Impaired Lung Dendritic Cell Activation in CCR2 Knockout Mice

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Dendritic cell (DC) recruitment is a hallmark event in antigen (Ag)-challenged lungs. We previously reported models for analyzing DC migration and activation in the lung after Th1- or Th2-eliciting pathogen Ag-bead challenge. To determine the role of chemokines in DC mobilization, we applied this analysis to CCR1, CCR2, CCR5, and CCR6 chemokine receptor knockout mice. Both *Mycobacterium bovis* protein Ags and helminthic, *Schistosoma mansoni* egg Ags elicited multiple chemokines, including CCR1, CCR2, CCR5, and to a lesser extent CCR6 ligands. DCs from wild-type lungs expressed transcripts for chemokine receptors, CCR1, CCR2, CCR5, and CXCR4. In all knockout strains, CD11c⁺ cells were recruited to Ag-beads likely because of receptor redundancy. However, DCs in CCR2^{-/-} mice had significantly decreased MHCII and CD40 expression. This was associated with abrogated cytokine production in draining lymph node cultures. Analysis of local innate inflammation revealed a 50% reduction in macrophage recruitment in CCR2^{-/-} mice. Bone marrow chimeras of mixed CCR2^{+/+} green fluorescent protein transgenic and CCR2^{-/-} green fluorescent protein-negative cells confirmed the DC maturation defect was only among the latter population. In conclusion, CCR2 knockout confers an intrinsic DC activation defect and CCR2 ligands likely promote the local activation/maturation of inflammatory DCs. (*Am J Pathol* 2004, 165:1199–1209)

Dendritic cells (DCs) are professional antigen (Ag)-presenting cells that dictate various types of immune responses including immune tolerance.¹ At different developmental stages cultured human and mouse DCs may express various chemokine receptors including CCR1, CCR2, CCR4, CCR5, CCR6, CCR7, CXCR1, and CXCR4. Many of these receptors have been identified in freshly isolated DCs from various anatomical sites.^{2–6} These findings support the notion that chemokine and chemokine receptor expression regulate DC trafficking.

Immature DCs are recruited rapidly to the lung during inflammatory responses elicited by a broad spectrum of stimuli including viral, bacterial, and soluble Ags.^{7,8} Recruited DCs reportedly move through the inflamed lung where they encounter Ag and inflammatory mediators, and then eventually migrate into the draining lymph node (DLN) where they function as mature Ag-presenting cells.^{7,9} Investigation of DC maturation and function has been hampered by the paucity of DCs found in tissues. Most studies have relied on *in vitro* culture. Unfortunately, the complexity and dynamics of the *in vivo* environment have not yet been possible to reproduce *in vitro*, therefore more *in vivo* analyses with relevance to disease conditions are required.

We previously described an experimental mouse model in which localized innate inflammatory responses in the lung were induced by embolized agarose beads coated with either Th1- and Th2-eliciting pathogen Ags derived from *Mycobacterium bovis* bacteria and *Schistosoma mansoni* ova, respectively.¹⁰ This approach allowed the study of synchronously recruited populations of DCs. Unlike active infection models in which lesions have a poorly predictable temporal appearance, the synchronized models are well suited for analyses of time-dependent events and can be used to study virtually all stages of the immune response. Furthermore, because of

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synchronicity, temporal events are amplified at any given point. For example, DCs isolated from the lung during asynchronous responses are at various stages of maturation, making analysis difficult, whereas in the Ag-bead-challenged lung, DCs are much more homogeneous in their activation status. Previous analysis showed that multiple chemokines were induced rapidly after Ag-bead challenge, which correlated well with the accumulation of CD11c+ DCs and other leukocytes around beads.¹⁰ Lesion-associated DCs displayed induction of MHCII and other co-stimulatory molecules when compared to DCs from unchallenged lungs, suggesting *in situ* DC maturation. In particular lesion-associated DCs dramatically increased levels of MHCII and CD40 expression and acquired Ag-presenting capability as demonstrated by adoptive transfer experiments.¹⁰

In the present study we used CCR1-, CCR2-, CCR5-, and CCR6-targeted gene knockout mice to determine the participation of chemokines in DC recruitment and activation *in situ* in the lung after pathogen Ag-bead challenge. Our results show that deletion of individual chemokine receptors fails to completely block DC recruitment. However, in CCR2-deficient mice, activation of DCs in the lung was significantly impaired as indicated by abrogated MHCII and CD40 expression. Further analysis revealed that in CCR2-deficient mice cytokine production was abrogated in DLNs and the local leukocyte recruitment to the lung was altered with a 50% reduction in macrophages. Transplantation of mixed CCR2+/+ green fluorescent protein (GFP) transgenic and CCR2-/- bone marrow cells confirmed the defect was only among the latter population. Hence, CD11c+CD11b+ DC recruitment is well protected by biological redundancy but CCR2 ligands play an important role in local DC maturation/activation.

Materials and Methods

Mice

CCR2-/- mice backcrossed to a C57/B6 background were originally generated as described.¹¹ CCR1-/- mice on 129xB6 background were generated as previously described.¹² CCR5-/- mice on 129xB6 background, 129xB6, C57/B6, and CBA mice were obtained from Jackson Laboratories, Bar Harbor, ME. Dr. Sergio Lira, Mount Sinai School of Medicine, New York, NY, generated GFP transgenic mice and CCR6-/- mice backcrossed to C57/B6 mice.^{13,14} Knockout status was confirmed before experimentation. All mice were maintained under specific pathogen-free conditions and provided food and water *ad libitum*.

Ag-Bead Challenge Protocol

Innate granulomatous responses, were elicited in naïve mice by intravenous injection of 6000 Sepharose 4B beads covalently coupled to *M. bovis* purified protein derivative (PPD) (Department of Agriculture, Veterinary

Division, Ames, IA) or to *S. mansoni* soluble schistosoma egg Ags (SEA) as previously described.¹⁵

Morphometry

Granulomas were measured blindly from formalin-inflated lungs that were paraffin-embedded, sectioned, and then stained with hematoxylin and eosin. Granuloma area was measured by computerized morphometry. A minimum of 20 lesions was measured per lung.

Immunohistochemistry

Frozen tissue sections (5 to 7 μ m thick) were mounted on poly-L-lysine-coated slides, fixed with acetone, and then rehydrated in phosphate-buffered saline (PBS). Sections were pretreated for 10 minutes with 0.03% H₂O₂ and then avidin and biotin. The sections were covered with 10 μ g/ml of biotinylated hamster anti-mouse CD11c mAb (BD Pharmingen, San Diego, CA). Biotinylated nonimmune hamster IgG served as a control. Slides were incubated for 1 hour at room temperature, then rinsed and overlaid with 1 μ g/ml of streptavidin peroxidase (Sigma, St. Louis, MO), and incubated another 30 minutes at room temperature. The slides were rinsed again in PBS and overlaid with a 1:20 dilution of AEC staining solution (Sigma) for 20 minutes at room temperature. Finally, the sections were rinsed, counterstained with Gill's hematoxylin, and mounted with coverslips.

Bone Marrow Transplantation

Recipient C57BL/6 wild-type or CCR2-/- mice received 10 Gy of total body irradiation (¹³⁷Cs source) split into two doses separated by 3 hours to minimize gastrointestinal toxicity. One hour after the last radiation dose, recipients were infused intravenously with 10 $\times$ 10⁶ freshly isolated bone marrow cells from either CCR2-/- or CCR2+/+ GFP transgenic C57BL/6 donors. Mice were housed in sterilized isolator cages and received sterile, acidified water. Three weeks after transplant mice were challenged with Ag-beads and lungs were subjected to analysis 3 days later. Successful grafting of GFP+ cells was confirmed by flow cytometric analysis of bone marrow samples.

CD11c+ DC Isolation

After perfusion with cold RPMI 1640, bead-challenged lungs were excised, placed in cold RPMI 1640 medium, and then homogenized in a Waring blender (EBERBACH, Ann Arbor, MI). Tissue fragments were collected over a no. 100 stainless steel mesh, rinsed with cold RPMI 1640, and then pelleted by centrifugation. Naïve lungs were similarly homogenized. Single cell suspensions were obtained by collagenase digestion as previously described.¹⁵ Briefly, homogenates were incubated with digestion medium containing RPMI (JRH Biosciences, Lenexa, KS), 10% fetal bovine serum (FBS) (Intergen,

Purchase, NY), 10 mmol/L glutamine, and 1000 U/ml type IV collagenase (Sigma). The digest was sieved through a stainless steel mesh (no. 100) and then washed four times by centrifugation in RPMI 1640. Single cell suspensions were also prepared from freshly harvested lymph nodes as previously described.^{15,16} CD11c⁺ DCs were isolated using MACS CD11c beads (Miltenyi Biotec, Auburn, CA). The enrichment of the recovered cells was confirmed by flow cytometric analysis and was routinely greater than 95%.

Flow Cytometry

Monoclonal antibodies used to identify mouse DC populations included fluorescein isothiocyanate-, phycoerythrin-, and cytochrome-conjugated anti-CD11c (HL3), phycoerythrin-conjugated anti-I-A^k (11-5.2), anti-CD8a (53-6.5), anti-CD11b/Mac-1a (M1/70), anti-CD40 (3/23) (all from BD PharMingen), anti-DEC-205 (NLDC-145) and anti-F4/80 (A3-1) (both from Serotec). All isotype controls and anti-CD16/CD32 (2.4G2) were from BD Pharmingen. After blocking with anti-CD16/CD32 for 5 minutes, cells were stained with fluorescent-labeled antibodies or isotype control antibodies in 2% FBS-PBS buffer. A FAC-Scan flow cytometer with CellQuest software (B-D, San Jose, CA) was used for data acquisition and analysis. Depending on the number of cells available, 10,000 to 100,000 events per sample were analyzed.

Lymph Node Cell Culture and Cytokine Assays

Mediastinal lymph nodes were collected aseptically at the time of lung harvest and teased into a single cell suspension. After washing, the cells were cultured in RPMI 1640 medium (JRH Biosciences) containing 10% FBS (Intergen), 10 mmol/L glutamine, 100 μ g/ml streptomycin, and 100 U/ml penicillin (RPMI-FBS) at 5×10^6 cells/ml in 100-mm dishes with 5 μ g/ml of PPD or SEA for 48 hours at 37°C in a humidified 5% CO₂ atmosphere. Supernates were collected by centrifugation and stored at -45°C before performing cytokine assays. All cytokines used in this study were obtained as purified carrier-free recombinant proteins from PreproTech Inc., Rocky Hill, NJ, and R&D Systems, Minneapolis, MN. Interleukin (IL)-2, IL-4, and interferon- γ were measured by standard enzyme-linked immunosorbent assay using commercially available reagents (R&D Systems and BD Pharmingen); sensitivities ranged from 15 to 50 pg/ml.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (PCR) Analysis of mRNA

Real-time reverse transcriptase-PCR analyses were performed as previously described.¹⁷ Poly(A)⁺ pure mRNA was isolated from cells using Poly(A)⁺ pure mRNA isolation kits (Ambion, Austin, TX) and reverse-transcribed using Reverse Transcription System kits (Promega, Madison, WI). Real-time PCR was performed on samples before and after reverse transcription to assess genomic DNA

contamination levels in a sample preparation. DNase treatment was applied whenever a significant contamination was detected. For each CK receptor the mRNA was considered detectable only when the signal was 10-fold more abundant than the contamination level. The ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) was used for real-time PCR analysis with comparative CT method as described by the manufacturer. TaqMan predeveloped reaction kits were used. In all cases, the thermal cycling condition was programmed according to the manufacturer's instructions. Data are expressed as arbitrary units as described previously.¹⁷

Statistics

Analysis of variance was used for intergroup comparisons with $P < 0.05$ considered to indicate significance. Pairwise tests were done with Dunnett error protection at 95% confidence interval.

Results

Chemokine Receptor Expression Profile of Ag-Bead Elicited CD11c⁺ DCs

Lung-embolized Ag-beads coated with either mycobacterial PPD or schistosomal egg Ags (SEA) elicit rapid innate DC recruitment and invoke polarized Th1 and Th2 responses in naïve mice as described.¹⁰ The recruited DCs were exclusively CD11b⁺ and hence of myeloid derivation. This approach was used to study the role of chemokine receptors in DC mobilization in the mouse lung.

Cultured human and mouse DCs reportedly express chemokine receptors, CCR1, CCR2, CCR4, CCR5, CCR6, CCR7, CXCR1, and CXCR4, at different developmental stages. Many of these receptors have been identified in freshly isolated DCs from various anatomical sites and are thought to mediate DC trafficking,²⁻⁶ however it was unknown what chemokine receptors were expressed by Ag-bead-elicited DCs. To approach this question we used real-time PCR analysis to characterize and compare chemokine receptor transcript expression among DC populations isolated from relevant tissue compartments. CD11c⁺ cells from DLNs and naïve or challenged lungs were isolated and analyzed. As shown in Figure 1, DCs of naïve lungs expressed CCR1, CCR5, and CXCR4 transcripts. Those of bead-challenged lungs likewise expressed these transcripts, but in addition showed CCR2 expression. Lung CD11c⁺ populations were different from those in the DLNs, which displayed mainly CCR4, CCR5, CCR6, CCR7, and CXCR4 transcripts. Transcripts for CXCR1 were not detected among any of the DC populations (data not shown). Interestingly, CCR6, which has been suggested to mediate the migration of DC and Langerhans cells to sites of Ag challenge in humans,¹⁸⁻²⁰ was not expressed at the transcript level among mouse lung DCs but was readily detected among the DLN populations.

Figure 1. Chemokine receptor transcript expression among CD11c⁺ populations purified from naïve lungs, challenged lungs, and DLNs. CD11c⁺ DCs were freshly isolated from naïve CBA/J mouse lungs (lung) or lungs (bead) and corresponding DLNs (node) from day 3 Ag-bead (SEA or PPD)-challenged CBA/J mice. Transcript expression was measured using real-time PCR. Bars are mean arbitrary units $\pm$ SD derived from three independent samples, in which each sample was generated from DCs isolated from a pool of five mice.

Ag-Bead-Challenged Lungs Display Appropriate Ligands for DC Chemokine Receptors

We next assessed transcript expression of CCR1/5, CCR2, and CCR6 agonists on days 1, 2, and 3 of Ag-bead challenge. Data are presented in Figure 2 and are expressed as fold-increases over naïve unchallenged lungs. As shown, CCR1/5 agonists, CCL3 (MIP-1 α) and CCL4 (MIP-1 β), and the CCR2 agonist, CCL2 (MCP-1), were the most dominant, whereas the CCR6 agonist, CCL20 (MIP-3 α) was only marginally induced in the PPD-bead and not in the SEA-bead response. We have shown previously that the CXCR4 agonist, CXCL12 (SDF-1 α), is expressed constitutively in the lung.²¹ These results indicated that CCR1, CCR2, and CCR5 agonists were locally induced after Ag-bead challenge and could potentially mediate the active recruitment of DCs based on the DC chemokine receptor profiles described above.

DC Recruitment Persists in CCR1-, CCR2-, CCR5-, and CCR6-Deficient Mice

Soluble Ags or microorganisms, when used for lung DC studies, are often introduced in a nonlocalized manner. The DCs that respond to Ag challenge under these con-

Figure 2. Time course and induction of chemokine transcripts in lungs with innate Ag-bead granuloma formation. Lung tissues were collected from naïve or day 1, 2, or 3 Ag-bead-challenged CBA/J mice. Transcript expression was measured using real-time PCR. Bars indicated fold increases over naïve unchallenged lungs and were derived from lungs of five individual mice.

ditions are not easily distinguished from those that are constitutively recruited in the lung *in situ*. Using Ag-coated beads that are much larger than the size of cells we have been able to localize Ag deposition and observe likely sites of Ag-DC encounter in the lung. Lodged in the microvasculature, Ag-beads attract large numbers of CD11c⁺ cells. By immunohistochemistry, electron microscopy, and flow cytometric analyses, we previously identified these cells as one of the earliest populations arriving at Ag-beads.¹⁰

To examine the potential role of chemokines in DC mobilization we elicited pathogen-Ag-bead responses in mouse strains with targeted knockout of CCR1, CCR2, CCR5, and CCR6. On day 3 the lungs were excised for histological and immunohistochemical analysis. Figure 3 shows the relative sizes of lesions in knockout and respective wild-type control strains. In the mycobacterial PPD response, lesions of CCR1^{-/-} and CCR2^{-/-} mice displayed a 30% decrease in cross-sectional area compared to controls, whereas CCR5^{-/-} and CCR6^{-/-} mice showed no changes. In the helminthic Ag (SEA) response, only CCR2 knockout had a significant effect, reducing lesion areas by ~40%. Thus CCR1 and CCR2 caused detectable effects on local innate inflammation.

To determine possible effects on DC mobilization, lungs were subjected to immunohistochemical staining for CD11c⁺ cells. Figure 4 shows representative immunohistochemical stains of Ag-bead-challenged lungs. Positively staining cells were readily detected in close proximity to beads. This was the case for all strains

Figure 3. Innate granuloma formation in chemokine receptor knockout mice. Day 3 Ag-bead granulomas were elicited in the various knockout strains as described in Materials and Methods. Lungs were then removed, prepared for histological sectioning, and subjected to morphometric analysis to determine average granuloma cross-sectional area. A minimum of 20 lesions was measured per mouse. Bars are means $\pm$ SEM. The **dashed line** indicates area occupied by the beads. Bars are grouped according to the background strain and are shown with the appropriate control strain (n = five to six mice per group). *, $P < 0.05$.

despite the effects on local inflammation. As discussed later this was further confirmed by flow cytometric analysis. Given the fact that circulating DCs or DC precursors are potentially capable of responding to multiple chemotactic factors persistent DC recruitment may not be unexpected when only individual chemokine receptors are deleted.

DC Activation in the Lung Is Impaired in CCR2-Deficient Mice

Immature CD11b+ DCs are derived from bone marrow from whence they migrate via the peripheral blood to virtually every tissue in the body where they potentially acquire maturation/activation markers after encounter with Ag and other stimuli including inflammatory mediators such as tumor necrosis factor- α and GM-CSF.¹ In our previous report, we showed that Ag-bead challenge induced a dramatic up-regulation of MHCII and CD40 expression by CD11c+CD11b+ cells in the lung.¹⁰ Significant numbers of lung DCs were found at sites of Ag-bead deposition. These cells presumably process Ags, undergo maturation, and acquire Ag presentation capacity *in situ*. Indeed, on adoptive transfer, Ag-bead-elicited CD11c+CD11b+ DCs induced Ag-specific T-cell differentiation in naïve recipient mice.¹⁰

We next evaluated the possible effect of chemokine receptor knockout on DC maturation events in the lungs because altered T-cell responses have been reported in several of these knockout strains.^{11,22–24} This analysis revealed that significant changes in DC maturational markers occurred only in CCR2-/- mice (Table 1). As we previously reported, compared to unchallenged lungs, Ag-bead challenge increased CD11c+ cells and the expression of MHCII and CD40 expression in wild-type mice.¹⁰ In CCR2-/- mice the proportion of CD11c+ cells was not significantly altered but the proportion of CD11c+MHCII+ and/or CD11c+CD40+ cells were reduced by 50 to 70% in PPD-bead-challenged lungs and 30 to 60% in SEA-bead-challenged lungs compared to wild-type controls.

Figure 4. Immunohistochemical and morphological detection of CD11c+ cells in Ag-bead granulomas elicited in control and chemokine receptor knockout strains. Frozen sections were obtained from day 3 bead-challenged lungs and stained for CD11c. Note red-brown staining cells within innate granulomas. CD11c+ cells were detectable in all knockout strains although overall cellularity was less in CCR2-/- mice. Wild-type B6X129 and CCR6 knockout mice (not shown) were similar to wild-type B6 controls (shown). Original magnifications, $\times 200$.

Table 1. Impaired DC Maturation in Ag-Bead-Challenged Lungs of CCR2 Knockout Mice

Experiment and strain	Percent of CD45+ cells (Mean fluorescent intensity)								
	Naïve lung			PPD-bead challenged			SEA-bead challenged		
	CD11c ⁺	CD11c ⁺ MHCII ⁺	CD11c ⁺ CD40 ⁺	CD11c ⁺	CD11c ⁺ MHCII ⁺	CD11c ⁺ CD40 ⁺	CD11c ⁺	CD11c ⁺ MHCII ⁺	CD11c ⁺ CD40 ⁺
Exp. 1									
CCR2 ^{+/+}	7 ± 3	4.0 ± 1.0 (1352 ± 194)	nd	24 ± 3.0	18 ± 3 (2421 ± 106)	nd	20 ± 2.0	15 ± 2.0 (1950 ± 422)	nd
CCR2 ^{-/-}	10 ± 1	5.6 ± 0.4 (1357 ± 338)	nd	20 ± 4.0	4.9 ± 0.1* (397 ± 72*)	nd	18 ± 2.0	6 ± 2.0* (686 ± 150*)	nd
Exp. 2									
CCR2 ^{+/+}	15 ± 1	7.1 ± 0.2 (839 ± 23)	1.2 ± 0.2 (17 ± 2)	23 ± 1.0	14 ± 2 (2134 ± 255)	6.3 ± 0.9 (57 ± 4)	25 ± 2.0	18 ± 0.5 (1850 ± 230)	7.0 ± 1.0 (42 ± 10)
CCR2 ^{-/-}	18	9.9 (1106)	1.7 (20)	19 ± 2.0	7 ± 3* (1014 ± 213*)	2.6 ± 0.4* (25 ± 6*)	23 ± 4.0	12 ± 3.0* (1303 ± 130*)	5.6 ± 0.2 (40 ± 8)

nd, not determined.
 Values in parentheses are MFI.
 **P* < 0.05.

The impaired DC maturation in challenged CCR2^{-/-} mice was also demonstrable at the level of average fluorescent intensity, which was comparably abrogated (Table 1). Interestingly, our analysis revealed different effects with PPD- and SEA-bead challenge such that CD40 up-regulation was reduced in the former but not in the latter indicating independent regulation of these co-stimulatory molecules (Table 1). Figure 5 shows scattergrams from a representative flow cytometry experiment showing the reduction of MHCII⁺ and CD40⁺ CD11c⁺ populations. Note that patterns of challenged CCR2^{-/-} mice tended to resemble that of unchallenged naïve mice. This effect was unique to CCR2^{-/-} mice and was not observed in CCR1^{-/-}, CCR5^{-/-}, or CCR6^{-/-} mice (data not shown). Taken together the data indicated that *in situ* maturation of DCs was impaired in Ag-bead-challenged CCR2^{-/-} mice.

Early DLN Cytokine Responses Are Impaired in Ag-Bead-Challenged CCR2^{-/-} Mice

Impaired cytokine responses have been reported in CCR2^{-/-} mice after adjuvant sensitization and bacterial infection.^{11,25–27} To determine whether Ag-bead-challenged, unsensitized CCR2^{-/-} mice likewise displayed impaired cytokine production during primary sensitization, draining mediastinal lymph nodes were dispersed and cultured with respective Ags 4 days after either PPD- or SEA-bead challenge and then levels of interferon- γ and IL-4 were measured. As shown in Table 2, cultures from PPD-bead-challenged CCR2^{-/-} mice displayed a 70% reduction in levels of interferon- γ . There was also a trend to reduced interferon- γ levels in the SEA-bead-challenged mice but this did not quite achieve statistical significance. However, the induction of IL-4 in SEA-bead-challenged mice was reduced to below assay detection limit in CCR2^{-/-} mice. This result was fully consistent with previous reports and demonstrated that cytokine defects could be detected in the early immune induction phase.

Local Innate Inflammation Is Altered in CCR2^{-/-} Mice

Because DC recruitment, maturation, and function may depend on signals from other cells in the microenvironment we analyzed Ag-bead-elicited inflammatory responses in the various knockout strains. Specifically, day 3 Ag-bead-challenged lungs were enzymatically dispersed and subjected to flow cytometric analysis to characterize recruited CD45⁺ leukocyte populations. The results of these studies in PPD- and SEA-bead-challenged mice are illustrated in Table 3. As well as showing a loss of mature CD11c⁺MHCII⁺ cells, CCR2^{-/-} mice displayed 60% and 50%, reductions of F4/80⁺ macrophages in PPD and SEA Ag-bead-challenged lungs, respectively. It was also observed that CCR1 knockout significantly reduced granulocytes. In addition, NK cells were reduced in the PPD response. The latter effect was similar to that which we reported for the secondary PPD-bead response.²³ The CCR5 and CCR6 knockout strains showed no significant differences from control mice. These results agreed with the morphometric findings showing reduced lesion sizes in both PPD- and SEA-bead-challenged CCR2^{-/-} mice and in PPD-bead-challenged CCR1^{-/-} mice (Figure 3). In summary, the local inflammatory microenvironment where the DCs were activated was altered by an impaired mobilization of F4/80⁺ cells in CCR2^{-/-} mice when challenged with either PPD- or SEA-beads. These cells represent a potentially rich source of cytokines and chemotactic factors.

CCR2^{-/-} Innate Granuloma DCs Display an Intrinsic Activation Defect

A possible explanation for the impaired activation of innate granuloma DCs was a paucity of activation signals from the reduced recruitment of ancillary F4/80 macrophages. Because CD11b⁺ DCs are bone marrow derived, we performed a series of bone marrow transplant

Figure 5. Flow cytometric scattergram analysis of CD11c⁺ populations in naive and challenged control and CCR2^{-/-} mice. The lungs of naive or day 3 Ag-bead-challenged CCR2^{+/+} controls or CCR2^{-/-} mice were enzymatically dispersed as described in Materials and Methods. The single cell suspensions from the lungs were then labeled with indicated antibodies and subjected to flow cytometric analysis. Note, the up-regulation of MHCII and CD40 observed in Ag-bead-challenged CCR2^{+/+} lungs failed to occur in the challenged lungs of CCR2^{-/-} mice (**top right quadrants**). Percentages of total cells are shown in **right quadrants**.

studies to test this hypothesis. To discern between CCR2^{-/-} and CCR2^{+/+} cells we used donor CCR2^{+/+} mice with transgene expression of GFP (GFP + CCR2^{+/+}). These donor cells were administered to irradiated CCR2^{+/+} or CCR2^{-/-} nontransgenic recipients independently, or mixed at a 1:1 ratio. Recipients were then challenged with PPD-beads 3 weeks after transplant. On day 3 after challenge the lungs were dispersed and subjected to morphometric and flow cytometric analysis.

Figure 6 illustrates the effect of bone marrow transplant on innate granulomatous inflammation (day 3). When CCR2^{-/-} bone marrow was transplanted to CCR2^{+/+} wild-type recipients the local inflammatory response was impaired to a degree similar to that of CCR2^{-/-} mice (Figure 3). Conversely the defect of CCR2^{-/-} mice was fully corrected by transplant of wild-type marrow cells, indicating that the recruitment defect of CCR2^{-/-} mice was because of defective hematopoietic cells. When CCR2^{-/-} and wild-type marrow was mixed (1:1) and transplanted, this also fully reconstituted the inflammatory defect.

We next performed flow cytometric analysis of dispersed granulomatous lungs to characterize relative MHCII expression among recruited CD11c⁺ cells. As shown in Table 4 wild-type mice receiving CCR2^{-/-} bone marrow, displayed a CD11c⁺ activation defect when compared to CCR2^{-/-} or CCR2^{+/+} receiving wild-type GFP⁺ bone marrow (group 1 versus groups 2 and 3). These groups indicated that MHCII activation status or lack thereof was attributed to the donor bone marrow cells and not the host environment. It also demonstrated that GFP⁺ transgenic CD11c⁺ cells activated normally on challenge.

If CCR2^{-/-} DCs required signals from ancillary CCR2^{+/+} cells to achieve maturation then mixing these populations might be predicted to correct the CCR2^{-/-} activation defect. Table 4 (group 4) shows the relative MHCII expression among the CD11c⁺ populations in recipients of combined CCR2^{+/+} and CCR2^{-/-} bone marrow. The CCR2^{+/+}CD11c⁺ cells, marked by GFP expression, showed normal activation but the CCR2^{-/-}CD11c⁺ cells displayed a persistent activation/maturation defect. Similar results were found for CD40 expression in which CCR2^{+/+} cells showed a ratio of CD40^{hi} to CD40^{lo} cells of 0.27 ± 0.01 but the CCR2^{-/-} cells were only 0.03 ± 0.01 . Figure 7 shows representative scattergrams displaying the different maturation states of the CCR2^{+/+} and CCR2^{-/-} CD11c⁺ cells co-existing in granulomatous lungs.

Table 2. Effect of CCR2 Deficiency on Day 4 DLN Cytokine Responses after Primary Antigen-Bead Challenge

Strain	IFN- γ (pg/ml)		IL-4 (pg/ml)	
	PPD	SEA	PPD	SEA
CCR2 ^{+/+}	2200 $\pm$ 30	210 $\pm$ 70	80 $\pm$ 20	170 $\pm$ 50
CCR2 ^{-/-}	550 $\pm$ 20*	70 $\pm$ 10	<50	<50*

Values are levels of cytokines in 48-hour supernates from antigen-stimulated DLN cultures. Lymph nodes were harvested on day 4, the time of earliest detectable Th cell differentiation.

* $P < 0.05$.

Table 3. Flow Cytometric Leukocyte Composition Analysis of Challenged Lungs from Chemokine Receptor Knockout Mice

Challenge and strain	F4/80+	Gr-1+	CD11c+MHCII+	CD3+CD4+	CD3+CD8+	DX5+(NK)
PPD-bead						
129xB6+/+	27 ± 8.5	15.7 ± 2.2	17 ± 6.0	8.1 ± 1.6	4.4 ± 0.5	10.6 ± 3.3
CCR1-/-	35 ± 8.8	7.8 ± 2.5*	22 ± 6.0	14 ± 2.5	5.0 ± 2.0	5.0 ± 2.0*
CCR2-/-	15.1 ± 0.9*	22 ± 8.6	5.9 ± 0.3*	6.4 ± 0.7	8.2 ± 2.7	22 ± 8.6
CCR5-/-	27 ± 8.5	15.7 ± 2.2	19 ± 3.0	8.1 ± 1.6	4.4 ± 0.5	15.7 ± 0.7
CCR6-/-	38 ± 3.5	18.6 ± 6.2	13.7 ± 3.0	4.4 ± 2.0	4.8 ± 0.6	11.2 ± 2.0
B6+/+	33 ± 6.4	11.4 ± 2.4	18.3 ± 6.0	7.0 ± 1.8	3.8 ± 1.2	11.4 ± 2.5
SEA-bead						
129xB6+/+	36 ± 4.0	18.5 ± 1.0	18 ± 1.0	8.1 ± 1.6	1.3 ± 1.0	9.4 ± 1.0
CCR1-/-	36 ± 4.5	6.0 ± 2.0*	24 ± 6.0	14.1 ± 2.5	5.0 ± 1.0	5.0 ± 2.0
CCR2-/-	16.4 ± 1.1*	9.3 ± 3.7	6.3 ± 2.3*	10.4 ± 2.0	7.8 ± 0.4	10.6 ± 1.1
CCR5-/-	49 ± 9.0	13 ± 1.5	18 ± 0.5	12 ± 0.5	3.8 ± 1.0	15 ± 0.5
CCR6-/-	30 ± 5.0	6.0 ± 2.0	13 ± 4.0	7.0 ± 2.0	4.4 ± 1.3	7.2 ± 1.2
B6+/+	30 ± 8.3	6.4 ± 3.5	16.3 ± 6	9.3 ± 2.2	5.5 ± 2.2	7.3 ± 3

Single cell suspensions from the lungs of day 3 PPD-bead-challenged CCR+/+ controls and the various knockout strains were labeled with indicated antibodies and subjected to flow cytometric analysis. The leukocyte populations are presented as percent of CD45+ cells. Values are means ± SD, four to six mice per group. Controls for both B6 and 129xB6 background strains are shown.

**P* < 0.05 as compared to respective background strain.

It should be noted that analyses of recipients of the 1:1 mixtures of CCR2-/- and CCR2+/+GFP+ bone marrow confirmed successful grafting with a ratio of CCR2-/- CD11b+ to CCR2+/+CD11b+ cells of 1.3 ± 0.4 within the marrow, but within challenged lungs this ratio was only 0.5 ± 0.1 fully consistent with impaired recruitment of CCR2-/- but not CCR2+/+ leukocytes. However, the ratio of CCR2-/-CD11b+ to CCR2+/+CD11b+ CD11c+ cells in challenged lungs was similar to the bone marrow, 1.3 ± 0.3, indicating that CCR2+/+ CD11c+ and CCR2-/-CD11c+ subpopulations were recruited equally, thus corroborating our immunohistochemical study. Hence, the maturation defect was intrinsic to the CCR2-/- cells and not because of a lack of extrinsic signaling from ancillary cells.

Figure 6. Innate granuloma formation in CCR2-/- and GFP transgenic bone marrow chimeric mice. Recipient CCR2+/+ wild-type or CCR2-/- mice were irradiated to ablate bone marrow and then reconstituted with CCR2-/-, GFP transgenic wild-type (*, CCR2+/+), or a 1:1 mixture of these. All recipients received a total of 10 million bone marrow cells. Three weeks after transfer, day 3 PPD-bead granulomas were elicited as described in Materials and Methods. Granulomatous lungs were then removed, prepared for histological sectioning, and subjected to morphometric analysis to determine average granuloma cross-sectional area. A minimum of 20 lesions was measured per mouse. The dashed line indicates area occupied by the beads. Bars are means ± SEM of three to four mice per group. *, *P* < 0.05.

Discussion

Rapid mobilization of DCs after Ag challenge in the lung is a well-described phenomenon.^{7,8} However, the underlying mechanisms are not well understood. CCR2 knockout mice have been analyzed extensively and a role for CCR2 in DC recruitment has been suggested.^{25,28} However, many questions remain. For example, it has been shown in a model of pulmonary *Cryptococcus neoformans* infection that DLNs from CCR2 knockout mice had fewer MHCII⁺CD11c⁺ cells compared to controls,²⁹ but the underlying mechanism was unknown. Fluorescein isothiocyanate-induced Langerhans cell migration to and within DLN was impaired in CCR2-/- mice.²⁶ Also the relocation of splenic MHCII⁺ DC during *Leishmania major* infection was reportedly blocked in CCR2-deficient mice.²⁶ However, there is no compelling evidence to show that CCR2 is directly involved in DC migration in lymphoid tissues. In fact, consistent with our findings, DCs found in lymphoid tissues are generally more mature and do not express CCR2.³⁰ DCs or DC precursors express CCR2,^{20,28} but the impairment of DC migration to the sites of Ag challenge has not been definitively shown in CCR2-deficient mice. In fact, Langerhans cell migration to the skin after fluorescein isothiocyanate painting was intact in CCR2-deficient mice,²⁶ which is not surprising given the fact that circulating DCs are responsive to multiple chemotactic factors. However, the development of T-cell responses is clearly impaired in CCR2-deficient mice resulting in compromised control of infection.^{27,31,32} Blocking of CCR2 function *in vivo* suppresses Th1 development.^{11,29,31,33,34} However, *in vitro* culture failed to show differences in Th1 differentiation between CCR2-deficient and wild-type control T cells.²⁵ Moreover, in a mouse autoimmune encephalitis model, adoptively transferred myelin oligodendrocyte glycoprotein 35-55-specific T cells lacking expression of CCR2 were able to induce experimental autoimmune encephalitis, whereas CCR2-/- recipients of wild-type T cells failed to develop disease.³⁵ These results indicate that CCR2 deficiency

Table 4. Impaired MHCII Expression in CCR2^{-/-} CD11c⁺ Cells

Group	Recipient	Bone marrow donor	Ratio (CD11c ⁺ MHCII ^{hi} to CD11c ⁺ MHCII ^{lo})	
			CCR2 ^{-/-}	CCR2 ^{+/+}
1	CCR2 ^{+/+}	CCR2 ^{-/-}	0.37 ± 0.08*	n/a
2	CCR2 ^{-/-}	CCR2 ^{+/+}	n/a	0.93 ± 0.19
3	CCR2 ^{+/+}	CCR2 ^{+/+}	n/a	1.3 ± 0.43
4	CCR2 ^{-/-}	CCR2 ^{-/-} + CCR2 ^{+/+}	0.43 ± 0.09*	1.0 ± 0.06

Values are ratios of MHCII^{hi} CD11c⁺ to MHCII^{lo}CD11c⁺ cells in day 3 PPD bead-challenged lungs among gated GFP⁻ and GFP⁺ cells representing CCR2^{-/-} and CCR2^{+/+} populations, respectively. All recipients were GFP-negative. Means ± SD derived from three to four individual mice.

**P* < 0.05.

n/a, not applicable.

among T cells is not responsible for observed defects in CCR2^{-/-} mice, thus pointing to another population. Although the effect on CCR2 deletion on DC migration is controversial, blocking CCR2 function is known to profoundly reduce the recruitment of F4/80⁺ macrophages to sites of inflammation in liver,³⁶ lung,^{11,31,37} central nervous system,³⁵ and peritoneal cavity.^{11,27,36} These findings are consistent with the fact that, in mice, CCR2 is homogeneously expressed on circulating monocytes but only on a small percentage of T cells.³⁸

A key event that has not been studied in CCR2-deficient mice is the *in situ* activation of Ag-presenting DCs at a site of Ag encounter. According to the current paradigm, DCs are Ag-loaded at sites of Ag encounter and

then activated by local inflammatory mediators.¹ This allows tissue-derived DCs to present Ag as well as direct T-cell maturation toward cytokine responses appropriate to the pathogen. In our study, we found that DC recruitment persisted after both PPD and SEA Ag-bead challenge in CCR1, CCR2, CCR5, and CCR6 knockout strains. However, in CCR2-deficient mice the *in situ* activation of DCs was impaired as indicated by abrogated MHCII and CD40 expression. Consistent with previous studies, CCR2-deficient mice had more than a 50% reduction of F4/80⁺ macrophages in inflammatory lesions as compared to the wild-type controls. This change was unique to CCR2^{-/-} mice and was not found in CCR1, CCR5, and CCR6-deficient mice although CCR1^{-/-} mice had reductions in nonmacrophage populations after PPD-bead challenge, but this was not associated with DC recruitment or maturation defects. It was previously reported that activation of lung DCs is required to prime Th1 development and without activation lung DCs favor Th2 development.³⁹ In accord with this, we recently showed in our models that the Ag-bead-associated DCs preferentially prime Th1 development.¹⁰ Thus, our current finding of impaired activation in CCR2^{-/-} DCs would explain the diminished Th1 response in CCR2^{-/-} mice. We have also reported that anamnestic Th2 cytokine responses are partially reduced in CCR2^{-/-} mice⁴⁰ and in the current study we detected impaired IL-4 induction after primary SEA-bead challenge, which may also relate to impaired DC activation. However, the induction of the Th2 response is clearly more complex and persistent or enhanced Th2 responses have been reported in CCR2^{-/-} mice.^{33,41} Hence, Th1 responses may have a net greater sensitivity to CCR2 knockout, which is intriguing in view of our observation that unlike with PPD challenge, CD40 induction was not as impaired in CCR2^{-/-} mice challenged with a Th2-eliciting Ag, SEA.

Macrophages are well-known sources of cytokines such as tumor necrosis factor- α and IL-1 that could promote DC activation and maturation. Therefore, decreased macrophage recruitment observed in CCR2^{-/-} mice was a possible cause for abrogated DC maturation. Because macrophages are also potential sources of chemokines their loss could also contribute to lessened overall DC recruitment, however CD11c⁺ cells were still readily detected around beads in CCR2^{-/-} mice. Using

Figure 7. CCR2^{-/-} DCs display a persistent maturation defect after co-transfer of CCR2^{+/+} and CCR2^{-/-} bone marrow. Recipient CCR2^{-/-} mice were irradiated to ablate bone marrow and then reconstituted with 1:1 mixture of CCR2^{-/-}, and CCR2^{+/+} GFP transgenic wild-type. Recipients received a total of 10 million bone marrow cells. Three weeks after transfer, day 3 PPD-bead granulomas were elicited as described in Materials and Methods. Granulomatous lungs were removed, enzymatically dispersed, and subjected to flow cytometric analysis. The mix of CCR2^{+/+} and CCR2^{-/-} cells was analyzed independently by gating on either GFP⁺ or GFP⁻ cells. Note in the **top right corner** of scattergrams the population shift from MHCII^{hi} and CD40^{hi} to low expression in CCR2^{-/-} mice. The dense population in **bottom left corner** of CCR2^{-/-} mice represents CD45-GFP⁻ cells derived from the bone marrow recipient. Scattergrams from a representative mouse are shown.

bone marrow transplantation using a GFP transgenic marker we were able to monitor the activation state of mixed CCR2+/+ and CCR2-/- DCs recruited to Ag-bead-challenged lungs. This study clearly showed that CCR2-/- DCs were effectively recruited but their maturation defect could not be corrected in a milieu of bystander wild-type cells. This provided direct evidence for an intrinsic activation defect and indicated a role for CCR2 ligands in local DC activation. Our findings would be fully consistent with the report of Mizumoto and colleagues⁴² showing enhanced DC activation in CCL2 transgenic mice. Previous studies have suggested that immune defects in CCR2-/- mice were because of impaired migration to lymph nodes, but those studies used DC activation markers to identify DCs and did not consider a potential intrinsic defect of DC maturation. In view of our current findings those studies may require reinterpretation.

Our transcript analysis of DC chemokine receptor profiles revealed differences between lymph node and lung and between challenged and naïve lung DC populations. These differences likely reflect effects of maturation, activation, and tissue distribution. CCR2 was expressed by DCs only with Ag challenge suggesting an increase in the proportion of locally recruited immature DCs.^{20,28} As our study demonstrated, this expression correlated with corresponding ligand induction and had functional significance.

We detected CCR6 transcripts only among DC populations in DLNs after Ag-bead challenge suggesting a role for CCR6 in DC interactions within the DLN, a possibility that is currently under investigation. In humans, *in vitro*-generated, immature DCs express CCR6 and that expression decreases progressively as DCs mature.^{6,18} However, recent studies showed that immature human peripheral blood CD11c+ DCs respond strongly only to CCR2 and CXCR4 ligands.²⁸ Freshly isolated epidermal Langerhans cells expressed CCR6 in humans⁴³ but not in mice.⁴⁴ In a murine system, CCR6 expression was detected among DCs found in the subepithelial dome region of reactive Peyer's patches.⁴⁵ Under steady state conditions splenic DC subsets did not express CCR6.⁴⁵ These results suggest that CCR6 expression in DCs is dynamic and dependent on the tissue microenvironment and possibly Ag challenge, but in our models CCR6 was not critical to local DC recruitment or maturation.

We identified CCR1 and CCR5 transcript expression among lung-derived DC populations but individually these receptors also did not seem to be essential for DC mobilization to Ag-beads. However, these receptors may play critical roles in DC responses to other stimuli. For example, daily treatment of adult rats with the selective CCR1 and CCR5 antagonist Met-RANTES reduced baseline numbers of tracheal intraepithelial DCs by 50 to 60%, and pretreatment of animals with Met-RANTES before inhalation of aerosol containing heat-killed bacteria abolished the rapid DC influx into the epithelium that occurred in untreated controls.⁴⁶ However, the same Met-RANTES treatment did not affect the levels of DC recruitment observed during airway mucosal Sendai virus infection and after aerosol challenge of sensitized animals with a

soluble recall Ag, ovalbumin. These findings indicate the existence of alternative DC recruitment pathways and suggest that the degree of CCR1 and CCR5 participation is dependent on the nature of the eliciting stimulus.⁴⁶ Clearly our Ag-bead challenge models invoke redundant DC recruitment pathways.

In conclusion, DCs or DC precursors are potentially able to respond to multiple chemotactic factors including CCR1, CCR2, and CCR5 agonists. Targeting a single chemotactic pathway may not completely block the recruitment of DCs to the lung when the challenge induces multiple chemotactic factors. However, we demonstrate that in addition to abrogating monocyte/macrophage recruitment, CCR2-/- results in a profound DC maturation defect. As this defect is intrinsic to the knockout cells it suggests that CCR2 ligands play an important role in local DC maturation/activation events.

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