Chemokine Receptor CCR2 but Not CCR5 or CCR6 Mediates the Increase in Pulmonary Dendritic Cells during Allergic Airway Inflammation


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Chemokine Receptor CCR2 but Not CCR5 or CCR6 Mediates the Increase in Pulmonary Dendritic Cells during Allergic Airway Inflammation


Increased numbers of pulmonary dendritic cells (DCs) are recruited to the lungs during allergic airway inflammation and contribute to the maintenance of the inflammatory immune response. The chemokine receptors that directly control DC accumulation into the lungs are largely unknown. To explore this issue, we generated mixed bone marrow chimeric mice containing both wild-type and knockout cells for a given chemokine receptor. After induction of allergic airway inflammation, we specifically tracked and compared chemokine receptor knockout vs wild-type DC populations through various lung compartments. Using this approach, we show that CCR2, but not CCR5 or CCR6, directly controls the accumulation of DCs into allergic lungs. Furthermore, the size of inflammatory monocyte populations in peripheral blood was strikingly CCR2 dependent, suggesting that CCR2 primarily mediates the release of monocytic DC precursors into the bloodstream. The Journal of Immunology, 2007, 178: 5305–5311.

Dendritic cells (DCs) are nowadays regarded as the most professional APCs (1). Originally derived from the bone marrow, these leukocytes circulate as monocytic precursors in blood and home to nonlymphoid tissues where they reside as immature cells. After Ag uptake in peripheral tissues, they migrate to draining lymph nodes (LNs) where they efficiently present Ag-derived peptides to naïve T cells (2). Besides their role in initiating inflammatory responses against invading pathogens, DCs maintain peripheral tolerance to self and harmless environmental Ags (3). Disruption of this fine-tuned balance between immunity and tolerance can lead to inflammatory disorders such as allergic disease.

Allergic asthma is an inflammatory lung disease where environmental Ags activate Th2 cells (4). This leads to peribronchial eosinophilia, goblet cell hyperplasia, airway hyperreactivity, production of IgE, and mast cell activation. In addition to their role in the sensitization and initial Th2 skewing of the airway immune response, pulmonary DCs are thought to be involved in the maintenance of inflammation during re-exposure to allergen (5–7). DCs are recruited in massive amounts to allergen-challenged airways where they locally activate effector T cells (8). In addition, airway DCs acquire a mature phenotype and rapidly transport allergen to the draining LNs (9). Interestingly, selective elimination of DCs in the effector phase attenuates the characteristic features of asthma (7). Therefore, interfering with DC recruitment to the lung might be a promising approach to control allergic disease. However, the mechanisms involved in the accumulation of DCs or their precursors into the lungs remain poorly defined.

Cell trafficking is largely mediated by the interaction of chemokines with their specific receptors expressed on the surface of leukocytes. Coordinated expression of inflammatory chemokines during the allergic response in lungs controls the influx of inflammatory cell types, thereby mediating various stages of allergic disease (10, 11). Selective expression of chemokine receptors on DCs tightly regulates the normal and inflammatory trafficking within lymphoid and nonlymphoid tissues (12, 13). To date, however, no conclusive data exist on which chemokine receptors direct DC movement to the lungs. Recently, several groups have focused on this issue when studying DC behavior and trafficking in chemokine receptor knockout (KO) mice. Experiments in CCR6 mice showed a reduced inflammatory response that was partially attributed to decreased pulmonary DC numbers (14). Another study reported a diminished early accumulation of lung DCs in challenged CCR2 and CCR6 mice (15). Activation and maturation of DCs was severely compromised in CCR2 mice (16). In addition, selective CCR1 and CCR5 antagonists were used to effectively block the recruitment of DC precursors into resting airway epithelium and upon bacterial challenge (17).

A limitation in using KO mice for these studies is that receptor deficiency affects a wide variety of cells potentially implicated in the inflammation (10). Consequently, the whole inflammatory climate is affected so that it becomes practically impossible to distinguish direct from indirect effects of chemokine receptor deficiency on DC trafficking.

Alternative strategies exist to address these issues. One approach involves adoptive transfer of chemokine receptor KO vs wild-type (WT) cells. However, we and others (18) have failed to...
detect significant numbers of DCs homing into the lung after i.v.
transfer, both in naive and in inflammatory conditions.

In this study, we circumvent this problem by creating chimeric mice with mixed bone marrow containing both WT and chemokine receptor KO donor cells. This allowed us to specifically track and compare, within the same animal, KO vs control (WT) cell populations through different lung compartments. Meanwhile, sufficient immunocompetent cells were present to sustain an inflammatory environment. Using this approach, we find that CCR2 but not CCR5 or CCR6 is required for the increase in DC numbers in the lung during allergic inflammation.

Materials and Methods

Mice

C57BL/6-CD45.1 mice were obtained from Charles River Laboratories and bred in our facility. CCR6+/- mice obtained from Harlan Sprague Dawley, CCR2+/- mice (19) and CCR5+/- mice (20) were originally obtained from Section of Molecular Genetics and Microbiology (Austin, TX) and bred in our facility. CCR6+/- mice (21) were from Schering-Plough and bred in our facility. The local ethics committee for animal experimentation of Ghent University approved all in vivo experiments.

Generation of mixed bone marrow chimeric mice

To generate mixed bone marrow chimeric mice, CD45.1 hosts were lethally irradiated (11 Gy in two doses) and transplanted within 24 h with bone marrow cells from CD45.1 mice and CD45.2 mice (1:1 mixture) as illustrated in Table I. CD45.1 bone marrow cells were derived from C57BL/6-CD45.1 mice and were always WT for a given chemokine receptor. CD45.2 bone marrow cells were prepared either from normal C57BL/6 WT mice (WT/WT chimeras) or from CCR2+/-, CCR5+/-, or CCR6+/- mice (WT/KO chimeras). Control WT/WT chimeras were always created in parallel to WT/KO chimeras. After 7–8 wk of engraftment, hemopoietic reconstitution was confirmed by analyzing different leukocyte populations in spleens of mixed (WT/WT and WT/KO) chimeras for the CD45.1/CD45.2 ratio, which ranged from 0.4 to 1.1 (data not shown). To compensate for the differences in engraftment between individual mice, the CD45.1/CD45.2 ratio calculated for pulmonary DCs was normalized to the CD45.1/CD45.2 ratio of an “engraftment control” cell population within the same mouse (22). This engraftment control ratio represented the reconstitution efficiency of the whole hemopoietic system and thereby should not be affected by the chemokine receptor deficiency. Therefore, splenic CD19+ B cells were used as control for CCR2 and CCR5 chimeras because these cells were stained negative for CCR2 and CCR5 in preliminary experiments. However, since splenic B cells stained abundantly positive for CCR6, we used splenic NK1.1+ NK cells (which are negative for CCR6) as engraftment control population in CCR6 chimeras. The ratio R was calculated for each mouse separately as R = (CD45.1/CD45.2)wt/(CD45.1/CD45.2)kat.

As such, R values > 1 indicate a relative disadvantage of CD45.2 cells (i.e., CCR-KO DCs in the WT/KO chimeras) compared with CD45.1 (WT) cells, whereas R ≤ 1 indicates no effect.

Allergen sensitization and challenge

Mice were sensitized by i.p. injection of 0.5 ml of PBS containing 10 μg of OVA (Sigma-Aldrich) adsorbed to 1 mg of Al(OH)3. Two weeks later, the animals were challenged daily with an aerosol of 1% (w/v) OVA in PBS during 30 min for 7 successive days. Controls received i.p. 1 mg of Al(OH)3 in PBS and were challenged with PBS aerosol.

Bronchoalveolar lavage (BAL) and cytospins

Twenty-four hours after the last aerosol, mice were sacrificed by a lethal dose of pentobarbital. A tracheal cannula was inserted, and BAL was performed by instillation of three times 300 μl of HBSS supplemented with BSA for cytokine analysis. Three additional instillations with 1 ml of HBSS plus EDTA were performed to achieve maximal recovery of BAL cells. BAL cells (50,000) were processed for cytospins and were stained with May-Grunwald-Giemsa for differential cell counting. Remaining cells were used for FACS analysis.

Preparation of single-cell suspensions

Lungs were perfused with saline plus EDTA through the pulmonary artery to remove contaminating blood cells. Lungs and mediastinal LNs were removed and digested as detailed previously (2). Briefly, minced lung pieces and LNs were incubated with 1 mg/ml collagenase and 20 μg/ml DNase I for 45 min at 37°C. RBCs were lysed using ammonium chloride buffer. Finally, cell suspensions were filtered through a 50-μm nylon mesh to remove undigested organ fragments. Spleen single-cell suspensions were obtained by mechanical disruption through a 100-μm nylon mesh. Peripheral blood was collected by cardiac puncture and transferred to EDTA-coated tubes (Vacutainer; BD Biosciences). After RBC lysis, platelets were discarded by centrifugation at 200 × g, and the remaining leukocyte fraction was used for cell surface staining.

DC bone marrow cultures

Bone marrow from C57BL/6, CCR2+/-, CCR5+/-, and CCR6+/- mice was cultured in the presence of 20 ng/ml GM-CSF. Culture medium was renewed every 3 days. After 8 days of culture, cells were harvested, counted and stimulated overnight with 1 μg/ml LPS (Sigma-Aldrich) or medium. After stimulation, cells were harvested and CD11c+ DCs were stained for apoptotic and dead cells using Annexin V-PE Detection Kit I (BD Biosciences).

Flow cytometry

All staining procedures were conducted in calcium and magnesium-free PBS containing 10 mM EDTA, 1% BSA (Dade Behring), and 0.1% sodium azide. One million cells were preincubated with anti-CD16/CD32 (2.4G2) to block FcRs. mAbs used to identify cell surface molecules were rat anti-CD11b FITC (M1/70), hamster anti-CD11c allopolyconjugate (HL3), mouse anti-mouse I-Aβ (AF6-120.1), mouse anti-CD45.1 PE (A20), biotinylated mouse anti-CD45.2 (104), biotinylated rat anti-CCR5 (C34-3448), and biotinylated Ly-6C (AL-21), all from BD Biosciences. Rat anti-CCR6 PE (140706) was purchased from R&D Systems. Biotinylated Abs were detected with streptavidin-PerCP (BD Biosciences). Unconjugated rat anti-CCR2 (MC-21; a gift from Dr. M. Mack, University of Regensburg, Regensburg, Germany) was revealed using Alexa488-conjugated chicken anti-rat Ab (In-vitrogen Life Technologies-Molecular Probes), followed by staining with directly conjugated Abs. Appropriate isotype control Abs were used to define background staining. DCs from lung tissue and BALF were characterized as CD11c+CD103−, low autofluorescent cells, whereas DCs from mediastinal LNs were identified as CD11c+ MHCIIhigh cells (2). 7-aminoactinomycin D (7-AAD) (BD Biosciences) was used to exclude nonviable cells from analysis. Analytical flow cytometry was conducted with a FACSCalibur (BD Biosciences), and data were processed with FlowJo software (Tree Star).

Determination of chemokines

 Supernatants of BAL fluid were used for determination of chemokines (CCL2/MCP-1, CCL5/RANTES, and CCL20/MIP-3α) protein levels using commercially available ELISA kits. All kits were purchased from R&D Systems.

Histology

Left lung was fixed in 4% paraformaldehyde and embedded in paraffin. Transversal sections of 3μm were stained with Congo Red (Klimpath) for the visualization of eosinophils and counterstained with hematoxylin (Sigma-Aldrich).

Statistical analysis

Reported values are expressed as mean ± SE. Statistical analysis was performed with Instat (GraphPad) using Student’s t test. Values of p < 0.05 were considered significant.

Table I. Generation of mixed bone marrow chimeric mice

<table>
<thead>
<tr>
<th>Mixed Chimera</th>
<th>Recipient Mice</th>
<th>Donor Bone Marrow</th>
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<tr>
<td>WT/WT</td>
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Statistical analysis

Reported values are expressed as mean ± SEM. Statistical analysis was performed with Instat (GraphPad) using Student’s t test. Values of p < 0.05 were considered significant.
Results

Mixed bone marrow chimeric mice develop airway inflammation

Mixed bone marrow chimeric mice were created as described in Materials and Methods and as illustrated in Table I. We first verified whether lethal irradiation and reconstitution with mixed congenic bone marrow cells allows the subsequent induction of allergic airway inflammation using i.p. immunization followed by aerosol exposure with OVA. As illustrated in Fig. 1, all chimeric combinations were conducive to the induction of allergic airway inflammation, as reflected by marked increases in BAL eosinophils, BAL DCs, lung DCs, and characteristic peribronchial eosinophilic and mononuclear cell infiltrates (Fig. 1, A–D). Also, production of relevant chemokine ligands was induced in the bronchoalveolar compartment of allergen-exposed chimeric mice (Fig. 1E).

These results clearly show that chimeric mice were capable of mounting an inflammatory airway response upon allergen sensitization and challenge.

Allergen-mediated increase in pulmonary DCs is CCR2 dependent

Having established that allergic immune responses can be induced in the airways and lungs of bone marrow chimeric mice, we next evaluated the contribution of CCR2, CCR5, and CCR6 to the increase in pulmonary DCs during inflammation. First, the ratio of CCR-WT and CCR-KO population sizes was calculated for DCs in the pulmonary tissue, in the BAL-fluid, and in draining LNs of the mixed chimeric mice (Fig. 2A). Each time, this quotient was corrected by a factor taking into account variations in reconstitution efficiency for both congenic bone marrow donor cells, thus yielding the ratio R (Fig. 2B) (22). By calculating this ratio R for various mixed chimeric mice, we found an important role for CCR2 in the increase in DCs in allergen-challenged lungs: the marked increase in the net ratio R reflects a relative disadvantage of CCR2 KO compared with CCR2 WT DCs (Fig. 2C). However, CCR2 deficiency did not seem to affect steady-state numbers of pulmonary DCs. By contrast, analysis performed in CCR5 and CCR6 WT/KO chimeras revealed no direct contribution of these chemokine receptors in the maintenance of pulmonary DC populations, whether in naive animals or during allergic airway inflammation (Fig. 2C). Furthermore, experiments examining DC ratios after 3 days of aerosol challenge confirmed that CCR5 and CCR6 were not additionally involved in pulmonary DC migration at more early stages in the development of allergic airway inflammation (data not shown).

Chemokine receptor expression of pulmonary DCs

When looking at chemokine receptor expression in C57BL/6 mice, we observed a preferential expression of CCR2 on the surface of pulmonary DCs (Fig. 3). Interestingly, allergic airway inflammation induced a partial loss of CCR2 on DCs from the BAL compartment. Meanwhile, expression of CCR5 on pulmonary DCs was relatively low (compared with control staining on splenocytes;
data not shown), and only a small subset of pulmonary DCs expressed CCR6. Although our analysis of CCR6 WT/KO chimeras indicates no direct involvement of this receptor in mediating the allergen-induced accumulation of DCs in the lung tissue or BAL compartment, we did observe an increase in the proportion of CCR6-expressing BAL DCs after allergen exposure.

**Apoptosis and proliferation of chemokine receptor-deficient DCs**

Absolute cell numbers found within tissues generally represent a dynamic balance between cell proliferation, migration, and cell loss (cell death or apoptosis). Accordingly, decreased numbers of CCR2-deficient DCs found within allergic lungs could as well be attributed to poor cell survival in the lungs. Therefore, we examined cell proliferation and apoptosis/cell death using a bone marrow-derived DC culture system. In contrast to the in vivo situation, this in vitro platform allowed us to study effectively the properties of CCR-deficient DCs in a well-controlled, standardized environment not biased by effects from CCR deficiency on other cells.

Using bone marrow from C57BL/6, CCR2<sup>+/−</sup>, CCR5<sup>+/−</sup>, and CCR6<sup>+/−</sup> mice, the results show that the total recovery of cells

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**FIGURE 2.** Analysis of pulmonary DC populations in mixed bone marrow chimeric mice. Mixed bone marrow chimeras were created as described in Table I. A and B. Representative dot plots for CD45.1 vs CD45.2 cells in OVA-challenged CCR2 mixed chimera gated for lung DCs (A) and splenic CD19<sup>+</sup> B cells (B) (reconstitution control). C. The ratio R was calculated according to the formula shown for DCs in lung tissue (lung DCs), in BAL fluid (BAL DCs), and in mediastinal LNs (LN DCs) in CCR2-, CCR5-, and CCR6-mixed chimeras. Lung and BAL DCs were characterized as CD11chigh low-autofluorescent cells, whereas LN DCs were identified as CD11c<sup>+</sup> MHCII<sup>high</sup> LN cells. Splenic CD19<sup>+</sup> B cells were used as reconstitution control population (ctrl) in CCR2- and CCR5-mixed chimaera, whereas splenic NK1.1<sup>+</sup> NK cells were used in CCR6-mixed chimera. R values were calculated for WT/WT chimeras (□) and WT/KO chimeras (○), in sham (PBS)- or allergen (OVA)-exposed groups, respectively (n = 5–7 mixed chimeric mice/group). R > 1 indicates a relative disadvantage for CD45.2 cells (i.e., CCR-KO DCs in the WT/KO chimeras) compared with CD45.1 cells. Error bars indicate SEM. Results are representative of two to three independent experiments (n = 5–6 mice/group).

**FIGURE 3.** Chemokine receptor expression on pulmonary DCs. Normal C57BL/6 mice were immunized and exposed to OVA or PBS to induce allergic airway inflammation. Single-cell suspensions derived from BAL fluid and enzymatically digested lung tissue were stained with mAbs for CD11c and CCR2, CCR5, or CCR6. Pulmonary DCs in BAL fluid (BAL DCs) and lung tissue (lung DCs) were identified as low-autofluorescent, CD11c<sup>high</sup> cells. Representative histograms (of two independent experiments, n = 5 mice/group) of CCR2, CCR5, and CCR6 expression (gray shaded) on pulmonary DCs are shown relative to isotype control staining (black line).
after 8 days of culture was comparable between groups (Fig. 4A). In addition, the proportion of Annexin V+/7-AAD− DCs did not differ substantially between groups, even after stimulation with LPS (Fig. 4B). Therefore, chemokine receptors on DCs themselves were not directly implicated in cell proliferation and survival/apoptosis.

Peripheral blood monocyte (PBM) populations are CCR2 dependent

Lastly, we sought to gain more insight into the level at which CCR2 mediates the increase in pulmonary DCs during allergen challenge. It is possible that CCR2 is directly involved in the extravasation of DCs or their precursors from the pulmonary circulation into the lung tissues. Alternatively, the number of DC precursors in the blood might be regulated through a CCR2-dependent mechanism. We examined PBM subpopulations in CCR2 WT/KO chimeric mice since it has been established that monocytes can differentiate in vivo into several types of DCs (23, 24). Based on previous studies, we could distinguish two main subpopulations of PBMs: one was Ly6C<sup>high</sup>/FSC<sup>high</sup> monocytes, described as an “inflammatory” subtype of monocyte (23), the other Ly6C<sup>low</sup>/FSC<sup>med</sup>, considered as a more mature form of the first subset (25) (Fig. 5A). Both subpopulations stained positive for CD115, the receptor for M-CSF (data not shown). Analysis of various CCR2 WT/KO ratios revealed that cell numbers of both blood monocyte subsets were critically dependent on CCR2 in steady state as well as during allergic airway inflammation (Fig. 5B). This suggests that the CCR2-dependent increase in lung DCs occurs before cell emigration at the pulmonary level (i.e., the release of bone marrow monocytes into the bloodstream).

Discussion

The generation of mixed bone marrow chimeric mice allowed us to determine the direct contribution of several CCRs to the recruitment of lung DCs during an allergic pulmonary response. We focused on CCR2, CCR5, and CCR6 because these receptors have been chiefly implicated in the mobilization of DCs toward Ag-exposed peripheral tissues. After induction of eosinophilic airway inflammation in these chimeric mice, analysis of WT/KO ratios revealed that cell numbers of both blood monocyte subsets were critically dependent on CCR2 in steady state as well as during allergic airway inflammation (Fig. 5B). This suggests that the CCR2-dependent increase in lung DCs occurs before cell emigration at the pulmonary level (i.e., the release of bone marrow monocytes into the bloodstream).
mediated by CCR6 (30, 31). However, our data indicate that CCR6 and its ligand CCL20 do not possess unique properties in attracting DCs toward the airways during full-blown allergic inflammation. By contrast, earlier studies reported reduced numbers of pulmonary DCs in CCR6−/− mice during allergic airway inflammation (14, 15). However, this effect might be secondary to an altered inflammatory environment since CCR6 deficiency can primarily affect other cell types than DCs. For example, defective trafficking of CD4 T cells into the airways has already been reported in CCR6−/− mice (14). Correspondingly, we also observed a preferential accumulation of CCR6-WT vs KO CD3+ T cells in the BAL compartment of CCR6 WT/KO mixed chimeric mice (data not shown), supporting a direct involvement of CCR6 in the recruitment of memory T cells to the lung (32).

The contribution of CCR2 to the allergen-mediated increase in pulmonary DCs correlated with high levels of CCR2 cell surface expression. Interestingly, these levels where down-regulated once DCs reached their Ag-exposed target organ. This might be due to receptor internalization by high local production of corresponding ligand (33) or, alternatively, to enhanced maturation of DCs in the airway lumen during inflammation (9, 12). Furthermore, we demonstrated that the preferential increase of CCR2-positive DCs in the airway lumen during inflammation (9, 12). Furthermore, we demonstrated that the preferential increase of CCR2-positive DCs in allergic lungs was not due to poor survival or proliferation of CCR2-deficient DCs.

According to a current paradigm, chemokines produced locally in the tissues attract circulating leukocytes bearing the corresponding receptor. Consequently, CCR2 ligands produced within the pulmonary environment would promote the attraction of CCR2+ blood monocytes, which would subsequently differentiate locally into DCs (23, 24). However, our data suggest that CCR2 is critically involved at a more upstream level, i.e., the release of monocytes from the bone marrow into the bloodstream could be CCR2 dependent. In support of this concept, Serbina and Pamer (34) recently showed in a model of Listeria monocytogenes infection that CCR2 controls the bone marrow egress of monocytes into the blood circulation rather than attracting monocytes into peripheral tissue. In this regard, high serum levels of CCL2 (34) would promote the eflux of DC precursors from the bone marrow. Thus, chemokines produced locally during allergic airway inflammation could not only activate (35) and recruit immune cells but could also act on a systemic level by stimulating bone marrow cells to extravasate into the blood circulation, as was shown for eotaxin (36). Therefore, therapeutic molecules designed to antagonize chemokine action locally could have profound side effects on blood cell composition.

In summary, we developed an in vivo mouse model in which cell migration was measured irrespectively of the levels of background allergic inflammation. By tracking and comparing chemokine receptor KO vs WT DCs in mixed bone marrow chimeric mice, we identified CCR2 as an essential chemokine receptor for the allergen-mediated increase in pulmonary DCs. Furthermore, these data show that CCR5 and CCR6 can only be indirectly involved in this process. Finally, our data suggest that the inflammatory expansion of pulmonary DC populations is dependent on a CCR2-mediated release of monocyte precursors into the bloodstream.

Acknowledgments

We dedicate this article to the memory of Prof. Romain Pauwels whose creativity and ideas greatly inspired this work. We thank Prof. L. Vakaet and his team for the use of the X-irradiation source. We thank Prof. M. Mack from the University of Regensburg for the gift of the MC-21 Ab. We also thank E. Castrique, C. Snaauwaert, G. Barbier, K. De Saedeleer, I. De Borle, A. Neesen, M. Mouton, E. Spruyt, and P. Degryze for excellent technical support.

Disclosures

The authors have no financial conflict of interest.

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