Functional Capacity of *Mycobacterium tuberculosis*-Specific T Cell Responses in Humans Is Associated with Mycobacterial Load

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High Ag load in chronic viral infections has been associated with impairment of Ag-specific T cell responses; however, the relationship between Ag load in chronic Mycobacterium tuberculosis infection and functional capacity of M. tuberculosis-specific T cells in humans is not clear. We compared M. tuberculosis-specific T cell-associated cytokine production and proliferative capacity in peripheral blood from adults with progressively higher mycobacterial loads—that is, persons with latent M. tuberculosis infection (LTBI), with smear-negative pulmonary tuberculosis (TB), and smear-positive TB. Patients with smear-positive TB had decreased polyfunctional IFN-γ IL-2 TNF-α and IL-2–producing specific CD4 T cells and increased TNF-α single-positive cells, when compared with smear-negative TB and LTBI. TB patients also had increased frequencies of TNF-α single-positive T cells compared with LTBI. M. tuberculosis-specific CD4 and CD8 T cell proliferative capacity was profoundly impaired in individuals with smear-positive TB, and correlated positively with ex vivo IFN-γ IL-2 TNF-α CD4 T cells, and inversely with TNF-α single-positive CD4 T cells. During 6 mo of anti-TB treatment, specific IFN-γ IL-2 TNF-α CD4 and CD8 T cells increased, whereas TNF-α and IFN-γ single-positive T cells decreased. These results suggest progressive impairment of M. tuberculosis-specific T cell responses with increasing mycobacterial load and recovery of responses during therapy. Furthermore, these data provide a link between specific cytokine-producing subsets and functional capacity of M. tuberculosis-specific T cells, and between the presence of specific CD8 T cells ex vivo and active TB disease. These data have potentially significant applications for the diagnosis of TB and for the identification of T cell correlates of TB disease progression.

I
nfection with Mycobacterium tuberculosis can persist for the lifetime of the host. In ∼10% of infected persons, clinical tuberculosis (TB) disease develops; ∼9 million new cases occurred worldwide in 2009, from which 1.5 million people died (1). Clinical disease does not develop in the majority of individuals infected with M. tuberculosis, providing compelling evidence that the human immune system is capable of controlling the pathogen. However, the precise mechanisms contributing to the loss of immune control and progression to active TB disease are not known.

Evidence from both animal and human models suggests an important role for both CD4 and CD8 T cells in successful immune control of M. tuberculosis infection. Mice lacking CD4 T cells show increased susceptibility to development of TB, compared with wild-type mice (2, 3). T helper 1 (Th1) cells are of particular importance, because IFN-γ–deficient mice and humans with IL-12 or IFN-γ receptor abnormalities demonstrate increased susceptibility to mycobacterial diseases (4–6). Numerous comparisons of IFN-γ production by specific CD4 T cells in latent M. tuberculosis infection (LTBI) and TB disease have yielded opposing associations with infection status (7, 8); this may relate to limitations when measuring IFN-γ alone, whereas the immune response to M. tuberculosis is more complex. For example, the importance of TNF-α is suggested by observations of reactivation of LTBI (9), and with reduced antimycobacterial activity of CD8 T cells, when patients with autoimmune disease have received therapeutic neutralizing anti–TNF-α monoclonal Abs (10).

M. tuberculosis–specific CD8 T cells are primed after transfer of mycobacterial Ags into the cytosol (11–13), or through cross-priming mediated by uptake of apoptotic vesicles from mycobacteria-infected macrophages by dendritic cells (14). Recent evidence suggests progressive dysfunction of CD8 T cells in chronic M. tuberculosis infection. For example, CD8 T cells in M. tuberculosis–infected mice gradually lose lytic potential during progression to the chronic phase of infection (15). Furthermore, CD8 T cells from individuals with pulmonary TB display decreased cytolytic activity and expression of cytotoxic molecules, compared with these cells from uninfected healthy controls (16, 17). An overwhelming body of evidence from animal and human models of chronic viral infections indicates that high levels of chronic
Ag stimulation leads to functional impairment of Ag-specific T cell responses, with reduced cytokine production, cytotoxicity, and proliferative capacity (18–23). The capacity of Ag-specific T cells to produce multiple cytokines simultaneously (i.e., polyfunctional cells) has been associated with superior functional capacity (24) and has been correlated with control of human chronic viral infections such as HIV (25–27) and hepatitis C virus (28). Moreover, polyfunctional T cells have been associated with protection against disease progression in mouse models of Leishmania major (29,30) and M. tuberculosis (31). Polyfunctional T cells producing IFN-γ, IL-2, and TNF-α have been described in studies of M. tuberculosis-infected adults (32–35), although with differing conclusions regarding the distribution of the various cytokine-producing CD4 T cell subsets in individuals with LTBI and TB.

Latent M. tuberculosis infection is characterized by the presence of M. tuberculosis-specific cellular immune responses in the absence of clinical symptoms. Pulmonary TB, the most common clinical manifestation of TB, is characterized by high levels of bacterial replication and the presence of bacilli in sputum that is directly detectable by sputum smear microscopy or culture. Although there is no direct quantitative measurement of bacterial load, previous clinical studies of immunocompetent adults have indicated that sputum smear grading at the time of diagnosis (negative, scanty, 1+, 2+, or 3+) correlates with sputum smear and culture conversion at two months of TB treatment, and is predictive of relapse and recurrence of TB disease (36,37). Moreover, smear grading correlates with disease severity by chest radiograph scoring (38). The aim of this study was to determine the relationship between mycobacterial load and quantitative and qualitative measurements of M. tuberculosis-specific T cell functional capacity. We hypothesized that high mycobacterial load in individuals with high-grade smear-positive TB would be associated with progressive dysfunction of M. tuberculosis-specific T cell responses, compared with lower mycobacterial load in individuals with smear-negative TB and LTBI.

Materials and Methods

Study population and sample collection

Participants were recruited for this study from the Cape Town region of South Africa. Participants were included when ≥18 y of age and sero-negative for HIV. Persons with LTBI and with pulmonary TB disease were enrolled. LTBI was defined by a positive response to CFP-10 or ESAT-6 peptides, or both, in a short-term whole blood intracellular cytokine staining (ICS) assay (see below), with no previous history of TB diagnosis or treatment. Diagnosis of pulmonary TB was based on epidemiologic history, signs and symptoms, and roentgenographic findings consistent with TB. All included patients had either positive sputum smear microscopy or positive culture for M. tuberculosis, or both. The number of bacilli in sputum smears was counted according to World Health Organization guidelines (39), and two groups of TB patients were enrolled: smear-negative (<10^6 bacilli/ml sputum) and 2+ or 3+ smear-positive (>10^6 bacilli/ml sputum) (40). Peripheral blood was obtained in sodium heparin Vacutainer tubes (BD Biosciences), and PBMCs were isolated by density centrifugation with Ficoll-Hypaque (Sigma-Aldrich), within 4 h of collection. Blood from individuals with TB was obtained prior to or within 7 d of starting standard course anti-TB treatment, which was provided according to South African national health guidelines. In a subset of individuals with TB, blood was obtained again at 2 and 6 mo after initiation of treatment. All study participants gave written, informed consent for the study, which was approved by the Human Research Ethics Committee of the University of Cape Town and the Western Cape Department of Health.

Ags

Peptides (15 mers overlapping by 10 aa) corresponding to the sequence of the M. tuberculosis Ags CFP-10 and ESAT-6 were pooled by protein, and used at 1.25 μg/ml per peptide in the whole blood ICS assay, and at 0.1 μg/ml in the 6-d PBMC proliferation assay. Tuberculin purified protein derivative (PPD; Statent Serum Institute) was used at 10 μg/ml in the whole blood ICS assay, and at 1 μg/ml in the 6-d PBMC proliferation assay. Staphylococcal enterotoxin B (SEB; Sigma-Aldrich; positive control) was used at 1 μg/ml in the whole blood ICS assay, and at 0.1 μg/ml in the 6-d PBMC proliferation assay.

Whole blood ICS assay

One milliliter heparinized blood was incubated with Ag within 2 h of blood collection, for 8 h at 37°C. Blood incubated with no Ag served as a negative control, and blood incubated with SEB served as a positive control. After 3 h, brefeldin A (10 μg/ml; Sigma-Aldrich) was added, and the incubation continued for an additional 5 h. Following the 8 h incubation, 2 mM EDTA was added for 10 min, followed by red cell lysis and white cell fixation in FACS Lysis Solution (BD Biosciences). Stained, fixed white blood cells were then cryopreserved in freezing medium and stored in liquid nitrogen until use, as described previously (41). Cryopreserved, fixed white cells were later thawed and washed once in PBS, followed by a second wash in Perm/Wash Buffer (BD Biosciences). Cells were stained with mAbs for CD3 Pacific Blue (UCHT1), anti-CD3 allophycocyanin-H7 (SK7), anti-CD8 PerCP-Cy5.5 (SK-1), anti–IFN-γ Alexa Fluor 700 (B27), and anti–IL-2 FITC (5344.111), all from BD Biosciences, and anti-CD4 QD605 (S3.5; Invitrogen) and anti–TNF-α–PE-Cy7 (Mab11; ebBiosciences).

Flow cytometry data analysis

Multiple-parameter flow cytometry data were analyzed using FlowJo software (v9.0.2; Treestar). Doublet cell populations were excluded by plotting forward scatter area versus forward scatter height. Single-stained anti-mouse Ig, k beads (BD Biosciences) were used to calculate compensation. Combinations of cytokine-producing cells were determined using Boolean gating in FlowJo, followed by further analysis using Pestle v1.6.2 (Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health) and Spice v5.1 (42). Background cytokine production in the negative control of ICS assays was subtracted from each Ag-stimulated condition; background proliferation in the negative control condition of the PBMC proliferation assay was subtracted from Ag-stimulated conditions. All donors responded to SEB in the ex vivo ICS assay and in the PBMC proliferation assay. Responses in ICS and proliferation assays were considered positive if the frequency of cytokine-producing or proliferating CD4 or CD8 T cells in the stimulated sample was greater than the median plus 3 times the median absolute deviation of the negative control samples. For analysis of the ICS assay, only individuals with positive T cell responses to M. tuberculosis Ags were included when calculating the proportion of each cytokine-producing subset contributing to the total M. tuberculosis-specific response.

Data analysis

Statistical testing was performed using GraphPad Prism v5.0a software. For analysis of whole blood ICS assay data, differences between the three participant groups was first assessed using the Kruskal–Wallis test, followed...
by the Dunn’s posttest. If significance was found \((p < 0.05)\), pairwise comparisons were analyzed using the Mann–Whitney \(U\) test. The Fisher’s exact test was used to assess differences in the frequency of recognition of \(M.\) \(tuberculosis\)-specific CD8 T cell responses among the three groups. For longitudinal analysis of \(M.\) \(tuberculosis\)-specific T cell responses on anti-TB treatment, differences between time points were first assessed using the Friedman test, followed by the Dunn’s posttest. If significance was found \((p < 0.05)\), pairwise comparisons were analyzed using the Wilcoxon matched pairs test. Correlations were assessed using Spearman’s rank correlation.

Results

Participants

To assess the relationship between mycobacterial load and the quantity and quality of the \(M.\) \(tuberculosis\)-specific T cell responses, we recruited three groups of adults from the Cape Town region of South Africa, where TB is endemic: 30 healthy, asymptomatic adults with LTBI, and 54 individuals with pulmonary TB stratified into two groups based on acid-fast bacilli (AFB) sputum smear results at the time of diagnosis. The first group had two successive 2+ or 3+ sputum smears (smear-positive TB; \(n = 38\)), whereas the second group had two successive AFB-negative smear positives, and smear cultures were positive for \(M.\) \(tuberculosis\) (smear-negative TB; \(n = 16\)). Compared with LTBI, TB patients were older and more likely to be male (Table I).

Smear-positive TB is associated with decreased polyfunctional and IL-2-producing, and increased TNF-\(\alpha\)-producing \(M.\) \(tuberculosis\)-specific CD4 T cells, compared with smear-negative TB and LTBI

CD4 T cell responses play a key role in immune control of \(M.\) \(tuberculosis\) infection, and polyfunctional cytokine production of Ag-specific Th1 cells has been associated with control of chronic bacterial and viral infections \((25, 26, 28–31)\). We used flow cytometry to measure the capacity of \(M.\) \(tuberculosis\)-specific CD4 T cells to produce cytokines in a short-term, whole blood ICS assay (Fig. 1A; Supplemental Figs. 1A, 2). Individuals with smear-positive TB displayed decreased proportions of PPD-specific IFN-\(\gamma\)IL-2\(^+\)TNF-\(\alpha\)^ and ESAT-6-specific IL-2\(^+\)TNF-\(\alpha\)^ CD4 T cells, and increased proportions of both ESAT-6- and PPD-specific IFN-\(\gamma\)TNF-\(\alpha\)^ and PPD-specific TNF-\(\alpha\)-producing single-positive CD4 T cells, compared with other groups (Fig. 1B). These data suggested a possible selective decrease in production of IL-2 by \(M.\) \(tuberculosis\)-specific CD4 T cells in individuals with smear-positive TB; therefore, we determined the relative contribution of IL-2-producing cells to the total CD4 T cell response. We found decreased proportions of IL-2-producing \(M.\) \(tuberculosis\)-specific CD4 T cells in individuals with smear-positive TB, compared with smear-negative TB; therefore, we determined the relative contribution of IL-2-producing cells to the total CD4 T cell response. We found decreased proportions of IL-2-producing \(M.\) \(tuberculosis\)-specific CD4 T cells in individuals with smear-positive TB, compared with smear-negative TB and LTBI (Fig. 1C). This decrease in IL-2 production was specific for \(M.\) \(tuberculosis\), as there was no difference in IL-2 production in response to SEB stimulation. These data indicate a shift in the cytokine production profiles of specific CD4 T cells with increasing mycobacterial load, characterized by progressive decreases in IL-2 and polyfunctional cytokine production capacity, coincident with increased TNF-\(\alpha\) production.

Increased \(M.\) \(tuberculosis\)-specific CD8 T cell responses in pulmonary TB

We next investigated the effect of mycobacterial load on \(M.\) \(tuberculosis\)-specific CD8 T cell responses in peripheral blood ex vivo. Individuals with TB had higher frequencies of IFN-\(\gamma\)- and TNF-\(\alpha\)-producing specific CD8 T cells, compared with LTBI (Fig. 2A, 2B). In all groups, the dominant cytokine-producing subsets of \(M.\) \(tuberculosis\)-specific CD8 T cells consisted of single- and double-positive IFN-\(\gamma\)- and TNF-\(\alpha\)-producing cells (Fig. 2C).

To determine whether the presence of \(M.\) \(tuberculosis\)-specific CD8 T cell responses was specifically associated with pulmonary TB disease status, we compared the number of individuals in each group with positive CFP-10 or ESAT-6 CD8 T cell responses, or both. Greater than 60% of individuals with smear-positive TB had detectable \(M.\) \(tuberculosis\)-specific CD8 T cell responses, compared with 38% and 20% of individuals with smear-negative TB and LTBI, respectively. Within \(M.\) \(tuberculosis\)-specific CD8 T cell responders, individuals responded to either CFP-10 or ESAT-6, with CFP-10–specific and ESAT-6–specific CD8 T cell responses within an individual rarely observed (Fig. 2D). These data strongly suggest Ag-driven expansion of \(M.\) \(tuberculosis\)-specific CD8 T cell responses that are detectable ex vivo in peripheral blood of individuals with pulmonary TB, compared with LTBI.

Increase in polyfunctional \(M.\) \(tuberculosis\)-specific T cell responses following reduction in bacterial load by anti-TB treatment

The above results suggest that increasing mycobacterial load may lead to progressive quantitative and qualitative changes in \(M.\) \(tuberculosis\)-specific CD4 and CD8 T cell responses. To determine whether reduction of bacterial load by antibiotic treatment was associated with enhanced functional capacity of \(M.\) \(tuberculosis\)-specific T cells, we followed 13 TB patients longitudinally after initiating anti-TB treatment; all were sputum smear-negative by 6 mo of treatment (Figs. 3A, 4A). With the exception of CFP-10–specific CD4 T cells, the total frequency of specific CD4 T cells producing any combination of cytokines was not different after 6 mo of TB treatment, compared with pretreatment values (Fig. 3B). All patients maintained a positive response to PPD at all time points; therefore, we analyzed longitudinally the proportion of each cytokine subset contributing to the total PPD response. The proportion of IFN-\(\gamma\)IL-2\(^+\) TNF-\(\alpha\)^ and IL-2\(^+\)TNF-\(\alpha\)^–PPD-specific CD4 T cells increased significantly on TB treatment, coincident with a decrease in IFN-\(\gamma\)TNF-\(\alpha\)^ and TNF-\(\alpha\)-single-positive cells (Fig. 3C, 3D). The

<table>
<thead>
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<th>Participant Group</th>
<th>AFB Sputum Smear Grade</th>
<th>TB tx, Days (Range)</th>
<th>Ager, Years (Range)</th>
<th>Sex (% Male)</th>
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<tr>
<td>LTBI</td>
<td>NA</td>
<td>NA</td>
<td>23 (18–50)</td>
<td>33</td>
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<tr>
<td>Smear-negative TB</td>
<td>Negative</td>
<td>0 (0–7)</td>
<td>45 (18–59)</td>
<td>77^</td>
</tr>
<tr>
<td>Smear-positive TB</td>
<td>3+ ((n = 32)); 2+ ((n = 6))</td>
<td>2 (0–7)</td>
<td>38 (19–60)</td>
<td>81^</td>
</tr>
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^Values denote median number of days on anti-TB treatment at first sample collection (range).

^Values denote median (range).

^\(p < 0.05\), compared with LTBI.

NA, not applicable.
A proportion of polyfunctional PPD-specific CD4 T cells increased in all subjects following 6 mo of TB treatment (Fig. 3D).

We next investigated the effect of TB treatment on the total frequency of *M. tuberculosis*-specific CD8 T cells. Although some individuals’ frequencies declined over time, overall there was no difference in the total frequency of cytokine-producing specific CD8 T cell responses (Fig. 4B). Seven of the 13 individuals followed longitudinally maintained positive *M. tuberculosis*-specific CD8 T cell responses at all three time points. In these patients, the proportion of IFN-γ+IL-2+TNF-α+ CD8 T cell responses increased over time (similar to specific CD4 T cell responses), coincident with a decrease in the proportion of IFN-γ single-positive
cells (Fig. 4C). These data strongly suggest that the cytokine production capacity of *M. tuberculosis*-specific CD4 and CD8 T cell responses is associated with mycobacterial load; moreover, increased specific polyfunctional T cells coincident with reduced IFN-γ or TNF-α–producing cells in particular may be indicative of successful response to TB treatment.

**Impaired proliferative capacity of *M. tuberculosis*-specific T cells in smear-positive TB compared with LTBI**

To further assess the functional capacity of *M. tuberculosis*-specific T cells in individuals with different mycobacterial loads, we determined these cells’ proliferative capacity in a 6-d, PBMC-based assay (Supplemental Fig. 1B). Individuals with smear-positive TB had significantly lower CD4 and CD8 T cell proliferative responses to *M. tuberculosis* Ags and SEB, compared with LTBI (Fig. 5). Overall, these data indicate that, although cytokine-producing *M. tuberculosis*-specific CD4 and CD8 T cell are detectable ex vivo in individuals with smear-positive TB, the proliferative capacity of *M. tuberculosis*-specific T cells is profoundly impaired in the context of high mycobacterial load.

**Proliferative capacity of *M. tuberculosis*-specific CD4 T cells correlates positively with polyfunctional IFN-γ+IL-2+TNF-α+ cells and inversely with TNF-α single-positive cells**

To establish whether there is a relationship between the ex vivo cytokine profile and the functional capacity of *M. tuberculosis*-specific T cells, we first correlated the total frequency of cytokine-producing *M. tuberculosis*-specific T cells with proliferative capacity, and we found a positive correlation for *M. tuberculosis*-specific CD4 T cell responses (Fig. 6A, 6B). No such association was found for *M. tuberculosis*-specific CD8 T cells (Fig. 6C), suggesting these two functions may be distinct in *M. tuberculosis*-specific CD8 T cells.

We next correlated profiles of individual or combined cytokine production of the *M. tuberculosis*-specific T cells ex vivo with proliferative capacity, and we found a positive correlation between the ex vivo proportion of polyfunctional IFN-γ+IL-2+TNF-α+ CD4 T cells and proliferative capacity, and an inverse correlation between the proportion of TNF-α single-positive CD4 T cells and proliferative capacity (Fig. 6D–F). None of the other subsets of cytokine-producing CD4 T cells correlated with the proliferative
capacity (data not shown). These data indicate an association between the cytokine production capacity ex vivo and T cell functionality in *M. tuberculosis* infection, and they indicate that specific IFN-γ*+IL-2*+TNF-α*+ and TNF-α single-positive CD4 T cells detected in peripheral blood ex vivo are predictive of proliferative capacity.

**Discussion**

In this study, we recruited adults with LTBI, smear-negative and smear-positive TB to determine the effect of mycobacterial load on the functional capacity of *M. tuberculosis*-specific T cell responses in peripheral blood. We found that, compared with individuals with lower mycobacterial load, high mycobacterial load in individuals with smear-positive TB was associated with decreased proliferative and IL-2-producing cells and increased TNF-α single-positive *M. tuberculosis*-specific CD4 T cells, as well as increased frequencies of specific (cytokine-positive) CD8 T cells, and impaired proliferative capacity of both *M. tuberculosis*-specific CD4 and CD8 T cell responses. During therapy, polymicrobial cytokine production capacity increased in both CD4 and CD8 T cells. Moreover, polymicrobial cytokine-producing capacity correlated with proliferative capacity. These data indicate that the functional capacity of *M. tuberculosis*-specific T cells is progressively impaired with increased mycobacterial load and recovers during therapy.

Recent studies have assessed differences in polymicrobial cytokine production profiles of *M. tuberculosis*-specific CD4 T cells in individuals with LTBI and with pulmonary TB (32–35), with some studies indicating increased *M. tuberculosis*-specific polymicrobial CD4 responses in TB patients (32, 33), and others indicating either decreased polymicrobial responses in TB patients (35) or no difference (34). Our findings of reduced polymicrobial cytokine production in individuals with smear-positive TB are consistent with progressive T cell dysfunction in the context of high mycobacterial loads, as has been described in human chronic viral infections (25–28). Furthermore, the decreased contribution of IL-2–producing specific CD4 T cells in individuals with smear-positive TB is consistent with previous reports of loss of IL-2–producing *M. tuberculosis*-specific T cells in HIV-coinfected individuals with high viral loads (43), as well as lower proportions of IL-2 single-positive *M. tuberculosis*-specific T cells in TB patients compared with household contacts (32). Moreover, the increased contribution of TNF-α single-positive CD4 cells in individuals with
smear-positive TB compared with LTBI is consistent with recent reports (32, 35), and it provides further rationale for measurement of this particular subset of M. tuberculosis-specific CD4 T cells for differentiating M. tuberculosis infection versus disease (35).

Previous studies of M. tuberculosis-specific T cell polyfunctionality in humans have not differentiated mycobacterial load or disease status within individuals with pulmonary TB. By using well-defined cohorts of individuals stratified by these parameters, we were able to determine specific associations with M. tuberculosis-specific T cell functional capacity and mycobacterial load. Interestingly, we did not find differences in quantitative or qualitative measurements of M. tuberculosis-specific CD4 T cell cytokine production between LTBI and smear-negative TB donors, suggesting that the qualitative differences observed in individuals with smear-positive TB are reflective of high mycobacterial load, rather than the presence of symptomatic active TB disease alone. Possible differences in the cytokine production profiles of M. tuberculosis-specific CD4 T cells found in this study compared with recent reports (32–35) may be due to differences in Ag specificity and type, methodological differences used for detection of cytokine-producing cells, and differences in study cohort characteristics.

Secreted immunodominant M. tuberculosis Ags can be processed by cytosolic pathways for presentation by MHC class I molecules (11, 12, 44), with cytosolic entry dependent on secretion of the M. tuberculosis virulence proteins CFP-10 and ESAT-6 (13). M. tuberculosis-specific CD8 T cell responses have been detected in individuals with latent and active disease (45), and we describe a novel association between higher frequencies of M. tuberculosis-specific CD8 responses and pulmonary TB disease in adults. IFN-γ release assays currently used for diagnosis of M. tuberculosis infection or disease do not discriminate between CD4 and CD8 T cell cytokine production (46); however, our results provide a rationale for measuring M. tuberculosis-specific CD8 T cell responses, in particular to further define the association between CD8 T cells and TB disease progression. Such studies may significantly aid the diagnosis of TB disease, particularly in populations such as children or immunocompromised individuals.
where it may be difficult to distinguish \textit{M. tuberculosis} infection from disease.

Further evidence of the association between the quality of \textit{M. tuberculosis}-specific T cell responses and mycobacterial load is supported by our longitudinal analysis of individuals on TB treatment, where we found increased polyfunctional cytokine production capacity along with reduced proportions of monofunctional cytokine-producing cells in both CD4 and CD8 T cell populations following successful anti-TB treatment. Our longitudinal analysis of patients receiving TB treatment extend initial observations by Millington et al. (47) where simultaneous measurement of IFN-\(\gamma\) and IL-2 production by \textit{M. tuberculosis}-specific CD4 T cells indicated an increase in IL-2-producing cells following treatment. By simultaneous measurement of IFN-\(\gamma\), IL-2, and TNF-\(\alpha\), we were able to determine that the increase in IL-2-producing cells after 6 mo of TB treatment is specifically within the polyfunctional IFN-\(\gamma\)-IL-2-TNF-\(\alpha\) subset that showed a positive correlation with proliferative capacity, thus providing further evidence that polyfunctional cytokine responses has largely come from animal models or chronic human viral infections (24, 29, 30, 50–52). To better understand the relationship between ex vivo cytokine production profiles and T cell functional capacity, we determined the proliferative capacity of \textit{M. tuberculosis}-specific T cells using freshly isolated PBMCs from the same blood samples used for assessment of ex vivo cytokine production profiles. Polyfunctional \textit{M. tuberculosis}-specific IFN-\(\gamma\)-IL-2-TNF-\(\alpha\) CD4 T cells were the only cytokine subset that showed a positive correlation with proliferative capacity, thus providing further evidence that polyfunctional cytokine production capacity may be associated with superior

**FIGURE 5.** Impaired proliferative capacity of \textit{M. tuberculosis}-specific T cells in smear-positive TB, compared with LTBI. Freshly isolated PBMCs from individuals with LTBI (\(n = 12\)) and smear-positive TB (\(n = 14\)) were labeled with Oregon Green (OG) and stimulated with CFP-10 and ESAT-6 peptide pools, PPD, or SEB for 6 d. A. Representative CD4 T cell proliferation assay data from an individual with LTBI (top row) and smear-positive TB (bottom row); plots are gated on Vivid\textsuperscript{low}CD3\textsuperscript{+}CD4\textsuperscript{+} T cells. The percentages indicate the frequency of proliferating (OG low) cells in the gated population. B, Representative CD8 T cell proliferation assay data from an individual with LTBI (top row) and smear-positive TB (bottom row); plots are gated on Vivid\textsuperscript{low}CD3\textsuperscript{+}CD8\textsuperscript{+} T cells. The percentages indicate the frequency of proliferating (OG low) cells in the gated population. Summary data of CD4 and CD8 T cell proliferative capacity in individuals with LTBI (closed symbols) and smear-positive TB (open symbols) are shown in C and D, respectively. Background proliferation in the negative control has been subtracted. Horizontal lines represent the median; differences were assessed by the Mann–Whitney U test.
functional capacity in the context of a chronic human bacterial infection. IL-2 is a T cell growth factor and important for proliferation of memory T cells after a repeated encounter with Ag (52, 53), and our results indicate that IL-2 production capacity specifically within the context of simultaneous IFN-γ and TNF-α production is indicative of Ag-specific CD4 T cell proliferative capacity.

In contrast to polyfunctional M. tuberculosis-specific CD4 T cell responses, the proportion of ex vivo TNF-α single-positive CD4 cells, which were increased in individuals with smear-positive TB, were inversely correlated with proliferative capacity. TNF-α plays an essential role in protection against TB (9, 54), and our results indicate that populations of M. tuberculosis-specific CD4 T cells producing TNF-α, in the absence of IFN-γ and IL-2 coexpression, are expanded under inflammatory conditions of high mycobacterial load and may identify a short-lived population of effector cells with limited survival and ability to expand upon a repeated encounter with Ag. Further study is warranted to determine particular phenotypes of M. tuberculosis-specific T cells from individuals with different stages of infection and disease are warranted to further define profiles that can predict risk of development of TB disease.

In summary, we have identified qualitative and quantitative associations between M. tuberculosis-specific T cell functional capacity and mycobacterial load in the context of human M. tuberculosis infection and disease. The immune correlates of protection from TB disease progression are not well defined, although results from this study provide rationale for future longitudinal studies of M. tuberculosis-infected individuals to determine whether progressive loss of polyfunctional or IL-2–producing M. tuberculosis-specific CD4 T cells and increasing frequencies of M. tuberculosis-specific CD8 T cells are predictive of development of clinical TB disease. Such data would have a significant effect on rates of M. tuberculosis transmission and TB-associated morbidity and mortality worldwide by facilitating early diagnosis of TB and targeted treatment intervention in M. tuberculosis-infected populations at risk for developing TB.

Disclosures
The authors have no financial conflicts of interest.

References


Figure S1. Flow cytometry gating strategies. (A) Gating strategy for the whole blood intracellular cytokine staining assay. Forward scatter area (FSC-A) versus forward scatter height (FSC-H) was used to select singlets, followed by FSC-A versus side scatter area (SSC-A) to select lymphocytes. CD3 T cells were gated within the lymphocyte population, followed by gating on CD4 or CD8 T cells. Total populations of IFN-γ, IL-2, and TNF-α-producing CD4 and CD8 T cells were selected, followed by Boolean gating to generate all possible combinations of cytokine-producing T cell populations. Representative data from whole blood of an LTBI donor stimulated for 8 hrs with SEB are shown. Percentages of cytokine-positive cells shown are after subtraction of background cytokine production in the unstimulated negative control. (B) Gating strategy for the PBMC proliferation assay. FSC-A versus forward FSC-H was used to select singlets, followed by FSC-A versus SSC-A to select lymphocytes. Live CD3 T cells were defined as CD3⁺VIVIDlow lymphocytes; CD4 or CD8 T cells were gated from the live CD3 T cell population. Proliferating cells were defined as Oregon Greenlow (OGlow) CD4 or CD8 T cells. Representative data from freshly isolated PBMC of an LTBI donor are shown for unstimulated (no antigen) and SEB-stimulated cells.
Figure S2. Frequency of cytokine-producing *Mtb*-specific CD4 T cell populations in whole blood *ex vivo*. Whole blood was stimulated for 8 hrs with overlapping CFP-10 and ESAT-6 peptide pools, PPD, or SEB. The frequencies of each CD4 T cell subset producing all possible combinations of IFN-γ, IL-2, and TNF-α are shown. Background cytokine production from the negative control sample has been subtracted. Statistical analysis between LTBI, smear− TB and smear+ TB donors was first performed by the Kruskal-Wallis test, and if significance was found (p<0.05), the Mann-Whitney test was used for comparisons between two groups.