

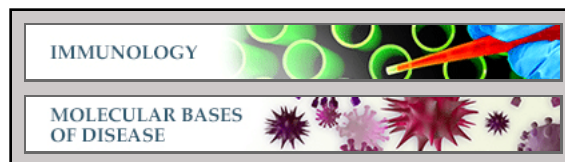
Immunology:

Signal Relay by CC Chemokine Receptor 2 (CCR2) and Formylpeptide Receptor 2 (Fpr2) in the Recruitment of Monocyte-derived Dendritic Cells in Allergic Airway Inflammation

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Signal Relay by CC Chemokine Receptor 2 (CCR2) and Formylpeptide Receptor 2 (Fpr2) in the Recruitment of Monocyte-derived Dendritic Cells in Allergic Airway Inflammation^{*S}

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Keqiang Chen[‡], Mingyong Liu[‡], Ying Liu[‡], Chunyan Wang[‡], Teizo Yoshimura[‡], Wanghua Gong[§], Yingying Le[¶], Lino Tessarollo^{||}, and Ji Ming Wang^{‡,1}

From the [‡]Laboratory of Molecular Immunoregulation, Cancer and Inflammation Program, Center for Cancer Research and the ^{||}Mouse Cancer Genetics Program, Center for Cancer Research, NCI, National Institutes of Health, Frederick, Maryland 21702,

[§]SAIC-Frederick, Frederick, Maryland 21702, and the [¶]Institute for Nutritional Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Background: Chemoattractant receptor Fpr2 interacts with host-derived agonists and mediates leukocyte trafficking.

Results: In the lung of allergic inflammation, chemokine receptor CCR2 elicits accumulation of monocyte-derived DC in perivascular region, but Fpr2 is critical for cell trafficking to peribronchiolar area.

Conclusion: CCR2 and Fpr2 sequentially guide DC trafficking in inflamed lung.

Significance: DC trafficking is controlled by multiple chemoattractant receptors, which are potential therapeutic targets.

Chemoattractant receptors regulate leukocyte accumulation at sites of inflammation. In allergic airway inflammation, although a chemokine receptor CCR2 was implicated in mediating monocyte-derived dendritic cell (DC) recruitment into the lung, we previously also discovered reduced accumulation of DCs in the inflamed lung in mice deficient in formylpeptide receptor Fpr2 (Fpr2^{-/-}). We therefore investigated the role of Fpr2 in the trafficking of monocyte-derived DCs in allergic airway inflammation in cooperation with CCR2. We report that in allergic airway inflammation, CCR2 mediated the recruitment of monocyte-derived DCs to the perivascular region, and Fpr2 was required for further migration of the cells into the bronchiolar area. We additionally found that the bronchoalveolar lavage liquid from mice with airway inflammation contained both the CCR2 ligand CCL2 and an Fpr2 agonist CRAMP. Furthermore, similar to Fpr2^{-/-} mice, in the inflamed airway of CRAMP^{-/-} mice, DC trafficking into the peribronchiolar areas was diminished. Our study demonstrates that the interaction of CCR2 and Fpr2 with their endogenous ligands sequentially mediates the trafficking of DCs within the inflamed lung.

Dendritic cells (DCs)² play critical roles in airway inflammatory responses (1, 2). Under inflammatory conditions, such as

viral infection, allergen challenge, or endotoxin inhalation, CD11b⁺ monocyte-derived DCs are rapidly recruited from the circulation. These cells express Ly6C as a marker of their monocytic descent (2, 3) and are essential for initiating and determining the severity of allergic airway inflammation. In mice, Ly6C^{high} conventional monocytes are CX3CR1^{low}, CCR2⁺, CD62L⁺, and CCR5⁻, whereas Ly6C^{low} monocytes are CX3CR1^{high}, CCR2⁻, CD62L⁻, and CCR5⁺ (4). Under inflammatory conditions, Ly6C^{high} monocytes differentiate into inflammatory DCs and acquire the capacity to prime T cell-mediated immune responses (5). In the lung, inflammatory stimuli such as pathogen-derived TLR ligands or exposure to environmental pollutants trigger the production of chemokines that recruit monocyte-derived inflammatory DCs in a CCR2-dependent manner (2, 3, 6, 7), presumably via interaction with the ligand CCL2. However, other chemokine receptors have also been implicated in the recruitment of monocyte-derived inflammatory DCs into the lung as evidenced by observations with CCR5 and CCR6 knock-out mice in cigarette smoke inhalation models (8, 9). After trafficking into the inflammatory sites, TLR agonists from pathogens or damaged tissues rapidly down-regulate the function of CCR1, 2, and 5 expressed on monocyte-derived DCs (10), and further cell recruitment was dependent on an increase in the expression of CCR7 (11). Thus, chemokine receptor-mediated monocyte-derived inflammatory DC trafficking has become accepted as the means by which cells are recruited and directed into inflammatory sites and draining lymph nodes (LN).

However, in addition to chemokine receptors, the importance of a group of classical chemoattractant receptors named formylpeptide receptors (FPRs) in leukocyte trafficking has been increasingly recognized. One of the FPR family members,

receptor; CRAMP, cathelin-related antimicrobial peptide; FPR or Fpr, formylpeptide receptor; LN, lymph nodes; OVA, ovalbumin.

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^S This article contains supplemental Figs. S1–S8.

¹ To whom correspondence should be addressed: Laboratory of Molecular Immunoregulation, Cancer and Inflammation Program, Center for Cancer Research, Frederick National Laboratory for Cancer Research, Bldg. 560, Rm. 31-76, Frederick, MD 21702. Tel.: 301-846-6979; Fax: 301-846-7042; E-mail: wangji@mail.nih.gov.

² The abbreviations used are: DC, dendritic cell; BAL, bronchoalveolar lavage fluid; BM, bone marrow; CCL, CC chemokine ligand; CCR, CC chemokine

Fpr1, was shown to mediate neutrophil accumulation in the core of injury in response to tissue-derived agonists, subsequent to the initial recruitment of the cells to the peripheral regions of the injury by chemokine receptors (12). We have shown that another FPR family member Fpr2 is critical for allergic airway inflammation, as well as T cell responses associated with accumulation of CD11c⁺ DCs in the inflamed airway and in draining LN (13). This is based on our recent findings of greatly reduced severity of allergic airway inflammation in Fpr2^{-/-} mice. However, the precise role of Fpr2 in DC trafficking remains to be determined. We hypothesized that Fpr2 may participate in a sequential chemoattractant signal relay required for the trafficking of Ly6C⁺ monocyte-derived inflammatory DCs in the inflamed lung. In this study, we demonstrate that Fpr2 and its endogenous ligand CRAMP play an important role in the accumulation of inflammatory DCs in the peribronchiolar tissues subsequent to CCR2-mediated cell trafficking into the perivascular regions in allergic airway.

EXPERIMENTAL PROCEDURES

Mice—Fpr2^{-/-} mice were generated as described (13). To generate CRAMP^{-/-} mice, CRAMP gene was retrieved from the mouse BAC clone RP23-77119 into pLMJ235 vector containing the thymidine kinase gene. The targeting vector was then electroporated into C57BL/6 mouse ES cells (14). Recombinant ES cells were injected into blastocysts of albino C57BL/6 mice to generate CRAMP flox-neo mice, which were crossed to β -actin Cre mice on a C57BL/6 background. Heterozygous CRAMP^{+/-} mice were mated to generate homozygous CRAMP^{-/-} mice.³ CCR2^{-/-} mice were a kind gift from Dr. Zack Howard (Frederick National Laboratory for Cancer Research). CCL2^{-/-} mice were previously reported (15). Age- and sex-matched mice were used in all experiments. Animal care was provided in accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals (National Research Council, 1996, National Academy Press, Washington D.C.).

Reagents—Purified hamster anti-mouse CD11c (isotype control: Armenian hamster IgG1, κ), purified hamster anti-mouse CD3 ϵ (isotype control: Armenian hamster IgG1, λ 2), and purified rat anti-mouse CD11b (isotype control: Rat IgG2b, κ) antibodies were from BD Biosciences.

Anti-Armenian hamster IgG biotin and anti-rat IgG biotin were from eBioscience (San Diego, CA). Streptavidin-FITC and -PE were from Biolegend (San Diego, CA). Fpr2 agonist peptide MMK-1 (LESIFRSLLFRVM) was synthesized at Colorado State University (Fort Collins, CO) (16). Anti-CRAMP (R-170) and anti-Fpr2 (M-73) antibodies were from Santa Cruz, Inc. (Santa Cruz, CA). The Fpr2 antagonist (WRW4) was purchased from Tocris Bioscience (R & D Systems, Minneapolis, MN). CCR2 antagonist (C₂₈H₃₄F₃N₅O₄S, CHF) was from Santa Cruz, Inc. Fluorescein-Formyl-Nle-Leu-Phe-Nle-Tyr-Lys (FITC-N-formyl peptide) was from Invitrogen.

Induction of Allergic Airway Inflammation—Allergic airway inflammation was induced as described (13). Briefly, the mice were immunized by intraperitoneal injection of 100 μ g of OVA

in 50 μ l of PBS mixed with 50 μ l of aluminum hydroxide. The mice were boosted 15 days later with OVA/aluminum hydroxide (20 μ g/50 μ l of PBS/50 μ l of aluminum hydroxide). After 10 days, the mice were challenged intranasally with 1% OVA containing 1 μ g of LPS/mouse for 3 days. Control mice were sensitized, and the airway was challenged with PBS. Mouse lungs were harvested 24 h after the last challenge and digested with collagenase and treated with ACK to remove erythrocytes. Lungs were harvested 24 h after the last challenge.

Leukocyte Count—Mouse blood was collected in tubes containing sodium heparin (BD Biosciences), and the number of leukocytes was counted using HEMAVET[®] HV950 (Drew Scientific, Inc., Dallas, TX).

Flow Cytometry—Mouse lung was digested with triple enzyme mix solution (10 $\times$ stock solution: 1 g of collagenase, 100 mg of hyaluronidase, 20,000 units of DNase in 100 ml of Hanks' balanced salt solution) and treated with ammonium-chloride-potassium lysing buffer (ACK) (Cambrex, East Rutherford, NJ) to remove erythrocytes. Heparin-treated blood was treated with ACK to remove erythrocytes. Lung cells and red cell-free blood leukocytes were preincubated in buffer (PBS with 1% FCS, 5 mM EDTA, and 0.1% NaN₃) containing anti-CD16/32 mAb for 20 min at 4 $^{\circ}$ C to eliminate nonspecific binding of mAb to the Fc γ II/IIIIR followed by hamster anti mouse CD11c-FITC or CD11c-PE (BD Biosciences), CD11b-PerCP-Cy5.5 or CD11b-FITC (BD Biosciences), I-A/I-E-PE (BD Biosciences), Ly6C-PerCP-Cy5.5 (eBioscience, San Diego, CA), CCR2-PE (R & D Systems), CCR5 (R & D Systems), and CCR6 (R & D Systems) antibodies for 30 min at 4 $^{\circ}$ C. For measuring CCR7, DCs were incubated with anti-CCR7-PE (eBioscience) in 37 $^{\circ}$ C water bath for 30 min. Armenian hamster IgG1, rat IgG2b, rat IgG2 α , rat IgG2c, rat IgG2B, mouse IgG1, and rat IgG2A were used as isotype controls. Cells were analyzed with a FACSCalibur flow cytometer using CELLQuest software (BD Biosciences).

Histopathology—Lung tissues fixed in formalin and embedded in paraffin were sectioned (5 μ m) and stained with hematoxylin and eosin. Peribronchial cells and perivascular cells were quantified using a five-point (scores 0–4) grading system as described (17). The scoring was: 0, no cells; 1, a few cells; 2, a ring of cells of 1 cell layer deep; 3, a ring of cells of 2–4 cells deep; and 4, a ring of cells with >4 cells deep. At least three fields of coded lung sections were examined by pathologists without knowledge of the sample identities. Mean scores were obtained from four or five mice after decoding the samples.

Isolation of Mouse Bone Marrow Cells and Generation of BM-derived Dendritic Cells (BMDC)—Bone marrow cells were obtained by flushing femurs with PBS as described. Red cells were lysed with ACK lysing buffer (Cambrex, NJ). Immature BMDCs were generated by culturing BM nucleated cells (10⁶ cells/well/3 ml) with GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) for 6 days (or indicated times). Immature BMDCs were stimulated with LPS (100 ng/ml or at indicated concentrations) for 24 h to obtain mature BMDCs.

Chemotaxis Assays—Chemotaxis of DCs was measured as described (18). The results are expressed as the means $\pm$ S.E. of the chemotaxis index, representing the fold increase in the number of migrated cells in response to chemoattractants over

³ T. Yoshimura, unpublished data.

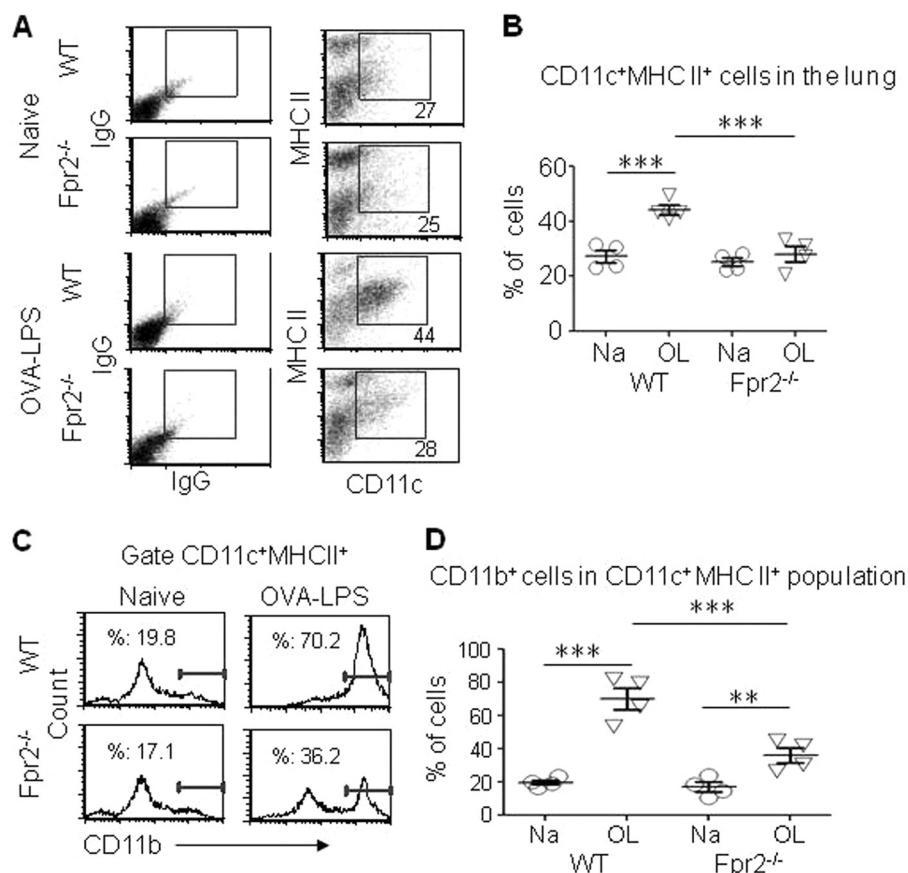


FIGURE 1. **Reduced accumulation of CD11c⁺MHC II⁺CD11b⁺ DCs in the inflamed airway in Fpr2^{-/-} mice.** A and B, accumulation of CD11c⁺MHC II⁺ cells in the inflamed lung. The results are expressed as the means $\pm$ S.E. $n = 5$ mice/group. ***, $p < 0.001$. C and D, the frequency of CD11b⁺ cells in the CD11c⁺MHC II⁺ cell population. The results are expressed as the means $\pm$ S.E. $n = 5$ mice/group. **, $p < 0.01$; ***, $p < 0.001$. Na, naïve; OL, OVA/LPS.

spontaneous cell migration (to control medium). For inhibition of BAL-induced DC chemotaxis by CCR2 or Fpr2 antagonist, DCs were pretreated with the Fpr2 antagonist WRW4 or the CCR2 inhibitor CHF alone or in combination for 30 min. The cells were then examined for migration in response to BAL.

RT-PCR—Total RNA was extracted using an RNeasy mini kit from lung tissues or DCs. The mRNA expression of Fpr2, CCR2, CCL2, and β -actin was examined by RT-PCR with previously reported primers (19, 20).

Western Immunoblotting—Whole lung homogenates were prepared by grinding tissue in 1 ml of radioimmune precipitation assay buffer (21). The samples were electrophoresed in 10% gradient SDS-PAGE gels, transferred to nitrocellulose membranes, and blocked with 3% skim milk in PBS. After incubation with primary anti-CRAMP Abs, the blots were incubated with a secondary antibody linked to HRP, and protein bands were visualized using ECL (SuperSignal West Pico Substrate; Pierce). The membranes were then stripped with Restore Western blot stripping buffer (Thermo) for detection of β -actin.

Bone Marrow Transplantation—BM transplantation was performed as previously described (13). BM cells harvested from donor mice were resuspended in serum-free medium (DMEM, 0.1% BSA, 1% penicillin-streptomycin, 1% L-glutamine, and 0.1% amphotericin B). Recipient mice received 1000 Rads of total body irradiation for 5 min. After 4 h, BM cells (1×10^7 cells/mouse) were administered by tail vein injection into

recipient mice. All mice with BM transplantation were given food and antibiotics in water for 2 weeks before induction of allergic airway inflammation and analysis of cells in the lung and blood.

Statistical Analysis—All of the experiments were performed at least three times. Representative and reproducible results are shown. Statistical analysis was performed with Prism software (GraphPad Software, La Jolla, CA). The values are expressed as the means $\pm$ S.E. The significance of the differences was assessed by Student's *t* test or one-way analysis of variance where appropriate. $p < 0.05$ was considered as statistically significant.

RESULTS

Reduced Recruitment of Monocyte-derived Inflammatory DCs into the Inflamed Airway in Fpr2^{-/-} Mice—We first investigated the identity of the receptors required for the accumulation of CD11c⁺MHC II⁺ cells in the airway in the secondary immune responses to the inhalation of OVA combined with a low dose of LPS in mice after systemic antigen immunization with OVA (22) CD11c⁺MHC II⁺ cells were increased in the lungs of WT mice immunized with OVA as compared with their naïve state (Fig. 1, A–D). These increased CD11c⁺MHC II⁺ cells in the inflamed lung expressed high levels of Ly6C as a marker of the monocytic origin (Fig. 2, A–E), suggesting that

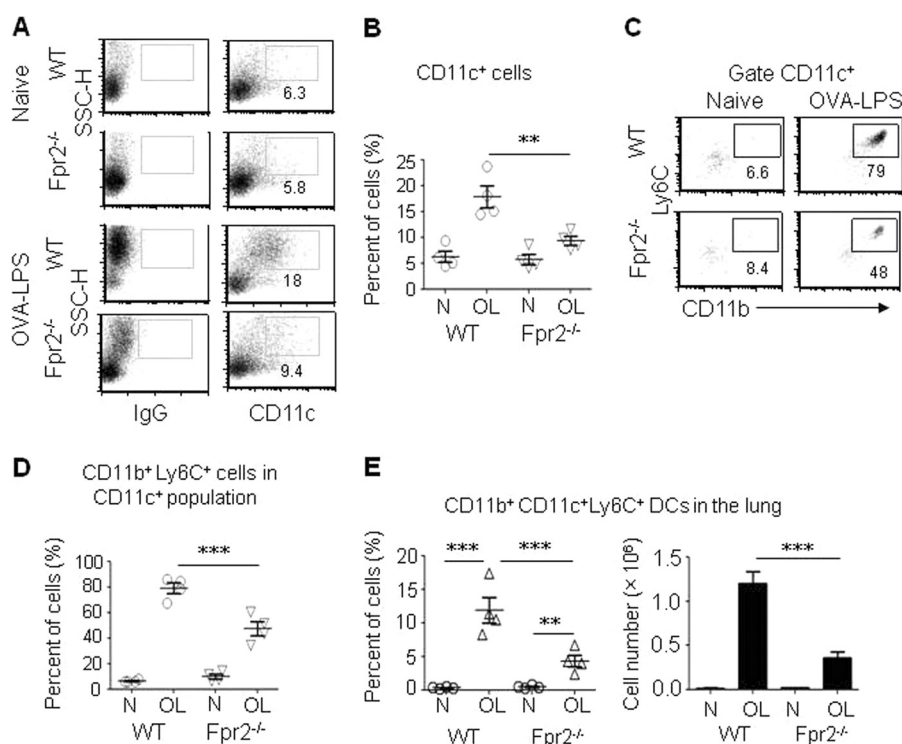


FIGURE 2. Reduced accumulation of monocyte-derived Ly6C⁺ DCs in the lung of Fpr2^{-/-} mice with allergic airway inflammation. A and B, accumulation of CD11c⁺ cells in the lung of mice with allergic airway inflammation. The results are expressed as the means $\pm$ S.E. $n = 5$ mice/group. **, $p < 0.01$. C and D, the percentage of CD11b⁺Ly6C⁺ cells in the CD11c⁺ cell population. The results are expressed as the means $\pm$ S.E. $n = 5$ mice/group. ***, $p < 0.001$. E, cumulative frequency of CD11b⁺Ly6C⁺CD11c⁺ cells in the lung. The results are expressed as the means $\pm$ S.E. $n = 5$ mice/group. **, $p < 0.01$; ***, $p < 0.001$. N, naive; OL, OVA/LPS.

they were differentiated from circulating CD11b⁺ monocyte precursors.

In contrast to WT mice, although CD11c⁺MHC II⁺ cells were also increased in the lungs of Fpr2^{-/-} mice with allergic airway inflammation as compared with the naïve state, the rate of such an increase was markedly lower as compared with WT mice (Fig. 1, A and B). The frequency of CD11b⁺ cells in the CD11c⁺MHC II⁺ cell population (Fig. 1, C and D), the number of CD11c⁺ and CD11b⁺Ly6C⁺ cells, and CD11b⁺Ly6C⁺CD11c⁺ subpopulations all were significantly reduced in the allergic airway of Fpr2^{-/-} mice as compared with WT mice (Fig. 2, A–E). Therefore, the trafficking of CD11b⁺ myeloid DCs into the inflamed lung of Fpr2^{-/-} mice was defective.

Reduced CCR2⁺ Population in Inflammatory DCs in the Inflamed Airway of Fpr2^{-/-} Mice—Because the chemokine receptors CCR2, CCR5, and CCR6 have been implicated in the recruitment of Ly6C⁺ inflammatory DCs from the blood to inflamed tissues (3, 7), we examined the frequency of CCR2⁺, CCR5⁺, and CCR6⁺ cells in the Ly6C⁺CD11c⁺ cell population in the lung of mice with allergic airway inflammation. Although the frequencies of CCR2⁺, CCR5⁺, and CCR6⁺ cells were increased in the inflamed lung of WT mice as compared with their naïve state (Fig. 3, A and B, and supplemental Fig. S1), the frequency of CCR2⁺ cells was the highest, similar to previous reports (7), indicating that the recruitment of monocyte-derived DCs into the allergic inflamed airway was in a CCR2-dependent manner. Interestingly, these cells were markedly reduced in the inflamed lung of Fpr2^{-/-} mice as compared with

WT mice (Fig. 3, A and B, and supplemental Fig. S1). These results suggest that Fpr2 deficiency is associated with reduced recruitment of CCR2⁺, CCR5⁺, and CCR6⁺ cells into the inflamed airway.

Retention of Ly6C⁺ Monocytes in the Blood of Fpr2^{-/-} Mice—Because CCR2⁺ inflammatory DCs were reduced in the inflamed lung of Fpr2^{-/-} mice, we hypothesized that Fpr2 deficiency may have resulted in impaired CCR2 function. However, after allergic stimulation, the number of CD11b⁺ and CCR2⁺CD11b⁺Ly6C⁺ cells in the blood was higher in Fpr2^{-/-} mice than in WT mice (Fig. 3, C and D, and supplemental Fig. S2A), and such cells expressed a similar level of CCR2 (supplemental Fig. S2B). Thus, Fpr2 deficiency did not reduce CCR2-dependent efflux of monocyte precursors from BM into the blood during allergic responses (23), but rather, the absence of Fpr2 appears to impair the subsequent cell trafficking to the lung.

Participation of Fpr2 in Trafficking of Inflammatory DCs from Perivascular Region to the Bronchioles—We therefore examined the participation of Fpr2 in the trafficking of inflammatory DCs within lung tissues. Histological examination revealed that in WT mice with allergic airway inflammation, monocyte-derived inflammatory DCs were recruited into the lung from blood vessels, followed by infiltration into peribronchiole tissues (Fig. 4, A–C). However, in the inflamed lung of Fpr2^{-/-} mice, the cells were mainly located in the perivascular regions and fewer cells localized in the area surrounding small airways (Fig. 4, A–C).

Fpr2 and CCR2 Control DC Trafficking in Asthmatic Lung

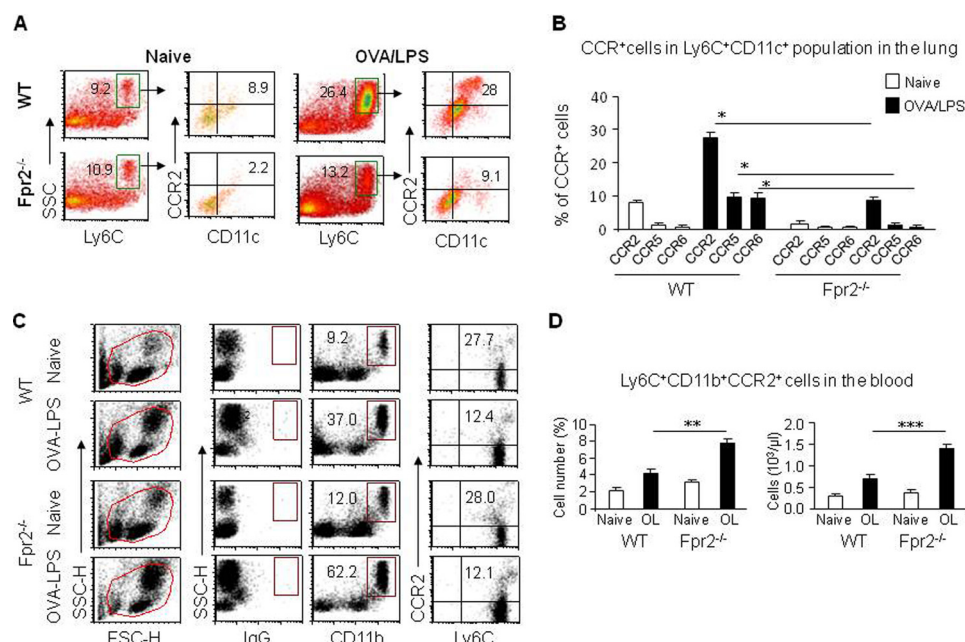


FIGURE 3. Monocyte-derived DC populations in the lung and blood of *Fpr2*^{-/-} mice with allergic airway inflammation. *A*, frequency of CD11c⁺CCR2⁺ cells in the Ly6C⁺ population in the inflamed lung of mice. *B*, cumulative results for CCR2⁺, CCR5⁺, and CCR6⁺ cells in the CD11c⁺Ly6C⁺ cell population in the inflamed lung. The results are expressed as the means $\pm$ S.E. *n* = 5 mice/group. The values are calculated as follows: percentage of CCR2⁺CD11c⁺Ly6C⁺ cell population = percentage of Ly6C⁺ cell population $\times$ percentage of CCR2⁺CD11c⁺/100. *, *p* < 0.05. *C*, accumulation of CD11b⁺ cells in the circulation. *D*, cumulative results of CCR2⁺CD11b⁺Ly6C⁺ cell frequencies in the blood. The results are expressed as the means $\pm$ S.E. *n* = 5 mice/group. OL, OVA/LPS. **, *p* < 0.01; ***, *p* < 0.001.

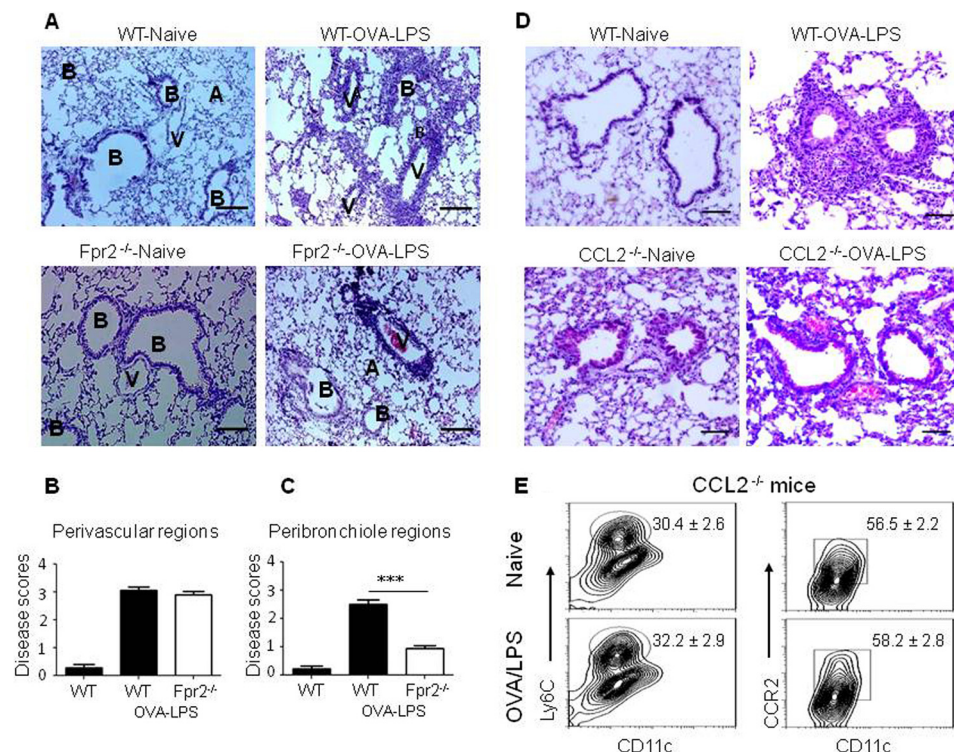


FIGURE 4. Reduced inflammatory cell infiltration in the peribronchiole regions of the inflamed lung of *Fpr2*^{-/-} mice and reduced recruitment of Ly6C⁺ cells into the inflamed lung in *CCL2*^{-/-} mice. *A*, inflammatory cell infiltration in the peribronchiole regions in the inflamed lung. A, alveolus; B, bronchiole; V, blood vessel. Scale bar, 100 μ m. The results from a representative experiment of four performed are shown. *B*, cumulative disease scores of perivascular regions in the lung. *C*, cumulative disease scores of the peribronchiole regions in the lung. The results are expressed as the means $\pm$ S.E. obtained from 45 blood vessels and 40 bronchioles of eight mice/group. ***, *p* < 0.001. *D*, reduced inflammatory cell infiltration in the lung of *CCL2*^{-/-} mice. Scale bar, 100 μ m. The results from a representative experiment of four performed are shown. *E*, absence of recruitment of Ly6C⁺ cells into the inflamed lung in *CCL2*^{-/-} mice. *CCL2*^{-/-} mice were immunized with OVA and aerosol-challenged with OVA/LPS. CD11c⁺Ly6C⁺ cell population and the percentage of CCR2⁺CD11c⁺ cells in the Ly6C⁺ population were analyzed with FACS. The data from a representative experiment of four performed are shown. The results are expressed as the means $\pm$ S.E., *n* = 5 mice/group.

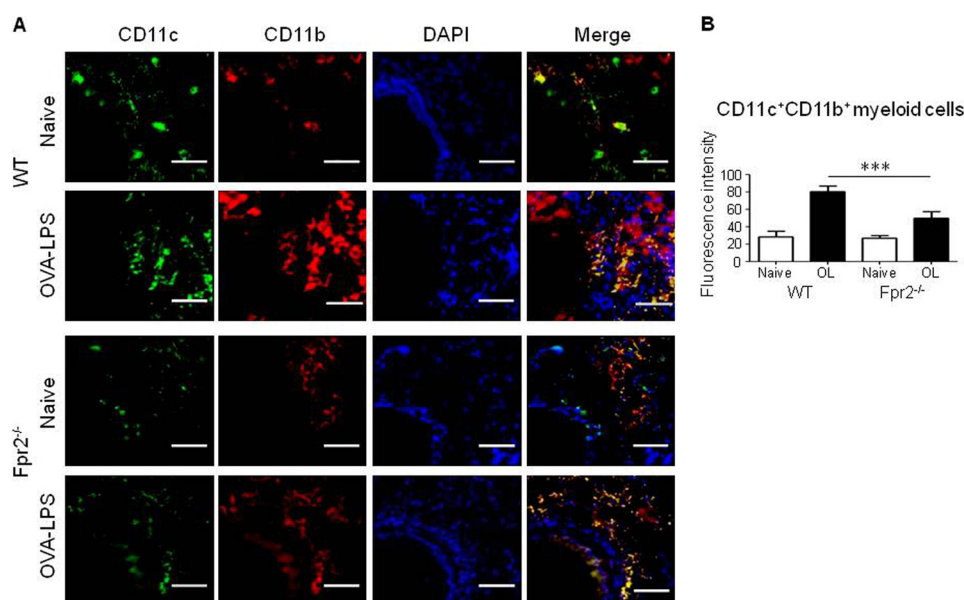


FIGURE 5. Reduced accumulation of CD11c⁺CD11b⁺ myeloid cells in the lung of Fpr2^{-/-} mice with allergic airway inflammation. A, reduced CD11c⁺CD11b⁺ myeloid cell infiltration in the inflamed lung of Fpr2^{-/-} mice. The mice were immunized with OVA and aerosol-challenged with OVA/LPS. Frozen lung sections were stained with hamster anti-mouse CD11c and CD11b antibodies followed by biotinylated anti-Ig antibodies and streptavidin-PE (red) or streptavidin-FITC (green). The nuclei were revealed by DAPI (blue). Hamster IgG was used as an isotype control. Scale bar, 50 μ m. The results from a representative experiment of three performed are shown. B, fluorescence intensity of CD11c⁺CD11b⁺ cells in the lung. The mice used were 8–12-week-old male littermates. ***, $p < 0.001$, significantly reduced CD11c⁺CD11b⁺ cells in the inflamed lung of Fpr2^{-/-} mice as compared with WT mice. CD11c⁺CD11b⁺ cells in the lung in mediastinal LN were identified by immunofluorescence. Frozen sections were stained with anti-mouse CD11c (BD Biosciences) and anti-mouse CD11b (BD Biosciences) followed by biotinylated anti-Ig Abs (BD Biosciences) with streptavidin-PE or streptavidin-FITC and DAPI (Invitrogen) or PE-anti-rabbit IgG antibody. Hamster IgG, rabbit IgG, or rat IgG2b was used as an isotype control. OL, OVA/LPS.

To verify the contribution of CCR2–CCL2 axis to DC recruitment, we used CCL2^{-/-} mice, in which only a very low level perivascular and peribronchiolar inflammation accompanied by congestion in blood vessels was observed (Fig. 4D). Ly6C⁺ monocyte-derived DC recruitment into the inflamed lung was completely absent in CCL2^{-/-} mice (Fig. 4E). This is consistent with the observation in CCR2^{-/-} mice with abolished recruitment of monocyte-derived DCs in the inflamed lung (7). These results clearly indicate the requirement for the CCR2–CCL2 axis to mediate the recruitment of monocyte-derived DCs from circulation into the inflamed lung. However, CCR2/CCL2 axes exert their roles only in cell accumulation in the perivascular region. The subsequent cell trafficking to the peribronchiolar area was mediated by Fpr2, as shown by the results from Fpr2^{-/-} mice. We further demonstrated that the cells infiltrating peribronchiolar tissues in the inflamed lungs of WT mice were CD11c⁺CD11b⁺, and such cells were markedly reduced in the peribronchiolar tissues of Fpr2^{-/-} mice (Fig. 5, A and B).

During immune responses, Ly6C⁺ monocyte-derived inflammatory DCs uptake antigens in the peripheral tissue and migrate via afferent lymphatics to the draining lymph nodes, where they produce cytokines (24–27). We confirmed that in immunized Fpr2^{-/-} mice, in addition to their reduction in the peribronchiolar region, the number of CD11c⁺ cells in the T cell zones of the mediastinal LNs was greatly diminished (supplemental Fig. S3, A and B). Thus, the capacity of monocyte-derived Ly6C⁺ inflammatory DCs in Fpr2^{-/-} mice to infiltrate the peribronchiolar region was impaired.

Restoration of the Trafficking of Inflammatory DCs in Fpr2^{-/-} Mice by Adoptive Transfer of WT Mouse BM Cells—To verify the involvement of Fpr2 in the trafficking of inflam-

matory DCs from the perivascular region to the inflamed small airway tissues, we transplanted BM cells from WT mice into Fpr2^{-/-} mice. The chimeric mice showed increased inflammatory cells in the peribronchiolar regions (Fig. 6, A and B, and supplemental Fig. S4A) with a considerable restoration of the accumulation of Ly6C⁺CD11c⁺ inflammatory DCs in the inflamed lung (Fig. 6C and supplemental Fig. S4, A and B). In contrast, in the blood of Fpr2^{-/-} mice with WT BM transfer, Ly6C⁺CD11b⁺CCR2⁺ inflammatory DC precursors were reduced as compared with the state without adoptive transfer (supplemental Fig. 4, C and D). These results further demonstrate an important role for Fpr2 in the trafficking of inflammatory DCs from perivascular regions to the inflamed bronchioles.

The participation of Fpr2 in the trafficking of monocyte-derived inflammatory DCs to the peribronchiolar tissues in the lung was also investigated by transplantation of carboxyfluorescein diacetate succinimidyl ester-labeled BM monocyte-derived DC precursors. After OVA immunization, the recipient WT mice were intravenously injected with carboxyfluorescein diacetate succinimidyl ester-labeled BM cells from WT or Fpr2^{-/-} mice, 2 days following intranasal OVA/LPS challenge. The recipient mice were then exposed to the aerosol allergen, and the appearance of carboxyfluorescein diacetate succinimidyl ester⁺ CD11b⁺ inflammatory DCs in the lung was assessed after 24 h. Compared with WT mouse cells, the recruitment of Fpr2^{-/-} mouse monocyte-derived inflammatory DCs into the inflamed lung of WT mice was minimal (supplemental Fig. S5, A and B). There was also a decreased recruitment of Fpr2^{-/-} mouse DCs into the mediastinal LN (supplemental Fig. S5, C and D). Thus, in the absence of Fpr2, although DC precursors were able to

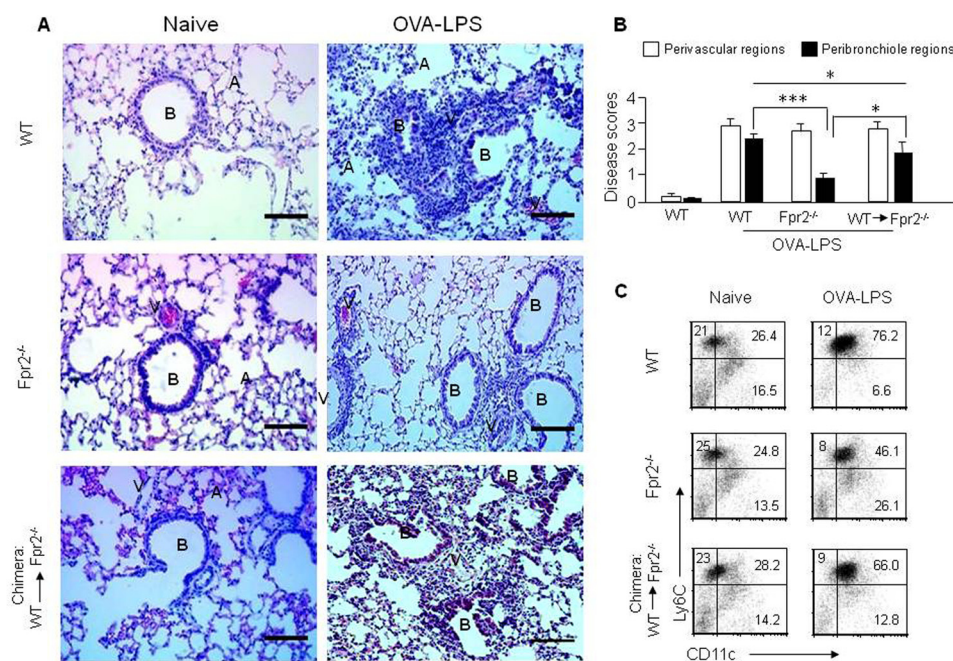


FIGURE 6. Restoration by adoptive transfer of WT mouse BM cells. A, cell infiltration in the lung tissues surrounding the inflamed bronchioles in chimeric mice. A, alveolus; B, bronchiole; V, blood vessel. Scale bar, 100 μ m. B, cumulative disease scores of the perivascular and bronchiole regions in the inflamed lung. The results are expressed as the means $\pm$ S.E. from 20 blood vessels and 25 bronchioles of 5 mice/group. *, $p < 0.05$; ***, $p < 0.001$, significantly reduced scores in the perivascular regions of the inflamed lung in Fpr2^{-/-} mice as compared with WT mice and significantly restored scores in the peribronchiole regions in the lung of Fpr2^{-/-} mice receiving transfer of WT BM cells as compared with Fpr2^{-/-} mice without BM transfer. C, restoration of CD11c⁺Ly6C⁺ cells infiltrating the inflamed lung tissues of chimera mice. The results from a representative experiment of four performed are shown.

accumulate in the perivascular region in the inflamed lung, they failed to complete the next phase of trafficking into the small airway region and subsequently into the draining lymph nodes.

Fpr2 and CCR2 Agonist Activity in BAL from Mice with Allergic Airway Inflammation—Because Fpr2 deficiency did not affect the recruitment of Ly6C⁺ monocyte-derived inflammatory DCs from circulation and accumulation in the perivascular regions in the inflamed lung, we hypothesized that Fpr2 may be critical for chemoattraction of monocyte-derived inflammatory DCs into the peribronchiole tissues. To test this, BM cells from WT, Fpr2^{-/-}, and CCR2^{-/-} mice were cultured in the presence of GM-CSF and IL-4 to generate immature DCs (Ly6C⁺CD11c⁻) (supplemental Fig. S6A). WT BM-derived immature DCs expressed both functional Fpr2 and CCR2. On the other hand, whereas BM-derived immature DCs from Fpr2^{-/-} mice expressed functional CCR2, CCR2^{-/-} mouse cells expressed functional Fpr2 (supplemental Fig. S6, B and C). We then tested whether inflamed lung produced agonists for Fpr2 and CCR2 that induce the chemotaxis of monocyte-derived inflammatory DCs. Indeed, BAL from WT mice with allergic airway inflammation contained chemoattractant activity specific for Fpr2 as well as the CCR2 agonist CCL2. As shown in Fig. 7A, the BAL from WT mice with airway inflammation induced potent chemotaxis of HEK293 cells transfected with Fpr2 as compared with parental HEK293 cells. Inflamed lung also expressed high levels of CCL2 mRNA and release CCL2 protein into the BAL (Fig. 7, B and C). An Fpr2 inhibitor WRW4 or a CCR2 inhibitor CHF each partially inhibited the migration of immature DCs from WT mice in response to the BAL (Fig. 7D). Combination of two inhibitors completely abolished cell responses (Fig. 7D). Immature DCs from Fpr2^{-/-}

mice or CCR2^{-/-} mice showed partially reduced chemotaxis in response to BAL from inflamed lung of WT mice (Fig. 7E), and the remaining cell responses were abrogated by the addition of either Fpr2 or CCR2 antagonist (Fig. 7, F and G).

Furthermore, because one of the reported host-derived Fpr2 ligands is CRAMP (28, 29), which is more highly expressed in the inflamed lung (21, 30) (Fig. 8A), we tested whether CRAMP might account for the Fpr2 agonist activity contained in the BAL. Fig. 8B shows that the antibody against CRAMP markedly reduced the chemotactic activity of the BAL for immature DCs, with remaining chemotactic activity attributed to CCL2. To support the role of CRAMP, in preliminary results obtained from CRAMP^{-/-} (Cnrip^{-/-}) mice with allergic airway inflammation, BAL contained reduced chemotactic activity for WT immature DCs (Fig. 8C). Although the BAL from inflamed lung of WT mice contained chemotactic activity for both Fpr2-transfected HEK 293 cells and the parental cells, the activity on Fpr2-transfected cells far exceeded that for the parental cells, indicating the presence of Fpr2-specific chemotactic agonist(s), presumably CRAMP. This was confirmed by the loss of the Fpr2-specific part of the chemotactic activity of BAL from CRAMP^{-/-} mice (Fig. 8D). The nature of the remaining chemotactic activity shown by BAL from WT and CRAMP^{-/-} mice remains to be elucidated. Histological examination revealed that in the inflamed lung of CRAMP^{-/-} mice, infiltrating leukocytes containing CD11b⁺CD11c⁺ DCs were mainly located in the perivascular regions with few cells accumulating in the area surrounding the small airways (supplemental Fig. S7 and data not shown). Thus, deficiency in CRAMP results in a phenotype similar to Fpr2^{-/-} mice in allergic airway inflammation. These results confirm the presence of

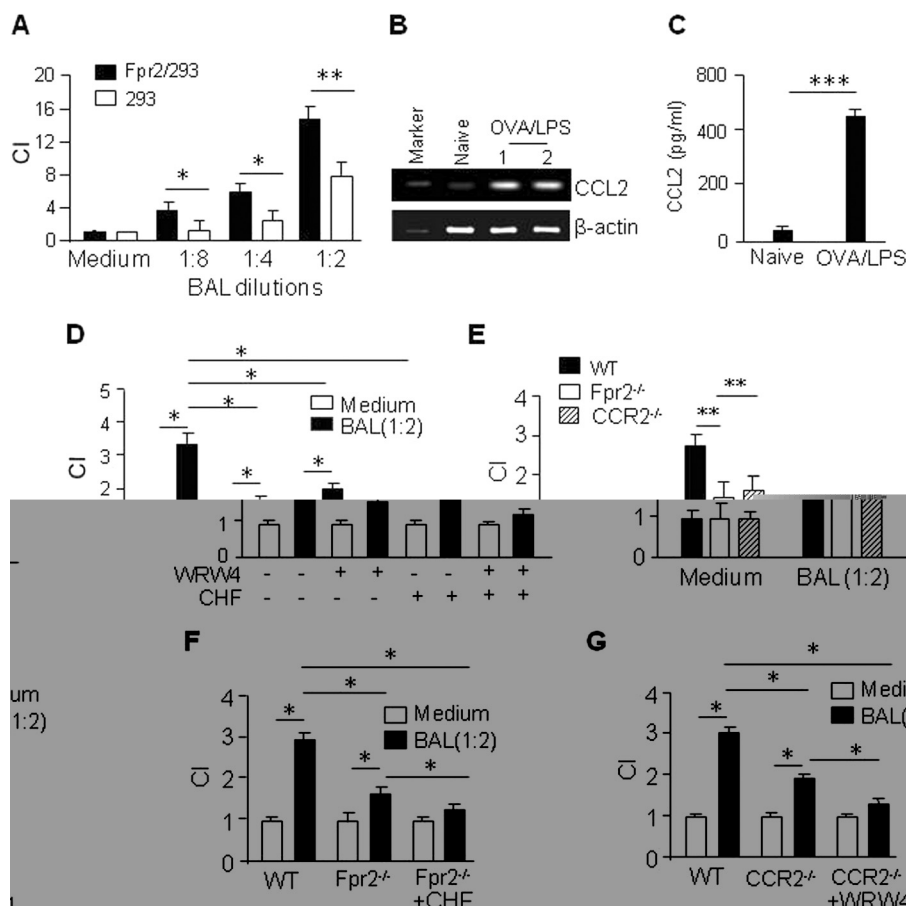


FIGURE 7. Fpr2 and CCR2 agonists contained in BAL from WT mice with allergic airway inflammation. A, parental 293 cells and 293 cells transfected with Fpr2 (Fpr2/293) were measured for migration induced by different concentrations of BAL from WT mice with airway inflammation. *, $p < 0.05$; **, $p < 0.01$. B, CCL2 mRNA expression in lung tissues. C, CCL2 in BAL. The results are expressed as the means $\pm$ S.E. $n = 5$ mice/group. ***, $p < 0.001$. D, inhibition of BAL-induced DC chemotaxis by CCR2 or Fpr2 antagonist. *, $p < 0.05$. E, reduced chemotaxis of DCs from Fpr2^{-/-} and CCR2^{-/-} mice in response to BAL. **, $p < 0.01$. F, CCR2 antagonist inhibits Fpr2^{-/-} cell migration to BAL. *, $p < 0.05$. G, inhibition of CCR2^{-/-} DC migration to BAL by Fpr2 antagonist. *, $p < 0.05$. C, chemotaxis index.

chemotactic agonists CCL2 and CRAMP in the inflamed lung, and they are responsible for mediating DC trafficking in the inflamed lung tissue.

The Sequential Expression of CCR2, Fpr2, and CCR7 by DCs during Allergic Airway Inflammation—Because a variety of bacteria-derived and endogenous TLR agonists exist in the lung tissues (31) and are capable of reducing the migration of monocyte-derived inflammatory DCs in response to chemokine ligands for CCR1, CCR2, and CCR5 (10), we examined the differential expression of chemoattractant receptors on the cells after TLR activation. We found that after stimulation with the TLR4 ligand LPS for 1 h (Fig. 9A), DCs migrated poorly in response to CCL2 but showed increased response to an Fpr2 agonist peptide MMK-1. Cells treated for 10 h with LPS showed a maximal chemotactic response to MMK-1, followed by a moderate reduction in response after LPS treatment for 16 h (Fig. 9B). Consistent with cell responses to MMK-1, the migration of DCs in response to BAL was maximal at 10 h after LPS treatment, with a reduction after 16 h (Fig. 9C). In addition, using DCs stimulated with LPS for 16 h, we found that cell chemotaxis in response to the BAL from inflamed lung of WT mice was substantially inhibited by the Fpr2, but not the CCR2, antagonist (Fig. 9D), suggesting that the infiltration of mono-

cyte-derived inflammatory DCs in the inflamed lung was mediated mainly by Fpr2 and its endogenous ligand CRAMP after TLR activation. We further found that LPS treatment of DCs for 1 h was sufficient to down-regulate CCR2 but up-regulate Fpr2 function with no effect on receptor gene expression.⁴ However, treatment for 10 h with LPS resulted in substantial reduction of CCR2 but enhancement of Fpr2 gene and cell surface expression in DCs (supplemental Fig. S8, A–C).

Concomitantly, the expression of the DC homing receptor CCR7 was progressively increased by DCs after TLR treatment (Fig. 9, E and F). These results demonstrate a sequential expression pattern of chemoattractant receptors by monocyte-derived inflammatory DCs in response to differentiation signals, which may account for their stepwise participation in trafficking of monocyte-derived inflammatory DCs during inflammatory responses.

DISCUSSION

Asthma is a chronic inflammatory airway disease with persistent homing of circulating monocyte-derived inflammatory DCs to the inflamed lung tissues (27). Monocyte-derived

⁴ K. Chen, data not shown.

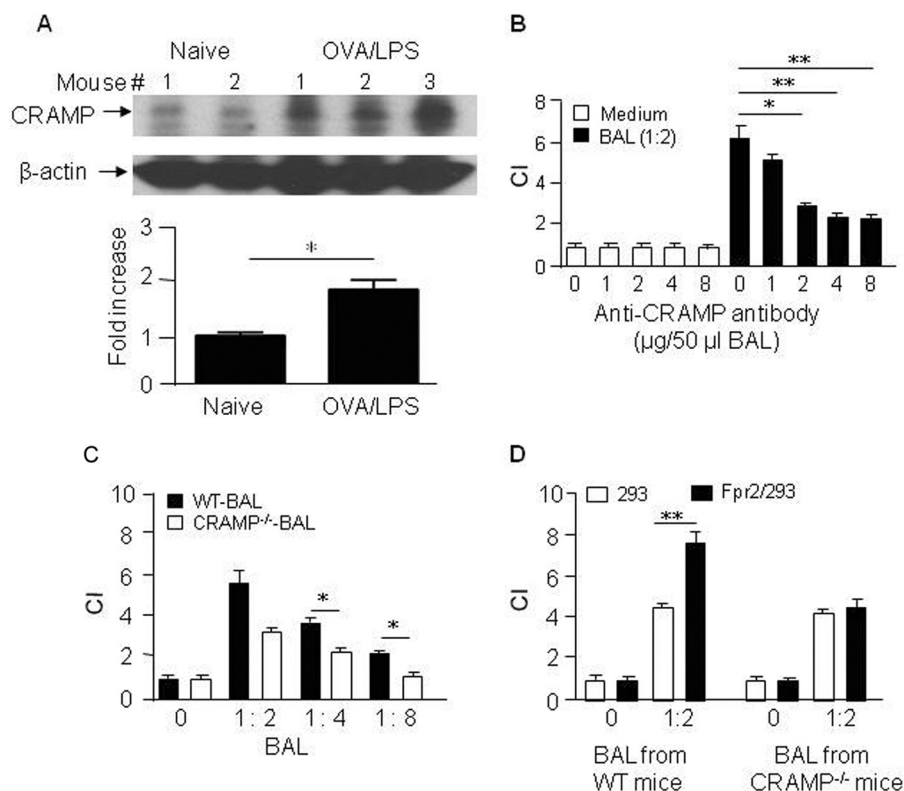


FIGURE 8. Chemotactic activity of CRAMP in the BAL of mice with allergic airway inflammation. A, CRAMP expression in the lung. Densitometry analysis shows significant increase of CRAMP in the inflamed lung of WT mice (*, $p < 0.01$). B, reduction of the chemotactic activity of BAL for DCs by anti-CRAMP antibody. The results are expressed as the means $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$. C, reduction of the chemotactic activity of BAL from CRAMP^{-/-} mice with allergic airway inflammation. *, $p < 0.05$. D, parental 293 cells and 293 cells transfected with Fpr2 (Fpr2/293) were measured for migration induced by the BAL from CRAMP^{-/-} mice with airway inflammation. **, $p < 0.01$. CI, chemotaxis index.

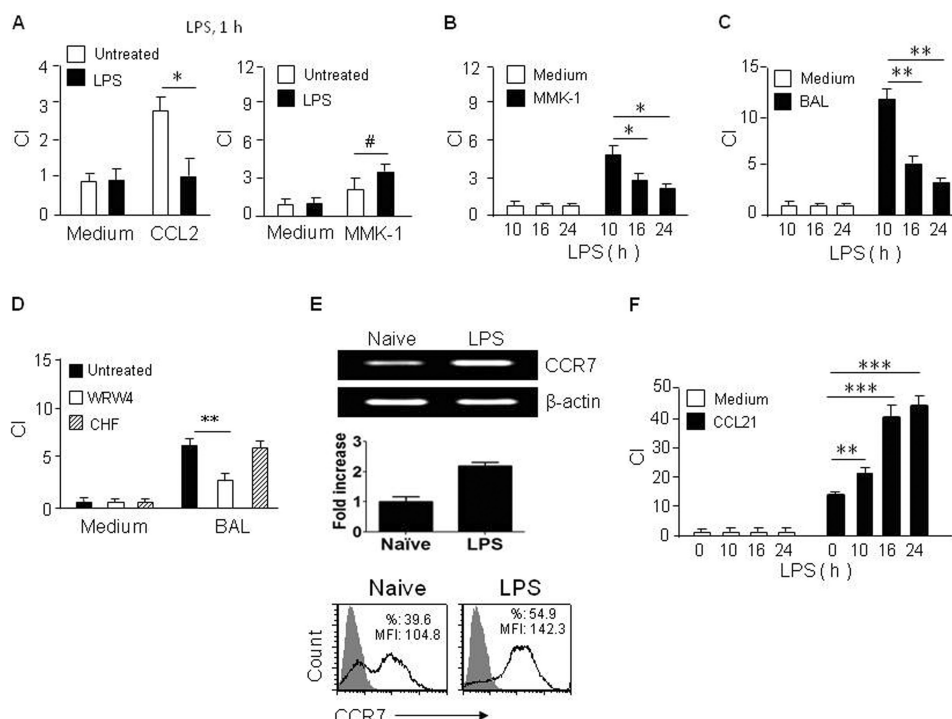


FIGURE 9. BAL-induced chemotaxis of WT mouse DCs activated with LPS (100 ng/ml). A, down-regulation of CCR2 function and increased Fpr2 function in DCs 1 h after LPS stimulation. *, $p < 0.05$; #, $p < 0.05$. B, time-dependent Fpr2 function after LPS stimulation. *, $p < 0.05$. C, time-dependent migration of DCs from WT mice in response to BAL (1:2) after LPS stimulation. **, $p < 0.01$. D, failure of CCR2 antagonist to inhibit the migration of LPS-treated DCs in response to BAL. **, $p < 0.01$. E, CCR7 expression in DCs after LPS stimulation. Top panel, CCR7 mRNA expression. Middle panel, fold increase of CCR7 mRNA expression after LPS stimulation. Bottom panel, CCR7 protein expression. MFI, Mean Fluorescence Intensity. F, increased CCR7 function in inflammatory DCs after LPS stimulation. **, $p < 0.01$; ***, $p < 0.001$. CI, chemotaxis index.

inflammatory DCs are critical in initiating Th2 responses, and their recruitment into the inflamed lung has been shown to depend on the chemokine receptor CCR2 (7). Our study confirmed that CCR2 is required for Ly6C^{high} monocytic DC precursor mobilization from BM into the circulation (7, 23) and from the blood to the inflamed lung (7). However, we further observed that after accumulating in the perivascular region, monocyte-derived inflammatory DCs lost the expression of functional CCR2, but Fpr2 is elevated to complete the further trafficking to the inflamed peribronchiole regions in response to the ligand CRAMP. This is supported by the observations that Fpr2-deficient DCs failed to infiltrate the inflamed bronchioles with retention in the circulation and perivascular regions, and Fpr2^{-/-} mice were unable to mount a high level of allergic responses (13). Similar to Fpr2^{-/-} mice, in the inflamed lung of CRAMP^{-/-} mice, inflammatory cells accumulated mainly in perivascular regions, and the cells failed to further migrate to the tissue surrounding small airways. Thus, our observations establish a key role for Fpr2/CRAMP axis in the signal relay that guides DC trafficking in the inflamed lung.

TLRs regulate the differentiation of DCs during inflammatory responses (31, 32). It has been shown that TLR2 activation by lipoteichoic acid from *Staphylococcus aureus* inhibits CCR1, CCR2, and CCR5 function on human monocytic cells and thus inhibits cell migration in response to chemokine agonists (10, 33). This down-regulation of the expression pattern of chemokine receptors by TLRs is important to retain infiltrating cells at sites of infection (10, 33). The down-regulation of chemokine receptor function on inflammatory cells by TLR agonists occurs in two manners: a slower (in hours) process with reduction of gene expression and mRNA stability associated with cell differentiation and a rapid (within minutes) interference in receptors function resembling “desensitization” (34, 35). For example, the TLR2 ligand Pam3CSK4 suppresses the migration of mouse monocytes by down-regulating CCR1, CCR2, and CCR5 genes, whereas another TLR2 ligand lipoteichoic acid interferes with the chemotaxis of mouse neutrophils by inhibiting the function of another chemokine receptor CXCR2 (33, 36, 37). Our study showed that CCR2 on Ly6C⁺ immature inflammatory DCs was rapidly down-regulated after LPS stimulation for 1 h with increased function of Fpr2. Thereafter, Fpr2 function in DCs progressively returned to the original level of nonstimulated state accompanied by an increased function of CCR7. This dynamic regulation of chemoattractant receptors on myeloid cells including DCs represents an important host response during disease states (38). In the inflamed lung, recruited inflammatory DCs immediately encounter a variety of bacteria- and host-derived TLR agonists such as LPS, PGN, HMGB1, fragment of fibronectin, and hyaluronic acid oligosaccharides (31, 39, 40), which reduce CCR2 but increase Fpr2 expression and function, enabling the cells to reach small airways in response to the Fpr2 agonist CRAMP produced in the inflamed tissue (41–44).

The diverse nature of chemoattractants contributes to the fine control of leukocyte trafficking *in vivo*, with sequential use of chemoattractants to accumulate at inflammatory sites (39). In this study, we confirmed the presence of CCL2 and CRAMP in the BAL of mice with allergic airway inflammation, which

enables the completion of the trafficking of DCs to the inflammatory sites within the lung via CCR2 followed by Fpr2. Changes in the expression pattern of chemoattractant receptors are critical for inflammatory responses that require immune cells to migrate through a complex milieu of microbial and host-derived chemotactic factors to reach the focus of the lesions (45, 46). This is reflected in our study that despite the presence of CCL2 and CRAMP in the airway, monocyte-derived inflammatory DC extravasated into the perivascular regions failed to further migrate into the inflamed small airway in the absence of Fpr2. Following prolonged exposure to TLR ligands, the expression of Fpr2 on inflammatory DCs started to reduce with concomitant increase of CCR7. Thus, CCR2-Fpr2-CCR7 signal relay is critical for completion of monocyte-derived inflammatory DC trafficking.

Chemoattractant signal relay was also demonstrated in other disease models. In sterile wounds of the liver, chemokine receptors and another FPR family member, Fpr1, mediate sequential trafficking of neutrophils from the circulation to the necrotic core of the wound (12). In autoantibody-induced murine arthritis, a lipid-cytokine-chemokine cascade orchestrates neutrophil recruitment into the joints (47). In a model of Th2 lymphocyte recruitment in asthma, activated mast cells release lipid mediators LTB4 and PGD2, which recruit the first wave of Th2 lymphocytes into the inflamed lung where the cells produce signature cytokines to activate resident lung cells that further release chemokines to recruit the second wave of Th2 lymphocytes (48). These new discoveries lay a basis for a paradigm of leukocyte recruitment in disease states that involve chemoattractants and receptors in sequence to initiate, amplify, and then resolve host responses. Our present observations also revealed a novel spectrum of the biological function of FPRs, which are not only important for antibacterial host defenses presumably in response to pathogen-derived chemotactic and activating peptides (49, 50) but also critical for inflammatory and immune responses mediated by host-derived agonists. Thus, FPRs demonstrate pattern recognition receptor properties based on their capacity to directly respond to bacterial formylated peptides as well as to endogenous PAMPs (pathogen-associated molecular patterns), DAMPs (damage-associated molecular pattern), and alarmins. They are therefore potential targets for the design of novel therapeutics for inflammatory diseases.

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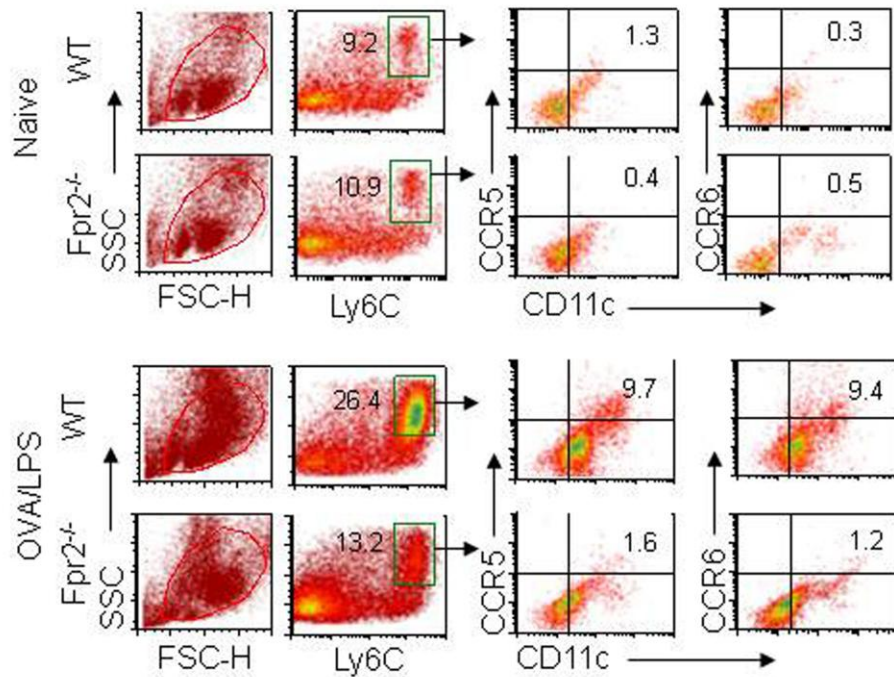
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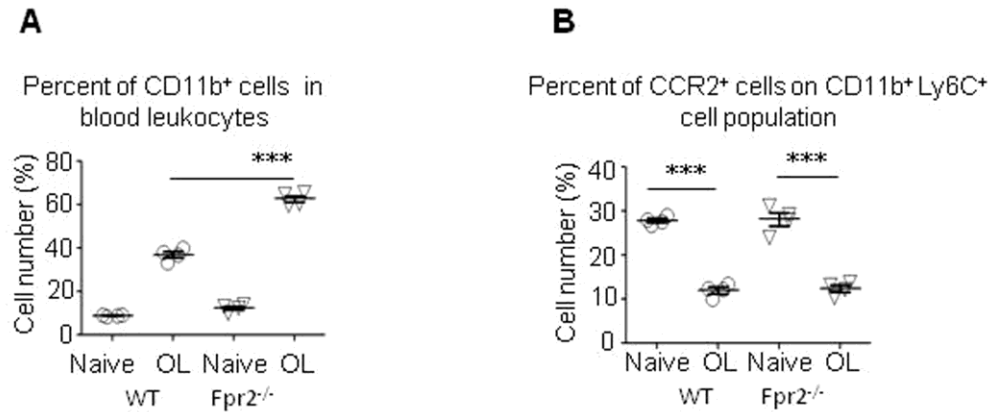
Supplemental Information:

Signal relay by CCR2 and Fpr2 in the recruitment of monocyte-derived dendritic cells in allergic airway inflammation

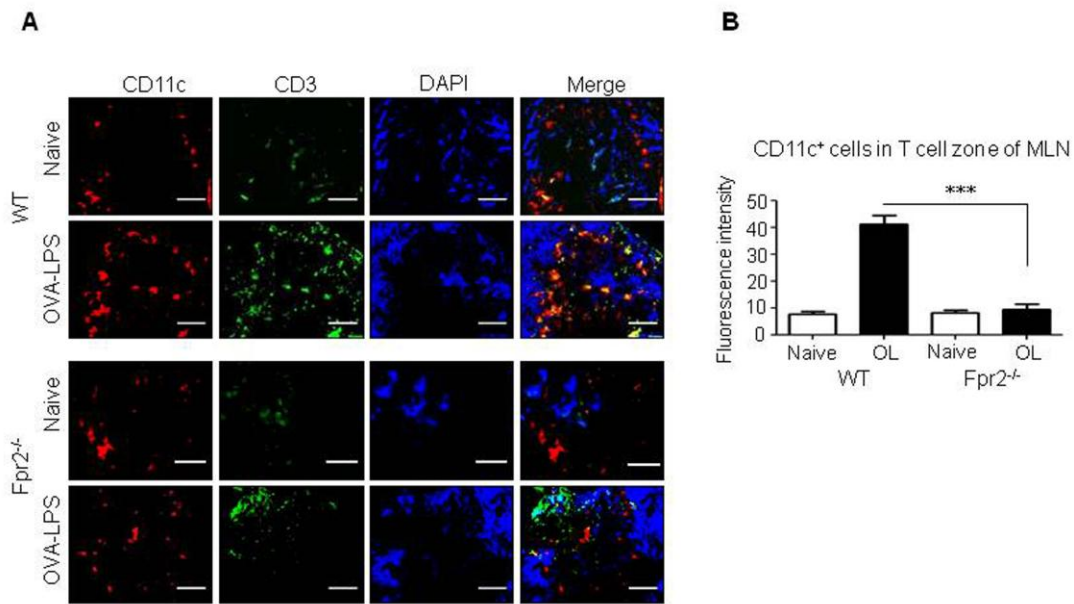
Keqiang Chen, Mingyong Liu, Ying Liu, Chunyan Wang, Teizo Yoshimura, Wanghua Gong, Yingying Le, Lino Tessarollo, Ji Ming Wang



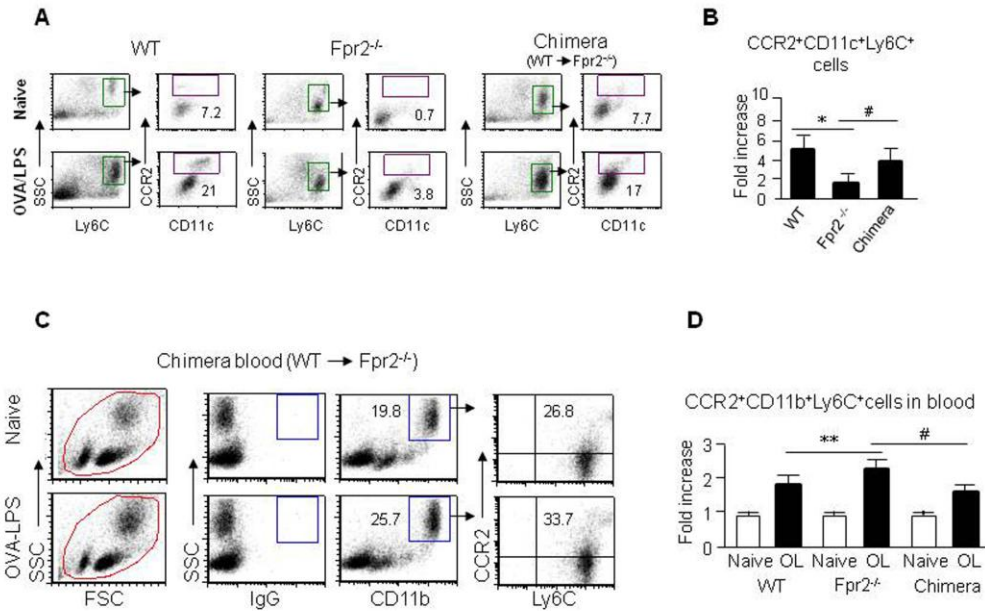
Supplemental Fig. S1: Reduced frequency of CD11c⁺CCR5⁺ and CD11c⁺CCR6⁺ cells in the Ly6C⁺ cell population in the inflamed lung of Fpr2^{-/-} mice—The frequency of CD11c⁺CCR5⁺ and CD11c⁺CCR6⁺ cells in the Ly6C⁺ cell population in the inflamed lung of mice. Mice were immunized and aerosol challenged with OVA/LPS. Lung cells were stained with anti-Ly6C, anti-CD11c and anti-CCR5 or anti-CCR6 antibodies and analyzed with FACS. Results from a representative experiment out of performed are shown. Mice used were 8-12 week old male littermates.



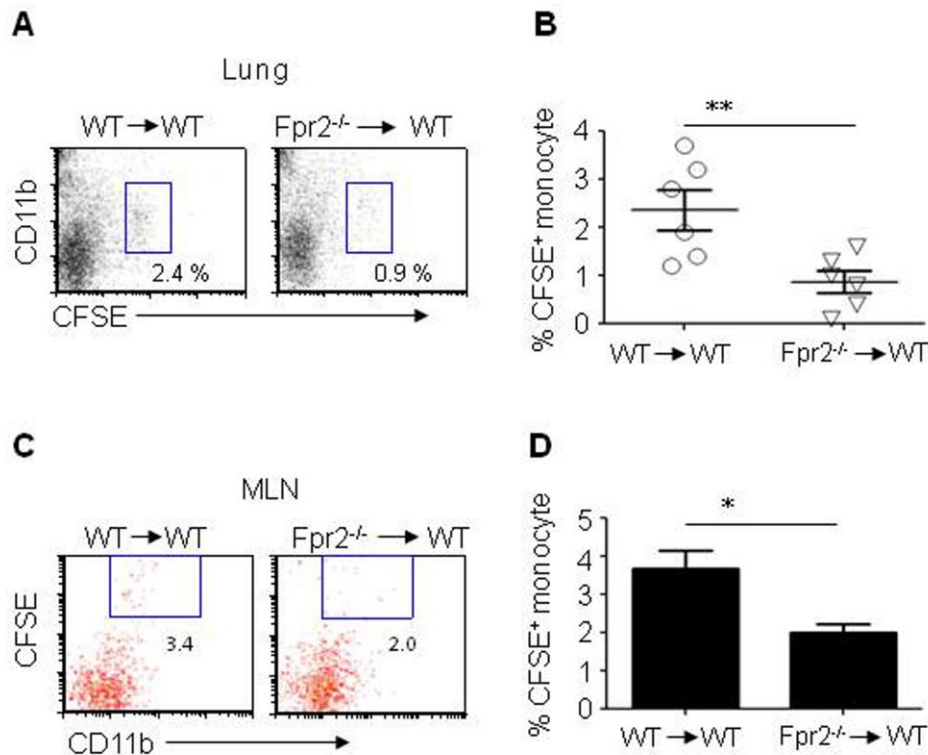
Supplemental Fig. S2: CD11b⁺ monocytes in the blood of mice with allergic airway inflammation and CCR2 expression by CD11b⁺Ly6C⁺ cells-Mice were immunized and aerosol challenged with OVA/LPS. After removal of erythrocytes, blood leukocytes were stained with anti-Ly6C, anti-CD11b and anti-CCR2 antibodies and analyzed by FACS. Results from a representative experiment out of 4 performed are shown. **A:** Cumulative results (mean $\pm$ SE) of CD11b⁺ cell frequency in the blood from 4 experiments. *** $P < 0.001$, significantly increased CD11b⁺ cell percent in the blood of Fpr2^{-/-} mice with allergic airway inflammation as compared to WT mice. **B:** Reduced expression of CCR2 on DC surface after LPS treatment. Cumulative results of CCR2⁺ cell frequency in the CD11b⁺Ly6C⁺ cell population. Mean values ($\pm$ SEM) of 4 independent experiments are shown. Na: naïve; OL: OVA/LPS. *** $P < 0.001$, significantly reduced CCR2⁺ cell frequency in the CD11b⁺Ly6C⁺ cell population in the lungs of mice with allergic airway inflammation as compared to their naïve states. Mice used were 8-12 week old male littermates.



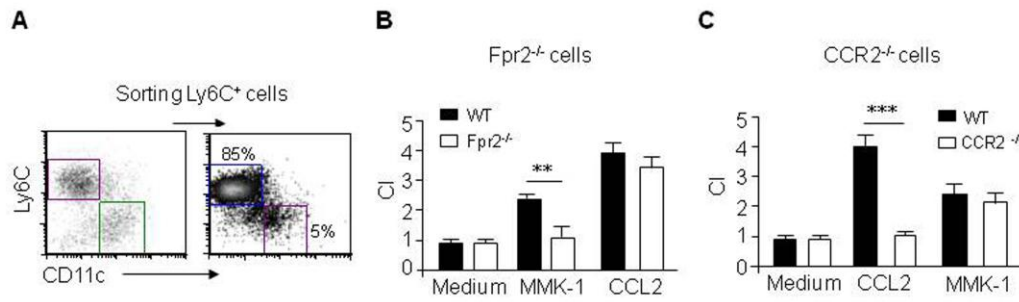
Supplemental Fig. S3: Reduced homing of mature DCs to the T cell zone of mediastinal lymph nodes (MLNs) in Fpr2^{-/-} mice with airway inflammation-**A:** Mice were immunized and aerosol challenged with OVA/LPS. CD11c⁺ cells and CD3⁺ cells in MLN were identified by immunofluorescence. Frozen sections were stained with anti-mouse CD11c (BD), anti-mouse CD3 Abs (BD) followed by biotinylated anti-Ig Abs (BD) with streptavidin-PE or streptavidin-FITC and DAPI (Invitrogen) or PE-anti-rabbit IgG antibody. Hamster IgG, Rabbit IgG or Rat IgG2b was used as an isotype control. Red: CD11c⁺ cells, Green: CD3⁺ cells, Blue: Nuclei stained with DAPI. Scale bar = 20 μ m. Mice: n = 4. **B:** The fluorescence intensity of CD11c⁺ cells in CD3⁺ T cell zone of MLN. Results are expressed as mean $\pm$ SEM. *** $P < 0.001$, significantly reduced CD11c⁺ cells in MLN of Fpr2^{-/-} mice as compared with WT mice. Mice used were 8-12 week old male littermates.



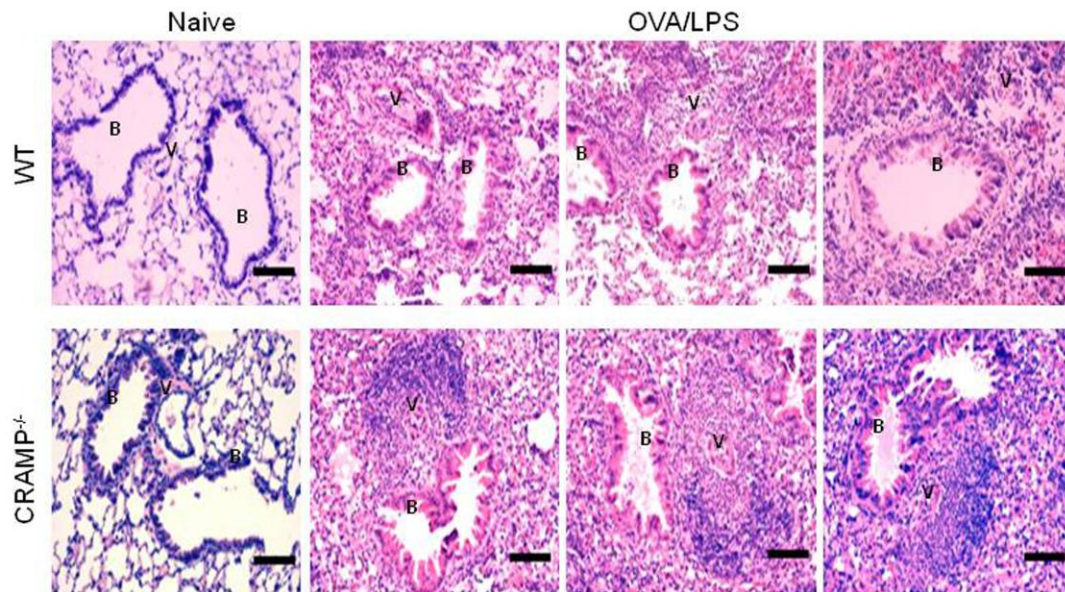
Supplemental Fig. S4: Restoration of monocyte-derived inflammatory DCs in the peribronchiole regions of inflamed lung in Fpr2^{-/-} mice by adoptive transfer of WT mouse BM cells-**A:** Inflammatory DCs in the inflamed lung of Fpr2^{-/-} mice with transfer of WT mouse BM. Lung cells from mice immunized and aerosol challenged with OVA/LPS were analyzed with FACS. Results from a representative experiment out of 4 performed are shown. **B:** Cumulative fold increase of inflammatory DCs in the lung of chimera mice. * $P < 0.05$, reduced cells in the lung of Fpr2^{-/-} mice compared with WT mice. # $P < 0.05$, increased cells in the lung of chimera mice compared with mice without BM transfer. **C:** The frequency of CCR2⁺Ly6C⁺CD11b⁺ DC precursors in the blood of chimera mice. Results from a representative experiment out of 4 performed are shown. **D:** Fold increase of CCR2⁺Ly6C⁺CD11b⁺ DC precursors in the blood of chimera mice with allergic airway inflammation. ** $P < 0.01$, significantly increased cells in the blood of Fpr2^{-/-} mice with airway inflammation compared with WT mice. # $P < 0.01$, significantly reduced cells in the blood of chimera mice compared to Fpr2^{-/-} mice. All mice used were 8-12 week old male littermates.



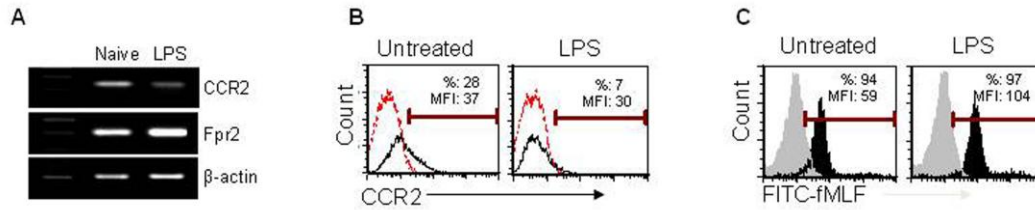
Supplemental Fig. S5. Reduced recruitment of Fpr2^{-/-} mouse CD11b⁺-CFSE cells into the lung and MLN of WT mice-BM cells were isolated from the femur and tibia from WT and Fpr2^{-/-} mice. Red blood cells were lysed by using ACK. The cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Invitrogen) at 37 °C for 10 minutes. The cells were then analyzed with flow cytometry (FACS) to adjust the concentration to 1×10⁸ CD11b⁺ cells/ml. The WT recipient mice were I.P. injected with OVA/Alum (100 µg OVA/50 µl PBS/50 µl Alum). Mice were boosted 15 d later with OVA/Alum (20 µg OVA/50 µl PBS/50 µl Alum). After 10 d, the mice were challenged intranasally with 1% OVA containing 1 µg LPS/mouse once a day for 2 d. On the third day, WT recipient mice were injected intravenously with 200 µl CFSE-labeled cells from WT (WT-WT) or Fpr2^{-/-} mice (KO-WT) and re-challenged. After 24 h, CFSE⁺CD11b⁺ cells in the lung and MLN were analyzed by FACS (Pool of cells from 6 mice). **A:** The frequency of CD11b⁺CFSE⁺ cells in the lung. **B:** Cumulative frequency (mean ± SE) of CD11b⁺CFSE⁺ cells in the lung. ** *P* < 0.01, significantly reduced cell number in the lung of WT mice receiving cells from Fpr2^{-/-} mice. **C:** The frequency of CD11b⁺CFSE⁺ cells in MLN. **D:** Cumulative frequency (mean ± SE) of CD11b⁺CFSE⁺ cells in MLN. * *P* < 0.05, significantly reduced cell number in MLN of WT mice receiving Fpr2^{-/-} cells. The experiments were performed 3 times. N= 6 mice per group. Results are expressed as the mean ± SEM.



Supplemental Fig. S6. The chemotactic activity of Fpr2 and CCR2 agonists for BM-derived Ly6C⁺ monocytes-A: Isolation of BM-derived Ly6C⁺ monocytes. BM cells from WT, Fpr2^{-/-} and CCR2^{-/-} mice were cultured in the presence of GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) for 4 d. The cells were sorted with CD11c (N418) MicroBeads to deplete CD11c⁺ cells. Ly6C⁺ cells with more than 85% purity were harvested. **B:** Migration of Ly6C⁺DC precursors to Fpr2 and CCR2 agonists. Results are expressed as the mean ± SE of CI, representing the fold increase in the number of migrated cells in response to chemoattractants over spontaneous cell migration (to control medium). ** *P* < 0.01, significantly reduced migration in response to the Fpr2 agonist peptide MMK-1 by cells from Fpr2^{-/-} mice as compared to WT mice. **C:** Migration of CCR2^{-/-}Ly6C⁺ DC precursors to Fpr2 and CCR2 agonists. *** *P* < 0.001, significantly reduced migration of cells from CCR2^{-/-} mice to CCL2 as compared to WT mouse cells. Results from a representative experiment out of 3 performed are shown.



Supplemental Fig. S7: Localization of infiltrating inflammatory cells in the perivascular regions in the inflamed lung of CRAMP^{-/-} mice-Mice were immunized and aerosol challenged with OVA/LPS. Lung tissues fixed in formalin and embedded in paraffin were sectioned (5 μ m) and stained with H&E. B: Bronchiole; V: Blood vessel. Scale bar = 100 μ m.



Supplemental Fig. S8. The expression of CCR2 and Fpr2 by inflammatory DCs during differentiation-A: CCR2 and Fpr2 mRNA expression by DCs. WT BM-derived immature inflammatory DCs were stimulated with LPS (10 ng/ml) for 10 h. The expression of CCR2, Fpr2 and β -actin mRNA was examined by RT-PCR. **B:** The expression of CCR2 on the surface of WT BM-derived inflammatory DCs. BM cells from WT mice were cultured in the presence of GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) for 4 d. Purified immature inflammatory DCs were stimulated with LPS (10 ng/ml) for 10 h. The expression of CCR2 on the cell surface was analyzed with FACS. The results were presented as the percentage of positive cells and mean fluorescence (MFI). Results from a representative experiment out of 3 performed are shown. **C:** Binding of fMLF by DCs. Purified inflammatory DCs stimulated with LPS (10 ng/ml) for 10 h were incubated with FITC-N-formyl peptide ($1 \mu\text{g}/0.5 \times 10^6$ cells) at 37°C for 30 minutes then were analyzed with FACS. Results were presented as the percentage of positive cells and MFI. Results from a representative experiment out of 3 performed are shown.