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Article

CCR2 signaling contributes to the differentiation of protective inflammatory dendritic cells in *Leishmania braziliensis* infection

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ABSTRACT

In vertebrate hosts, *Leishmania braziliensis* parasites infect mainly mononuclear phagocytic system cells, which when activated by T helper cell type 1 cytokines produce nitric oxide and kill the pathogens. Chemokine (C–C motif) receptor 2 is a chemokine receptor that binds primarily chemokine (C–C motif) ligand 2 and has an important role in the recruitment of monocytic phagocytes. Although it has been reported that *Leishmania braziliensis* infection induces CCR2 expression in the lesions, the role of CCR2 during *Leishmania braziliensis* infection remains unknown. Here, we showed that CCR2 has a role in mediating protection against *Leishmania braziliensis* infection in mice. The absence of CCR2 resulted in increased susceptibility to infection and was associated with low amounts of Ly6C$^+$ inflammatory dendritic cells in the lesions, which we found to be the major sources of tumor necrosis factor production and induced nitric oxide synthase expression in C57BL/6 mice lesions. Consequently, CCR2$^{-/-}$ mice showed decreased tumor necrosis factor production and induced nitric oxide synthase expression, resulting in impaired parasite elimination. We also demonstrated that CCR2 has a role in directly mediating the differentiation of monocytes into inflammatory dendritic cells at the infection sites, contributing to the accumulation of inflammatory dendritic cells in *Leishmania braziliensis* lesions and subsequent control of parasite replication. Therefore, these data provide new information on the role of chemokines during the immune response to infections and identify a potential target for therapeutic interventions in cutaneous leishmaniasis.
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Introduction

Upon transmission through the bite of an infected insect vector, *Leishmania* parasites infect phagocytic cells, mainly macrophages [1], which, along with other APCs, can present parasite antigens associated with MHC class II molecules to CD4$^+$ T lymphocytes. Activated APCs produce cytokines, such as IL-12, which drive the differentiation of Th1 cells [2, 3], which primarily produce IFN-γ. These cytokines trigger the activation of the enzyme iNOS in macrophages, resulting in NO synthesis and parasite killing [4, 5]. Activated APCs also produce TNF, which acts synergistically with IFN-γ to potentiate iNOS activation and NO production [6].

During the inflammatory response initiated by the insect vector bites, the major sources of macrophages are monocytes that migrate from the bloodstream to the tissue [7, 8]. The monocytes proceed toward a gradient of chemoattractant molecules, one of the most important being CCL2, which binds to the cell surface receptor CCR2 [9, 10]. In the *Leishmania major* model of murine infection, the recruitment of blood monocytes to infected tissues through the CCL2–CCR2 axis is important for the protective responses mediated by monocytes themselves and by macrophages derived from monocytes [11, 12]. In the *Leishmania braziliensis* murine model of infection, CCL2 is produced soon after parasite inoculation, and the chemokine production and CCR2 expression are associated with increased inflammatory responses at the lesion sites [13]. In humans infected with *L. braziliensis*, the expression of CCL2 in lesions and the production of TNF by CCR2$^+$ monocytes are also associated with increased inflammatory response [14].

Different than *L. major*, *L. braziliensis* has the ability to induce strong Th1 responses in most strains of mice [15]. Moreover, contrary to what is seen in *L. major* infection, where Th1 response is beneficial, the exacerbation of this type of response driven by *L. braziliensis* may be detrimental to the host, causing more-severe

Abbreviations: DC = dendritic cell, LN = lymph node, MDSC = myeloid-derived suppressor cell, wpi = weeks postinfection, WT = wild type

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lesions and disease [16, 17]. Therefore, it remains unknown whether the role of CCR2 signaling is beneficial or detrimental during *L. braziliensis* infection. Also, the role of CCR2 in the maturation and differentiation of monocytes at the infectious site is poorly understood.

Using a murine model of *L. braziliensis* cutaneous leishmaniasis, we found that CCR2 mediates protection against the infection. Our results show that the accumulation of TNF/iNOS-producing inflammatory DCs in *L. braziliensis* infection sites is dependent on CCR2 expression and controls parasite replication. Finally, we describe CCR2 as having a direct role in the induction of monocyte differentiation into inflammatory DCs at the sites of infection. Thus, these data bring new light to the role of chemokines in immune response to infectious diseases, which is not restricted to chemotaxis, but can also effectively mediate cell activation and differentiation during control of infection.

MATERIALS AND METHODS

Mice

Female C57BL/6 and CCR2$^{-/-}$ mice, 6 to 8 wk old, were used in all experiments. The animals were bred and maintained at the animal facilities of Ribeirão Preto Medical School, University of São Paulo. The local Ethics Committee in Animal Research from Ribeirão Preto Medical School, University of São Paulo, approved all procedures (process HCRP 2534/2003).

Parasites

Leishmania braziliensis parasites, isolate LTCP393 [18], were used in all experiments. Promastigotes were grown at 25°C in Schneider insect medium (Sigma, Saint Louis, MO, USA), supplemented with 20% heat-inactivated FCS, 2 mM L-glutamine, 4 mM $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco, Grand Island, NY, USA), and 2% v/v male human urine. Parasites in the stationary phase of culture were used for all experiments.

Experimental infections, lesion kinetics determination, and estimation of parasite load

Mice were inoculated in the right ear dermis with 1×10^6 stationary-phase *L. braziliensis* LTCP393 promastigotes in 10 μl of sterile PBS using 30-gauge needle syringes. Lesion thickness was measured weekly using a caliper (Mitutoyo, Suzano, SP, Brazil) and was defined as the difference between the thickness of the infected and the noninfected, contralateral ear. Parasite load was determined using a quantitative limiting dilution assay, described previously [19].

Cell isolation from lesions, LNs, and bone marrow

Ears of infected mice were initially incubated at 37°C for 2 h in RPMI 1640 without serum (Gibco) containing 200 μg/ml Liberase TL (Roche, Basel, Switzerland) and then processed in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (all from Gibco) (complete RPMI medium) along with 2 mM EDTA. Cells were then filtered through a 70-μm nylon membrane. Retromaxillar LNs from infected mice were macerated through a nylon membrane with 70-μm pores. Bone marrow cells were isolated from the femurs and tibiae of uninfected mice, by flushing with PBS. In all procedures, cell viability was assessed by trypan blue exclusion before adjusting cell concentration.

Flow cytometry

Cells were stained with antibodies specific for CD4 (clone GK1.5), CD8 (clone 53-6.7), CD11b (clone M1/70), CD11c (clone N418), Ly6C (clone A21), IFN-γ (clone XMG 1.2), and TNF (clone MP6-XT22), conjugated to FITC, PE, PerCP, PECy7, APC, or APCCy7 fluorochromes (BD Bioscience and eBioscience, San Diego, CA, USA). For iNOS staining, a rabbit polyclonal antibody was used (Santa Cruz Biotechnologies, Dallas, TX, USA), followed by staining with a secondary antibody conjugated to Alexa Fluor 647 (Life technologies, Grand Island, NY, USA). For intracellular staining, the Cytofix/Cytoperm kit was used (BD Biosciences). Cell acquisition was performed using a FACSCanto II flow cytometer and FACSDiva software (BD Biosciences). Data were plotted and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Cytokine production quantification

Lymph node cells (5×10^5/well) were seeded in 96-well plates in 200 μl of complete RPMI 1640 medium (Gibco) and stimulated or not with live, stationary phase, *L. braziliensis* promastigotes (5 parasites per cell, 2.5×10^6 parasites/well). Cells were incubated at 37°C, 5% CO_2 atmosphere, for 48 h to detect TNF and IL-12p70 production, or for 72 h to detect IFN-γ, IL-4, and IL-10 production in supernatants, all using ELISA commercial kits (BD Biosciences and R&D Systems, Minneapolis, MN, USA). Ear lesion cells (2×10^6 cells/well) were seeded into 24-well plates in 500 μl of complete RPMI medium (Gibco). For detecting IFN-γ production by flow cytometry, cells were stimulated for 6 h with PMA (20 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug (brefeldin) (BD Biosciences), according to the manufacturer's instructions. To detect TNF and iNOS production by flow cytometry, cells were stimulated with live, stationary phase, *L. braziliensis* promastigotes (5 parasites per cell, 1×10^7 parasites/well), and Golgi Plug (BD Biosciences) was added in the last 6 h of culture, according to the manufacturer's instructions.

Cell transfer experiments

Bone marrow cells from uninfected C57BL/6 and CCR2$^{-/-}$ mice were stained for CD11b, CD11c, and Ly6C. Cellular debris, leukocytes, and granulocytes were excluded by size and granularity characteristics. The cells in the monocytes gate exhibiting the CD11c$^-$CD11b$^+$Ly6C$^+$ phenotype were isolated with a FACSAria III sorter (BD Biosciences) and stained with CFSE before transfer. A total of 3×10^6 purified cells from C57BL/6 or CCR2$^{-/-}$ mice were transferred i.v. into CCR2$^{-/-}$ mice, which were infected 4 wk earlier with *L. braziliensis*. Three days later, spleens, bone marrow, blood, retromaxillar LNs, and ear lesions were collected from the transferred mice, and the percentage of CD11b$^+$CFSE$^+$ cells, as well as those that had become CD11c$^+$ or that remained CD11c$^-$ were analyzed by flow cytometry using a FACSCanto II cytometer (BD Biosciences) and FlowJo software (Tree Star).

DC differentiation and culture

Bone marrow cells from uninfected C57BL/6 mice were cultured with recombinant murine GM-CSF (20 ng/ml) (PeproTech, Rocky Hill, NJ, USA) in the presence or absence of recombinant murine CCL2 (100 ng/ml) (R&D Systems). On day 3, additional supplemented medium containing the same concentration of stimuli was added, and on day 6, the nonadherent cells were collected. The cells that were differentiated under the 2 different conditions presented similar CD11c$^+$ expression patterns of around 90%. Next, 5×10^5 cells/well were distributed in 24-well plates in 300 μl of complete RPMI medium and cultured at 37°C, 5% CO_2 atmosphere, in the presence of medium alone; live, stationary phase, *L. braziliensis* promastigotes; stationary phase, *L. braziliensis* promastigotes; stationary phase, *L. braziliensis* promastigotes plus recombinant murine IFN-γ (10 ng/ml); or stationary phase, *L. braziliensis* promastigotes plus recombinant murine CCL2 (100 ng/ml) plus recombinant murine IFN-γ (10 ng/ml). For TNF quantification, 5 parasites/cell were used as stimulus, whereas 8 parasites/cell were used for NO quantification and parasite-killing quantification. Supernatants were removed after 24 h to quantify TNF production by ELISA assay or after 48 h for indirect quantification of NO production using the Griess method [20]. For quantifying parasite killing, cells were removed from plates, centrifuged onto microscope slides in a Shandon Cytospin3

centrifuge (Thermo Scientific, Waltham, MA, USA) and stained with Giemsa; after which, the number of amastigotes per 100 infected DCs was determined.

Statistical analyses

In all experiments, the differences between the groups were analyzed using Student's *t* test. Differences were considered significant when $P < 0.05$. All analyses were performed using Prism software (GraphPad Software, La Jolla, CA, USA).

RESULTS

CCR2$^{-/-}$ mice are more susceptible to *L. braziliensis* infection

Lesion development in WT and CCR2$^{-/-}$ mice after *L. braziliensis* infection was similar until 3 wpi, and lesion size remained similar between the groups until 4 wpi. After 3 wpi, lesions in WT mice started to heal, whereas CCR2$^{-/-}$ mice developed more-severe lesions, particularly between 4 and 5 wpi, which remained constant in size until 8 wpi (**Fig. 1A**). The larger lesions found in CCR2$^{-/-}$ mice were associated with impaired control of parasite growth in ear lesions and draining LNs at 8 wpi (Fig. 1B). Therefore, these data demonstrate that CCR2 has a role in the control of *L. braziliensis* infection.

Leishmania braziliensis–infected CCR2$^{-/-}$ mice present fewer Ly6C^{+} DCs in lesions

Because CCR2 is important for recruitment of monocytes/macrophages and DCs [9], we analyzed the phenotypes of these cells in WT and CCR2$^{-/-}$ mice lesions and retromaxillar draining LNs. Both the frequency (**Fig. 2A** and **B**) and total number (Fig. 2C) of CD11b^{+}CD11c^{+} cells (DCs) were drastically reduced in lesions of CCR2$^{-/-}$ mice compared with WT mice at 4 and 8 wpi. At the draining LNs, the frequency of DCs was only modestly lower in CCR2$^{-/-}$ mice at 4 wpi (Fig. 2A and B), and no differences in the total number of these cells were observed (Fig. 2C). In contrast, the frequency (Fig. 2A and D) and total number (Fig. 2E) of CD11b^{+}CD11c^{-} cells (macrophages) were increased in the lesions of CCR2$^{-/-}$ mice at 4 wpi, whereas, at 8 wpi, the frequency and number were lower in CCR2$^{-/-}$ compared with lesions from WT mice. At the draining LNs, however, there was no difference in the frequency and number of macrophages between CCR2$^{-/-}$ and WT mice (Fig. 2A, D, and E). Because CCR2 mediates monocyte chemotaxis to inflamed tissues, and DCs generated from inflammatory monocytes express Ly6C [21], we evaluated Ly6C expression in DCs from *L. braziliensis*–infected WT and CCR2$^{-/-}$ mice. We found that both the frequency and total number of Ly6C^{+} DCs were higher in lesions from WT compared with CCR2$^{-/-}$ mice at 4 and 8 wpi (Fig. 2F, G, and H). In the draining LNs, we also found a lower frequency of Ly6C^{+} DCs in CCR2$^{-/-}$ mice at 4 and 8 wpi (Fig. 2F and G), and the total number of these cells was higher in WT mice at 4 wpi (Fig. 2H). However, the magnitude of the difference observed in the draining LNs was not as striking as that observed in the lesions. Therefore, these results indicate that the increased susceptibility of CCR2$^{-/-}$ mice to *L. braziliensis* infection is associated with reduced amounts of Ly6C^{+} DCs, mainly at the lesion sites.

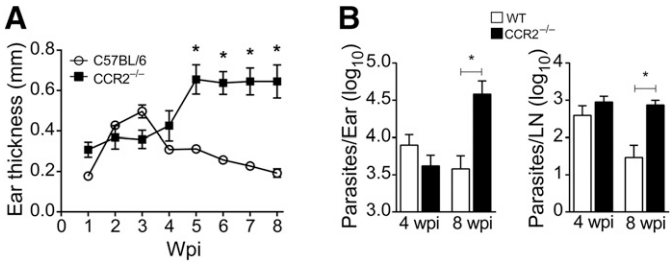

Figure 1. Kinetics of lesion development and parasite load in *L. braziliensis*–infected mice. C57BL/6 (WT) (black square) and CCR2$^{-/-}$ (white circle) *L. braziliensis*–infected mice were monitored by 8 wk. (A) Lesion thickness in millimeters, determined as the difference between the infected ear and the contralateral, noninfected ear. (B) Parasite load in the ear lesions and retromaxillar draining LNs from C57BL/6 (WT) (white bars) and CCR2$^{-/-}$ (black bars) at 4 and 8 wpi. Results are expressed as the means ± SEM and are representative of 3 independent experiments; 4 mice/group in each experiment. $^{*}P < 0.05$.

CCR2$^{-/-}$ mice present impaired TNF production and iNOS expression

We next asked whether the pattern of cytokines produced in response to infection would also be affected by CCR2 deficiency. We found that the production of IFN-γ (**Fig. 3A**), IL-4 (Fig. 3B), IL-10 (Fig. 3C), and IL-12p70 (Fig. 3D) by draining LN cells stimulated with live *L. braziliensis* promastigotes were similar in WT and CCR2$^{-/-}$ mice, whereas TNF production by CCR2$^{-/-}$ mice cells was lower than WT mice cells only at 4 wpi (Fig. 3E). Because neither Th1 nor Th2 responses in the LNs were affected by the absence of CCR2, the TNF source, in this case, is presumably innate immune cells. To evaluate the cytokines produced by inflammatory cells at the site of the *L. braziliensis* infection, we stimulated lesion-derived cells with PMA and ionomycin. IL-4 production by CD4^{+} and CD8^{+} T cells was similarly low in both groups (Supplemental Fig. 1A and B), indicating that CCR2 deficiency did not result in differences in Th2 response at the lesion sites. IL-10 production by the same cell populations was also similarly low in both groups (Supplemental Fig. 1A and B), and no differences in the frequency of CD4^{+}FoxP3^{+} cells in lesions and draining LNs were observed (Supplemental Fig. 1C and D), indicating that CCR2 deficiency did not result in changes in T_{reg} responses. In both groups, primarily CD4^{+} T cells produced IFN-γ, and the frequency of IFN-γ producing CD4^{+} and CD8^{+} T cells was also similar in WT and CCR2$^{-/-}$ mice (**Fig. 4A** and **B**). To detect cytokine production by myeloid cells, we stimulated the lesion-derived cells for 24 h with live parasites and found that the frequency of CD11b^{+}TNF^{+} cells was lower in CCR2$^{-/-}$ mice (Fig. 4C and D). We then evaluated the contribution of DCs and macrophages to TNF production in lesions of mice from both groups. In WT mice, we observed that among the CD11b^{+}TNF^{+} cells, DCs (CD11c^{+} cells) were the major population producing TNF at 4 and 8 wpi, whereas, in CCR2$^{-/-}$ mice, TNF production was evenly distributed between DCs and macrophages (CD11c^{-} cells) at 4 wpi, but DCs were the major cell population producing TNF at 8 wpi (Fig. 4C and E). We next analyzed the frequency of Ly6C^{+} and Ly6C^{-} subsets among the TNF-producing DCs (CD11b^{+}TNF^{+}CD11c^{+} cells) in both groups and found that Ly6C^{+}

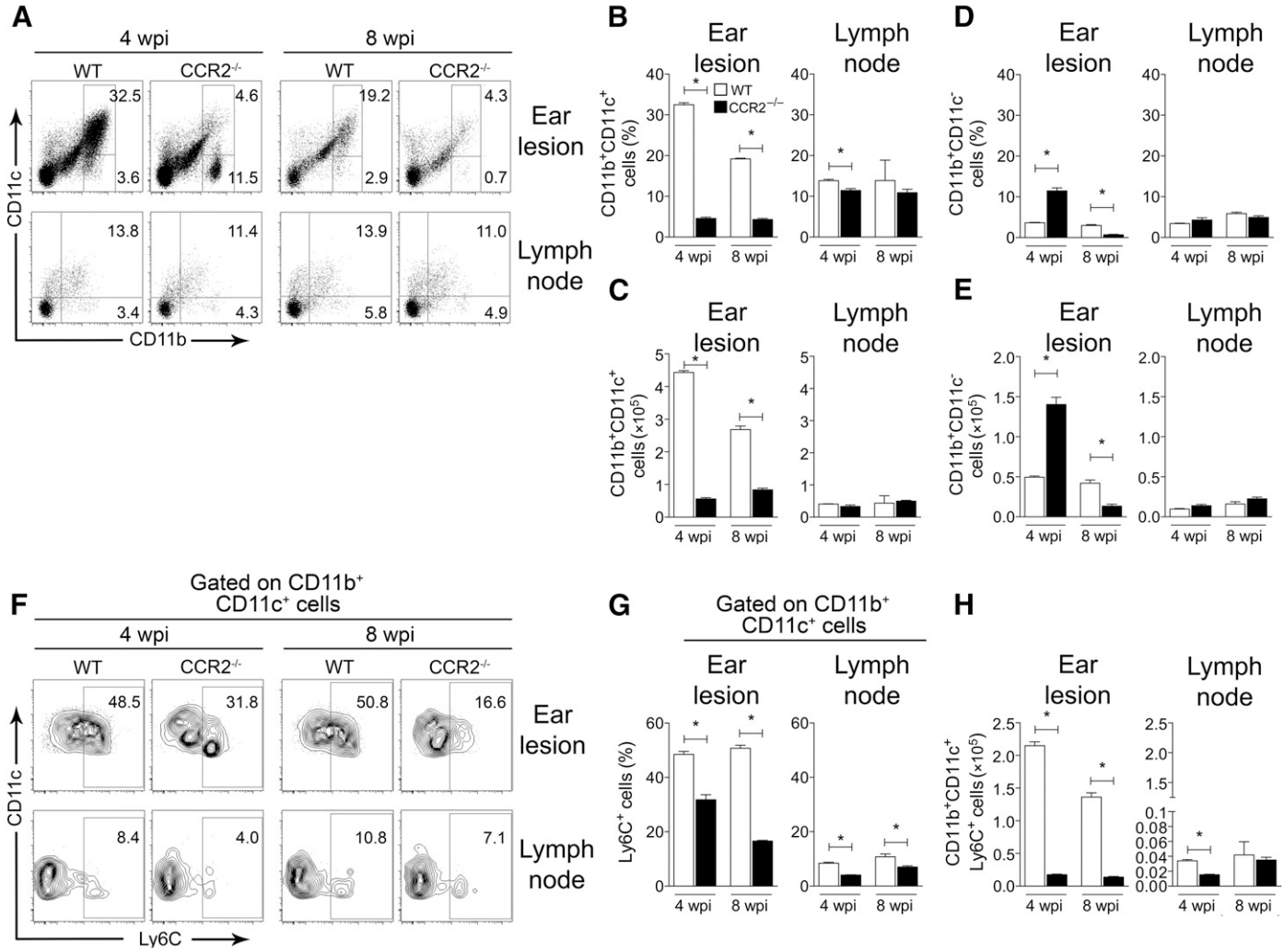

Figure 2. Quantification of macrophages and DCs in lesions and LNs of *L. braziliensis*–infected mice. The frequency of leukocytes in the ear lesions and retromaxillar draining LNs of C57BL/6 (WT) (white bars) and CCR2$^{-/-}$ (black bars) *Leishmania braziliensis*–infected mice were quantified by flow cytometry at 4 and 8 wpi. (A) Representative dot plots showing the frequency of macrophages (CD11b^{+}CD11c^{-}) and DCs (CD11b^{+}CD11c^{+}). Graphs show the frequency (B) and total number (C) of DCs (CD11b^{+}CD11c^{+}); and the frequency (D) and total number (E) of macrophages (CD11b^{+}CD11c^{+}). (F) Representative dot plots showing the frequency of Ly6C^{+} DCs (gated in CD11b^{+}CD11c^{+} cells). Graphs showing the frequency DCs expressing Ly6C^{+} (G) and the total number of Ly6C^{+} DCs (H) (both gated in CD11b^{+}CD11c^{+} cells). The cells were initially gated according to size and granularity characteristic monocytic cells (exclusion of granulocytic and lymphocytic gates). Results are expressed as representative dot plots or as means ± SEM and are representative of 3 independent experiments, 8 mice/group in each experiment. *$P < 0.05$.

cell subset was the major population of DCs producing TNF in both WT and CCR2$^{-/-}$ mice at both time points (Fig. 4C and F). Similar to what was observed regarding TNF production, the frequency of CD11b^{+}iNOS^{+} cells was also lower in lesions of CCR2$^{-/-}$ mice compared with WT mice (Fig. 4G and H). We found that, among the CD11b^{+}iNOS^{+} cells, DCs (CD11c^{+} cells) were the major population expressing iNOS in both groups of mice, at 4 and 8 wpi (Fig. 4G and I). Among the iNOS-expressing

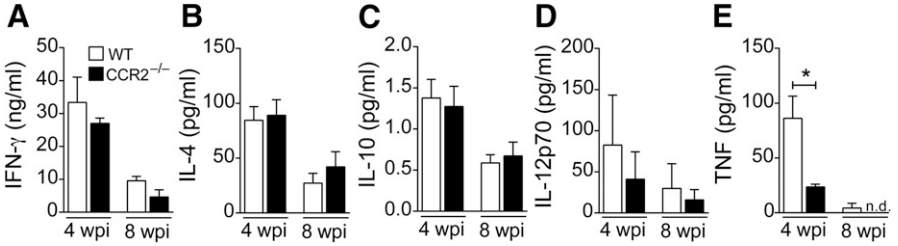

Figure 3. Cytokine production by LN cells isolated from *L. braziliensis*–infected mice. Concentrations of IFN-γ (A), IL-4 (B), IL-10 (C), IL-12p70 (D), and TNF (E) in supernatants of cultures of retromaxillar LN cells from *L. braziliensis*–infected C57BL/6 (WT) (white bars) and CCR2$^{-/-}$ (black bars) mice, harvested at 4 and 8 wpi and stimulated with live promastigotes. Results are expressed as means ± SEM and are representative of 2 independent experiments, 4 mice/group in each experiment. *$P < 0.05$. n.d., not detected.

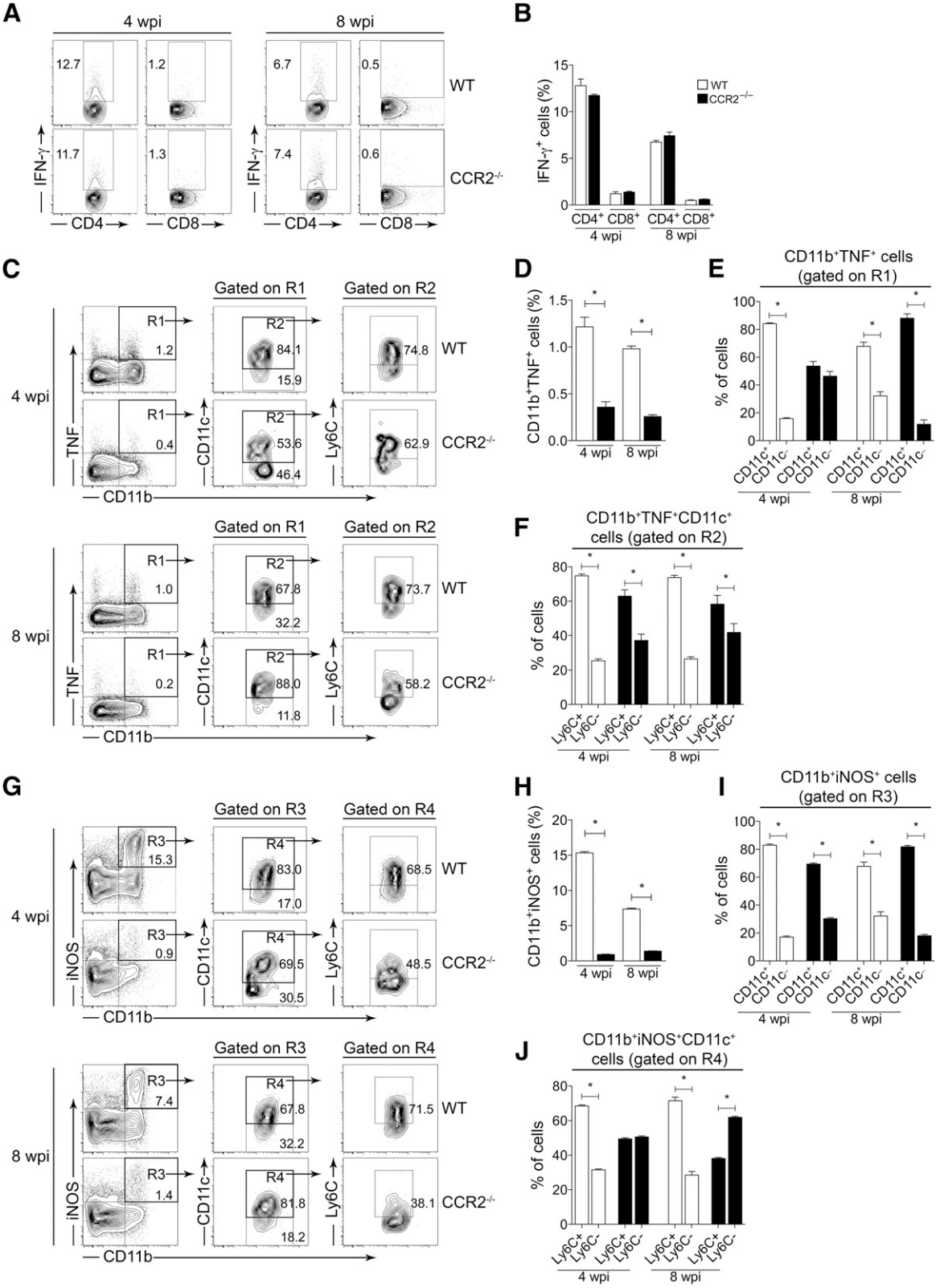

Figure 4. Cytokine expression and iNOS activation in leukocytes from the ear lesions of *L. braziliensis*–infected mice. Leukocytes from ear lesions of C57BL/6 (WT) (white bars) and CCR2$^{-/-}$ (black bars), *L. braziliensis*–infected mice were isolated at 4 and 8 wpi. Representative dot plots (A) and graph (B) showing IFN-γ production by CD4$^+$ and CD8$^+$ T cells. (C) Representative dot plots showing phenotypic characterization of TNF-producing

(*continued on next page*)

DCs ($CD11b^+iNOS^+CD11c^+$ cells), the $Ly6C^+$ subset was the main source of iNOS in WT mice at 4 and 8 wpi, whereas, in $CCR2^{-/-}$ mice, both $Ly6C^+$ and $Ly6C^-$ cells contributed equally to the iNOS expression by the DCs at 4 wpi, but at 8 wpi, the $Ly6C^+$ subset was the major DC population to express iNOS (Fig. 4G and J). These results show that during *L. braziliensis* infection, the absence of CCR2 results in impaired production of TNF and expression of iNOS by myeloid cells from the lesions. They also show that $Ly6C^+$ DCs are the main source of TNF and iNOS in the lesions of *L. braziliensis*–infected WT mice, whereas, in lesions of $CCR2^{-/-}$ mice, although present in drastically decreased number, $Ly6C^+$ DCs still provided an important contribution to the observed residual production of TNF and iNOS. Therefore, the impaired TNF production and iNOS expression found in $CCR2^{-/-}$ mouse lesions is most likely directly associated to the profound decrease in the number of $Ly6C^+$ DCs found in this group.

CCR2 contributes to $CD11b^+Ly6C^+$ monocyte differentiation into DCs in *L. braziliensis* lesions

Next, we asked whether CCR2 is involved in the recruitment of $CD11b^+Ly6C^+$ monocytes from the bloodstream to infectious foci or whether CCR2 signaling has a direct role in the differentiation of $CD11b^+Ly6C^+$ monocytes into DCs in inflamed tissues. To address this question, we adoptively transferred CFSE-stained bone marrow $CD11b^+CD11c^-Ly6C^+$ monocytes (Supplemental Fig. 2A and B), purified from uninfected C57BL/6 or $CCR2^{-/-}$ mice, to $CCR2^{-/-}$ mice infected with *L. braziliensis* 4 wk earlier. The monocyte precursors MDP (macrophage dendritic cell progenitor) and cMoP (common monocyte progenitor) undergo constant proliferation at the bone marrow, but after their differentiation into monocytes, the mature cells are not expected to proliferate in the blood or in peripheral sites of inflammation they migrate to and where they perform their effector functions [22]. Therefore, CFSE staining of monocytes can be used as a method to track the migration of these cells, without decay of the CFSE staining from proliferation. Using this approach, we observed that 3 d after cell transfer, very few $CD11b^+CFSE^+$ cells were found in the spleens, bone marrow, blood, and retromaxillar LNs of mice from either group (**Fig. 5A**). However, the frequency of $CFSE^+$ cells in the ear lesions of both groups of mice was significantly greater than that observed in the other organs analyzed, and the frequency of $CFSE^+$ cells was similar in lesions of mice that received WT or $CCR2^{-/-}$ cells (Fig. 5B and C). These results demonstrate that monocyte migration to the lesions of *L. braziliensis*–infected mice is not impaired in the absence of CCR2. Because most transferred cells were located in the lesions, we next quantified the percentage of these cells that became $CD11c^+$ at that site. We found higher amounts of transferred cells that became $CD11c^+$ in the lesions of mice that received WT monocytes than in mice that received $CCR2^{-/-}$ monocytes (Fig. 5B and D). Therefore, these results suggest that CCR2 deficiency compromises the conversion of monocytes into DCs at the infection site and that CCR2 signaling has a direct role in the differentiation of $CD11b^+Ly6C^+$ monocytes into DCs in *L. braziliensis* lesions.

CCL2 induces differentiation of DCs with proinflammatory profiles

Because CCL2 is the major CCR2 ligand, we next asked whether CCL2 is necessary during DC differentiation to induce a proinflammatory profile. To answer this question, we performed in vitro DC differentiation in the presence or absence of CCL2 and evaluated the production of TNF and NO as well as the leishmanicidal activity of these cells 6 d later. The addition of increasing concentrations of CCL2 (1–100 ng/ml) during DC differentiation did not change the pattern of expression of CD11b and CD11c (Supplemental Fig. 3A and B) nor Ly6C (Supplemental Fig. 3C and D) in the resulting DCs. Even the highest concentration of CCL2 used did not result in accelerated in vitro development of DCs (Supplemental Fig. 3E). However, DCs differentiated in the presence of GM-CSF and CCL2, then challenged with *L. braziliensis* promastigotes and either CCL2, IFN-γ, or IFN-γ plus CCL2, produced higher levels of TNF than did those differentiated in the presence of GM-CSF alone (**Fig. 6A**). NO production was detected only when the DCs were challenged with *L. braziliensis* plus IFN-γ or plus IFN-γ and CCL2, and in both cases, differentiation in the presence of CCL2 resulted in increased NO production (Fig. 6B). We further observed that DCs differentiated in the presence of CCL2 exhibited increased leishmanicidal activity when stimulated with IFN-γ or IFN-γ plus CCL2 (Fig. 6C); therefore, the higher production of NO by DCs differentiated in the presence of CCL2 was translated into enhanced microbicidal activity. Also, greater TNF and NO production and leishmanicidal activity were observed only when CCL2 was present during the differentiation phase of the DCs. The addition of CCL2 and IFN-γ to differentiated cells did not result in enhancement of TNF or NO production compared with stimulation with IFN-γ alone (Figs 6A, B, and C). The addition of pertussis toxin, an irreversible inhibitor of G protein-coupled receptors, which blocks the effects of CCL2 on chemokine receptors, to the differentiation media resulted in complete loss of the cells' ability to produce TNF and NO upon further stimulation, even in those cells that were differentiated in the presence of GM-CSF alone. However, the addition of increasing doses of CCL2 to the differentiation media resulted in dose-dependent increases in the production of TNF and NO by the DCs (not shown). We also evaluated CCR2 expression in bone marrow cells and observed that the receptor is expressed by common DC precursors (Supplemental Fig. 3F)

$CD11b^+$ cells. (D) Graph showing frequency of TNF-producing $CD11b^+$ cells. (E) Frequency of DCs ($CD11c^+$) and macrophages ($CD11c^-$) among $CD11b^+TNF^+$ cells (R1 gate from Fig. 4C). (F) Frequency of $Ly6C^+$ and $Ly6C^-$ subsets among $CD11b^+TNF^+CD11c^+$ cells (R2 gate from Fig. 4C). (G) Representative dot plots showing phonotypic characterization of iNOS-expressing $CD11b^+$ cells. (H) Graph showing frequency of iNOS-expressing $CD11b^+$ cells. (I) Frequency of DCs ($CD11c^+$) and macrophages ($CD11c^-$) and among $CD11b^+iNOS^+$ cells (R3 gate from Fig. 4G). (J) Frequency of $Ly6C^+$ and $Ly6C^-$ subsets among $CD11b^+iNOS^+CD11c^+$ cells (R4 gate from Fig. 4G). The cells were initially gated according to size and granularity characteristics of the lymphocytic cells (A and B) or monocytic cells (exclusion of granulocytic and lymphocytic gates). Results are expressed as representative dot plots or as means ± SEM and are representative of 2 independent experiments, 8 mice/group in each experiment. $*P < 0.05$.

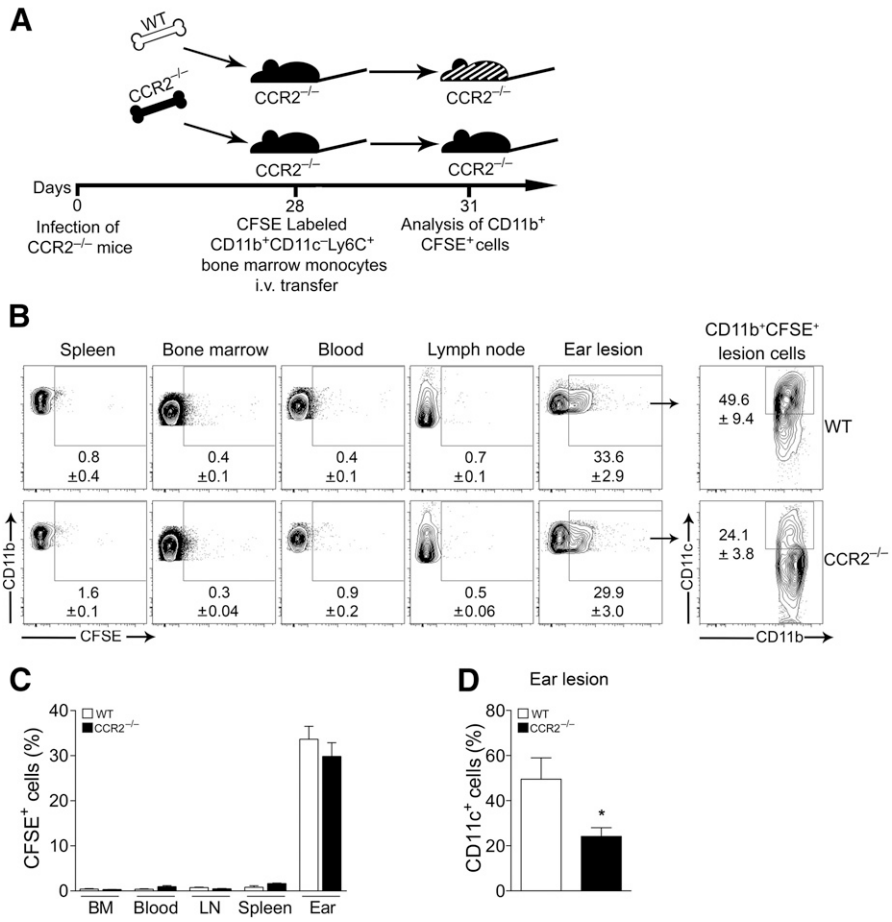

Figure 5. Outcomes of WT and CCR2$^{-/-}$ bone marrow Ly6C$^+$ monocytes transferred into *L. braziliensis*–infected CCR2$^{-/-}$ mice. (A) CD11c$^-$CD11b$^+$Ly6C$^+$ monocytes from C57BL/6 (WT) (white bars) or CCR2$^{-/-}$ (black bars), uninfected mice were stained with CFSE and transferred i.v. into CCR2$^{-/-}$ mice, which were infected with *L. braziliensis* 4 wk earlier. Three days later, transferred cells were recovered from different sites and analyzed. (B) Representative dot plots showing the frequency of CD11b$^+$CFSE$^+$ cells recovered from different sites, as well as the frequency of CD11c$^+$ population among CD11b$^+$CFSE$^+$ cells recovered from the ear lesions. Graphs showing the frequency of CD11b$^+$CFSE$^+$ cells recovered from different sites 3 d after transfer (C); and the frequency of CD11c$^+$ population among CD11b$^+$CFSE$^+$ cells recovered from the ear lesions at the same time point (D). Results are expressed as representative dot plots or as means ± SEM and are representative of 2 independent experiments, 5 mice/group in each experiment. *$P < 0.05$.

and CD11c$^+$Ly6C$^+$ monocytes (Supplemental Fig. 3G), both of which can be differentiated into DCs, and it is also expressed by differentiated DCs (Supplemental Fig. 3H). Because CCR2 is considered to be the only identified receptor for CCL2 in myeloid cells, except from decoy receptor D6 [23–25], the effect of CCL2 in DCs in this case is presumably mediated by signaling through CCR2. Therefore, these results show that during the DC differentiation phase, CCL2 induces the generation of DCs with an inflammatory profile and enhanced microbicidal activity. In addition, this suggests that in vivo, the CCL2 production at sites of *L. braziliensis* infection can also induce the differentiation of DCs with similar characteristics, resulting in more-efficient control of parasite replication.

DISCUSSION

Leishmania braziliensis infection induces an intense inflammatory infiltration characterized by the presence of macrophages and DCs and massive Th1 polarization in patients and mice [18, 26–28]. The CCL2/CCR2 axis is classically associated with mononuclear phagocyte recruitment and Th1 responses [29],

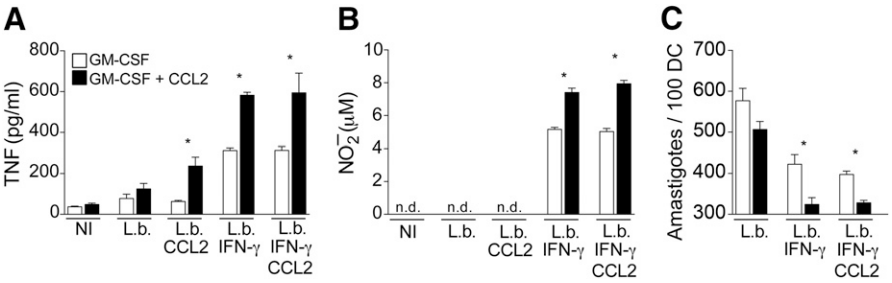

Figure 6. Production of TNF, NO, and parasite killing by in vitro, differentiated DCs. Bone marrow cells from C57BL/6 (WT) mice were isolated and differentiated in vitro in the presence of GM-CSF (20 ng/ml) (white bars) or GM-CSF (20 ng/ml) plus CCL2 (100 ng/ml) (black bars). Afterward, cells were stimulated with medium alone, medium plus live, stationary phase, *L. braziliensis* promastigotes (L.b.), L.b. plus CCL2 (100 ng/ml), L.b. plus IFN-γ (10 ng/ml), or L.b. plus both IFN-γ (10 ng/ml) and CCL2 (100 ng/ml), and the production of TNF (A) and NO (B) was determined 72 and 48 h later, respectively. (C) Cells were stimulated with L.b., L.b. plus IFN-γ (10 ng/ml), or L.b. plus both IFN-γ (10 ng/ml) and CCL2 (100 ng/ml) for 48 h; stained with Giemsa; and the number of *L. braziliensis* amastigotes per 100 DCs was determined. Results are expressed as means ± SEM and are representative of 2 independent experiments, both performed in triplicate. *$P < 0.05$; n.d., not detected; NI, non infected.

and although *L. braziliensis* infection is known to trigger CCL2 production [13], the role played by CCR2 signaling during *L. braziliensis* infection was unknown. However, in the present work, we characterized how CCR2 signaling contributes to the generation of a protective host immune response against infection with *L. braziliensis* in mice.

The development of efficient Th1 responses is widely known to be critical for protection against *Leishmania* infection [5]. However, the greater susceptibility to parasite replication, and consequently, the increased severity of the lesions that we observed in $CCR2^{-/-}$ mice upon *L. braziliensis* infection, was not related to impaired Th1 responses, increased production of Th2 cytokines, or increased frequency of T_{regs} in lesions and draining LNs. In *L. major* infection, CCR2 deficiency results in susceptibility to infection, which is associated with a skewed T cell response toward a Th2 pattern. This is caused by alterations in Langerhans cell migration and maturation, which result in inefficient Th1 cell priming and differentiation [30]. *Leishmania braziliensis*, however, is known to promote enhanced DC activation in comparison to *L. major* [31]. Therefore, it is possible that, during *L. braziliensis* infection, there is an up-regulation in the expression of other chemokine receptors in addition to CCR2, which may mediate proper Langerhans cell migration and activation, even in the absence of CCR2, resulting in efficient Th1 cell polarization.

The susceptibility of $CCR2^{-/-}$ mice to *L. braziliensis* infection was, in fact, associated with a profound decrease in the frequency and total number of $Ly6C^+$ DCs in the lesions, as well as less TNF production and iNOS expression compared with that of WT mice, in which $Ly6C^+$ DCs were the major iNOS-expressing and TNF-producing myeloid cell population found in the lesions. $Ly6C^+$ inflammatory DCs have previously been found to be the major source of TNF production and iNOS expression in *L. major* murine infection [32]. Therefore, these results suggest that the profound reduction in the levels of $Ly6C^+$ DCs found in the lesions of $CCR2^{-/-}$ mice resulted in the lessened production of TNF and iNOS that was observed, leading to impaired parasite elimination in comparison to WT mice, because TNF and iNOS have critical roles in resistance against *L. braziliensis* infection [33]. Moreover, in separate experiments, we found that DCs start to accumulate in the lesions of WT, but not $CCR2^{-/-}$, mice between 2 to 3 wpi (not shown). Importantly, as shown in Fig. 1, WT mouse lesions start to heal after 3 wpi, whereas, in $CCR2^{-/-}$ mice, this happens much later (around 8 wpi). Therefore, the association observed between the kinetics of the accumulation of DCs in lesions of WT mice with the initiation of lesion healing suggests that these cells have an important role in controlling parasite replication.

In contrast to most DC subsets that are derived primarily from a bone marrow precursor called *common DC precursor* [34, 35], this inflammatory DC subset was first described as being derived from inflammatory monocytes that migrate from the bloodstream to inflamed tissues and differentiate into DCs via stimuli, such as IFN-γ [21] and GM-CSF [36]. This subset of inflammatory DCs is phenotypically characterized by the expression of the molecule Ly6C on the DC surface [37], and because they have been shown to promote protection against intracellular pathogens, like *Listeria monocytogenes* [38] and *Trypanosoma brucei* [21], through the production of NO and TNF, they have become known as *TNF/iNOS-producing DCs*. The involvement of CCR2 in the migration of $Ly6C^+$ DCs and of monocytes that will differentiate into $Ly6C^+$ DCs at sites of inflammation has already been described [39]. In addition, it has been shown that CCR2 mediates the release of $CD11b^+Ly6C^+$ monocytes from the bone marrow to the bloodstream; therefore, $CCR2^{-/-}$ mice naturally have a limited availability of cells in the blood that can migrate and differentiate into inflammatory DCs at the inflamed tissues [40].

However, another possible mechanism by which CCR2 might be involved in the accumulation of $Ly6C^+$ DCs at sites of inflammation, which has not, to our knowledge, been previously addressed, is that CCR2 signaling could be directly participating in the differentiation of monocytes into inflammatory DCs at the site of infection. To test this hypothesis, we adoptively transferred CFSE-stained $CD11c^-CD11b^+Ly6c^+$ monocytes from WT or $CCR2^{-/-}$ mice directly into the bloodstream of recipient $CCR2^{-/-}$ mice previously infected with *L. braziliensis*, to overcome the bone marrow–bloodstream migration barrier that is affected by CCR2 deficiency [40]. In fact, 3 d after transfer, few $CFSE^+$ cells were recovered from bone marrow, showing that the cells did not reenter this site, and few $CFSE^+$ cells were recovered from spleens, blood, and draining LNs. In both groups, most transferred cells migrated to the lesions, and there was no difference in the frequency of transferred cells recovered from this site between the groups. However, the frequency of transferred cells that differentiated into DCs in the lesions was greater among WT than it was among $CCR2^{-/-}$ transferred cells. Therefore, CCR2 deficiency does not affect the ability of inflammatory monocytes to migrate to *L. braziliensis* cutaneous lesions. In fact, these results indicate that CCR2 has a direct role in monocyte differentiation into DCs at the sites of infection with *L. braziliensis*. Moreover, the observation that the addition of CCL2 to the culture during DC differentiation resulted in cells with a greater capacity to produce TNF and NO and to kill intracellular parasites, strengthened our conclusions that the CCL2/CCR2 axis has a direct role in the differentiation/activation of DCs with inflammatory properties. Although the major factor causing impairment in accumulation of inflammatory $Ly6C^+$ DCs in lesions of *L. braziliensis*–infected, $CCR2^{-/-}$ mice is probably the naturally few $Ly6C^+$ monocytes present in the circulation of these animals [40–42], our results bring evidence of an additional mechanism by which CCR2 signaling contributes to the accumulation of inflammatory $Ly6C^+$ DCs at infection sites, which helps to promote control of parasite replication.

CCR2 is an important chemokine receptor expressed by a variety of myeloid cell subsets [25], including immature myeloid cells, such as the myeloid-derived suppressor cells. These cells are known to suppress the activity of effector T lymphocytes [43], but in addition to this characteristic, they have been shown to exert a protective role during murine infection with *L. major* [44]. Because myeloid-derived suppressor cells can express CCR2 [45], it is possible that the absence of this receptor may also have effects on the dynamics of migration and activation of this cell subset during *L. braziliensis* infection, which was not investigated. Therefore, additional studies are required to evaluate this

subject, as well as the role of myeloid-derived suppressor cells during *L. braziliensis* infection, which is unknown.

We, therefore, conclude that CCR2 has an important role in host protection against *L. braziliensis* infection by mediating accumulation of $Ly6C^+$ DCs, which produce TNF and NO, which have an important role in the inhibition of parasite growth. Additionally, our data show that one of the mechanisms by which CCR2 contributes to the accumulation of $Ly6C^+$ DCs at infection sites, and the consequent protection against *L. braziliensis*, is by directly mediating the differentiation/maturation of monocytes into inflammatory DCs. Thus, these data shed new light on the role of chemokines during infections as active inducers of cell activation and differentiation in addition to cell migration.

AUTHORSHIP

D.L.C., D.S.L.J., M.S.N., L.A.S., R.P.A., V.C., and J.S.S. designed the experiments; D.L.C., D.S.L.J., M.S.N., and L.A.S. performed the experiments; D.L.C., D.S.L.J., M.S.N., L.A.S., V.C., and J.S.S. analyzed the data; D.L.C., R.P.A., and J.S.S. contributed materials, reagents, and analysis tools; and D.L.C. and J.S.S. wrote the article.

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DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
cutaneous leishmaniasis · monocytes · TNF · iNOS