Early Depletion of *Mycobacterium tuberculosis*–Specific T Helper 1 Cell Responses after HIV-1 Infection

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**Background.** The acid-fast bacillus *Mycobacterium tuberculosis* is often the first manifestation of acquired immunodeficiency syndrome in patients infected with human immunodeficiency virus (HIV). This study was conducted to better understand the mechanism underlying *M. tuberculosis*–specific pathogenicity early after onset of HIV infection.

**Methods.** *M. tuberculosis*–specific T helper 1 (Th1) cells were studied in HIV negative (*n* = 114) and chronically HIV infected (*n* = 68) Tanzanian subjects by using early secreted antigenic target 6 (ESAT6) protein or tuberculin (purified protein derivative) with interferon-γ ELISPOT and intracellular cytokine staining. In a longitudinal study, the effect of acute HIV infection on *M. tuberculosis*–specific Th1 cells was determined by polychromatic flow cytometric analysis in 5 subjects with latent *M. tuberculosis* infection who became infected with HIV.

**Results.** In tuberculosis (TB)–asymptomatic subjects (i.e., subjects with unknown TB status who did not show clinical signs suggestive of TB), chronic HIV infection was associated with a decreased percentage of subjects with detectable *M. tuberculosis*–specific Th1 cells (*P* = .001), a decrease which was not observed among subjects with active TB. Acute HIV infection induced a rapid depletion of *M. tuberculosis*–specific Th1 cells in 4 subjects remained TB asymptomatic, whereas the population of these cells remained stable in subjects who remained HIV negative (*P* < .01).

**Conclusions.** Taken together, these data suggest a mechanism of rapid *M. tuberculosis*–specific Th1 cell depletion that may contribute to the early onset of TB in individuals with latent *M. tuberculosis* infection who become HIV infected.

HIV infection is characterized by a progressive depletion of CD4+ T cells that eventually leads to AIDS, as defined by the onset of opportunistic infections. The mechanism of the pathogenesis underlying HIV-associated immune damage may differ between the acute and chronic phases of infection. It is widely accepted that ongoing viral replication and virus-induced cell death are responsible for the massive depletion of memory CD4+ T cells from mucosal sites during acute simian immunodeficiency virus (SIV) infection and HIV infection [1–5]. Yet, despite this rapid depletion of memory CD4+ T cells early during HIV infection, most opportunistic infections typically cause complications only after extended periods of HIV disease progression. Few pathogens cause disease early after onset of HIV infection. One such pathogen of high clinical relevance is the acid-fast bacillus (AFB) *Mycobacterium tuberculosis*. During the first year of coinfection with HIV and *M. tuberculosis*, the risk of developing active tuberculosis (TB) increases dramatically [6, 7]. TB
disease occurs in HIV-infected persons at all CD4+ T lymphocyte counts [7], and especially in developing nations, pulmonary TB frequently is the first manifestation of AIDS [7], suggesting that the pathology of M. tuberculosis infection in HIV-positive individuals may differ from that observed for most other opportunistic infections.

M. tuberculosis commonly causes latent infection in the lungs that is tightly controlled by the M. tuberculosis–specific cellular immune response in healthy individuals but results in disease during periods of immunosuppression. Secretion of interferon (IFN)–γ by CD4+ Th1 cells can activate M. tuberculosis–infected macrophages and contribute to the containment of the intraphagosomal pathogen [8, 9]; hence, these cells are important in the control of M. tuberculosis infection. Optimal activation of infected macrophages also may require involvement of costimulatory cell-surface proteins [10] and therefore is dependent on cellular contact with M. tuberculosis–specific lymphocytes. Interestingly, the same interactions also contribute to maximal HIV-1 replication inside alveolar macrophages [11] and may lead to efficient transmission of HIV from macrophages coinfected with M. tuberculosis and HIV to M. tuberculosis–specific CD4+ T cells. However, despite the fact that M. tuberculosis–specific immunity is apparently greatly suppressed in individuals coinfected with both pathogens, the effects of HIV infection on M. tuberculosis–specific CD4+ T cells are not well understood. M. tuberculosis–specific CD4+ T cell responses are present in subjects with pulmonary TB regardless of HIV infection status [11, 12], yet HIV infection is associated with a reduced delayed-type hypersensitivity reaction to the tuberculin skin test; the latter observation suggests that HIV affects M. tuberculosis–specific Th1 cell responses in vivo.

The region of difference 1 (RD1) antigens, early secreted antigenic target 6 (ESAT6) protein and culture filtrate protein 10 (CFP10), are more specific for M. tuberculosis than tuberculin (purified protein derivative [PPD]) and have more recently been used to identify M. tuberculosis–specific cellular immune responses [12–16]. The ESAT6 and CFP10 antigens, especially in combination with PPD, are therefore suitable for more detailed study of M. tuberculosis–specific CD4+ T cell responses and the effect of HIV on these responses.

To investigate the mechanisms underlying the early onset of pulmonary TB often observed after HIV infection, we studied the effect of chronic HIV infection on M. tuberculosis–specific Th1 cell responses in TB-asymptomatic subjects (i.e., subjects with unknown TB status who did not show clinical signs suggestive of TB) and subjects with active pulmonary TB. We further dissected the events occurring early after HIV infection onset by following up 5 women from a cohort of commercial sex workers in Tanzania who had latent M. tuberculosis infection before they became infected with HIV. We analyzed the M. tuberculosis–specific CD4+ T cell response during and up to 12 months after HIV seroconversion in these 5 women and determined their clinical outcome by means of a final interview that took place 3 years after the last study follow-up visit.

SUBJECTS, MATERIALS, AND METHODS

Study subjects. For analysis of M. tuberculosis–specific Th1 cell responses, volunteers were recruited from 3 ongoing studies that have been described elsewhere [17–19]. This substudy received a separate ethical clearance from the local and national institutional review boards, and a new consent form was signed by the volunteers in case additional specimens were obtained. For cross-sectional analysis of M. tuberculosis–specific Th1 cell responses, 14 patients who had positive AFB smear results and clinical symptoms of pulmonary TB (defined as productive cough for >4 weeks, night sweats, weight loss, and loss of appetite) were recruited from an ongoing TB diagnostic trial [18]. During the study, all participants received health care for acute medical problems. Patients with diagnosed TB were referred for TB treatment that accorded with World Health Organization standards. During the course of this study, all individuals were antiretroviral naive. TB was diagnosed on the basis of positive AFB staining results for 3 independent sputum samples and a clinical diagnosis. Latent infection with M. tuberculosis was defined on the basis of cellular responses to RD1 antigens in the absence of clinical symptoms suggestive of TB. HIV infection status was determined by using 2 diagnostic HIV-specific enzyme-linked immunoassays (Enzygnost Anti HIV1/2 Plus [Dade Behring] and Determine HIV 1/2 [Abbott]). CD4+ cell counts were determined in fresh whole blood samples by use of the BD multitest kit (Becton Dickinson).

Antigens. ESAT6 and CFP10 antigens (Lionex) and PPD for in vitro testing (Statens Serum Institut) were used at a final concentration of 10 μg/mL. Peptides that overlapped by 11 amino acids were designed for ESAT6 (AF420491.1) and CFP10 (AAC83445) by using the PeptGen peptide generator from the HIV Molecular Immunology Database (available at: http://www.hiv.lanl.gov/content/immunology/).

IFN-γ ELISPOT assays. Freshly isolated peripheral blood mononuclear cells (PBMCs) were screened for responses specific for recombinant ESAT6 and PPD by stimulation of 2.5 × 10^5 PBMCs/well in duplicate overnight. The assay was performed as previously described [20]. Responses with ≥20 spot-forming cells (sfc) per 10^6 PBMCs and ≥3 times the value for the negative control sample were scored as positive. Discordant findings, defined as results in which only 1 of the duplicate wells was scored as positive, were excluded from analysis.

Conjugated antibodies for flow cytometric analysis. The following antibodies were used: CD3–fluorescein isothiocyanate, IFN-γ–fluorescein isothiocyanate, and CCR5–phycoerythrin (PE) (BD Biosciences); CD27–Cy5PE and CD45RO–Texas red–PE (Beckman Coulter); and CD4–PECy5.5 (Caltag). The following antibodies were conjugated in-house in accordance with standard protocols (available at: http://drmr.com/abcon/index.html): CD8–quantum dot 655 and tumor necrosis factor (TNF)–α–Alexa680.

Stimulation and flow cytometric analysis of PBMCs. Cell stimulation and staining were performed by using a modified
A total of 182 subjects were tested in a cross-sectional study with an IFN-γ ELISPOT assay for recognition of recombinant ESAT6 protein. Table 1 summarizes CD4+ T cell counts, the number of M. tuberculosis–specific CD4+ T cells detected, and the results of AFB staining for HIV-negative and HIV-positive TB-asymptomatic subjects and for HIV-negative and HIV-positive subjects with pulmonary TB.

**HIV infection is associated with significantly decreased M. tuberculosis–specific CD4+ T cell responses in individuals with no clinical signs of TB.** HIV infection was associated with a significant decrease in the frequency of detectable IFN-γ ELISPOT responses to ESAT6 in patients with no clinical signs of TB (P < .001, by Fisher’s exact test). Whereas 50 (47%) of 106 HIV-negative, TB-asymptomatic subjects responded to ESAT6 (5 subjects were excluded because of discordant results between duplicate wells), only 10 (19%) of 54 of HIV-positive, TB-asymptomatic subjects had detectable responses (3 subjects were excluded because of discordant results between duplicate wells). Intracellular cytokine staining demonstrated that detectable IFN-γ responses were mediated by CD4+ T cells (figure 1A).

Comparison of the overall median magnitude of responses for 106 HIV-negative subjects (18 sfc/10⁶ PBMCs) with that for 54 HIV-positive subjects (2 sfc/10⁶ PBMCs) revealed that HIV infection was associated with a 9-fold reduction in the number of M. tuberculosis–specific CD4+ T cells (P < .01) (figure 2A). PPD-specific responses followed an identical trend (P < .001) (figure 2B). Contrary to our expectation, there was no linear correlation between M. tuberculosis–specific CD4+ T cells and the total CD4+ T cell count (figure 2B and 2D). Taken together, these results demonstrate that at least half of the study population from the Mbeya region probably had latent M. tuberculosis infection, and HIV infection was greatly decreasing the M. tuberculosis–specific Th1 cell responses in these subjects.

**In HIV-infected individuals, active TB is associated with detectable ESAT6 responses.** To study the differences between individuals with latent TB and those with active TB, we measured the level of ESAT6–specific Th1 cells in HIV-negative and HIV-positive individuals who had a positive AFB smear result. In line with the results of a previous report [12], 10 (91%) of 11 HIV-positive patients with pulmonary TB had detectable ESAT6 responses, with a median of 118 sfc/10⁶ PBMCs (figure 2A). In agreement with our previous observation, detection of M. tuberculosis–specific CD4+ T cell responses did not correspond to the total CD4+ cell count. Three (100%) of 3 HIV-negative patients with pulmonary TB responded to ESAT6. Taken together, our results suggest that the M. tuberculosis–specific Th1 cells present during latent infection are depleted after HIV infection and their level increases only after M. tuberculosis reactivation or de novo infection, independent of the total CD4+ T cell count. We therefore hypothesize that detection of M. tuberculosis–specific Th1 cells in HIV-positive individuals is a marker for immune exposure to new M. tuberculosis antigen and therefore indicates at least transient M. tuberculosis reactivation.
High levels of HIV coreceptor CCR5 expression on M. tuberculosis–specific Th1 cells. Most newly transmitted HIV viruses infect cells that express surface viral receptors CD4 and CCR5 [23]. To study whether M. tuberculosis–specific Th1 cells are potentially susceptible to viral infection during primary HIV infection, we next analyzed CCR5 expression in 9 HIV-negative subjects with latent M. tuberculosis infection and compared these cells (gated in figure 3A) to different CD4+ T cell subsets defined by their expression of the T cell memory markers CD27 and CD45RO (figure 3C). Naive CD4+ T cells (CD27+CD45RO−) that did not express CCR5 were used to determine cutoff values (figure 3B). As expected, a higher fraction of CD27−CD45RO+ CD4+ T cells (median, 50.7%) expressed CCR5, compared with CD27−CD45RO+ CD4+ T cells (median, 18.7%). A median of 58.8% of M. tuberculosis–specific Th1 cells (range, 29.4%–69.3%) expressed CCR5, constituting a 2-fold increase compared with total memory CD4+ T cells (median, 27.9%). The level of CCR5 cell surface expression was also significantly increased (P < .01). Taken together, these data demonstrate that a large fraction of M. tuberculosis–specific Th1 cells from subjects with latent M. tuberculosis infection express the cellular receptors required for HIV entry for most newly transmitted viruses.

Rapid depletion of M. tuberculosis–specific Th1 cells during early during HIV infection. Rapid depletion of memory CD4+ T cells is a hallmark of acute SIV and HIV infection [1–5]. Despite this significant decrease of memory CD4+ T cells, Candida albicans–specific, CMV–specific, and tetanus toxoid–specific CD4+ T cells can usually still be detected early in HIV infection [24, 25]. In particular, the lack of correlation between M. tuberculosis–specific Th1 cells and the total CD4+ cell count led us to hypothesize that M. tuberculosis–specific CD4+ T cells are depleted early in HIV infection. To clarify this, we studied the dynamics of RD1-specific and PPD-
specific CD4+ T cell responses in 5 subjects with latent TB who acquired HIV infection during the HIV Superinfection Study [19].

Before HIV infection, Th1 cell responses targeting either one of the RD1 peptide sets (ESAT6 or CFP10) and PPD were detected in all 5 subjects (figure 4). As expected, during the last HIV-seronegative follow-up visit, RD1-specific responses (range, 0.09%–0.21% of memory CD4+ T cells; background subtracted) and PPD-specific responses (range, 0.1%–0.8% of memory CD4+ T cells) were of relatively low magnitude. Although the RD1 peptide sets were specifically designed to study M. tuberculosis–specific CD8+ T cell responses, none were detected in these or any other HIV-negative subjects with latent M. tuberculosis infection who were tested throughout this study. In 4 of 5 subjects with latent infection, M. tuberculosis–specific responses were rapidly depleted within the first year after HIV seroconversion. In contrast, M. tuberculosis–specific Th1 cell responses did not fluctuate notably in subjects with latent M. tuberculosis infection, who remained HIV seronegative (P < .01 [data not shown]). Importantly, none of these 4 subjects received a diagnosis of active TB within 4 years after HIV infection.

The fifth subject (H19) had the most dramatic decrease in CD4+ T cell counts (a 58% decrease from 810 cells/μL to 343 cells/μL) and a high viral load continuously >100,000 RNA copies/mL. In this subject, M. tuberculosis–specific Th1 responses increased after HIV infection and in particular, the population of PPD-specific CD4+ T cells expanded dramatically 1 year after HIV seroconversion (figure 4). In addition, a strong CD8+ T cell response targeting CFP10 emerged after HIV infection, reaching 0.57% of CD8+ T cells. Sub-

Figure 2. Chronic HIV infection and Th1 cell responses to early secreted antigenic target 6 (ESAT6) and purified protein derivative (PPD) in tuberculosis (TB)–asymptomatic subjects and subjects with pulmonary TB. Shown are the spot-forming cells per 10^6 peripheral blood mononuclear cells (PBMCs) responding to recombinant ESAT6 protein (A) and PPD (C) for each subject and the corresponding total CD4+ cell counts for HIV-infected individuals in (B and D). Blue circles, HIV-negative, TB-asymptomatic subjects (n = 110); red circles, HIV-negative subjects with pulmonary TB (n = 3); blue diamonds, HIV-infected, TB-asymptomatic subjects (n = 55); red diamonds, HIV-positive subjects with pulmonary TB (n = 11); horizontal black lines, median values. The cutoff value was 20 sfc/10^6 PBMCs (red dotted line). Statistical analysis for panels A and C was performed by using the Mann-Whitney test; for panels B and D, the Spearman rank test was used.
object H19 received a diagnosis of active TB, and was treated for it, within 15 months after HIV seroconversion.

**DISCUSSION**

The present study was primarily designed to dissect the underlying mechanism associated with the dramatic increase in the risk of developing pulmonary TB shortly after HIV infection for subjects who are latently infected with *M. tuberculosis*. We addressed the following 3 questions: (1) Does chronic HIV infection affect *M. tuberculosis*-specific cellular immunity in individuals with latent *M. tuberculosis* infection? (2) Do these cells express the HIV coreceptor CCR5? (3) How does acute HIV infection affect the *M. tuberculosis*-specific cellular immune responses?

The high prevalence of ESAT6-specific responses observed among HIV-negative, TB-asymptomatic subjects suggests that at least half of the study population from the Mbeya region was latently infected with *M. tuberculosis*. However, the absence of these cells in many HIV-positive subjects does not exclude latent *M. tuberculosis* infection, as demonstrated by the detection of *M. tuberculosis*-specific Th1 cell responses before HIV infection and their disappearance thereafter. Taken together, these data suggest that typically, coinfected individuals in TB-endemic regions are already latently infected with *M. tuberculosis* before HIV infection. Although not a sufficient cause, this may contribute to the early manifestation of TB associated with HIV infection.

Chronic HIV infection was associated with a reduced percentage of subjects who responded to *M. tuberculosis* antigen, indicating that cellular immunity to *M. tuberculosis* was reduced by chronic HIV infection. The rapid depletion of *M. tuberculosis*-specific responses early after HIV infection and their presence in HIV-positive subjects with pulmonary TB suggests that the population of these cells (re)expands after ongoing or transient reactivation of mycobacterial growth or de novo exposure. This interpretation is further supported by 2 recent studies [26, 27].

Our results support a scenario in which early *M. tuberculosis*-specific Th1 cell depletion could be caused by direct HIV infection. Active HIV viral replication indeed is a potent suppressor of *M. tuberculosis*-specific Th1 cell responses, as demonstrated by the dramatic expansion of this cell population observed after initiation of antiretroviral therapy [28, 29]. High levels of surface expression of the viral coreceptor CCR5 should contribute to direct HIV infection. PPD antigen alone can trigger productive HIV-1 infection of CD4+ T cells from HIV-1-positive

**Figure 3.** Proportion of *Mycobacterium tuberculosis*-specific Th1 cells expressing HIV coreceptor CCR5. Shown is the cell surface expression of CCR5 (B) on gated interferon (IFN)-γ positive CD4+ T cells (A) detected after 6 h of stimulation with region of difference 1 peptides. C and D, the proportion of CCR5+ CD4+ T cells in different CD4+ T cell subsets for 9 HIV-negative subjects who were latently infected with *M. tuberculosis*. Statistical analysis was performed by using the Wilcoxon matched pairs test.
cocultured dendritic cells in vitro [30], and it is sufficient to induce HIV replication and cell death in PBMCs from HIV-positive subjects who have positive tuberculin skin test results [31]. Alveolar macrophages are easily infected with HIV in vitro and are also infected in vivo during the acute and final phase of SIV infection [32]. In addition, anti--M. tuberculosis cellular immunity maximizes HIV replication inside alveolar macrophages [11], which could enhance “M. tuberculosis–specific” HIV transmission to M. tuberculosis–specific Th1 cells. A state of complete metabolic quiescence during latent M. tuberculosis infection may undermine this specific mode of transmission. However, it has been suggested that rather than being caused by metabolic quiescence, M. tuberculosis latency is the result of a “continuous cross talk between the host immune system and the persisting pathogens,” as indicated by active sites of cell proliferation and follicle-like structures within affected parts of the lungs from latently M. tuberculosis infected individuals [33, p. 89]. The high viral load found in bronchoalveolar lavage (BAL) fluid from sites of active TB disease, but not from unaffected sites [34, 35], further sup-

Figure 4. Depletion of Mycobacterium tuberculosis–specific Th1 cells early during HIV infection. Shown are the frequencies of purified protein derivative (PPD)–specific (upper panel) and region of difference 1–specific (lower panel) CD4+ T cells as a percentage of memory CD4+ T cells, for each HIV-infected subject studied (H228, H590, H140, H80, and H19). The upper left set of panels show representative dot plots after stimulation with medium alone or early secreted antigenic target 6 (ESAT6). All other panels show the percentage of M. tuberculosis–specific memory CD4+ T cells detected before and after HIV infection (red arrow) for 5 subjects who became HIV infected. The percentage of memory CD4+ T cells was determined by CD27 and CD45RO expression. Naive CD4+ T cells (CD27-CD45RO cells) were excluded from analysis of memory CD4+ T cells. Interferon (IFN)–γ positive memory CD4+ T cells were detected after overnight stimulation with PPD, ESAT6, or culture filtrate protein 10 (CFP10). The background was subtracted.
ports the hypothesis that the proinflammatory microenvironment caused by local \textit{M. tuberculosis} infection enhances HIV transmission to \textit{M. tuberculosis}–specific CD4$^+$ T cells in vivo.

The importance of Th1 cytokines in mediating protection from TB is well documented [36, 37]. Rapid depletion of \textit{M. tuberculosis}–specific Th1 cells early in HIV infection could therefore be key to the tremendous increase in the risk of developing TB. In contrast, CMV–specific CD4$^+$ T cells persist until the late stages of HIV infection [25], and typically, CMV–associated pathology does not occur until the very late stages of AIDS, further supporting our hypothesis that rapid depletion of \textit{M. tuberculosis}–specific CD4$^+$ T cells is important in the specific pathologic interactions of \textit{M. tuberculosis} and HIV. However, despite the depletion of this important T cell subset, only a minority of coinfected subjects develop TB soon after HIV infection. Specific CD8$^+$ T cells, nonconventional T cell subsets, or alternative sources of IFN–γ and TNF–α, such as NK cells, may compensate for some of the effector functions of the \textit{M. tuberculosis}–specific Th1 cells [8, 38]. In addition, Th17 cells that do not produce IFN–γ or TNF–α have been shown to participate in \textit{M. tuberculosis}–specific immunity [39]. Such responses may still be sufficient in most cases to control \textit{M. tuberculosis} after depletion of \textit{M. tuberculosis}–specific Th1 cells. Alternatively, host genetic polymorphisms affecting anti–\textit{M. tuberculosis} immunity could account for differences in susceptibility to disease. Finally, differences in pathogen load or virulence may also contribute to different TB disease outcomes in subjects coinfected with HIV and \textit{M. tuberculosis}. Interestingly, early in HIV infection the pulmonary and nondisseminated form of TB predominates, whereas in patients with AIDS who have very low CD4$^+$ cell counts, disseminated and extrapulmonary TB disease is frequently observed, indicating that even in such subjects there is residual anti–\textit{M. tuberculosis} immunity left after acute HIV infection.

In conclusion, our results demonstrate that acute HIV infection is associated with the rapid loss of \textit{M. tuberculosis}–specific Th1 cells in the peripheral blood. This contrast with the gradual decline of the total CD4$^+$ cell count during the chronic phase of HIV infection and may be caused by direct HIV infection of these cells. Taken together, these data suggest a mechanism of rapid \textit{M. tuberculosis}–specific Th1 cell depletion that may contribute to the early onset of TB that is often observed in latently infected individuals who become HIV infected.

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