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Simian Immunodeficiency Virus-Induced Changes in T Cell Cytokine Responses in Cynomolgus Macaques with Latent Mycobacterium tuberculosis Infection Are Associated with Timing of Reactivation

Joshua T. Mattila,* Collin R. Diedrich,* Philana Ling Lin,† Jiayao Phuah,* and JoAnne L. Flynn*

Understanding the early immunologic events accompanying reactivated tuberculosis (TB) in HIV-infected individuals may yield insight into causes of reactivation and improve treatment modalities. We used the cynomolgus macaque (Macaca fascicularis) model of HIV–Mycobacterium tuberculosis coinfection to investigate the dynamics of multifunctional T cell responses and granuloma T cell phenotypes in reactivated TB. CD4+ and CD8+ T cells expressing Th1 cytokines (IFN-γ, IL-2, TNF) and Th2 cytokines (IL-4 and IL-10) were followed from latent M. tuberculosis infection to reactivation after coinfection with a pathogenic SIV. Coinfected animals experienced increased Th1 cytokine responses to M. tuberculosis Ags above the latent-response baseline 3–5 wk post-SIV infection that corresponded with peak plasma viremia. Th2 cytokine expression was not Ag specific, but strong, transient IL-4 expression was noted 4–7 wk post-SIV infection. Animals reactivating <17 wk post-SIV infection had significantly more multifunctional CD4+ T cells 3–5 wk post-SIV infection and more Th2-polarized and fewer Th0-, Th1-polarized CD8+ T cells during weeks 1–10 post-SIV infection than animals reactivating >26 wk post-SIV infection. Granuloma T cells included Th0-, Th1-, and Th2-polarized phenotypes but were particularly rich in cytolytic (CD107+) T cells. When combined with the changes in peripheral blood T cells, these factors indicate that events during acute HIV infection are likely to include distortions in proinflammatory and anti-inflammatory T cell responses within the granuloma that have significant effects on reactivation of latent TB. Moreover, it appears that mycobacteria-specific multifunctional T cells are better correlates of Ag load (i.e., disease status) than of protection. The Journal of Immunology, 2011, 186: 3527–3537.

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early a third of the world’s population is estimated to be infected with Mycobacterium tuberculosis, a pathogenic bacterium that causes tuberculosis (TB). Individuals with normal immune systems can maintain subclinical infections for decades in an asymptomatic state known as latent TB (1, 2). Infection with HIV strongly predisposes individuals with latent TB to experience reactivated disease (3). Although HIV-infected individuals with low CD4+ T cell numbers are at severe risk of developing TB (4, 5), individuals with better preserved CD4+ T cell numbers are also at increased risk of primary or reactivated TB well before the onset of AIDS (6). The combined effects of HIV and M. tuberculosis coinfection have an unfortunate and deadly synergy and make TB the leading cause of death among HIV-infected individuals (6).

Cytokine-mediated communication between innate and adaptive immune systems in the granuloma is required to mount protective anti-mycobacterial responses. The cytokines most commonly associated with protection are IFN-γ (7) and tumor necrosis factor-α (TNF) (8), both of which are released by M. tuberculosis-specific T cells and cause macrophage activation and bactericidal activity (9, 10). IL-2 acts in conjunction with IFN-γ and TNF (Th1 cytokines) by promoting T cell survival and proliferation (11). The roles of Th2 cytokines such as IL-4 and IL-10 are less understood and more controversial but correlated with diminished protection against TB and poor prognosis (12–18). IL-4 polarizes naive T cells toward Th2 responses (12) possibly causing decreased IFN-γ and macrophage activation (13), and abundant IL-4+CD4+ bronchoalveolar lavage (BAL) T cells are present in cavitary TB (14). The relationship between IL-10 and TB is also unclear, but IL-10-expressing T cells are associated with anergy (15) and delayed responses to drug therapy (16). Moreover, IL-10 may downregulate monocyte and macrophage costimulatory molecules, thereby impairing CD4+ and CD8+ T cell-mediated cytotoxicity in TB patients (19). The importance of anti-inflammatory factors, such as IL-10 and regulatory T cells (Tregs), in the granuloma as a means of limiting pathology also must be considered. We previously demonstrated that Tregs rapidly migrated from blood to lungs after M. tuberculosis infection and that higher levels of peripheral Tregs prior to infection correlated with better outcome of infection (20). However, other groups have...
demonstrated in mice that substantial reduction in Treg CD4+ T cells results in higher bacterial burden (21). The available data support that the balance of proinflammatory and anti-inflammatory factors in the granuloma may play a major role in control of infection.

The push to develop T cell-based vaccines has led to questions on what defines a protective T cell response, how T cell responses change after infection, and how to drive responses in directions favoring protection. Multifunctional T cells, which express combinations of multiple cytokines (22–25), are of particular interest in vaccine development. Multifunctional T cells expressing Th1 cytokines have been described as immune correlates for protection against intracellular pathogens including HIV (26), SIV (27), and leishmania (11). In contrast to these responses, however, multifunctional CD4+ T cell responses in humans with TB have been correlated with active disease or higher bacterial burden rather than protection (28–30). The divergence in multifunctional responses between HIV and M. tuberculosis and the interest in development of T cell-based vaccines necessitates a better understanding of T cell responses to coinfection, yet the number of studies examining multifunctional CD4+ and CD8+ T cell responses in HIV–M. tuberculosis coinfection remain limited (29, 31, 32). Day et al. (32) found that the majority of CD4+ T cells in the blood of HIV-positive individuals with latent TB are multifunctional, that the frequency of IL-2–expressing cells inversely correlated with viral load, and that these cells are among the first populations depleted by HIV. Moreover, responses in CD4+ BAL T cells from HIV-positive individuals are less multifunctional than T cells from HIV-negative individuals with TB (33). Although controversial, multifunctional T cells expressing both IFN-γ and Th2 cytokines, referred to as the Th0 phenotype, are associated with active TB (34, 35). Initiation of anti-tuberculous drug treatment has been associated with decreased frequencies of Th0-polarized T cells and shifts toward protective Th1-polarized T cells, suggesting a link between Th2 cytokine expression and active disease (35–38).

Despite the consequences of HIV–M. tuberculosis coinfection, much of the basic biology underlying interactions between these pathogens and the host remain poorly understood. Studying human coinfections is inherently difficult and complicated by numerous uncontrollable variables. Non-human primates offer alternative systems for studying human coinfections under experimental conditions. We recently used cynomolgus macaques, which accurately represent latent and active TB (39), with tissue mechanically homogenized into single-cell suspensions for immunologic analysis with Medimachines (BD Biosciences, San Jose, CA). Intracellular cytokine staining and flow cytometry

Materials and Methods

Experimental animals

Eighteen M. tuberculosis-, SIV-, simian HIV-, or simian retrovirus D-free adult (>4 y of age) cynomolgus macaques (Macaca fascicularis) were used for these studies (Covance, Alice, TX; USA Valley Biosystems, West Sacramento, CA). The experimental design used in these studies is presented in Supplemental Fig. 1. The SIV-only macaques (11507, 11807, 1808, 1907, 2007, and control SIV–M. tuberculosis) macaques (1207, 1407, 1807, 1907, 2407, 3007, 10405) are the same as those previously reported in Diedrich et al. (40). SIV-infected animals were housed under BSL-2 conditions, and M. tuberculosis-infected animals were housed under BSL-3 conditions. BAL, gastric aspirate, and other procedures were performed as previously described (16, 41). Blood from monkeys 3708, 3809, 4908, 5008, 5508, and 5608, and 19408 were used as SIV-negative controls for active TB. Similarly, granulomas from monkeys 3809 and 19408 were controls for SIV-negative monkeys with active TB. These studies followed all animal experimentation guidelines, and all experimental manipulations and protocols were approved by the University of Pittsburgh’s Institutional Animal Care and Use Committee.

M. tuberculosis and SIV infection

Cynomolgus macaques were infected with ~25 CFU Erdman strain M. tuberculosis via intrabronchial instillation as previously described (41). Monkeys 3708, 4908, 5008, 5508, and 5608, referred to as high-dose (hd) M. tuberculosis monkeys, and similarly infected SIV Erdman strain M. tuberculosis and developed active TB. Infection was confirmed by conversion of negative to positive tuberculin skin test and PBMC responses elevated from baseline in lymphocyte proliferation and ELISPOT assays (39). Animals were classified as latent or active 8–10 mo postinfection, and seven animals with latent infections were selected for SIV infection (36). Latent monkeys are defined as TST positive without signs of clinical disease (23, 41). All animals were infected with SIV mac251 (provided by Dr. Keith Reimann, Beth Israel Deaconess Medical Center, Harvard University). Concentrated SIV stock was diluted in RPMI 1640 medium, and 106 to 107 tissue culture-infective dose (TCID50) units were injected intravenously. Two groups of animals were infected: four M. tuberculosis-uninfected macaques (SIV-only control group) and seven monkeys with latent TB coinfection groups. The criteria for determining reactivated TB included weight loss, increased ESR, M. tuberculosis-positive gastric aspirates or BAL cultures, and/or abnormal chest radiograph and was confirmed at necropsy as higher bacterial burdens and gross pathology scores (40).

PBMC isolation and necropsy procedures

Blood was drawn from hd M. tuberculosis monkeys at the time of infection and subsequently 9 wk post-M. tuberculosis infection, at which point these animals were transferred to other studies. Blood was sampled from SIV-only and coinfected animals every week for the first 8 wk postinfection. Thereafter, sampling from M. tuberculosis–SIV coinfected animals was done every other week and from SIV-only animals monthly. PBMCs were isolated via Percoll gradient centrifugation as previously described (42). Quantitative RT-PCR was performed on plasma as described (40), and results were previously reported by Diedrich et al. (40). Animals were humanely euthanized and necropsies performed as previously described (41) when reactivated TB occurred. Blood and tissues were processed as described (39), with tissue mechanically homogenized into single-cell suspensions for immunologic analysis with Medimachines (BD Biosciences, San Jose, CA).

Intracellular cytokine staining and flow cytometry

Freshly isolated PBMCs were resuspended in RPMI 1640 media supplemented with 10% human AB serum (Gemini Bio-Products, West Sacramento, CA), 1% L-glutamine (Sigma-Aldrich, St. Louis, MO), and 1% HEPES (Sigma) at a density of 3 × 10^6 to 3 × 10^7 cells/ml and transferred to FACS tubes for stimulation with mycobacterial peptides with 11 amino acid overlaps between sequential peptides. Phorbol 12,13-dibutyrate and ionomycin (50 nM and 10 μM final concentration, respectively; Sigma) were used as positive controls. Sham-stimulated (media-only) cells were
used as negative (unstimulated) controls. Cells were stimulated for 1 h at 37°C in 5% CO₂ prior to addition of brefeldin A (GolgiPlug; BD Biosciences) and incubated for another 4.5–5 h at 37°C in 5% CO₂ prior to being stained. Surface CD107 was used as an indicator of degranulation and cytotoxicity (43). Tissue cells and PBMCs were stained with Abs against CD107a (clone eBioH4A; eBioscience, San Diego, CA) that were conjugated with QD565 (Invitrogen, Carlsbad, CA), or a similarly conjugated isotype Ab (eBioscience), in the presence of monensin (BD Bioscience) and brefeldin A while being stimulated 4.5–5 h at 37°C in 5% CO₂. PBMCs and tissue cells were stained for viability with Blue Viability Dye (Invitrogen) and then stained for CD3 (clone SP34-2; BD Biosciences), CD4 (clone L200; BD Biosciences), CD8 (clone DK25 [Dako, Carpinteria, CA] or clone ORT8 [eBioscience]) and CD20 (clone 2H7; eBioscience). Cells were fixed and permeabilized with Fix-Perm reagents (BD) and stained intracellularly for IFN-γ (clone B27; BD Bioscience), IL-2 (clone MQ1-17H12; eBioscience), and TNF (clone mab11; eBioscience). Cells were stained for CD3, CD4, and CD8 by staining with PE-CD3 (clone SP34-2; BD Biosciences), CD4 (clone 4D10; eBioscience) and CD8 (clone 53-6.7; BD Biosciences) and then stained for CD3 (clone SP34-2; BD Biosciences), CD4 (clone DK25 [Dako, Carpinteria, CA] or clone ORT8 [eBioscience]) and CD20 (clone 2H7; eBioscience). Cell phenotypes were read with an LSR II flow cytometer (BD Biosciences) with the objective of acquiring 30,000 viable CD3⁺ events per tube. Only data from freshly isolated cells are presented because of inconsistencies in cytokine production by cryopreserved cells.

Data analysis
Flow cytometry data were analyzed with the FlowJo software package (Tree Star, Ashland, OR). Cytokine-positive gates were based on unstimulated (media-only) controls set so the frequency of events in the cytokine-positive gate was generally <0.05%. Pie charts depicting multifunctional responses were plotted with SPICE (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Data were analyzed using Prism (GraphPad Software, San Diego, CA). Statistical comparisons were done using nonparametric statistics to account for data that were not normally distributed. Pairwise comparisons were performed using Mann–Whitney U rank sum test and the Kruskal–Wallis one-way ANOVA where multiple groups were to be compared with \( p < 0.05 \) considered statistically significant.

Results

M. tuberculosis and SIV infection

Seven cynomolgus macaques with latent

\( M. \) tuberculosis infection were infected with SIVmac251 as described by Diedrich et al. (40). All animals experienced peak viremia with ~10⁶ virus copies/ml plasma between 3 and 5 wk postinfection, a time frame coinciding with the most severe depletion of peripheral CD4⁺ T cells (40). After the acute phase of virus infection (1–8 wk post-SIV infection, a period consistent with 1*³⁰ to 1*³⁵ virus copies/ml plasma), viral loads decreased substantially, and T cell populations rebounded to numbers within normal ranges for these monkeys (pre-SIV infection range for coinfectected animals on the date of SIV infection: 208–1170 CD4⁺ T cells/ml, 367–1357 CD8⁺ T cells/ml). Indications of reactivated TB were observed in three animals (1207, 1407, 2407) 4–6 wk post-SIV infection, and necropsies were performed on these monkeys 12–17 wk post-SIV infection (40). Four other monkeys (1807, 1907, 3007, 10405) showed indications of reactivation 16–47 wk post-SIV infection and were euthanized 26–48 wk post-SIV infection (40). Based on this, the monkeys are separated into two groups according to their time to necropsy, with animals undergoing necropsy <17 wk post-SIV infection considered early reactivators and animals undergoing necropsy >26 wk post-SIV infection considered late reactivators. The monkeys displayed a spectrum of disease ranging from a few lung granulomas in late reactivators to widely disseminated pathology in early reactivators (40).

M. tuberculosis-specific Th1 cytokine responses are perturbed during acute SIV infection

Th1 (IFN-γ, IL-2, TNF) and Th2 (IL-4, IL-10) responses in CD4⁺ and CD8⁺ T cells were measured to determine how SIV and \( M. \) tuberculosis–SIV coinfection modified Ag-specific responses (Supplemental Fig. 2). IL-10 is a cytokine with pleomorphic effects, and for the purposes of this study IL-10 was considered a Th2 cytokine because it has the capacity to downregulate Th1 responses (44). \( M. \) tuberculosis-specific T cell responses varied widely among animals and within the same animal. The hd \( M. \) tuberculosis animals were used to monitor responses to acute \( M. \) tuberculosis infection in \( M. \) tuberculosis- and SIV-naive animals and showed small numbers of IFN-γ- and IL-2–producing T cells in the periphery after CFP stimulation (Figs. 1, 2). SIV-only animals demonstrated SIV-specific responses but negligible \( M. \) tuberculosis-specific responses (Figs. 1, 2). Animals with latent infection, which were subsequently infected with SIV, showed low \( M. \) tuberculosis-specific responses that were similar to responses observed in the hd \( M. \) tuberculosis monkeys. SIV infection of these animals led to substantially increased \( M. \) tuberculosis-specific responses. These animals experienced significant increases in the frequency of \( M. \) tuberculosis-specific IFN-γ- and IL-2–expressing T cells 2–5 wk post-SIV infection that were above pre-SIV expression levels (Figs. 1, 2). After the initial spike in Ag-specific T cells, the frequencies of IFN-γ- and IL-2–expressing T cells decreased to low levels. The initial spike in responses was not observed in the hd \( M. \) tuberculosis animals, suggesting the large peak in peripheral responses was unique to monkeys with previously primed anti-mycobacterial responses. The frequency of TNF⁺ T cells in the coinfected period was variable and similar to the pre-SIV infection period of latent disease (Figs. 1, 2).

Significant numbers of IL-4–expressing T cells are present for a short period postinfection

Th2 cytokines have been linked to diminished resistance to mycobacterial infection (12–18, 45) so we assessed the effect of SIV infection on IL-4 and IL-10 expression in the macaques. IL-10⁺ T cells were present at low frequencies on a constitutive basis over the course of infection, but their frequency did not increase above baseline levels in response to stimulation with CFP or Gag/Pol Ags (data not shown). However, the macaques experienced a transient pulse of IL-4–expressing T cells that was significantly above baseline IL-4 expression (\( p < 0.05 \); Kruskal–Wallis test) after infection with \( M. \) tuberculosis or SIV (SIV-only or coinfected monkeys) (Fig. 3). IL-4 expression was largely restricted to CD3⁺ T cells with only a small fraction of CD20⁺ B cells expressing IL-4 (Fig. 3A). This pulse of IL-4 expression included more CD8⁺ T cells than CD4⁺ T cells (Fig. 3B) and was not repeated over the course of study in either SIV-only or coinfected macaques (Fig. 3B, 3C). The quantity of IL-4 on a per cell basis, as measured by mean fluorescence units, was similar between CD4⁺ and CD8⁺ T cells (Fig. 3D). The cause of this sudden and short-lived burst of IL-4⁺ cells remains to be determined, but it temporally correlated with a period of reduced IFN-γ and IL-2 expression (Figs. 1, 2) in coinfected animals, suggesting that it downregulated proinflammatory cytokine expression.

Multifunctional Th1 cytokine responses increase with acute SIV infection

Multifunctional T cells are postulated to be immune correlates of protection (11). Given this, we measured the multifunctionality of mycobacteria-specific T cell responses to determine whether higher frequencies of multifunctional T cells correlated with protection against reactivated TB. Most cytokine-positive cells were positive for only a single cytokine with a period of abundant multifunctional CD4⁺ T cells temporally consistent with peak plasma viremia (Fig. 4). There was a drop in single-positive cells concurrent with increased frequencies of multifunctional cells, suggesting these multifunctional cells differentiated from single-
function cells as proposed by Seder et al. (46). Significant differences in two- and three-function CD8\(^+\) T cell frequencies were not observed. Early reactivating monkeys had significantly more IFN-\(\gamma\)+IL-2+TNF+CD4\(^+\) T cells over weeks 3–5, when aggregated together, than late reactivating monkeys (\(p = 0.021\), Mann–Whitney U) over the same time period (Fig. 4). The early reactivating animals had greater bacterial burdens and gross pathology at necropsy (40), and the abundance of Ag-specific IFN-\(\gamma\)+IL-2+TNF+CD4\(^+\) T cells during acute SIV infection suggests multifunctional T cell proliferation may relate to increased levels of mycobacterial Ags. Separating the multifunctional analysis into discrete functional units (e.g., IFN-\(\gamma\)+IL-2+ compared with IFN-\(\gamma\)+TNF+) demonstrated the remarkably diverse response elicited by CFP stimulation (Fig. 5). CD4\(^+\) T cell responses during weeks 1–3 post-SIV infection included increased frequencies of IFN-\(\gamma\)+CD4\(^+\) T cells (Fig. 5A, orange pie slices) relative to the pre-SIV latent period. CD8\(^+\) T cell responses appeared more diverse than CD4\(^+\) T cell responses, especially regarding the IFN-\(\gamma\)+TNF+CD8\(^+\) T cells (Fig. 5B, green pie slices) in late reactivating monkeys. Notably, weeks 6–8 were characterized by an unusual lack of CD8\(^+\) T cell multifunctionality where all monkeys demonstrated an abundance of TNF-only responses (Fig. 5B, royal blue slices, red box). This trend was less evident in CFP-stimulated CD4\(^+\) T cells (Fig. 5A).

**CD8\(^+\) T cell polarization during acute SIV infection correlates with reactivation**

There is some evidence that individuals with less severe TB have more robust peripheral Th1-cytokine responses than those of individuals with more severe disease who have T cells biased toward Th2-cytokine production (19, 47). Similarly, untreated TB may be accompanied by mixed proinflammatory and anti-inflammatory responses mediated by Th0-polarized T cells that shift toward proinflammatory Th1 cytokine production after successful drug therapy (35–37). To investigate the contributions of Th0- and Th2-polarized cells to SIV-induced reactivated TB, we evaluated the phenotypes of cells stained for IFN-\(\gamma\), IL-4, and IL-10 to compare frequencies of Th0- (IFN-\(\gamma\)+IL-4+IL-10+), Th1- (IFN-\(\gamma\)+), and Th2- (IL-4+IL-10+, IL-4+, IL-10+) polarized T cells in early and late reactivating monkeys. CD4\(^+\) T cell polarization was not significantly different between early and late reactivating monkeys (Fig. 6A). In contrast to CD4\(^+\) T cell responses, we found late reactivating monkeys had fewer Th2-polarized CD8\(^+\) T cells and more Th0- and Th1-polarized

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**FIGURE 1.** Pathogen-specific CD4\(^+\) T cell responses increase during the acute phase of infection. Data represent the mean frequency of cytokine-positive CD4\(^+\) T cells ± SEM; only upper error bars have been shown for clarity. Early reactivators are animals reactivating <17 wk post-SIV infection, and late reactivators are animals reactivating >26 wk post-SIV infection. Pre-SIV infection data from monkey 10405 were not available. hdMtb, high-dose *M. tuberculosis*.
T cells (Fig. 6B). When the responses from the weeks 1–10 post-SIV infection were aggregated and compared, the early reactivating animals had more Th2-polarized \((p = 0.023, \text{Mann–Whitney } U)\), fewer Th0-polarized \((p = 0.043, \text{Mann–Whitney } U)\), and fewer Th1-polarized \((p < 0.001, \text{Mann–Whitney } U)\) CD8\(^+\) T cells than late reactivating animals (Fig. 6C). In contrast, polarization states of CD4\(^+\) T cells from early and late reactivating monkeys were not significantly different (data not shown). These data suggest a predominance of purely Th2-polarized CD8\(^+\) T cells coupled with a lack of Th1 cytokine production had detrimental effects on the maintenance of latency.

**Mycobacteria-specific responses in the granuloma differ from those of the PBMCs**

Granuloma-containing tissues from *M. tuberculosis*-only and coinfected monkeys were homogenized into single-cell suspensions and the T cell responses assessed by flow cytometry. Each granuloma is unique, in terms of bacterial numbers and even cellular composition (39), and we found the T cell cytokine responses among granulomas were highly variable and different from those of the peripheral blood. Granuloma T cells tended to have high frequencies of *M. tuberculosis*-specific Th1-polarized T cells (Fig. 7A). IL-4– and IL-10–expressing cells were observed (Fig. 7A), but similar to responses in the peripheral blood, expression of these cytokines was not upregulated by CFP stimulation. CD107\(^+\) cells were observed and generally more abundant than the cytokine-positive cells (Fig. 7A). In a multifunctional analysis examining the frequency of Th0- (IFN-\(\gamma\)+IL-4+IL-10\(^+\)), Th1- (IFN-\(\gamma\)+), and Th2- (IL-4+ IL-10\(^+\), or IL-4+IL-10\(^+\)) polarized T cells, the majority of granuloma T cell responses were found to be Th0- and Th2-polarized (Fig. 7B). Separating the cytokine-positive cells into distinct multifunctional populations demonstrated the heterogeneity in responses between monkeys and between PBMC and tissue cells within a monkey (Supplemental Table I). Th1 cytokines were upregulated after stimulation, and expression of these cytokines was compared against upregulated responses from the peripheral blood. Single positive IL-2–producing T cells were the dominant phenotype,
with single positive cells expressing TNF being the other major cytokine-secreting phenotype (Fig. 7C). Inclusion of surface CD107 expression, an indicator of T cell cytolytic activity (43), indicated significant numbers of T cells in the granuloma were positive for only CD107 (Fig. 7D, brown pie slides), with cytokine-producing cells making up smaller fractions of the total response (Fig. 7D). These data suggest that T cell responses in the granuloma are balanced between Th1 and Th2 responses and

FIGURE 3. Transient IL-4 expression occurs in macaques after infection. All animals experienced a pulse of IL-4+ T cells after SIV or *M. tuberculosis* infection. FACS plots showing IL-4 expression are from monkey 3007, a coinfected animal. A, IL-4 expression is largely restricted to CD3+ T cells. B and C, Peak periods of IL-4 expression included a significant proportion of CD4+ and CD8+ T cells and were not repeated. Greater frequencies of CD8+ T cells expressed IL-4 compared with frequencies of CD4+ T cells that expressed IL-4. Pre-SIV infection IL-4 data for coinfected animals were not available. Data represent the mean frequency of IL-4+ T cells ± SEM; only upper error bars are shown for clarity. Asterisks indicate statistically significant increases at week 6 in coinfected animals and at weeks 2–6 in SIV only animals compared with postpeak IL-4 time points (*p* < 0.05, Kruskal–Wallis one-way ANOVA) and at weeks 8–9 in *M. tuberculosis*-only animals compared with other time points (*p* < 0.001, Kruskal–Wallis one-way ANOVA). D, IL-4 expression on a per-cell basis was not significantly different between CD4+ or CD8+ T cell populations. Data are the mean values from each monkey of the unstimulated and CFP-stimulated (coinfected animals) or SIV Gag/Pol-stimulated (SIV only group) T cells. IL-4 expression in unstimulated and stimulated T cells was not significantly different. Data were acquired on different dates with different FACS compensation settings, thus were not directly comparable. Mtb, *M. tuberculosis.*
substantially different from those of peripheral blood T cells, particularly with respect to the abundance of cytolytic T cells.

Discussion

Imune events associated with HIV coinfection of individuals with latent TB are challenging to investigate through clinical studies, and the lack of a biologically relevant coinfection model has limited the research that can be performed. We recently developed and characterized an animal model of HIV-induced reactivation of latent TB using cynomolgus macaques to address this need. Although there was an initial peak in viral loads, the cynomolgus macaques did not recapitulate high plasma virus set-point observed in human HIV infections (40, 48), but even at low peripheral viral loads, these animals were sufficiently immuno-suppressed by SIV that they all developed reactivated TB (40). Previously, we identified significant correlations between CD4+ T cell numbers and time to reactivation (40) but did not focus on the changes in cytokine expression (outside of ELISPOT-assessed IFN-γ production) after SIV infection that may have influenced reactivation. Considering this, we sought to address this question by measuring CD4+ and CD8+ T cell cytokine responses from latent TB through the early events after coinfection with SIV and development of reactivated TB.

Our data demonstrate that SIV infection causes remarkable immunologic flux in cynomolgus macaques with latent TB. Increased cytokine responses reminiscent of amplified cytokine responses observed during acute HIV infection (49) included significantly elevated frequencies of M. tuberculosis-specific IFN-γ, IL-2, and TNF-expressing T cells. We have postulated that SIV depletes T cells coordinating anti-mycobacterial responses in stable granulomas during acute infection, releasing immune pressures that normally limit bacterial replication, and leading to an increased abundance of mycobacterial Ags (40). The data presented in this study suggest that the increased Ag load stimulates proliferation of M. tuberculosis-specific T cells, which are detected as Th1 cytokine-positive cells in the peripheral blood. Thus, the abundance of M. tuberculosis-specific multifunctional T cells during acute SIV infection may represent higher Ag loads, and we note that the earliest reactivating animals had both the highest frequencies of IFN-γIL-2TNFCD4+ T cells during acute infection and the highest bacterial burden at necropsy (40). Moreover, later reactivating animals showed increasing frequencies of three-function CD4+ T cells toward the end of the study period. In humans, multifunctional IFN-γIL-2TNFCD4+ T cells have been associated with active TB (29), and it is noted that frequencies of IFN-γIL-2TNFCD4+ T cell frequencies decrease after antituberculous drug therapy (28). Considering these data, it appears that multifunctional T cells in TB are peripheral indicators of higher Ag or bacterial loads rather than of protection. Whether the presence of multifunctional cells present prior to infection, for example from a vaccination protocol, could limit mycobacterial infection, as suggested by Darrah et al. (11), remains to be seen. The fate of the large number of cytokine-producing cells, as shown by their disappearance after acute infection, is unknown. It has been demonstrated that HIV selectively depletes M. tuberculosis-specific T cells prior to the loss of T cells specific for opportunistic pathogens including Candida albicans and CMV (50), and these cells may be eliminated by virus infection. Alternatively, T cell exhaustion is prominent in HIV infection (51), and the decreased frequencies of cytokine-producing cells may indicate exhaustion. MHC tetramers are not available for cynomolgus macaques, and staining for PD-1, a cell-surface receptor associated with exhaustion in HIV patients (52), has been inconclusive (data not shown), thus the processes leading to diminished Th1 cytokine expression remain unclear. Finally, it is possible that these cells migrate from the periphery to the site of infection and therefore are no longer available for serial sampling.

Peripheral blood T cell responses can be sampled with relative ease, but understanding T cell function in the granuloma is necessary to determine how infection is controlled at the local level. Moreover, data on factors constituting appropriate and protective granuloma T cell responses will be important for designing cell-based vaccines against M. tuberculosis. Although protective correlates in the granuloma are largely unknown, the greatest pro-

![FIGURE 4. Frequency of cytokine-positive T cells positive for one, two, or three cytokines in coinfected macaques correlated with plasma viral loads. Data from cytokine-positive CFP-stimulated T cells were Boolean gated and grouped into three categories: IFN-γIL-2TNF+ 3-function cells (open squares), 2-function cells (filled circles) expressing two cytokines, and 1-function cells expressing only IFN-γ, IL-2, or TNF (open triangles). Plasma viral loads (open circles) were previously reported by Diedrich et al. (40). Data represent the mean frequency of cytokine-positive cells for each functional group with error bars omitted for clarity. Asterisks indicates a statistically significant difference (p = 0.021, Mann–Whitney U) in the mean frequency of 3-function CD4+ T cells between early (n = 3) and late (n = 4) reactivating animals for the pooled data from weeks 3, 4, 5 post-SIV infection (shaded box).]
Detection will likely be achieved by balancing responses that activate macrophages (IFN-γ and TNF), stimulate proliferation (IL-2), reduce inflammation, and induce apoptosis in M. tuberculosis-infected cells. Our data indicate that M. tuberculosis-specific T cells in the granuloma have lytic functions or produce IL-2 and are less likely than peripheral blood T cells to express IFN-γ or TNF. We reported a similar distinction in the CD8+ T cell subset in mice infected with M. tuberculosis, where CD8+ T cells were more likely to produce IL-2 and less likely to produce IFN-γ.

**FIGURE 5.** CFP stimulation induces multifunctional phenotypes in CD4+ and CD8+ T cells from coinfected macaques. Boolean gated data are presented as pie charts with each slice representing a percentage of the total number of cytokine-positive cells. Time points where data were not available are represented by open circles, and points where responses in CFP-stimulated tubes were less than or equal to the unstimulated tube are indicated by a horizontal dash. The dotted blue box indicates T cell responses on the day of SIV infection. The solid red line indicates a 3 wk period rich in CD8+ T cells only producing TNF. Monkeys 1207, 1407, and 2407 are early reactivating animals, whereas 1807, 1907, 3007, and 10405 are late reactivating animals. The time pre- and post-SIV infection is indicated in weeks; monkeys 1207, 1407, and 1807 were sampled at −21, −14, −6, and −2 wk pre-SIV infection, and monkeys 1907, 2407, and 3007 were sampled at −20, −15, −8, and −4 wk pre-SIV infection. N, T cell responses at the time of necropsy. A, CD4+ T cell responses. B, CD8+ T cell responses.

**FIGURE 6.** Early and late reactivating animals show differences in frequencies of Th0-, Th1-, and Th2-polarized CD8+ T cells. Responses of CFP-stimulated cells were combined to reflect populations of Th1-polarized (IFN-γ), Th2-polarized (IL-4+IL-10−, IL-4−, or IL-10−), or Th0-polarized (IFN-γ−IL-4+IL-10+, IFN-γ−IL-4−, or IFN-γ−IL-4+IL-10−) T cells. A, CD4+ T cell responses. B, CD8+ T cell responses. Data represent the mean frequency of cytokine-expressing cells ± SEM; lower error bars have been omitted for clarity. C, CD8+ T cell phenotypes were determined as previously described and the response for weeks 1–10 obtained by pooling the responses for early and late reactivating monkeys. Pre-SIV phenotypes were not significantly different between groups of animals (data not shown). Early reactivating animals had significantly fewer Th0- and Th1-polarized CD8+ T cells (p = 0.004 and p = 0.018, respectively; Mann–Whitney U) and more Th2-polarized CD8+ T cells (p < 0.001; Mann–Whitney U).
either cytotoxic or IFN-γ producing, but rarely both (53). This suggests that the T cell response in the granuloma during active TB is predominately focused on lysis of infected cells, with a minority of T cells having roles activating macrophages or stimulating T cell proliferation.

Excessive inflammation may negatively affect protection by damaging lung tissue peripheral to the granuloma; consequently, balancing proinflammatory Th1 cytokine expression with appropriate expression of Th2 cytokines or anti-inflammatory factors may also be protective. In concordance with this, we found granulomas from SIV-negative and coinfected animals contained both Th1- and Th2-polarized cells as well as Th0-polarized T cells. Peripheral blood CD8+ T cells in early reactivating monkeys had responses skewed toward IL-4 and IL-10 expression, whereas late reactivating monkeys had lower frequencies of Th2-polarized CD8+ T cells and higher frequencies of IFN-γ-expressing CD8+ T cells during acute SIV infection, indicating a balance between Th1 and Th2 responses may be critical in maintenance of latency. Moreover, appropriately timed expression of Th2 cytokines may also be important and function to dampen tissue-damaging inflammation after acute responses to pathogens. The brief pulse of IL-4–expressing T cells in M. tuberculosis-only, SIV-only, and M. tuberculosis–SIV coinfected macaques may reflect a systemic effort to downregulate inflammatory processes, but as of yet, mechanisms regulating this pulse of IL-4 and its function remain unclear. We found that the Th1 and Th2 cytokine profiles of...
granuloma T cells from SIV-negative and coinfected animals were highly variable, even between granulomas from the same animal. In all cases, these responses were measured during active disease and it is possible that after reactivation has occurred, granuloma T cell responses from SIV-infected monkeys may be similar to responses from SIV-negative monkeys with active TB. Consequently, it may be difficult retrospectively to link reactivated TB to cytokine expression of a single cell subset after reactivation has already occurred. Instead, latent granulomas, or granulomas in the early stages of SIV coinfection, should be studied in the future to address these questions.

Our results indicate that immunologic events during the acute phase of HIV infection are critically important in determining susceptibility to early reactivation of latent TB. These data indicate that the immune dysregulation occurring after HIV infection, even in the absence of severe CD4+ T cell depletion and high viral loads, correlates with reactivated TB. Based on our data from these SIV-infected cynomolgus macaques, we conjecture that HIV-induced distortions in T cell populations mediating proinflammatory and anti-inflammatory cytokine responses at the granuloma level may distort the equilibrium between bacterial growth and immune pressure in favor of reactivation. Furthermore, our data highlight that alterations in the relative abundance of activated CD4+ T helper type 1 and 2 (Th1 and Th2; CD4+ IFN-γ+ versus CD4+ IL-4+) is associated with reactivation of TB. We expect that studies using models of HIV–M. tuberculosis coinfection will enable a better understanding of the basic biology of host–pathogen interaction and allow development of novel therapies and vaccines fostering protective responses that diminish the likelihood of reactivated TB in immunocompromised individuals.

Acknowledgments
We thank Dr. Edwin Klein and Dr. Chris Janssen for performing necropsies, Mark Rodgers, Catherine Cochran, and Kelly Wyatt for superb technical assistance, and Jennifer Kerr, Melanie O’Malley, Jamie Tomko, Dan Fillmore, and Paul Johnston for outstanding animal care and procedures.

Disclosures
The authors have no financial conflicts of interest.

References
5. Djoba Siawaya, J. F., M. Ruhwald, J. Eugen-Olsen, and G. Walzl. 2007. Correlation of cytokine expression of a single cell subset after reactivation has already occurred. Instead, latent granulomas, or granulomas in the early stages of SIV coinfection, should be studied in the future to address these questions.

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The authors have no financial conflicts of interest.


**Supplemental Figure Legends**

**Supplemental Figure 1. Experimental design.** Three groups of animals were used: one group only infected with Mtb, one group only infected with SIV, and one group first infected with Mtb, allowed to become latent and then co-infected with SIV. Mtb-only animals were: 3708, 3809, 4908, 5008, 5508, 5608, and 19408. PBMCs from 3708, 4908, 5008, 5508, and 5608 (also referred to as hdMtb monkeys) were sampled once a week for the first 8 weeks of Mtb infection to examine responses during acute infection and then were transferred to other studies. PBMCs and tissues from Mtb-only animals 3809 and 19408, which were originally parts of other studies, were obtained at necropsy for use as Mtb-only controls. SIV-only animals, 11507, 11807, 19506, and 19706, were used to define the response of cynomolgus macaques to SIVmac251 infection. Co-infected animals were initially infected with Mtb, allowed to establish latent infections, and then monkeys 1207, 1407, 1807, 1907, 2407, 3007 and 10405 were co-infected with SIV. All animals experienced weekly blood draws during the first 8 weeks of infection (acute infection), and then either every other week for co-infected animals, or once a month for SIV-infected animals.

**Supplemental Figure 2. FACS gating strategy.** A. Data from stained cells were obtained by acquiring 20,000-30,000 CD3+ events per tube. Freshly isolated PBMCs were not run with a viability stain, but tubes containing cells from granulomas included a viability stain to exclude dead cells. The CD3+ population contained small numbers of CD3+CD20+ cells which were excluded from analysis. CD3+CD20- were cells subsequently separated into CD4+ or CD8+ populations. B. Gates defining cytokine-positive CD4+ and CD8+ T cell populations were based
on the unstimulated media-only tube for each monkey. Duplicate tubes of CFP and SIV Gag/Pol
stimulated cells were prepared, with the Th1 tube stained for IFNγ, IL-2, and TNF, and the Th0/2
tube stained for IFNγ, IL-4 and IL-10. IL-10 was included in the Th2 cytokine group because of
its capacity to downregulate Th1 responses. At necropsy, a tube of granuloma cells and PBMCs
were stained for CD107, IFNγ, IL-2 and TNF to determine the frequency of cytolytic (CD107+)
or cytokine-producing cells. Boolean gates were established based on quadrant containing
CD4+CD8-CD20-cytokine+ or CD4-CD8+CD20-cytokine+ cells. The FACS plots shown here
are from CFP-stimulated granuloma cells from monkey 19408.
Supplemental Table 1. Expression of IFNγ, IL-10 and IL-4 by CD4+ and CD8+ T cells from peripheral blood and granulomas.

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