CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 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 (cfs1r, 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/H11002/H11002) have a milder phenotype than the Csf1r-deficient mice (8). Other cytokines, such as GM-CSF, FLT3, LT-αβ2 (LT-α) (9–15), and chemokines (16, 17) have also been shown to control the development and homeostasis of the macrophage and DC networks.

Cellular cloning and transplantation studies have shown that many macrophage subsets, most of the conventional DCs (cDCs) in the secondary lymphoid organs of mice, and at least a fraction of the DCs in the mouse thymus probably originate from myeloid progenitors (18–20).
Granulocyte-macrophage progenitors (GMPs [reference 21]) include a clonogenic BM macrophage/DC precursor (MDP) that gives rise to spleen cDCs (both the CD11c+ CD8α+ CD11b- and CD11c+ CD8α- CD11b+ 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 DC generation. However, MDPs and CDPs are both included in the CD115+ lin- 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 CX3CR1 is not expressed on early hematopoietic progenitors and is first detected on MDPs. CX3CR1 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 CX3CR1 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- IL7Ra- CD117+ CD135+ CD115+ CX3CR1+). 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. CX3CR1 deficiency decreased the recruitment into the spleen of CD115+ Gr1+ 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 CX3CR1 in Gr1+ 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 CX3CR1 in GMPs (Lineage- CD117+ Sca1- IL7Ra- CD34- CD16/32- 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- precursors by flow cytometry (Fig. 1 a), and the results indicated that most CDPs expressed CX3CR1 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, MDPs in spleen express a CSF-1R-EGFP transgene (2), and we observed that PDCs also expressed CX3CR1 (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20081385/DC1) and that MDP gave rise to a population of CD11b+ CD11c+ CX3CR1+ splenocytes that are distinct from cDCs and monocyte/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 MDP, purified as previously described (Fig. S2) (22), after i.v. adoptive transfer. BM MDP from CX3CR1+/+ 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+ CD11c+ CD11b+ CX3CR1+ PDCs, as well as to CD11c+CD11b- CX3CR1- and CD11c-CD11b+ CX3CR1+ cDCs and to CD11b+ CD11c- CX3CR1+ 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+ CD11b+ cDCs (Fig. 2 c). However, CD11b- PDCs were easily distinguishable from CD11b- cDCs by their expression of CX3CR1 and PDCA1 and their low expression of CD11c (Fig. 2, a and b). MDP-derived CD11b+ CD11c- monocytes expressed high levels of CX3CR1 (Fig. 2 a), which unambiguously distinguished them from granulocytes which do not express CX3CR1 (29).

Because the anti-CD115 antibody (AFS98) used to purify MDP (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 in to 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 × 10⁴ 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 studies of the fate of MDP in competitive adoptive transfer, in which 10⁴ 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 ($3 \times 10^5$ Lm; Fig. 4 e) and low ($7 \times 10^3$ 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. (a–c) MDPs from Cd45.1/2 or Cd45.2/Cx3cr1gfp/+ 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 CD8α antibodies. R1 corresponds to PDCs (Lin− CD11cint CD11b− CX3CR1+ cells), R2 and R3 correspond to cDCs (lin− CD11chigh CD11b− CX3CR1− cells and lin− CD11cint CD11b− CX3CR1+ cells), and R4 corresponds to monocytes (lin− CD11b+ CD11c+ CX3CR1+ cells). The experiment was repeated five times with two to three mice per group and with similar results. (b) Donor-derived Lin− CD11cint CD11b− CX3CR1+ cells express PDCA1. (c) Role of anti-CD115 Ab. The number of donor-derived cells per spleen are represented after adoptive transfer of MDP, purified with or without 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 CX3CR1-deficient animals and in controls (Fig. S6), indicating that the apoptosis rate of CX3CR1-deficient Gr1+ 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 CX3CR1-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 CX3cr1−/− 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 CX3CR1 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 CX3CR1 deficiency (Fig. 4 d), indicating that CX3CR1 is dispensable for the recruitment of monocytes in the spleen in the steady state but important during acute inflammation.

CD115+ Gr1+ 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 CX3CR1-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+ Gr1+ subset (Fig. S7, available at http://www.jem.org/cgi/content/full/jem.20081385/DC1). In spleen, lineage−/Ly6G, NK1.1, CD3, and CD19) CD11b+ CD11c+ splenocytes were identified as monocytes based on their expression of CD115, Ly6C/Gr1, and F4/80 (Fig. 5 a) (32).

We observed that these spleen CD115+ Gr1+ 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 CX3CR1-deficient animals and in controls (Fig. S6), indicating that the apoptosis rate of CX3CR1-deficient Gr1+ monocytes during Lm infection was not increased in comparison with controls.

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Figure 3. CX3CR1 is important for the development of CD11b+ CD11c+ monocytes. (a) MDP numbers in BM from CX3CR1+/+ (black) and CX3CR1−/− (white) mice. Data are the mean ± SD of five mice per group. (b–d) Competitive adoptive transfer of MDP into irradiated host (900 rad). Equal numbers (10^4) of MDPs from Cd45.1/2 mice (blue) and Cd45.2/2 mice (red) were mixed and adoptively transferred into a Cd45.1 congenic recipient. Splenocytes of recipient mice were analyzed at day 7 after transfer by flow cytometry, using lineage marker (NK1.1 CD3 CD19), CD11b, CD11c, and CD8−/− antibodies. (b and c) When both Cd45.1/2 and Cd45.2/2 donor mice were of the CX3cr1+/+ genotype, both donors contributed equally to NK1.1− CD3− CD19− CD11c亮 CD11b+ (R1, cDC) and NK1.1− CD3− CD19− CD11c亮 CD11b− (R2, cDC), NK1.1− CD3− CD19− CD11c亮 CD11b+ (R3, PDC), and NK1.1− CD3− CD19− CD11b− CD11c+ splenocytes (R4). In contrast, when Cd45.1/2 donor mice were of the CX3cr1+/+ genotype and Cd45.2/2 donor mice were of the CX3cr1−/− genotype (CX3CR1 deficient), both donors contributed equally to CD11c+ cDCs in R1 and R2 but Cd45.1/2 CX3cr1−/− MDPs were 10× more efficient than Cd45.2/2 CX3CR1-deficient MDPs in generating CD11b+ CD11c+ splenocytes in R3. Results in b are from one representative experiment out of three, with two to three mice per experimental group, and bar graphs in c and d represent the mean and SD from three independent experiments. The asterisk indicates a significant difference between groups (P < 0.05 using the Wilcoxon test).
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. 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 × 10^5) of BM monocytes from Cx3cr1+/− CD45.2+/− mice (blue) and Cx3cr1−/− CD45.2+/− mice (red) were mixed and adoptively transferred into a Cx45.1 congenic recipient. CX3CR1-expressing CD45.1+ Gr1+ monocytes are recruited in the spleen of irradiated host 10x 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 × 10^5), 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 × 10^5 Lm, and monocytes were enumerated after 24 h in the spleen of infected mice. (f) C57BL/6 Cx3cr1+/− and Cx3cr1−/− mice (seven per group) were injected with 7 × 10^4 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 significative 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 × 10^7 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 × 10^7). 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 10^8 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 CX3C1−/− 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 Cx3cr1+ and Cx3cr1−/− 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 monocyte/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

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**Figure 5. Functions of spleen CD115+ Gr1+/TipDCs.**

(a) CD11b+ spleen cells from Cx3cr1 GFP+ reporter mice negative for lineage marker (NK1.1, CD3, B220, CD19, and CD11c) were analyzed for Ly6G, Ly6C, F4/80, CD115, and CX3CR1 (gfp) expression by multicolor flow cytometry. (b) TNF production by lineage− CD11c− CD11b+ cells 24 h after i.v. infection with Lm. Gated cells (circled) are displayed in red. Data show representative FACS profiles from three experiments. (c and d) Production of TNF-α, iNOS, and ROI by lineage− CD11c− CD11b+ Gr1+/Ly6C+ spleen cells from C57BL/6 mice 24 (c) and 48 h (d) after i.v. infection with 7 × 10^5 bacteria and 7 × 10^3 bacteria, respectively. The experiment was repeated five times with similar results. Error bars show SD. (e) iNOS+ cells in the spleen of BALB/c mice 24 h after infection with 10^5 WT Lm (red) express CD11b and Ly6C and are found in the perifollicular area and T cell area. B cell follicles are labeled with anti-B220 antibodies (green) and T cells are labeled with anti-CD3 antibodies (blue). Bar, 100 μm. The experiment was repeated five times with similar results.
reference 9). MDP can give rise to both DCs (PDCs and cDCs) and monocytes/macrophages but do not have the potential to give rise to granulocytes. Thus, they have a more restricted differentiation potential than CMPs, which can give rise to all myeloid cells, and GMPs, which can give rise to granulocytes, macrophages, and DCs (Fig. 2d). CDP has been reported to give rise to cDCs and PDCs but not macrophages and, thus, has a more restricted differentiation potential than MDPs and may be placed downstream (Fig. 2d). Further studies will be needed to investigate the developmental relationship between MDPs and CDPs.

CX3CR1 is a later marker than CSF-1R in myeloid lineage commitment. In this study, we investigated the role of CX3CR1/CX3CL1 in the development of MDP-derived cells by studying mice lacking CX3CR1 or its ligand CX3CL1/fractalkine during acute and chronic inflammation and in a sensitive competitive adoptive transfer model. This study revealed an important role of CX3CR1 in the recruitment of CD115+ Gr1+ inflammatory monocytes, which are immediate precursors to TipDCs, to the spleen during infection and for the control of bacterial growth during primary infection in two different strains of mice (C57BL/6 and BALB/c).

Impaired recruitment of CX3CR1-deficient Gr1+ monocytes/TipDCs is the most likely mechanism underlying our observations for the following reasons: in situ hybridization experiments (Fig. 4 and Fig. S4) indicated that fractalkine is expressed in the marginal zone/T cell areas of the spleen, where TipDC accumulate during infection (Fig. 5) (44); short-term adoptive transfer of BM monocytes into irradiated hosts decreased the accumulation of CX3CR1-deficient monocytes in the spleen by a factor of ten (see Fig. 4); during acute Lm infection, the number of monocytes was higher in the blood of infected CX3CR1-deficient mice but decreased in the spleen (Fig. 4), and the frequency of annexin-Vhigh monocytes was not increased in spleen from infected CX3CR1-deficient mice (Fig. S6); CX3CR1 deficiency does not appear to influence the survival of CD115+ Gr1+ inflammatory monocytes in the periphery (Fig. S9); and finally, we have found that CX3CR1 was redundant for MDP development, survival, homing to the spleen, and differentiation in vivo and in vitro (Fig. S10) and MDP-derived spleen cDCs and PDCs appear to develop normally.

It is of note that although CD115+ Gr1 (CX3CR1low) inflammatory monocytes were present in normal numbers in both CX3CR1- and CX3CL1-deficient mice, the present data confirm that CX3CR1 deficiency also slightly decreases the survival of the CD115+ Gr1+ monocytes (Fig. S9). This result was expected and consistent with the conclusions that we made from our earlier adoptive transfer experiments (29) and with a more recent study (47). CX3CR1 has been previously shown to be important for the adhesion of CD115+ Gr1 (CX3CR1high) monocytes to the endothelium of blood vessels (38, 42). The mechanism underlying the selective survival defect of CD115+ Gr1+ CX3CR1high monocytes may thus be related to the unique patrolling behavior of these cells, which attach themselves to the luminal side of the endothelium via a CX3CR1- and LFA1-dependent mechanism, crawling onto the endothelium for considerable periods of time without extravasation (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 CX3CL1 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 Lm infection and die of infection within a few days (46), whereas CX3CR1-deficient hosts exhibit a reduced clearance of Lm 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 Cd45.2 or Cd45.1 congenic backgrounds were obtained from Charles River Laboratories. Cx3cr1−/− reporter mice on the C57BL/6 and BALB/c background were obtained from D. Littman’s laboratory (Skirball Institute, New York, NY). Cx3cr1−/− C57BL/6 mice were crossed with WT Cd45.1 mice to produce Cx3cr1−/−+ mice on a mixed Cd45.1/Cd45.2 background for competitive adoptive transfer experiments. C57BL/6 Cx3cl1−/−, Cx3cr1−/−, Cx3cl1−/− and Cx3cr1−/− and BALB/c Cx3cr1−/− and Cx3cl1−/− were bred and maintained in the specific pathogen-free animal facility of the Institut Fédéral de Recherche (BF) Necker-Enfants Malades and of the Institut National de la Santé et de la Recherche Médicale U924. C57BL/6 Cx3cr1−/−+ and Cx3cl1−/− and Cx3cr1−/− and ApoE−/− Cx3cl1−/− and Cx3cr1−/− mice were maintained in the laboratory of I.F. Chrou (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. 4-wk-old C57BL/6 mice on the Cd45.1 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-FCγRIIIb/II (CD32/16, clone 2.4G2); PE–APC–, Pp–, or biotin-labeled anti-CD11b (M1/70); PE– APC–, or biotin-labeled anti-CD11c (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-CD6 (AL-21); PE– or APC-labeled anti-Ly6C (AL-21); PE–Cy7–labeled anti-CD8−α (53–6.7); PerCP-Cy7–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 IgG1, mAb. The following antibodies were purchased from ebioscience: PE–Cy7–labeled anti-CD45.1 (A20),
APC–Alexa Fluor 750-labeled anti-CD45.2 (104), PE- or PE-Cy5-labeled anti-Flk (A293), and PE- or biotin-labeled anti CD115 (AF508). Anti-CD169 (MOMA-1) and APC- or biotin-labeled F4/80 clone CLA-1 were purchased from AbD Serotec. mPDCA1 antibody was purchased from Miltenyi Biotec. Anti-NOS-2 (M-19) polyclonal rabbit was purchased from Santa Cruz Biotechnology, Inc. Goat anti-rabbit Alexa Fluor 647 was purchased from Invitrogen. Hydroethidine was purchased from Polysciences, Inc. Antibodies used for depletion, including TER-119 (erythrocytes), RB6-8C5 (Ly6-C/G), and GK1.5 (CD4), were supernatants of hybridomas donated by B. Rocha (IFR Necker, Paris, France). Streptavidin Pacific Blue was purchased from Invitrogen. Dead cells were gated out by PI labeling (Invitrogen). APC-labeled annexin V was purchased from BD. Recombinant mouse macrophage CSF-1 and recombiant mouse fractalkine (CX3CL1) were purchased from R&D Systems. Recombinant mouse GM-CSF was purchased from PeproTech.

**Mouse blood phenotyping.** Blood was drawn according to institutional guidelines, and red blood cells were lysed in 5 vol of red blood cell lysis buffer (155 mM NH4Cl, 10 mM NaHCO3, and 0.1 mM EDTA). Cell suspensions were filtered using a 40-μm filter (BD), washed twice in PBS 0.5% BSA, and then stained with anti-mouse antibodies specific for CD11b (PE-Cy7), 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 monocytic cells were identified as CD115+, CD11b+, SSC<sup>hi</sup>, NK1.1<sup>+</sup>, or Ly6c<sup>hi</sup> cells. Annexin V staining was performed according to the manufacturer’s instructions. Absolute numbers were calculated using cell percentages and total white blood cells counts. Data on blood monocytes counts were obtained from littermate animals of the C57BL/6J background and from C57BL/6J<sup>m剽/剽</sup> mice as described in the Animals section, were deposited into wells of 96-well round-bottom plates, coated with fractalkine or BSA as control, and grown in decreasing concentrations of CSF-1. After 5 d in culture, cells were harvested from individual wells and analyzed for origin by CD45.1 and CD45.2 staining by flow cytometry. At least 10 wells were analyzed per experiment.

**Phenotyping of splenocytes.** Splenocytes were removed, triturated in RPMI 2% FCS at 4°C with the end of a 3-ml syringe, and then passed through a 100-μm cell strainer (BD) in one well of a 6-well plate. Cell suspensions were filtered using a 40-μm filter, washed twice in PBS 0.5% BSA, blocked with anti-mouse FcRII/III (2.4G2), and cells were labeled using antibodies against lineage markers (IL7Rα, CD11c, CD11b, Ly6C, mPDCA1, NK1.1, and CD115). 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).

**Phenotyping, isolation, and adoptive transfer of MDP and CDP.** BM cells from femurs and tibias of C57BL/6<sup>m剽/剽</sup> mice aged 6 wk were flushed with serum-free RPMI. Fe receptors were blocked with antibody to FcγRIII/II (2.4G2), and cells were labeled using antibodies against lineage markers (IL7Rα, CD11c, CD11b, NK1.1, CD3, Ter119, CD19, and Gr1), CD117, CD115, and CD135. For purification of MDP, red blood cells and Gr1-expressing cells were removed using Ter119 and RB68C5 antibodies as previously described (22). MDP (defined as IL7Rα<sup>－</sup>lineage<sup>－</sup>g<sup>pf</sup> [CXCRIK1] CD117<sup>+</sup> expressing CD115; Fig. S2), CDP (defined as IL7Rα<sup>－</sup>lineage<sup>－</sup>CD135<sup>－</sup>CD117<sup>+</sup> Fig. S3), and BM monocytic cells (defined as g<sup>pf</sup> CD11b<sup>+</sup> CD115<sup>－</sup> NK1.1<sup>－</sup> CD117<sup>－</sup>) were purified by flow cytometry using FACSVantage or FACSaria cell sorters (BD). For competitive adoptive transfers, monocyes or MDP were purified from Cx3cr1<sup>gfp/gfp</sup> mice on a C57B6 background and from Cx3cr1<sup>gfp/gfp</sup> mice on a C57B6.2 background, unless otherwise indicated. MDP-purified cells from each genetic background were mixed at a 1:1 ratio and were transferred i.v. to 4-wk-old recipient mice on a C57B6.1 background. Recipients were either irradiated with 10 Gy or were not irradiated. For analysis of cell populations in WT mice, Cx3cr1<sup>gfp/gfp</sup> reporter mice, and adoptive transfer recipients, blood and organs were harvested from anaesthetized mice as described previously (29), and spleens were digested with collagenase d, homogenized, and filtered. Erythrocytes were lysed using red blood cell lysis buffer for quantification of cell populations in reporter mice or were depleted by density gradient centrifugation (LSM 1077, Eurobio) for analysis of adoptive transfer recipients. PBMC or splenic mononuclear cell suspensions were enumerated with a Guava Viacount analyzer. Fe receptors were blocked, and then cells were labeled using fluorochrome- or biotin-conjugated antibodies and analyzed by five, six, or seven-color flow cytometry using a Cyan ADP.

For in vivo proliferation assays, splenocytes or FACS-sorted MDP were labeled using BODIPY far-red cell tracker (Invitrogen), transferred to congenic recipients, and analyzed by flow cytometry after 5 d. Cell division of MDP-derived cells was assessed by comparison with nondividing B220<sup>－</sup> B lymphocytes.

**In vitro analysis of cell survival, proliferation, and differentiation potential.** MDPs from Cx3cr1<sup>gfp+/－</sup> or Cx3cr1<sup>gfp+/－</sup> were purified by FACs, as described in the previous section, and plated at one cell per well into 96-well plates containing Opti-MEM (Invitrogen) supplemented with 10% FCS (PAN biotech), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) along with CSF-1, GM-CSF, or both (10 ng/ml each unless otherwise indicated) in the presence or absence of recombinant CX3CL1. Resulting colonies were enumerated after incubation at 37°C in 5% CO<sub>2</sub> for 7 d. For differentiation analysis, colonies were recovered from wells by vigorous pipetting and were immunolabeled for cell surface markers as described in the previous section. For determination of proliferation potential, individual cells from colonies arising from single cell clones were counted using a 10 or 5× objective on a microscope (Axiovert; Carl Zeiss, Inc.). Counting accuracy was validated by digital analysis of random colonies of various sizes photographed using a camera (CoolSnap ES; Roper Scientific) and Photoshop CS (Adobe) for marking of individual cell bodies.

For analysis of proliferation under competitive conditions, 100 MDPs each from Cx3cr1<sup>gfp+/－</sup> and Cx3cr1<sup>gfp+/－</sup> on different CD45 congenic backgrounds, as described in the Animals section, were deposited into wells of 96-well round-bottom plates, coated with fractalkine or BSA as control, and grown in decreasing concentrations of CSF-1. After 5 d in culture, cells were harvested from individual wells and analyzed for origin by CD45.1 and CD45.2 staining by flow cytometry. At least 10 wells were analyzed per experiment.

**Lm infection.** The Lm 10403s WT strain was used in these experiments. This strain exhibits an LD<sub>50</sub> of 7 × 10<sup>8</sup> in C57BL/6 mice. Bacteria were prepared from clones grown from organs of infected mice. Stocks of bacteria were kept frozen at −80°C. For infections, bacteria were grown to a logarithmic phase (OD<sub>600</sub> = 0.05–0.15) in Broth Heart Infusion medium (Sigma-Aldrich), diluted in PBS, and injected i.v. into lateral tail vein with 0.1–10× LD<sub>50</sub> of WT Lm per mouse. To study production of TNF and iNOS, organs were cut in small pieces and incubated at 37°C for 20 min in HBSS medium (Invitrogen) containing 4,000 U/ml collagenase I (Invitrogen) and 0.1 mg/ml DNase I (Roche). Red blood cells were lysed for 2–3 min in 170 mM NH4Cl and 17 mM Tris HCl, pH 7.4. Cells were stained with the specified antibodies in 100 ml PBS containing 0.5% of BSA (FACS buffer). For the TNF-α intracellular staining, splenocytes were incubated at 37°C and 5% CO<sub>2</sub> for 3–4 h in RPMI 1640 (Invitrogen) containing 5% FCS and 2 mg/ml Golgi Plug (BD) with or without 5 × 10<sup>5</sup> HKLM/ml. Cells were incubated for 20 min on ice with the indicated cell surface marker mAbs, fixed in 1% PFA FACS buffer for 20 min on ice, and permeabilized for 30 min in Perm/Wash (BD). For intracellular staining of TNF-α, cells were incubated for 20 min on ice in FACS buffer containing anti–TNF-α or control rat IgG<sub>1</sub>. For intracellular staining of iNOS, cells were incubated for 20 min on ice in FACS buffer containing anti–iNOS rabbit polyclonal or control normal goat IgG<sub>1</sub>, and staining was revealed using goat anti-rabbit Alexa Fluor 647 mAb. In all cases, cells were washed, fixed for 30 min in 1% PFA FACS buffer, and analyzed on a FACS Calibur cytometer (BD). To study the production of ROI, 5–10×10<sup>5</sup> splenocytes were incubated for 3 h at 37°C and 5% CO2 in 5% FCS RPMI 1640 with 5 × 10<sup>8</sup> HKLM/ml and 160 μM hydroethidine. Hydroethidine is oxidized by ROI in red fluorescent ethidium bromide, therefore allowing for the detection.
of ROI-producing cells. Cells were washed in FACS buffer and stained for expression of cell surface markers. To measure bacterial titer in the spleen, mice were injected i.v. into the lateral tail vein with 10⁵ WT Las 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 × 10⁴ bacteria. 48 h later, spleen and leg bones were harvested. BM were flushed with RPMI 1% FCS and spleen were treated with collagenase/DNase. After lysis of red blood cells, cells suspensions were separately enriched for CD11b (MACs) and stained for CD11b, Ly6c, DX5, CD3, CD19, and Ly6G surface markers. Monocytes (CD11b+ 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 RNase 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 0.05 M phosphate buffer, 0.1 M L-lysine, pH 7.4, 2 mg/ml NaIO₄, and 50 μg/ml PI in PBS complemented with 50 μg/ml RNase at 37°C for 30 min. Samples were then analyzed by flow cytometry.

**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'-GATCTGTGCGACAAGATGACCTCA-3' and reverse, 5'-TTCCAATGGTGTCTGAT-3' (455-bp product); and cytolphin forward, 5'-TGTCAGACCCCGCTGTTCT-3' and reverse, 5'-TCCAGCATTTGGCATTGACAAAG-3' (455-bp product).
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