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Long-Term Control of *Mycobacterium tuberculosis* Infection Is Mediated by Dynamic Immune Responses

Vanja Lazarevic, Dawn Nolt, and JoAnne L. Flynn

The primary goal of this study was to determine how chronic exposure to Ag influences the functionality of *Mycobacterium tuberculosis*-specific T cell responses. The frequency of IFN-γ-producing effector CD4⁺ and CD8⁺ T cells dynamically changed during persistent *M. tuberculosis* infection. CD8⁺ T cells used differential effector functions during acute and chronic phases of the immune response, where CD8⁺ T cells produced negligible amounts of IFN-γ early in infection, but switched to cytokine production during the chronic stage of infection. Using limiting dilution analysis, CD8⁺ T cells isolated during the initial phase of infection demonstrated lytic potential, but this waned in the chronic stage. The apparent loss of cytotoxic activity was not associated with the lack of perforin. Ag dose could potentially govern the functional program of CD8⁺ T cells. Collectively, these results depict a host immune response mounted against *M. tuberculosis* of a significantly more dynamic nature than previously recognized. *The Journal of Immunology*, 2005, 175: 1107–1117.

Over the past 20 years, work using gene-knockout mice has proven essential for understanding the contributions of various immune cell types and effector arms in the successful long-term control of *Mycobacterium tuberculosis* infection (reviewed in Ref. 1). IL-12 production by *M. tuberculosis*-infected dendritic cells is essential for the priming of potent Th1 responses characterized by IFN-γ and TNF production by CD4⁺ and CD8⁺ T cells (2, 3). Elaboration of proinflammatory cytokines by T cells leads to the induction of bacteriostatic and bactericidal mechanisms by infected macrophages mediated via reactive nitrogen intermediates and reactive oxygen species pathways (reviewed in Ref. 1). Coincident with the onset of *M. tuberculosis*-specific T cell mediated immunity, bacterial growth is suppressed and maintained under strict control, resulting in life-long containment and latency within infected individuals. However, in 10% of infected individuals, spontaneous reactivation occurs, usually as a consequence of waning immune function.

It has been particularly challenging to establish adequate animal models of human latent tuberculosis. Mice infected with *M. tuberculosis* typically do not display overt signs of disease (4), and reactivation occurs when experimental manipulations, such as CD4⁺ T cell depletion, NO synthase inhibition, or TNF neutralization, are implemented (5–8). Unlike humans, bacterial loads are maintained at relatively high levels (∼1 × 10⁶ CFU/lung), which can lead to progressive immunopathology. Therefore, the murine model of tuberculosis is more reflective of persistent *M. tuberculosis* infection, a feature that can be exploited to study the influence of chronic exposure to Ag on effector functions of *M. tuberculosis*-specific CD4⁺ and CD8⁺ T cells.

It has been shown in viral persistent infections caused by HIV, CMV, EBV, Friend virus, and clone 13 lymphocytic choriomeningitis virus infections that chronic exposure of CD8⁺ T cells to viral Ags leads to functional abnormalities, including impaired cytotoxicity and cytokine production, reduced proliferative capacity, and clonal deletion or exhaustion (9–17). Infection with a high dose of lymphocytic choriomeningitis virus clone 13 can also lead to aberrant CD4⁺ T cell responses, marked by reduced IFN-γ and IL-2 production (13, 14). From these findings a model has emerged in which chronic exposure to high viral load culminates in functionally weak CD4⁺ T cell responses and defective CD8⁺ T cell responses, leading to persistent viral infection (13, 14). Therefore, the role of Ag levels in shaping the functional competency and developmental skewing of T cell responses has become recognized as a key factor in chronic disease processes.

In this study we sought to determine the effect of prolonged exposure to bacterial infection on the quality of effector CD4⁺ and CD8⁺ T cell responses during a 5- to 7-mo period of *M. tuberculosis* infection. The results from our study reveal several important findings: 1) CD4⁺ and CD8⁺ T cells did not show signs of replicative senescence; 2) although CD4⁺ T cells were the primary source of IFN-γ during acute infection, CD8⁺ T cells equally contributed to IFN-γ production during chronic infection; 3) the frequency of IFN-γ-producing CD4⁺ and CD8⁺ T cells dynamically changed during protracted *M. tuberculosis* infection; and 4) there was differential regulation of CD8⁺ effector functions during different phases of infection. Collectively, our findings depict a dynamic host immune response during persistent *M. tuberculosis* infection characterized by quantitative and qualitative differences in the effector functions of CD4⁺ and CD8⁺ T cell responses that were previously unrecognized.

**Materials and Methods**

*Mice and infections*

C57BL/6 wild-type mice were purchased from Charles River Laboratories. All mice were kept under specific pathogen-free conditions in a biosafety level 3 facility, and animal protocols were approved by the university institutional animal care and use committee. Mice were infected with a low dose of *M. tuberculosis* (Erdman strain; Trudeau Institute) at 5 × 10⁷/ml using a nose-exposure only aerosolizer unit (Intox). The dose received was...
estimated by plating whole lung homogenates of three mice 24 h after aerosol infection. Twenty mice were used for each time point, with five mice per experimental group.

**Cytotoxicity determination**

Lung homogenates were serially diluted in PBS/0.05% Tween 80 and plated on 7H10 agar plates (Difco). Plates were incubated at 37°C in 5% CO2 for 21 days before counting colonies.

**Bone marrow-derived macrophages and dendritic cell cultures**

In ELISPOT and limiting dilution assays, bone marrow-derived dendritic cells were cultured in the presence of GM-CSF supernatant at 1/200 dilution (gift from Dr. B. Lu, University of Pittsburgh, Pittsburgh, PA) and 20 ng/ml IL-4 (Peprotech), and macrophages were cultured in the presence of L cell supernatant as a source of CSF-1 using standard procedure, described previously (18).

**Preparation of lung and lymph node cells**

For all experiments performed in this study, we treated lung samples with 1 mg/ml collagenase A and 25 U/ml DNase (Roche) at 37°C for 30 min. The lungs were crushed with a 5-ml syringe plunger through a 70-μm pore size cell strainer to obtain a single-cell suspension. RBC were lysed with NH4Cl/Tris solution for 2 min at room temperature. After one wash with 1× PBS, cells were suspended in T cell medium (DMEM, 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES, and 50 μM 2-ME (Sigma-Aldrich)) and counted using the trypan blue exclusion method. Comparisons with numerous other studies in our laboratory in which colagenase and DNase were not used revealed that this treatment does not necessarily release more cells and that the cells are identical in function regardless of the method of preparation (data not shown). Each mediastinal lymph node was dissociated separately, and cells were prepared as described above (no collagenase or DNase was used).

**Flow cytometry**

Lung single-cell suspensions were stained as described previously (19). Cells were stained with anti-CD4 (clone H129.19), anti-CD8 (clone 53-6.7), anti-CD69 (clone H1.2F3), anti-Ly6C (clone AL-21), anti-CD44 (clone IM7), and anti-CD62L (clone MEL-14) fluorescently conjugated Abs. All Abs were purchased from BD Pharmingen and used at a 0.2 μg/ml concentration. Cells were collected on a FACSCalibur (BD Biosciences) and analyzed by CellQuest (BD Biosciences) or FlowJo (TreeStar) software.

**Proliferation of T cells in the lungs of infected mice**

Sixteen hours before each experimental time point, mice were injected i.p. with saline containing 1 mg of BrdU (Sigma-Aldrich). Lung cells were stained for cell surface markers CD4 and CD8 at room temperature for 20 min before a fixation step with 4% formaldehyde (PFA, 200 μl/tube) on ice for 20 min. Cells were washed with tissue culture PBS at 470 × g, and cell pellets were suspended in ice-cold 0.15 M NaCl (100 μl/tube), followed immediately by dropwise fixation with ice-cold 95% ethanol (200 μl/tube) on ice for 30 min. After a PBS wash, cells were permeabilized and fixed with 200 μl/tube 0.4% saponin and 2% PFA for 1 h at room temperature. Cells were washed with PBS and incubated in 200 μl/tube 0.15 M NaCl and 4.2 mM MgCl2 (pH 5) containing 250 U/ml DNase I (Roche) for 30 min in 37°C water bath. Cells were washed with PBS and incubated with 5 μl/tube Annexin V FITC and 5 μl/tube 7-AAD for 15 min in the dark at room temperature. Cells were washed with 2 ml/tube 1× binding buffer to remove any unbound Annexin V FITC and 7-AAD and were fixed with 4% PFA in 1× binding buffer. Samples were analyzed within 30 min.

**IFN-γ production**

For intracellular cytokine staining, lung and lymph node cells were incubated in medium or stimulated with anti-CD3 (BD Pharmingen; clone 145-2C11; 0.1 μg/ml) and anti-CD28 (BD Pharmingen; clone 37.51; 1 μg/ml) Abs for 4 h in the presence of 3 μM monensin (Sigma-Aldrich). At the end of the stimulation period, cells were stained for CD4 and CD8, fixed, permeabilized, and stained for IFN-γ expression. For ELISPOT assay, lung and lymph node cells were stained in anti-IFN-γ Ab-coated (BD Pharmingen; clone R4-6A2) plates (MAIP54510; Millipore at 80,000 cells/well and 150,000 cell/well, respectively). Cells were incubated in duplicate wells with medium, Con A (10 μg/ml; Sigma-Aldrich), and uninfected and M. tuberculosis-infected dendritic cells (multiplicity of infection, 3; overnight) to estimate the number of total number of IFN-γ-producing T cells, and M. tuberculosis-infected dendritic cells were incubated with the blocking anti-MHC class I (BD Pharmingen; clone 8F12) or anti-MHC class II (BD Pharmingen; clone M5/114.15.2) Abs at 10 μg/ml to estimate the number of IFN-γ-producing CD4+ and CD8+ T cells, respectively. In addition, dendritic cells were pulsed with early secretory Ag target 6 protein (re- ceived from National Institutes of Health) at 10 μg/ml overnight. All dendritic cells were added to lung and lymph nodes cells at a 1:2 ratio, and the cultures were supplemented with IL-2 (Peprotech) at a final concentration of 20 U/ml. After 40-h incubation, the IFN-γ-producing T cells were visualized after stepwise incubation of plates with biotinylated anti-IFN-γ Ab (BD Pharmingen; clone XMG 1.2), streptavidin-conjugated enzyme (PK-6100; Vector Laboratories), and 3-amino-9-ethyl-carbazole substrate (SK-4200; Vector Laboratories). The spot-forming units were enumerated using an ELISPOT reader (Cellular Technology).

**Limiting dilution assay (LDA)**

Effector cells were derived from the lungs and lung draining lymph nodes of M. tuberculosis-infected mice at designated time points. Freshly isolated cells were plated in 2-fold serial dilutions from 40,000 to 1250 cells/well in V-bottom, 96-well plates (24 replicates/input number) supplemented with IL-2 at 20 U/ml. Lung and lymph node cells were incubated with M. tuberculosis-infected dendritic cells (500 cells/well) for 7 days. After incubation, 100 μl of old medium was removed from each well, and lung cells were cultured for another round of stimulation with M. tuberculosis-infected macrophages (1000 macrophages/well) and IL-2 (20 U/ml) to allow for expansion of CTL precursors. Flow cytometric analysis revealed that 75–95% of cells were CD8+ T cells after 2 wk of culture. Cytotoxicity was determined in each well by a standard 51Cr release assay with M. tuberculosis-infected macrophages as targets. Positive wells were defined as being greater than the mean ± 3 SD of spontaneous target cell release. The frequency of CTL precursors (CTLp) was determined using a zero-order Poisson equation (ln Y = −Fx + ln A, where x is the number of effector cells/well, Y is the percentage of negative wells, A is the y-axis intercept, and F is CTLp frequency, defined by the negative slope of the line. All calculations were performed using software program fitted to the equation by y2 minimization analysis (gift from Dr. C. A. Keever-Taylor, Medical College of Wisconsin, Milwaukee, WI).

**Intracellular perforin and IFN-γ staining**

To determine perforin and IFN-γ expression within CD8+ T cells, we adapted the technique for combined intracellular staining of perforin and IFN-γ as previously described (20). Freshly isolated lung and lymph node cells were incubated with the stimulating anti-CD3 (BD Pharmingen; clone 145-2C11; 0.1 μg/ml) and anti-CD28 (BD Pharmingen; clone 37.51; 1 μg/ml) Abs for 4 h in the presence of 3 μM monensin (Sigma-Aldrich). At the end of the stimulation period, cells were fixed with 2% PFA for 20 min on ice, 0.4% PFA washed, and permeabilized with 0.1% saponin/1% FBSC/0.1% PFA. Cells were incubated with a 1/200 dilution of anti-perforin Ab (clone KM 585 (P1-8); Kamiya Biomedical) in 0.3% saponin/5% normal goat serum/Ca2+-free PBS. For intracellular cytokine staining, lung and lymph node cells were stained with annexin V-FITC and 7-aminoactinomycin (7-AAD) reagents (BD Pharmingen) according to the manufacturer’s instructions. Brefeldia, cells were stained in 100 μl/tube 1× binding buffer

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3 Abbreviations used in this paper: PFA, paraformaldehyde; 7-AAD, 7-aminoactinomycin; CTLp, CTL precursor; LDA, limiting dilution assay; MFI, mean fluorescent intensity.
Immunohistochemistry

To determine M. tuberculosis antigenic load in the lung sections, we stained sections with a 1/1000 dilution of polyclonal rabbit anti-Mycobacterium bovis Ab (DakoCytomation), which is known to be cross-reactive with >100 Ags common to mycobacteria, including M. tuberculosis (DakoCytomation). In addition, lung sections were stained with rabbit IgG control (Accurate Chemical & Scientific) for 1 h at room temperature. After a series of washes, sections were then stained with biotinylated, goat anti-rabbit IgG as a secondary Ab (PK-6101; Vector Laboratories) for 1 h at room temperature. Ab binding was visualized using the ABC kit (Vector Laboratories) and 3-amino-9-ethyl-carbazole substrate (SK-4200; Vector Laboratories) according to the manufacturer’s instructions.

Statistics

Statistically significant differences in the numbers of effector T cells between the two time points were determined using unpaired, two-sided, Student’s t test. A value of p < 0.05 was considered significant. The degree of correlation was determined by Spearman’s rank correlation test.

Results

Generation of immune response follows bacterial replication kinetics

After a low dose aerosol infection (20–50 CFU/lung), M. tuberculosis replicated exponentially in the lungs of infected mice, peaking 3 wk after infection and then stabilizing at 4 wk after infection (Fig. 1A). Enhanced replication of mycobacteria early in infection was followed by increased infiltration of immune cells into the lung, which peaked and stabilized at 4 wk after infection (Fig. 1B). This control of M. tuberculosis replication coincided with the formation of organized structures called granulomas, consisting of T cells, B cells, macrophages, and dendritic cells (data not shown). Thus, with the induction of the immune response, growth of mycobacteria was hindered and maintained under control conditions during long-term persistent infection.

Unlike many well-studied viral or bacterial infections, there does not seem to be an immunodominant Ag recognized by murine CD8+ T cells in M. tuberculosis infection in C57BL/6 (H-2b) mice. Although there is a dominant M. tuberculosis Ag recognized by CD4+ T cells early in infection (21) (see below), MHC class II multimer reagents for this Ag are not readily available. These limitations preclude the identification of M. tuberculosis Ag-specific T cells by flow cytometry. In this section we analyzed the complete T cell population in the lungs during M. tuberculosis infection. Because the lung is not a lymphoid organ and normally contains very few lymphocytes, the majority of cells observed are present due to the infection.

The number of CD4+ and CD8+ T cells in the lungs increased 10-fold from 2 to 4 wk after infection (Fig. 1, C and D). The number of CD4+ T cells was 2-fold higher than the number of CD8+ T cells during the peak of the response, which was followed by a retraction phase in both T cell populations once bacterial numbers stabilized (Fig. 1, C and D). During chronic infection, the numbers of both bacteria and immune cells were maintained at a steady state level.

Turnover of CD4+ and CD8+ T cells in the lungs of M. tuberculosis-infected mice

To gain insight into the turnover of CD4+ and CD8+ T cells during acute and chronic infection, we measured the amounts of proliferation and apoptosis using BrdU incorporation and annexin V-7-AAD staining, respectively. BrdU was injected 16 h before each time point to obtain an accurate estimation of proliferating cells in infected lungs. Although we cannot exclude the possibility that these BrdU+ T cells migrated to the lungs within the 16-h labeling period, it is also possible that this was the shortest time required to incorporate BrdU into the DNA of slowly replicating T cells. Shorter labeling periods (<12 h) resulted in few BrdU+ cells in the lungs or lymph nodes. Lung cells were harvested at the indicated time points, and cells from the same mice were stained with anti-CD4, anti-CD8, and anti-BrdU Abs or with annexin V, 7-AAD, anti-CD4, and anti-CD8 Abs to quantify proliferating and apoptotic cells, respectively. Combined staining of cells with annexin V and one of the fluorescent intercalative DNA dyes, such as 7-AAD, propidium iodide, or YOPRO-1, is commonly used to differentiate between early and late apoptotic cells (22, 23). Fig. 2A depicts the percentages of BrdU−CD4+ and BrdU+ CD8+ T cells within the lymphocyte gate. Initially during the peak of the response, there was a 6-fold increase in the percentages of
BrdU+/CD4+ and BrdU+/CD8+ T cells. Once bacterial numbers stabilized, the percentage of proliferating cells decreased and remained low until the late stage of infection. Apoptosis occurring within CD4+ and CD8+ T cell populations followed similar kinetics as BrdU incorporation; there was an initial increase in the percentage of apoptotic cells during the peak of the response, followed by a stabilization phase with more apoptotic CD4+ than CD8+ T cells. The amount of apoptosis increased during the late stage of chronic infection (Fig. 2B).

The graphs in Fig. 2, C and D, directly compare the amounts of proliferation and apoptosis within CD4 and CD8 T cell gates. These experiments produced two important results. First, the 10-fold increase in the numbers of CD4+ and CD8+ T cells during the peak of response at 4 wk after infection (Fig. 1, C and D) was mainly due to infiltration of primed effector T cells into the inflamed lung, rather than proliferation of the effector cells, because only 5% of proliferating CD4+ and CD8+ T cells were detected at this time (Fig. 2, C and D). Second, the retraction phase was brought about by increased apoptosis and reduced proliferation of T cells as infection progressed into the chronic phase (Fig. 2, C and D).

CD4+ and CD8+ T cells display activated effector phenotype in the face of chronic M. tuberculosis infection

To determine the activation status of CD4+ and CD8+ T cells during acute and chronic infections, we isolated cells from the lungs of infected mice at the indicated time points and stained cells for the expression of CD4, CD8, Ly6C, CD62L, and CD69 (Fig. 3A). Ly6C is expressed on CD44high memory CD8+ T cells (24, 25), whereas CD69, an early activation marker, is detected on the surface of recently activated T cells (26, 27). CD62L is expressed on naive T cells, but is quickly down-regulated upon T cell activation (28). The vast majority of CD4+ and CD8+ T cells isolated from the lungs of infected mice displayed high levels of CD44 as early as 2 wk after infection (data not shown). In addition, most CD4+ and CD8+ T cells up-regulated and remained Ly6C<sup>high</sup> and down-regulated CD62L. 20–30% of T cells up-regulated CD69 over the course of infection, suggesting that T cells exhibited a persistently activated status during chronic M. tuberculosis infection (Fig. 3B).

Priming T cells specific for M. tuberculosis in lung-draining lymph nodes

The priming of M. tuberculosis-specific IFN-γ T cell responses in lymph nodes of infected mice was determined by ELISPOT. We
and others have shown previously that T cells recognizing *M. tuberculosis* Ags are first detected in the mediastinal lymph nodes and then in the lungs of infected mice, indicating that priming of T cell responses is occurring in the draining lymph nodes (3, 29). T cells isolated from mediastinal lymph nodes were stimulated with infected wild-type dendritic cells that were incubated with either blocking anti-MHC class I or anti-MHC class II Abs to estimate the frequency of IFN-γ-producing CD4+ and CD8+ T cells, respectively. In addition, wild-type dendritic cells were pulsed with ESAT-6 protein, an Ag exclusively recognized by CD4+ T cells in C57BL/6 (H-2b) mice (21), to estimate the frequency of ESAT-6-specific CD4+ T cells. The number of IFN-γ-producing T cell responses peaked in the lung draining lymph nodes at 2 wk after infection (Fig. 4A). Early in infection the priming of IFN-γ-producing CD4+ T cells was prominent, and strong ESAT-6 responses were detected early in infection (Fig. 4A). Surprisingly very few CD8+ T cells were primed to produce IFN-γ in acute infection (Fig. 4B). The most important finding of these experiments was that during the plateau stage when the number of bacteria and immune cells reached the steady state (Fig. 1), the frequency of IFN-γ-producing effector cells dynamically changed (Fig. 4A and B, study 1). Both CD4+ and CD8+ T cell IFN-γ responses waxed and waned over the course of infection, suggesting bursts in the immune response. Due to such unexpected findings, we decided to summarize the results from two to nine independent studies with 1- to 2-wk intervals between the time points (6 –28 mice/time point). The cumulative results from these studies demonstrated similar patterns of increases and decreases in the frequency of IFN-γ-producing CD4 and CD8 T cells (Fig. 4, A and B, studies 2–9), confirming the data from study 1.

Because IFN-γ production is a key player in protection against tuberculosis, we next asked how many IFN-γ-producing T cells inside the infected lung were specific for *M. tuberculosis*. To address this question we directly compared the number of IFN-γ-producing CD4+ and CD8+ T cells, as estimated by intracellular cytokine staining and ELISPOT (Fig. 4D). Nonspecific stimulation

**FIGURE 3.** CD4+ and CD8+ T cells exhibit an activated phenotype in the lungs during persistent *M. tuberculosis* infection. A, Single lung cell suspensions were stained with anti-CD4, anti-CD8, anti-Ly6C, anti-CD62L, and anti-CD69 Abs, and the relative expression of the indicated markers within CD4 and CD8 gates was estimated by flow cytometry. The solid line represents background staining with the isotype control Ab. B, The graphs illustrate the percentage of Ly6C+, CD62L+, and CD69+ cells within CD4 and CD8 gates. The cells exhibit an activated phenotype characterized by down-regulation of CD62L and up-regulation of CD69. All cells were CD44high within 2 wk after infection (data not shown) and remained Ly6C+ high during persistent *M. tuberculosis* infection.
of T cells with anti-CD3/CD28 Abs will trigger IFN-γ production by all T cells that were primed to secrete IFN-γ. However, incubation of T cells with *M. tuberculosis*-infected dendritic cells will stimulate IFN-γ secretion only by *M. tuberculosis*-specific T cells. A 40-h incubation of lung T cells with *M. tuberculosis*-infected dendritic cells was required for detection of IFN-γ production by
stimulated T cells, whereas a 4-h incubation of lung T cells with anti-CD3/CD28 Abs was sufficient to trigger IFN-γ production by stimulated T cells. CD8⁺ T cells showed a strong positive correlation between the two assays, indicating that most CD8⁺ T cells that were primed to produce IFN-γ were specific for M. tuberculosis (Fig. 4D). Although CD4⁺ T cells also showed a positive correlation, a significant portion of CD4⁺ T cells were activated bystanders (Fig. 4D).

Differential regulation of CD8⁺ effector functions in different phases of immune response against M. tuberculosis

The cytotoxic potential of CD8⁺ T cells during M. tuberculosis infection was analyzed using LDA with M. tuberculosis-infected macrophages as targets. It has been demonstrated in other systems that LDA underestimates the actual frequency of CTLs. However, due to technical limitations in our system, LDA was the most applicable for our analyses. The frequency of CTLp is generally low in the lungs of M. tuberculosis-infected mice, and it cannot be detected by less sensitive methods, such as ex vivo chromium release assay. In C57BL/6 mice, no major CD8⁺ T cell-recognized epitopes of mycobacterial Ags have been identified, which also limits the ability to detect in vivo cytotoxic activity. Nonetheless, although an imperfect assay, LDA allowed comparison of a similar phenomenon (CTLp frequency) among serial time points.

As shown in Fig. 5, CD8⁺ T cells isolated from the lungs during acute infection were cytotoxic in this assay; however, the frequency of CTLp declined as infection progressed into the chronic stage. The CD8⁺ T cells that were primed early in the lymph node

**FIGURE 5.** The frequency of cytotoxic CD8⁺ T cell precursors decreases as the frequency of IFN-γ-producing CD8⁺ T cells increases during chronic M. tuberculosis infection. A. The frequency of CTLp was estimated by LDA, using χ² minimization analysis as described in Materials and Methods. B and C. The median number of CTL precursors per million lymph node and lung cells. D. An individual mouse. The line represents the median number of CTLp for all mice in each group (four or five mice). D. The median number of IFN-γ-producing CD8⁺ T cells per million lung cells. The experiment was repeated twice.
had cytotoxic potential, and these cells also had cytotoxic potential in the lungs up to 4 wk postinfection (Fig. 5, B and C). However, by 8 wk postinfection, the CD8⁺ T cells in the lymph nodes and lungs had essentially no cytolytic potential (Fig. 5, B and C). As the frequency of CTLp decreased, the frequency of IFN-γ-producing CD8⁺ T cells increased during the chronic infection, suggesting that there was a differential regulation of CD8⁺ T cell effector functions during different phases of the immune response (Fig. 5D).

To further evaluate the functional program of CD8⁺ T cells on a per cell basis during acute and persistent M. tuberculosis infection, we measured the mean fluorescent intensity (MFI) and percentage of perforin-producing cells within CD8⁺IFN-γ⁺ and CD8⁺IFN-γ⁻ populations using intracellular perforin staining (Fig. 6). Ex vivo isolated lung and lymph node cells were incubated in medium or stimulated with anti-CD3/CD28 Abs in the presence of monensin for 4 h. After incubation, cells were fixed, permeabilized, and stained for perforin, IFN-γ, and CD8 marker. Stimulation of CD8 T cells with anti-CD3 and anti-CD28 Abs did not cause these cells to degranulate in vitro, because stimulated CD8 T cells had similar or even higher perforin content than unstimulated T cells (data not shown). During acute infection, when CD8⁺ T cells exhibited cytotoxic potential, perforin detection was low within the CD8⁺ T cell population (Fig. 6, C and D). The lack of perforin staining could be associated with in vivo degranulation of CD8⁺ T cells during acute infection. Perforin expression was detected almost exclusively within IFN-γ⁻CD8⁺ T cells during chronic infection, suggesting that these CD8⁺ T cells were clearly capable of synthesizing perforin at a time when their cytolytic activity was minimal (Fig. 6, C and D). Neither an increase in the

**FIGURE 6.** Perforin staining was limited only to the IFN-γ-negative CD8⁺ T cell population. There was minimal cytotoxic activity despite significant perforin expression during chronic infection. To determine the percentage of perforin-positive cells within IFN-γ⁺ positive and IFN-γ⁻negative CD8⁺ T cells, we adapted the method from Slifka et al. (20). Single-cell suspensions from lymph nodes and lungs were stimulated ex vivo with anti-CD3 and anti-CD28 Abs in the presence of monensin for 4 h at 37°C. After stimulation, cells were fixed-permeabilized and stained with anti-perforin, anti-IFN-γ, and anti-CD8 Abs as described in Materials and Methods. Incubation of CD8 T cells with anti-CD3/CD28 Abs did not cause in vitro perforin degranulation, because Ab-stimulated CD8 T cells expressed equal or higher levels of perforin compared with unstimulated CD8 T cells. The populations were gated as shown in A. The graph in B depicts the MFI and the percentage of perforin-expressing cells within CD8⁺IFN-γ⁺ and CD8⁺IFN-γ⁻ cells relative to isotype controls (n = 8; wk 20). The graphs in C and D summarize the mean percentage ± SEM of perforin-positive cells (CD8 gate) isolated from lymph nodes (C) and lungs (D) of M. tuberculosis-infected mice.
MFI compared with the isotype control (Fig. 6B) nor an increase in the percentage of perforin-positive cells (Fig. 6, B–D) within the CD8<sup>+</sup>/H11001 IFN-γ/H9253/H11001 population was observed. In contrast, a 5-fold increase in the MFI and an increase in the percentage of perforin-positive cells were observed within CD8<sup>+</sup>/H11001 IFN-γ/H9253/H11002 cells (Fig. 6B).

Because exposure of CD8<sup>+</sup>/H11001 T cells to low or high dose of Ags can determine the functional program of CD8<sup>+</sup>/H11001 T cells (30–34), we performed direct comparison between bacterial load, as determined by the number of CFU and the frequency of CTLp in the lungs of infected mice. We did not find a direct correlation between the total number of live replicating bacteria and cytotoxic activity of CD8<sup>+</sup>/H11001 T cells. However, mycobacteria that are killed by the immune response or even by drugs are not quickly cleared (35, 36), so the cumulative Ag load may contribute to the change in CD8<sup>+</sup> T cell function. To assess the Ag load in the lungs of mice during acute and chronic phases of infection, we stained lung sections with polyclonal rabbit anti-M. bovis Ab, which is cross-reactive with M. tuberculosis Ags common to mycobacteria, including M. tuberculosis (Fig. 7). Although there was no statistically significant difference in the numbers of viable bacteria from 4–16 wk after infection (Fig. 1A), immunohistochemical staining indicated that there was extensive accumulation of mycobacterial Ags over time, which may contribute to the switch in effector function of CD8<sup>+</sup> T cells (Fig. 7).

**Discussion**

The primary aim of this study was to determine how chronic exposure to Ag dose influences the functionality of M. tuberculosis-specific T cell responses. In contrast to models of chronic viral infection, we found no evidence of paralysis or permanent loss of effector functions during chronic M. tuberculosis infection. Although CD4<sup>+</sup> T cells were the predominant source of IFN-γ during acute infection, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells equally contributed to IFN-γ production during chronic infection. There were reproducible dynamic changes in the frequency of IFN-γ-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells during the stable chronic phase of infection. Surprisingly, CD8<sup>+</sup> T cells exhibited differential effector functions at different phases of infection; they produced negligible amounts of IFN-γ early in infection, but switched to IFN-γ production during chronic infection. In contrast, our results using limiting dilution assay were consistent with the cytotoxic potential of the CD8<sup>+</sup> T cells in the early phase, but not during the chronic phase, of infection. The lack of cytotoxic potential was not associated with the loss of perforin staining, suggesting that CD8<sup>+</sup> T cells were restrained from using their cytotoxic effector functions.

In viral models of chronic infections, it has been shown that T cells are incapable of mounting adequate effector functions to facilitate viral clearance despite being persistently activated. Our data demonstrate that the interaction of M. tuberculosis with the immune system, in which a chronic infection is established, is very different from the viral systems studied to date.

A common feature of persistent viral infections is that after exposure to high Ag doses, virus-specific T cells undergo accelerated and excessive T cell turnover, resulting in replicative senescence. Our results using staining for BrdU incorporation and annexin V/7AAD expression on the same lung cells suggest that over the 7
mo of *M. tuberculosis* infection, CD4⁺ and CD8⁺ T cells did not lose the capacity to proliferate. Both cell populations underwent two successive, low magnitude bursts of replication in the lungs. The level of apoptosis occurring within the lung paralleled the proliferation kinetics, by which increased apoptosis of the cells was accompanied by increased proliferation. Once cellular numbers were stabilized, increased apoptosis and reduced proliferation contributed to the contraction phase of the T cell responses. However, the stabilization of T cell numbers during chronic infection was accompanied by steady levels of apoptosis, suggesting that infiltration of new effector T cells into the lungs is likely to be ongoing during chronic infection.

Proliferation of *M. tuberculosis*-specific IFN-γ responses peaked at 2 wk after infection. IFN-γ-specific ELISPot and intracellular cytokine staining showed that CD4⁺ T cells were responsible for the majority of IFN-γ production during acute infection, as suggested previously (21). ESAT-6 appeared to be the dominant Ag in acute infection. However, during chronic infection, the frequency of ESAT-6-specific CD4⁺ T cells in the lungs decreased, indicating that other mycobacterial Ags were being recognized. The priming and the presence of IFN-γ-producing CD8⁺ T cells in the infected lungs were negligible until 8 wk postinfection. Although CD8⁺ T cells contributed minimally to IFN-γ production during acute infection, the frequency of IFN-γ-producing CD8⁺ T cells was equivalent to that of IFN-γ-producing CD4⁺ T cells during chronic infection.

A surprising result was that the frequency of IFN-γ-producing CD4⁺ and CD8⁺ T cells dynamically waxed and waned during persistent *M. tuberculosis* infection. These findings were confirmed when we summarized the results from two to nine independent studies with 1- to 2-wk intervals between the study points. Although the changes in the frequency of IFN-γ-producing effector cells were not of great magnitude (2- to 3-fold), they were reproducible in nine independent studies. These changes could have substantial consequences within the local level of the granuloma. For example, at 12 wk after infection, the average number of IFN-γ-producing T cells in the lungs of infected mice was 12,500 (1 of 337 cells). At 16 wk postinfection, the average number of IFN-γ-producing T cells decreased to 6,000 (1 of 863 cells). This may mean that there were half as many effector T cells interacting with the same number of infected macrophages. The observations of waxing and waning of T cell effector function could be explained by dynamic responsiveness of the immune system to periodic and transient bursts of mycobacterial replication inside infected lungs and a fine-tuning of the response to control the infection without inducing substantial pathology. During chronic infection, mycobacteria are probably mostly quiescent; however, they may transiently undergo bursts of replication. These changes in bacterial numbers are sensed by the host immune system, which responds, in turn, by rapidly increasing the numbers of IFN-γ-producing T cells until bacterial growth is brought under control. In this manner, bacterial growth will be strictly controlled, with minimal immunopathology that otherwise would be induced by persistently elevated levels of inflammatory cytokines, such as IFN-γ. This explanation suggests a dynamic equilibrium achieved between the pathogen and the host’s immune system that persists throughout the course of infection. Our current level of detection of bacterial numbers in the local environment of each granuloma does not permit us to directly test this hypothesis at this time.

Longitudinal analysis of CD8⁺ T cell effector functions revealed that there was a switch in the functional program of CD8⁺ T cells during the course of infection. Early in infection, CD8⁺ T cells were predominantly cytotoxic, but during chronic infection, CD8⁺ T cells switched to cytokine production. The dual staining for IFN-γ and perforin within the CD8⁺ T cell population revealed two important findings. First, the split between cytokine production and cytotoxic functions was clearly evident on a per cell basis, because perforin staining segregated to only the IFN-γ-negative T cells. Second, although CD8⁺ T cells exhibited minimal cytotoxicity in chronic infection, their ability to synthesize perforin remained unimpaired. In fact, when measurable cytotoxic function was high, it was difficult to detect intracellular perforin staining, suggesting that perforin was efficiently released from the cells in vivo. Therefore, it appears that the lack of CTL activity was not a result of CD8⁺ T cell exhaustion, but, rather, was a consequence of factors that dictate the functional program of CD8⁺ T cells during persistent *M. tuberculosis* infection. The clearest candidate accounting for these observations is Ag dose.

Several studies reported that the level of TCR occupancy can determine the development fate of CD8⁺ T cells, i.e., whether CD8⁺ T cells will be cytotoxic or produce cytokines (2, 30, 31, 33, 34). At low Ag doses, an immature immunologic synapse is formed, which is sufficient to trigger cytotoxicity, but not cytokine production or proliferation (33, 34). Conversely, at high levels of TCR occupancy, a mature immunological synapse is formed that, due to sustained and heightened levels of Ca²⁺ intracellular signaling, will lead to preferential cytokine production by CD8⁺ T cells (33, 34). Most of these studies were performed on CD8⁺ T cells clones and by pulsing APCs with different peptide concentrations. Very few reports exist on the behavior of primary CD8⁺ T cells during in vivo infection. Betts et al. (32) showed that in primary human HIV- and CMV-specific CD8⁺ T cells, Ag dose was the sole determinant of the cytokine vs cytotoxic nature of virus-specific CD8⁺ T cell responses. Using *M. tuberculosis*-specific human CD8⁺ T cell clones, Lewinsohn et al. (37) demonstrated that CD8⁺ T cell clones preferentially lysed heavily infected cells. These CD8⁺ T cell clones were generated from the peripheral blood of latently infected individuals, in whom it is believed the presence of *M. tuberculosis* Ag is minimal or undetectable. Results from several epidemiological studies indicate that the cytotoxic activity of CD8⁺ T cells was dependent on the clinical stage of tuberculosis patients. De la Barrera et al. (38) reported that although cytotoxic activity was readily detectable in healthy purified protein derivative-positive individuals, the cytotoxic potential of CD4⁺ and CD8⁺ T cells was significantly diminished in patients with active tuberculosis. Therefore, cell lines and clones from healthy purified protein derivative-positive individuals may not represent the physiological state of CD8⁺ T cells in patients with active or chronic tuberculosis, in whom CD8⁺ T cells may be exposed to a high Ag dose over long periods of time.

We were unable to correlate CD8⁺ CTLp frequency with the total number of viable bacteria in the lungs. However, determination of CFUs is a crude estimate of the antigenic load to which CD8⁺ T cells may be exposed in vivo. It measures only the number of replication-competent mycobacteria. We attempted to address the amount of Ag in the lungs of infected mice at different stages of the immune response by staining lung sections with Ab that recognizes >100 mycobacterial Ags. Immunohistochemical analysis indicates that there was substantial accumulation of mycobacterial Ags during the course of infection, although the number of viable mycobacteria did not significantly change. Hence, the cumulative Ag dose could have influenced the functional program of CD8⁺ T cells, a hypothesis that merits additional investigation.

Overall, our study indicates that the long-term control of *M. tuberculosis* is achieved through dynamic immune responses that persist even during the chronic phase of infection, including waxing and waning of cytokine production and shifts in effector phenotypes. We showed previously that CD4⁺ T cells are required for cytotoxic functions of CD8⁺ T cells, suggesting that the immune response to *M. tuberculosis* is cross-regulatory. Understanding the
in vivo mechanisms that regulate the effector functions of T cells in the face of persistent *M. tuberculosis* infection is pivotal for the improved design of antituberculosis vaccine strategies.

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**Disclosures**

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