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Maintenance of Peripheral T Cell Responses during Mycobacterium tuberculosis Infection

William W. Reiley,* Susan T. Wittmer,* Lynn M. Ryan,* Sheri M. Eaton,* Laura Haynes,* Gary M. Winslow,*,† and David L. Woodland*,†

Fully functional T cells are necessary for the maintenance of protective immunity during chronic infections. However, activated T cells often undergo apoptosis or exhaustion upon chronic stimulation mediated by Ag or inflammation. T cell attrition can be compensated for by the production of thymus-derived T cells, although the new naive T cells must undergo T cell priming and differentiation under conditions different from those encountered during acute infection. We used a murine model of Mycobacterium tuberculosis infection to address how the activation and differentiation of new thymic emigrants is affected by chronic inflammation, as well as whether the newly developed effector T cells help to maintain peripheral T cell responses. Although new thymic emigrants contributed to the peripheral T cell response early during acute M. tuberculosis infection, the relative contribution of new effector T cells to the peripheral CD4 and CD8 T cell pools declined during chronic infection. The decline in new T cell recruitment was a consequence of quantitative and/or qualitative changes in Ag presentation, because during chronic infection both the priming and expansion of naive T cells were inefficient. Thus, although thymic tolerance is not a major factor that limits protective T cell responses, the chronic environment does not efficiently support naive T cell priming and accumulation during M. tuberculosis infection. These studies support our previous findings that long-term protective T cell responses can be maintained indefinitely in the periphery, but also suggest that the perturbation of homeostasis during chronic inflammatory responses may elicit immune pathology mediated by new T cells. The Journal of Immunology, 2012, 189: 000–000.

Infection with a pathogen results in the orchestrated priming, expansion, and differentiation of a T cell response. T cells first encounter Ag in secondary lymphoid tissues, enter the circulation after a period of proliferation, and are subsequently recruited to sites of infection, where they exert their effector functions. For Mycobacterium tuberculosis, studies in the mouse showed that Ag-specific naive T cells are primed in the lung-draining lymph node ~10 d after aerosol infection (1). Primed Ag-specific T cells undergo extensive proliferation, emigrate from the lymph nodes, and are recruited to the lungs and other sites of inflammation (2, 3). The activity of these T cells (i.e., production of IFN-γ) is essential for the control of bacterial replication and survival of the host (4, 5).

Although much is known regarding the early adaptive immune response generated against M. tuberculosis, less is known about how T cell responses are maintained during chronic infection. Chronic T cell responses are affected by the inflammatory environment, which, for M. tuberculosis, includes the granuloma, a hallmark of tuberculosis (6). The granuloma is highly ordered and is continually evolving at the structural and cellular level throughout M. tuberculosis infection (7, 8). It is unclear how these changes in the granuloma (i.e., the chronic environment) impact T cell immunity. The granuloma may limit T cell migration and/or access to Ag, because it was shown that effector T cell functions are diminished, in part, as the result of limiting Ag (9, 10). This apparent change in Ag availability could, in part, allow for bacterial persistence.

Persistant Ag stimulation during other chronic infections often leads to T cell exhaustion (11, 12). Pathogens, such as M. tuberculosis, establish life-long chronic infection; the host must maintain a stable population of functioning effector T cells. Many mechanisms may contribute to the maintenance of T cells during chronic infection, and these likely are pathogen specific. We proposed that T cell responses are maintained during M. tuberculosis infection by an uncharacterized self-renewing effector cell population, because T cells do not appear to undergo clonal exhaustion (13, 14), and highly proliferative CD4 T cells undergo rapid turnover in the lung throughout chronic infection (14, 15). Indeed, peripheral T cell responses are maintained for up to 4 mo postinfection in thymectomized mice (15). Nevertheless, these data do not exclude the possibility that newly generated T cells (i.e., recent thymic emigrants [RTEs]) also contribute to the maintenance of peripheral T cell responses. Some chronic viruses, including polyoma virus and murine CMV, use RTEs to maintain protective T cell responses (16, 17), but the relative contribution of RTEs during M. tuberculosis infection has not been investigated. Moreover, studies of Mycobacterium avium-infected mice demonstrated that infection of the thymus resulted in T cell tolerance and prevented the generation of effector T cell responses to pathogen-specific Ags (18). The findings from the M. avium studies suggested that RTEs do not contribute to peripheral immunity during chronic M. tuberculosis infections.

In the current study, we investigated whether the infected environment within the mouse was capable of supporting the activation of RTEs, thereby allowing recently generated effector T cells...
to contribute to the peripheral T cell response during *M. tuberculosis* infection. We demonstrate that new thymus-derived Ag-specific T cells contribute to peripheral immunity, revealing that central tolerance is not a major factor limiting peripheral T cell responses during *M. tuberculosis* infection. However, the contribution of RTEs diminishes during chronic infection, likely a consequence of the diminished capacity of the chronic environment to support the activation and accumulation of new effector T cells. These studies extend our understanding of how T cell responses are maintained in a chronic inflammatory environment during persistent bacterial infections.

**Materials and Methods**

**Animals**

C57BL/6J and B6.SJL-Ptp<sup>cre</sup> Peps<sup>3β</sup>/BoyJ (CD45.1) mice were purchased from The Jackson Laboratory. C57BL/6 mice were crossed with B6.SJL-Ptp<sup>cre</sup> Peps<sup>3β</sup>/BoyJ mice to generate B6.SJL-Ptp<sup>cre</sup> Peps<sup>3β</sup> mice (indicated as CD45.1/CD45.2). The early secreted antigenic target 6 (ESAT6)-transgenic mice, which are specific for ESAT<sub>6α</sub>-20 presented by I-<sup>Aα</sup>, were described previously (1). Thy1<sup>+</sup> SMI-transgenic mice, which contain CD4 T cells specific for *Salmonella* flagellin<sub>427–441</sub> were also described previously (19). T cell transfers were described previously and used 1 × 10<sup>6</sup> or 2 × 10<sup>6</sup> naive transgenic T cells, which were transferred by i.v. injection into recipient mice (1). All mice were maintained in specific pathogen-free facilities at the Trudeau Institute. Experimental mice were age and sex matched and infected between 8 and 12 wk of age. This study was carried out in strict accord with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were handled according to all applicable institutional, state, and federal animal care guidelines under animal care protocols approved by the Trudeau Institute. Veterinary technicians or laboratory staff assessed animal health daily. Moribund mice were humanely euthanized.

**Bacterial infections**

The H37Rv strain of *M. tuberculosis* was grown in Proskauer and Beck medium containing 0.05% Tween 80 to mid-log phase and was preserved in 1-ml aliquots at −70°C. For aerosol infections, animals were infected with a low dose of bacteria (≈75 CFU), using a Glasc-Col airborne infection system, as described previously (20). Bacteria in the lungs were measured by counting viable CFU in homogenized tissue, as described (20).

**Lymphocyte isolation and flow cytometry**

Lung tissue was prepared for analysis by injecting lungs with a 0.5-mg/ml ammonium chloride solution to lyse erythrocytes. Lymphocytes were isolated and cultured previously and used 1 × 10<sup>6</sup> or 2 × 10<sup>6</sup> naive transgenic T cells, which were transferred by i.v. injection into recipient mice (1). All mice were maintained in specific pathogen-free facilities at the Trudeau Institute. Experimental mice were age and sex matched and infected between 8 and 12 wk of age. This study was carried out in strict accord with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were handled according to all applicable institutional, state, and federal animal care guidelines under animal care protocols approved by the Trudeau Institute. Veterinary technicians or laboratory staff assessed animal health daily. Moribund mice were humanely euthanized.

**In vitro Th1 effector cell generation**

Effector T cell populations were generated by stimulating naive ESAT<sub>6α</sub>-transgenic T cells with 2 μM ESAT<sub>6α</sub>-20 peptide presented by mitomycin C-treated B cell blasts. Cells were cultured and expanded in Th1-polarizing conditions (IL-2, 11 ng/ml; IL-12, 10 μg/ml; and anti–IL-4, 10 μg/ml). The cells were used for experiments 6–8 d postactivation.

**Intracellular cytokine-detection assays**

Lymphocytes were isolated and incubated in a 96-well plate, at a concentration of 3 × 10<sup>5</sup> cells/well, in the presence of the ESAT<sub>6α</sub>-20 or Sendai HN<sub>421–436</sub> peptides (5 μg/ml) for 2 h at 37°C; brefeldin A (50 μg/ml) was added, and the incubation was continued for an additional 4 h. Surface staining for CD4, CD8 was performed, after which the cells were fixed and permeabilized using a Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences). For detection of intracellular IFN-γ and TNF-α, the cells were incubated for 30 min in Perm/Wash Buffer (BD Biosciences) with anti–IFN-γ and anti–TNF-α; the cells were analyzed by flow cytometry.

**Generation of bone marrow chimeras**

Myeloablative drug treatment and bone marrow reconstitution were performed, as previously described (16), by treatment of naive or *M. tuberculosis*-infected CD45.1/CD45.2 mice, or allogeneic congenic mice, with busulfan i.p. (0.6 mg/animal; generously provided by Otsuka America Pharmaceutical, Rockville, MD). One day later, mice were administered, via i.v. injection, 1.5 × 10<sup>7</sup> C57BL/6 bone marrow cells that had been depleted of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells.

**Statistical analysis**

Statistical significance between groups was determined with Prism5 GraphPad software using a two-way ANOVA test. Differences with a p value < 0.05 were considered significant.

**Results**

**The thymus is a site of tuberculosis infection**

To assess whether the chronic infection allows for the development of RTEs and whether infection of the thymus impacts thymic tolerance during *M. tuberculosis* infection, we first examined the kinetics of bacterial dissemination to the thymus after aerosol infection. Similar to observations made by Nobrega et al. (23), we observed that *M. tuberculosis* colonizes the thymus, but infection in that tissue reached a maximum only after day 60 postinfection, much later than in the lung and secondary lymphoid tissues (Fig. 1A). We investigated whether the change in the log linear growth of *M. tuberculosis* within the thymus correlated with the presence of an effector T cell response. Indeed, the apparent delay in the control of bacterial replication within the thymus correlated with a delay in the accumulation of ESAT<sub>6α</sub>-17/K<sup>Aα</sup> tetramer-positive CD4 T cells and Tb10.44<sub>11</sub>/K<sup>a</sup> tetramer-positive CD8 T cells (Fig. 1B, 1C). The thymic effector T cells were functional, because they produced IFN-γ and TNF-α following Ag encounter in vitro (Fig. 1D). These results demonstrate that *M. tuberculosis* infects the thymus and results in the recruitment of effector T cells to the site of infection, albeit later in infection relative to other tissues.

**RTEs contribute to the peripheral T cell response during acute infection**

Infection of the thymus by *M. tuberculosis* raised the possibility that developing thymocytes could become tolerized against bacterial Ags. Therefore, to determine whether new effector T cells generated from RTEs were tolerized or could contribute to the maintenance of the T cell response during *M. tuberculosis* infection, we transferred 1.5 × 10<sup>7</sup> C57BL/6 T cell-depleted bone marrow cells into congenic recipient mice that had been infected with *M. tuberculosis* (on either day 30 or 90 postinfection). Recipient animals were treated 1 d prior to bone marrow transfer with a minimally myeloablative drug (i.e., busulfan) (Fig. 2A). The loss of bone marrow cells caused by busulfan treatment promotes the engraftment of the donor bone marrow cells that subsequently give rise to thymus-derived naive T cells (16); donor cells can be distinguished from host T cells based on allelic differences. Drug treatment did not impact bacterial burdens in mice that had been treated with busulfan and did not detectably affect the host T cell response, because the frequency and number of ESAT<sub>6α</sub>-17/K<sup>Aα</sup> tetramer-positive host CD4 T cells remained normal (Fig. 2B, Supplemental Fig. 1).
Engraftment of donor bone marrow was observed in all mice, and thymus-derived CD4+ CD45.2+ donor T cells were detected in the lungs and secondary lymphoid organs as early as 5 wk postdonor bone marrow transfer. Sixty-three days after donor bone marrow transfer, we examined whether the donor cells were contributing to the peripheral T cell response. A population of donor-derived ESAT6–17/Ab tetramer-positive CD4 T cells was detected in mice that had been reconstituted on day 30 postaerosol infection, indicating that new thymus-derived T cells contributed to the Ag-specific T cell response. In contrast, few, if any, donor-derived Ag-specific T cells were detected within the lungs of mice that had received donor bone marrow on day 90 postinfection (Fig. 2B).

**FIGURE 1.** The thymus is a target of *M. tuberculosis* infection. Mice were infected via the low-dose aerosol route with *M. tuberculosis* (strain H37Rv), and bacterial titers and effector T cell responses were measured. (A) Colonization of the thymus, lung, MLN, spleen, and liver after aerosol *M. tuberculosis* infection. The data shown were accumulated from two experiments. (B) The flow cytometric gating strategy used to analyze ESAT6–17/Ab and Tb10.4–11/Kb Ag-specific T cells in the thymus, on days 11, 33, 68, and 98 postinfection, is shown. (C) The number of ESAT6–17/Ab and Tb10.4–11/Kb Ag-specific T cells detected in the thymus during *M. tuberculosis* infection. (D) Representative dot plots indicate the frequency of IFN-γ- and TNF-α-producing CD4 and CD8 single-positive thymic T cells detected on days 31, 67, and 100 postinfection. The data are representative of three experiments of similar design (n = 5 mice/group).

Engraftment of donor bone marrow was observed in all mice, and thymus-derived CD4+ CD45.2+ donor T cells were detected in the lungs and secondary lymphoid organs as early as 5 wk postdonor bone marrow transfer. Sixty-three days after donor bone marrow transfer, we examined whether the donor cells were contributing to the peripheral T cell response. A population of donor-derived ESAT6–17/Ab tetramer-positive CD4 T cells was detected in mice that had been reconstituted on day 30 postaerosol infection, indicating that new thymus-derived T cells contributed to the Ag-specific T cell response. In contrast, few, if any, donor-derived Ag-specific T cells were detected within the lungs of mice that had received donor bone marrow on day 90 postinfection (Fig. 2B).

**Priming and expansion of naive CD4 T cells decline during chronic infection**

Although tolerance of developing lymphocytes may explain the failure to detect thymus-derived effector T cells after busulfan treatment on day 90 postinfection, an alternative explanation is that the chronic environment was insufficient to drive efficient naive T cell priming and/or expansion. Inefficient T cell activation could have been caused by sequestration of Ag, changes in tissue organization, exclusion of T cells within the granuloma, or insufficient Ag. Support for this was demonstrated in two recent reports, in which the ability of Ag 85b-specific effector cells to respond to Ag in vivo was low during chronic infection (9, 10). To address whether and when Ag was available to prime naive T cells, we transferred 10^6 naive ESAT61–20/Ab-specific transgenic CD4 T cells into infected CD45- and Thyl-congenic recipient mice; 12 h later, primed T cells were identified by their expression of the early activation marker CD69 (Fig. 3A) (1, 24). This approach allowed us to determine whether Ag was present that was capable of stimulating naive T cells. Transferred Ag-specific CD4 T cells were activated within the mediastinal lymph node (MLN), beginning on day 10 postinfection (Fig. 3B) (1). T cell activation was specific, because flagellin427–441/Ab-specific SM1 transgenic T cells transferred into infected mice were not activated (Fig. 3). T cell priming in the MLN was highest during acute infection (i.e., days 18–25), and it declined markedly thereafter. T cell priming was less efficient after day 30, because only ~10% of the donor T cells were activated within 12 h; nevertheless, Ag was available at this time, because the frequency of primed T cells in the MLN continued to increased 36 h posttransfer (Supplemental Fig. 2A). Slightly delayed kinetics of naive T cell priming were observed in the spleen, although higher frequencies of T cells were primed, relative to the MLN, after day 30 postinfection (Fig. 3B). Although T cell priming predominantly occurred in the spleen and MLNs, priming was also observed in the cervical lymph nodes, mesenteric lymph nodes, and lung, albeit at reduced efficiencies (Supplemental Fig. 2B). These results indicate that Ag is available during chronic infection, although it is less efficient at priming T cells.

Although T cell priming decreased during chronic infection, it was not possible in the above studies to determine whether this decrease was due to limiting Ag or whether APCs lacked the capacity to efficiently stimulate naive T cells. To resolve this issue, we used in vitro-differentiated ESAT61–20/Ab-specific Th1 effector cells; such activated T cells are highly sensitive to Ag and can detect Ag presented on many different APCs in the absence of costimulation. We transferred 10^6 in vitro-differentiated ESAT61–20/Ab-specific Th1 effector cells into CD45-congenic recipient mice at several times postinfection; 12 h later, effector T cells that had responded to Ag were identified by their expression of CD69. Transferred
effector T cells readily detected Ag in the spleen, MLNs, and lungs at all time points examined (Fig. 4). In contrast to naive T cells, effector T cells detected similar levels of Ag within the spleen and lungs, at time points as long as 325 d postinfection. Additionally, effector T cells readily detected Ag in the lung (Fig. 4, Supplemental Fig. 2B), a finding that supports our previous studies that concluded that the lung is not an efficient environment for naive T cell priming (1). Effector T cells also detected Ag in the MLN, although, like naive T cells, the effector T cells were stimulated less efficiently in this tissue during chronic infection, suggesting that Ag presentation progressively declined within the MLN during chronic infection. Taken together, these results in-

FIGURE 2. RTEs contribute to the T cell response during M. tuberculosis infection. (A) Experimental protocol. CD45.1/CD45.2 mice were either left uninfected or were infected, via the low-dose aerosol route, with M. tuberculosis (strain H37Rv); 30 or 90 d later, the mice were adminis-

FIGURE 3. Naive T cell priming during M. tuberculosis infection. Naive ESAT6- and SM1-transgenic T cells were isolated from mice by negative magnetic bead selection; 1 × 10^6 cells were injected, at a 1:1 ratio, into infected CD45- and Thy1-congenic recipient mice. Mice were analyzed 12 h after cell transfer on the days indicated. (A) Representative flow cytometric analysis of CD69 expression on ESAT6- or SM1-specific CD45^hi thymus-derived donor and host CD4 T cells were quanti-

FIGURE 4. Effector T cells detect similar levels of Ag during M. tu-

FIGURE 5. Expansion of naive transgenic T cells during acute and chronic M. tuberculosis infection. Naive ESAT6-transgenic T cells (2 × 10^3) were injected i.v. into CD45-congenic mice that had been aerosol infected 11, 30, 60, or 90 d prior. The number of transgenic donor cells in the spleen, MLN, and lung was determined on days 2, 7, and 10 post-

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dicate that the loss of naive T cell priming observed after day 30 postinfection was not completely a result of declining Ag availability within infected tissues.

Because the capacity of the immune system to prime naive T cell responses declined over time following *M. tuberculosis* infection, we next asked how this decline affected the accumulation and/or expansion of RTEs. For these studies, we transferred $2 \times 10^3$ ESAT61–20/I$\alpha$-specific T cells into mice that had been infected with *M. tuberculosis* for 11, 30, 60, or 90 d; donor T cells were enumerated 2, 7, and 10 d later. When transfer was performed on day 11 or 30 postinfection, donor T cells accumulated rapidly in the spleen, MLN, and lung (Fig. 5). In contrast, donor T cells underwent limited expansion and/or accumulation when transferred on day 60 or 90 postinfection. No further increase in donor T cells was detected after day 10 posttransfer, regardless of when the T cells were transferred postinfection. The decline in T cell expansion observed during chronic infection was not due to a failure of the donor cells to differentiate into effector cells, because effector cell phenotype, proliferation, and cytokine production all occurred normally in those cells (Supplemental Fig. 3).

**RTEs accumulate during chronic infection**

Our results demonstrate that, although Ag is presented throughout *M. tuberculosis* infection and is capable of initiating T cell priming, expansion, and differentiation, T cell priming is less efficient during chronic infection. Thus, the failure to detect the accumulation of effector T cells generated from RTEs after day 90 postinfection is likely a consequence of poor T cell priming and a slower accumulation of these cells. To address this hypothesis, we examined RTEs at later times following hematopoietic reconstitution. Mice were treated with busulfan; reconstituted on day 30, 60, or 90 postinfection; and Ag-specific effector RTEs were quantitated in lungs 49, 63, 84, and 140 d postdonor bone marrow transfer. Mice that were reconstituted on day 30 postinfection contained a sizable population of Ag-specific effector RTEs as early as 49 d postdonor bone marrow transfer (Fig. 6A). As the time following reconstitution increased, so did the accumulation of Ag-specific effector RTEs in the lung. In contrast, few Ag-specific effector RTEs were detected when busulfan was administered 60 d postinfection and analysis was performed 49 d postdonor bone marrow transfer. However, when these mice were analyzed 63 d postbone marrow transfer, significant numbers of Ag-specific donor T cells were detected in most mice. The accumulation of Ag-specific effector RTEs was delayed further in mice that had been treated with busulfan on day 90 postinfection, because Ag-specific effector RTEs were detected only after 84 d postdonor bone marrow transfer; however, by day 140 postreconstitution, thymus-derived T cells had accumulated to similar numbers as in mice that were treated with busulfan on either day 30 or day 60 postinfection.

**FIGURE 6.** RTEs contribute to the peripheral CD4 T cell response during chronic infection. Infected CD45-congenic mice were administered 0.6 mg of busulfan on day 30, 60, or 90 postinfection. One day later, $1.5 \times 10^7$ T cell-depleted bone marrow cells from C57BL/6 mice were injected, and ESAT61–20/I$\alpha$–specific CD4 T cells were enumerated in the lungs 49, 63, 84, and 140 d after reconstitution. (A) The number of ESAT61–20/I$\alpha$–specific host and donor CD4 T cells is shown. (B) Representative dot plots of IFN-γ– and TNF-α–producing donor or host CD4 T cells and the frequencies of ESAT61–20/I$\alpha$–specific CD4 T cells from busulfan-treated mice that were analyzed 140 d postreconstitution. Intracellular and tetramer staining were performed on two independent samples from the same animals. (C) The frequencies of ESAT61–20/I$\alpha$–tetramer-positive cells within the donor- and host-expressing populations were plotted against the frequency of ESAT61–20/I$\alpha$ cells that specifically produced IFN-γ for each busulfan-treated mouse within each group; the mice received busulfan on either day 30 or 90 postinfection, because Ag-specific effector RTEs were detected only after 84 d postdonor bone marrow transfer; however, by day 140 postreconstitution, thymus-derived T cells had accumulated to similar numbers as in mice that were treated with busulfan on either day 30 or day 60 postinfection.
Although effector RTEs were detected in chronically infected mice, it was possible that the cells were functionally impaired (i.e., anergic). However, this was not the case because T cells from mice that had been treated with busulfan on day 30 or 90 post-infection produced IFN-γ and TNF-α in response to specific peptide Ag when assayed 140 d postreconstitution (Fig. 6B). The lower frequency of effector RTEs producing cytokine compared with host cells was a result of a difference in the frequencies of Ag-specific T cells within the two populations and was not due to the inability of donor cells to produce cytokines (Fig. 6B). This was evident from comparison of the frequencies of host and donor ESA064-20/Kb tetramer-specific CD4 T cells with the abilities of these cells to produce IFN-γ in response to ESA061-20 peptide (Fig. 6C). These results indicate that the Ag-specific effector RTEs were functional and could contribute to the control of bacterial replication during M. tuberculosis infection. Our findings indicate that the failure to detect CD4 effector RTEs in the periphery of chronically infected busulfan-treated mice was a consequence of their delayed priming and slow accumulation.

These results demonstrate that RTEs contribute to the maintenance of Ag-specific CD4 T cells during chronic M. tuberculosis infection. However, CD4 and CD8 T cells could be maintained by different mechanisms during acute and chronic infections (25). To determine whether RTEs could contribute to the maintenance of Ag-specific CD8 T cells, we examined whether Tb10.44-11/Kb Ag-specific CD8 T cells were present in mice 63 d after donor bone marrow reconstitution on day 30 or 90 post-M. tuberculosis infection (Fig. 7A). Similar to our studies of CD4 T cells, mice that were reconstituted on day 30 postinfection contained appreciable numbers of effector RTEs that had expanded and become part of the ongoing Ag-specific T cell response. However, few, if any, detectable Tb10.44-11/Kb Ag-specific CD8 T cells were present in mice 63 d after donor bone marrow reconstitution on day 30 or 90 post-M. tuberculosis infection (Fig. 7B). Therefore, RTEs can contribute to the maintenance of both the peripheral CD4 and CD8 Ag-specific T cell response during M. tuberculosis infection.

**Discussion**

Our data demonstrate that thymus-derived T cells contribute to the maintenance of T cell responses during acute and chronic M. tuberculosis infection and that thymic tolerance is not a major mechanism impacting the maintenance of protective immunity during chronic infection. The slow accumulation of effector RTEs during infection does not appear to be due to thymic tolerance or anergy, but a consequence of inefficient Ag presentation in the periphery during chronic infection, which limits the accumulation of Ag-specific effector T cells.

The thymus, once thought of as an immune-privileged site, can also be infected by viruses (e.g., HIV, lymphocytic choriomeningitis virus, and measles), parasites (Trypanosoma cruzi), and fungi (Paracoccidioides brasiliensis) (26). The most common consequence of chronic infection is thymic atrophy, but it is not known whether infection of the thymus by such diverse pathogens leads to central tolerance. Our studies indicate that thymic tolerance is not an inevitable outcome of thymic infection, even though a persistent infection is present in the thymus during murine M. tuberculosis infection. One explanation for the apparent lack of central tolerance during M. tuberculosis infection is that bacterial Ags are not expressed by cells in the thymus that are capable of inducing tolerance in developing T cells. Alternatively, the control of infection by day 60 postaerosol may limit the expression of bacterial Ags within the thymus, thereby limiting the induction of thymic tolerance. Although our studies used a mouse model, M. tuberculosis has been detected in the thymi of infected humans (27, 28), although it is not known whether this is a common occurrence in tuberculosis patients. Our results suggest that, even if M. tuberculosis colonizes the thymi in chronically infected humans, developing T cells are able to circumvent central tolerance and contribute to the peripheral immune response.

Our data differ from those in a report that proposed thymic tolerance as a major mechanism of immune evasion during M. avium infection (18). In that study, thymi were transplanted from M. avium-infected TCR-α−/− mice into nude recipient mice prior to infection; under these conditions, the investigators failed to detect effector T cell responses to M. avium Ags ex vivo. Opposing findings from the studies likely derive from differences in the species of Mycobacterium used, routes of infection, the sensitivity of the techniques used to identify RTEs, and the experimental approaches (i.e., transplantation of infected thymi versus the use of busulfan). Another possibility is that the numbers of bacteria or the expression of bacterial Ags were higher within the transferred thymus used in the study by Nobrega et al. (18) relative to M. tuberculosis-infected thymi, where effector T cell responses were observed. We cannot exclude the possibility that some developing T cells were tolerized, because we could not directly
examine whether Ag-specific T cells were deleted from the thymus of chronically infected mice that were treated with busulfan. However, our studies provide definitive evidence that new Ag-specific T cell responses can develop during chronic *M. tuberculosis* infection in the mouse.

A requirement for RTEs in other chronic infections has been documented (16, 17). However, if recruitment of RTEs was the sole mechanism responsible for maintaining the Ag-specific T cell response during chronic infections, eventually all Ag-specific cells would be replaced by donor-derived cells. Our data demonstrate that effector RTEs contribute to the peripheral T cell response, yet they are not solely required for maintenance, because the donor-derived cells never fully replaced host cells. These data support our previous findings that the thymus was not required for the maintenance of Ag-specific T cell responses, for at least as long as 125 d postinfection (15). Thus, RTEs provide only one mechanism by which peripheral T cell responses are maintained during *M. tuberculosis* infection.

The capacity of T cells to be primed during chronic infection is diminished, and this is likely due to changes in the infected environment, a consequence of host control of bacterial replication. The temporal changes in T cell responses could be due to reduced Ag availability, changes in the APC populations and/or their expression of costimulation molecules, the cytokine milieu, the ability of naive RTEs to interact with APC populations, and the ability of naive RTEs to sustain prolonged contact with APCs. We observed differences in the capacity of lymphoid tissues to present *M. tuberculosis* Ags, because both the priming and expansion of naive T cells were decreased in the MLN during chronic infection relative to the spleen. These differences are unlikely to be a consequence of tissue bacterial loads, because these were similar between the two lymphoid organs after day 30 postinfection (Fig. 1A) (14). Indeed, effector T cells responded similarly to Ag during both acute and chronic infection. One possibility is that chronic tuberculosis infection changes the architecture of the spleen and MLN and that this disruption may alter the capacity of APCs to prime naive T cells. Differences among the types or strength of costimulatory molecules, the cytokines being produced, and/or the quantity and quality of available Ag in the different lymphoid tissues could influence the ability of APCs to prime and expand naive T cells (29).

Our finding that naive T cells accumulate to a lesser extent during chronic *M. tuberculosis* infection has potential clinical implications for *M. tuberculosis*-infected individuals with active disease. Our results suggest that it may be advantageous to use additional strategies that invoke an efficient naive T cell activation, in addition to current chemotherapeutic treatments, because such a strategy could lead to decreased periods of chemotherapy treatment. The ability to effectively stimulate new naive T cell responses during chronic *M. tuberculosis* infection may be a mechanism used by the therapeutic RUTI vaccine, which was shown to increase effector T cell responses in *M. tuberculosis*-infected animals that are undergoing chemotherapy (30). Thus, thymic selection and peripheral T cell priming occur throughout chronic *M. tuberculosis* infection in the mouse and contribute to the maintenance of protective T cell responses. Although infection of the thymus occurs in mice and humans, our data also argue that thymic tolerance is not a major mechanism that limits T cell-mediated immunity to *M. tuberculosis* infection.

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**Disclosures**

The authors have no financial conflicts of interest.

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