Depletion of Treg cells from PBMC isolated from individuals with active tuberculosis has been shown to increase cytokine production in response to mycobacterial protein (10, 43); however, it is unclear whether blunted immune responses from Treg cell expansion are responsible for active disease or if this Treg cell expansion is simply part of the host inflammatory response to active infection. The evaluation of factors that predispose individuals to extrapulmonary tuberculosis may not be accurate in the setting of active disease, because active tuberculosis is associated with aberrations in the host immune response that resolve after therapy (25, 56).

We reasoned that evaluation of individuals after completion of therapy would more likely reflect their baseline immune status before exposure to *M. tuberculosis*. The results of a recent study of *M. tuberculosis*-infected cynomolgus macaques suggest that higher preinfection frequencies of Treg cells are associated with a higher likelihood of latent TB (21). While these results differ from the results of our study, the strategy for gating Treg cells by the

investigators included all FoxP3⁺ cells and therefore may have included populations of recently activated (non-Treg) CD4⁺ T cells. Also, the animals were not evaluated after successful tuberculosis treatment to determine whether Treg cell frequencies after eradication of infectious organisms returned to preinfection levels. Further studies in a well-characterized animal infection model such as this will be quite useful. In humans, Ribeiro-Rodrigues et al. found that frequencies of Treg cells persisted at high levels in individuals with pulmonary tuberculosis compared to control subjects, even at the end of a treatment course (43). However, this group used only the CD25 marker to measure Treg cell frequencies, and this may simply reflect increased immune activation through the course of therapy. Chen et al. used a combination of the CD25 and FoxP3 markers to identify Treg cells, and they found that the frequencies of Treg cells in individuals 2 years after successful treatment for pulmonary tuberculosis were similar to those in controls (10). In addition to using a combination of markers thought to be highly specific for Treg cells, our study differs from prior work in that we studied persons with treated extrapulmonary tuberculosis. We evaluated individuals for up to several months after the completion of therapy and found that subjects with prior extrapulmonary tuberculosis had the highest frequency of Treg cells compared to controls with pulmonary tuberculosis, latent *M. tuberculosis* infection, or no *M. tuberculosis* infection. Our results suggest that an increased frequency of Treg cells is associated with and may predispose one to extrapulmonary tuberculosis.

Generalized immune activation is observed in chronic infections such as HIV infection and tuberculosis (11, 19, 29, 37, 44, 50). The perturbation of the immunologic environment during

M. tuberculosis infection is felt to contribute to HIV disease severity and progression in coinfecting persons (20). Previous studies have reported increased activation of CD4⁺ and CD8⁺ T lymphocytes in active tuberculosis and latent infection (4, 24, 43, 44, 54). These studies have either studied only persons with pulmonary tuberculosis or evaluated pulmonary and extrapulmonary tuberculosis together as one entity. Our study is unique in that we characterized immune activation in four distinct groups that represent the spectrum of tuberculosis disease. We found that among the four groups studied, persons with prior extrapulmonary tuberculosis had evidence of global activation of CD4⁺ T lymphocytes and, to a lesser extent, CD8⁺ T lymphocytes.

We found a strong correlation between immune activation and Treg cell frequency in uninfected persons, persons with previous pulmonary tuberculosis, and persons with previous extrapulmonary tuberculosis; the latter correlation was not statistically significant, likely because of fewer study subjects in this group. A significant correlation was not observed for persons with latent *M. tuberculosis* infection. Our findings differ from a previous study that recently found a positive correlation between Treg cell frequency and the frequency of CD4⁺ CD38⁺ HLA-DR⁺ T cells in healthy uninfected controls but not among subjects with active tuberculosis disease or latent *M. tuberculosis* infection (54). One reason that we may have observed different results could be because we compared immune activation between persons with pulmonary and extrapulmonary tuberculosis, whereas the referenced study combined these two patient groups. Also, the other study evaluated immune activation only in persons with active tuberculosis, whereas we studied persons after they had completed therapy.

The clinical significance of our findings is unclear but may reflect increased expansion of Treg cells as a response to generalized immune activation in persons with tuberculosis, which persists for up to several months after the completion of therapy. Another explanation for this finding is that increased immune activation in the presence of an increased frequency of Treg cells in persons with previous extrapulmonary tuberculosis could be a marker of subtle immune dysregulation that was present prior to infection and that may predispose individuals to this form of tuberculosis. We favor this explanation because these findings were observed several months after tuberculosis treatment. Also, these findings are consistent with our previous studies that found decreased numbers of CD4⁺ T lymphocytes and decreased *in vitro* cytokine production by peripheral blood mononuclear cells in persons with previous extrapulmonary tuberculosis (1, 47), indicating a possible subtle immune defect in these individuals.

Interestingly, we found that persons with previous extrapulmonary tuberculosis had a lower median absolute neutrophil count than the other study groups. A clear role for neutrophils in the immune response to tuberculosis has not been defined. Previous studies have reported variously that neutrophils are important in the early defense against *M. tuberculosis* infection (41), that they contribute to pathology rather than protection (15), and that they have no role in the immune response (45). In a previous study, we found that macrophages from persons with previous extrapulmonary tuberculosis produced lower levels of interleukin-8 (IL-8), an important chemoattractant for neutrophils, than did those from latently infected persons (16, 47). These findings suggest that the role of neutrophils in the immune response to *M. tuberculosis* may be complex and should be studied further.

Our study had some limitations. First, the sample size was small, which limited our ability to perform detailed subgroup analyses. Second, extrapulmonary tuberculosis is a complex disease, and different sites of disease manifestation may have different pathophysiologies (13). A larger study that assesses Treg cell frequency and activation according to the site of extrapulmonary disease is warranted. Also, immune activation in the periphery may differ from the immune response at the site of disease. Third, although we were unable to assess whether skin test positivity was affected by BCG vaccination or exposure to nontuberculous mycobacteria, only one person in the latent infection group had received BCG in the past. Furthermore, we used stringent criteria for latent infection and included subjects only if they had been exposed to a person with culture-positive pulmonary tuberculosis and were TST positive. We identified subjects who had completed therapy, and samples were not available from these individuals before and during active disease to document changes in immune activation and Treg cell frequency before, during, and after the course of treatment. For identification of Treg cells, we used the currently accepted combination of CD25^{high} CD127^{low} FOXP3⁺ staining (36, 40). Recent studies have identified the intracellular protein GARP as a potentially important marker for identification of activated T regulatory cells (49, 53). Future studies will be required to determine whether this will increase the specificity for Treg cells. We had insufficient cells to perform functional studies of Treg cells, but it would be interesting to evaluate whether Treg cells provide greater suppression of *M. tuberculosis*-specific T cell responses in individuals with prior extrapulmonary tuberculosis. Finally, we were unable to control for other factors, such as age (52) and nutrition (39), that may affect Treg cell frequency. However, there was no difference in median age between the study groups.

In conclusion, this study has several important findings. We found evidence of an increased frequency of regulatory T lymphocytes and generalized CD4⁺ and CD8⁺ T lymphocyte activation among persons with previously treated extrapulmonary tuberculosis. We also found a correlation between increased immune activation and an increased frequency of Treg cells in persons who had completed treatment for tuberculosis. The presence of persisting immune activation and correspondingly high frequencies of regulatory T lymphocytes may reflect immune dysregulation that predisposes individuals to clinical tuberculosis, specifically to extrapulmonary tuberculosis. Further prospective studies before infection and after therapy should be undertaken in order to better understand the regulation of the host immune response during *M. tuberculosis* infection.

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