Reduced Th17 Response in Patients with Tuberculosis Correlates with IL-6R Expression on CD4+ T Cells

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Rationale: Although it is well recognized that CD4+ T cells and T helper (Th) 1 cytokines are critical in the cell-mediated response to Mycobacterium tuberculosis, it is also clear that this immunity alone is not enough. Understanding the roles of other T cell subsets and cytokines is essential for vaccine design and clinical immunotherapy against tuberculosis (TB).

Objectives: To investigate the clinical significance and possible regulatory mechanism of Th17 responses in human TB.

Methods: The frequencies of IFN-γ–, IL-4–, IL-17–, FoxP3– and IL-6 receptor (IL-6R)–expressing CD4+ T cells in blood and/or pleural effusion samples of healthy donors, subjects with latent TB infection, and patients with active TB were analyzed by flow cytometry. Cytokines, transforming growth factor-β and IL-6, in plasma and pleural fluid samples were determined by ELISA.

Measurements and Main Results: The frequency of Th17 cells in patients with active TB is significantly lower than those in healthy donors and individuals with latent TB infection. Correlation analysis showed that reduced Th17 responses observed in patients with active TB was significantly correlated with the decreased expression of IL-6R on CD4+ T cells, but did not correlate with the concentrations of the cytokines, transforming growth factor-β and IL-6. Consistently; in vitro study showed that M. tuberculosis products inhibit the expression of IL-6R on CD4+ T cells.

Conclusions: Our results demonstrate that reduced Th17 responses were associated with the clinical outcome of M. tuberculosis infection. Suppression of Th17 response through down-regulation of IL-6R expression may be an important mechanism in the development of active TB.

Keywords: T helper 17 response; IL-6 receptor; Mycobacterium tuberculosis

Tuberculosis (TB) caused by Mycobacterium tuberculosis is one of the three major killers among infectious diseases, with an estimated 8–9 million new cases and 2–3 million deaths from TB annually (1). Despite the high infectivity of M. tuberculosis, only 5–10% of infected individuals develop active disease with clinical symptoms (2). In addition, the manifestation among patients with active disease varies substantially, from mild symptoms to severe or fatal TB, with the latter often characterized by large cavitary lung lesions, military TB, or meningitis (3). These facts indicate that protective immunity against M. tuberculosis infection plays an important role in disease development (2). Although it is well recognized that CD4+ T cells and T helper (Th) 1 cytokines are critical in the cell-mediated response to M. tuberculosis (2, 4–8), it is also clear that this immunity alone is not enough (2, 7). The roles of other T cell subsets and cytokines are only beginning to be elucidated (8–10).

Th17 cells belong to a CD4+ T cell subset that is distinct from Th1 and Th2 subsets; Th17 cells have significant proinflammatory functions via production of the cytokines, IL-17A and IL-17F (11). Th17 cells have been reported to play a central role, not only in the development of autoimmune and inflammatory diseases (12–14), but also in protection against intracellular pathogens (15, 16). Although initial studies suggested that γδ T cells are a primary source of IL-17 in response to M. tuberculosis infection (17, 18), recent studies have indicated that Th17 cells are the major IL-17–producing cells and participate in the protective immunity against M. tuberculosis (9, 19–22). Khader and colleagues (9, 23) reported an indirect role for the Th17 response in protective immunity in mouse models of M. tuberculosis infection. Human Th17 response is inducible by M. tuberculosis infection and a reduced M. tuberculosis antigen-specific Th17 response, which may be due to suppression by Th1 cytokines, has been observed in patients with active disease compared with healthy donors (21). However, the clinical significance and regulation mechanism of Th17 responses in development of active TB have not been well elucidated.

In this study, we compared Th17 responses in healthy donors, individuals with latent TB infection (LTBI), and patients with TB. We found that the magnitude of Th17 response is associated with clinical outcome of M. tuberculosis infection, as well as the severity of diseases. Reduced Th17 response may be due to down-regulation of IL-6 receptor (IL-
6R) expression on CD4+ T cells induced by \textit{M. tuberculosis} infection and bacterial products. Thus, our study provides evidence that interaction between \textit{M. tuberculosis} and host cells plays a critical role in shaping the Th17 response, which in turn determines the clinical outcome of \textit{M. tuberculosis} infection.

**METHODS**

See the online supplement for an extended version of the METHODS.

**Subjects and Samples**

Healthy adults (\(n = 94\)) with no history of TB disease were recruited at the Shenzhen Third People’s Hospital and Shenzhen Polytechnic College in Shenzhen, China. All participants had received bacillus Calmette-Guerin (BCG) vaccination at birth. A previously established \textit{M. tuberculosis}–specific IFN-\(\gamma\) enzyme-linked immunospot (ELISPOT) assay was used to differentiate individuals with LTBI (\(n = 27\)) from true healthy donors without LTBI (HD; \(n = 66\)) (24). HIV-uninfected patients (\(n = 62\)) with different manifestations of active TB were recruited at clinics in the Shenzhen Third People’s Hospital. Among them, 35 were diagnosed with pulmonary TB (PTB), 13 with severe PTB complicated with tuberculous meningitis (STB), and 14 were diagnosed with pleural TB (TP). The ages of each group (median [interquartile range]) were: HD, 28.0 (25.0–32.0); LTBI, 28.0 (25.0–37.0); PTB, 30.0 (25.0–44.5); STB, 32.0 (20.0–48.0); and TP, 30.0 (25.0–35.0). The male:female ratio of each group was: HD, 29:37; LTBI, 13:14; PTB, 27:8; STB, 10:3; and TP, 8:6.

Clinical specimens from patients with TB were collected within 1 week after anti-TB treatment was initiated under the protocols that were approved by the Institutional Review Board of Shenzhen Third People’s Hospital. Written informed consent was obtained from all participants. Heparinized whole blood was collected by venipuncture from the populations mentioned above, and pleural fluid samples were collected from patients with TP.

**Intracellular Cytokine Staining and Flow Cytometric Analysis**

To analyze cytokine production in different cell subsets, 1 ml heparinized whole blood or \(1 \times 10^6\) pleural fluid mononuclear cells (PFMCs) in 1 ml RPMI 1,640 containing 10% fetal bovine serum were incubated with phorbol myristate acetate (PMA; 50 ng/ml; Sigma-Aldrich, St. Louis, MO)/ionomycin (1 \(\mu\)g/ml; Sigma-Aldrich), or \textit{M. tuberculosis}–specific antigens (10 \(\mu\)g/ml) at 37\(^\circ\)C. Cultured cells were then harvested.

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**Figure 1.** Different CD4+ T cell response profiles in healthy donors (HDs), individuals with latent tuberculosis (TB) infection (LTBI), and patients with active TB. (A) Enzyme-linked immunospot assay of IFN-\(\gamma\) production by peripheral blood mononuclear cells (PBMCs) in response to \textit{Mycobacterium tuberculosis}–specific antigens early secreted antigenic target 6 kDa protein (left panel) and early secreted antigenic target 6 kDa/culture filtrate protein 10 kDa (ESAT-6/CFP-10) peptides (right panel) in HDs (\(n = 66\)), LTBI (\(n = 27\)), and TB (\(n = 62\)). Each data point represents the number of spot forming cells (SFCs) per \(2 \times 10^5\) PBMCs from one individual. Horizontal bars represent the mean (this same convention is used hereafter). (B) Flow cytometric detection of expression of IL-17 and IFN-\(\gamma\), IL-17 and IL-4, IL-17, and FoxP3 by CD4+ T cells from HD, LTBI, and TB. Plots shown were gated on CD3-CD4+ T cells in whole blood after 6-hour stimulation with phorbol myristate acetate/ionomycin. Numbers in the quadrants refer to percentage of positive cells in that quadrant. Results shown are typical for the donor populations. (C) Frequencies of IL-17–, IFN-\(\gamma\), IL-4–, and FoxP3–expressing CD4+ T cells in HD, LTBI, and TB. Differences between groups were compared with the one-way analysis of variance/Newman-Keuls multiple comparison test; \(P\) values are indicated.
for surface and intracellular staining with antibodies against CD3, CD4, IL-17, IFN-γ, IL-4, and FoxP3. At least 0.2 million cells were acquired for analysis using FACSDiva software (BD Biosciences, San Jose, CA).

Analysis of the Effect of Mycobacterial Antigens on IL-17 Production and IL-6R Expression by PBMCs and PFMCs

One million PBMCs and/or PFMCs in RPMI 1,640 containing 10% fetal bovine serum were cultured in the absence or presence of early secreted antigenic target 6kDa (ESAT-6), live Mycobacterium bovis BCG and M. tuberculosis H37Rv lysate. Supernatant was harvested after 72 hours for assay of IL-17 production by ELISA. Cells were collected for flow cytometry analysis for IL-6R expression.

Statistical Analysis

All statistical tests were performed with Prism 3.0 (GraphPad, La Jolla, CA). The one-way analysis of variance/Newman-Keuls multiple comparison test was used for statistical analyses to compare the differences among multiple groups. The unpaired t test was used to analyze the difference between two groups. The Wilcoxon matched pair t test was used to analyze the effect of M. tuberculosis antigens on IL-6R expression on CD4+ T cells in vitro. Pearson’s r test was used to analyze the correlation between the IL-6R expression on CD4+ T cells and the frequency of Th17. Differences were considered significant for P values less than 0.05.

RESULTS

Active TB Disease Is Associated with Lower Frequency of Th17 Cells in Circulation

After exposure to M. tuberculosis, there are basically three different outcomes: no infection, LTBI, and active TB. Active TB is recognized as a failure of immune control of M. tuberculosis infection in comparison to LTBI. To put this in the context of Th17 responses, we first compared the frequency of nonspecific Th17 responses in circulation of HDs, subjects with LTBI, and patients with active TB to evaluate the role of Th17 cells. By using a previously developed M. tuberculosis ESAT-6/culture filtrate protein 10 kDa (CFP-10) antigens–specific IFN-γ ELISPot assay, we differentiated individuals with LTBI from HDs, as the former have positive IFN-γ responses either to ESAT-6 protein antigen or ESAT-6-CFP-10 peptide antigens (Figure 1A). Flow cytometry analysis showed that the frequency of nonspecific Th17 response was significantly lower in patients with active TB disease than in HDs and in those with LTBI (Figures 1B and 1C). Although reduced Th17 responses were also observed in individuals with LTBI, the differences between HD and LTBI were not statistically significant. Consistent with previous findings (21, 25), the frequencies of IFN-γ- and IL-4–producing cells, as well as FoxP3+ cells, were also significantly different in various comparisons (Figures 1B and 1C).

Suppressed Th17 Response Is Associated with Severity of Active TB Disease

The finding that Th17 responses are suppressed in TB prompted us to investigate whether Th17 responses are associated with the severity of disease, as different manifestations of TB have different adaptive immune profiles (24, 26). We therefore compared IFN-γ and Th17 responses in subgroups of patients with TB—namely, patients with PTB complicated with tuber-
culous meningitis, a manifestation of STB, and those patients with PTB only. As shown in Figure 2A, IFN-γ responses to *M. tuberculosis*-specific antigens are reduced in PBMCs from patients with more severe disease. Also as expected, the frequency of Th17 cells was significantly lower in patients with STB than those with PTB (Figure 2B, left panel). Surprisingly, the frequencies of nonspecific (PMA/ionomycin–stimulated) IFN-γ– and IL-4–producing cells in patients with STB were not significantly different from those with PTB (Figure 2B, right panel; Figure 2C, left panel). In contrast, the frequency of regulatory T cells (Tregs) (CD4+FoxP3+ T cells) was reduced in patients with STB compared with those with PTB (Figure 2C, right panel). Therefore, polyclonal Th17 responses may be more relevant than polyclonal Th1 responses in the context of the severity of TB disease.

**Decreased Nonspecific Th17 Responses in Circulation Are Not Due to Th17 Cell Migration to the Site of Infection in Patients with TP**

A previous study has suggested that the lower frequency of *M. tuberculosis* antigen–specific Th17 cells may be due to their migration to the site of infection (21). To investigate whether this is the case for the reduced nonspecific Th17 responses that we observed (described previously here), we simultaneously measured antigen-specific and nonspecific T cell responses in peripheral blood and pleural effusion fluid in 14 patients with TP. Consistent with previous finding (19, 25), *M. tuberculosis* antigen–specific IFN-γ and IL-17 production were significantly enhanced in PFMCs compared with PBMCs (Figures 3A and 3B). Notably, in PBMCs, Th17 cell responses to mycobacterial antigens were almost undetectable, whereas the frequencies of *M. tuberculosis* antigen–specific Th17 responses were significantly increased in PFMCs (Figure 3C). Unexpectedly, the frequency of nonspecific Th17 cells in PFMCs was significantly lower than that in PBMCs (Figure 3D). The frequency of nonspecific Th1 cell was also lower in PFMCs than in PBMCs, but the difference was not significant (Figure 3D). Thus, the reduced nonspecific Th17 cell population in circulation is not due to their migration into the pleural fluid compartment. Similar to previous observations, PFMCs have a higher percentage of Tregs (CD4+FoxP3+ T cells) and a reduced frequency of Th2 cells (IL-4–producing CD4+ T cells) than PBMCs (Figure 3D).

**Figure 3.** Differential antigen-specific and nonspecific CD4+ T cell responses in peripheral blood mononuclear cells (PBMC) and pleural fluid mononuclear cells (PFMC) in patients with tuberculous pleurisy (TP). (A) Parallel enzyme-linked immunospot assays for IFN-γ production by PBMCs and PFMCs in response to *Mycobacterium tuberculosis*–specific early secreted antigenic target 6 kDa (ESAT-6) protein (left panel) and ESAT-6/culture filtrate protein 10 kDa (CFP-10) peptides (right panel) in patients with TP (n = 14). (B) IL-17 concentration in supernatants of PBMCs and PFMCs from patients with TP cultured in the presence of *M. tuberculosis* lysate (n = 14). (C) Flow cytometric detection of production of intracellular IL-17 and IFN-γ by CD4+ T cells in PFMCs and PBMCs in response to the annotated mycobacterial antigens in a patient with TP. Plots shown were gated CD3+CD4+ T cells, and results are typical of the patients studied. (D) Frequencies of IL-17+, IFN-γ+, IL-4+, and FoxP3-expressing CD4+ T cells in PBMCs and PFMCs of patients with TP (n = 14) after 6-hour stimulation with phorbol myristate acetate/ionomycin. Differences between PBMCs and PFMCs were compared with paired t test; P values are indicated.
Imbalance of Transforming Growth Factor–β/IL-6 Production in Patients with Active TB Disease

The observation that reduced Th17 responses coincident with increased Treg responses in both peripheral blood and pleural fluid suggests that a skewed cytokine environment caused by *M. tuberculosis* infection may contribute to the reduced Th17 responses. This is a particularly attractive hypothesis given that transforming growth factor (TGF)–β is important for Treg development, and TGF-β in the presence of IL-6 facilitates Th17 development from naive T cells. Therefore, we determined the concentration of TGF-β and IL-6 in plasma and/or pleural fluid by ELISA. We found that both TGF-β and IL-6 production were increased in plasma of patients with active TB compared with LTBI; the increased levels of TGF-β were more prominent over IL-6 concentrations, resulting in an increased TGF-β:IL-6 ratio (Figure 4A, right panel). However, the TGF-β:IL-6 ratio is decreased in plasma of patients with tuberculous meningitis (STB) compared with PTB (Figure 4B, right panel). In contrast, TGF-β production was decreased, whereas IL-6 production was significantly increased in pleural fluid, and thus there was a more profound decrease of the TGF-β:IL-6 ratio compared with plasma from the cohort of patients with TP (Figure 4C). Therefore, neither the production of TGF-β nor the TGF-β:IL-6 ratio correlated with the reduced Th17 response and increased Treg frequencies.

Decreased IL-6R Expression on CD4+ T Cells in Patients with Active Disease

Because production of both IL-6 and TGF-β, which are critical for development of Th17 cells, are not inhibited in patients with active TB, the reduced Th17 response is probably not due to the effects of insufficient IL-6 and TGF-β production. However, it remains possible that an impairment of signal transduction pathways related to IL-6 and/or TGF-β (e.g., IL-6R) caused by *M. tuberculosis* infection suppressed the development of Th17 cells. Because the initial step in that pathway is binding of IL-6 to its cognate receptor, we investigated the surface expression IL-6R on CD4+ T cells, a key component to mediate the IL-6 signal. In support of reduced IL-6/IL-6R signaling, we found that expression of IL-6R on CD4+ T cells in patients with active TB was significantly lower than those of HDs and individuals with LTBI (Figures 5A and 5B). In addition, the proportion of IL-6R–expressing CD4+ T cells in PFMCs was significantly lower than that in parallel PBMCs (Figure 5C). Furthermore, the proportion of IL-6R+ CD4+ T cells in blood is significantly correlated with the frequencies of Th17 in patients with active TB (Figure 5D).

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*p values are indicated.*

![Figure 4](image-url)
with PTB (Figure 5D). Thus, the decreased expression of IL-6R on CD4+ T cells is correlated with the suppressive Th17 response in patients with active TB disease.

**M. tuberculosis** Down-Regulates IL-6R Expression on CD4+ T Cells *In Vitro*

To confirm the in vivo findings that *M. tuberculosis* infection inhibits IL-6R expression on CD4+ T cells, we studied the effects of *M. tuberculosis* products on regulation of IL-6R expression on CD4+ T cells *in vitro*. Incubation of PBMCs in the presence of *M. tuberculosis* lysate or live *M. bovis* BCG down-regulated surface expression of IL-6R on CD4+ T cells (Figure 6A). Interestingly, *M. tuberculosis* lysate has more profound effects in down-regulation of IL-6R expression compared with BCG (Figure 6A). This inhibitory effect begins at 24 hours, and peaks at 72 hours after incubation in a dose-dependent manner (Figure 6B). Finally, PBMCs from patients with active TB are more sensitive to IL-6R down-regulation by *M. tuberculosis* lysate than are HD PBMCs, as the percentage of reduction is significantly higher in patients with TB than in HDs (Figure 6C).

**DISCUSSION**

Recent studies show that *M. tuberculosis* infection in humans can induce antigen-specific Th17 T cell responses, which is diminished in patients with active TB disease manifestations compared with mycobacteria-exposed healthy donors. In the present study, we extended this observation by finding that nonspecific Th17 responses are also suppressed in patients with active TB diseases. Inhibition of Th17 responses is associated with severity of TB diseases, as patients with STB have significantly lower frequencies of Th17 cells than patients with PTB. In contrast, Th1 responses, which are recognized as critical immune effectors against *M. tuberculosis* infection, are also reduced in patients with active disease, but we saw no difference in Th1 responses between STB and TB. Taken together, these findings substantially support the concept that Th17 cells play an important role in protective immunity against development of active TB disease, but may not protect against getting *M. tuberculosis* infection itself.

A previous study on antigen-specific Th17 responses in TB suggested that the low frequency of Th17 cells in blood is probably due to the migration of those cells to the site of infection (21). This is supported by evidence that human IL-17-producing T cells express CCR6, which may facilitate T cell homing to inflamed tissues (16, 27, 28). If this is also the case for Th17 responses in TB, higher numbers of Th17 cells or elevated production IL-17 should be expected in specimens collected at the site of infection (e.g., bronchoalveolar lavage fluid or pleural fluid) as is observed for Th1 responses (25). However, the production of IL-17 was undetectable in bronchoalveolar lavage fluid in patients with active TB diseases (21). Similarly, we found that the frequency of Th17 cells in pleural effusion is even...
lower than in blood. Thus, suppressed Th17 responses in circulation observed in our study are probably not due to Th17 cell migration to the site of infection.

Another probable mechanism underlying the suppressed Th17 response would be the inhibitory effect of Tregs (21, 29). This is particularly attractive, not only because Th17 cells and Tregs are recognized to be reciprocally regulated during differentiation by a shared developmental requirement for the cytokine, TGF-β, but also because Tregs can inhibit Th17 cell development (29–32). Consistent with this, we found that suppression of Th17 responses in blood and pleural fluid of patients with TB was coincident with increased frequencies of Tregs. However, neither the production of TGF-β nor the TGF-β:IL-6 ratio is correlated with the frequencies of Th17 responses. Additionally, high amounts of IL-6 present in plasma and/or pleural fluid of patients with active TB diseases also argue against suppressive effects of Tregs on Th17 cells, as high IL-6 damps Treg-suppressive function on Th17 cells, and facilitates differentiation of Th17 cells (33, 34).

Although other mechanisms, such as the inhibitory functions of Th1 and Th2 cytokines, as well as nature killer group 2D (NKG2D) expressed on CD4 T cells, may be involved in regulating Th17 responses in M. tuberculosis infection, these are mostly related to regulation of the differentiated antigen-specific, but not nonspecific, Th17 responses (20, 21, 35, 36). Our finding, that down-regulation of IL-6R expression on CD4+ T cells in patients with active TB diseases, suggests that it may be an important mechanism driving the suppressed, nonspecific Th17 responses in patients with TB. This was further supported by the finding that M. tuberculosis products down-regulate IL-6R expression on CD4+ T cells in vitro. However, this may be challenged by the report by Paidipally and colleagues (20), which indicated that, in individuals with M. tuberculosis infection, IL-6 is not required for T cells to produce IL-17 in response to mycobacterial antigens. Nevertheless, the expansion of antigen-experienced Th17 cells evaluated by Paidipally and colleagues is different from differentiation of Th17 subsets from naive T cells—a process in which IL-6R–mediated signaling is required. Detection of nonspecific Th17 responses in this study is more likely to involve Th17 cells derived from naive CD4+ T cells. In accordance with our findings, Fujimoto and colleagues (13) have demonstrated that treatment with anti–IL-6R–blocking monoclonal antibody (MR16-1) markedly suppressed the induction of Th17 responses (determined as PMA/ionomycin–stimulated, IL-17–producing CD4+ T cells in the same protocol as in our study) from naive T cells in vitro and in vivo; antibody treatment had no effect on expansion of differentiated antigen-specific Th17 cells (13). Specifically, the
Inhibitory effect of antibody MR16-1 is directly on CD4+ T cells, because it significantly inhibited Th17 cell differentiation from purified CD4+CD62L<sup>high</sup>CD25<sup>-</sup> naïve T cells activated by plate-bound anti-CD3 and anti-CD28 antibodies (13). Similarly, a study by Xiao and colleagues (37) also demonstrated that Th17 development can be suppressed by retinoic acid via inhibition of IL-6R expression. Therefore, we propose that down-regulation of IL-6R expression on CD4+ T cells is an important mechanism used by <i>M. tuberculosis</i> to suppress Th17 cell development, through which the protective immunity mediated by Th17 responses is impaired, and allowing reactivation of TB disease.

Decreased IL-6R expression on CD4<sup>+</sup> T cells has been observed upon binding of IL-6 to its receptor, or by T cell activation via T cell receptor (TCR) engagement (38, 39). Because TCR engagement with <i>M. tuberculosis</i> antigen is expected with proliferation of antigen-specific T cells (including Th17 cells), and the decreased IL-6R expression is correlated with reduced nonspecific Th17 response in our study, this mechanism does not seem to account for decreased IL-6R expression in patients with active TB in this study. On the other hand, down-regulation of IL-6R by IL-6 can still take place in naïve or immature CD4<sup>+</sup> T cells (e.g., thymic CD4<sup>+</sup>CD5<sup>-</sup> T cells) in the absence of TCR activation (38). Therefore, decreased IL-6R expression might be due to internalization and degradation upon binding of IL-6, the production of which is efficiently induced by <i>M. tuberculosis</i> infection (40–44). <i>M. tuberculosis</i> and its products activate the expression of IL-6 gene at the transcriptional level through interactions with nuclear factors NF-IL6 and NF-κB to induce IL-6 expression (41). In addition, the capability of virulent <i>M. bovis</i> to induce IL-6 expression is significantly higher than that of BCG (45). Consistent with this, our <i>in vitro</i> study showed that <i>M. tuberculosis</i> suppressed IL-6R expression more efficiently than BCG, which probably reflects the different capability of <i>M. tuberculosis</i> and BCG in inducing IL-6 production. It is possible that other mechanisms, perhaps even expulsion of the receptor via exosomes or microvesicles, are also involved in the down-regulation of IL-6R. Thus, further investigations are warranted to understand the mechanism underlying the decreased IL-6R expression on CD4<sup>+</sup> T cells, as well as the suppressive Th17 responses in patients with active TB.

In summary, Th17 responses are suppressed in patients with active TB disease. The association between Th17 response and the clinical outcome of <i>M. tuberculosis</i> infection suggests that Th17 cells are more likely to play an important role in prevention of disease development and progression, but these cells have a less important role in resistance to the primary infection. Although a complete understanding of the mechanisms underlying the suppression of Th17 responses warrants further investigation, down-regulation of IL-6R expression on CD4<sup>+</sup> T cells by <i>M. tuberculosis</i> is an important mechanism.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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