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Persistence and Turnover of Antigen-Specific CD4 T Cells During Chronic Tuberculosis Infection in the Mouse

Gary M. Winslow, Alan D. Roberts, Marcia A. Blackman, and David L. Woodland

CD4 T cells are critical for resistance to Mycobacterium tuberculosis infection, but how effective T cell responses are maintained during chronic infection is not well understood. To address this question we examined the CD4 T cell response to a peptide from ESAT-6 during tuberculosis infection in the mouse. The ESAT-6Iα-specific CD4 T cells that responded in vitro expressed activation markers characteristic of chronically activated effector cells and used a limited VB repertoire that was clonally stable in vivo for at least 12 wk. 5-Bromo-2-deoxyuridine incorporation studies indicated a relatively high rate of cell division among both total CD4 and ESAT-6Iα-specific CD4 T cells during acute infection, but the degree of 5-bromo-2-deoxyuridine incorporation by both the CD4 T cells and the Ag-specific cells declined at least 3-fold during chronic infection. The data indicate that the peripheral ESAT-6Iα-specific CD4 T cell response to M. tuberculosis is characterized during the acute phase of infection by a period of extensive proliferation, but once bacterial control is achieved, this is followed during chronic infection by an extended containment phase that is associated with a persistent response of activated, yet more slowly proliferating, T cells. The Journal of Immunology, 2003, 170: 2046–2052.

CD4 T cells play an instrumental role in the control of tuberculosis infections in both humans and mice (for reviews, see Refs. 1 and 2). Chief among the functions of CD4 T cells is the production of IFN-γ, which is required for resistance to fatal infection, as both CD4+ and IFN-γ-deficient mice rapidly succumb to infection (3–6). An important function of IFN-γ produced by CD4 T cells is the induction of nitric oxide synthesis in macrophages that, in turn, controls bacterial infection (5, 7–9). This control occurs within about 3 wk in mouse lungs after aerosol inoculation with Mycobacterium tuberculosis H37Rv (10, 11). A stationary phase of bacterial colonization subsequently occurs that is limited by αβ T cells until the mice succumb to infection, which in C57BL/6 mice typically occurs between 200 and 250 days postinfection (11). It is not known why the mice eventually succumb, although it is possible that death is a consequence of a loss of cellular immunity or is due to chronic T cell-mediated pathology.

The details of the CD4 T cell responses during extended chronic infection are not well understood. Previous studies in the mouse have analyzed phenotypic characteristics of T cells as populations (12–14). These studies showed that by 8 wk after aerosol infection with M. tuberculosis H37Rv, lung CD4 T cells expressed high levels of CD44, CD69, and CD11a and low levels of CD45RB and CD62L, a phenotype characteristic of activated T cells. Ag-specific T cells were not identified in these studies, however, and it is possible that the magnitude and duration of the responses to specific mycobacterial T cell epitopes vary during chronic infection. Nonspecific T cells also may be recruited to sites of infection and alter the responses of the bulk T cell population. Therefore, to further explore the behavior of T cells during tuberculosis infection in the mouse, we have enumerated Ag-specific CD4 T cells throughout chronic infection and have examined their capacity for proliferation. Our studies revealed that although the frequencies of particular Ag-specific T cells remain remarkably stable during chronic infection, the magnitude of their proliferative responses decreases.

Materials and Methods

Mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (Germantown, NY). Mice were aerosol-infected at 8 wk of age. All studies were performed on cells pooled from the tissues of four or five mice. Adult thymectomies were performed at 5 wk of age. Mice were anesthetized, and a 2- to 3-cm mid-line longitudinal incision was made over the suprasternal notch, the sternum was cut, the chest cavity was opened with forceps, and the thymus was removed using a suction cannula. The forceps were then removed, and the skin was closed and secured with wound clips. Control mice in some, although not all, experiments were sham-thymectomized, but no differences were observed between the two groups of control mice.

Bacteria and infections

M. tuberculosis strain H37Rv was provided by Dr. R. North (Trudeau Institute). All infections were via the aerosol route using an aerosol infection apparatus as described previously (11). Bacteria were enumerated in infected lungs by sampling pooled aliquots of cells and the disaggregated lung tissue remaining at the end of the lymphocyte isolation procedure. The samples were homogenized with a motorized Teflon pestle in PBS containing 0.01% Tween, and 10-fold serial dilutions were plated on enriched Middlebrook 7H11 agar and incubated at 37°C for 3 wk. Colony counts from the samples of cells and tissue were combined and used to calculate the total bacteria titer.
Lymphocyte isolation

Lymph nodes and spleens were harvested from infected mice, mechanically disrupted, passed through a 100-μm pore size cell strainer, and treated with buffered ammonium chloride solution to lyse the erythrocytes. Spleen lymphocytes were further enriched on discontinuous 80/40% Percoll (Pharmacia Biotech, Uppsala, Sweden) gradients for 30 min at 400 × g. The cells were isolated from the interface, washed, resuspended in balanced salt solution (BSS), and then further purified by centrifugation over lymphocyte separation medium (LSM; ICN Pharmaceuticals, Costa Mesa, CA). The lungs were isolated, minced with a scalpel, and treated with collagenase (Roche, Indianapolis, IN) for 30–60 min at 37°C. The remaining tissue was passed through a 100-μm pore size cell strainer, the erythrocytes were lysed, and the cells were further purified on Percoll and LSM gradients as described above. In some experiments nonadherent cells from lung, mediastinal lymph nodes (MLN), and spleen, were enriched by panning on plastic tissue culture plates for 30–60 min at 37°C. For the intra-cellular cytokine assays, the LSM gradient was omitted.

ELISPOT

The frequencies of Ag-specific cells in the lung, spleen, and MLN were determined in a standard ELISPOT system (15). Multiscreen MAHA mono-nucleotrode plates (Millipore, Bedford, MA) were coated with rat anti-mouse IFN-γ (clone R4-6A2; BD PharMingen, San Diego, CA) overnight at 4°C (100 μl/well) at a concentration of 10 μg/ml. The plates were subsequently blocked with complete tumor medium containing 10% FCS (16) for at least 2 h. Serial dilutions of lymphocytes obtained from infected tissues (starting at 1 × 10^5/well) were incubated with irradiated spleen APCs from uninfected C57BL/6 mice (1 × 10^5/well) in the presence or the absence of the ESAT-6_1–20 peptide (New England Peptide, Fitchburg, MA) in complete tumor medium (containing 10% FCS) and containing 10% FCS. All cultures were performed in triplicate. After 40 h the cells were washed with PBS containing 0.05% Tween 20. The plates were then incubated with bixinylated rat anti-mouse IFN-γ Ab (clone XM1.2; BD PharMingen) at 5 μg/ml in PBS/Tween 20 containing 1% FCS at 4°C for 18–24 h. The plates were washed five times with PBS/Tween and incubated with streptavidin–alkaline phosphatase (DAKO, Carpinteria, CA) for 1 h at room temperature. The plates were washed with PBS/Tween and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich, St. Louis, MO) as a substrate. The wells were photographed with an Olympus C-3040 digital camera (New Hyde Park, NY), images were printed, and the spots were enumerated. Specific responses were quantitated by subtracting the number of spots, if any, detected in the absence of the specific peptide.

The frequencies of ESAT-6_1–20/IAα-specific CD4 T cells were determined by dividing the number of Ag-specific spots per well by the number of CD4 T cells plated per well. The number of CD4 T cells was determined after measuring the frequency of CD4 T cells in the population by flow cytometry.

Flow cytometric detection of intracellular IFN-γ, T cell activation markers, and TCR Vβ elements

CFSE (Sigma-Aldrich) was dissolved at 5 mM in DMSO and used to label spleen APCs in BSS at a concentration of 0.5 μM for 10 min in the dark. The cells were subsequently washed in BSS and used at 1 × 10^6/ml in the in vitro stimulation assays. Lymphocytes were isolated from infected mice and were incubated in 48-well microtiter plates at a concentration of 1 × 10^6/ml with equal numbers of CFSE-labeled syngeneic spleen APCs in the absence or the presence of the ESAT-6_1–20 peptide (New England Peptide, Fitchburg, MA) in complete tumor medium containing 10% FCS. All cultures were performed in triplicate. After 40 h the cells were washed with PBS containing 0.05% Tween 20. The plates were then incubated with bixinylated rat anti-mouse IFN-γ Ab (clone XM1.2; BD PharMingen) at 5 μg/ml in PBS/Tween 20 containing 1% FCS at 4°C for 18–24 h. The plates were washed five times with PBS/Tween and incubated with streptavidin–alkaline phosphatase (DAKO, Carpinteria, CA) for 1 h at room temperature. The plates were washed with PBS/Tween and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich, St. Louis, MO) as a substrate. The wells were photographed with an Olympus C-3040 digital camera (New Hyde Park, NY), images were printed, and the spots were enumerated. Specific responses were quantitated by subtracting the number of spots, if any, detected in the absence of the specific peptide.

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Results

Frequency of ESAT-6_1–20-specific T cells during M. tuberculosis infection

CD4 T cells make responses to a large number of M. tuberculosis Ags in both mice and humans (18, 19). A particularly strong response against the ESAT-6 Ag has been described previously in mice (20) and humans (21–24), and a major epitope of ESAT-6 was identified in C57BL/6 (B6; H-2b) mice as ESAT-6_1–20/IAα. ESAT-6_1–20/IAα-specific splenic CD4 T cells were found at a frequency of ~0.1% during a recall response to tuberculosis in B6 mice (20).

We have extended these studies by examining the frequency of ESAT-6_1–20/IAα-specific CD4 T cells in the lungs, MLN, and spleens of B6 mice at various times after aerosol infection with M. tuberculosis H37Rv (Fig. 1). High frequencies of CD4 T cells were detected within the lungs of infected mice within 14 days postinfection, and the absolute number of CD4 T cells reached a peak on about day 28 postinfection, at ~1.5 × 10^7/lung, which was consistent with previous studies (13, 14). The ESAT-6_1–20/IAα-specific CD4 T cell responses were first detected on day 21 postinfection by ELISPOT assay for IFN-γ. By day 28 the responses accounted in the lung for ~2% of the total CD4 T cells or, in absolute numbers, ~3.6 × 10^5 ESAT-6_1–20/IAα-specific CD4 T cells. The percentages and numbers of both total CD4 T cells and...
Figure 1. Frequency of CD4 T cells and ESAT-6-20/IAα-specific CD4 T cells in M. tuberculosis-infected mice. Organs from aerosol-infected C57BL/6 mice were pooled from groups of four or five mice, and the frequency of CD4 T cells (left panels) was determined by flow cytometry. ELISPOT assays were performed on the indicated days postinfection to determine the frequency of ESAT-6-20/IAα-specific CD4 T cells (right panels). The mean absolute numbers of CD4 T cells in the lung during chronic infection (after day 28) was 8.3 x 10^6, and the mean absolute number of ESAT-6-20/IAα-specific CD4 T cells during this same period was 1.4 ± 1.1 x 10^5, 3.0 ± 2.3 x 10^5, and 1.0 ± 0.7 x 10^5 in the lungs, MLN, and spleen, respectively. Error bars from data pooled from two or more experiments indicate the SE and are shown when data were available from replicate experiments.

ESAT-6-20/IAα-specific T cells declined after day 28 postinfection, reaching stable levels that persisted for at least 166 days. The percentages and numbers of ESAT-6-20/IAα-specific CD4 T cells in the MLN and spleen followed a pattern similar to that observed in the lungs. Although the overall frequencies in the MLN and spleen were lower, no detectable decline in the magnitude of the responses was detected (Fig. 1). Thus, the Ag-specific T cell response was prolonged and stable in the lungs, MLN, and spleen during chronic tuberculosis infection, which for the purposes of this study we have defined as the period when bacterial colonization is controlled by the immune response.

Expression of activation markers by ESAT-6-specific T cells

T cells that respond to Ags during tuberculosis infection produce IFN-γ mRNA, and will secrete IFN-γ upon in vitro stimulation (14). Flow cytometric detection of intracellular IFN-γ was therefore used as an alternative means of identifying the ESAT-6-20/IAα-specific CD4 T cells, and other markers were used to determine their activation state. Mononuclear cells from the lungs of day 21 infected mice were challenged in vitro with ESAT-6-20, for 5 h in the presence of brefeldin A, and activation marker expression was evaluated on the IFN-γ-expressing CD4 T cells. This approach limits the analysis to Ag-specific cells that respond in vitro, so the technique may not identify the entire Ag-specific T cell population, and we cannot rule out the possibility that some changes in marker expression occurred in vitro. CD44 expression, a marker for activated T cells, was expressed at high levels on most of the responding ESAT-6-20/IAα-specific CD4 T cells (Fig. 2). The ESAT-6-20/IAα-specific CD4 T cells also expressed uniformly low levels of CD62L (L-selectin), where low expression is characteristic of activated T cells. Expression of CD69 and CD25, typical markers of acutely activated T cells, were low within the responding population. The activation marker CD43 was expressed on ~60% of the Ag-specific cells, typical of the expression pattern found during viral respiratory infections (25). Low to intermediate levels of CD45RB were also observed on the Ag-specific cells, and similar observations were made when several of the markers were examined on Ag-specific cells on day 49 postinfection (data not shown). The frequency of CD44+CD25+ cells among the bulk CD4 T cell population increased between days 21 and 49, consistent with the findings of previous studies (data not shown) (13). Thus, ESAT-6-20/IAα-specific CD4 T cells exhibited characteristics of chronically activated effector T cells.

Figure 2. Expression of activation markers on lung ESAT-6-20/IAα-specific CD4 T cells. Lung T cells from day 21 infected mice were stimulated in vitro for 5 h, with or without specific peptide, in the presence of syngeneic spleen APCs, then harvested, and CD4 T cells were analyzed by flow cytometry for intracellular IFN-γ expression and expression of the indicated activation markers. The spleen APCs were labeled with CFSE and were excluded from the flow cytometric analyses. The experiment was repeated three times with similar results. The CD25 data were from a separate experiment performed using day 34 infected mice.
Repete analysis of ESAT-61/IAb–specific CD4 T cells

Although the ELISPOT analyses indicated that the ESAT-61/IAb–specific CD4 T cell responses persisted during chronic infection, it was not known whether the clonality of the responding T cells was maintained during this time, or whether clonal populations predominating at a given time were replaced by others at different times during infection (26). This question was addressed by analysis of the Vβ repertoire of the Ag-specific T cells, which can detect gross changes in the T cell repertoire. Lung Ag-specific CD4 T cells were identified by in vitro stimulation with ESAT-61, followed by detection of intracellular IFN-γ expression. Ten available Vβ Abs (Vβ2, -3, -4, -5, -6, -7, -8, -10, -11, -14) identified >70% of the CD4 T cells in the lungs of day 34 infected mice (data not shown). Forty-seven percent of ESAT-61/IAb–specific CD4 T cells, identified as IFN-γ-secreting cells, together used four different Vβ chains (Vβ3 (9.6%), Vβ6 (24.1%), Vβ8 (7.1%), and Vβ14 (7.1%)), indicating a relatively oligoclonal T cell response to this epitope (Fig. 3a). To determine whether this oligoclonal repertoire was maintained during chronic infection, the analysis was repeated on day 125 postinfection. As shown in Fig. 3b, the same Vβ elements were used by the responding Ag-specific T cells at this time, indicating little change in the T cell repertoire. As the frequency of IFN-γ-producing cells within the total CD4 T cells population was relatively low, it is possible that some Vβ elements that had been used at lower frequencies (≤5%) may not have been detected. Nevertheless, the overall clonal nature of the T cell response in the lungs of chronically infected mice was stable for at least 90 days.

Turnover of lung CD4 T cells during chronic infection

Our ELISPOT data indicated that the mice mounted persistent CD4 T cell responses during chronic infection, but it was unclear how long individual T cells persisted in peripheral tissues during what may be continual exposure to bacterial Ags. To address the rate of T cell proliferation and turnover, 5-bromo-2-deoxyuridine (BrdU), a nucleotide analog that is incorporated into the DNA of dividing cells, was administered to infected mice. Cells that incorporate BrdU can be readily detected by flow cytometry (17). One dose of BrdU (0.8 mg) was administered to infected mice via the peritoneum. Under these conditions BrdU is incorporated by dividing cells for only ~1 day. Once BrdU is incorporated, cells that no longer continue to divide can survive and be detected months later, in some cases (D. L. Woodland, unpublished observations). In contrast, cells that continue to proliferate lose their label in a relatively short period. Thirty-three percent of lung CD4 T cells from day 21 infected mice incorporated BrdU within 24 h of BrdU administration (Fig. 4). When similarly treated mice were examined 6 days later, only 3% of the cells that had incorporated BrdU during the effective 24-h pulse period could be detected, indicating that the cells that had incorporated BrdU had divided, died, or emigrated from the lungs. Thus, the effective life span of a lung CD4 T cell was <6 days at 21 days postinfection.

To determine whether the extent of BrdU incorporation by CD4 T cells changed during chronic infection, we performed similar 24-h pulse experiments on mice at various intervals postinfection. The percentage of lung CD4 T cells that had incorporated BrdU reached maxima at 21 days postinfection (29%), declined rapidly until about day 56, and then stabilized at a level of ~8%, for as long as 250 days postinfection (Fig. 4b). Thus, the degree of BrdU incorporation of CD4 T cells in the lung was of greatest magnitude during the acute response, but declined to lower, but stable, levels during the period of chronic infection. By way of comparison, the frequency of ESAT-61/IAb–specific CD4 T in the draining MLN was ~3% on day 111 postinfection (data not shown).

Turnover of Ag-specific T cells

The BrdU incorporation studies described above were unable to identify the Ag-specific T cells within the population of CD4 T cells in the infected lungs. Therefore, ESAT-61/IAb–specific CD4 T cells were identified by assay for intracellular IFN-γ following in vitro peptide stimulation, and BrdU incorporation was measured using a modified protocol that permitted simultaneous detection of both IFN-γ and BrdU by flow cytometry. In these experiments BrdU was administered continuously to mice via the drinking water for a period of 1–14 days. This approach was used to measure the time required for all CD4 T cells to incorporate...
BrdU incorporation by CD4 T cells during infection. Approximately 2% of the CD4 T cells analyzed on day 21 postinfection were found to respond to ESAT-6–20 in vitro by producing IFN-γ, similar to the frequency detected by ELISPOT assay. Nearly 100% of the ESAT-6–20-specific CD4 T cells had incorporated BrdU after continuous treatment for 7 days (Fig. 5a). High levels of BrdU incorporation also were observed when BrdU was administered continuously for 14 days (Fig. 5b). However, only 37% of the ESAT-6–20-specific CD4 T cells also had incorporated BrdU within 1 day following i.p. administration. This indicated that most ESAT-6–20-specific CD4 T cells had not undergone cell division within the previous 24-h period. The lack of concordance between proliferation and IFN-γ secretion suggested that some cells ceased proliferation before expressing effector functions.

To determine whether the rate of BrdU incorporation within the ESAT-6–20 IA^b^-specific CD4 T cell population declined during chronic infection, a phenomenon that was observed in the total CD4 T cell population, we compared BrdU incorporation after 24-h pulse labeling on days 21 and 125 postinfection. Lung T cells were stimulated in vitro with ESAT-6–20, and BrdU and IFN-γ were measured simultaneously as described above. Only 11% of the ESAT-6–20 IA^b^-specific CD4 T cells incorporated BrdU on day 125 (Fig. 5c) compared with 37% on day 21 postinfection, indicating that the decline in the rate of incorporation observed in the CD4 T cell population was also observed in ESAT-6–20 IA^b^-specific CD4 T cells.

Lack of a role for thymus

Our studies indicated that the ESAT-6–20 T cell response was maintained for several months in chronically infected mice, suggesting that either the peripheral T cells possessed an essentially unlimited proliferative potential or that the pools of peripheral T cells were maintained by an influx of naive T cells from the thymus. To discriminate among these hypotheses, ELISPOT analyses were performed using both normal and adult-thymectomized B6 mice on day 49 postinfection. The frequency of both lung CD4 T cells and ESAT-6–20 IA^b^-specific CD4 T cells was similar in normal and thymectomized mice (Fig. 6a). The thymectomized mice did not display any enhanced morbidity or mortality (data not shown), and lung bacterial titers were similar (Fig. 6a). To exclude the possibility that the thymectomized mice compensated for lowered T cell frequencies by increasing their proliferation, BrdU incorporation was measured in normal and thymectomized mice 24 h after administration at several different intervals postinfection. The level of BrdU incorporation in CD4 T cells in thymectomized mice was similar or lower than those in control mice, indicating that the pools of Ag-specific CD4 T cells were maintained in thymectomized mice in the absence of any significant change in their degree

**FIGURE 4.** BrdU incorporation by CD4 T cells during infection. a. BrdU was administered to C57BL/6 mice on day 21 postinfection, and pulsed lung lymphocytes from four or five mice were analyzed for BrdU incorporation in CD4 T cells 1 day (left panel) or 7 days (right panel) later. b. Percentages of lung CD4 T cells that incorporated BrdU 1 day after i.p. administration on the indicated days postinfection. Error bars, indicating the SE, are shown for analyses that were conducted two or more times.

**FIGURE 5.** BrdU incorporation by lung ESAT-6–20 IA^b^-specific CD4 T cells during infection. a. BrdU was administered on day 21 postinfection via the drinking water continuously for 7 days, after which time lung lymphocytes were cultured in vitro with spleen APCs in the absence or the presence of specific peptide for 5 h, as described in Fig. 2. Analysis was performed simultaneously for intracellular IFN-γ expression and BrdU incorporation in CD4 T cells as described in Materials and Methods. Cells that expressed IFN-γ and incorporated BrdU are those visible in the *upper right quadrants* of the dot plots. b. Day 21 infected mice were treated continuously with BrdU for 1–14 days, and the percentages of BrdU-positive ESAT-6–20 IA^b^-specific (double-positive) CD4 T cells were determined as described in a. c. Percentages of ESAT-6–20 IA^b^-specific CD4 T cells that incorporated BrdU 1 day following i.p. administration on days 21 and 125 postinfection. The data from day 21 were also included in Fig. 4b.
of proliferation. Thus, the prolonged ESAT-6₁⁻₂₀-specific response was probably derived entirely from existing pools of peripheral T cells.

Discussion

*M. tuberculosis* infection in the mouse is characterized by a period of relatively rapid bacterial growth for 3–4 wk, followed by a prolonged period of chronic infection, during which time further bacterial expansion is limited by CD4 T cells. Exactly how the CD4 T cells are able to control the infection under what appears to be continual Ag challenge is not well understood. Our studies suggest that T cells achieve this control by maintaining stable pools of activated and proliferating Ag-specific T cells in the lungs and secondary lymphoid organs. These findings were enabled by current approaches available to detect Ag-specific cells and by our development and application of a technique that allowed the simultaneous detection of IFN-γ secretion and BrdU incorporation in an Ag-specific T cells.

Previous studies demonstrated that ESAT-6₁⁻₂₀/IAb-specific CD4 T cells could be found at a frequency of ~0.1% among total T cells during a recall response in the spleen, but the frequencies of Ag-specific cells during primary or chronic infection in the lung were not determined (20). Other studies in the mouse have typically measured CD4 and CD8 T cell responses at the population level. For example, it has been shown that the frequencies of total lung CD4 T cells from *M. tuberculosis*-infected mice that produced IFN-γ after polyclonal activation in vitro increased dramatically during the first 3 wk after i.v. or aerosol infection (14, 27). Although these approaches have been highly informative, they lack resolution, as it is possible either that Ag-nonspecific T cells are recruited to the lung during chronic *M. tuberculosis* infection (28) or that the responses of T cells specific for particular Ags do not mirror the response of the CD4 T cell population. We focused on the ESAT-6₁⁻₂₀/IAb-specific CD4 T cells and have extended previous studies by demonstrating that these activated Ag-specific CD4 T cells were maintained at nearly constant levels in the lung, MLN, and spleen for an extended period (at least 24 wk) during chronic infection. Similar observations were made previously in studies of the polyclonal CD4 T cell response after aerosol inoculation (13). The Ag-specific responses in the lungs paralleled the frequency of total CD4 T cells, indicating that ESAT-6₁⁻₂₀/IAb-specific CD4 T cells undergo similar response kinetics as the polyclonal CD4 T cell population. This outcome was not certain, however, as it was possible that the magnitude and quality of the CD4 T cell responses to particular Ags, such as ESAT-6, might vary during chronic infection depending on both the development of the host response and physiological changes that might occur in the bacteria during the period of chronic colonization of host macrophages. The function and timing of expression of ESAT-6 by *M. tuberculosis* in vivo is not known, so it was possible that CD4 T cell responses to this Ag might not have followed the responses of the bulk CD4 population to all bacterial Ags. A complete understanding of the cellular responses to *M. tuberculosis* will require knowledge of the T cell responses to many different Ags. Indeed, if a large proportion of the T cells recruited to the lung are *M. tuberculosis* specific, our data indicate that T cells that recognize many different Ags are involved in the response, because only 2% of the population recognized ESAT-6₁⁻₂₀. Information regarding the full spectrum of Ags recognized will be critical for the evaluation of different vaccination strategies, because candidate vaccines might vary in their abilities to elicit responses to particular protective Ags.

How are the ESAT-6₁⁻₂₀/IAb-specific CD4 T cell responses maintained during what may be continuous stimulation by Ag? The T cell repertoire of the Ag-specific population was remarkably stable, and the response was not driven to clonality, anergy (29, 30), or exhaustion (31). Moreover, an influx of naive Ag-specific cells from the thymus was not required for maintenance of the pool of lung T cells. In contrast to acute infections, where massive cell death accompanies clearance of infectious agents, a significant contraction of the T cell response was not observed during chronic tuberculosis infection. It is likely instead that a balance between CD4 T cell proliferation and death during chronic tuberculosis infection is actively maintained in the inflammatory environment of the infected lung by cytokines and/or other factors (32–34).

Both the bulk CD4 and ESAT-6₁⁻₂₀/IAb-specific lung T cells showed a significant capacity for proliferation. Thirty-five percent of the total CD4 T cells present in the lungs during the acute infection had incorporated BrdU within 24 h, indicative of a relatively high degree of cell division, comparable to levels observed during acute respiratory viral infection (L. Cauley, and D. L. Woodland, unpublished observations). Although the frequencies of ESAT-6₁⁻₂₀/IAb-specific CD4 T cells were nearly constant during chronic infection, our studies also revealed that the peak proliferation and turnover of both the bulk CD4 population and the Ag-specific cells occurred by day 28 postinfection and thereafter declined rather sharply, reaching stable levels by approximately day 56 postinfection. Although it is not known what factor(s) regulates the change in BrdU incorporation by CD4 T cells, it is likely that ESAT-6 production, or Ag presentation, is maximal during acute infection. The bacteria reach the end of their log-phase growth at ~3–4 wk postinfection in aerosol-infected mice (10, 11), so more ESAT-6₁⁻₂₀ could be more available for presentation by APCs at this time. Once immunological control is achieved, the
expression of the ESAT-6 Ag may decrease, perhaps due to a decrease in bacterial proliferation or to bacterial differentiation. Alternatively, the numbers, type, or physiology of the APC could change during infection in the lung or secondary lymphoid organs. Trafficking of CD4 T cells during tuberculosis infection is not well understood, and it is not known where or on which APCs the T cells encounter Ag. Thus, alterations in APC function or T cell localization may be responsible for the changes in the rate of BrdU incorporation that we observed. Alternatively, the inflammatory environment in the lung or secondary lymphoid tissues might be more conducive to T cell proliferation during the acute phase of the infection.

When the BrdU incorporation studies were extended to examine both proliferation and cytokine secretion simultaneously, we observed that only 38% of the ESAT-6 specific CD4 T cells that produced IFN-γ in vitro had also incorporated BrdU in the preceding 24 h. One interpretation of these data is that proliferation and cytokine secretion are not necessarily linked, as was observed in an experimental T cell transgenic model system (35). Thus, during tuberculosis infection, some lung T cells might terminate cell division before expressing particular effector functions. After 7 days of continuous BrdU treatment, all the Ag-specific cells in the lungs had incorporated BrdU, indicating that all the lung T cells that responded in vitro had undergone cell division during this time. However, as the population was defined by its ability to respond to Ag in vitro, it is not yet known whether all the Ag-specific cells actually responded to Ag in vivo. Such an analysis will require tetramer reagents to identify the Ag-specific cells in a manner that is independent of their function.

Our findings, taken together, reveal a remarkable persistence of the ESAT-6 specific CD4 T cell response during long periods of chronic tuberculosis infection. This prolonged response is clonally stable, is maintained entirely by the existing pools of peripheral T cells, and is accompanied by a decline in the rate of T cell turnover. The factors that regulate the changes in T cell function probably occur as a consequence of changes in bacteria or host physiology, so their identification will probably offer insights into immune regulation during infection by this intracellular bacterial pathogen.

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