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CCR8 Is Not Essential for the Development of Inflammation in a Mouse Model of Allergic Airway Disease

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Chemokine receptors play an important role in the trafficking of various immune cell types to sites of inflammation. Several chemokine receptors are differentially expressed in Th1 and Th2 effector populations. Th2 cells selectively express CCR3, CCR4, and CCR8, which could direct their trafficking to sites of allergic inflammation. Additionally, increased expression of the CCR8 ligand, TCA-3, has been detected in affected lungs in a mouse model of asthma. In this study, CCR8-deficient mice were generated to address the biological role of CCR8 in a model of allergic airway disease. Using two different protocols of allergen challenge, we demonstrate that absence of CCR8 does not affect the development of pulmonary eosinophilia and Th2 cytokine responses. In addition, administration of anti-TCA-3-neutralizing Ab during allergen sensitization and rechallenge failed to inhibit airway allergic inflammation. These results suggest that CCR8 does not play an essential role in the pathogenesis of inflammation in this mouse model of allergic airway disease. The Journal of Immunology, 2003, 170: 581–587.
substrain. F₁ mice were generated by breeding chimeric mice to C57BL/6 females. Heterozygous and homozygous mutant animals were obtained by intercrossing males and females from the F₁ generation. The genotype of the mice was identified by PCR analysis of tail genomic DNA using the following primers: 5’(E)-GGAGGTTTGAAGGGACTCACTTG; GG(T,E)-CAGGTTCAAGGGTATATCTGTG; Neo(T) GGGGATC GATCCGTCCTGTAAAGTC. The expected sizes for the endogenous and targeting alleles were 305 and 606 bp, respectively. Eight- to twelve-week-old CCR8⁻/⁻ and wild-type (WT)² littermates from F₂ generation were used for further characterization. To confirm the absence of CCR8 message in homozygous mutant mice, RT-PCR analysis was performed on RNA prepared from thymus.

Mouse models of airway allergic inflammation

To induce airway inflammation, WT and CCR8⁻/⁻ mice were immunized with OVA using two different protocols. In protocol A, mice were immunized with i.p. injection of 100 μg of OVA in alum (1:1 in alum in 0.1 ml final volume) on days 0 and 14, as previously described (22). On days 14, 24, 25, and 26, mice were challenged intranasally with 100 μg of OVA in 0.1 ml of normal saline. In protocol B, mice were immunized on days 0 and 5 with 8 μg of OVA in alum (1:1 in alum in 0.1 ml), as described earlier (23). On day 12, mice received 100 μg of OVA in 0.1 ml of normal saline. For Ab neutralization studies, 6- to 8-wk-old BALB/c mice (D&K, Seattle, WA) were immunized according to protocol A. Fifty micrograms of anti-TCA-3 Ab or control goat Ab was administered 1 h before Ag challenge on days 14, 24, 25, and 26. All animal procedures for this part of the study were approved by the University of Washington Animal Care Committee.

Analysis of bronchoalveolar lavage (BAL) fluid

Two days after the last OVA challenge in protocols A and B, BAL fluid was collected according to a previously described protocol (22). Briefly, the left lung was ligated with a suture thread, and the right lung was lavaged three times with 0.4 ml of normal saline each. The cells were pelleted by centrifugation at 1500 × g for 5 min, and the supernatants from all three dishes were pooled and later used to quantify different Th2 cytokines. The total number of cells in the pellet was counted, and cytospin slides were stained using Diff-Quik stain set for estimating the differential counts of various cell types. A total of 200 cells were counted in each slide to calculate the percentages of eosinophils in the BAL from different groups of mice. Cytokine levels in the BAL fluid were quantified using 50 μl of BAL fluid (assayed in duplicate) using IL-4, IL-5, and IL-13 ELISA kits, according to the manufacturer’s instructions. The lower level of sensitivity for all three cytokines was 10 pg/ml. Plasma IgE levels were measured using a commercial kit (BD PharMingen, San Diego, CA). Results were expressed as mean +/− SD values for each group.

Lung histology

Left lung from WT or CCR8⁻/⁻ mice was fixed in 10% neutral buffered Formalin. Tissues were cut into 5-μm sections and stained using H&E. The number of eosinophils were counted at ×400 magnification.

Quantitative RT-PCR analysis

Total RNA from lung was prepared using the TRIzol method (Invitrogen, San Diego, CA). One-step quantitative RT-PCR was performed using real-time fluorogenic 5’-nuclease PCR using an ABI Prism 7700 Sequence BiosDector (PE Biosystems, Foster City, CA), according to the manufacturer’s instructions (TaqMan; PerkinElmer, Foster City, CA). Primers and probes for murine CCR8 and GAPDH were purchased from ABI/Applied Biosystems (Foster City, CA). Cycling conditions were 30 min at 48°C for the reverse-transcription step, and 12 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Control experiments established >95% efficiency over serial 10-fold dilutions for each of the primer pairs and probes. Reactions were set up concurrently for CCR8 and GAPDH genes, and the expression of the CCR8 gene was normalized to GAPDH abundance. The specific signals for each of the transcripts were at least 45-fold (5.5 cycles) over nonspecific background from RNA without prior reverse transcription.

Migration assay

The neutralizing ability of anti-TCA-3 Ab was tested by its ability to block TCA-3-dependent migration in a BI-CD4 T cell lymphoma cell line (kind gift from C. Miceli, University of California School of Medicine, Los Angeles, CA). Migration assays were performed using 96-well migration chambers (5 μM pore size; Neuroprobe, Gaithersburg, MD). TCA-3 (10 ng/ml) was preincubated in the presence of 10 or 100 μg/ml of anti-TCA-3 Ab for 30 min at 37°C. A total of 32 μl of ligand (preincubated in the

FIGURE 1. Expression of CCR8 in a mouse model of allergic airway inflammation, and generation of CCR8-deficient mice. BALB/c mice (n = 4 per group) were sensitized with OVA on days 0 and 14 and subsequently challenged on 3 consecutive days (days 25, 26, and 27) with the same Ag (protocol A). Lungs were collected at various time points, and CCR8 expression was determined using quantitative RT-PCR analysis. Results are presented as fold expression over unimmunized mice (A). Gene-targeting strategy to generate CCR8-deficient mice (B). Mice were screened to identify heterozygous or homozygous mutant animals using PCR-based analysis of tail genomic DNA (C). RT-PCR analysis of thymus RNA was performed to confirm the absence of CCR8 message in homozygous deficient mice (D).

² Abbreviations used in this paper: WT, wild type; BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase.
FIGURE 2. Lack of effect of CCR8 deficiency on allergic airway inflammation. WT and CCR8\(^{-/-}\) (KO) mice (\(n = 6–8\) per group) were immunized with OVA, as described in Fig. 1. BAL fluid was collected on day 29, and total cell numbers were enumerated (A), and percentages of eosinophils were calculated based on differential counts on cytospin slides (B). Plasma IgE levels were measured by ELISA (C). Data represent mean ± SD from each group of mice.

FIGURE 3. WT and CCR8\(^{-/-}\) (KO) mice (\(n = 6–8\) per group) were sensitized with OVA in alum on days 0 and 5, and challenged with a single dose of the same Ag in PBS on day 12 (protocol B). On day 14, BAL fluids were collected, and the total number of cells was determined (A). Differential counts were done on cytospin slides to calculate the percentage of eosinophils (B). EPO levels were measured from lung homogenates, as described in Materials and Methods (C).
The presence or absence of anti-TCA-3 Ab was added to the lower chamber, and the filter was assembled on the top of this chamber. Fifty microliters of cells (5 × 10^6/ml) were placed on top of the filters and incubated at 37°C for 2 h. After removing the filter, 5 μl of Alamar blue was added to each well and incubated for an additional 30 min at 37°C. The relative fluorescence was measured at 530- to 580-nm excitation wavelength and 590-nm emission wavelength. The number of cells that migrated to TCA-3 stimulation was 10–15% of the total input of cells.

Eosinophil peroxidase (EPO) assay

Whole lungs were homogenized in a buffer A containing 5% HBSS diluted in 1× PBS (pH 7.2). Samples were centrifuged at 2000 × g for 10 min, and the supernatant was discarded. The pellet was resuspended in 1 ml of RBC lysis buffer and centrifuged at 3000 × g for 5 min at 4°C. Next, the pellet was resuspended in buffer A containing 0.5% (w/v) hexadecyltrimethyl ammonium bromide and snap frozen in LN2. The samples were then thawed in a 37°C water bath. After a total of three freeze-thaw cycles, samples were centrifuged at 3000 × g for 10 min, and 50 μl of supernatant was incubated with 50 μl of substrate (1.5 mM o-phenylenediamine and 6.6 mM hydrogen peroxide in 0.05 M Tris-HCl, pH 7.5) for 10 min at room temperature. Reactions were stopped by addition of 4 M sulfuric acid, and the absorbance was read at 490 nm to determine EPO activity.

FIGURE 4. Lack of effect of CCR8 deficiency on lung inflammation. Lung sections from saline (A and B) or OVA (C and D)-challenged mice were stained with H&E (original magnification ×400). The number of eosinophils per high power field (HPF) was counted for each group of mice (n = 4–5 per group), and the results were expressed as mean ± SD.

FIGURE 5. Lack of effect of CCR8 deficiency on Th2 cytokines in BAL fluid of OVA-treated mice. WT and CCR8+/− (KO) mice (n = 6–8 per group) were sensitized with OVA according to protocol B. BAL fluid was collected from allergen-challenged mice (n = 4–5 per group). IL-4 (A), IL-5 (B), and IL-13 (C) levels were measured using ELISA. Data represent mean ± SD of each group of mice.


**Results and Discussion**

**CCR8 expression in OVA model of allergic airway inflammation**

We used an established mouse model of allergic asthma to examine the expression of CCR8 during the course of induction of an airway inflammatory response using real-time quantitative RT-PCR analysis. BALB/c mice were sensitized with OVA on days 0 and 14, and were subsequently rechallenged with the same Ag on days 24, 25, and 26 (protocol A). Very low levels of CCR8 expression were detected in nonchallenged lungs (Fig. 1A). In contrast, three consecutive rechallenges of OVA resulted in a consistent 2-fold increase in CCR8 expression in the inflamed lungs compared with lungs obtained from unchallenged mice (Fig. 1A).
Generation of CCR8-deficient mice

To understand the in vivo function of CCR8 in allergic airway inflammation, CCR8-deficient mice were generated using homologous recombination (Fig. 1, B and C). The lack of expression of CCR8 in homozygous deficient mice was confirmed by RT-PCR analysis of thymus RNA (Fig. 1D). Phenotypic analysis revealed that CCR8−/− mice were viable and indistinguishable from heterozygous or WT littermate controls. The total cell numbers and percentages of different subpopulations in the spleen, thymus, and lymph nodes were comparable between WT and CCR8−/− mice (data not shown).

Characterization of allergic inflammation in CCR8−/− mice

WT and CCR8 homozygous deficient mice were sensitized and challenged with OVA, as described above (protocol A). Forty hours after the last OVA challenge, BAL fluid was collected from CCR8−/− and WT control mice for further analysis. In contrast to saline-treated mice, Ag-challenged mice had a significant infiltration of inflammatory cells in BAL fluid, composed primarily of eosinophils (70–90% of total cells). The total number of infiltrating cells and the percentage of eosinophils in the BAL fluid were comparable in CCR8−/− mice and WT mice (Fig. 2, A and B). There was no difference in the number of lymphocytes or macrophages present in the BAL fluid between both groups of mice (data not shown). Additionally, the amount of IL-5 protein detected in the BAL fluid of allergen-challenged animals was comparable between CCR8−/− and WT mice (data not shown). Histological analysis of affected lungs revealed significant perivascular and peribronchiorial infiltration of inflammatory cells, primarily of mononuclear cells and eosinophils, which was indistinguishable between CCR8−/− and WT littermates (data not shown). In addition, there was no difference in the elevated IgE levels in response to OVA challenge between both groups of mice (Fig. 2C).

Effect of Ag dose on allergic airway inflammation in CCR8−/− mice

The dosage and timing of OVA immunization can affect the magnitude and nature of allergic airway inflammation in mouse models of asthma (24). For example, a critical role for mast cells for the development of airway hyperresponsiveness was abolished when mice were challenged with multiple doses of OVA (24). Therefore, we examined whether the requirement for CCR8 to affect the outcome of airway inflammation was masked by the dosage and timing of Ag challenge. In protocol B, mice were immunized with lower doses of Ag, as described previously (23). CCR8−/− mice and WT littermates were sensitized on days 0 and 5 with 8 µg of OVA, and later challenged with a single intranasal dose of OVA on day 12. On day 14, mice were sacrificed, and the total number of infiltrating cells in the BAL fluid was enumerated. As shown in Fig. 3, A and B, there was no significant difference in the total number of cells or percentage of eosinophils present in the BAL fluid in OVA-challenged CCR8−/− mice compared with WT mice. EPO activity was also measured in lung homogenates after allergen challenge as an additional parameter of pulmonary eosinophilia. Lung EPO levels were elevated to similar levels between WT and CCR8−/− mice (Fig. 3C). EPO activity was not detected in saline-treated animals (data not shown). Histological examination of lung sections also did not reveal any differences in the degree of lung inflammation and tissue eosinophilia between the two groups of mice (Fig. 4, A–E). Furthermore, the levels of BAL Th2 cytokines (IL-4, IL-5, and IL-13) were also similar between CCR8−/− and WT mice (Fig. 5, A–C).

Effect of TCA-3 neutralization on OVA-induced allergic inflammation

TCA-3 has been identified as the ligand for CCR8 based on its ability to bind CCR8 and induce migration of CCR8-expressing cells (12). Increased expression of TCA-3 has also been observed in the affected lungs, after adoptive transfer of Ag-specific Th2 cells to induce airway inflammation (13). To address the role of TCA-3 in allergic asthma, we examined the effect of neutralizing anti-TCA-3 Ab in the mouse asthma model. Initially, we tested the ability of anti-TCA-3 Ab to block CCR8-dependent migration under in vitro stimulation conditions. As shown in Fig. 6A, the presence of excess amounts of anti-TCA-3 Ab completely inhibited TCA-3-dependent migration.

We next examined the effect of TCA-3 neutralization on the development of allergic airway inflammation. BALB/c mice were administered with control Ab or anti-TCA-3 Ab 1 h before Ag challenge on days 14, 24, 25, and 26. As shown in Fig. 6, B and C, there was no detectable difference in the total number of infiltrating cells or percentage of eosinophils in the BAL fluid of anti-TCA-3 or control Ab-treated mice. Moreover, there was no reduction in the levels of IL-5 and IL-13 in the BAL fluid between the two groups of mice (Fig. 6, D and E). Administration of TCA-3 Ab also failed to inhibit methacholine-induced airway hyperreactivity in OVA-challenged mice as determined by whole body in vivo plethysmography (25) (data not shown).

In summary, the present study was conducted to explore the in vivo biological role of CCR8 and its ligand TCA-3, in allergic airway inflammation. Using two different protocols of Ag challenge, we were unable to demonstrate an essential role for CCR8 in the mouse model of allergen-induced airway inflammation. Although CCR8 is selectively expressed by Th2 cells and Ag-activated eosinophils, we were not able to detect any impairment in pulmonary Th2 cytokine responses and eosinophilia in CCR8−/− mice. Studies using the neutralizing anti-TCA-3 Ab also provided additional support for the lack of an important role for CCR8 in allergic airway inflammation.

When this study was in progress, Chensue et al. (21) reported that CCR8−/− mice had impaired pulmonary eosinophilia and Th2 responses in a mouse asthma model. Although we have used an OVA immunization protocol (protocol B) and mouse strain background (C57Bl/6×129/OlaHsd Fl) similar to their report, no defect was observed in the development of allergic airway inflammation in CCR8−/− mice. At present, we are unable to resolve this discrepancy. Although CCR8-positive T cells have been localized in the lungs from asthma patients, it is not clear whether this population represents the pool of Th2 cells (15). It is possible that other receptors involved in Th2 cell migration such as CCR3, CCR4, or CRTh2 could compensate for the absence of CCR8 in promoting recruitment of Th2 cells to sites of allergic inflammation. Future studies are necessary to simultaneously disrupt the function of these receptors to elucidate the mechanisms that drive Th2-mediated inflammation in asthma and allergy.

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References


