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Polyfunctional CD4+ and CD8+ T Cell Responses to Tuberculosis Antigens in HIV-1–Infected Patients before and after Anti-Retroviral Treatment

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Tuberculosis (TB) kills 2 million people per year and infection with HIV is the most potent known risk factor for progression to active TB. An understanding of the immune response to TB Ags in HIV-infected patients is required to develop optimal TB vaccines and diagnostics. We assessed polyfunctional (IFN-γ+IL-2+TNF-α+) T cell responses to TB Ags in three groups of HIV-1–infected patients dependent on their TB status, CD4 counts, and anti-retroviral exposure. We found that although the proportion of IFN-γ cells in response to TB Ags was higher in patients with low CD4 counts, the responding cells changed from a polyfunctional CD4+ to a monofunctional CD8+ response. The overall polyfunctionality of the cells was restored by 12 mo of anti-retroviral therapy and primarily involved CD4+ T cells with an effector memory phenotype. These findings have major implications for diagnosis of TB and in vaccine development strategies for TB in HIV-1–infected patients. The Journal of Immunology, 2010, 184: 6537–6544.

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Abbreviations used in this paper: ART, anti-retroviral therapy; CM, central memory; EC, ESAT-6/CFP-10 fusion protein; EM, effector memory; IRIS, immune reconstitution inflammatory syndrome; NA, not applicable; ND, not done; PFT, polyfunctional T cell; PPD, purified protein derivative; RT, room temperature; TB, tuberculosis; TE, terminal effector; TST, tuberculin skin test.

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Tuberculosis (TB) is a global health problem with ~2 billion people infected worldwide. Of these, 10% will progress to active TB disease resulting in almost 2 million deaths per year (1). Infection with HIV is the most potent known risk factor for progression to active TB; patients with advanced HIV are 100 times more likely to reactivate latent TB compared with infected immune competent individuals (1, 2).

The immune response to TB is complex and the outcome is heavily influenced by the Mycobacterium itself. As such it has been difficult to determine the requirements for a protective immune response to TB although this knowledge is essential for development of improved vaccine strategies. It is known that CD4+ T cells are required to mount an effective immune response to TB although CD8+ T cells are also important in more advanced stages of disease (3). IFN-γ and TNF-α are key cytokines in fighting TB infection (4, 5). However, although IFN-γ is crucial in the protective immune response to TB, it is not sufficient on its own (4). This may explain the recent finding that relative levels of IFN-γ have been shown to increase in response to TB Ags in HIV-1–infected patients with advanced immunosuppression who are most susceptible to development of TB (6). Assessment of other factors alongside IFN-γ is vital and requires analysis of so-called “polyfunctional” T cells (PFT). Interestingly, although PFT can provide vaccine-induced protection (7) and have also been shown to protect against disease progression in HIV (8), we have shown the converse to be true in TB (9), adding further complexity to the analysis of immune responses in TB/HIV coinfection.

A TB vaccine for use in HIV-1–infected patients is urgently needed but additionally, the major problems associated with TB/HIV coinfection is lack of an accurate diagnostic test for TB and a deficiency in our understanding of the optimal treatment protocol for both diseases. In regard to diagnostics, the currently available tests involve the use of IFN-γ production as a marker for TB infection but these are not able to discriminate between TB infection and active disease and are not indicated for use in HIV-1–positive patients (10). Not surprisingly, this appears to relate to CD4 counts with detection of latent infection by ELISPOT retained in HIV-infected patients but dependent on the degree of immunosuppression (11). Of the IFN-γ release assays currently on the market, the T-SPOT.TB test appears to have the greatest potential for use in HIV-infected patients as it is relatively independent of the level of CD4 T cell depletion (12). In regard to treatment protocols, anti-retroviral therapy (ART) for HIV infection has been shown to reduce TB incidence by up to 90% (13) but TB is also one of the most predominant pathogens associated with immune reconstitution inflammatory syndrome (IRIS) seen during early stages of ART (14, 15). Increasing our knowledge of which patients may be susceptible to HIV- and/or ART-induced TB requires a better understanding of the underlying immunological responses to TB Ags. Thus, the current study assessed PFT responses to TB Ags in three groups of HIV-1–infected patients: 1) ART-naïve patients stratified according to CD4 counts; 2) ART-naïve patients with latent or confirmed TB disease; and 3) a longitudinal analyses of patients pre-ART and at 3, 6, and 12 mo of ART. We found that although the proportion of IFN-γ cells in response to TB Ags was higher in patients with low CD4 counts,
the responding cells changed from a polyfunctional CD4+ to a monofunctional CD8+ response. The overall polyfunctionality of the cells was restored by 12 mo of ART and primarily involved CD4+ T cells with an effector memory (EM) phenotype. These findings have major implications for diagnosis of TB and also in vaccine development strategies for TB in HIV-infected patients.

Materials and Methods

Patients

Three separate cohorts of patients were consented from the Genitourinary Medicine clinic at Medical Research Council Laboratories (Fajara, The Gambia) (Table I). All patients were seropositive for HIV-1 and had no prior or current symptoms suggestive of TB unless otherwise stated. In the anti-

<table>
<thead>
<tr>
<th>Group</th>
<th>Category</th>
<th>Treatment</th>
<th>No.</th>
<th>Age</th>
<th>Sex (M/F)</th>
<th>CD4 Count*</th>
<th>Viral Load (Copies/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARV naive HIV+low CD4</td>
<td>Pre-ART</td>
<td>14</td>
<td>38 (27–47)</td>
<td>3:11</td>
<td>90 (50–155)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>HIV+medium CD4</td>
<td>Pre-ART</td>
<td>15</td>
<td>34 (28–40)</td>
<td>6:9</td>
<td>390 (310–435)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>HIV+high CD4</td>
<td>Pre-ART</td>
<td>12</td>
<td>44 (28–50)</td>
<td>5:7</td>
<td>680 (533–770)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ARV treated HIV+</td>
<td>Pre-ART</td>
<td>16</td>
<td>38 (27–47)</td>
<td>3:13</td>
<td>58 (20–98)</td>
<td>340,098 (121,343–878,430)</td>
<td></td>
</tr>
<tr>
<td>12 mo ART</td>
<td>NA</td>
<td>16</td>
<td>39 (32–45)</td>
<td>9:6</td>
<td>200 (93–299)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TB disease HIV+TB+</td>
<td>NA</td>
<td>14</td>
<td>39 (32–45)</td>
<td>9:6</td>
<td>200 (93–299)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>HIV+TB+</td>
<td>NA</td>
<td>16</td>
<td>33 (24–41)</td>
<td>12:3</td>
<td>286 (10–451)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Controls&lt;sup&gt;b&lt;/sup&gt;</td>
<td>HIV+TB+ (TST+)</td>
<td>NA</td>
<td>36</td>
<td>25 (21–36)</td>
<td>20:16</td>
<td>682 (476–1109)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Data expressed as median (interquartile range).
*TST+ household contacts.
NA, not applicable; ND, not done.
confirmed TB disease (positive chest X-ray and sputum smear; confirmed to be HIV-1 infected. Patients were divided into those with monia (one patient). For analysis of TB/HIV coinfection, patients were included thrush (two patients), Kaposi’s sarcoma (one patient), and pneumonia (one patient). For analysis of TB/HIV coinfection, patients were recruited as part of our TB case-contact platform and were subsequently confirmed to be HIV-1 infected. Patients were divided into those with confirmed TB disease (positive chest X-ray and sputum smear; n = 14) or Mycobacterium-exposed patients without active disease (HIV and TB negative; n = 16). HIV seronegative patients were also recruited from the same cohort (n = 36). For ART-naive patients, fresh whole blood was used and cryopreserved samples used for all other patients. Ethical approval was obtained from the Gambia Government/Medical Research Council joint ethics committee. Although isoniazid preventative therapy is recommended by the World Health Organization it has not yet become implemented as part of the TB control strategy in The Gambia.

Flow cytometry

**Overnight antigenic stimulation.** For fresh samples, 200 μl blood was added per stimulation to a polypropylene tube (Dako, Carpinteria, CA). For cryopreserved samples, PBMCs were thawed, washed, and aliquoted into tubes (between 1–3 million PBMCs per tube) and rested for 6–8 h in RPMI 1640 and 5% HS and 0.01% DNase. Each sample was cultured in the following conditions: negative (media alone), positive (anti-CD3; final concentration 5 μg/ml; BD Pharmingen, San Diego, CA), HIV-1 p24 gag peptidc (final concentration 2 μg/ml; Prospeccio, Rehovot, Israel), PPD (final concentration 10 μg/ml; SSI, Copenhagen, Denmark), and ESAT-6/CFP-10 fusion protein (EC; final concentration 10 μg/ml; kindly supplied by Prof. Tom Ottenhoff, LUMC, Leiden, The Netherlands). Purified anti-CD49d and anti-CD28 were added to all tubes at a final concentration of 1 μg/ml each (BD Pharmingen). Tubes were vortexed, covered, and placed at 37˚C, 5% CO2 overnight (16 h). Brefeldin A (Sigma-Aldrich, Dorset, U.K.) was added after 2 h (final concentration 10 μg/ml).

**Intracellular cytokine staining.** After overnight stimulation, 20 μl of previously titrated surface marker mixture was added to each tube. Abs used were CD3-APC, IFN-γ-PacificBlue, TNF-α-PacificBlue, IL-2–PE, and CD8–PECy7 (all from BD Biosciences). Tubes were vortexed after staining with anti-TNF-α–PECy7, and IL-2–PE (all from BD Biosciences!). Tubes were again vortexed and incubated for 30 min at room temperature (RT) for whole blood, or 15 min in 4˚C for PBMC. After this, 2 ml FACSlysing solution (BD Pharmingen) was added to whole blood tubes and incubated for 9 min, at RT in the dark, followed by centrifugation for 5 min at 600 × g. For PBMCs, cells were washed and resuspended in 150 μl Fix/Perm solution (BD Pharmingen) for 15 min, 4˚C. For permeabilization of the cells, 500 μl one times FACS Perm2 solution (BD Pharmingen) was added to fresh cells or one times Perm/Wash (BD Pharmingen) to PBMCs, tubes vortexed and incubated for 20 min at RT, in the dark. After centrifugation, the supernatant was carefully removed, and 20 μl cytokine mixture added (CD3-APC, IFN-γ-PacificBlue, TNF-α–PECy7, and IL-2–PE [all from BD Biosciences]). Tubes were again vortexed and incubated for 30 min, at RT, in the dark. Tubes were then washed, the supernatant removed, and cells resuspended in 1% paraformaldehyde prior to acquisition (all tubes were acquired within 4 h of staining).

**Flow cytometry acquisition and analysis.** Between 200,000 and 500,000 lymphocytes were acquired with a CyAn ADP (Beckman Coulter) flow cytometer after gating according to 90˚ forward and side scatter plots. FACSDiva software was analyzed using FlowJo software (version 6.1) (TreeStar, Ashland, OR). After gating on CD3+ cells, the percent frequencies of the different combinations of IL-2, TNF-α, and IFN-γ cells after antigenic stimulation were calculated within the total population of CD4+ and/or CD8+ T cells and background subtracted (as determined from the medium alone control). Nonspecific background was extremely low when more than one cytokine was examined. A cutoff of 0.01% was used as described previously (7); values lower than this were set to zero. Combinatorial cytokine data were analyzed with PESTLE (version 1.5.4) and SPICE (version 4.1.5) software obtained from M. Roederer (National Institutes of Health, Bethesda, MD).

### Statistical analysis

Group medians and distributions were analyzed using a Mann-Whitney U test or Kruskal-Wallis ANOVA with Dunn’s posttest comparison. Pre- and post-ART responses were analyzed using Wilcoxon matched pairs analysis. All analyses were performed with GraphPad Prism software version 5 (Software MacKiev, GraphPad, San Diego, CA).

### Results

**Changes in T cell phenotype and magnitude of response to TB Ags with increasing immunosuppression**

With decreasing CD4 counts, the Mycobacterium tuberculosis-specific response shifted from a predominantly CD4+ response in retroviral naive group, a total of 41 patients were recruited: low (CD4 < 200 cells/μl blood; medium (CD4 200–500/μl), and high (CD4 >500/μl). Of 41 patients, 14 patients had a tuberculin skin test (TST) performed with two recording a positive score (>5 mm). No significant difference in age or sex was seen between the groups. An additional 16 patients were analyzed prior to ART and at 3, 6, and 12 mo of ART. Two of 16 had a positive TST score. These patients also had clinical symptoms of TB and were started on TB treatment 2 mo prior to the start of ART. Both patients were sputum smear and culture negative at all time points. Firstline ART consisted of stavudine, lamivudine, and a non-nucleotide reverse transcriptase inhibitor (predominantly efavirenz). Treatment compliance was high with >90% of patients achieving plasma viral loads <100 copies/ml. No patients showed symptoms of IRIS during the study period but other clinical manifestations included thrush (two patients), Kaposi’s sarcoma (one patient), and pneumo

**FIGURE 2.** Comparison of T cell phenotype and function in HIV+TB+ and HIV+TB- patients. A–D, PBMCs were stimulated overnight with PPD (A, B) and EC (C, D) and analyzed for proportion of IFN-γ, IL-2, or TNF-α positive cells within CD4+ (left) or CD8+ (right) T cells. Line indicates median of 14 HIV+TB+ (filled circles) and 16 HIV+TB- (open circles) patients. Data were analyzed using Mann-Whitney U tests and p values are indicated. E, Analysis of the multifunctional profile of CD4+ and CD8+ T cells after overnight stimulation with anti-CD3/CD28, PPD, or EC. Cytokine positive CD4+ or CD8+ T cells were analyzed for different combinations of IFN-γ, TNF-α, and IL-2. Pies were analyzed according to slice color using ANOVA and p values indicated.
patients with CD4 counts >500/µl to a reliance on CD8+ T cells in patients with CD4 count <200/µl (Fig. 1A). The CD8+ response to purified protein derivative (PPD) stimulation in the patient with a high CD4 count is limited to IFN-γ or TNF-α with very few IFN-γ+TNF-α+ cells. In contrast, the response from the CD4+ cells shows a distinct IFN-γ+TNF-α+ double-positive population (Fig. 1A, bottom panel). In the patient with low CD4 count, there is an absence of a CD4+ response (although some TNF-α alone is present), whereas the CD8+ response is maintained but without the double-positive cytokine population. Because of the shift in T cell responses with increasing immunosuppression, we have based our comparison of M. tuberculosis-specific responses with differing CD4 counts on the total T cell population rather than separating into CD4+ and CD8+ T cell subsets.

No difference in the proportion of IFN-γ positive T cells was seen with decreasing CD4 counts after positive control (anti-CD3/CD28) or PPD stimulation (Fig. 1B, 1C). However, a significantly increased proportion of IFN-γ+ cells was seen after EC stimulation in patients with CD4 counts <200/µl compared with both CD4 high (>500/µl) and HIV-negative controls (p < 0.01; p < 0.001 respectively; Fig. 1D). Interestingly, all HIV-positive patients and 25 of 36 (69%) of the HIV negative controls showed a positive response to EC stimulation indicating they were sensitized to M. tuberculosis. No difference in total TNF-α or IL-2 production was observed (data not shown).

Analysis of IL-2 and TNF-α production alongside IFN-γ was used to determine the breadth of the T cell response to TB Ags in HIV-1 infection. With decreasing CD4 counts, there was a contraction of the T cell response observed after EC stimulation (Fig. 1E): the CD4 high group had significantly more T cells producing more than one cytokine simultaneously (p = 0.01) compared with both the CD4 med and CD4 low groups (Fig. 1E). The response to PPD was more conserved than to EC even in patients with CD4 count >500/µl: no significant differences were seen between the groups for both PPD and anti-CD3/28 stimulation.

Comparison of TB-specific responses in HIV/TB coinfection

We next assessed the differences in responses to M. tuberculosis Ags between HIV-1–positive patients who had confirmed TB disease (HIV+TB+) and those without TB disease (HIV+TB–). Analysis of these two groups was based on similar CD4 counts for both groups (median [interquartile range] 200 [93–299] cells/µl for TB+ and 286 [110–451] cells/µl for TB–; Table I), thus CD4+ and CD8+ subsets were analyzed separately in this group of patients. After
both PPD and EC stimulation, HIV+TB+ patients had a significantly higher proportion of CD4+IL-2 cells compared with HIV+TB- patients (p = 0.032 and p = 0.042, respectively; Fig. 2A, 2C). In addition, HIV+TB+ patients had a significantly higher proportion of CD4+TNF-α+ cells after PPD stimulation compared with HIV+TB- patients (p = 0.041; Fig. 2A). No difference in the proportion of CD4+IFN-γ+ cells was observed. There was a significantly higher proportion of CD8+ TNF-α+ cells after both PPD and EC stimulation in HIV+TB+ (p = 0.040; p = 0.007, respectively) and CD8+IFN-γ+ cells after EC stimulation (p = 0.002) (Fig. 2B, 2D).

Analysis of the polyfunctional responses after stimulation showed very similar profiles between HIV+TB+ and HIV+TB- patients for both CD4+ and CD8+ subsets (Fig. 2E). These findings indicate that changes in the PFT cell response to TB Ags relate more to CD4 count than TB status. Interestingly, individual subset analysis based on expression of different combinations of IFN-γ, IL-2, and TNF-α showed significantly higher proportions of CD4+IFN-γ+TNF-α+ IL-2+ cells in HIV+TB+ compared with HIV+TB- but also CD8+ cells expressing only IL-2 (p = 0.044, p = 0.032, respectively) after PPD stimulation (data not shown).

**Restoration of CD4+ T cell responses after ART**

We hypothesized that with reduction of viral load and subsequent increase in CD4+ T cell levels following ART that we would see a change in the CD4+ T cell responsiveness to TB Ags. Flow cytometry analysis of TNF-α and IFN-γ production after anti-CD3/CD28 or PPD stimulation showed a return of the CD4+ response to both Ags by 6 mo of ART, with retention of the CD8+ response (Fig. 3A–H). Furthermore, there was a distinct CD4+TNF-α+ IFN-γ+ population evident after PPD stimulation which was not present in the responding cells (CD4+ or CD8+) prior to treatment (Fig. 3B, 3G).

Analysis of the polyfunctional CD4+ T cell responses at 3, 6, and 12 mo of ART showed a significant increase by 6 mo of ART after EC stimulation and as early as 3 mo after PPD stimulation compared with pretreatment findings (p = 0.023 and p = 0.028, respectively; Fig. 3I). After EC stimulation, these results were due to significant increases in cells expressing IL-2 and TNF-α+ or IFN-γ+ and a significant decrease in cells expressing IFN-γ alone (p = 0.04 for all; data not shown). The changes after PPD stimulation were due to increased triple-positive cells (p = 0.03) and reduction in cells expressing IFN-γ alone (p = 0.02; data not shown). Interestingly, at 3 mo of treatment, a significant increase in polyfunctionality was observed in response to anti-CD3/28 stimulation (p = 0.07) that was reduced again at 6 and 12 mo of treatment (Fig. 3I, top row). This was due to a significant increase in cells expressing all three cytokines (p = 0.005) and reduction in cells expressing only IFN-γ (p = 0.04; data not shown). No difference in the CD4+ profile (either overall or individually) to HIV-1 stimulation was observed for the time-points included in this study (Fig. 3I, third row).

We also analyzed the absolute number of cytokine positive CD4+ T cells (per milliliter blood) at 12 mo of ART compared with pre-ART using Wilcoxon matched pairs analysis (Fig. 4). The major differences were seen with the positive control (anti-CD3/CD28 stimulation with highly significant increases in IFN-γ+ (p = 0.001), IL-2+ (p = 0.003), and TNF-α+ (p = 0.002) CD4+ T cells (Fig. 4A). We saw no difference in absolute levels of cytokine-producing CD4+ T cells after stimulation with HIV-1 at 12 mo of ART (Fig. 4B). The total number of cytokine positive cells was significantly increased after EC stimulation (p = 0.0334; Fig. 4C) but this could not be attributed to any particular subset of cells, despite higher levels of both CD4+IL-2+ and CD4+TNF-α+ cells (Fig. 4C). The converse was true for PPD stimulation with no significant difference in total levels of cytokine-positive cells at 12 mo of ART despite a significant increase in CD4+TNF-α+ absolute cell number (p = 0.031; Fig. 4D).

**Maturational phenotype of the responding cells after ART**

We divided the CD4+ T cells according to surface marker expression of CD27 and CD45RO into naive (CD27+CD45RO+), central memory (CM) (CD27+CD45RO+), EM (CD27+CD45RO-), and terminal effector (TE) (CD27-CD45RO-) subsets. Cytokine production within each subset was assessed to determine the phenotype of the major cytokine-producing cells (Fig. 5A). This analysis was performed for PPD stimulation but not EC stimulation due to the higher number of responding cells with PPD stimulation and also the more multifunctional phenotype seen at 12 mo compared with EC stimulation. The percentages in the dot plots shown are the frequency of the “grandparent” population; that is, we have taken into account the relative percentage of the CD4+ subset to

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Analysis of absolute CD4+ cytokine+ cell numbers pre- and post-ART. PBMCs from patients prior to and at 12 mo of ART were stimulated with EC, PPD, HIV, or anti-CD3/CD28 overnight and analyzed for absolute levels of CD4+IFN-γ+, IL-2+, TNF-α+, or total cytokine-positive cells. A–D. Absolute number of cytokine positive cells after anti-CD3/CD28 (A), HIV-1 (B), EC (C), or PPD (D) stimulation. Bar indicates median of 16 patients (pre-ART, filled circles; post-ART, open circles). Data were analyzed using a Wilcoxon matched t test and p values are indicated.
accurately reflect the relative levels of cytokine-producing cells. We found few cytokine-producing cells in the naive compartment as expected. Because of our use of an overnight assay, the majority of responding cells were within the EM rather than the CM compartment (Fig. 5A, 5B). A very similar pattern was seen at all time-points for TNF-α+IFN-γ+ and TNF-α+IFN-γ- cells but although there were significant differences in TNF-α+IFN-γ- cells between the subsets at 3 and 6 mo post-ART, this was not seen at 12 mo. A significant increase in TNF-α+ cells within the CM (p = 0.048), EM (p = 0.025), and TE (p = 0.0039) subsets and a significant increase in TNF-α+IFN-γ- cells within the EM compartment was seen at 12 mo compared with 3 mo of ART (Fig. 5B).

Discussion
This study provides a detailed analysis of PFT cell responses to TB Ags in HIV-1 infection with decreasing CD4+ T cell levels and longitudinally after initiation of ART and subsequent increase in CD4+ T cells. We found that the proportion of IFN-γ+ T cells in response to TB Ags was higher in HIV-1–positive patients with lower CD4 counts but that this response was reliant on CD8+ T cells. There was also a significant reduction in the polyfunctional profile of the cells that was restored by 12 mo of ART and primarily involved CD4+ T cells with an EM phenotype.

After stimulation with EC, we found a significant increase in the proportion of IFN-γ T cells in patients with CD4 counts <200/μl compared with those with CD4 counts >500/μl or HIV-negative patients. We did not see a difference in the proportion after positive control or HIV-1 stimulation indicating this was a TBSpecific change. When 12 mo ART responses were analyzed in the same patients, we saw no difference in the absolute number of CD4+IFN-γ+ cells/ml blood after TB and HIV antigenic stimulation. However, the quality of the immune response changed from that of a predominantly functionally restricted CD8+ response to a polyfunctional CD4+ T cell profile after ART with an increase in both TNF-α+ and IL-2+ cells. This is of fundamental importance because CD4+ T cells are absolutely required for an optimal protective immune response to TB (4). The role of CD8+ T cells in immune protection to TB is still controversial in humans but they have been shown to preferentially recognize heavily infected cells in vitro that suggests a role for CD8+ T cells in immune surveillance (3). Targeting these cells may be required in development of vaccines to be used for prevention of reactivation of latent infection (16) as seen in HIV-1 infection. However, the reliance on CD8+ T cells in HIV-infected patients with very low CD4+ T cell counts is not optimal and may explain the increase in TB despite the increase in proportion of IFN-γ-producing cells with decreasing CD4 counts. Moreover, the increase in the polyfunctional profile of the circulating cells associated with a reduction in TB prevalence with ART suggests an important role for this population in protecting against new TB infections.

Analysis of HIV-1–infected patients with confirmed TB (HIV+TB+) compared with those with comparable CD4 levels but no symptoms of TB (HIV-TB-) showed similar profiles in regard to the overall complexity of the responding CD4+ T cells. However, a significantly higher proportion of both CD4+TNF-α- and CD8+ TNF-α- cells were seen in HIV+TB+ compared with HIV-TB-, which we have also seen in HIV negative TB cases (9). The role of TNF-α in the protective immune response to TB infection is shown by the increased incidence of TB during treatment with anti-TNF Abs (17). TNF-α acts by promoting apoptosis of the M. tuberculosis-infected macrophages; a mechanism that has been shown to be impaired in HIV-1 infection (18) However, the role of TNF-α has also been postulated to be dose dependent with too high levels associated with increased TB pathology (19). Furthermore, it has been shown in animal models that with increasing TB severity the intracellular bacteria can cause deregulation of macrophage activity, possibly by increasing production of TNFR2 and thus neutralizing the effects of the TNF (20). Unfortunately, it is not possible to accurately determine bacterial load in human TB infection but it is likely that this will directly influence the ability

![Figure 5](http://www.jimmunol.org/)
of the macrophages to contain the infection, particularly in HIV-1 coinfection.

The regeneration of the CD4+ response to PPD by 12 mo of ART was predominantly by cells with an EM phenotype, particularly in regard to simultaneous production of TNF-α and IFN-γ. EM T cells have been shown to respond to Ag re-encounter more rapidly than CM cells (21), which is clearly important in the control of TB. Interestingly, we saw no correlation with levels of CD4 cells prior to treatment and the ability to rejuvinate an immune response after, indicating that the increase in polyfunctional cells as early as 3 mo posttreatment (particularly for PPD responses) are most likely due to a redistribution of sequestered cells during HIV-1 infection (22). By 12 mo post-ART, the response would also be contributed to by thymic-derived naive CD4+ T cells (23, 24) encountering the Ag for the first time and eliciting a robust polyfunctional response important for the generation of protective immunity to TB. Interestingly, we have previously shown that a polyfunctional CD4+ T cell response does not correlate with protection against disease protection in HIV-negative individuals with active TB (9). In light of our findings post-ART, this suggests that the PFT cell response to TB Ags is protective but that the outcome of the response is influenced by the negative effects of the bacteria (i.e., on macrophage function) in subjects with active TB disease, which will not occur in the asymptomatic cohort (with presumed low bacterial load) we used to assess pre- and post-ART responses. These findings support the current literature showing a protective role for PFT cells in HIV and vaccine-induced immunity (7, 8).

The timing of ART has been debated for many years, particularly in regard to intermittent therapy and the juxtaposition of TB-associated IRIS development (25–28). ART has been available in The Gambia since 2004, so it is hard to know if there has been a reduction in TB incidence, but a recent study in Iran showed a significant reduction in TB incidence when ART was initiated for patients with CD4 counts <100/μl (29). The World Health Organization guidelines recommend ART initiation when CD4 counts drop below 300/μl and our data suggest this is crucial in the context of retaining an adequate response to TB Ags (polyfunctional responses were lost in patients with counts below 500/μl). The development of a new TB vaccine is urgently needed and would appear to be most effective if given to HIV-positive individuals after restoration of CD4+ T cell responses to TB Ags with ART. This has important public health benefits in controlling the spread of TB throughout HIV-infected communities (30, 31). Our findings also raise questions in regard to the development of TB diagnostics for use in HIV-infected patients. In this regard, the T-SPOT.TB test appears to have the greatest potential of the IFN-γ–based diagnostics in HIV-infected patients as it is relatively independent of the level of CD4 T cell depletion, possibly because it can stimulate both a CD8+ and CD4+ T cell response (12).

In conclusion, our data provides a detailed immunological profile of responses to M. tuberculosis Ags as ART reduces susceptibility of HIV-positive patients to TB and implies that a polyfunctional CD4+ T cell profile could be protective against TB in HIV-1 infection. These results have important public health implications for TB diagnostics and vaccine design for use in HIV-positive patients.

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Disclosures
The authors have no financial conflicts of interest.

References


