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Distinct functions of CXCR4, CCR2, and CX3CR1 direct dendritic cell precursors from the bone marrow to the lung

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Abstract

Precursors of dendritic cells (pre-DCs) arise in the bone marrow (BM), egress to the blood, and finally migrate to peripheral tissue, where they differentiate to conventional dendritic cells (cDCs). Upon their activation, antigen-bearing cDCs migrate from peripheral tissue to regional lymph nodes (LNs) in a manner dependent on the chemokine receptor, CCR7. To maintain immune homeostasis, these departing cDCs must be replenished by new cDCs that develop from pre-DCs, but the molecular signals that direct pre-DC trafficking from the BM to the blood and peripheral tissues remain poorly understood. In the present study, we found that pre-DCs express the chemokine receptors CXCR4, CCR2, and CX3CR1, and that each of these receptors has a distinct role in pre-DC trafficking. Flow cytometric analysis of pre-DCs lacking CXCR4 revealed that this receptor is required for the retention of pre-DCs in the BM. Analyses of mice lacking CCR2 or CX3CR1, or both, revealed that they promote pre-DC migration to the lung at steady state. CCR2, but not CX3CR1, was required for pre-DC migration to the inflamed lung. Thus, these multiple chemokine receptors cooperate in a step-wise fashion to coordinate the trafficking of pre-DCs from the BM to the circulation and peripheral tissues.

cell migration chemokine pre-DCs monocytes inflammation

Introduction

cDCs are potent Ag-presenting cells that are distributed throughout the body. They serve as sentinels of the immune system by acquiring endogenous or exogenous proteins in peripheral tissue, migrating to regional LNs, and presenting protein-derived peptides to Ag-specific T cells [1⇓–3]. Mature cDC migration from peripheral tissue to LNs proceeds at a constant rate during steady state and increases in response to inflammation [4]. To maintain cDC homeostasis in peripheral tissues, the emigration of these cells must be balanced by new cDCs that develop from bloodborne precursors [5, 6]. These pre-DCs develop in the BM from CDPs in a manner dependent on the growth factor FLT3L [7⇓⇓⇓⇓–11]. Pre-DCs egress from the BM to the blood then migrate to peripheral tissues, where they ultimately develop into mature cDCs [6, 7]. Although pre-DC trafficking is critical to maintaining the cDC network and, therefore, immunoresponsiveness, the molecular mechanisms that direct their trafficking remain poorly understood.

DCs are classically identified by their surface display of the integrin α X/CD11c [12]. cDCs

are derived exclusively from FLT3L-dependent pre-DCs [2, 7⇓⇓⇓–10] and are, therefore, developmentally distinct from monocyte-derived cells, which arise independent of FLT3L. However, assignment of specific functions to various CD11c-expressing cells has been complicated by the fact that some monocyte-derived cells also display CD11c [13⇓–15]. The recent identification of lineage-specific molecules has allowed discrimination between cDCs and monocyte-derived $CD11c^+$ cells. For example, the transcription factor Zbtb46, the c-type lectin CLEC9A, and cell-surface proteins CD24 and CD26 are associated with cDCs [16⇓⇓⇓⇓–20], whereas CD14, CD64, and CD88 are primarily associated with monocyte-derived cells [14, 15, 17, 21]. Strategies using the differential expression of these molecules to resolve these cell types have revealed that cDCs, but not monocyte-derived cells, can migrate from tissue to draining LNs. Similarly, cDCs, but not monocyte-derived cells, can activate naïve T cells [16, 17, 22].

Monocyte trafficking has been extensively studied, and their migration to inflamed tissues is directed primarily by the chemokine receptor CCR2 and its ligands CCL2 and CCL7 [23⇓⇓⇓–26]. However, the molecules that direct pre-DC trafficking are unknown. CCR1 and CCR6, as well as their respective ligands CCL9 and CCL20, direct DCs to specific regions within Peyer's patches [27⇓–29], but this occurs after these tissue-resident DCs have already differentiated from pre-DCs. A recent report [30] demonstrated that $CCR9^-$ pre-DCs give rise to cDCs in peripheral tissue, but it is not known whether a specific chemokine receptor is required for pre-DC trafficking. Upon migration to peripheral tissue, pre-DCs differentiate into mature cDCs, which are a heterogeneous population, comprising multiple subsets that can be distinguished by differential display of several other cell-surface proteins [7]. In nonlymphoid organs, 2 major $CD11c^+$ cDC subsets can be identified, based on their reciprocal display of the integrins α E/CD103 ($CD103^+$ cDCs) and α M/CD11b ($CD11b^{hi}$ cDCs) [2, 31]. $CD103^+$ cDCs in nonlymphoid organs are very similar to $CD8^+$ cDCs found in secondary lymphoid organs, such as the spleen and LNs [32, 33]. The number of mature cDCs in any tissue is determined by multiple factors, including recruitment of pre-DCs to that tissue, survival, proliferation, and differentiation of those progenitors, and survival and cell death of the cDCs themselves. In the present study, we sought to identify the chemokines that govern pre-DC trafficking from the BM to the lung. Our studies revealed pre-DC trafficking is exquisitely controlled by the sequential induction and action of multiple chemokine receptors. CXCR4 acts to retain pre-DCs in the BM, CCR2 and CX3CR1 direct migration of pre-DCs to the lung at steady state, and CCR2 directs that migration during inflammation.

MATERIALS AND METHODS

Mice

C57BL/6J, $Ccr2^{-/-}$ (B6.129S4-$Ccr2^{tm1Ifc}$/J), $Cd11c^{Cre}$ (B6.Cg-Tg [*Itgax-cre*] *1-1Reiz*/J), CD45.1 (B6.SJL-*Ptprca* $Pepc^b$/BoyJ), $Cxcr4^{fl/fl}$ (B6.129P2-$Cxcr4^{tm2Yzo}$/J), and $Rosa26^{Tomato}$ (B6.Cg-Gt$(ROSA)26Sor^{tm9(CAG-tdTomato)Hze}$/J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). $Cx3cr1^{-/-}$ (B6.129-$Cx3cr1^{tm1Zm}$) mice were purchased from Taconic Biosciences (Germantown, NY, USA) [34]. $Ccr7^{gfp}$ knock-in mice (C57BL/6-$Ccr7^{tm1/Dnc}$/J) were generated previously in our laboratory [21]. $Ccr2^{-/-}Cx3cr1^{-/-}$ DKO mice were generated by crossing the $Ccr2^{-/-}$ and $Cx3cr1^{-/-}$ strains. CXCR4-CKO mice were generated by crossing the $Cd11c^{Cre}$ and $Cxcr4^{fl/fl}$ strains. For fate mapping of CD11c-producing cells, $Cd11c^{Cre}$ mice were crossed with $Rosa26^{Tomato}$ mice. CD45.1 × CD45.2 F1 mice were generated by crossing C57BL/6J (CD45.2) and CD45.1 mice. Mice were bred and housed in specific pathogen-free conditions at the NIEHS and used between 6 and 12 wk of age, in accordance with guidelines provided by the Institutional Animal Care and Use Committees.

Flow cytometric analysis

Cells were diluted to 1–2 × 10^6/100 μl and incubated with a nonspecific-binding blocking reagent cocktail of anti-mouse CD16/CD32 (2.4G2), normal mouse and rat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Cell surface Ags were stained with fluorochrome allophycocyanin, APC-Cy7, Alexa Fluor 488, Alexa Fluor 647, Brilliant Violet 510, Brilliant Violet 605, Brilliant Ultra Violet 395, eFluor 450, eFluor 605 NC, FITC, PerCP-Cy5.5, or phycoerythrin- or biotin-conjugated Abs against mouse CD3 (145-2C11), CD4 (GK1.5 and RM4-5), CD8α (53-6.7), CD11b (M1/70), CD11c (N418 and HL3), CD19 (605), CD45R-B220 (RA3-6B2), CD45.1 (A20), CD45.2 (104), CD49b (DX5), CD88 (20/70), CD103 (M290), CD115 (AFS98), CD135 (A2F10), CD172a (P84), Ly-6A/E

(D7), Ly-6C (AL-21), Ly-6G (1A8), Ly6-C/G (RB6-8C5), MHC class-II I-A^b (AFb.120), Siglec-H (eBio440c or 551), and TER119 (TER-119) (BD Biosciences, San Jose, CA, USA; BioLegend, San Diego, CA, USA; and eBioscience, San Diego, CA, USA). Allophycocyanin- or phycoerythrin-conjugated Abs to CCR2 (475301), CXCR4 (2B11), and CX3CR1 (SA011F11) were purchased from R&D Systems (Minneapolis, MN, USA), eBioscience, and BioLegend, respectively. Staining with biotinylated Abs was followed by fluorochrome-conjugated streptavidin. Stained cells were analyzed on an LSR-II flow cytometer (BD Biosciences), and the data were analyzed using FACS Diva (BD Bioscience) and FlowJo software (Tree Star, Ashland, OR, USA). Only single cells were analyzed, and dead cells were excluded based on their forward and side scatter.

Preparation and purification of DCs and pre-DCs

Airway inflammation was induced by oropharyngeal aspiration of 50 µl PBS containing 0.1 µg LPS (Sigma-Aldrich, St. Louis, MI, USA), following isoflurane anesthesia [35, 36], and mice were euthanized with i.p. injection of sodium pentobarbital (Vortech Pharmaceuticals, Dearborn, MI, USA). RBCs in BM cells were lysed using ACK (ammonium-chloride-potassium) buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA). Cells from heparinized blood were centrifuged on a Histopaque 1083 gradient (Sigma-Aldrich), and RBCs were lysed. Lungs were perfused by PBS injection into the right ventricle. For DC preparation, minced tissues were digested for 30 (spleen) or 60 min (lung) with Liberase TM, collagenase XI, hyaluronidase I-S, and DNase I (Sigma-Aldrich) [37]. To enrich DCs, low-density cells from the lung and spleen were collected by gradient centrifugation using 16% Nycodenz (Accurate Chemical, Westbury, NY, USA). To prepare pre-DCs from the lung or spleen, single-cell suspensions were prepared from minced tissues without digestion, and pre-DCs were enriched by gradient centrifugation using Histopaque. In some experiments, pre-DCs ($B220^-CD3^-CD11b^-CD11c^+CD19^-CD49b^-CD135^+CD172a^{low/intermediate\ (lo/int)}I\text{-}A^-Ly\text{-}6A/E^-Ly\text{-}6G^-TER119^-$) were purified using a magnet-activated cell sorter (AutoMACS, Miltenyi Biotec, Auburn, CA, USA) and an FACS ARIA-II cell sorter (BD Bioscience), as previously described [11, 17]. Purity was consistently >95%.

Chemotaxis assay

BM cells were suspended in complete RPMI-10 (1×10^6/100 µl) and added to the upper well of a transwell support (pore size, 5.0 µm) on a 24-well plate containing various concentrations of recombinant mouse CCL2 (R&D Systems). After a 2-h incubation at 37°C in a CO_2 incubator, cells were collected from the bottom well, stained with Abs, and analyzed by flow cytometry.

Mixed BM competition assay

To generate mixed BM chimera mice, BM cells were prepared from CD45.1 WT and CD45.2 KO mice, mixed at a ratio of 1:1, and 1×10^7 total cells injected i.v. into sex-matched, γ-ray (9 Gy)-irradiated CD45.1 × CD45.2 F1 mice. Following euthanasia and perfusion of the lungs 4–8 wk after BM transplantation, genotype-specific pre-DCs or cDCs in the lung and BM were evaluated by flow cytometry using anti-CD45.1 and CD45.1 mAbs, and the abundance of lung pre-DCs or cDCs derived from WT and KO donors was normalized to BM pre-DCs of the same genotype as follows: percentage of lung pre-DCs/percentage BM pre-DCs and percentage of lung cDCs/percentage of BM pre-DCs.

In vivo pre-DC migration assay

BM cells were prepared from *Cd11c*^{Cre}*Rosa26*^{Tomato} mice 7–9 d after transplant of FLT3L-producing B16 melanoma cells [38]. Pre-DCs were purified from the BM and injected into the tail vein of splenectomized C57BL/6 mice (3.5×10^6 cells/recipient). Some recipient mice were given 0.1 µg LPS into the airway 1 h before the pre-DC transfer. Recipient mice were euthanized 12 h after pre-DC injection, and lung slices of the right superior lobe were made using a precision-cut tissue slicer VF-300 Compresstome (Precisionary Instruments, Greenville, NC, USA) at 150 µm thickness without perfusion, as previously described [39]. The slices were stained with Alexa Fluor 647-conjugated anti–E-cadherin (clone DECMA-1; eBioscience) and FITC-conjugated anti-CD31 Abs (clone 390; BD Bioscience) and analyzed using a multiphoton laser-scanning microscope Zeiss 880 (Carl Zeiss, Thornwood, NY, USA) and Zen software (Bitplane, Concord, MA, USA).

Gene expression analysis

Total RNA was extracted using Trizol (Thermo Fisher Scientific, Waltham, MA, USA) and converted to cDNA using oligo dT primers and a SuperScript III First Strand kit (Thermo Fisher Scientific). Quantitative PCR amplification was performed using SYBR Green Master Mix (Thermo Fisher Scientific) on a Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA, USA) using the primers shown in Supplemental Table 1. Relative expression of each gene was normalized to that of the house-keeping genes *Gapdh* or *Ppih*.

Statistics

Data are presented as means ± SEM. Statistical differences between groups were calculated using a 2-tailed Student's *t* test, unless indicated otherwise. $P < 0.05$ was considered significant.

RESULTS

Pre-DCs display the chemokine receptors CCR2, CXCR4, and CX3CR1

To investigate mechanisms that direct pre-DC trafficking from the BM to the blood and peripheral tissues, we purified lineage$^-$CD135$^+$CD11c$^+$I-A$^-$CD172a$^{lo/int}$ pre-DCs [11, 17] from mouse BM (**Fig. 1A**) and measured mRNAs encoding 20 different chemokine receptors. In parallel, we also measured chemokine receptor expression in mature splenic DCs. As previously reported [32, 40], splenic DCs expressed *Ccr7* and *Xcr1*, whereas pre-DCs expressed high amounts of *Ccr2* and *Cxcr4* and moderate amounts of *Cx3cr1* (Fig. 1B). Flow cytometric analyses using specific Abs against CXCR4, CCR2, and CX3CR1 confirmed that these receptors are displayed on pre-DCs from WT mice (Fig. 1C and D).

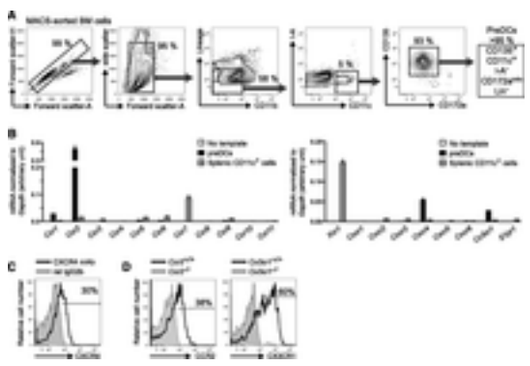

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Figure 1. Chemokine receptor expression by pre-DCs.

(A) Gating strategy for purifying pre-DCs. (B) Chemokine receptor expression in purified BM pre-DCs and in CD11c$^+$ splenic DCs, as measured by quantitative PCR. The expression levels were normalized to *Gapdh* expression. Means ± SEM are shown. (C and D) Chemokine receptor display on pre-DCs. (C) Staining by anti-CXCR4 mAbs (black line) or rat IgG_{2b} isotype control (shaded gray area) by flow cytometry. (D) Surface display of CCR2 and CX3CR1 on BM pre-DCs from WT mice (black line) or gene-targeted mice lacking those receptors (gray shaded area). The percentage of receptor-positive cells within the population is indicated. Results shown are from 1 of 3 experiments yielding similar results.

CXCR4 retains pre-DCs in the BM

CCR2 is required for the mobilization of monocytes from the BM to the blood at a steady state and during inflammation [24, 41], but a role for this receptor in pre-DC biology has not, to our knowledge, been reported. We therefore tested whether CCR2 deficiency affects the number of pre-DCs in the BM, where these cells differentiate from their progenitor CDPs [6]. We found that BM of WT and *Ccr2*$^{-/-}$ mice contained similar numbers of pre-DCs (**Fig. 2A**). This result indicates that CCR2 is dispensable for the development of pre-DCs. Likewise, WT and *Cx3cr1*$^{-/-}$ mice also had similar numbers of pre-DCs in their BM, ruling out a requirement for CX3CR1 in pre-DC development (Fig. 2A).

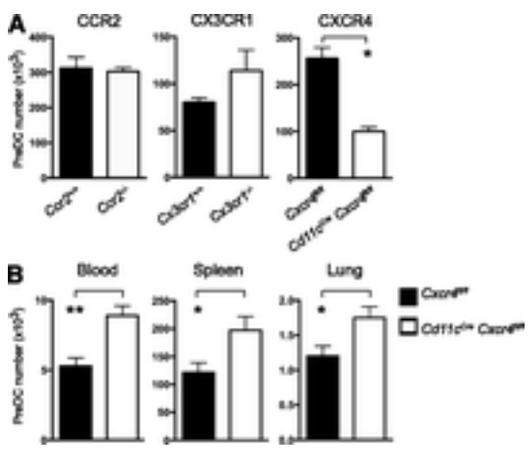

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Figure 2. Roles of CCR2, CX3CR1 and CXCR4 in BM pre-DC homeostasis.

(A) Number of pre-DCs in the BM of the indicated mouse strains, as determined by flow cytometry. (B) Pre-DC number in the blood, spleen, and perfused lung from *Cd11c*^{Cre}*Cxcr4*$^{fl/fl}$ mice and control *Cxcr4*$^{fl/fl}$ mice. Means ± SEM are shown (N = 3–4). *P < 0.05, **P < 0.01. Results shown are from 1 of 2 experiments yielding similar results.

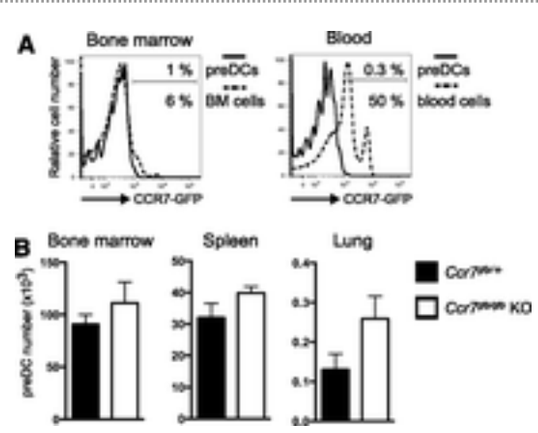
We next tested whether CXCR4 deficiency affects the number of pre-DCs in the BM. Ubiquitous deletion of *Cxcr4* in mice results in embryonic lethality from cardiac, neuronal, and hematopoietic defects [42], precluding the use of these mice for studying pre-DCs in postnatal animals. Interactions between CXCR4 and its ligand CXCL12 are critical for the retention of HSCs and CDPs in the BM [43]. However, these early progenitor cells do not express *Cd11c* [44, 45], and we reasoned that mice lacking *Cxcr4* in pre-DCs (but not in HSCs or CDPs) could be generated by crossing transgenic mice expressing *Cre* recombinase gene under the control of *Cd11c* promoter (*Cd11c*^{Cre} mice) to mice bearing a *loxP*-flanked (floxed) *Cxcr4* gene (*Cxcr4*^{fl}) [46]. Analysis of BM from the resulting *Cd11c*^{Cre}*Cxcr4*$^{fl/fl}$ mice revealed that pre-DCs were dramatically decreased compared with their counterparts in *Cxcr4*$^{fl/fl}$ control mice (Fig. 2A). This finding suggests that a CXCR4-dependent signal is critical for maintaining the number of pre-DCs in the BM by contributing to their development, expansion, or retention. We hypothesized that a requirement of CXCR4 for pre-DC development or expansion in BM would lead to reduced numbers of pre-DCs in the circulation of the *Cd11c*^{Cre}*Cxcr4*$^{fl/fl}$ mice, whereas a requirement of CXCR4 for pre-DC retention in BM would result in an increased number of circulating pre-DCs. We, therefore, analyzed pre-DCs in the blood, spleen, and lung. Compared with *Cxcr4*$^{fl/fl}$ control mice, *Cd11c*^{Cre}*Cxcr4*$^{fl/fl}$ animals had significantly greater numbers of pre-DCs in the blood and spleen (Fig. 2B). Standard enzymatic digestion of the lung decreased FLT3 display on the cell surface, thereby confounding our analysis of pre-DCs (Supplemental Fig. 1A and B). However, FLT3 was retained on the cell surface of cells in minced, but undigested, lung tissue, and using that approach, we found that pre-DC numbers were greater in lungs of *Cd11c*^{Cre}*Cxcr4*$^{fl/fl}$ mice compared with *Cxcr4*$^{fl/fl}$ mice (Fig. 2B). Together, these results suggest that CXCR4 is required for pre-DC retention in the BM but is dispensable for the development of those cells and for their accumulation in peripheral tissues.

CCR7 is dispensable for pre-DC trafficking

CCR7 directs the migration of mature, activated cDCs from peripheral tissue through the lymphatics to regional LNs [21, 47-49]. *Ccr7* expression in CDPs has been reported [43], suggesting that this receptor might also contribute to pre-DC trafficking. Although we did not detect *Ccr7* mRNA expression in pre-DCs isolated from mouse BM (Fig. 1B), we nonetheless tested the function of CCR7 in this regard. Analysis of *Ccr7* expression using *Ccr7*^{gfp} reporter (*Ccr7*$^{gfp/+}$ heterozygous) mice [21] revealed that, although GFP$^+$ cells could be found in the blood of the reporter mice, there were very few GFP$^+$ cells in the BM. Moreover, almost no pre-DCs in BM or blood were GFP$^+$ (**Fig. 3A**), suggesting that CCR7 does not have a major role in directing pre-DC trafficking. In support of that result, the numbers of pre-DCs in the BM, spleen, and lung were similar in WT and CCR7-deficient (*Ccr7*$^{gfp/gfp}$) mice (Fig. 3B). These results demonstrate that CCR7 is dispensable for pre-DC development in the BM, for mobilization to the blood, and for migration to peripheral tissues.

Figure 3. CCR7 is dispensable for pre-DC seeding in the lung.

(A) CCR7-GFP reporter fluorescence in pre-DCs (solid line) and in total cells (dashed line) from the BM or blood of *Ccr7*$^{gfp/+}$ mouse. Percentages of CCR7-GFP$^+$ cells are indicated. (B) Pre-DC numbers in the BM, spleen, and perfused lung of CCR7-deficient



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($Ccr7^{gfp/gfp}$) and CCR7-sufficient ($Ccr7^{gfp/+}$) mice at steady state. Means ± SEM are shown (N = 3). Results shown are from 1 of 2 experiments yielding similar results.

CCR2 and CX3CR1 mediate pre-DC accumulation at steady state

CCR2 directs the migration of monocytes to inflamed tissues that produce its ligands [23-26], but a role for this receptor in pre-DC migration has not been reported. Our gene expression analysis of chemokine receptors in pre-DCs had revealed that *Ccr2* and *Cx3cr1* are highly expressed (Fig. 1), and flow cytometric analysis confirmed that CCR2 and CX3CR1 protein are displayed on the surface of pre-DCs in the BM, blood, spleen and lung (**Figs**. 1D and **4A** and **C**). To test the potential roles of these receptors on pre-DC trafficking, we compared numbers of these cells in the blood, spleen, and lungs of WT, $Ccr2^{-/-}$, $Cx3cr1^{-/-}$ [34], and $Ccr2^{-/-}Cx3cr1^{-/-}$ DKO mice lacking both receptors (Fig. 4E). At steady states, no genotype-specific differences were found between WT mice and either of the single-KO mice in any tissue tested (Fig. 4B and 4D), but pre-DCs were significantly decreased in the lungs of DKO mice (Fig. 4F). Pre-DC numbers in the BM and spleen were similar in WT and DKO mice, and although there was a trend toward fewer pre-DCs in the blood of DKO mice compared with WT mice, that difference was not statistically significant (Fig. 4F). These data suggest that at a steady state, CCR2 and CX3CR1 have compensatory roles in the accumulation of pre-DCs in the lung.

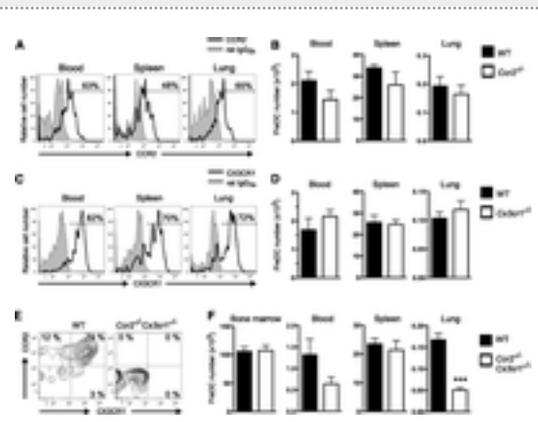

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Figure 4. CCR2 and CX3CR1 promote pre-DC accumulation in the lung.

(A) Representative histograms of pre-DCs from the blood, spleen, and lung were stained with anti-CCR2 mAb (solid line) or rat IgG_{2b} isotype control (shaded). Percentages of positive cells are indicated. (B) Number of pre-DCs in the blood, spleen, and perfused lungs of CCR2-sufficient or -deficient mice at steady state. (C) Representative histograms of pre-DCs from the blood, spleen, and lung stained with anti-CX3CR1 mAb (solid line) or rat IgG_{2a} isotype control (shaded). (D) Number of pre-DCs in the blood, spleen, and perfused lungs of CX3CR1-sufficient or -deficient mice at steady state. (E) Representative contour plots of splenic pre-DCs from WT or $Ccr2^{-/-}Cx3cr1^{-/-}$ DKO mice stained with anti-CCR2 and -CX3CR1 mAbs. (F) Number of pre-DCs in the BM, blood, spleen, and perfused lungs of WT and $Ccr2^{-/-}Cx3cr1^{-/-}$ DKO mice at steady state. Means ± SEM are shown (N = 3–6). ***P < 0.001. Results shown are from 1 of 2 experiments yielding similar results.

In view of a previous report showing that pre-DCs can proliferate in peripheral tissues [11], we considered the possibility that these cells might proliferate more in $Ccr2^{-/-}$ or $Cx3cr1^{-/-}$ mice than in WT mice, thereby masking the individual roles of these receptors in pre-DC recruitment to the lung. To address that, we performed a sensitive pre-DC competition assay. Donor BM cells from WT (CD45.1) and KO (CD45.2) mice were mixed at a 1:1 ratio and adoptively transferred into irradiated CD45.1 × CD45.2 F1 recipient mice (**Fig. 5A**). The frequencies of donor pre-DCs in the lungs of recipient mice were assessed by flow cytometry and normalized to the number of pre-DCs in the BM (Fig. 5B). Almost no endogenous $CD45.1^+CD45.2^+$ double-positive pre-DCs were observed in the lungs or BM of recipient animals, indicating that recipient pre-DCs were nearly completely

reconstituted with donor cells. In mice receiving mixtures of WT and *Ccr2*$^{-/-}$ BM cells, the percentages of WT pre-DCs were greater in the lung than in the BM, whereas the percentages of KO pre-DCs were reversed (Fig. 5C). After normalizing lung pre-DCs to the percentages of pre-DCs in the BM, we found that *Ccr2*$^{-/-}$ pre-DCs in the lung were significantly reduced compared with their WT counterparts (Fig. 5C). No reduction of *Cx3cr1*$^{-/-}$ pre-DCs was observed compared with WT pre-DCs in recipient mouse lungs (Fig. 5D). Mice receiving mixtures of WT and *Ccr2*$^{-/-}$*Cx3cr1*$^{-/-}$ DKO BM had markedly fewer DKO pre-DCs than WT pre-DCs in the lung (Fig. 5E), indicating that these 2 receptors have partially redundant roles and that both receptors can contribute to the recruitment of pre-DCs to the lung. Notably, we did not observe a requirement for either CCR2 or CX3CR1 in the recruitment of pre-DCs in the spleen (data not shown), suggesting that a different molecular mechanism is responsible for pre-DC accumulation in lymphoid tissues.

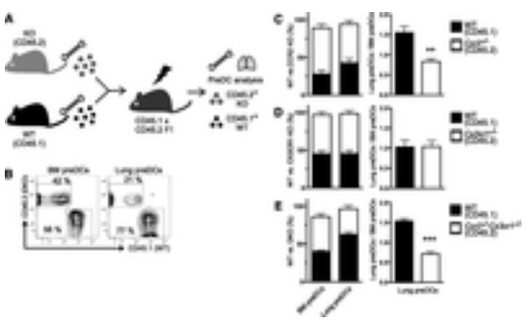

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Figure 5. CCR2- and CX3CR1-dependent pre-DC accumulation in the lung.

(A) Experimental design for pre-DC competition assay in mixed BM chimera mice. BM cells isolated from WT (CD45.1) and chemokine receptor KO mice (CD45.2) were mixed at a 1:1 ratio and transferred to irradiated CD45.1 × CD45.2 F1 recipient mice. pre-DCs in the BM and lungs from the recipient mice were analyzed by flow cytometry. (B) Representative contour plots of pre-DCs in the BM and lungs of mice receiving mixed BM donor cells from WT and *Ccr2*$^{-/-}$*Cx3cr1*$^{-/-}$ DKO mice. Percentage of pre-DCs derived from WT or DKO BM displaying CD45.1 or CD45.2 on their surface, respectively, is shown. (C–E) Effect of CCR2 and CX3CR1 on the ability of pre-DCs to competitively repopulate lungs of irradiated recipient mice. Shown are percentages of WT- and KO-derived pre-DCs in the BM and lungs of recipient mice (left); and lung pre-DCs of each genotype normalized to pre-DCs of that same genotype in recipient BM (right). Means ± SEM are shown ($N = 4$). $^{**}P < 0.01$, $^{***}P < 0.001$. Results shown are from 1 of 2 experiments yielding similar results.

Mature cDCs accumulation in the lungs of mice lacking CCR2 and CX3CR1

Because pre-DCs differentiate to cDCs after migration from the blood to peripheral tissue, we analyzed the numbers of mature cDCs in the lungs of mice lacking CCR2 or CX3CR1 or both. To restrict our analyses to pre-DC–derived cDCs, we excluded CD88^{hi} macrophages (formerly called monocyte-derived DCs) and Ly-6C^{+} monocytes from our gate of CD11c^{+}I-A^{+} lung cells [17]. In mice receiving mixtures of WT and *Ccr2*$^{-/-}$ BM, *Ccr2*$^{-/-}$ BM-derived DCs were underrepresented in both CD11b^{hi} and CD103^{+} cDCs subsets compared with their WT counterparts (**Fig. 6A**), in agreement with our finding that *Ccr2*$^{-/-}$ pre-DCs are also decreased in the lungs of mixed BM chimera mice (Fig. 5C). In mice receiving mixtures of WT and *Cx3cr1*$^{-/-}$ BM cells, CD11b^{hi} cDCs derived from *Cx3cr1*$^{-/-}$ donor BM cells were modestly, but significantly, decreased compared with those derived from WT donors, whereas no genotype-specific differences were seen for CD103^{+} cDCs (Fig. 6B). In mice receiving mixtures of WT and *Ccr2*$^{-/-}$*Cx3cr1*$^{-/-}$ DKO BM, the numbers of mature CD11b^{hi} and CD103^{+} cDCs derived from *Ccr2*$^{-/-}$*Cx3cr1*$^{-/-}$ DKO BM cells were markedly decreased compared with those derived from their WT counterparts (Fig. 6C). Thus, the homeostasis of mature cDCs in the lung at a steady state is directly influenced by pre-DC accumulation, which is, in turn, directed by CCR2 and CX3CR1.

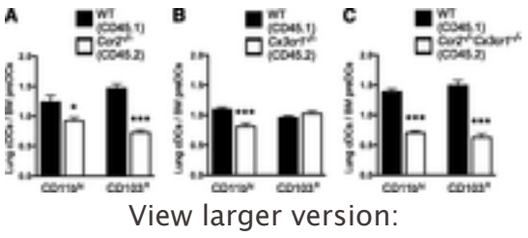

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Figure 6. CCR2- and CX3CR1-dependent cDC accumulation in the lung.

Relative abundance of WT- and KO-derived CD11b^{hi} cDCs and

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CD103$^+$ cDCs in lungs of mice receiving 1:1 mixtures of WT and KO BM. Lung cDCs of each genotype were normalized to numbers of pre-DCs of that same genotype in recipient BM. Shown are means ± SEM (N = 4). *P < 0.05, ***P < 0.001. Results shown are from 1 of 2 experiments yielding similar results.

CCR2 mediates pre-DC migration during inflammation

Inhalation of LPS increased the number of pre-DCs in the lung compared with those seen at steady state (**Fig. 7A**). To better understand the molecular mechanisms underlying that increase, we measured the expression of genes encoding ligands for the CXCR4, CX3CR1, and CCR2. *Cxcl12* and *Cx3cl1*, which encode ligand chemokines of CXCR4 and CX3CR1, respectively, showed little or no increase in expression in the lung after LPS inhalation (Fig. 7B). By contrast, expression levels of *Ccl2* and *Ccl7*, which encode CCR2 ligands, were dramatically up-regulated in the lung after LPS inhalation (Fig. 7B), suggesting that these ligands recruit pre-DCs to the site of inflammation. Furthermore, ex vivo chemotaxis assays also supported a role for CCR2 ligands in directing pre-DC migration (Fig. 7C). To directly test the role of CCR2 in pre-DC accumulation in inflamed lung, we performed in vivo assays with WT and *Ccr2*$^{-/-}$ mice. Pre-DC numbers in the BM and blood were comparable between WT and *Ccr2*$^{-/-}$ mice and did not change dramatically after LPS inhalation, although pre-DCs were modestly reduced in the blood of LPS-treated *Ccr2*$^{-/-}$ mice compared with their WT counterparts (Fig. 7D). Importantly, lung pre-DC numbers were dramatically increased in WT mice as well as *Cx3cr1*$^{-/-}$ mice after LPS inhalation, but the same treatment failed to increase pre-DC numbers in *Ccr2*$^{-/-}$ mouse lungs (Fig. 7D and E). These results suggest that CCR2 and its ligands have a major role in the recruitment of pre-DCs to the inflamed lung, whereas CX3CR1 is dispensable.

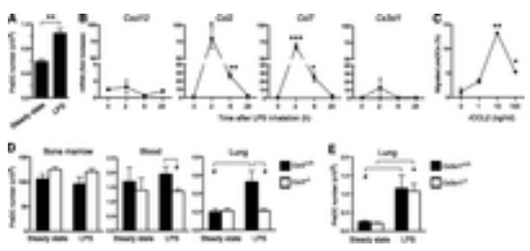

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Figure 7. CCR2-dependent pre-DC accumulation in the inflamed lung.

(A) Number of pre-DCs in lungs of C57BL/6 mice at steady state and 16 h after LPS inhalation (N = 3). (B) mRNA for *Cxcl12*, *Ccl2*, *Ccl7*, and *Cx3cl1* normalized to *Ppih* mRNA in whole lungs at the indicated times after LPS inhalation. Values shown represent the fold increase compared with baseline (0 h) (N = 3). (C) Dose–response of pre-DCs to CCL2 as measured in a transwell chemotaxis assay. Shown are the percentages of pre-DCs migrating from the top to bottom wells at the indicated concentration of CCL2. (D) Number of pre-DCs in the BM, blood, and lungs of CCR2-sufficient or -deficient mice at steady state and 16 h after LPS inhalation. (E) Number of pre-DCs in the perfused lungs from CX3CR1-sufficient or -deficient mice at steady state and 16 h after LPS inhalation. Means ± SEM are shown (N = 3). $^{\#}P$ = 0.06, *P < 0.05, **P < 0.01, ***P < 0.001. Results shown are from 1 of 2 experiments yielding similar results.

Localization of pre-DCs in the lung during inflammation

In the inflamed lung, CCL2 is produced by multiple cell types, including vascular endothelial cells, airway epithelial cells, smooth muscle cells, and alveolar macrophages [50, 51]. However, it is not known where in the lung pre-DCs localize upon extravasation from the vasculature. To study this, we purified pre-DCs from BM of *Cd11c*^{Cre} *Rosa26*^{Tomato} mice and transferred the cells into splenectomized C57BL/6 mice. Adoptively transferred pre-DCs, identifiable by their red fluorescence (TdTomato), were readily detected in precision-cut lung slices by confocal microscopy. Although some TdTomato$^+$ pre-DCs were seen in close association with alveolar walls throughout the lung at a steady state (**Fig. 8A–C**), many more pre-DCs were observed in lungs of LPS-treated recipients (Fig. 8D-F). The latter animals displayed an accumulation of pre-DCs in the vascular endothelium, subendothelial area (Fig. 8F), and subpleural areas (Fig. 8D). Pre-DCs in close proximity to alveolar walls were also increased, and some pre-DCs were

associated with the airway mucosa (Fig. 8E). This diverse localization of pre-DCs within the lung is consistent with the multiple sources of CCL2 in the lung and with the ability of CCR2-ligand chemokines to attract pre-DCs.

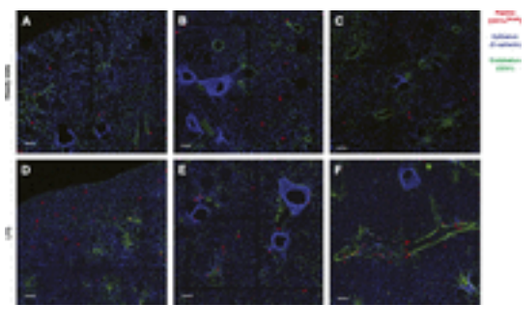

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Figure 8. Microscopic imaging of pre-DC recruitment to the lung.

Images of 150-μm-thick lung slices freshly prepared from animals 12 h after adoptive transfer of purified pre-DCs (TdTomato^{+}, red) to unsensitized (A–C) or LPS-instilled recipient mice (D–F). Slices are stained with Abs against E-cadherin (blue) and CD31 (green). Images of whole lung slices were acquired using a multiphoton laser-scanning microscope with a ×20 objective lens for 7 h. Tissue images, including subpleural area (A and D), subepithelial area (B and E), and subendothelial area (C and F), are shown. White bar denotes 100 μm.

DISCUSSION

Constitutive migration of cDCs from peripheral tissues to regional LNs during steady state reduces the number of cDCs in the lung, and that migration is accelerated during inflammation. To maintain immune homeostasis, emigration of cDCs from the lung must be balanced by a commensurate recruitment to the lung of pre-DCs that can differentiate into new cDCs. The present study sought to identify molecular mechanisms that control that important event. We found that 3 different chemokine receptors—CXCR4, CCR2, and CX3CR1—coordinate pre-DC trafficking and accumulation. Analysis of mice lacking CXCR4 in *Cd11c*-expressing cells revealed that CXCR4 functions to retain pre-DCs in the BM. CXCR4 and its ligand CXCL12 have been previously shown to act as retention factors for HSCs and CDPs in the BM [43], but a role for CXCR4 in pre-DC localization has not, to our knowledge, been reported. Because HSCs and CDPs lack *Cd11c* expression [44, 45], use of *Cd11c*^{cre}*Cxcr4*$^{fl/fl}$ CKO mice to restrict CXCR4 deficiency to *Cd11c*-expressing cells ensured that our findings for pre-DCs did not simply reflect the abnormalities of their progenitors.

In pre-DC competition assays, *Ccr2*$^{-/-}$ pre-DCs were underrepresented compared with their WT counterparts in the lungs of irradiated BM chimera mice, indicating that CCR2 contributes to pre-DC accumulation in the lung. Paradoxically, however, intact WT and *Ccr2*$^{-/-}$ mice had similar numbers of pre-DCs in the lung. One explanation to account for both observations is that increased proliferation of pre-DCs in the lungs of *Ccr2*$^{-/-}$ mice can compensate for impaired migration of those cells to that organ. Interestingly, however, pre-DCs in intact *Ccr2*$^{-/-}$*Cx3cr1*$^{-/-}$ DKO mice were markedly reduced compared with those in WT mice, indicating that increased local proliferation in DKO mice cannot fully restore the numbers of pre-DCs to the levels seen in WT mice. It is possible that one of these two chemokine receptors is primarily involved in recruitment and that the other primarily controls proliferation or survival in the lung. Additional studies will be required to test those possibilities.

Recently, Schlitzer et al. [52] reported that Siglec-H^{lo} pre-DCs can be categorized according to their display levels of Ly-6C. They found that Ly-6C^{hi} pre-DCs display relatively high levels of CCR2 and give rise to CD11b^{hi} cDCs, whereas Ly-6C^{lo} pre-DCs display CX3CR1 and give rise to CD103^{+} cDCs. Our observation that CD11b^{hi} and CD103^{+} cDC subsets were both dependent on CCR2 in pre-DC competition assays suggests that CCR2 has a functional role in the accumulation of precursors of both DC subsets. Thus, a minor population of CCR2^{+}Ly-6C^{lo} pre-DCs might give rise to CD103^{+} cDCs. Alternatively, CD103^{+} cDC precursors displaying CCR2 at low levels might increase their display of CCR2 in a tissue-specific manner as they mature. Our observation that CCR2 is highly displayed on both CD11b^{hi} and CD103^{+} cDCs in the lung, but not in the spleen, supports the latter possibility (unpublished observations). By contrast, WT and *Cx3cr1*$^{-/-}$ BM gave rise to similar numbers of pre-DCs in the competition assays, and it remains to be determined what role CX3CR1 has in pre-DC biology.

Pre-DCs were reduced, but not absent, in the lungs of *Ccr2*$^{-/-}$*Cx3cr1*$^{-/-}$ DKO mice. It is possible, therefore, that a third chemokine receptor can also direct recruitment of pre-DCs, at least in the absence of CCR2 or CX3CR1. Alternatively, increased self-renewal of pre-DCs and cDCs in *Ccr2*$^{-/-}$*Cx3cr1*$^{-/-}$ DKO mice might partly compensate for the impaired pre-DC recruitment in these animals. The latter idea was supported by a previous report showing that pre-DCs and cDCs have the capacity to proliferate in the spleen, LNs, and lung [11, 53]. The extent to which each of those possible mechanisms affects cDC numbers in the lung remains uncertain. It is likely that the number of cDCs in the lung is also affected by proliferation and cell death of the cDCs themselves, as well as by emigration from the lung to draining LNs. Our goal in the present study was to identify which chemokines and receptors orchestrate the trafficking of pre-DCs to the lung. We found that this occurs in a coordinated, step-wise fashion, with CXCR4 and CXCL12 promoting the retention of pre-DCs in the BM and CCR2 and CX3CR1 directing the movement of pre-DCs from the circulation to the lung. Finally, the induction of CCR7 in activated, mature cDCs guides them from the lung to regional LNs to stimulate naïve T cells [21]. Thus, the trafficking of cDCs and their precursors is exquisitely controlled by the sequential induction of multiple chemokine receptors, culminating in the arrival of mature, Ag-bearing cDCs in regional LNs.

In addition to directing pre-DC trafficking, CCR2 and CX3CR1 also promote monocyte migration [23, 54]. Therefore, pre-DCs and monocytes likely colocalize in tissues producing ligands of these chemokine receptors. Upon their entry into inflamed tissue, blood-derived monocytes rapidly differentiate into CD11c^{+}MHC-II^{+} inflammatory monocytes [17]. CCR2-dependent pathogenesis, including that associated with viral infection, atherosclerosis, and Th1 cell differentiation, has been largely attributed to the function of monocytes and their progeny [55⇓⇓–58]. However, monocyte-derived cells do not stimulate T cells as efficiently as cDCs do [17], and it seems likely that the T cell–mediated aspect of CCR2-dependent pathology in various disease models is at least partly due to the migration of pre-DCs to inflamed tissue. Although CCR2 antagonists have been effective in some inflammatory settings, such as influenza infection and atherosclerosis [59, 60], small molecules that differentially inhibit the migration of monocytes and pre-DCs will be important to build on our current understanding and to improve treatment of CCR2-mediated pathology.

AUTHORSHIP

H.N. and D.N.C. conceived and designed the project. H.N., M.R.L.C., G.S.W., and K.N. performed experiments. H.N. and D.N.C. wrote the manuscript.

DISCLOSURES

The authors declare no conflicts of interest.

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Footnotes

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

BM
bone marrow
cDC

conventional dendritic cell

CDP
common dendritic cell progenitor

CKO
conditional knockout

DKO
double knockout

FLT3L
FLT3 ligand

HSC
hematopoietic stem cell

KO
knockout

LN
lymph node

NIEHS
National Institute of Environmental Health Sciences

ppih
peptidylprolyl isomerase H

pre-DC
dendritic cell precursor

WT
wild-type

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