

Regulatory T Cells Modulate Th17 Responses in Patients with Positive Tuberculin Skin Test Results

Subash Babu,^{1,3} Sajid Q. Bhat,¹ N. Pavan Kumar,¹ V. Kumaraswami,^{1,2} and Thomas B. Nutman⁴

¹National Institutes of Health—International Center for Excellence in Research and ²Tuberculosis Research Center, Chennai, India; ³Science Applications International, National Cancer Institute, Frederick, and ⁴Laboratory of Parasitic Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Background. The factors governing latency in tuberculosis are not well understood but appear to involve both the pathogen and the host. We have used tuberculin skin test (TST) positivity as a tool to study cytokine responses in latent tuberculosis.

Methods. To identify the host factors that are important in the maintenance of TST positivity, we examined mycobacteria-specific immune responses of TST-positive (latent tuberculosis) or TST-negative individuals in South India, where TST positivity can define tuberculosis latency.

Results. Although purified protein derivative-specific and *Mycobacterium tuberculosis* culture filtrate antigen-specific Th1 and Th2 cytokines were not statistically significantly different between the 2 groups, the Th17 cytokines (interleukin 17 and interleukin 23) were statistically significantly decreased in TST-positive individuals, compared with those in TST-negative individuals. This Th17 cytokine modulation was associated with statistically significantly increased expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) and Foxp3. Although CTLA-4 blockade failed to restore full production of interleukin 17 and interleukin 23 in TST-positive individuals, depletion of regulatory T cells significantly increased production of these cytokines.

Conclusion. TST positivity is characterized by increased activity of regulatory T cells and a coincident down-regulation of the Th17 response.

Although *Mycobacterium tuberculosis* infects ~2 billion people worldwide, 90% of *M. tuberculosis*-infected individuals resist developing overt disease (active tuberculosis) and manifest only latent infection [1–3]. Latent tuberculosis is defined as the presence of live *M. tuberculosis* that persists within an infected host without causing disease. It is characterized by a delayed-type hypersensitivity response to purified protein derivative (PPD) mediated by mycobacteria-specific T cells [4], although in areas of low *M. tuberculosis* transmission, quantiferon assay positivity has been suggested to be a better predictor of latency [5]. In South India, however,

the tuberculin skin test (TST) has been conclusively shown to be an accurate diagnostic tool for latent tuberculosis [6].

Latent tuberculosis can be maintained for the lifetime of the individual unless the immunologic balance between host and pathogen is perturbed, resulting in reactivation of *M. tuberculosis* and active disease [7]. The host and environmental factors that can compromise the ability to contain latent infection are human immunodeficiency virus coinfection, malnutrition, aging, stress, type 2 diabetes mellitus, use of immunosuppressive agents, and genetic factors [7]. On the pathogen side, latency is thought to reflect a transition from replicating to nonreplicating dormant bacilli, with this transition being influenced by a variety of factors, including oxygen deprivation and nitric oxide [7, 8]. In vitro and in vivo models of latency, combined with genomewide transcriptome profiling, have led to identification of *M. tuberculosis* genes that are highly expressed during latency, called *dosR* or *devR* (dormancy) genes [7, 8]; however, each of the host- and pathogen-related factors controlling resistance and/or susceptibility to tuberculosis awaits complete elucidation.

Received 27 April 2009; accepted 20 August 2009; electronically published 24 November 2009.

Potential conflicts of interest: none reported.

Financial support: Intramural Research Program of the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Reprints or correspondence: Dr Subash Babu, NIH-ICER, Chetpet, Chennai, Tamil Nadu 600031, India (sbabu@mail.nih.gov).

The Journal of Infectious Diseases 2010;201:20–31

© 2009 by the Infectious Diseases Society of America. All rights reserved.

0022-1899/2010/20101-0005\$15.00

DOI: 10.1086/648735

As demonstrated using murine models, immunity to *M. tuberculosis* requires Th1 responses and (to a lesser extent) Th17 responses. Thus, interleukin 12 (IL-12), interferon γ (IFN- γ), and tumor necrosis factor α (TNF- α), as well as IL-17 and IL-23, all play important roles in induction and maintenance of protective immune responses against tuberculous [7, 9–11]. Recently, a number of regulatory factors, including regulatory T (Treg) cells, IL-10, transforming growth factor β (TGF- β), cytotoxic T lymphocyte antigen 4 (CTLA-4), and programmed death 1 (PD-1), have been implicated in establishment of chronic viral, bacterial, and parasitic infections [12–14]. To study the roles of the major T cell subsets and potential regulatory factors, we examined *M. tuberculosis* antigen-specific induction of Th1, Th2, Th17, and Treg responses as well as expression of IL-10, TGF- β , CTLA-4, and PD-1 in TST-positive or TST-negative individuals. We observed that TST positivity is characterized by increased expression of Treg markers and coincident down-regulation of Th17 cells, thus providing a novel mechanism by which Th17 responses are negatively regulated in human infection.

METHODS

Study population. We studied a group of 25 individuals who were TST positive and 25 who were TST negative in Tamil Nadu, South India. The age distribution of the TST-positive group was 19–50 years (median age, 32 years) and that of the TST-negative group was 15–48 years (median age, 30 years). Each of the 2 groups comprised 15 men and 10 women. The first 30 patients were recruited for cytokine and messenger RNA (mRNA) studies, whereas the remaining 20 patients were recruited for blockade and depletion studies. All TST-positive patients had positive skin test reactivity to intradermal tuberculin (2 TU). A positive TST result was defined as an induration at the site of inoculation of at least 12 mm in diameter to account for the high prevalence of environmental mycobacteria. This cutoff was based on a rigorous multivariate analysis of 280,000 patients followed up in South India for 15 years, in which patients with TST indurations of 8–11 mm in reaction to PPD standard had a risk for development of culture-positive tuberculosis that was low (and no different from that of patients with 0–7-mm indurations) but in which patients with indurations of ≥ 12 mm, in comparison, had a 3-fold greater relative risk and a 6.1% lifetime risk for development of active tuberculosis [6]. All TST-negative patients had indurations < 5 mm in diameter. All patients had normal chest radiograph findings. None of the patients had pulmonary symptoms (cough, fever, chest pain, or hemoptysis) or sputum that tested positive for *M. tuberculosis* by smear microscopy and culture. All patients had been given the BCG vaccine at birth. All individuals were examined as part of a clinical protocol (NCT 01-I-N261) ap-

proved by institutional review boards both of the National Institute of Allergy and Infectious Diseases and of the Tuberculosis Research Center. Informed consent was obtained from all participants.

Isolation of peripheral blood mononuclear cells (PBMCs). Heparinized blood was collected and PBMCs were isolated by Ficoll diatrizoate gradient centrifugation in lymphocyte separation medium (ICN Biomedicals). Erythrocytes were lysed using ACK lysis buffer (Biosource International). Cells were then washed and cultured in Roswell Park Memorial Institute 1640 medium (BioWhittaker) supplemented with 20 mmol/L glutamine (BioWhittaker), 10% heat-inactivated fetal calf serum (Harlan Bioproducts for Science), and 50 $\mu\text{g}/\text{mL}$ gentamycin (Mediatech).

Antigens. Mycobacterial PPD (Statens Serum Institute, Copenhagen, Denmark) and culture filtrate antigen from *M. tuberculosis* strain H37 Rv (gift of Dr P. Selvaraj, Tuberculosis Research Center, Chennai, India) were used as antigenic stimuli, and anti-CD3 antibody was used as positive control. The final concentrations were 10 $\mu\text{g}/\text{mL}$ for PPD and *M. tuberculosis* culture filtrate antigen (Mtb CFA) and 5 $\mu\text{g}/\text{mL}$ for anti-CD3.

In vitro culture. PBMCs were cultured with PPD, Mtb CFA, or anti-CD3 in 24-well tissue culture plates (Corning) at concentrations of 5×10^6 per well. After 24 h, culture supernatants were collected and analyzed for cytokines. For depletion of Treg cells, we positively selected CD4⁺CD25⁺ T cells by use of a Treg isolation kit (Miltenyi-Biotec) and used the negatively selected cells for culture. For CTLA-4 blocking experiments, we cultured cells in the presence of CTLA-4 immunoglobulin (10 $\mu\text{g}/\text{mL}$) or control immunoglobulin (10 $\mu\text{g}/\text{mL}$) (Ancell).

Enzyme-linked immunosorbent assay (ELISA). Levels of cytokines in the culture supernatants were measured using the Bioplex multiplex cytokine assay system (Bio-Rad). The cytokines analyzed were IL-2, IFN- γ , TNF- α , IL-12, IL-4, IL-5, IL-10, IL-13, IL-17, IL-23, IL-6, and IL-1 β . ELISA for IL-23 was performed using a commercial kit (R&D Systems).

RNA preparation. PBMCs were lysed using the reagents of a commercial kit (QIAshredder; Qiagen). Total RNA was extracted according to the manufacturer's protocol (RNeasy Mini kit; Qiagen), and RNA was dissolved in 50 μL of ribonuclease-free water.

Complementary DNA (cDNA) synthesis. RNA (1 μg) was used to generate cDNA by use of TaqMan reverse transcription reagents, according to the manufacturer's protocol (Applied Biosystems). Briefly, random hexamers were used to prime RNA samples for reverse transcription (MultiScribe).

Real-time reverse-transcription polymerase chain reaction (RT-PCR). Real-time quantitative RT-PCR was performed in an ABI 7500 sequence detection system (Applied Biosystems) using TaqMan Assays-on-Demand reagents for CTLA-4, PD-1, TGF- β , Foxp3, ROR γt , and an endogenous 18s ribosomal

RNA control. Relative transcripts were determined by the formula $CT_{\text{target}} - CT_{\text{control}}$, where CT is the threshold cycle during the exponential phase of amplification.

Statistical analysis. Comparisons were made using either the Mann-Whitney *U* test (unpaired comparisons) or the Wilcoxon signed rank test (paired comparisons). The Spearman rank correlation was used to examine relationships between variables. All statistics were performed using Prism software (version 5; GraphPad) for Windows.

RESULTS

No association between TST positivity and alterations in Th1 or Th2 cytokines. To determine the impact of TST positivity on Th1 responses, we stimulated PBMCs from TST-positive or TST-negative individuals with PPD, Mtb CFA, or anti-CD3 for 24 h and measured levels of prototypical Th1 cytokines by ELISA (Figure 1A). There were no intrinsic differences between the 2 groups in the levels of IFN- γ , TNF- α , IL-2, or IL-12 produced spontaneously or in response to anti-CD3. In addition, in response to either PPD or Mtb CFA, there were no differences between the 2 groups in the levels of IFN- γ (geometric mean [GM] for PPD, 608.2 and 418.5 pg/mL in TST-positive and TST-negative groups, respectively; GM for Mtb CFA, 669.1 and 835.7 pg/mL in TST-positive and TST-negative groups, respectively), TNF- α (GM for PPD, 2343 and 2329 pg/mL in TST-positive and TST-negative groups, respectively; GM for Mtb CFA, 2464 and 2488 pg/mL in TST-positive and TST-negative groups, respectively), IL-2 (GM for PPD, 353.1 and 296 pg/mL in TST-positive and TST-negative groups, respectively; GM for Mtb CFA, 479.8 and 392.6 pg/mL in TST-positive and TST-negative groups, respectively), or IL-12 (GM for PPD, 28.99 and 48.45 pg/mL in TST-positive and TST-negative groups, respectively; GM for Mtb CFA, 15.43 and 147.7 pg/mL in TST-positive and TST-negative groups, respectively).

To determine the impact of TST positivity on mycobacterial antigen-specific Th2 responses, we stimulated PBMCs from TST-positive or TST-negative individuals with PPD, Mtb CFA, or anti-CD3 for 24 h, measured the levels of IL-4, IL-5, and IL-13, and compared them to the levels produced spontaneously. As with the Th1 cytokines, there were no statistically significant differences in spontaneous or anti-CD3-stimulated production of IL-4, IL-5, or IL-13. In addition, neither PPD nor Mtb CFA induced statistically significant differences in the production of IL-4, IL-5, or IL-13 between the 2 groups (Figure 1B).

Association between TST positivity and down-regulation of IL-17 and IL-23 production. To determine the impact of TST positivity on mycobacterial antigen-specific Th17 responses, we stimulated PBMCs from TST-positive or TST-negative individuals with PPD, Mtb CFA, or anti-CD3 for 24 h and measured

the levels of IL-17 and its inducing cytokines, IL-23, IL-6, and IL-1 β . There were no statistically significant differences between the 2 groups in either spontaneous or anti-CD3-stimulated production of IL-17, IL-23, IL-6, or IL-1 β . In contrast, in response to both PPD and Mtb CFA, compared with individuals in the TST-negative group, those in the TST-positive group produced statistically significantly less IL-17 (GM for PPD, 105.2 and 823.7 pg/mL in TST-positive and TST-negative groups, respectively [$P < .001$]; GM for Mtb CFA, 186.3 and 937 pg/mL in TST-positive and TST-negative groups, respectively [$P < .001$]) and statistically significantly less IL-23 (GM for PPD, 167.9 and 920.9 pg/mL in TST-positive and TST-negative groups, respectively [$P < .001$]; GM for Mtb CFA, 143.7 and 1146 pg/mL in TST-positive and TST-negative groups, respectively [$P = .001$]) (Figure 2). There were no statistically significant differences in the production of IL-6 and IL-1 β in response to PPD or Mtb CFA between the TST-positive and TST-negative groups.

Association between TST positivity and enhanced Foxp3 and diminished ROR γ t expression. Because Treg and Th17 cells exhibit mutually reciprocal inhibition in murine T cells and because Foxp3 and ROR γ t are the transcription factors driving Treg and Th17 cell differentiation, respectively [15, 16], we isolated RNA from cells stimulated with PPD, Mtb CFA, or anti-CD3 and estimated the RNA levels of Foxp3 and ROR γ t by quantitative RT-PCR. Although no differences between baseline and anti-CD3-induced Foxp3 or ROR γ t expression were observed, both PPD and Mtb CFA induced a statistically significantly higher expression of Foxp3 (GM fold change for PPD, 1.897 and 0.7604 in TST-positive and TST-negative groups, respectively [$P < .001$]; GM for Mtb CFA, 4.302 and 0.7105 in TST-positive and TST-negative groups, respectively [$P < .001$]) and a statistically significantly lower expression of ROR γ t (GM fold change for PPD, 0.9443 and 2.463 in TST-positive and TST-negative groups, respectively [$P < .001$]; GM for Mtb CFA, 0.9491 and 2.630 in TST-positive and TST-negative groups, respectively [$P < .001$]) in TST-positive individuals compared with TST-negative individuals (Figure 3). Furthermore, there was a striking inverse relationship between expression of Foxp3 following PPD and Mtb CFA stimulation and that of ROR γ t ($r = -0.7937$ and $P < .001$ for PPD; $r = -0.7005$ and $P < .001$ for Mtb CFA by Spearman rank), suggesting that the induction of Treg expression was modulating Th17 cells in the context of mycobacterial antigen stimulation.

No association between TST positivity and changes in the expression or production of TGF- β and IL-10. Because TGF- β and IL-10 are associated with down-regulation of immune responses, we investigated the expression patterns of TGF- β and IL-10 in latent tuberculosis. We used RT-PCR to examine the expression of TGF- β , and we used ELISA to examine the production of IL-10. As shown in Figure 4, we observed no

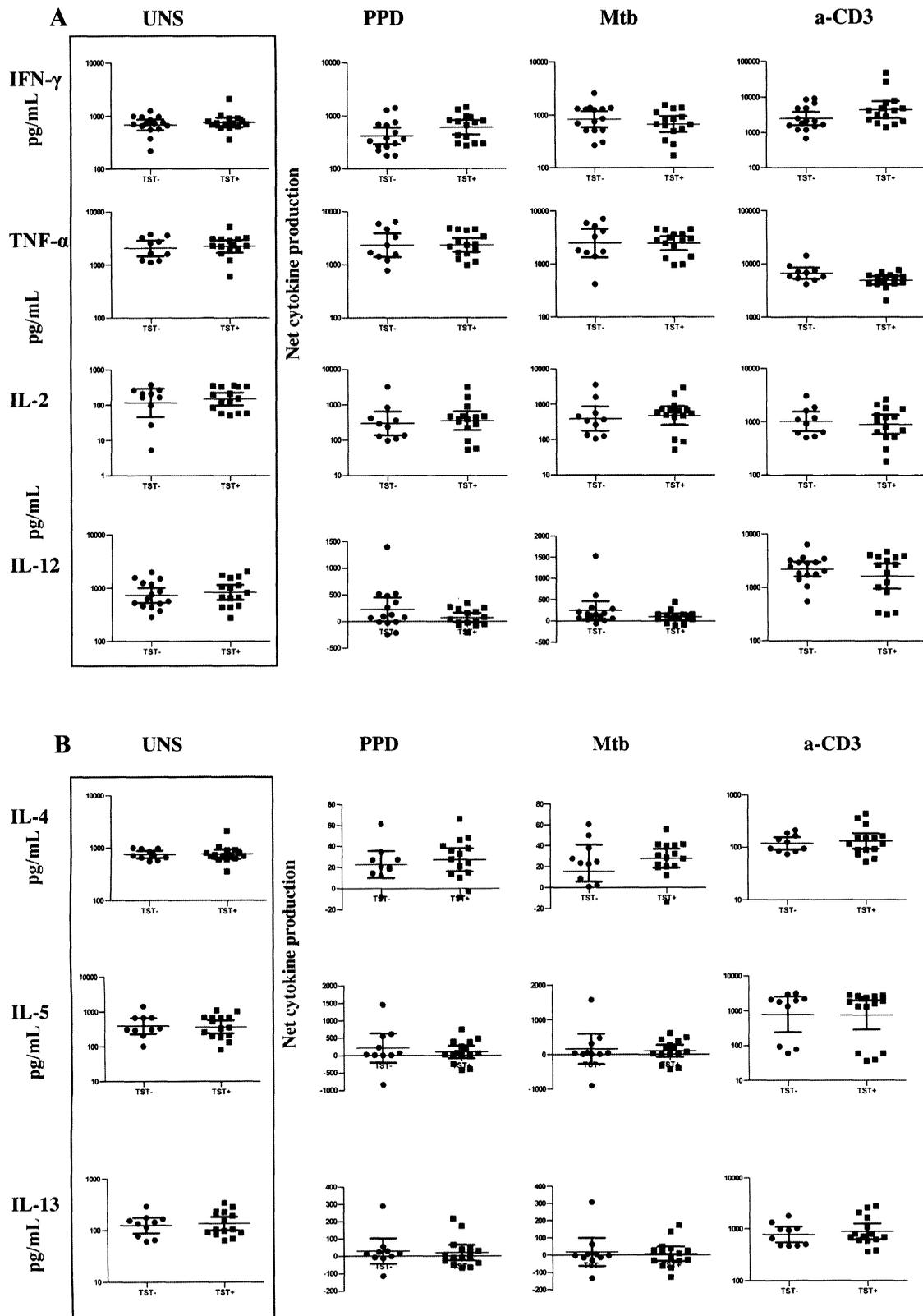


Figure 1. Results showing that tuberculin skin test (TST) positivity is not associated with alteration in Th1 or Th2 cytokines. Peripheral blood mononuclear cells from TST-positive (TST⁺) ($n = 15$) and TST-negative (TST⁻) ($n = 10$) patients were stimulated with purified protein derivative (PPD) ($10 \mu\text{g/mL}$), *Mycobacterium tuberculosis* (Mtb) culture filtrate antigen ($10 \mu\text{g/mL}$), or anti-CD3 (a-CD3) ($5 \mu\text{g/mL}$) for 24 h, and levels of Th1 cytokines interleukin 2 (IL-2), interferon γ (IFN- γ), tumor necrosis factor α (TNF- α), and IL-12 (A) as well as levels of Th2 cytokines IL-4, IL-5, and IL-13 (B) were measured by enzyme-linked immunosorbent assay. Results are shown as net cytokine production above the control levels. P values were calculated using the Mann-Whitney U test. UNS, unstimulated.

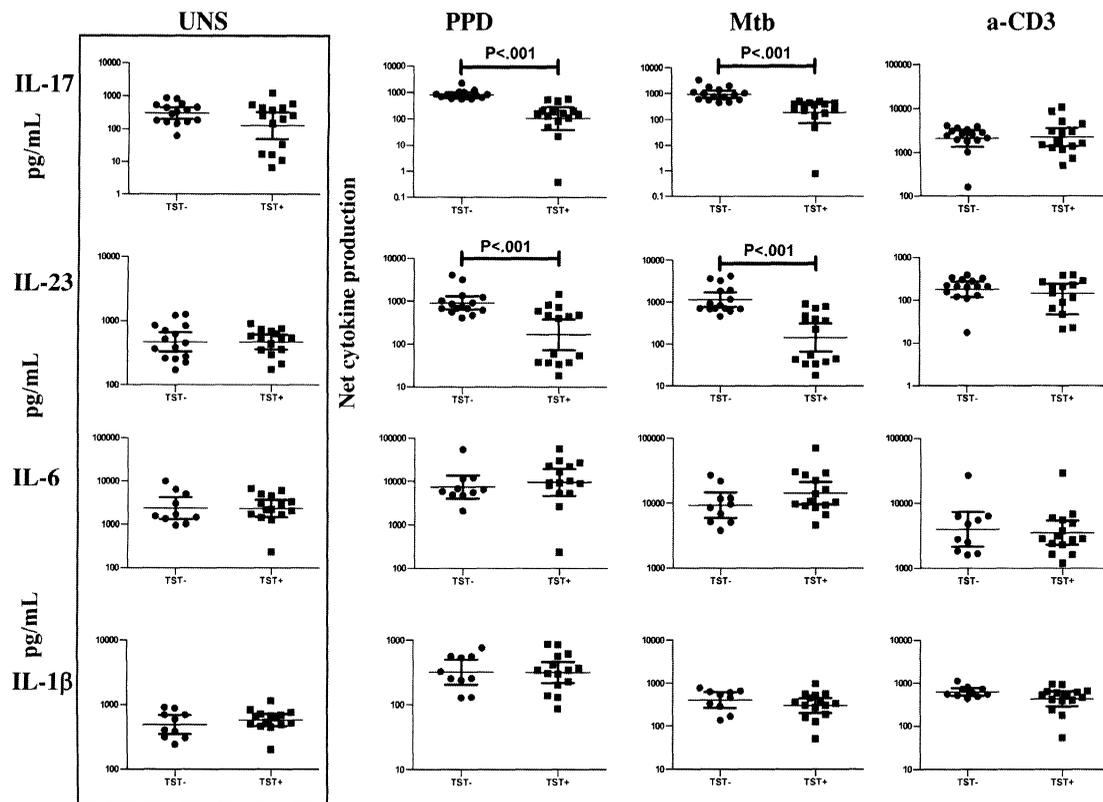


Figure 2. Results showing that tuberculin skin test (TST) positivity is associated with down-regulation of antigen-specific interleukin 17 (IL-17) and IL-23. Peripheral blood mononuclear cells from TST-positive (TST⁺) ($n = 15$) and TST-negative (TST⁻) ($n = 10$) patients were stimulated with purified protein derivative (PPD) (10 μ g/mL), *Mycobacterium tuberculosis* (Mtb) culture filtrate antigen (10 μ g/mL), or anti-CD3 (a-CD3) (5 μ g/mL) for 24 h, and IL-17, IL-23, IL-6, and IL-1 β cytokine levels were measured by enzyme-linked immunosorbent assay. Results are shown as net cytokine production above the control levels. P values were calculated using the Mann-Whitney U test, and only statistically significant P values are shown. UNS, unstimulated.

statistically significant differences between the expression at baseline and that following stimulation by mycobacterial antigen or anti-CD3.

Association between TST positivity and increased expression of CTLA-4 but not PD-1. Because CTLA-4 and PD-1 have been shown to be involved in the regulation of immune responses in active tuberculosis [17, 18], we isolated RNA from cells stimulated with PPD, Mtb CFA, or anti-CD3 and measured the expression of CTLA-4 and PD-1. As shown in Figure 5, we observed an increase in the expression of PPD- and Mtb CFA-induced CTLA-4 mRNA in the TST-positive individuals, compared with TST-negative individuals (GM fold change for PPD, 2.052 and 0.4525 in TST-positive and TST-negative groups, respectively [$P < .001$]; GM for Mtb CFA, 2.048 and 0.8518 in TST-positive and TST-negative groups, respectively [$P < .002$]), but no increase in PD-1 mRNA (GM fold change for PPD, 1.225 and 1.276 in TST-positive and TST-negative groups, respectively; GM for Mtb CFA, 1.577 and 2.012 in TST-positive and TST-negative groups, respectively). No difference between

the baseline and the anti-CD3-induced levels of CTLA-4 and PD-1 mRNA was observed.

Antigen-specific Th1 and Th17 responses significantly enhanced by depletion of Treg cells in TST-positive individuals.

To determine the functional relationship between the increased expression of Foxp3 and the down-regulated Th17 responses observed in TST-positive individuals, we cultured cells from these patients with PPD or Mtb CFA after depletion of CD4⁺CD25⁺ T cells and measured the levels of IFN- γ , IL-17, and IL-23 by ELISA (Figure 6A). Depletion of CD4⁺CD25⁺ T cells completely abrogated the induction of Foxp3 mRNA in antigen-stimulated cultures (data not shown). We also observed statistically significantly increased (2–3-fold) PPD- and Mtb CFA-induced production of IFN- γ (GM for PPD, 253.1 and 100.6 pg/mL in the absence and presence of Treg cells, respectively [$P < .001$]; GM for Mtb CFA, 135.3 and 79.26 pg/mL in the absence and presence of Treg cells, respectively [$P = .002$]), IL-17 (GM for PPD, 248 and 126.3 pg/mL in the absence and presence of Treg cells, respectively [$P = .005$]; GM for Mtb

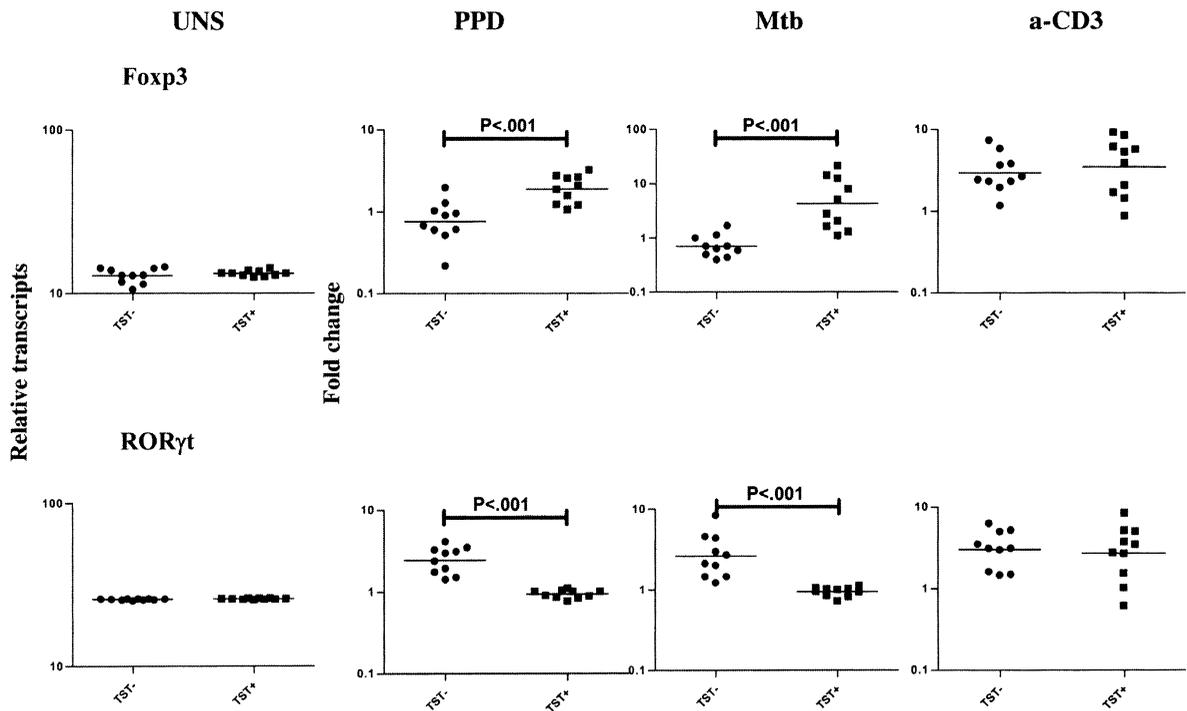
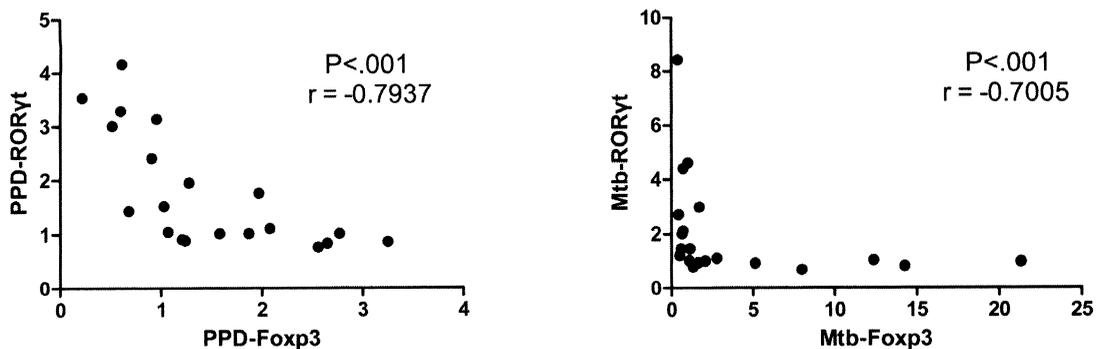
A**B**

Figure 3. Results showing that tuberculin skin test (TST) positivity is associated with up-regulation of Fxp3 messenger RNA (mRNA) and down-regulation of ROR γ t. *A*, Fxp3 and ROR γ t mRNA levels measured by real-time reverse-transcription polymerase chain reaction. Peripheral blood mononuclear cells from TST-positive (TST⁺) ($n = 10$) and TST-negative (TST⁻) ($n = 10$) patients had been stimulated with purified protein derivative (PPD) (10 μ g/mL), *Mycobacterium tuberculosis* (Mtb) culture filtrate antigen (10 μ g/mL), or anti-CD3 (a-CD3) (5 μ g/mL) for 24 h. Results are shown as fold change above the control levels. *P* values were calculated using the Mann-Whitney *U* test. *B*, Correlation between PPD- and *M. tuberculosis*-induced levels of Fxp3 and ROR γ t mRNA, shown as X-Y scatter plots. *P* values were calculated using the Spearman rank correlation, and only statistically significant *P* values are shown. UNS, unstimulated.

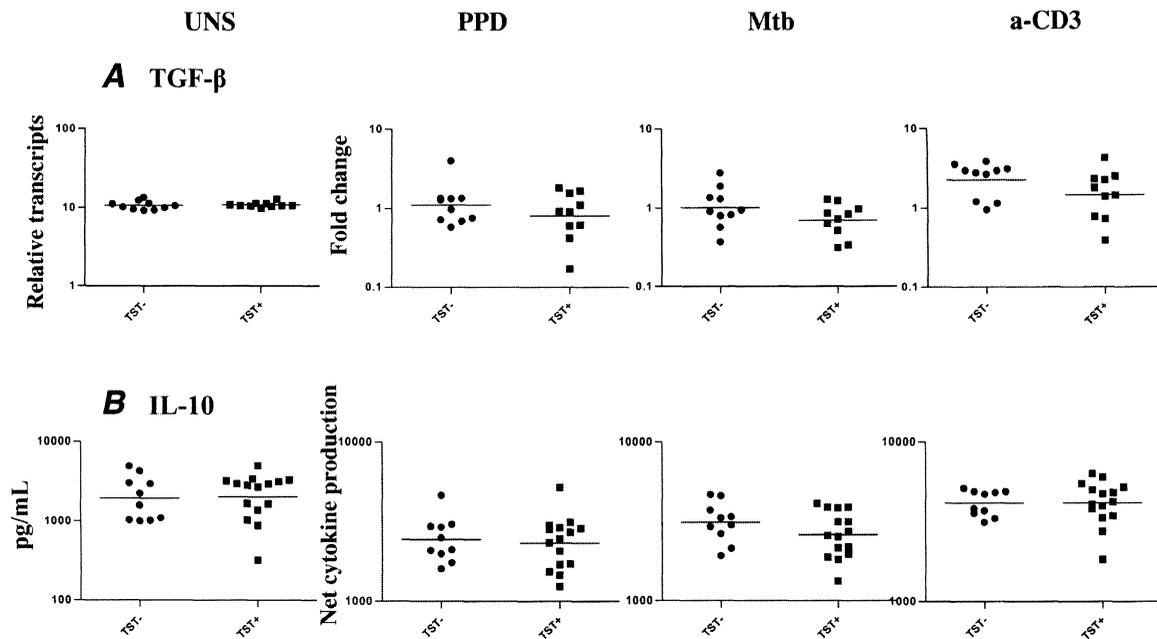


Figure 4. Results showing that tuberculin skin test (TST) positivity is not associated with changes in the expression of transforming growth factor β (TGF- β) or interleukin 10 (IL-10). Peripheral blood mononuclear cells from TST-positive (TST⁺) ($n = 10$ –15) and TST-negative (TST⁻) ($n = 10$) patients were stimulated with purified protein derivative (PPD) (10 μ g/mL), *Mycobacterium tuberculosis* (Mtb) culture filtrate antigen (10 μ g/mL), or anti-CD3 (a-CD3) (5 μ g/mL) for 24 h, and TGF- β messenger RNA and IL-10 cytokine levels were measured by real-time reverse-transcription polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. Results are shown as fold change or as net cytokine production above the control levels. P values were calculated using the Mann-Whitney U test. UNS, unstimulated.

CFA, 294.8 and 118.5 pg/mL in the absence and presence of Treg cells, respectively [$P = .001$]), and IL-23 (GM for PPD, 80.11 and 26.36 pg/mL in the absence and presence of Treg cells, respectively [$P = .010$]; GM for Mtb CFA, 41.85 and 21.18 pg/mL in the absence and presence of Treg cells, respectively [$P = .006$]) after Treg depletion in TST-positive individuals.

To ensure that Treg depletion was not just enhancing Th1 and Th17 immune responses nonspecifically, we cultured cells from TST-negative individuals and used ELISA to examine the production of IFN- γ , IL-17, and IL-23 (Figure 6B) after the removal of Treg cells. We observed no statistically significant changes in PPD- and Mtb CFA-induced production of IFN- γ , IL-17, and IL-23 in the presence or absence of Treg cells in TST-negative individuals. Collectively, these data suggest that Treg cells play an important role in the down-regulation of mycobacterial antigen-specific Th1 and Th17 responses in TST-positive individuals.

Antigen-specific Th1 and Th17 down-regulation not reversed by CTLA-4 blockade in TST positivity. To determine the relationship between increased expression of CTLA-4 and the down-regulated Th17 responses that were observed in TST-positive individuals, we cultured cells from these patients with PPD or Mtb CFA in the presence of CTLA-4 immunoglobulin or control immunoglobulin and used ELISA to measure the levels of IFN- γ , IL-17, and IL-23. As shown in Figure 7, we

observed no significant changes in PPD- and Mtb CFA-induced production of IFN- γ , IL-17, and IL-23 after CTLA blockade. Collectively, these data suggest that CTLA-4 does not play a major role in down-regulation of mycobacterial antigen-specific Th17 responses.

DISCUSSION

Infection with *M. tuberculosis* can produce a variety of outcomes, including active infection, which manifests either as primary fulminant tuberculosis or as chronic, slowly progressing tuberculosis, and latent infection, which occurs when the initial infection is controlled but not completely eliminated [9]. Active tuberculosis is characterized by increased Treg responses and decreased Th1 responses, with both factors contributing to the establishment of active infection [19–21]; however, the roles played by Treg cells and by Th1, Th2, and Th17 cells in latent tuberculosis have not been elucidated. Because latent tuberculosis afflicts the overwhelming majority (90%) of the ~2 billion people who have tuberculosis, the study of factors influencing the development of latent infection is of great importance. To this end, we studied 2 groups of individuals from the same community: 1 group tested positive for the tuberculin skin reaction, and the other group tested negative. Our study of antigen-driven T cell responses in these individuals unrav-

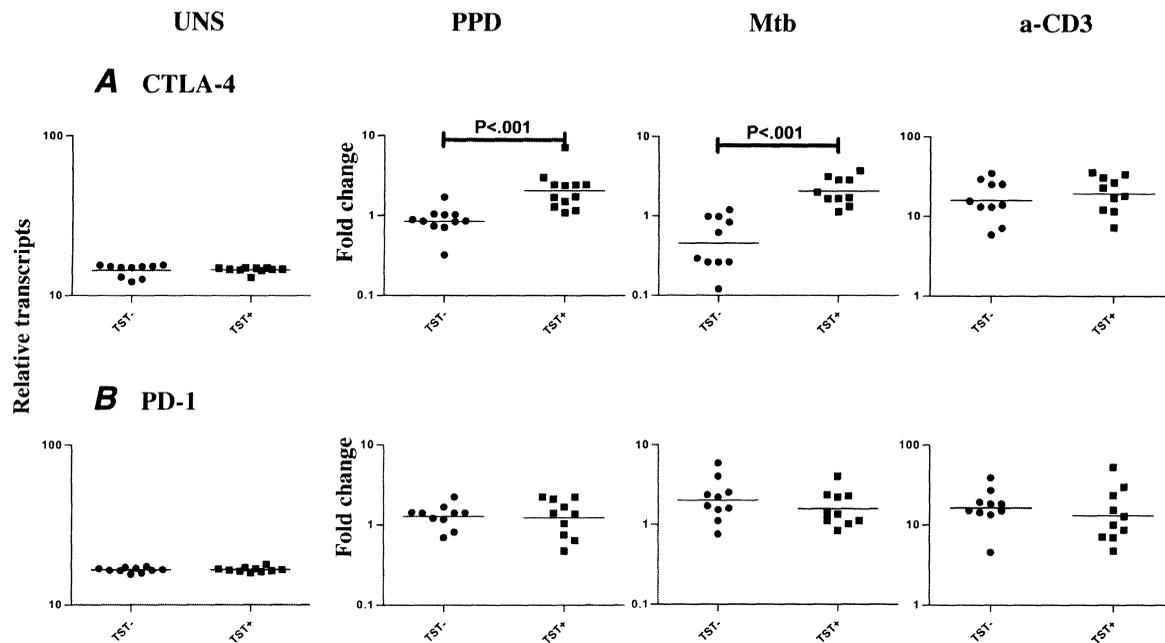


Figure 5. Results showing that tuberculin skin test (TST) positivity is associated with up-regulation of CTLA-4 but not PD-1 messenger RNA. Peripheral blood mononuclear cells from TST-positive (TST⁺) ($n = 10$) and TST-negative (TST⁻) ($n = 10$) patients were stimulated with purified protein derivative (PPD) (10 $\mu\text{g}/\text{mL}$), *Mycobacterium tuberculosis* (Mtb) culture filtrate antigen (10 $\mu\text{g}/\text{mL}$), or anti-CD3 (a-CD3) (5 $\mu\text{g}/\text{mL}$) for 24 h, and CTLA-4 and PD-1 levels were measured by real-time reverse-transcription polymerase chain reaction. Results are shown as fold change above the control levels. P values were calculated using the Mann-Whitney U test, and only statistically significant P values are shown. UNS, unstimulated.

els several interesting factors that govern TST skin positivity, as discussed below.

T cell differentiation into Th1 and Th2 lineages on the basis of their cytokine profile and transcription factor expression has served as the foundation of our understanding the pathogenesis of a variety of infectious and allergic diseases [22]. With the advent of newer techniques, T cell differentiation has expanded into several subsets, including Treg cells, Th17 cells, and poly-functional T cells, among others [14, 23–26]. Th1 cells are essential for resistance to tuberculosis both in mice and in humans. Deficiencies in the IL-12–IFN- γ –Stat-1 pathway leads to disseminated mycobacterial infection in humans and to abrogation of resistance in mice [27, 28]. In addition, TNF- α , another Th1 cytokine, is of almost equal importance, as treatment with biologics (eg, anti-TNF- α antibody) for inflammatory disorders such as rheumatoid arthritis has caused reactivation of tuberculosis in some individuals [29]. We observed no differences in expression of Th1 cytokines both at baseline and after antigen-specific stimulation between the TST-positive and TST-negative individuals. Although induction of antigen-specific IFN- γ production is usually a hallmark of tuberculosis infection, it has been shown elsewhere that no significant differences in IFN- γ expression were observed when healthy but potentially exposed individuals were separated on the basis of TST reactivity [30, 31]. In addition, another potential expla-

nation might be that all individuals in the study had been given the BCG vaccine at birth, and although memory T cell–IFN- γ responses to mycobacterial antigen wane with time, these responses can be significantly higher than in individuals who have not been given the BCG vaccine [32]. The absence of differences between the 2 groups in our study with regard to Th1 response suggests that development of latent tuberculosis is not primarily dependent on Th1 responses.

Increased Th2 responses have also been postulated to play a role in susceptibility to tuberculosis, because IL-4 and IL-13 can undermine Th1-mediated immunity and drive inappropriate alternative activation of macrophages [33]. Our study reveals that Th2 cytokines play a very small role in TST positivity, because both TST-positive and TST-negative individuals had equivalent (and low) Th2 responses both at baseline and after antigen stimulation.

We also examined the induction of Th17 responses in TST-positive and TST-negative individuals. IL-17 is a potent inflammatory cytokine induced by *M. tuberculosis* infection; its induction is dependent, in general, on the presence of IL-1 β , IL-6, TGF- β , IL-21, and IL-23. The response of IL-17 to *M. tuberculosis* is largely dependent on IL-23 [10, 24], with Th17 differentiation requiring induction of transcription factor ROR γ t [16, 34]. Although Th17 cells are not as important as Th1 cells in mediating protection against primary *M. tuber-*

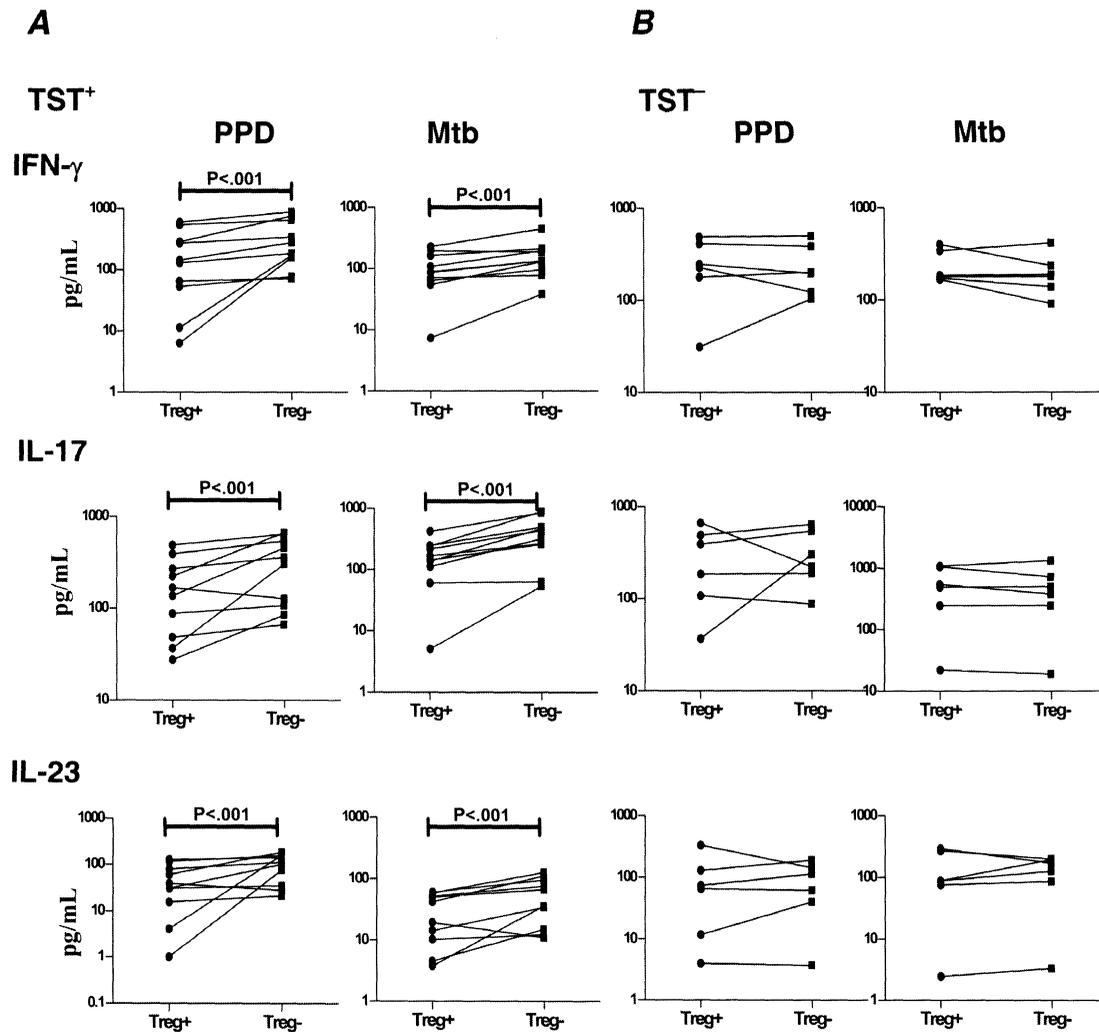


Figure 6. Results showing that CD4⁺CD25⁺ T cell depletion causes up-regulation of interferon γ (IFN- γ), interleukin 17 (IL-17), and IL-23 production in individuals with positive tuberculin skin test (TST) results. Peripheral blood mononuclear cell production of IFN- γ , IL-17, and IL-23 after 24-h stimulation with purified protein derivative (PPD) (10 μ g/mL) or *Mycobacterium tuberculosis* (Mtb) culture filtrate antigen (10 μ g/mL) in the presence of CD4⁺CD25⁺ T cell depletion in TST-positive (A) or TST-negative (B) individuals is shown as line graphs, where each line represents a single patient ($n = 6$ –10). Results are shown as net cytokine production above the control levels. P values were calculated using the Wilcoxon signed rank test, and only statistically significant P values are shown. Treg⁺, in the presence of regulatory T cells; Treg⁻, in the absence of regulatory T cells.

tuberculosis infection, IL-17 appears to be critical to the induction of *M. tuberculosis*-specific memory response and the mediation of protection against challenge infections and during vaccinations [35–38]. In addition, the IL-23–IL-17 axis has been found to be important in the human immune response to *M. tuberculosis* [31, 39–41]. Our finding that TST-positive individuals have significantly impaired IL-17 and IL-23 production suggests that lack of Th17 up-regulation is a main feature of TST positivity. In addition, diminished expression of ROR γ t suggests that lack of Th17 induction may be mediated at the transcriptional level.

A variety of factors have recently been discovered to play important roles in dampening immune responses in infectious

diseases, autoimmunity, and tumor biology. The most important and well studied are Treg cells, characterized by surface expression of markers CD4 and CD25 and intracellular expression of Foxp3 [14]. In addition, other factors that act independently from or in concert with Treg cells have also been studied, including negative costimulatory molecules CTLA-4, PD-1, and B and T lymphocyte attenuator indoleamine; inhibitory cytokines IL-10 and TGF- β ; metabolic enzyme 2,3-dioxygenase; ubiquitin ligases cbl-b and gene related to anergy in lymphocytes (GRAIL); perforin-dependent and/or granzyme B-dependent killing; and IL-2 consumption [42]. We examined the role of IL-10, TGF- β , and PD-1 and found no statistically significant differences in their expression or production be-

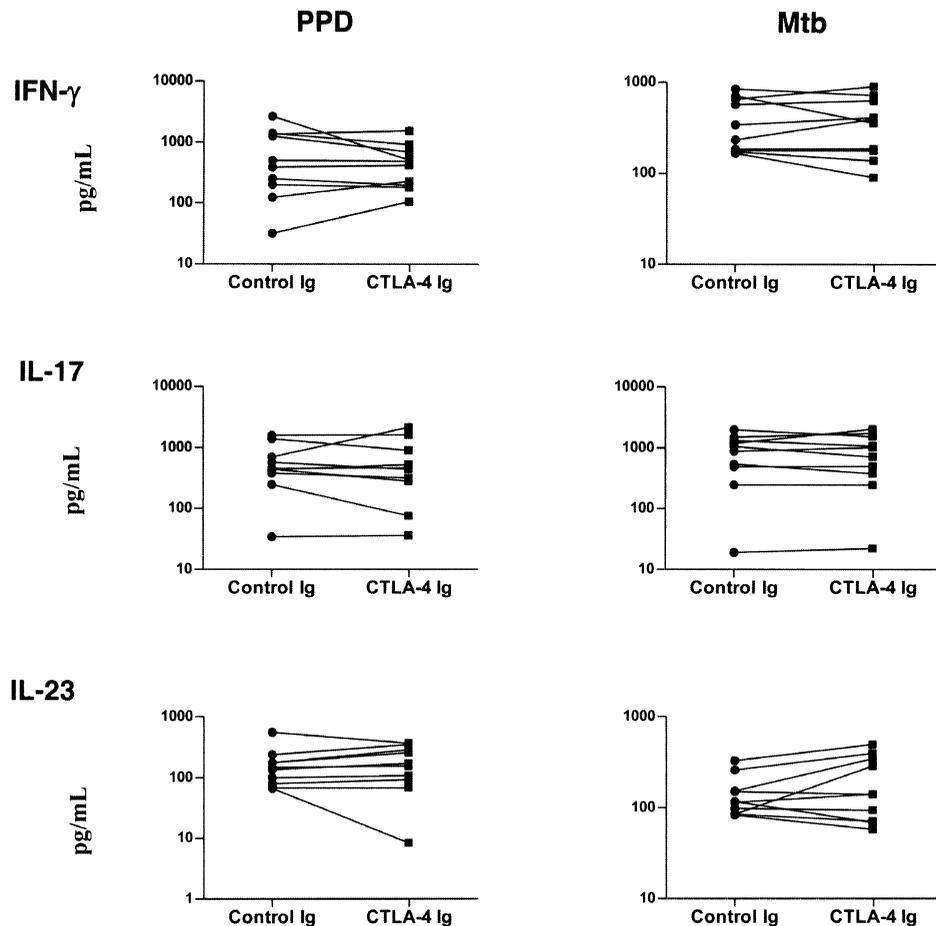


Figure 7. Results showing that CTLA-4 blockade does not alter the production of interferon γ (IFN- γ), interleukin 17 (IL-17), or IL-23 in individuals with positive tuberculin skin test (TST) results. Peripheral blood mononuclear cell production of IFN- γ , IL-17, and IL-23 after 24-h stimulation with purified protein derivative (PPD) (10 μ g/mL) or *Mycobacterium tuberculosis* (Mtb) culture filtrate antigen (10 μ g/mL) in the presence of CTLA-4 immunoglobulin (CTLA-4 Ig) or control immunoglobulin (control Ig) is shown as line graphs, where each line represents a single TST-positive patient ($n = 10$). Results are shown as net cytokine production above the control levels. P values were calculated using the Wilcoxon signed rank test.

tween TST-positive and TST-negative individuals; however, we did observe statistically significantly increased expression of Foxp3 (indicating enhanced Treg expression) and CTLA-4 (indicating enhanced coinhibitory activity by T cells) in TST-positive individuals. Inhibition of CTLA-4 interaction with its ligands CD80 and CD86 by CTLA-4 immunoglobulin administration had no effect on antigen-driven Th1 and Th17 responses, indicating that down-regulated Th17 responses in TST-positive individuals were not an effect of CTLA-4.

Treg cells are known regulators of Th17 responses in mice [43, 44]. Indeed, reciprocal regulation of Th17 and Treg cells is an established feature of T cell differentiation and is governed by the presence of specific cytokines [43, 44]. TGF- β is a known inducer of Treg cells as well as of Th17 cells [34, 43–48]. The presence of accessory cytokines IL-6 or IL-21, however, skews development of Th17 cells at the expense of the Treg lineage [34, 43–48]. This interdependent regulation of Th17 and Treg

cells is less well characterized in humans than is the association of Th17 up-regulation and Treg suppression that has been described in only a few human autoimmune and inflammatory disorders [49, 50]. Our study reveals that, in an infectious disease setting (latent tuberculosis as defined by TST positivity), there is a clear inverse relationship between Th17 cells and Treg cells and that depletion of CD4⁺CD25⁺ T cells reverses *M. tuberculosis*-specific Th17 inhibition in TST-positive but not TST-negative individuals.

Although we have used ELISA to measure cytokine responses and RT-PCR to measure mRNA expression of costimulatory and other molecules, further evaluation by using surface and intracellular cytokine flow cytometry is necessary. Nevertheless, this study highlights the differential immune responses that characterize susceptibility to latent tuberculosis and suggests that the balance between Th17 responses and Treg cells might play a vital role in mediating resistance to latent infection.

Elucidation of the mechanism by which Treg cells down-regulate Th17 responses and by which lack of Th17 cells or Th17-mediated cytokines predisposes humans to latent tuberculosis would provide valuable information for designing better therapeutics and vaccines for this highly prevalent infection.

Acknowledgments

We thank the Epidemiology Unit of the Tuberculosis Research Center, Tiruvallur, India, for valuable assistance in recruiting the patients for this study, and the National Institute of Allergy and Infectious Diseases intramural editor, Brenda Rae Marshall, for assistance.

References

- Comstock GW, Livesay VT, Woolpert SF. The prognosis of a positive tuberculin reaction in childhood and adolescence. *Am J Epidemiol* **1974**; 99:131–8.
- Lillebaek T, Dirksen A, Baess I, et al. Molecular evidence of endogenous reactivation of *Mycobacterium tuberculosis* after 33 years of latent infection. *J Infect Dis* **2002**; 185:401–4.
- Wayne LG, Sohaskey CD. Nonreplicating persistence of *Mycobacterium tuberculosis*. *Annu Rev Microbiol* **2001**; 55:139–63.
- Edwards PQ, Edwards LB. Story of the tuberculin test from an epidemiologic viewpoint. *Am Rev Respir Dis* **1960**; 81:1–47.
- Vinton P, Mhrshahi S, Johnson P, et al. Comparison of QuantiFERON-TB Gold In-Tube Test and tuberculin skin test for identification of latent *Mycobacterium tuberculosis* infection in healthcare staff and association between positive test results and known risk factors for infection. *Infect Control Hosp Epidemiol* **2009**; 30:215–21.
- Radhakrishna S, Frieden TR, Subramani R. Association of initial tuberculin sensitivity, age and sex with the incidence of tuberculosis in south India: a 15-year follow-up. *Int J Tuberc Lung Dis* **2003**; 7:1083–91.
- Lin MY, Ottenhoff TH. Not to wake a sleeping giant: new insights into host-pathogen interactions identify new targets for vaccination against latent *Mycobacterium tuberculosis* infection. *Biol Chem* **2008**; 389:497–511.
- Locht C, Rouanet C, Hougardy JM, Mascart F. How a different look at latency can help to develop novel diagnostics and vaccines against tuberculosis. *Expert Opin Biol Ther* **2007**; 7:1665–77.
- Kaufmann SH. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol* **2001**; 1:20–30.
- Khader SA, Cooper AM. IL-23 and IL-17 in tuberculosis. *Cytokine* **2008**; 41:79–83.
- North RJ, Jung YJ. Immunity to tuberculosis. *Annu Rev Immunol* **2004**; 22:599–623.
- Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol* **2005**; 23:515–48.
- Li MO, Flavell RA. Contextual regulation of inflammation: a duet by transforming growth factor- β and interleukin-10. *Immunity* **2008**; 28:468–76.
- Sakaguchi S. Naturally arising CD4⁺ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* **2004**; 22:531–62.
- Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor *Foxp3*. *Science* **2003**; 299:1057–61.
- Ivanov II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor ROR γ t directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell* **2006**; 126:1121–33.
- Jurado JO, Alvarez IB, Pasquinelli V, et al. Programmed death (PD)-1:PD-ligand 1/PD-ligand 2 pathway inhibits T cell effector functions during human tuberculosis. *J Immunol* **2008**; 181:116–25.
- Kirman J, McCoy K, Hook S, et al. CTLA-4 blockade enhances the immune response induced by mycobacterial infection but does not lead to increased protection. *Infect Immun* **1999**; 67:3786–92.
- Li L, Wu CY. CD4⁺CD25⁺ Treg cells inhibit human memory $\gamma\delta$ T cells to produce IFN- γ in response to *M. tuberculosis* antigen ESAT-6. *Blood* **2008**; 111:5629–36.
- Roberts T, Beyers N, Aguirre A, Walzl G. Immunosuppression during active tuberculosis is characterized by decreased interferon- γ production and CD25 expression with elevated forkhead box P3, transforming growth factor- β , and interleukin-4 mRNA levels. *J Infect Dis* **2007**; 195:870–8.
- Scott-Browne JP, Shafiani S, Tucker-Heard G, et al. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *J Exp Med* **2007**; 204:2159–69.
- Murphy KM, Reiner SL. The lineage decisions of helper T cells. *Nat Rev Immunol* **2002**; 2:933–44.
- Fontenot JD, Rudensky AY. Molecular aspects of regulatory T cell development. *Semin Immunol* **2004**; 16:73–80.
- McGeachy MJ, Cua DJ. Th17 cell differentiation: the long and winding road. *Immunity* **2008**; 28:445–53.
- Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* **2008**; 8:247–58.
- Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* **2007**; 25:821–52.
- Flynn JL. Lessons from experimental *Mycobacterium tuberculosis* infections. *Microbes Infect* **2006**; 8:1179–88.
- Holland SM. Interferon γ , IL-12, IL-12R and STAT-1 immunodeficiency diseases: disorders of the interface of innate and adaptive immunity. *Immunol Res* **2007**; 38:342–6.
- Jacobs M, Samarina A, Grivennikov S, et al. Reactivation of tuberculosis by tumor necrosis factor neutralization. *Eur Cytokine Netw* **2007**; 18:5–13.
- Liebshuetz S, Bamber S, Ewer K, et al. Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. *Lancet* **2004**; 364:2196–203.
- Scriba TJ, Kalsdorf B, Abrahams DA, et al. Distinct, specific IL-17- and IL-22-producing CD4⁺ T cell subsets contribute to the human anti-mycobacterial immune response. *J Immunol* **2008**; 180:1962–70.
- Weir RE, Gorak-Stolinska P, Floyd S, et al. Persistence of the immune response induced by BCG vaccination. *BMC Infect Dis* **2008**; 8:9.
- Kahnert A, Seiler P, Stein M, et al. Alternative activation deprives macrophages of a coordinated defense program to *Mycobacterium tuberculosis*. *Eur J Immunol* **2006**; 36:631–47.
- Manel N, Unutmaz D, Littman DR. The differentiation of human T_H17 cells requires transforming growth factor- β and induction of the nuclear receptor ROR γ t. *Nat Immunol* **2008**; 9:641–9.
- Khader SA, Bell GK, Pearl JE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4⁺ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat Immunol* **2007**; 8:369–77.
- Khader SA, Pearl JE, Sakamoto K, et al. IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN- γ responses if IL-12p70 is available. *J Immunol* **2005**; 175:788–95.
- Umemura M, Yahagi A, Hamada S, et al. IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis* bacille Calmette-Guérin infection. *J Immunol* **2007**; 178:3786–96.
- Wozniak TM, Ryan AA, Triccas JA, Britton WJ. Plasmid interleukin-23 (IL-23), but not plasmid IL-27, enhances the protective efficacy of a DNA vaccine against *Mycobacterium tuberculosis* infection. *Infect Immun* **2006**; 74:557–65.
- Dheda K, Chang JS, Lala S, et al. Gene expression of IL17 and IL23 in the lungs of patients with active tuberculosis. *Thorax* **2008**; 63:566–8.
- Pasquinelli V, Townsend JC, Jurado JO, et al. IFN- γ production during

- active tuberculosis is regulated by mechanisms that involve IL-17, SLAM, and CREB. *J Infect Dis* **2009**;199:661–5.
41. Peng MY, Wang ZH, Yao CY, et al. Interleukin 17-producing $\gamma\delta$ T cells increased in patients with active pulmonary tuberculosis. *Cell Mol Immunol* **2008**;5:203–8.
 42. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* **2008**;8:523–32.
 43. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector T_H17 and regulatory T cells. *Nature* **2006**;441:235–8.
 44. Zhou L, Lopes JE, Chong MM, et al. TGF- β -induced Foxp3 inhibits T_H17 cell differentiation by antagonizing ROR γ t function. *Nature* **2008**;453:236–40.
 45. Korn T, Bettelli E, Gao W, et al. IL-21 initiates an alternative pathway to induce proinflammatory T_H17 cells. *Nature* **2007**;448:484–7.
 46. Mangan PR, Harrington LE, O'Quinn DB, et al. Transforming growth factor- β induces development of the T_H17 lineage. *Nature* **2006**;441:231–4.
 47. Mucida D, Park Y, Kim G, et al. Reciprocal T_H17 and regulatory T cell differentiation mediated by retinoic acid. *Science* **2007**;317:256–60.
 48. Yang L, Anderson DE, Baecher-Allan C, et al. IL-21 and TGF- β are required for differentiation of human T_H17 cells. *Nature* **2008**;454:350–2.
 49. Cheng X, Yu X, Ding YJ, et al. The Th17/Treg imbalance in patients with acute coronary syndrome. *Clin Immunol* **2008**;127:89–97.
 50. Nistala K, Moncrieffe H, Newton KR, et al. Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum* **2008**;58:875–87.