CX(3)CR1(+) CD115(+) CD135(+) common macrophage/DC precursors and the role of CX(3)CR1 in their response to inflammation

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Monocytes, macrophages, and DCs form networks of phagocytic cells throughout most tissues, the development of which are dependent on the CSF-1 receptor (csf1r, also known as CD115, c-fms, and M-CSF receptor) (1, 2). These cells, which are sometimes referred to as the mononuclear phagocyte system, play major roles in development, scavenging, inflammation, and antipathogen defenses (3, 4). They are highly heterogeneous in phenotype, tissue distribution, and function (3, 5, 6). Considerable attention is currently focused on the characterization of their progenitors and precursors, the signals driving their development in the BM, their migration to tissues, and their homeostasis in peripheral tissues. CSF-1R and its two known ligands, M-CSF and IL34 (7), are critical for the development of this lineage because M-CSF–deficient mice (op/op and csf1−/−) have a milder phenotype than the Csf1r-deficient mice (8). Other cytokines, such as GM-CSF, FLT3, LT-α1β2 (LT-α) (9–15), and chemokines (16, 17) have also been shown to control the development and homeostasis of the macrophage and DC networks.

CX3CR1 expression is associated with the commitment of CSF-1R+ myeloid precursors to the macrophage/dendritic cell (DC) lineage. However, the relationship of the CSF-1R+ CX3CR1+ macrophage/DC precursor (MDP) with other DC precursors and the role of CX3CR1 in macrophage and DC development remain unclear. We show that MDPs give rise to conventional DCs (cDCs), plasmacytoid DCs (PDCs), and monocytes, including Gr1+ inflammatory monocytes that differentiate into TipDCs during infection. CX3CR1 deficiency selectively impairs the recruitment of blood Gr1+ monocytes in the spleen after transfer and during acute Listeria monocytogenes infection but does not affect the development of monocytes, cDCs, and PDCs.
Granulocyte-macrophage progenitors (GMPs [reference 21]) include a clonogenic BM macrophage/DC precursor (MDP) that gives rise to spleen cDCs (both the CD11c<sup>+</sup> CD8α<sup>+</sup> CD11b<sup>-</sup> and CD11c<sup>+</sup> CD8α<sup>-</sup> CD11b<sup>+</sup> subsets) directly, with no monocytic intermediate, and to monocytes and macrophages (9, 22, 23). The MDP has no significant granulocytic potential, and initial studies failed to detect a plasmacytoid DC (PDC) potential (9, 22). Another precursor, common DC precursor (CDP), was recently shown to give rise to cDCs and PDCs but not to monocytes, and it did not respond to CSF-1 (24, 25). This result was interpreted as indicating the existence of two pathways for cDC generation. However, MDPs and CDPs are both included in the CD115<sup>-</sup> Lin<sup>-</sup> fraction of BM progenitors (9) and could represent different stages of differentiation along the same pathway. It is also possible that differences in differentiation potential between these cells reported by different groups may reflect differences in experimental protocols rather than intrinsic properties of the cells.

The chemokine receptor and adhesion molecule CX<sub>3</sub>CR1 is not expressed on early hematopoietic progenitors and is first detected on MDPs. CX<sub>3</sub>CR1 is therefore associated with the commitment of myeloid progenitors to the monocyte/macrophage/DC lineage (22). However, its role in the development and homeostasis of cells of the mononuclear phagocyte system remains unknown.

In this paper, we therefore reevaluated the differentiation potential of the MDP and the possible roles of CX<sub>3</sub>CR1 in the differentiation of mononuclear phagocytes in mice using adoptive transfer and disease models. We found that MDPs can give rise to PDCs, as well as to cDCs and monocytes, after adoptive transfer and that MDPs and CDPs share a similar surface phenotype (Lin<sup>-</sup> IL7Ra<sup>-</sup> CD117<sup<int></int></sup> CD135<sup+</sup> CD115<sup+</sup> CX<sub>3</sub>CR1<sup+</sup>). The use of AFS98, an antibody designed to block CSF-1 binding to its receptor CD115, and CSF-1–dependent proliferation (26–28) to purify MDP did not impair the ability of MDP to give rise to monocytes, cDCs, or PDCs in vivo. Because MDP can give rise to PDCs, cDCs, and monocytes/macrophages, whereas CDP only give rise to PDCs and cDCs (24, 25), MDP appears to exhibit a broader differentiation potential than CDP and may represent an earlier precursor. CX<sub>3</sub>CR1 deficiency decreased the recruitment into the spleen of CD115<sup+</sup> Gr1<sup+</sup> monocytes (TipDC precursors) after irradiation and during acute Listeria monocytogenes infection and decreased the efficiency of bacterial clearance but did not affect the development of cDCs or PDCs. The results from this study, therefore, clarify the family tree of mononuclear phagocytes and uncover the role of CX<sub>3</sub>CR1 in Gr1<sup+</sup> monocyte recruitment to the spleen during inflammation and infection.

RESULTS

MDPs and CDPs are phenotypically overlapping cell populations in mouse BM

Expression of the chemokine receptor CX<sub>3</sub>CR1 in GMPs (Lin<sup>-</sup> IL7Ra<sup>-</sup> CD117<sup+</sup> Scα<sup-</sup> IL7Ra<sup-</sup> CD134<sup+</sup> CD16/32<sup+</sup> BM cells) (21) characterizes the MDP (22) and is thus associated with the commitment of myeloid progenitors toward the macrophage/DC lineage. The MDP is also characterized by a low expression of CD117 (c-kit, the receptor for stem cell factor), as compared with GMPs and CDPs (22), and expression of functional CSF-1R (CD115) and FLT3 (CD135) (9, 22). More recently, a precursor common to cDCs and PDCs (CDP) was reported and proposed to be distinct from the MDPs because the CDP gave rise to both cDCs and PDCs but not to monocytes/macrophages, whereas the MDPs give rise to cDCs and monocytes/macrophages but not to PDCs (24, 25). We performed an analysis of mouse BM Lin<sup-</sup> precursors by flow cytometry (Fig. 1 a), and the results indicated that most CDPs expressed CX<sub>3</sub>CR1 at levels similar to its level of expression on MDPs, and that most MDPs expressed both CSF-1R/CD115 and CD135 at levels similar to their expression on CDPs (Fig. 1). These data confirm the data from Waskow et al. (9) suggesting that MDPs and CDPs had an overlapping phenotype in the BM, and we therefore sought to reevaluate the differentiation potential of MDPs.

MDPs give rise to monocytes, cDCs, and PDCs

Initial studies failed to identify PDCs in the progeny of MDPs after in vivo transfer into C57BL/6 mice (9, 22, 23). However, PDCs in spleen express a CSF-1R-EGFP transgene (2), and we observed that PDCs also expressed CX<sub>3</sub>CR1 (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20081385/DC1) and that MDP gave rise to a population of CD11b<sup+</sup> CD11c<sup+</sup> CX<sub>3</sub>CR1<sup+</sup> splenocytes that are distinct from cDCs and monocytes/macrophages and may correspond to PDCs after in vivo transfer into irradiated hosts (Fig. S1). In addition, the availability of PDCA1 antibody now permits a better phenotypic definition of PDC (24, 25). We thus investigated the differentiation potential in vivo of MDPs, purified as previously described (Fig. S2) (22), after i.v. adoptive transfer. BM MDP from CX<sub>3</sub>CR1<sup+/-</sup> of the Cd45.1/Cd45.2 genotypes was injected i.v. into Cd45.2-irradiated hosts, and splenocytes were analyzed by flow cytometry 6–7 d after transfer. Results indicated that MDP gave rise to donor-derived PDCA1<sup+</sup> CD11c<sup+</sup> CD11b<sup+</sup> CX<sub>3</sub>CR1<sup+</sup> PDC, as well as to CD11c<sup+</sup> CD11b<sup+</sup> CX<sub>3</sub>CR1<sup+</sup> cDC and to CD11b<sup+</sup> CD11c<sup-</sup> CX<sub>3</sub>CR1<sup+</sup> monocytes (Fig. 2, a–c). As expected, the frequency of donor-derived PDCs and monocytes in the spleen was 2–3-fold lower than that of CD11b<sup+</sup> CD11c<sup+</sup> cDCs (Fig. 2 c). However, CD11b<sup+</sup> PDCs were easily distinguishable from CD11b<sup-</sup> cDCs by their expression of CX<sub>3</sub>CR1 and PDCA1 and their low expression of CD11c (Fig. 2, a and b). MDP-derived CD11b<sup+</sup> CD11c<sup-</sup> monocytes expressed high levels of CX<sub>3</sub>CR1 (Fig. 2 a), which unambiguously distinguished them from granulocytes which do not express CX<sub>3</sub>CR1 (29).

Because the anti-CD115 antibody (AFS98) used to purify CDP (25), and MDP in one study (9), was originally screened for its ability to block the binding of CSF-1 to its receptor (26–28, 30, 31), we investigated whether labeling of MDPs with AFS98 would affect the differentiation of MDPs in our experimental model. We observed that, in vitro, the cloning
efficiency of single MDP seeded into 96-well plates in the presence of CSF-1, as well as the size of colonies, was reduced when AFS98 was added to the antibody cocktail used for cell sorting (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20081385/DC1). However, addition of AFS98 to the antibody cocktail used for cell sorting did not affect the differentiation potential of MDP in vivo (Fig. 2 c).

These data indicate that MDP and CDP share expression of CX3CR1, CD115, and FLT3 and have an otherwise overlapping phenotype in the BM, whereas MDP appears to have a broader differentiation potential than CDP after adoptive transfer in vivo because MDP gave rise to both DCs (PDCs and cDCs) and monocyte/macrophages. It is of note that addition of AFS98 to the antibody cocktail used for cell sorting decreased the proliferative response of MDP to CSF-1 in vitro but did not affect their differentiation into DCs or monocytes in vivo.

CX3CR1-deficient MDPs have a decreased potential to give rise to spleen monocytes
Because expression of CX3CR1 is associated with the commitment of myeloid progenitors to the monocyte/macrophage/DC lineage, we investigated the role of CX3CR1 in the homeostasis of this lineage. The number of MDPs in the BM was not affected by CX3CR1 deficiency (Fig. 3 a) and, on average, 6.5 × 10^4 MDPs were recovered per femur from CX3CR1−/− and CX3CR1−/−− mice, suggesting that CX3CR1 is dispensable for MDP development in the BM. We thus investigated the role of CX3CR1 in DC and monocyte development from MDP by studying the fate of MDP in competitive adoptive transfer, in which 10^4 MDP from each of two different donors of the Cd45.2 and Cd45.2/Cd45.1 genotype were coinjected i.v. into a Cd45.1 congenic recipient (Fig. 3 b). When both donor mice were of the CX3CR1+/− genotype, CD45.2 and CD45.2/CD45.1 MDPs contributed equally to cDCs, PDCs, and CD11b+ CD11c− monocytes (Fig. 3, b and c). When CD45.2 MDPs were of the CX3CR1−/− genotype and CD45.2/CD45.1 MDPs were of the CX3CR1+/− genotype, they contributed equally to spleen DCs and PDCs (Fig. 3, b and d). However, CX3CR1-deficient MDP generated spleen CD11b+ CD11c− cells with an efficiency of one fifth to one tenth that of the control (Fig. 3, b and d). MDP-derived CD11b+ CD11c− splenocytes were negative for NK1.1, CD3, CD19, and Ly6G and expressed high levels of CX3CR1 and intermediate levels of F4/80, and most of these cells expressed Gr1 (Fig. 3 e). These characteristics are similar to those of CD115+ Gr1+ blood monocytes (29) and of spleen monocytes, as recently described by Nahrendorf et al. (32). Thus, we concluded that CX3CR1 may be selectively involved in the development, recruitment, proliferation, and/or survival of CD11b+ CD11c− monocytes in the spleen.

CX3CR1 is important for the recruitment of blood Gr1+ monocytes to the spleen during infection and for the clearance of Lm in mice
The only known ligand of CX3CR1 is the transmembrane chemokine fractalkine/CX3CL1, which is expressed in neurons, endothelial cells, and DCs (33–37). CX3CR1 is involved in the adhesion of leukocytes, including monocytes in particular, to endothelial cells (38–42) and in the migration of microglial cells (43). Fractalkine is expressed in the T cell areas of lymph nodes (37). PCR and in situ hybridization experiments indicated that CX3CL1 was also expressed in the spleen (Fig. 4, a and b; and Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20081385/DC1). Spleen CX3CL1-producing cells were located at the periphery of the B cell follicle.
which correspond to the marginal zone/T cell area (Fig. 4, a and b). The precise identification of CX3CL1-expressing cells in the spleen will require the availability of specific antibodies; however, localization of Cx3cl1 messenger RNA is compatible with the expression of CX3CL1 in the marginal zone/T cell area of the spleen and, thus, with a role of CX3CL1 in the recruitment of blood monocytes.

We tested the role of CX3CR1 for the recruitment of monocytes in the spleen by adoptive transfer of monocytes and during infection with Lm. Short-term competitive transfer of BM monocytes from donors of Cd45.2 and Cd45.2/ Cd45.1 genotype into irradiated Cd45.1 congenic recipients indicated that CX3CR1-deficient monocytes were only one tenth as efficient as control monocytes at accumulating in the spleen (Fig. 4 c). During Lm infection in CX3CR1-deficient BALB/c mice, the number of monocytes was increased in the blood, whereas the number of monocytes in the spleen was decreased in comparison with controls (Fig. 4 d). A similar phenomenon was observed in C57BL/6 mice (Fig. 5, e and f) both for high (3 × 105 Lm; Fig. 4 e) and low (7 × 103 Lm; Fig. 5 f) numbers of bacteria. To investigate whether the proliferation of blood monocytes in the spleen of Lm-infected mice was involved in their accumulation in the spleen, monocytes from the BM and spleen of infected mice at 16 and 48 h after infection were isolated by flow cytometry and analyzed for DNA content using propidium iodide (PI) staining (Fig. 4 g and Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20081385/DC1). Results indicated that BM monocytes, but not spleen monocytes, actively proliferate at 16 and 48 h.

![Figure 2. Differentiation potential of MDP in vivo.](http://www.jem.org/cgi/content/full/jem.20081385/DC1)

(a–c) MDPs from Cd45.1/2 or Cd45.2 Cd3cr1 gfp/+ reporter mice were purified as described in Fig. S2 (available at http://www.jem.org/cgi/content/full/jem.20081385/DC1) and were adoptively transferred into irradiated (900 rad) C57BL/6 Cd45.2 congenic recipients. Spleens of recipient mice were analyzed at day 7 after transfer by flow cytometry, using lineage marker (NK1.1 CD3 CD19), CD11b, CD11c, and CD8-α antibodies. R1 corresponds to PDCs (Lin− CD11cint CD11b− CX3CR1+ cells), R2 and R3 correspond to cDCs (Lin− CD11cint CD11b− CX3CR1− cells and Lin− CD11clow CD11b+ CX3CR1− cells), and R4 corresponds to monocytes (Lin− CD11b+ CD11c− CX3CR1− cells). The number of donor-derived cells per spleen are represented after adoptive transfer of MDP, purified with or without anti-CD115 antibody (n = at least 3 mice per group from two experiments). Error bars show SD. (d) The flow diagram represent the Lin− CD117+ CD115+ CD135+ CX3CR1+ MDPs that give rise to monocytes, cDCs, and PDCs and their putative relationship with other myeloid precursors.
after infection and, thus, that accumulation of spleen monocytes during infection is not a result of their proliferation in the spleen. In addition, the proportion of splenic monocytes that bind annexin-V in *Lm*-infected mice was similar in CX<sub>3</sub>CR1-deficient animals and in controls (Fig. S6), indicating that the apoptosis rate of CX<sub>3</sub>CR1 monocytes during *Lm* infection was not increased in comparison with controls.

Early control of *Lm* growth in the spleen was also significantly less efficient in CX<sub>3</sub>CR1-deficient mice on both BALB/c and C57BL/6 backgrounds (Fig. 4, h and i). Bacterial load in the spleen 24 h after an i.v. infection was 4× higher in BALB/c CX<sub>3</sub>cr<sup>1−/−</sup> mice in comparison with controls (Fig. 5 h) and at least twice as high in C57BL/6 (Fig. 4 i).

Altogether, these data indicate that CX<sub>3</sub>CR1 is important for the recruitment of monocytes to the spleen in irradiated host and during *Lm* infection, and for the early control of bacteria growth. It is of note that the frequency of monocytes in the spleen of noninfected mice were not affected by CX<sub>3</sub>CR1 deficiency (Fig. 4 d), indicating that CX<sub>3</sub>CR1 is dispensable for the recruitment of monocytes in the spleen in the steady state but important during acute inflammation.

CD115<sup>+</sup> Gr1<sup>+</sup> monocytes recruited to the spleen during *Lm* infection differentiate into effector cells (TipDCs) that produce TNF, inducible nitric oxide synthase (iNOS), and reactive oxygen intermediates (ROIs)

Because the decreased recruitment of monocytes in the spleen during *Lm* infection in CX<sub>3</sub>CR1-deficient mice correlated with a decreased bacterial clearance, we investigated whether monocytes recruited to the spleen may be involved in the control of *Lm* growth and express effector activities important for *Lm* clearance such as TNF-α secretion, iNOS expression, and reactive oxygen production. Monocytes have already been proposed to be precursors of TipDCs that accumulate to the spleen of mice infected with *Lm*, produce TNF-α and iNOS, and are needed for the control of primary infection (44–46). Recruited monocytes were the majority (90%) of the CD115<sup>+</sup> Gr1<sup>+</sup> subset (Fig. S7, available at http://www.jem.org/cgi/content/full/jem.20081385/DC1).

We observed that these spleen CD115<sup>+</sup> Gr1<sup>+</sup> monocytes produced TNF-α, iNOS, and ROI in mice during infection after infection and, thus, that accumulation of spleen monocytes during infection is not a result of their proliferation in the spleen. In addition, the proportion of splenic monocytes that bind annexin-V in *Lm*-infected mice was similar in CX<sub>3</sub>CR1-deficient animals and in controls (Fig. S6), indicating that the apoptosis rate of CX<sub>3</sub>CR1 monocytes during *Lm* infection was not increased in comparison with controls.
with *Lm* (Fig. 5, b–d; and Fig. S8) and were localized in the T cell area and in the perifollicular area of the spleen (Fig. 5 e). Altogether, these data therefore indicate that MDPs give rise to blood CD115⁺ Gr1⁺ monocytes, which are recruited to the spleen during infection via a process that involves CX₃CR1, and that these cells expressed effector functions that are important for *Lm* elimination.

**Gr1⁺ monocyte survival and MDP survival and proliferation are normal in CX₃CR1-deficient mice**

Other mechanisms that might be responsible for the impaired accumulation of CX₃CR1-deficient CD115⁺ Gr1⁺ monocytes in the spleen include decreased survival or proliferation of monocytes or decreased survival, proliferation, or differentiation of MDP. As indicated by Fig. 4 g and Fig. S6, CX₃CR1-deficient Gr1⁺ monocytes did not exhibit increased apoptosis rate during *Lm* infection, and the proliferation of monocytes in the spleen is not responsible for their accumulation. Total blood monocyte counts (CD115⁺ CD11b⁺ NK1.1⁻) and Gr1⁺ monocyte counts performed in CX₃CR1⁻/⁻, CX₃CR1⁺/⁻, CX₃CL1⁻/⁻, and CX₃CL1⁺/⁻ mice did not show any difference between CX₃CR1-deficient or CX₃CL1-deficient mice and control mice (Fig. S9, a–c), and the proportion of Gr1⁺ blood monocytes that bind annexin-V in the steady state was also similar in CX₃CR1-deficient animals and in controls (Fig. S9 d). However, there was a 20% decrease in the numbers with *Lm* (Fig. 5, b–d; and Fig. S8) and were localized in the T cell area and in the perifollicular area of the spleen (Fig. 5 e).

![Figure 4](http://www.jem.org/cgi/content/full/jem.20081385/DC1)

**Figure 4.** CX₃CR1 is important for the recruitment of CD11b⁺ CD11c⁺ monocytes to the spleen during infection. (a) and (b) Mouse fractalkine/CX₃CL1 transcripts are detected in spleen tissue sections from WT mice around B cells follicles in the marginal zone/T cell area. Bars: (a) 500 μm; (b) 200 μm. (c) Short-term adoptive transfer of BM monocytes into irradiated WT recipient mice. Equal numbers (2 × 10⁵) of BM monocytes from CX₃CR1⁺/⁺, CD45.1/2 mice (blue) and CX₃CR1⁻/⁻, CD45.1/2 mice (red) were mixed and adoptively transferred into a CD45.1 congenic recipient. CX₃CR1-expressing CD45.1/2 Gr1⁺ monocytes are recruited in the spleen of irradiated host 10× more efficiently than CX₃CR1-deficient monocytes. The experiment was performed three times with two to three mice per group with similar results. (d–f) Monocyte recruitment in the spleen of infected mice. (d) BALB/c CX₃CR1⁺/⁺ and CX₃CR1⁻/⁻ mice (seven per group) were infected i.v. with live *Lm* (3 × 10⁵), and monocytes (CX₃CR1⁺ gfp⁺ CD11b⁺ Ly-6C⁺ Ly-6G⁻, CD19⁻, CD3⁻, and NK1.1⁻) were enumerated after 24 h in the blood and spleen of infected and control uninfected mice. (e) C57BL/6 CX₃CR1⁺/⁺ and CX₃CR1⁻/⁻ mice (seven per group) were injected with 7 × 10⁵ *Lm*, and monocytes were enumerated after 24 h in the blood and spleen of infected mice. (f) C57BL/6 CX₃CR1⁺/⁺ and CX₃CR1⁻/⁻ mice (seven per group) were injected with 7 × 10¹ *Lm*, and monocytes were enumerated after 48 h in the spleen of infected mice. *Lm* growth in the spleen of infected mice is shown. The asterisks indicate a significant difference between groups (P < 0.05). (g) Absence of proliferation of monocytes in the spleen of *Lm*-infected mice. BALB/c mice (n = 6 per group) were infected with 3 × 10⁵ bacteria, spleen and leg bones were harvested 16 and 48 h later, and cells were processed as indicated in Fig. S5 (available at http://www.jem.org/cgi/content/full/jem.20081385/DC1). Data indicate the percentage of BM precursors and spleen monocytes in G0/G1 and in G2+S as analyzed by flow cytometry after DNA labeling with PI. The experiment was performed three times with similar results. (h) BALB/c CX₃CR1⁺/⁺ and CX₃CR1⁻/⁻ mice were infected i.v. with live *Lm* (3 × 10⁵). Data show the number of bacteria (mean ± SE) in the spleen 24 h after infection. (i) C57BL/6 CX₃CR1⁺/⁺ and CX₃CR1⁻/⁻ mice were infected with 10⁶ *Lm*. Data show the number of bacteria (mean ± SE) in the spleen 24 h after infection. Circles represent individual mice.
of blood Gr1− monocytes in CX3CR1-deficient and CX3CL1-deficient mice in comparison with controls (Fig. S9, a–c), and the frequency of apoptotic Gr1− monocytes was higher in CX3CL1−/− mice in comparison with WT mice (Fig. S9 d).

The clonogenic and proliferation potentials of MDPs were then studied in vitro in the presence or absence of CX3CL1/fractalkine. CX3CR1+/CX3CL1 did not confer any advantage in terms of growth or survival, even in competition with WT cells (Fig. S10, a–c, available at http://www.jem.org/cgi/content/full/jem.20081385/DC1). The role of CX3CR1 in the proliferation of MDP-derived cells in vivo was investigated by labeling MDP and CD19+ B cells, as a control, with Cell Tracker 633 (BODIPY 630/650 MeBr) and carrying out the adoptive transfer of these cells into irradiated recipients. 6 d later, 100% of both CXε1+/− and CXε1−/− MDP-derived cDCs and monocytes were no longer labeled (Fig. S10 d), indicating they had undergone multiple rounds of division. Together with results from the adoptive transfer experiments depicted in Fig. 4, these data indicate that CX3CR1 deficiency does not significantly affect the proliferation and differentiation potential of MDP.

**DISCUSSION**

This study investigated the differentiation potential of the CX3CR1+ MDPs and the role of CX3CR1 in their development. First, our data confirmed that CX3CR1 was associated with the commitment of myeloid progenitors to the macrophage/DC lineage. The recently described CDP (24, 25) also expresses CX3CR1, CD115, and FLT3, and its phenotype was overlapping with the MDP (this study and...
shown to be important for the adhesion of CD115+ Gr1+ monocytes to the endothelium of blood vessels (38, 42). Human CX3CR1high cells also adhere to the endothelium via a CX3CR1-dependent mechanism (38), and adhesion is known to increase survival as has been shown for CX3CR1-expressing cells binding to CX4CL1 via a mechanism involving an Akt/GSK-3β-mediated antiapoptotic signaling pathway (48, 49).

The role of CX3CR1 for the recruitment of CD115+ Gr1+ monocytes/TipDC precursors is reminiscent of the phenotype of CCR2-, MCP1-, and MCP3-deficient mice (45, 50). However, the disruption of the CCR2 axis results in a much more dramatic phenotype because both Gr1+ monocytes exit from the BM into the bloodstream, and the recruitment into tissues of the few monocytes that exit the BM is impaired in these mice (45, 46, 50). Thus CCR2-deficient mice have reduced numbers of TipDCs. In contrast, BM output is intact in CX3CR1-deficient mice, the number of blood Gr1+ monocytes are normal, and only their recruitment is impaired. Similarly, CCR2-deficient mice have a dramatically impaired innate response to LPS infection and die of infection within a few days (46), whereas CX3CR1-deficient hosts exhibit a reduced clearance of LPS at 24 h after infections (Fig. 3), but at later time points we did not observe significant differences in the survival of CX3CR1-deficient and control mice (not depicted). We therefore propose that CX3CR1 may play an important role in mediating the recruitment of CD115+ Gr1+ inflammatory monocytes in the white pulp of the spleen during acute inflammation, possibly through adhesion to the capillary endothelial cells of the marginal zone of the spleen.

**MATERIALS AND METHODS**

**Animals.** C57BL/6 mice on C4d5.2 or C4d5.1 congenic backgrounds were purchased from Charles River Laboratories. Cx3cr1gfp reporter mice on the C57BL/6 and BALB/c background were obtained from D. Littman’s laboratory (Skirball Institute, New York, NY). Cx3cr1gfp/gfp C57BL/6 mice were crossed with WT C4d5.1 to produce Cx3cr1gfp/gfp mice on a mixed C4d5.1/C4d5.2 background for competitive adoptive transfer experiments. C57BL/6 Cx3cr1gfp+/−, Cx3cr1gfp+/−, and Cx3cr1gfp+/− and BALB/c Cx3cr1gfp+/− and Cx3cr1gfp−/− were bred and maintained in the specific pathogen-free animal facility of the Institut National de la Santé et de la Recherche Médicale (I.F. Charro/Necker Enfants Malades and of the Institut National de la Santé et de la Recherche Médicale U924). C57BL/6/Cx3cr1gfp+/− and Cx3cr1gfp+/− and ApoE−/− Cx3cr1gfp+/− and Cx3cr1gfp−/− mice were maintained in the laboratory of I.F. Charro (Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, San Francisco, CA). All mice were maintained according to institutional guidelines and used at the age of 8–14 wk old. C57BL/6 Cx3cr1gfp+/− mice on the C4d5.1 background were used as adoptive transfer recipients. Experimental protocols using mice were examined and approved by the Direction Départementale des Services Vétérinaires de Paris (Paris, France).

**Antibodies and recombinant proteins.** The following purified or conjugated antibodies were purchased from BD; purified anti-FcyRIII/II (CD32/16, clone 2.4G2); PE-, APC-, PE- and biotin-labeled anti-CD11b (M1/70); PE-, APC-, or biotin-labeled anti-CD117 (cKit; 2B8); PE- or APC-labeled anti-NK1.1 (PK136); FITC-, PE-, or APC-labeled anti-CD11c (HL3); PE- or APC-labeled anti-TCR-B (H57-597); PE- or APC-labeled anti-B220 (RA3-6B2); biotin-labeled anti-CD45.2 (104); FITC- or biotin-labeled anti-Ly6C (AL-21); PE-labeled anti-Ly6G (1A8); PE-Cy7-labeled anti-CD8-α (53–6.7); PerCP-labeled anti-CD4 (RM4-5); PE-labeled anti-CD3 (145–2C11); PE-labeled anti-CD19 (MB19-1); APC-labeled anti-TNF-α (MP6-XT22); and control rat IgG, mAb. The following antibodies were purchased from eBioscience: PE-Cy7-labeled anti-CD45.1 (A20),
were filtered using a 40-μm filter, washed twice in PBS 0.5% BSA, blocked and then stained with anti-mouse antibodies specific for CD11b, Ly6C (biotin), NK1.1 (APC), and CD115 (PE). Biotinylated antibodies were revealed by subsequent staining with streptavidin–Pacific blue. Cells were analyzed on a nine-color CYAN ADP flow cytometer (Dako) using the Summit 4.3 software (Dako), and monocytoids were identified as CD115+, CD11b+, SSC^hi, NK1.1^-, or Ly6c^-^-^-^-^^-^-^-^-^-^-^-^-^-_-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^...
of ROI-producing cells. Cells were washed in FACS buffer and stained for expression of cell surface markers. To measure bacterial titters in the spleen, mice were injected i.v. into the lateral tail vein with 10^4 WT Lm and organs were harvested and dissociated on metal screens in 10 ml of 0.1% Triton X-100 (Sigma-Aldrich). Serial dilutions were performed in the same buffer, and 50 μl was plated onto BHI media plates.

For cell cycle analysis, 6-wk-old BALB/c mice were infected with 3 x 10^3 bacteria. 48 h later, spleen and leg bones were harvested. BM were flushed with RPMI 5% FCS and spleen were treated with collagenase/DNase. After lysis of red blood cells, cell suspensions were separately enriched for CD11b (MACs) and stained for CD11b, Ly6c, DX5, CD3, CD19, and Ly6G surface markers. Monocytes (CD11b^hi Ly6c^- DX5^- CD3^- CD19^- Ly6G^-) were sorted, and splenic monocytes from individual mice were treated independently for cell cycle, whereas monocytes from BM were pooled. Cell cycle is defined as the following: cells were washed in PBS and fixed in 70% EtOH at 4°C for 30 min. Samples were then stained with 50 μg/ml PI in PBS complemented with 50 μg/ml RNaše at 37°C for 30 min. Samples were then analyzed by flow cytometry.

**Immunofluorescence.** Spleens were fixed for 1 h in medium containing 0.05 M phosphate buffer, 0.1 M l-lysine, pH 7.4, 2 mg/ml NaIO4, and RT PCR.

**RT PCR.** Standard methods were used for the preparation of total cellular RNA and first-strand complementary DNA from spleens and brains of WT C57BL/6 mice. The following primer pairs were used: Cx3cl1 forward, 5'-CATGTTGCAGAGATGACCTCA-3', and reverse, 5'-TTCCATAGCTCTTGAGGGTTAGC-3' (455-bp product); and cyclophilin forward, 5'-TGGTCAACCCACCAGGTGTTCCG-3', and reverse, 5'-TCCAGCATTTGGCATTGACAGA-3' (455-bp product).

**In situ hybridization.** The 60-mer oligonucleotide probes were synthesized and purified by Invitrogen. The oligonucleotides were 3' end labeled with [35S]dATP (PerkinElmer) using 15 U/ml of terminal deoxyribonucleotidyl transferase (Invitrogen) to a specific activity of ~7 x 10^6 cpm/μg. The probes were purified on BioSpin columns (Bio-Rad Laboratories) before use. The Cx3cl1 probes were chosen according to the human Cx3cl1 complementary DNA sequence (available under GenBank accession no. NM_009142; http://www.ncbi.nlm.nih.gov/Genbank). The sequences of the probes were 5'-CCGAGGAGCCACAGCTACTTACTCTATCAGACACAGAGCTGCTCCAGC-3' for the Cx3cl1 sense probe (position 1443–1503) and 5'-AGCTGAAGGTCAACCTGCT-CTGTCTCTGTAAGTGATGGTCTCAGATGGACCTCAGC-3' for the Cx3cl1 antisense probe (position 1503–1443).

The hybridization cocktail contained 50% formamide, 4× SSC, Denhardt’s solution, 0.25 mg/ml of yeast transfer RNA, 0.25 mg/ml of sheared herring sperm DNA, 0.25 mg/ml poly A, 10% dextran sulfate (Sigma-Aldrich), 100 mmol DTT, and [35S]dATP-labeled probes (10^6 cpm/100 μl final concentration). 100 μl of hybridization solution was applied to each section. Sections were covered with a parafilm coverslip and incubated in a humidified chamber at 43°C for 20 h. After hybridization, the slides were washed twice for 15 min in SSC supplemented with 10 mM DTT at 55°C, twice for 15 min in 0.5× SSC supplemented with 10 mM DTT at 55°C, and, finally, in 0.5× SSC supplemented with 10 mM DTT for 15 min at room temperature. The sections were dipped in water, dehydrated by incubation in a series of graded concentrations of ethanol, placed against x-ray film (Hyperfilm Betamax; GE Healthcare) for 10 d, and then against photographic emulsion (NTB2; Eastman Kodak) for 2 mo at 4°C. Sections were developed, counterstained with toluidine blue (0.2% in 0.2 M sodium acetate, pH 4.3), covered with a coverslip, and examined under bright- or dark-field illumination with a light microscope (DNRB2; Leica). Both bright- and dark-field images were collected by a charge-coupled device camera (Nikon) connected to a computer.

**Online supplemental material.** Fig. S1 shows the expression of CxCR1 by PDCs. Fig. S2 shows the sorting gates for MDPs. Fig. S3 shows the expression of the AFS08 anti-CD115 antibody on the response of MDP to M-CSF in vitro. Fig. S4 shows the expression of CxCR1, CxCL1 in the spleen. Fig. S5 shows analysis of the proliferation of monocytes in the spleen and BM. Figs. S6 and S7 show the ratio of Gr1+ and Gr1- monocytes in the spleens of infected and control mice and the frequency of annexin-V+positive cells. Fig. S8 describes the analysis of the production of TNF, NOx2, and ROI by Gr1+ monocytes in the spleen of infected and controls mice. Fig. S9 indicates the absolute numbers of Gr1+ and Gr1- monocytes in the blood of mice deficient in CxCR1 or CxCL1 in comparison to controls. Fig. S10 describes the role of CxCR1-CxCL1 in the proliferation of MDP in vitro and in vivo. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081385/DC1.

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