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Tissue Requirements for Establishing Long-Term CD4+ T Cell–Mediated Immunity following *Leishmania donovani* Infection

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Organ-specific immunity is a feature of many infectious diseases, including visceral leishmaniasis caused by *Leishmania donovani*. Experimental visceral leishmaniasis in genetically susceptible mice is characterized by an acute, resolving infection in the liver and chronic infection in the spleen. CD4+ T cell responses are critical for the establishment and maintenance of hepatic immunity in this disease model, but their role in chronically infected spleens remains unclear. In this study, we show that dendritic cells are critical for CD4+ T cell activation and expansion in all tissue sites examined. We found that FTY720-mediated blockade of T cell trafficking early in infection prevented Ag-specific CD4+ T cells from appearing in lymph nodes, but not the spleen and liver, suggesting that early CD4+ T cell priming does not occur in liver-draining lymph nodes. Extended treatment with FTY720 over the first month of infection increased parasite burdens, although this associated with blockade of lymphocyte egress from secondary lymphoid tissue, as well as with more generalized splenic lymphopenia. Importantly, we demonstrate that CD4+ T cells are required for the establishment and maintenance of antiparasitic immunity in the liver, as well as for immune surveillance and suppression of parasite outgrowth in chronically infected spleens. Finally, although early CD4+ T cell priming appeared to occur most effectively in the spleen, we unexpectedly revealed that protective CD4+ T cell–mediated hepatic immunity could be generated in the complete absence of all secondary lymphoid tissues. *The Journal of Immunology*, 2014, 192: 3709–3718.

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The function of the spleen to remove infectious agents from the circulation also makes it a site of residence for many persistent viruses, bacteria, and parasites, and immune responses targeted at these pathogens may lead to gross splenic pathology. For example, both mice and humans with visceral leishmaniasis (VL), caused by infection with the protozoan parasite *Leishmania donovani*, develop significant splenomegaly accompanied by dysruptions to splenic lymphoid tissue architecture and immune function (reviewed in Refs. 6, 7). Strikingly, splenic pathology develops in infected mice concurrent with the onset of immunity directed toward parasites in the liver. This observation, together with similar findings in other infectious disease models, led to an increasing awareness of organ-specific compartmentalization within the immune system following pathogen challenge (8).

The infection of genetically susceptible mice with *L. donovani* results in parasite growth in the liver, spleen, lymph nodes, and bone marrow (BM) (9–11). Although a chronic infection is established in the latter three tissue sites, a CD4+ T cell–dependent immune response develops in the liver, resulting in the formation of proinflammatory granulomas around infected Kupffer cells associated with control of infection, although sterilizing immunity is rarely, if ever, observed (12–14). Remarkably, despite the presence of high parasite burdens in other infected tissues, the liver remains immune to reinfection by mechanisms dependent on CD4+ T cells (15). However, the requirements for the generation...
and maintenance of hepatic CD4+ T cell–mediated immunity during experimental VL remain poorly understood. Therefore, we investigated the anatomical and migratory requirements for the generation of CD4+ T cell responses and establishment of immunity in the liver following L. donovani infection of C57BL/6 mice.

Materials and Methods

Mice

Inbred female C57BL/6 and B6.SJL-Ptprca (B6.CD45.1) mice were purchased from the Australian Resource Centre (Canning Vale, WA, Australia) and maintained under conventional conditions. B6.RAG1−/− (16), B6.LTα−/− (17), B6.SJL-Ptprca × OT II (18), and B6.CD11c-DTR (19) mice were bred and maintained at the Queensland Institute of Medical Research (QIMR). All mice used were matched for age and sex and were housed under specific pathogen–free conditions. Chimeric mice were prepared by irradiating mice with two doses of 5.5 Gy and then engrafting with 2 × 106 fresh B6.SJL-Ptprca BM cells i.v. via the lateral tail vein. Mice were maintained on antibiotics for 2 wk after engraftment and infected with L. donovani 8–12 wk after receiving BM, as previously described (20). In some experiments, mice underwent a splenectomy procedure, whereby they were injected with buprenorphine (0.1 mg/kg s.c.) 30 min before surgery and then anesthetized with 5% (v/v) isoflurane in 100% (v/v) oxygen. Once unconscious, mice were placed on a heat mat, and anesthesia was maintained by isoflurane (0.5–1.5% [v/v]) delivered by face mask. Fur was removed using clippers, and skin was sterilized by swabbing with chlorhexidine in 70% (v/v) ethanol. A 7-mm left paralumbar incision was made with a scalpel parallel to the last rib, and the three layers of abdominal muscle were split by blunt dissection to expose the spleen. The spleen was exteriorized and removed using a cauterizing pen. The abdominal muscles were closed and sutured in one layer, followed by closing of the skin and suturing. Surgical control mice underwent the same procedure, except that the spleen was not removed. Mice were allowed to regain consciousness in a warm cage and rested for 3–4 wk prior to infection. Depletion of DCs, using diphtheria toxin (DT), was carried out on B6.CD11c-DTR BM chimeric mice, as previously described (21).

All animal procedures were approved and monitored by the QIMR Animal Ethics Committee. This work was conducted under QIMR animal ethics approval number A02-634M, in accordance with the “Australian Code of Practice for the Care and Use of Animals for Scientific Purposes” (Australian National Health and Medical Research Council).

FIGURE 1. Ag-specific CD4+ T cell activation is first observed in the spleen following L. donovani infection. C57BL/6 mice received 2 × 106 CFSE-labeled OT II cells 2 h prior to infection with OVA-transgenic L. donovani. At the times indicated, spleen, liver, lymph nodes (celiac, portal, and mesenteric), and BM were removed, and CFSE dilution of OT II was analyzed by FACS. The gating strategy used is shown (top panels). Graphs show CFSE staining of individual animals in each group that have been overlaid. The arrow indicates the first detected proliferating OT II cells on day 2 in the spleen. CFSE-labeled OT II cells in control mice 5 d.p.i. with wild-type (LV9) L. donovani (i.e., no non-specific CFSE dilution) (far right panels). Bar graphs show percentage and number of proliferating OT II cells. Each bar represents the mean ± SEM. Data are representative of two independent experiments (n = 4 mice/group).
Parasites and infections

L. donovani (LV9) and OVA-transgenic LV9 (PINK LV9) (22) were maintained by passage in B6.RAG1−/− mice, and amastigotes were isolated from the spleens of chronically infected mice. Mice were infected by injecting 2 × 10⁷ amastigotes i.v. via the lateral tail vein, killed at the times indicated by CO₂ asphyxiation, and bled via cardiac puncture. Spleens and perfused livers were removed at the times indicated, and parasite burdens were determined from Diff-Quick–stained impression smears (Lab Aids, Narrabeen, Australia) and expressed as Leishman–Donovan units (number of amastigotes/1000 host nuclei × organ weight [g]) (23). Liver tissue was also preserved in Tissue-Tek O.C.T. compound (Sakura, Torrance, CA). Hepatic, splenic, lymph node, and BM mononuclear cells were isolated as previously described (24).

Anti-CD4 mAb, anti-CD8β mAb, and FTY720 treatment

Ab-producing hybridomas were grown in 5% (v/v) FCS and RPMI 1640 containing 10 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Purified Ab was prepared as previously described (25). Mice were depleted of CD4+ and CD8+ T cells with anti-CD4 (YTS191.1) and anti-CD8β (53-5.8) mAb, respectively, as previously reported (23). Briefly, mice received 0.5 mg mAb via the i.p. route every 3 d at the times indicated. Depletion of T cells was confirmed at the completion of experiments by assessing T cell numbers in the spleen by flow cytometry. More than 95% of CD4+ and CD8+ T cells were depleted by Ab treatment. In all experiments, control mice received the same quantities of the appropriate control rat IgG (Sigma-Aldrich). FTY720 (Cayman Chemicals, Ann Arbor, MI) was diluted in saline and injected i.p. at 1 mg/kg in 150 μl volume on alternate days.

CD4+ T cell proliferation

To assess Ag-specific T cell proliferation in vivo, mice were infected with OVA-transgenic PINK LV9 (22). Splenic OVA-specific OT II T cells were isolated and labeled with CFSE, as previously described (26). CFSE-labeled OT II cells (2 × 10⁶) were adoptively transferred into mice 2 h prior to infection with LV9 or PINK LV9. Expansion of CFSE+ cells in tissues was monitored by FACS at the indicated times. In all of these experiments, control animals were included that received the same number of CFSE-labeled OT II cells but were infected with wild-type parasites. No OT II proliferation was observed in these animals.

Assessment of granuloma formation

The maturation of granulomas was scored around infected Kupffer cells in acetone-fixed liver sections, as previously described (23, 24).

FIGURE 2. DCs are critical for Ag-specific CD4+ T cell activation following L. donovani infection. Chimeric B6.CD11c-DTR mice received 2 × 10⁶ CFSE-labeled OT II cells 2 h prior to infection with OVA-transgenic L. donovani. Half of the animals were treated with DT to deplete DCs, whereas the other half received saline, as indicated. Four days later, spleen, liver, and lymph nodes (celiac, portal, and mesenteric) were removed, and CFSE dilution of OT II was analyzed by FACS. Graphs show CFSE staining of individual animals in each group that have been overlaid. The gating strategy to measure proliferating OT II cells is the same as shown in Fig. 1. Bar graphs show the percentage and number of proliferating OT II cells in control and DT-treated mice. Each bar represents the mean ± SEM. Data are representative of three independent experiments (n = 4 mice/group). *p < 0.05.
Flow cytometry

Allophycocyanin-conjugated anti–TCRβ-chain (H57-597), anti-CD11c (clone N418), PE-Cy5–conjugated anti-CD4 (GK1.5), FITC-conjugated anti-CD45.1 (clone A20), anti–TCRβ-chain (H57-597), Brilliant Violet 421–conjugated anti–IFN-γ (clone XMG1.2), allophycocyanin-Cy7–conjugated anti-NK1.1 (clone PK136), Alexa Fluor 700–conjugated anti-CD8α (clone 53-6.7), Pacific Blue–conjugated anti–CD45R/B220 (clone RA3-6B2), and PerCP-Cy5.5–conjugated anti-CD11b (clone M1/70) were purchased from BioLegend (San Diego, CA) or BD Biosciences (Franklin Lakes, NJ). Dead cells were excluded from the analysis using LIVE/DEAD Fixable Aqua Stain or LIVE/DEAD Fixable Near I-R Stain (Invitrogen-Molecular Probes, Carlsbad, CA), according to the manufacturer’s instructions. The staining of cell surface Ags and intracellular cytokine staining were carried out as described previously (25). FACS was performed on a FACSCanto II or LSRFortessa (BD Biosciences), and data were analyzed using FlowJo software (TreeStar, Ashland, OR). Gating strategies used for analysis are shown in Figs. 1 and 4.

Statistical analysis

Statistical differences between groups were determined using the Mann–Whitney U test by GraphPad Prism version 4.03 for Windows (GraphPad, San Diego, CA); p < 0.05 was considered statistically significant. The distribution of hepatic histological responses was compared using the χ2 analysis with Microsoft Excel software. All data are presented as the mean ± SE, unless otherwise stated.

Results

Early Ag-specific CD4+ T cell expansion occurs in the spleen

To identify where and when Ag-specific CD4+ T cell activation first occurs during experimental VL, we transferred 2 × 10^6 CFSE-labeled OT II cells into C57BL/6 mice infected with OVA-transgenic *L. donovani* and measured proliferation in the spleen, lymph nodes, liver, and BM each day for the first 5 d of infection (Fig. 1). The celiac, portal, and mesenteric lymph nodes were removed for examination in these experiments, because they drain the liver (27). Notably, OT II proliferation was detected only in the spleen 2 d postinfection (p.i.) (indicated by arrow in Fig. 1). The following day, dividing OT II cells were observed in all tissues examined. We included the BM in this analysis because it is a site of *L. donovani* infection (28, 29) and is potentially involved in T cell priming (30). However, after 5 d of infection, the frequency of OT II cells in BM was less than 10, 2, and 0.5% of that found in the liver, lymph nodes, and spleen, respectively, suggesting that this tissue site played a limited role, if any, in T cell priming. Together, these data indicate that the first round of Ag-specific CD4+ T cell division occurs in the spleen 2 d p.i., prior to the presence of dividing cells in other infected tissue sites.
Conventional DCs are critical for Ag-specific CD4+ T cell activation following L. donovani infection

Despite the widely recognized importance of DCs for Ag-specific activation of naive CD4+ T cells (31), this has not been demonstrated in experimental VL. To formally test this, we generated BM chimeric mice in which expression of the DT receptor was strictly confined to hematopoietic CD11c\(^{hi}\) cells (19). These mice were treated with DT or saline, given 2 \times 10^6 CFSE-labeled OT II cells, and infected with OVA-transgenic L. donovani. OT II proliferation in the spleen, liver, and lymph nodes was measured on day 4 p.i. (Fig. 2). As previously reported (21), DT treatment resulted in >90% depletion of conventional DCs (cDCs; CD11c\(^{hi}\) MHCII\(^{hi}\)), but not plasmacytoid DCs (CD11c\(^{int}\) B220/BST2\(^{+}\)), in the spleen (data not shown). Proliferating OT II cells were found in all tissues from control mice, whereas no OT II cell proliferation was observed in any tissue of mice depleted of cDCs (Fig. 2).

These findings formally demonstrate that cDCs are critical for Ag-specific CD4+ T cell activation following L. donovani infection. Ag-specific CD4+ T cell activation occurs in the liver of L. donovani--infected mice

To test whether Ag-specific CD4+ T cells divide in the spleen before migrating to other tissues, we again transferred OT II cells into C57BL/6 mice infected with OVA-transgenic L. donovani, and in addition, treated them with FTY720 to prevent lymphocyte egress from the spleen and all other secondary lymphoid tissues (SLTs) (32, 33). As expected, dividing cells were observed in all tissues from control mice 3 d p.i. (Fig. 3). However, although FTY720 treatment had no effect on OT II proliferation in the spleen, it significantly inhibited the appearance of proliferating OT II cells in lymph nodes, suggesting that Ag-specific CD4+ T cells are not activated in these tissues but, once activated, can traffic through them. However, it should be noted that this blockade was not complete, and some proliferation of Ag-specific CD4+ T cells was still observed in lymph nodes of drug-treated mice. In contrast, FTY720 had minimal impact on OT II division in the liver, indicating that Ag-specific CD4+ T cell activation occurs in this organ. Together, these data indicate that Ag-specific CD4+ T cell activation following L. donovani infection primarily occurs in the spleen and liver, but the spleen appears to play a greater role in this process, as indicated by a larger number of proliferating, Ag-specific CD4+ T cells in this tissue site.

FTY720 treatment prevents CD4+ T cell–mediated immunity from being established in the liver

Our results above indicate that antiparasitic CD4+ T cell responses were generated in the liver. To examine whether these responses were capable of mediating efficient antiparasitic immunity, we next assessed hepatic parasite burdens in mice continuously treated with FTY720 to block T cell trafficking from the spleen and lymph nodes to the liver over the first month of infection (Fig. 4). As expected from previous studies (34), depletion of CD4+ T cells over this time caused increased parasite growth in mice by day 28 p.i. (Fig. 4A). Importantly, mice treated with FTY720 also had elevated parasite burdens by day 28 p.i. compared with control animals (Fig. 4A), suggesting that parasite-specific CD4+ T cells had to traffic through SLTs and back into the liver to control hepatic infection. In support of this, we found reduced numbers of liver mononuclear cells (Fig. 4C), total CD4+ T cells (Fig. 4D), and IFN-γ–producing CD4+ T cells (Fig. 4B, 4E) at day 28 p.i. in mice treated with FTY720 compared with control animals. Although FTY720 reduced the number of IFN-γ–producing CD4+ T cells (Fig. 4E), the frequency of these cells was not different (Fig. 4B).

Thus, we cannot exclude the possibility that the drug might reduce CD4+ T cells in the liver without impacting upon the ability to prime antiparasitic CD4+ T cell responses.

FTY720 was reported to alter the positioning of DCs within the spleen (35), as well as reduce splenic DC number and frequency of...
responder T cells in a model of graft-versus-host disease (36). Therefore, a more detailed analysis of the kinetics of CD4+ T cell responses in the liver and spleen of L. donovani–infected mice treated for 28 d with FTY720 was undertaken (Fig. 5), including examination of expression of CD49d and CD11a activation markers on CD4+ T cells, because these were reported to mark Ag-experienced CD4+ T cells (37). We found increased hepatic parasite burden (Fig. 5A) and decreased hepatic mononuclear cell (Fig. 5B) and CD4+ T cell (Fig. 5C) numbers, accompanied by a reduction in the number of Ag-experienced CD4+ T cells (Fig. 5D) in FTY720-treated mice compared with control mice, as well as similar effects in the spleen at day 28 p.i. (Fig. 5, right panels). Thus, these data suggest that FTY720 has other effects on CD4+ T cell activation following L. donovani infection not related to blocking egress of lymphocytes out of SLTs.

**Maintenance of hepatic immunity and control of parasite growth in the chronically infected spleen require CD4+ T cells**

We next investigated the impact of CD4+ and CD8+ T cell depletion on the maintenance of antiparasitic immunity at day 56 p.i., after parasite-specific CD4+ T cells had been generated and controlled infection in the liver but not the spleen. Depletion of CD4+ T cells in mice between days 56 and 70 p.i. caused increased parasite growth in the liver and spleen (Fig. 6A). This confirmed a key role for these cells in maintaining immunity in the liver (15) and showed that they mediate control over parasite growth in the spleen during chronic infection. In contrast, depletion of CD8+ T cells at this time had no impact on parasite burdens. We also delayed CD4+ T cell depletion until a later time (days 90–104 p.i.) to establish whether protective CD4+ T cell–mediated immunity diminished. However, we again found that depletion of CD4+ T cells over this time caused increased parasite growth in the liver and spleen (Fig. 6B), confirming a critical role for this cell population in the maintenance of hepatic immunity and control of parasite growth in the chronically infected spleen.

*Hepatic immunity can be generated in the complete absence of SLT following L. donovani infection*

Finally, as an alternative way to test whether antiparasitic immunity in the liver required any SLT involvement, we generated mice lacking a spleen and/or lymph nodes by engrafting wild-type BM into lethally irradiated B6.LTα−/− mice, which lack all lymph...
nodes (17), and then splenectomizing or subjecting them to a mock surgical procedure. Mice lacking a spleen, lymph nodes, or both were able to control \textit{L. donovani} infection in the liver with similar kinetics to control animals (Fig. 7A). Furthermore, no differences in hepatic granuloma development, a critical \textit{CD4}+ T cell–dependent, antiparasitic mechanism (34), were observed by day 28 p.i. (Fig. 7B). Importantly, when mice were reinfected at day 56 p.i., parasite growth was controlled rapidly in all groups (Fig. 7A, left panel) relative to age-matched, naive C57BL/6 mice (Fig. 7A, right panel). In addition, there were increased numbers of liver mononuclear cells, total \textit{CD4}+ T cells, and IFN-γ-producing \textit{CD4}+ T cells (Fig. 7C–E) in all groups after both primary and secondary infection. Together, these data clearly indicate that antiparasitic immunity in the liver can be generated and maintained in the absence of SLTs.

\textbf{Discussion}

\textit{L. donovani} infects and resides in tissue macrophages of the spleen, liver, lymph nodes, and BM. However, the extent of disease in these different tissue sites varies considerably among human VL patients (38). C57BL/6 mice experimentally infected with this parasite display one pattern of this disease profile, whereby the spleen and BM harbor relatively high parasite burdens for life, associated with considerable tissue pathology, whereas the liver is a site of resolving infection that becomes immune to reinfection (7, 8, 34). As such, this experimental model provides the opportunity to study the establishment of immunity in liver, as well as factors that promote chronic infection and disease pathogenesis in the spleen in the same infected animal. Given the importance of secondary lymphoid structure for the activation of \textit{CD4}+ T cells (39), as well as the maintenance of \textit{CD4}+ T cell memory by enabling appropriate lymphocyte movement (40), we examined whether protective \textit{CD4}+ T cell immunity could develop and be maintained in the liver. Our findings indicate that Ag-specific \textit{CD4}+ T cell responses can be generated and maintained in the liver of genetically susceptible mice following \textit{L. donovani} infection. Furthermore, we also found that \textit{CD4}+ T cells played a critical role in controlling parasite growth in chronically infected spleens.

Previous reports indicated that \textit{CD4}+ T cells from VL patients were refractory to Ag stimulation, as indicated by a lack of response in the leishmanin skin test and a failure of PBMCs to proliferate and produce IFN-γ in response to parasite Ags (41). However, more recent work showed that \textit{CD4}+ T cells from VL patients can respond to parasite Ag in whole-blood assays, suggesting that sample processing may have contributed to earlier results (42–44). Our results showing that \textit{CD4}+ T cells were important for controlling parasite burden in chronically infected spleens of \textit{L. donovani}–infected mice (Fig. 6) support the idea that \textit{CD4}+ T cells in chronically infected tissues are not refractory to Ag stimulation and potentially can be targeted for therapeutic advantage. IL-10 has been identified as an important suppressor of parasite-specific \textit{CD4}+ T cell responses in VL patients (44, 45); therefore, transient blockade of this cytokine or similar immune-suppressive molecules may be one approach to harnessing the antiparasitic potential of \textit{CD4}+ T cells in infected individuals.

Previous work with alymphoplastic mice suggested that \textit{CD4}+ and \textit{CD8}+ T cell responses can be generated in the liver in the absence of SLTs (46). Similarly, lymphoid tissues in the lung (BALB) were reported to generate primary T cell responses against influenza (47), supporting the idea that naive T cells can be activated in nonlymphoid tissues. However, in both of these studies, the importance of nonlymphoid T cell activation in tissue-intact animals was not fully addressed. Furthermore, the possibility that T cell activation might occur in the BM, as reported by other investigators (30), was not excluded. Nevertheless, elegant studies using liver transplantation in mice with SLTs showed that Ag-specific \textit{CD8}+ T cells could be activated in recipient mice, even though the recipients’ MHC class I molecules were unable to present the antigenic peptide (48), thus providing strong evidence for the activation of naive \textit{CD8}+ T cells in nonlymphoid tissues.

Our results indicate that there were 10–100-fold more Ag-specific \textit{CD4}+ T cells in the spleen compared with the liver in the first few days of \textit{L. donovani} infection (Fig. 1), suggesting that this tissue is normally the major site for early \textit{CD4}+ T cell activation in this model. However, the liver is a site where relatively large numbers of NKT cells reside (49), and we showed previously that these cells expand and become activated following \textit{L. donovani} infection, although they only play a minor role in controlling parasite growth (24). Our studies in mice lacking lymph nodes and spleen indicate that activation of conventional, Ag-specific \textit{CD4}+ T cells also can occur in the liver during experimental VL. In addition, in FTY720-treated animals, when Ag-specific \textit{CD4}+ T cell trafficking to lymph nodes had been blocked, albeit not completely (Fig. 3), Ag-specific \textit{CD4}+ T cell activation was able to occur in both the spleen and liver, confirming that Ag-specific \textit{CD4}+ T cell activation could occur in the liver. The finding that hepatic immunity could be generated in mice lacking a spleen, lymph nodes, or both (Fig. 7) further supports this conclusion and also indicates that SLTs were not required for the establishment and maintenance of this immunity.

The ability of conventional, antiparasitic \textit{CD4}+ T cells activated in the liver to maintain residency in this organ was difficult to assess because of the potential effects of long-term FTY720 treatment not relating to blockade of lymphocyte egress from SLTs (Figs. 4, 5). T cells move through the liver via sinusoids, and intravital two-photon imaging studies on Ag-specific \textit{CD8}+ T cell movements through the liver in \textit{L. donovani}–infected mice show that these cells leave the sinusoids and enter granulomas formed...
around infected Kupffer cells, where they appear to sample cell surfaces before exiting the granuloma structure back into the sinusoids (50). Whether Ag-specific CD4+ T cells exhibit a similar pattern of cell movement in this model is not known. However, when hepatic immunity has been established, they are critical for controlling persisting parasites in this tissue site. Whether these cells are true liver-resident cells has not been formally established; however, if this were the case, one possible location for them would be in the hepatic granulomas that remain intact long after parasite growth has been controlled in this tissue ($180$ d; C.R. Engwerda, unpublished observations). It should be emphasized that sterilizing immunity is not established in this model, and that at least some of these remaining hepatic granulomas are likely to be sites for low numbers of persisting parasites, as borne out in recent computational modeling of granuloma diversity (51). Such granulomas may provide the location for sustained local Ag presentation to parasite-specific CD4+ T cells. Thus, our findings suggest the existence of a tissue-resident CD4+ T cell population responsible for maintaining immunity in the liver. Importantly, a large-scale genome-wide association study (52) on populations from India and Brazil recently identified HLA class II region polymorphisms as important genetic risk factors for VL, indicating that CD4+ T cells are likely to play key roles in determining the outcome of VL in humans.

As mentioned above, when FTY720 was administered early during infection (days 0–28 p.i.), there appeared to be drug effects not related to blocking lymphocyte egress out of SLTs, because antiparasitic immunity, including numbers of Ag-experienced CD4+ T cells, was diminished in the spleen, as well as the liver (Fig. 5). If FTY720 was simply preventing lymphocyte egress from SLTs, then one might predict an accumulation of lymphocytes in these tissue sites. Previous work (36) in a model of graft-versus-host disease reported similar findings, which may be related to the effects of FTY720 on DCs’ ability to move in the spleen and position themselves for efficient CD4+ T cell activation (35). Our results indicate a similar effect; however, because the drug did not interfere with Ag-specific CD4+ T cell proliferation in the first 3 d of infection (Fig. 3), these effects appear to impact upon DCs recruited to tissues p.i. In experiments involving FTY720 treatment during established infection, we also observed decreased mononuclear cell numbers in the liver and spleen (data not shown), consistent with previous reports showing that long-term drug treatment results in decreased lymphocyte numbers in the body (53). Thus, we were unable to assess the requirements for tissue migration of CD4+ T cells on the maintenance of antiparasitic immunity in infected organs. However, it was clear from our findings in allogeneic mice (Fig. 7) that potent hepatic anti-
parasitic CD4+ T cell responses could be generated and maintained in the absence of SLTs.

One unexpected finding from our studies was the amount of time taken before CD4+ T cell responses were able to mediate control of parasite growth in the liver. The impact of CD4+ T cell depletion was not observed until after day 14 p.i. (Fig. 4A), whereas previous studies in B6.RAG1−/− mice indicated a clear defect in the control of parasite growth by this time point (26, 54, 55). Hence, either immune regulatory pathways are initiated soon p.i. that can suppress antiparasitic CD4+ T cell development or some early antiparasitic activity can be generated in the absence of CD4+ T cells. In support of the former possibility, we observed that Ag-specific (OT II) CD4+ T cell numbers increased for 3 d following infection in the spleen and 4 d in the liver and lymph nodes and then stopped (Fig. 1). The reason for this halt in cell expansion is not clear, but likely reflects a mechanism of immune modulation contributing to inefficient control of parasite growth in the liver and the establishment of a chronic infection in the spleen and BM. Regardless, CD4+ T cells ultimately are able to control parasite growth in the liver (Fig. 4) and are required for continued surveillance in this tissue site (Fig. 6) (15), as well as containing parasitemia in the spleen during the chronic phase of infection (Fig. 6).

In conclusion, we demonstrate that DCs are critical for CD4+ T cell activation following L. donovani infection and that antiparasitic CD4+ T cell responses can be generated and maintain immunity in the liver. If we can better understand how these protective CD4+ T cell responses are generated, we may be able to target vaccines or therapies for their more efficient development. Our finding that CD4+ T cells play an active role in controlling parasite growth in the spleen during chronic infection also indicates that they can be targeted to better eliminate parasites from this tissue. Thus, our work identifies new approaches to try and control an important parasitic disease of humans.

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