Lack of CXCR3 Delays the Development of Hepatic Inflammation but Does Not Impair Resistance to *Leishmania donovani*  

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CXCR chemokine receptor 3 (CXCR3) ligands CXCL9 and CXCL10 are produced at high levels in mice and humans infected with *Leishmania donovani*, but their contribution to host resistance against *L. donovani* is not clear. Here, using CXCR3−/− mice, we demonstrate that, although CXCR3 regulates early immune cell trafficking and hepatic inflammation during *L. donovani* infection, it is not essential for immunity against *L. donovani*, unlike *L. major*. CXCR3−/− C57BL/6 mice show a delayed onset of hepatic inflammation and granuloma formation after *L. donovani* infection. However, they mount an efficient T helper cell type 1 response, recruit T cells to the liver, and control parasite growth as efficiently as do CXCR3+/+ C57BL/6 mice.

*Leishmania donovani* is an intracellular protozoan parasite that causes visceral leishmaniasis (VL). VL is characterized by dissemination of parasites into the spleen, liver, lymph nodes, and bone marrow, resulting in hepatosplenomegaly, fever, abdominal pain, and weight loss. The disease is fatal if left untreated, because of complications such as secondary bacterial infection, anemia, and malnutrition [1].

CXCR3 is expressed on a variety of leukocytes, including CD4+ Th1 cells. Three of the main ligands for CXCR3 are CXCL9 (also known as “monokine induced by interferon [IFN]–γ,” or MIG), CXCL10 (also known as “IFN-inducible protein 10,” or IP-10), and CXCL11 (also known as “IFN-inducible T cell α-chemoattractant,” or I-TAC), and their induction is closely associated with Th1-dominated responses [2]. CXCR3 and its ligands play a critical role in the recruitment of leukocytes to inflammatory sites in several diverse models of disease [3]. Additionally, some experiments have shown that CXCR3 ligands also regulate T cell activation and IFN-γ production [4].

Experiments conducted in our laboratory have shown that CXCR3 is required for the resolution of primary *L. major* infection [5]. In the absence of CXCR3, *L. major*-resistant C57BL/6 mice mount a Th1 response but recruit fewer CD4+ and CD8+ T cells to infected skin, produce less IFN-γ locally, and develop lesions full of parasites [5]. In contrast, wild-type mice effectively control parasite growth and show a significant increase in CXCR3-expressing T cells in both the regional lymph nodes and the lesions [5]. These findings indicate that CXCR3 plays a nonredundant role in the trafficking of IFN-γ-producing effector T cells to infected skin during cutaneous leishmaniasis.

CXCR3 and its ligands also regulate recruitment of T cells to the liver during inflammation [6]. Furthermore, patients with VL showed elevated levels of CXCL9 and CXCL10 in their serum during active infection [7]. Despite these observations, it is not clear whether CXCR3 and its ligands contribute to host resistance against *L. donovani*. Therefore, we examined the in vivo role played by CXCR3 in mediating host resistance against VL caused by *L. donovani* by use of CXCR3−/− C57BL/6 mice. Our results demonstrate that, although CXCR3 may contribute to the development of hepatic inflammation initially, it is not essential for Th1 development, leukocyte recruitment, and resolution of *L. donovani* infection.

**Materials and methods.** Eight- to 10-week-old, sex-matched CXCR3−/− C57BL/6 and wild-type C57BL/6 mice (Harlan) were infected intravenously with 1 × 107 *L. donovani* (1 Sudan strain) amastigotes harvested from the spleens of infected hamsters. Mice were housed and maintained in conformity with the institutional guidelines for animal research at the Ohio State University. Disease progression was monitored by measuring parasite loads in livers and spleens 15, 30, and 60 days after infection, as described elsewhere [8]. Also, hematoxylin-eosin–stained tissue sections from the livers were examined at these time points, to enumerate granulomas as described elsewhere [8]. Furthermore, spleens were removed from these mice, and parasite-specific T cell proliferation was
assessed by Alamar Blue assay (Biosource International). Levels of IFN-γ, interleukin (IL)–12p70, IL-4, and IL-10 in supernatants were measured by ELISA, as described elsewhere [8].

For flow cytometry analysis, single-cell suspensions of liver leukocytes were obtained as described elsewhere [8]. Cells (1 × 10^6–2 × 10^6) were stained with fluorescein isothiocyanate– and phycoerythrin-conjugated antibodies purchased from Biolegend and BD PharMingen. Single-cell suspensions of splenocytes were obtained as described for the proliferation assay and were stained in a manner similar to the liver cells. Flow cytometry analysis was then performed using a FACSCalibur flow cytometer and CellQuest Pro software (Becton Dickinson).

Total RNA was extracted from infected livers by use of the SV Total RNA Isolation System (Promega). mRNA was reverse transcribed, and cDNA was amplified in an Opticon2 DNA Engine (BioRad) using SYBR Green Taq polymerase. The primers used to amplify the cDNA in this semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) were found using the PrimerBank Web site (http://pga.mgh.harvard.edu/primerbank/index.html). In all of the experiments, Student’s unpaired t test was used to determine the statistical significance of differences in the values observed. \(P < 0.05\) was considered to be significant.

**Results and discussion.** We have previously demonstrated that CXCR3 is required for immunity against cutaneous leishmaniasis caused by *L. major* [5]. In the present study, however, CXCR3−/− C57BL/6 mice did not show increased susceptibility to *L. donovani* and did control *L. donovani* growth in the liver and spleen as efficiently as resistant wild-type C57BL/6 (figure 1A and 1B). Interestingly, 30 days after infection, CXCR3−/− mice showed more-rapid resolution of parasite burden in their spleens than did CXCR3+/+ mice, indicating that a lack of CXCR3 facilitates parasite clearance from the spleen (figure 1B). Together, these findings demonstrate that CXCR3 is not required for the control of *L. donovani* infection in mice. Furthermore, they also suggest that a CXCR3-dependent mechanism may be involved in promoting *L. donovani* growth in the spleen.

CXCR3 controls the migration of CD4+ and CD8+ T cells to the site of inflammation in several diseases [3, 9], and CXCR3−/−
C57BL/6 mice show a significant impairment of T cell recruitment to the skin during L. major infection [5]. Effector CD4+ and CD8+ T cells recruited to the liver during L. donovani infection are critical for the host defense against L. donovani, but they also contribute to granuloma formation and induce liver disease. We therefore analyzed hepatic inflammation and enumerated granulomas in the livers of L. donovani–infected CXCR3+/+ and CXCR3−/− C57BL/6 mice on days 15, 30, and 60 after infection. The onset of hepatic inflammation and granuloma formation was delayed in CXCR3−/− mice, and these mice had significantly fewer foci of inflammation and granulomas in their livers than did CXCR3+/+ mice on days 15 and 30 after infection (figure 1C and 1D). However, both groups eventually developed similar hepatic inflammation and contained comparable numbers of granulomas by day 60 (figure 1C and 1D). Flow cytometry analysis showed that the infected livers of CXCR3−/− mice contained 50% less CD11b+ macrophages, CD4+ T cells, and CD8+ T cells on day 15 than did CXCR3+/+ mice, but both groups contained comparable numbers of these cell types in their livers at later time points (data not shown). These findings indicate that, despite a delayed onset of cellular infiltration and inflammation, CXCR3−/− mice ultimately recruit sufficient effector immune cells to the site of L. donovani infection. Our observations are similar to those of a previous study, which reported that a lack of CXCR3 impairs early granuloma formation during Mycobacterium tuberculosis infection in mice but has no effect on the final outcome of the infection; furthermore, this study also found that neutrophils control granuloma formation via a CXCR3-dependent pathway [10]. However, it is unlikely that a lack of neutrophil recruitment to the liver impaired early granuloma formation in CXCR3−/− mice during L. donovani infection, given that flow cytometry analysis showed that both CXCR3+/+ and CXCR3−/− mice recruited comparable proportions of Gr1+ neutrophils to their livers during infection (data not shown). Nevertheless, our data show that, although CXCR3 may be involved in the control of early hepatic inflammation and granuloma formation after L. donovani infection, it is not essential for the recruitment of immune cell types in the livers of infected mice.
cells to the liver during the late stage of the infection. Additionally, these findings also suggest that other T cell–associated C-C and CXC chemokine receptors may be involved in the migration of effector CD4⁺ and CD8⁺ T cells to the liver and spleen. One candidate is CXCR6, which interacts with CXCL16 and mediates T cell recruitment to the inflamed liver. Further study of this chemokine–chemokine receptor interaction during VL should prove worthwhile in the exploration of liver-specific T cell migration.

There may be several reasons why a lack of CXCR3 impairs the migration of T cells to the skin during L. major infection but has no effect on T cell trafficking to the liver and spleen during L. donovani infection. First, L. major and L. donovani cause different diseases in humans and mice and trigger complex immune responses that determine the outcome of the disease in the host. Second, it is becoming increasingly evident that chemokine receptors play organ-specific roles in the regulation of leukocyte trafficking. For example, selective recruitment of naive lymphocytes to the secondary lymphoid organs is mediated largely by the action of CCR7 and its ligands, whereas CCR9 is involved in T cell development in the thymus. Similarly, among the so-called inflammatory chemokine receptors, CCR4 and CCR10 have been implicated in the selective recruitment of certain T cell subsets to the skin [11]. Our findings in the present study, together with our previous findings, demonstrate clearly that, even though CXCR3 is necessary for optimal recruitment of T cells to the skin, other chemokine-dependent mechanisms efficiently recruit T cells to spleen and liver tissue during inflammation.

IL-12–driven CD4⁺ Th1 lymphocytes play a critical role in immunity to L. donovani [12]. However, the clear Th1-Th2 pattern of disease development shown for L. major is not observed in VL caused by L. donovani in mice and humans, because Th2 cytokines (such as IL-4 and IL-13) do not determine susceptibility [13, 14]. In fact, IL-4⁺ and IL-4Rα⁻/⁻ mice are more susceptible to L. donovani, indicating that IL-4 may play a disease-protective role during VL [15]. Previous studies have shown that CXCR3 plays a critical role in T cell activation. Similarly, CXCL9 and CXCL10 also stimulate T cell proliferation and effector cytokine production [4]. We therefore analyzed Th1 and Th2 responses in L. donovani–infected CXCR3⁻/⁻ and CXCR3⁻/⁻ mice by determining titers of L. donovani antigen (LdAg)–specific Th1-associated IgG2a and Th2-associated IgG1 and by measuring proliferation and cytokine production in spleen cells after in vitro stimulation with LdAg. Additionally, we compared levels of tumor necrosis factor (TNF)–α, IL-12, IFN-γ, IL-4, and IL-10 mRNA in infected livers by real-time RT-PCR. Throughout the course of infection, both groups displayed similar titers of L. donovani–specific Th1-associated IgG2a and Th2-associated IgG1 (data not shown). On days 15, 30, and 60 after infection, LdAg-stimulated spleen cells from L. donovani–infected CXCR3⁻/⁻ and CXCR3⁻/⁻ mice displayed comparable proliferation responses (data not shown) and produced comparable amounts of IL-12, IFN-γ, IL-4, and IL-10 (figure 2A–2D). At all time points, infected livers from both groups contained comparable amounts of TNF-α, IL-12, IFN-γ, and IL-10 mRNA. Levels of IL-4 mRNA were also similar in CXCR3⁻/⁻ and CXCR3⁻/⁻ mice on days 15 and 30; however, CXCR3⁻/⁻ mice consistently contained significantly more IL-4 mRNA in their livers on day 60 (figure 2E–2I). Additionally, no significant difference in CXCL9 and CXCL10 mRNA levels was found in the livers of CXCR3⁻/⁻ and CXCR3⁻/⁻ mice at all time points assayed (data not shown). These results demonstrate that a lack of CXCR3 does not alter T cell activation, production of Th1 and Th2 cytokines, and recruitment of IFN-γ–producing cells to the liver and spleen during L. donovani infection.

In sum, although CXCR3⁻/⁻ C57BL/6 mice show delayed formation of liver granulomas after L. donovani infection, they eventually recruit sufficient numbers of T cells to the liver and spleen and control L. donovani infection as efficiently as do CXCR3⁺/⁺ C57BL/6 mice. Furthermore, resolution of L. donovani infection by CXCR3⁻/⁻ mice is associated with the development of an efficient Th1 response. These results also suggest that, although the CXCR3 ligands CXCL9 and CXCL10 are produced at high levels during L. donovani infection, they may play a minor role in the host defense against this parasite.

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References


