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Absence of CCR8 Does Not Impair the Response to Ovalbumin-Induced Allergic Airway Disease[1]

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Interaction of chemokines with their specific receptors results in tight control of leukocyte migration and positioning. CCR8 is a chemokine receptor expressed mainly in $CD4^+$ single-positive thymocytes and Th2 cells. We generated CCR8-deficient mice ($CCR8^{-/-}$) to study the in vivo role of this receptor, and describe in this study the $CCR8^{-/-}$ mouse response in OVA-induced allergic airway disease using several models, including an adoptive transfer model and receptor-blocking experiments. All $CCR8^{-/-}$ mice developed a pathological response similar to that of wild-type animals with respect to bronchoalveolar lavage cell composition, peripheral blood and bone marrow eosinophilia, lung infiltrates, and Th2 cytokine levels in lung and serum. The results contrast with a recent report using one of the OVA-induced asthma models studied here. Similar immune responses were also observed in $CCR8^{-/-}$ and wild-type animals in a different model of ragweed allergen-induced peritoneal eosinophilic inflammation, with an equivalent number of eosinophils and analogous increased levels of Th2 cytokines in peritoneum and peripheral blood. Our results show that allergic diseases course without critical CCR8 participation, and suggest that further work is needed to unravel the in vivo role of CCR8 in Th2-mediated pathologies. *The Journal of Immunology,* **2003, 170: 2138–2146.**

The complex trafficking pathways that characterize a leukocyte subpopulation throughout its life stages are tightly controlled by chemokines, which bind and activate specifically seven transmembrane receptors expressed in their target cells (1–6). Chemokine control of leukocyte movement is also exerted in pathological settings, and has an important effect on cell recruitment to inflammation sites (7). Following interaction with Ags, T cells differ in their activation and polarization states. T cell polarization to Th1 and Th2 cells constitutes a paradigm of effector T cell responses (8); Th1 and Th2 cells are distinguished by the distinct cytokines they produce and the different types of protective or pathogenic responses they mediate. Th1 cells produce IFN-γ and participate in host defense against pathogens, whereas Th2 cells are IL-4 and IL-5 producers and are associated to allergic reactions (8). Chemokine receptors are also differentially expressed in Th cells, as CCR1, CCR5, and CXCR3 are preferentially expressed by Th1 cells, whereas Th2 cells express CCR3, CCR4, and CCR8 (9–15).

CCR8 is the receptor for C-C chemokine ligand (CCL)[3] 1 (human I-309, murine T cell-activated gene 3 (TCA3)) (15–18). Expression of the CCR8 message is maximal in the thymus (15, 18–20), in which the CCR8 protein shows finely regulated expression that increases throughout differentiation of the $CD4^+$ lineage (21). This regulated expression suggests that CCR8, in addition to its role in Th2 cells, may have another role in thymocyte maturation. Human CCL1/I-309 also has angiogenic activity in both in vitro and in vivo assays (22, 23).

To study the in vivo function of CCR8, we generated mice deficient for this chemokine receptor ($CCR8^{-/-}$). In this study, we report data obtained from a study of the $CCR8^{-/-}$ mouse response in different models of OVA-induced allergic airway disease, a Th2-mediated pathology widely used as an asthma model (24). Our analysis includes two models that differ in OVA dose and administration protocol, as well as an adoptive transfer model, and blocking experiments with an anti-mouse CCR8 neutralizing mAb. The results show that development of OVA-induced disease in $CCR8^{-/-}$ mice did not differ significantly from that observed in wild-type (WT) animals in any case studied. This contrasts with recently published data (25) using one of the OVA models analyzed in this study. The behavior of $CCR8^{-/-}$ mice in a model of ragweed allergen-induced peritoneal eosinophilic inflammation was also studied; again, a similar number of eosinophils was observed in the peritoneum and peripheral blood of $CCR8^{-/-}$ and WT animals. All together, our data suggest that targeting CCR8 is not sufficient to critically alter the development of allergic pathologies in vivo, and that other chemokine receptor(s) may act in a compensatory fashion.

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[3] Abbreviations used in this paper: CCL, C-C chemokine ligand; TCA3, T cell-activated gene 3; WT, wild type; ES, embryonic stem; SP, single positive; BAL, bronchoalveolar lavage; LN, lymph node.

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Materials and Methods

Gene targeting

Gene targeting was performed according to established methods (26). The genomic clone λ301 (18), which contains a 14-kbp genomic DNA fragment spanning the CCR8 coding sequence and flanking regions, was used as starting material to subclone 3-kb *Xho*I-*Bam*HI and 3.5-kb *Bam*HI-*Bam*HI DNA fragments from the CCR8 gene 5′ and 3′ regions, respectively. These DNA fragments were then subcloned at either end of a neomycin resistance gene, under the control of the phosphoglycerate kinase promoter. The *Herpes simplex* thymidine kinase gene was also fused at the 5′ end of the cloned CCR8 sequences in the replacement targeting construct. The resulting plasmid was linearized by *Not*I digestion and electroporated into the 129 SvJ (27) embryonic stem (ES) cell line. A total of 249 gancyclovir- and G418-resistant clones were selected, and 15 μg of genomic DNA from each clone were *Kpn*I-*Xba*I digested, subjected to gel electrophoresis, and blotted onto Hybond-N^+ membranes (Amersham Pharmacia Biotech, Piscataway, NJ). High stringency hybridizations with CCR8-specific ^{32}P-labeled probes were performed in Rapid-Hyb buffer (Amersham Pharmacia Biotech). To produce chimeric mice, four independent correctly targeted ES clones were injected individually into CD1 morulae, which were transferred to pseudopregnant CD1 females as described (26). Chimeric males were bred to C57BL/6 females; offspring genotype was analyzed by Southern blotting and PCR with specific primers. The null phenotype was confirmed by RT-PCR analysis of CCR8 transcripts in total thymocyte samples and by flow cytometric analysis of CCR8 protein expression in $CD4^+$ single-positive (SP) thymocytes. Age- and sex-matched 10- to 12-wk-old 129 SvJ $CCR8^{-/-}$ animals that had been backcrossed onto the C57BL/6 genetic background three (F_3), four (F_4), or five (F_5) times were used in this study; WT 129 SvJ × C57BL/6 F_3, F_4, or F_5 $CCR8^{+/+}$ littermates were used as controls. The genotype of each animal included in the study was verified by a standard PCR procedure using tail DNA.

CCR8 activity assays

The response of WT and $CCR8^{-/-}$ CD8-depleted thymocytes to mouse CCL1 was analyzed in adhesion and migration assays. Adhesion assays were performed on heparan sulfate-coated 24-well plates (Costar, Cambridge, MA). Cells (10^6/ml) were resuspended in RPMI 1640 supplemented with 20 nM HEPES and 0.75% BSA. At $t = 0$, cells and CCL1, prepared in the same medium, were mixed; 600-μl aliquots were dispensed into wells and incubated (20 min, 37°C). After incubation, 600 μl were recovered from each well and cells were counted in an EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL) to establish the nonadherent fraction. Adhered cells were recovered by incubating wells with EDTA (15 min, 37°C) and were counted similarly.

Migration assays were performed in fibronectin-coated 5-μm pore Transwell inserts (Costar). Thymocytes were resuspended in RPMI 1640 with 1% BSA and 25 mM HEPES (10^7 cells/ml), and 100-μl aliquots were loaded into upper inserts. Aliquots (600 μl) of mouse CCL1, prepared in the same medium, were placed in lower wells. After incubation (2 h, 37°C), inserts were removed and migrated cells were counted in the flow cytometer (Beckman Coulter). Two or more replicate wells were used for each point in both assays. Adhesion and migration indices were established by the following ratio: cells induced to adhere or migrate by CCL1 to cells induced to adhere or migrate by buffer.

Flow cytometry analysis

The following rat mAbs were used: protein G-purified anti-mouse CCR8 mAb 8F4 (21), FITC-anti-mouse CD4 (clone RM4-5; BD PharMingen, San Diego, CA), Spectral Red-labeled-anti-mouse CD8 (clone 53-6.7; Southern Biotechnology Associates, Birmingham, AL), and PE-anti-mouse B220 (clone RA3.6B2; Southern Biotechnology Associates). Cell staining and flow cytometry were conducted in an EPICS XL flow cytometer (Beckman Coulter) according to standard protocols.

OVA-induced allergic airway disease: multichallenge model

$CCR8^{-/-}$ and WT mice (10- to 12-wk-old) were immunized with OVA as follows: on day 0, 15 μg of OVA (Sigma-Aldrich, St. Louis, MO) in 100 μl of PBS were mixed with 100 μl of alum (Pierce, Rockford, IL) according to supplier's instructions, and injected i.p. to sensitize mice. On days 14–18, animals were placed in a metacrylate box (35 × 28 × 15 cm) and challenged for 25 min with aerosolized 1.5% OVA in PBS; control animals received aerosolized PBS. Mice were sacrificed for analysis on days 15–19, 16 h after challenge. To study the effect of the rat anti-mouse CCR8 neutralizing mAb 8F4; in some experiments, WT mice received daily i.p. injections of 200 μg of 8F4 in 400 μl of PBS, or a control isotype-matched irrelevant rat mAb (clone A95-1; BD PharMingen) on days 14–18, 4 h before OVA challenge.

OVA-induced allergic airway disease: adoptive transfer model

WT and $CCR8^{-/-}$ mice were immunized i.p. with 10 μg of OVA in alum. Five days later, spleens were removed, $CD4^+$ T cells were purified and cultured in complete RPMI 1640 with OVA (10 μg/ml) and mitomycin C-treated splenocytes at a 1:5 ratio. These cultures were established in polarizing conditions to generate Th1 (IL-12 (10 ng/ml) and anti-IL-4 mAb (11B11; 40 μg/ml, R&D Systems, Minneapolis, MN)) or Th2 cells (IL-4 (10 ng/ml) and anti-IL-12 mAb (TOSH 2; 3 μg/ml, Endogen, Woburn, MA)). After 5 days, cells were washed, purified using a Ficoll gradient, and resuspended in PBS. Naive C57BL/6 mice received 2 × 10^6 Th1 or Th2 cells i.v. and, 24 h later, mice were exposed to a daily OVA aerosol (50 mg/ml, 20 min) for 7 consecutive days. At 48 h after the last challenge, bronchoalveolar lavage (BAL) was performed and infiltrating leukocytes in the BAL fluid assessed as described below.

OVA-induced allergic airway disease: two-challenge model

$CCR8^{-/-}$ and control WT mice (10- to 12-wk-old) were OVA immunized as described (25). Briefly, on day 0, 15 μg of OVA in 200 μl of alum were injected i.p. to sensitize mice. On day 5, the animals received another i.p. injection of 15 μg of OVA in 200 μl of alum and, on day 12, were challenged with aerosolized 0.5% OVA in PBS (2 challenges of 60 min each, 4 h apart). Control animals were aerosolized with PBS. On day 14, 40 h after the second OVA challenge, mice were sacrificed for analysis.

Mouse model of peritoneal eosinophilic inflammation

$CCR8^{-/-}$ and control WT mice (10- to 12-wk-old) were immunized with a ragweed allergen essentially as described (28). Briefly, mice received a series of five s.c. injections of a 1/1000 dilution of a ragweed pollen extract (Stallergenes, Antony, France) on days 0 and 1 (100 μl), and days 6, 8, and 14 (200 μl). On day 20, mice were challenged by i.p. injection (200 μl) of ragweed allergen extract; control animals received 200 μl of PBS. At 48 h after challenge, mice were sacrificed for analysis.

Analysis of leukocyte subpopulations in BAL, peritoneum, peripheral blood, and bone marrow

Lungs from sacrificed mice were lavaged three times through a tracheal cannula with 800 μl of RPMI 1640 (Life Technologies, Paisley, U.K.), supplemented with 50 μM 2-ME, 10% FCS, and antibiotics (comRPMI); cells in the peritoneal cavity were collected by washing the peritoneum twice with 5 ml of the same medium. Combined BAL or peritoneal fluid was centrifuged (5 min, 200 × *g*, 4°C), cells were washed in PBS, resuspended in 1 ml PBS, and counted on a hemocytometer. To analyze BAL cell composition and peritoneal fluid eosinophilia, aliquots of 2 × 10^5 cells in 600 μl of PBS were applied to glass slides by cytocentrifugation (3 min, 80 × *g*, room temperature), air-dried for 2 h, and differentially stained with Diff-Quik (Dade Behring, Düdingen, Switzerland). Proportions of lymphocytes, neutrophils, eosinophils, and macrophages were established by counting at least 600 cells per sample in eight different fields. Before sacrifice, mice were bled from the retro-orbital plexus; blood smears were prepared on glass slides, air-dried for 1 h, and Diff-Quik-stained. Leukocyte subpopulations were determined as for BAL, examining the complete smear preparation. Bone marrow was obtained by injecting comRPMI into mouse femurs, and processed as described for BAL to determine the eosinophil to neutrophil ratio. All samples were analyzed in a blind fashion.

Cytokine and chemokine analysis

Real-time PCR analysis of cytokine, CCL1/TCA3 and chemokine receptor levels was performed essentially as described (29). Briefly, snap-frozen lung lobes or peritoneal cells were dispersed in Tri-reagent (Sigma-Aldrich), and total cellular RNA was extracted. Five micrograms of total RNA were reverse-transcribed using random hexamers and 100 U of Superscript II RT (Life Technologies). Real-time PCR was performed with a LightCycler (Roche, Mannheim, Germany) or an ABI PRISM 7700 (Applied Biosystems, Foster City, CA), using FastStart DNA Master SYBR Green I mix (Roche) or SYBR Green PCR Core Reagents (Applied Biosystems), respectively. Final $MgCl_2$ concentration was 4.5 μM and primers were used at 0.4 μM. Reactions were incubated 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 25 s at 68°C, and 5 s at 80°C in the LightCycler, or 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 90 s at 67°C, in the ABI PRISM 7700. The corresponding specific primers were designed to amplify sequences spanning different exons, except for the 28S rRNA pair. For each mRNA, relative expression was determined using

normalized standard curves. Fluorescence values obtained with cDNA from a randomly chosen CCR8$^{+/+}$ Ag-treated mouse were used as reference values and assigned 100 arbitrary units. Fluorescence of cDNAs from the remaining animals were converted to arbitrary units by comparison with the reference values, and mean ± SD for each animal group was calculated. Differences in cDNA load were corrected by the amount of amplified 28S rRNA. When indicated, mRNA expression was also given as ΔCt values, which express the cycle threshold difference between the indicated primer pair and the 28S rRNA pair (30). IL-5 content in mouse serum was analyzed using the mouse IL-5 ELISA kit (Endogen, Woburn, MA), as recommended by the supplier.

Immunohistochemistry

Lungs from PBS- or OVA-treated mice were inflated through the trachea with 50% Jung tissue freezing medium (Leica, Nussloch, Germany) in PBS. The apex of the right caudal lobe and the medial part of the right cephalic lobe were trimmed, embedded, frozen, and stored at −80°C. Ten-micrometer cryosections were dried and fixed in 4% PBS-buffered formaldehyde for H&E staining or in cold acetone for immunohistochemistry. For immunohistochemical studies, sections were blocked with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) and incubated in 5% normal goat serum with the following biotinylated Abs: F4/80 (Southern Biotechnology Associates), anti-CD11b, -CD11c, -CD4, -CD8, -CD54, and -CD106 (BD PharMingen). Vectastain ABC or ABC-AP kits (Vector Laboratories) were used to visualize these Abs, with the exception of F4/80, which was visualized with streptavidin-Cy3 (Amersham Pharmacia Biotech). The rat anti-mouse CCR8 mAb 8F4 (21) and the isotype-matched control rat mAb (BD PharMingen) used in neutralization experiments were detected with anti-rat HRP (DAKO, Glostrup, Denmark) in acetone-fixed lung cryosections blocked with 0.3% H_2O_2 in TBS and 20% normal goat serum. Anti hamster-HRP (DAKO) was used as a control.

Results

Generation of CCR8$^{-/-}$ mice

The homologous recombination strategy used to delete mouse CCR8 eliminated the 508 bp preceding the initial ATG and the first 658 bp within the coding sequence (Fig. 1*A*). Deletion was confirmed by Southern blot analysis of DNA from correctly targeted ES clones (Fig. 1*B*); in addition, RT-PCR analysis of total RNA from thymus (Fig. 1*C*) and flow cytometric analysis of mouse CD4$^+$ SP thymocytes (Fig. 1*D*) confirmed the lack of CCR8 mRNA and protein in CCR8$^{-/-}$ animals. Consistent with this, CD8-depleted CCR8$^{-/-}$ thymocytes were unable to respond to mouse CCL1 in cell adhesion (Fig. 1*E*) or migration assays (Fig. 1*F*). Mice maintained under barrier isolation were healthy and bred according to Mendelian inheritance patterns. Flow cytometric analysis was performed of CD4$^+$, CD8$^+$, and B220$^+$ lymphocyte subpopulations from several tissues including thymus, lymph node (LN), spleen, and peripheral blood, and no significant differences were observed between CCR8$^{-/-}$ and WT mice (not shown).

Similar responses of WT and CCR8$^{-/-}$ mice in a model of OVA-induced allergic airway disease

As CCR8 expression has been associated to Th2 cells (14, 15), we studied the effect of in vivo CCR8 deletion in an OVA-induced allergic airway disease model, which generates a predominantly Th2 response. Animals were sensitized to OVA, then challenged with aerosolized Ag or PBS, as described in *Materials and Methods* (Fig. 2*A*). Analysis of inflammatory cell recruitment in BAL of PBS-challenged WT and CCR8$^{-/-}$ mice on day 19 showed that macrophages predominated, with no differences between WT and CCR8$^{-/-}$ mice (Fig. 2*A*). The situation was markedly different in OVA-sensitized and -challenged animals; a clear increase was observed in total cell number, due to increases in neutrophils, lymphocytes, macrophages and especially, eosinophils. WT and CCR8$^{-/-}$ mice nonetheless showed similar cell numbers, with no significant differences. These results suggest that CCR8$^{-/-}$ animals have a normal complement of resident BAL cells, and are able to recruit inflammatory cells to the same extent, quantitatively and qualitatively, as WT mice.

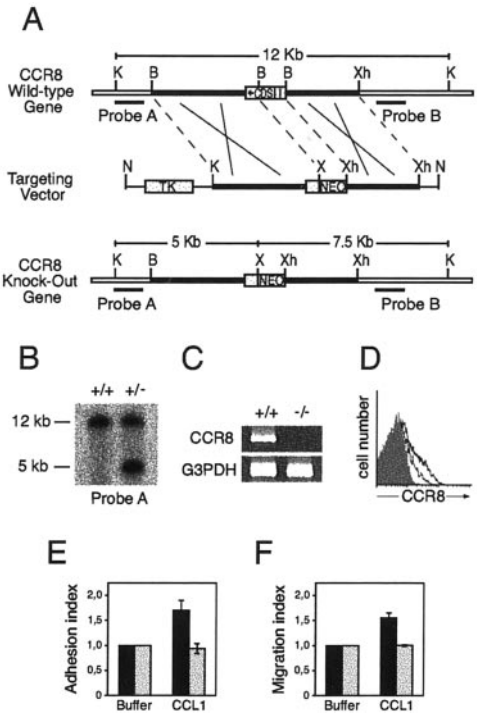

FIGURE 1. Generation of CCR8$^{-/-}$ mice. *A*, CCR8 WT locus with partial restriction map, targeting vector, and strategy. The CCR8 coding sequence (CDS) and intron (I), the thymidine kinase (TK), and neomycin-resistance (NEO) genes are shown as open boxes. Thick black bars show probes A and B, used in Southern blot analysis of genomic DNA for screening purposes. Restriction enzymes are B, *Bam*HI; K, *Kpn*I; N, *Not*I; X, *Xba*I; Xh, *Xho*I. *B*, Representative Southern blot analysis of *Kpn*I- and *Xba*I-digested DNA from a WT (+/+) and a correctly targeted, heterozygous (+/−) ES cell, using probe A. *C*, RT-PCR analysis of CCR8 mRNA expression in total thymocyte samples from CCR8 WT (+/+) and knockout (−/−) mice. *D*, Flow cytometric analysis of CCR8 protein expression in CD4 SP thymocytes from CCR8 WT (+/+, bold line histogram), heterozygous (+/−, dashed line), and homozygous (−/−, gray histogram) animals. The gray histogram is also representative of the results obtained with an isotype-matched irrelevant rat mAb. CD8-depleted, CCR8-deficient thymocytes (▨) do not respond to 10 nM mouse CCL1 in cell adhesion (*E*) or 0.5 nM mouse CCL1 in migration (*F*) assays. ■, Values for positive controls.

Analysis of peripheral blood cells on day 19 showed that the main difference between control and OVA-treated mice was the clear increase in eosinophils in the latter animals, but again the effect was similar in WT and CCR8$^{-/-}$ mice (Fig. 2*A*). In addition, day 19 lung sections from control and OVA-treated animals were prepared and H&E-stained to study OVA-induced histological changes. Consistent with the lack of differences observed in BAL and peripheral blood, the results showed the presence of similar peribronchial and perivascular infiltrates in OVA-treated CCR8$^{+/+}$ and CCR8$^{-/-}$ animals, which were not present in the lungs of control animals (Fig. 2*B*). Lung sections stained to detect macrophages (F4/80$^+$), granulocytes (CD11b$^+$), dendritic cells (CD11c$^+$), and lymphocytes (CD4$^+$ and CD8$^+$) revealed no significant differences between CCR8$^{+/+}$ and CCR8$^{-/-}$ animals, as was also the case when sections were stained to visualize expression of ICAM-1 and VCAM-1, two adhesion molecules important for eosinophil rolling and firm adhesion (28, 31) (not shown).

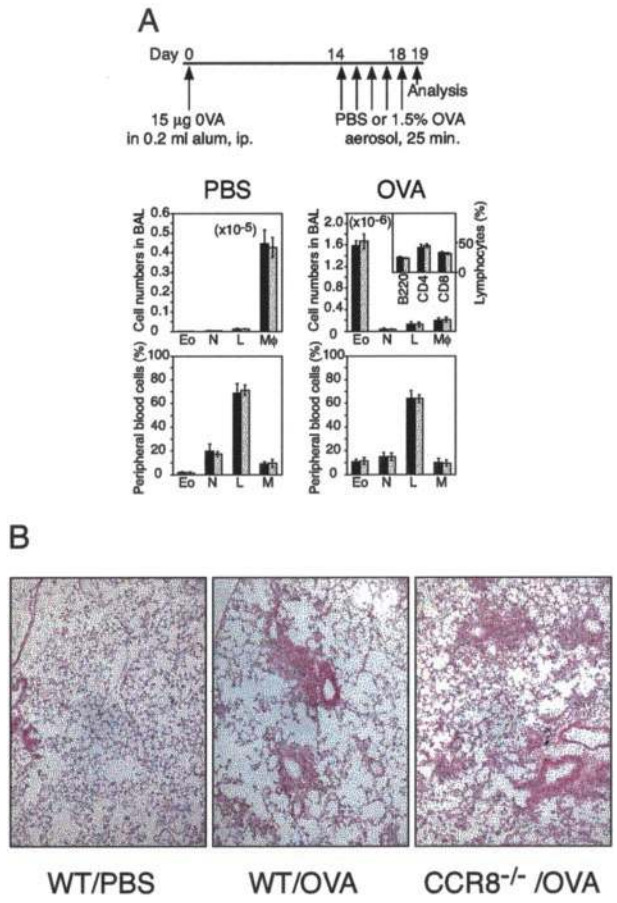

FIGURE 2. Analogous responses of CCR8$^{+/+}$ and CCR8$^{-/-}$ mice in OVA-induced allergic airway disease. CCR8$^{+/+}$ (n = 8 PBS, n = 27 OVA, ■) and CCR8$^{-/-}$ (n = 8 PBS, n = 27 OVA, ▨) mice were OVA-sensitized, then challenged with PBS or OVA following the protocol in *A*. On day 19, animals were sacrificed and leukocyte subsets in peripheral blood and BAL were analyzed. Eosinophils (Eo), neutrophils (N), lymphocytes (L), monocytes (M), and macrophages (Mϕ). Analysis is also shown of lymphocyte subpopulations in BAL from OVA-treated mice (*inset*). Bars represent mean ± SD of results obtained in one of two experiments performed. *B*, Representative H&E staining of day 19 lung sections from mice with the indicated genotypes and treatments, showing similar peribronchial and perivascular infiltrates in OVA-treated CCR8$^{+/+}$ and CCR8$^{-/-}$ animals. Original magnification, ×10. The results observed in PBS-treated CCR8$^{+/+}$ mice, showing lack of infiltrating leukocytes, are also representative of PBS-treated CCR8$^{-/-}$ mice.

As Th2 cytokines are required for pulmonary eosinophilia (32, 33), we performed real-time quantitative RT-PCR analysis of cytokine expression in the lung on day 19 (Table I). Messenger RNA levels of Th2 cytokines such as IL-4, IL-5, and IL-13 were clearly and similarly increased in the lungs of OVA-treated WT and CCR8$^{-/-}$ mice, compared with the cytokine levels in control animal lungs. Conversely, the mRNA levels of IFN-γ, a Th1 cytokine, were decreased 3-fold in OVA-treated lungs. We also analyzed lung expression of CCR8 and its ligand, CCL1/TCA3. CCL1/TCA3 mRNA levels were increased ~20-fold in the lungs of OVA-treated animals, with no significant differences between WT and CCR8$^{-/-}$ mice (Table I). For CCR8, a 4-fold increase in mRNA levels was detected in OVA-treated WT mice compared with WT control lungs. Results of analysis of serum IL-5 levels were consistent with observations in lung. OVA-treated mice showed increased serum IL-5 levels compared with those of untreated control animals, with no significant difference between WT and CCR8$^{-/-}$ mice (Table I). Real-time quantitative RT-PCR studies of BAL cells from OVA-treated WT animals, and flow cytometric analysis of these cells with gating in either eosinophil or lymphocyte subsets showed that CCR8 expression was associated to a CD4$^+$ T cell subpopulation and not to eosinophils (not shown). In vitro proliferation assays in which mediastinal LN cells from OVA-treated WT and CCR8$^{-/-}$ mice were OVA-stimulated yielded similar results in both animal groups, suggesting that both mouse types developed similar numbers of reactive T cell species (not shown).

Parallel time-course of OVA-induced effects in CCR8$^{+/+}$ and CCR8$^{-/-}$ mice

It was possible that WT and CCR8$^{-/-}$ mouse responses to OVA differed in early phases of the treatment, which were no longer detectable by day 19. To address this question, similar experiments were performed, and groups of WT and CCR8$^{-/-}$ mice (n = 6/group) were analyzed daily on days 15–19, 16 h after OVA challenge. Analysis of BAL cells showed that with the increasing number of OVA challenges, eosinophil numbers were augmented greatly; increases were also observed in the number of neutrophils, macrophages and lymphocytes (Fig. 3*A*). Changes in BAL cell composition were nonetheless similar in WT and CCR8$^{-/-}$ mice. The number of eosinophils in peripheral blood increased throughout OVA treatment, with no differences between WT and CCR8$^{-/-}$ mice (Fig. 3*B*). This is consistent with the increased eosinophil generation found in bone marrow as a consequence of repeated OVA challenges (Fig. 3*C*). In addition, sections were prepared from lungs of animals sacrificed on days 15–19, and stained to analyze the tissue infiltrates. Increasing perivascular and peribronchial infiltrates were observed throughout treatment, with predominant increases in eosinophils (Fig. 3*D*). Among the infiltrating lymphocytes, B and T cells, the latter mainly CD4$^+$ cells, were observed (not shown). Consistent with the earlier analyses performed, immunohistochemistry confirmed that WT and CCR8$^{-/-}$ mice responded similarly to OVA-induced allergic airway inflammation.

Table I. *Analysis of cytokine, CCL1, and CCR8 expression in day 19 mouse lungs and sera*[a]

Genotype/Treatment	IL-4 (L)	IL-5 (L)	IL-5 (B)	IL-13 (L)	IFN-γ (L)	CCL1 (L)	CCR8 (L)
CCR8$^{+/+}$/PBS	17.96 ± 5.74	21.48 ± 19.40	24.61 ± 1.52	10.65 ± 5.17	326.87 ± 56.98	4.41 ± 1.40	25.38 ± 5.82
CCR8$^{-/-}$/PBS	13.25 ± 5.48	18.14 ± 9.06	24.78 ± 2.81	8.45 ± 4.63	309.35 ± 93.11	5.01 ± 1.30	
CCR8$^{+/+}$/OVA	114.30 ± 43.81	81.37 ± 47.40	117.59 ± 34.25	74.71 ± 23.33	96.22 ± 50.14	124.46 ± 65.86	90.88 ± 22.22
CCR8$^{-/-}$/OVA	115.40 ± 31.92	110.68 ± 50.19	109.93 ± 56.51	80.47 ± 35.77	113.66 ± 27.72	105.65 ± 36.53	

[a] Mice were OVA-sensitized, then challenged with either aerosolized PBS or OVA (*Materials and Methods*). On day 19, lungs (L) and blood (B) were collected. RNA (lung) and sera (blood) were prepared for RT-PCR or ELISA analysis, respectively. Values in arbitrary units (RT-PCR) or picograms per milliliter (ELISA) are mean ± SD from 8–10 individual animals in each group.

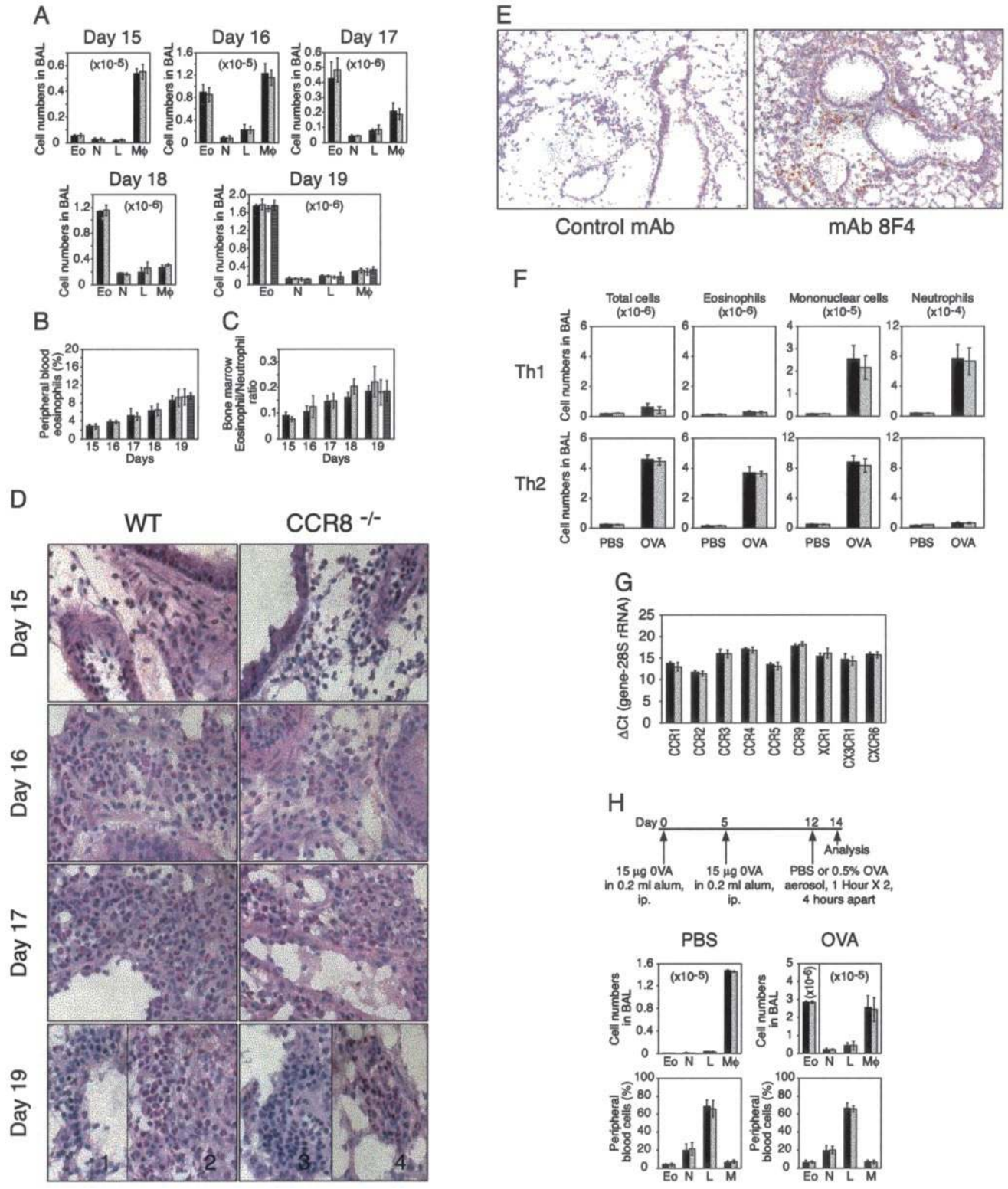

FIGURE 3. CCR8 deficiency has no significant effect in the response to allergen challenge in the lung or peritoneum. *A*, Parallel time-course development of OVA-induced allergic airway disease in CCR8$^{+/+}$ and CCR8$^{-/-}$ mice. Mice were OVA-sensitized on day 0 and challenged with aerosolized OVA on days 14–18. On days 15–19, 16 h after each OVA challenge, groups of CCR8$^{+/+}$ (n = 6, ■ throughout the figure) and CCR8$^{-/-}$ (n = 6, ▨ throughout the figure) mice were sacrificed and analyzed. Results are also shown on day 19 for OVA-treated WT mice receiving daily injections of rat anti-mouse CCR8 neutralizing mAb 8F4 (n = 8, □), or an isotype-matched irrelevant rat mAb (n = 4, ▤). BAL fluids were prepared and the number of eosinophils (Eo), neutrophils (N), lymphocytes (L), and macrophages (Mϕ) established using standard morphological criteria. *B*, Similar eosinophil increases in CCR8$^{+/+}$ and CCR8$^{-/-}$ mice. Mice were bled from the retro-orbital plexus on days 15–19 and the percentage of eosinophils in peripheral blood was determined (*Materials and Methods*). *C*, Similar eosinophil-neutrophil ratio in CCR8$^{+/+}$ and CCR8$^{-/-}$ bone marrow. Bone marrow was obtained from mouse femurs on days 15–19 and the number of neutrophils and eosinophils was evaluated. *D*, CCR8$^{+/+}$ and CCR8$^{-/-}$ mice develop similar lung infiltrates. On the days indicated, lung sections were prepared and H&E-stained. Both animal groups developed similar lung infiltrates, which also showed parallel progression with the increasing number of OVA challenges. Increasing eosinophil-rich infiltrates were observed on (*Figure legend continues*)

Anti-CCR8 neutralizing mAb 8F4 did not attenuate the response to OVA in WT mice

Despite the similar responses in WT and CCR8$^{-/-}$ mice, increases in CCL1/TCA3 and CCR8 expression were observed in OVA-treated WT mouse lung (Table I). We thus performed CCR8 blocking experiments to study the role of this receptor in the response to OVA in an alternative manner, using the rat anti-mouse CCR8 neutralizing mAb 8F4 (21). mAb 8F4 was injected i.p. in mice on days 14–18, 4 h before Ag challenge, and the cell profile of BAL fluid was analyzed on day 19. No significant differences were detected compared with the profile of mice treated with an isotype-matched irrelevant rat mAb, or those of WT or CCR8$^{-/-}$ animals to which no Ab had been administered (Fig. 3*A*). Peripheral blood eosinophilia was also analyzed (Fig. 3*B*), as was the eosinophil/neutrophil ratio in bone marrow (Fig. 3*C*); again, similar results were observed in the different animal groups. Staining of lung sections confirmed that mAb 8F4 did not provoke a differential effect (not shown). Some lung sections were stained with an anti-rat IgG, confirming the presence of 8F4 in infiltrating cells in the lungs (Fig. 3*E*). Although we cannot formally exclude the possibility that in vivo the CCR8-neutralizing mAb does not block the receptor on lung Th2 cells, taken together our results strongly suggest that absence of CCR8 activity does not impair the OVA-induced allergic airway response in the models studied.

Th2 cells from WT and CCR8$^{-/-}$ mice induce a similar OVA-dependent allergic airway pathology using an adoptive transfer model

To assess the role of CCR8 in Th2 cell-mediated inflammation more directly, we used an adoptive transfer model in which Ag-specific Th1 or Th2 cells are generated in vitro, then transferred back to a naive animal. CCR8$^{-/-}$ and WT mice were immunized with OVA (*Materials and Methods*). Five days after immunization, CD4$^+$ T cells were isolated from LN and spleen, and polarized for 4 days to Th1 or Th2 cells in the presence of APC and OVA. The Th1 or Th2 cells thus obtained were transferred (2×10^6 cells/mouse) to WT recipients, which were challenged daily for 7 days with OVA or control PBS. Two days after the last challenge, animals were sacrificed for analysis. In agreement with previous studies (34), allergen exposure of WT Th1-recipient animals resulted in greater neutrophilic inflammation than that observed in WT Th2-recipient animals, which developed an eosinophilic mucosal inflammatory response following OVA challenge. Neither Th1- nor Th2-mediated inflammation was modified in mice that received CCR8$^{-/-}$ Th1 or Th2 cell populations, as assessed by analysis of the BAL fluid (Fig. 3*F*) or by histology in the airway tissue (not shown).

Chemokine receptor expression during the allergic response

The mouse *CCR8* gene is located on chromosome 9 in the proximity of CCR4 and CX3CR1, near the chemokine receptor cluster formed by CCR9, CXCR6, XCR1, CCR1, CCR3, CCR2, and CCR5. As some of these receptors are reported to play a role in asthma, we used a real-time quantitative RT-PCR procedure to analyze their expression in day 19 lungs of OVA-treated mice. The results showed that, in CCR8$^{-/-}$ animals, the expression level of all chemokine receptors neighboring the CCR8 locus was similar to that of the corresponding WT animals (Fig. 3*G*). Expression of CCR3 and CCR4, two Th2 cell-associated chemokine receptors, was ~10- and ~3-fold higher, respectively, in the lungs of OVA-treated mice compared with that in lungs of PBS-treated controls, with no differences between WT and CCR8$^{-/-}$ mice (not shown). These results suggest that CCR8$^{-/-}$ mice do not show defective expression of other chemokine receptors, and appear to rule out possible local alterations in other chemokine receptor loci caused when deleting the *CCR8* gene to generate our mouse strain.

Analogous responses of WT and CCR8$^{-/-}$ mice in a two-challenge model of OVA-induced allergic airway disease

Chensue et al. (25) recently reported that CCR8$^{-/-}$ mice have reduced pulmonary eosinophilic infiltrates after sensitization and inhalation of aerosolized OVA. We applied this model to our mice to determine whether the difference in results was due to the distinct models studied. According to the model used by Chensue et al. (25), WT and CCR8$^{-/-}$ animals were OVA-sensitized twice on days 0 and 5, challenged with aerosolized OVA or PBS twice on day 12, and analyzed 40 h after the last challenge (Fig. 3*H*). When BAL cell composition from control and OVA-challenged mice was compared, the latter showed greater numbers of cells, with increases in macrophages, neutrophils, lymphocytes, and especially in eosinophils (Fig. 3*H*). In contrast to the results of Chensue et al. (25), the response of WT and CCR8$^{-/-}$ mice was nonetheless remarkably similar. Consistent with these data, analysis of peripheral blood showed analogous numbers of eosinophils in WT and CCR8$^{-/-}$ mice, which were 2-fold higher in animals receiving OVA at day 12 compared with eosinophils in control animal blood.

Mouse model of peritoneal eosinophilic inflammation

We further examined eosinophil production and infiltration in our CCR8$^{-/-}$ mice in a different mouse model of allergic disease, using a peritoneal eosinophilic inflammation model. CCR8$^{-/-}$ and control WT mice were immunized repeatedly with ragweed pollen extract, then challenged with an i.p. injection of the same allergen following the protocol described (28) (Fig. 4). Mice were sacrificed for analysis on day 22, 48 h after the last challenge. Allergen-challenged animals had greater numbers of circulating blood eosinophils than did control animals (Fig. 4). This was also observed in peritoneal lavages, in which eosinophils recovered from the peritoneum of challenged animals constituted 18–20% of total cells, and their numbers were 10-fold higher than the eosinophils recovered from control animals (Fig. 4). As in the previous models

days 15–19. Examples of day 19 lymphocyte- (1, WT mice, and 3, CCR8$^{-/-}$ animals) and eosinophil-rich (2, WT mice, and 4, CCR8$^{-/-}$ animals) OVA-treated lung infiltrates are shown. *E*, Lung delivery of rat anti-mouse CCR8 neutralizing mAb 8F4. CCR8 blocking experiments were performed as described (*Materials and Methods*). On day 19, lung sections were prepared and stained with an anti-rat IgG to show the presence of the 8F4 mAb in the lungs. *F*, Th2 cells from WT and CCR8$^{-/-}$ mice ($n = 5–6$ per group) induce comparable eosinophilic inflammation of the airways. Ag-specific Th1 or Th2 cells from WT and CCR8$^{-/-}$ mice were adoptively transferred to naive recipient animals that were then exposed to PBS or OVA aerosol for 7 consecutive days. At 48 h after the last aerosol challenge, BAL fluid was obtained and leukocyte subsets were evaluated. *G*, Chemokine receptor expression during the allergic response. A real-time quantitative RT-PCR procedure was used to analyze the expression of all chemokine receptors neighboring the CCR8 chromosome locus in day 19 lungs of OVA-treated mice. In all cases, the expression level detected in WT and CCR8$^{-/-}$ animals was similar. *H*, Similar responses of WT and CCR8$^{-/-}$ mice in a two-challenge model of OVA-induced allergic airway disease. CCR8$^{+/+}$ ($n = 8$ PBS, $n = 12$ OVA) and CCR8$^{-/-}$ ($n = 8$ PBS, $n = 12$ OVA) mice were OVA-sensitized, then challenged with PBS or OVA, following the protocol depicted, as described (25). On day 14, animals were sacrificed and leukocyte subsets in peripheral blood and BAL were analyzed. Bars represent mean ± SD of individual results obtained.

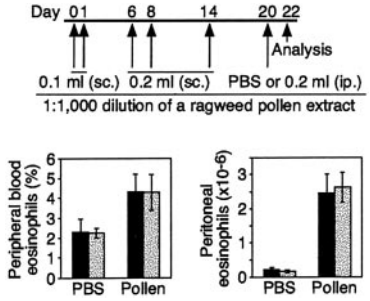

FIGURE 4. Comparable responses of WT and CCR8$^{-/-}$ mice in a mouse model of peritoneal eosinophilic inflammation. WT (n = 11) and CCR8$^{-/-}$ (n = 11) mice were immunized following the protocol depicted, as described (28), and then challenged by i.p. injection of the same allergen (n = 7) or control PBS (n = 4). On day 22, transmigrated peritoneal eosinophils and peripheral blood eosinophils were counted. Data are presented as mean ± SD of individual results obtained.

studied, the eosinophil response of CCR8$^{-/-}$ and control WT mice was very similar.

In their study, Chensue et al. (25) reported an impairment of eosinophil production in their CCR8$^{-/-}$ mice, apparently related to a systemic IL-5 production defect in these animals. In the different OVA models, we observed no significant defect in bone marrow eosinophilopoiesis in our CCR8$^{-/-}$ mice during the Ag challenge phase, or in the number of circulating and lung-infiltrated eosinophils (Figs. 2 and 3). We also analyzed cytokine production in mice treated with ragweed pollen extract. The results showed that the levels of IFN-γ, a Th1 cytokine, did not differ significantly between control and pollen-treated mice (Table II). Nonetheless, allergen-treated mice showed clear increases in the levels of Th2 cytokine mRNA (IL-4, IL-5, IL-13) and protein (IL-5) in the cells recruited to peritoneum and in serum, respectively; once again, analogous responses were observed in WT and CCR8$^{-/-}$ mice.

Discussion

The selective expression of CCR8 in thymocyte subsets (21) and Th2 cells (14, 15) suggests important roles for this β-chemokine receptor in T cell maturation and Th2-mediated immune responses. As human and mouse CCR8 are orthologous receptors with a very similar expression pattern (18), we generated CCR8$^{-/-}$ mice to study the role of CCR8 in vivo.

Considerable in vitro evidence links CCR8 to Th2 cells (14, 15). A recent study of CCR8$^{-/-}$ mice showed that these animals have a defective Th2 response and impaired eosinophil recruitment in OVA- and cockroach Ag-induced allergic airway inflammation models, as well as in a model of *Schistosoma mansoni* soluble egg Ag-induced granuloma formation (25). In contrast to that report, in this study we present data from three different models plus an in vivo blocking study, which clearly show that the response to OVA-induced allergic airway inflammation is not critically CCR8-dependent. In our study, we found similar lung infiltrates, BAL cell composition, bone marrow and peripheral blood eosinophilia, as well as lung and serum Th2 cytokine levels in WT and CCR8$^{-/-}$ mice. Remarkably, we detect increased levels of CCR8 (in WT mice) and its ligand CCL1 (WT and CCR8$^{-/-}$ mice) in the lungs of OVA-treated animals, suggesting a role for this receptor/ligand pair in asthma development, probably in the control of lymphocyte infiltration into lung. Administration of a CCR8-neutralizing mAb (21) to OVA-treated WT animals had no significant effect, however, consistent with the results obtained in CCR8$^{-/-}$ mice. Expression of CCR3 and CCR4, two Th2 cell-associated chemokine receptors, was also increased in the lungs of OVA-challenged mice, with no differences between WT and CCR8$^{-/-}$ mice. Therefore, it is tempting to speculate that the activity of some of these receptors may compensate for the absence of CCR8, as was recently described for CCR4 and CCR10 in cutaneous inflammation (35). Daily analysis of various parameters after each OVA challenge showed that the Ag response developed similarly in both animal groups. This appears to rule out the possibility that WT and CCR8$^{-/-}$ mice respond differently to the Ag, but achieve a similar response level by day 19.

In addition, no differences were observed in an adoptive transfer model of asthma, in which naive WT animals that had received Ag-specific Th2 cells from WT or CCR8$^{-/-}$ mice developed a similar eosinophilic mucosal inflammatory response after challenge with aerosolized OVA. Notably, when the model used by Chensue et al. (25) was studied, the BAL infiltrates and peripheral blood eosinophilia developed by our CCR8$^{-/-}$ mice was analogous to those observed in control WT mice. Although the number of infiltrated eosinophils accumulated in BAL varied substantially among the models studied, CCR8$^{-/-}$ mice showed the same eosinophilic response observed in WT mice in all cases. Despite the increases in CCL1/TCA3 and CCR8 levels detected in the lungs of Ag-challenged animals, we therefore conclude that the lack of this chemokine receptor does not notably alter disease development in CCR8$^{-/-}$ mice. Of note, similar immune responses were also observed in CCR8$^{-/-}$ and wild-type animals in a different allergic disease model of ragweed allergen-induced peritoneal eosinophilic inflammation, with an equivalent number of eosinophils and analogous increased levels of Th2 cytokines in peritoneum and peripheral blood.

The main explanations for the distinct results obtained with the two CCR8$^{-/-}$ mouse strains are related to differences in the models studied, the genetic background of the animals used, certain environmental factors, or any combination of these. In addition, the possibility of a second, undesired genetic hit in the ES cell used to generate the CCR8-deficient mice should also be considered. OVA is used extensively as an asthma-inducing agent, in a remarkable variety of protocols (see Ref. 24 for a recent review). Although model choice is critical (24), the fact that we obtained similar results in all models studied, including that applied by

Table II. *Analysis of cytokine expression in the peritoneum and sera of pollen extract-treated mice*[a]

Genotype/Treatment	IL-4 (P)	IL-5 (P)	IL-5 (B)	IL-13 (P)	IFN-γ (P)
CCR8$^{+/+}$/PBS	21.74 ± 2.02	18.93 ± 5.76	41.41 ± 3.43	4.55 ± 2.91	72.78 ± 4.99
CCR8$^{-/-}$/PBS	21.24 ± 2.49	18.09 ± 1.77	42.42 ± 6.86	4.20 ± 2.09	71.99 ± 9.15
CCR8$^{+/+}$/pollen	86.60 ± 18.95	79.74 ± 18.01	58.16 ± 2.91	74.32 ± 23.72	88.85 ± 10.16
CCR8$^{-/-}$/pollen	86.44 ± 28.29	82.58 ± 2.58	60.31 ± 6.11	65.86 ± 2.65	88.44 ± 7.79

[a] Mice were sensitized with a ragweed pollen extract, then challenged with either aerosolized PBS or allergen (*Materials and Methods*). Cells in peritoneum (P) and blood (B) were collected and RNA (peritoneum) and sera (blood) were prepared for RT-PCR or ELISA analysis, respectively. Values in arbitrary units (RT-PCR) or picograms per milliliter (ELISA) are mean ± SD from four to five individual animals in each group.

Chensue et al. (25) to their animals, nonetheless appears to rule out model differences as the main cause of these discrepancies.

The CCR8$^{-/-}$ animals used by each group had a different genetic background, as Chensue et al. (25) used 129Sv and 129Sv × C57BL/6 F_2 animals, whereas we used 129SvJ × C57BL/6 F_3, F_4, and F_5 mice. These differences can clearly influence the immune response of the animals, as the 129SvJ mouse strain shows a profound cell recruitment defect (36). Migration of macrophages, which are critical effector cells in asthma, is greatly diminished, and this migration defect is lost with progressive backcrossing to the C57BL/6 background (36). Differences in genetic background might also affect IL-5 production, reported to be defective in the Chensue et al. (25) CCR8$^{-/-}$ mouse strain. Our results using Ags of very different complexity as inducers of eosinophilic inflammation, in different anatomical compartments such as lung or peritoneum, suggest that our CCR8$^{-/-}$ mice have no IL-5 production defect in the models studied.

Differences in models and mouse genetic backgrounds may also contribute to the controversial results on the in vivo role of other chemokine receptors, using OVA-induced allergic airway inflammation models. CCR4 is another Th2 cell-expressed receptor (10). Whereas the response of CCR4-deficient mice in an OVA-induced asthma model was similar to that of WT animals (37), studies neutralizing CCL22/macrophage-derived chemokine (38) or CCL17/thymus and activation-regulated cytokine (39) showed that blocking either of these CCR4-specific ligands attenuated allergic airway disease development. Conflicting results have also been published in studies of mice deficient in CCR2, a β-chemokine receptor reported not to be critical for development of OVA-induced pulmonary inflammation (40), or to modulate the corresponding immune response (41), whereas neutralization of the CCR2 ligand CCL2/monocyte chemoattractant protein-1 was reported to diminish the inflammation drastically (42).

Whereas there is increasing evidence that chemokines and their receptors have important effects on pulmonary allergic responses, experimental differences nonetheless generate conflicting results and make assignment of specific roles to individual genes difficult. This may be especially important when analyzing complex diseases such as asthma, in which expression waves of different chemokines appear to act in a coordinated manner, contributing to the pathology (42, 43). Increasing knowledge of the biology of chemokine receptors also suggests that additional physiologically relevant subpopulations remain to be defined among cells bearing a given complement of chemokine receptors (44, 45). In addition, the microenvironmental homing of leukocytes is a multistep process in which different chemoattractant receptors and other receptor types, all expressed on the leukocyte membrane, are sequentially engaged. Although it would be useful to find a critical step in this chain of events, the emerging concept in the treatment of multifactorial diseases such as asthma is that multitargeted approaches may prove to be most promising. As to the in vivo role of CCR8 in allergic diseases, our results in the OVA-induced asthma and peritoneal eosinophilic inflammation models clearly indicate that, although CCR8 may have a role in the development in these allergic diseases, its deficiency alone has no significant effect on the development of these pathologies.

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