Aberrant In Vivo T Helper Type 2 Cell Response and Impaired Eosinophil Recruitment in CC Chemokine Receptor 8 Knockout Mice

By Stephen W. Chensue,* Nicholas W. Lukacs,* Tong-Yuan Yang,** Xiaozhou Shang,* Kirsten A. Frait,* Steven L. Kunkel,* Ted Kung,** Maria T. Wiekowski,** Joseph A. Hedrick,‖ Donald N. Cook,** Alessandra Zingoni,‡ Satwant K. Narula,** Albert Zlotnik,¶ Franck J. Barrat,¶ Anne O'Garra,¶ Monica Napolitano,§ and Sergio A. Lira**

*From the *Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48105; the ‡Department of Experimental Medicine and Pathology, University of Rome, 00167 Rome, Italy; the §Instituto Dermopatico dell'Immacolata-IRCCS, 00167 Rome, Italy; ‖Human Genome Research, Palo Alto, California 94304; the ¶DNAX Research Institute for Cellular and Molecular Biology, Palo Alto, California 94304; and the **Department of Immunology, Schering-Plough Research Institute, Kenilworth, New Jersey 07033*

Abstract

Chemokine receptors transduce signals important for the function and trafficking of leukocytes. Recently, it has been shown that CC chemokine receptor (CCR)8 is selectively expressed by Th2 subsets, but its functional relevance is unclear. To address the biological role of CCR8, we generated CCR8 deficient (−/−) mice. Here we report defective T helper type 2 (Th2) immune responses in vivo in $CCR8^{-/-}$ mice in models of *Schistosoma mansoni* soluble egg antigen (SEA)-induced granuloma formation as well as ovalbumin (OVA)- and cockroach antigen (CRA)-induced allergic airway inflammation. In these mice, the response to SEA, OVA, and CRA showed impaired Th2 cytokine production that was associated with aberrant type 2 inflammation displaying a 50 to 80% reduction in eosinophils. In contrast, a prototypical Th1 immune response, elicited by *Mycobacteria bovis* purified protein derivative (PPD) was unaffected by CCR8 deficiency. Mechanistic analyses indicated that Th2 cells developed normally and that the reduction in eosinophil recruitment was likely due to systemic reduction in interleukin 5. These results indicate an important role for CCR8 in Th2 functional responses in vivo.

Key words: chemokine receptors • chemokines • T helper type 2 cells • allergy • granulomas

Introduction

Numerous chemokines with similar structure and diverse in vitro activities have been described but their physiologic role remains to be fully established (1). Although the primary function of chemokines is considered to be chemotactic, many studies suggest that they have complex regulatory functions that extend beyond leukocyte trafficking (2–5). To clarify the physiologic functions of chemokines, recent efforts have focused on defining the cellular expression and distribution of their G protein–coupled receptors. To date, several of these receptors have been characterized and evidence suggests differential expression by leukocyte subpopulations (6, 7). In particular, lymphocyte subpopulations are reported to display restricted expression of chemokine receptors, raising the possibility of receptor-based immune manipulation (8). Intriguingly, this differential receptor expression includes both human and mouse type 1 (Th1) and type 2 (Th2) Th cells. Specifically, Th1 cells are associated with CXC chemokine receptor 3 (CXCR3)[1] expression,

Address correspondence to S.A. Lira, Department of Immunology K15 D-210C, Schering-Plough Research Institute, 2015 Galloping Hill Rd., Kenilworth, NJ 07033-0539. Phone: 908-740-3088; Fax: 908-740-3084; E-mail: sergio.lira@spcorp.com

[1]*Abbreviations used in this paper:* ANOVA, analysis of variance; BAL, bronchoalveolar lavage; CCR, CC chemokine receptor; CRA, cockroach antigen; CXCR, CXC chemokine receptor; EPO, eosinophil peroxidase; ES, embryonic stem; FBS, fetal bovine serum; PPD, purified protein derivative; RT, reverse transcription; SEA, schistosome egg antigen; TCA, T cell activation–specific gene 3.

whereas Th2 cells preferentially express CC chemokine receptor (CCR)3 and CCR4 (9, 10). More recently, the CCR8 is reportedly among those preferentially expressed on Th2 cells (11, 12). Indeed, the ligands for this receptor, I-309 in humans and T cell activation–specific gene 3 (TCA3) in mice, are chemotactic for Th2 cells in vitro (11). However, the in vivo function of CCR8 and its ligands is unknown.

To assess the role of CCR8 in vivo, we examined multiple immune and inflammatory parameters in mice with a targeted deletion of the CCR8 gene. These studies revealed impaired Th2 cytokine production and eosinophil recruitment during Th2-mediated responses, with no abrogation of the Th1 response. Moreover, Th2 cell development in vitro was normal, suggesting that the defect in Th2 responses was functional in nature. Taken together, these findings suggest that CCR8 is required for optimal Th2 responses in vivo. Thus, CCR8 may provide a potential target for selective therapeutic manipulation of Th2-mediated hypersensitivity states.

Materials and Methods

Gene Targeting. A 1.2-kb BglII DNA fragment of the mCCR8 gene containing the 5′ region of homology, and a 6.5-kb BglII-HindIII fragment containing the 3′ region of homology were sequentially cloned into pOS Dupdel plasmid (a gift of Dr. O. Smithies, University of North Carolina at Chapel Hill, Chapel Hill, NC). This targeting vector was designed so that the entire coding sequences of the murine CCR8 gene was replaced with the neomycin (neo) gene (see Fig. 1 A). This targeting vector was linearized with NotI restriction digestion and electroporated into embryonic stem (ES) cells. Neomycin-resistant ES cell clones were screened for homologous recombination by PCR with the primers TY118 (5′-CACGCTGTTCCATTGCTCTGGAG-3′) and TY70 (5′-GGGTTTGCTCGACATTGGGTGG-3′). A total of five positive clones were identified. Confirmation of the recombinant ES cells was done by Southern blot analysis of PstI-digested genomic DNA hybridized to a 0.5-kb 5′ end probe detecting 2.5-kb and 1.7-kb fragments corresponding to the wild-type and mutant alleles, respectively (see Fig. 1, B and C).

Mice. Four CCR8 targeted clones were injected into C57Bl/6J blastocysts. Chimeras from these clones were mated with C57Bl/6J females. Germline transmission of the targeted CCR8 allele was detected in animals generated from three independent clones. Heterozygous mice were bred and live offspring of the expected +/+, +/−, and −/− CCR8 genotypes were identified. Confirmation of the null phenotype was done by reverse transcription (RT)-PCR using RNA derived from lungs of CCR8 +/+ and −/− mice (see Fig. 1 D). Mice lacking CCR8 were generated in the 129Sv and in the 129Sv × C57BL/6 backgrounds. Controls consisted of littermate $CCR8^{+/+}$, $CCR8^{+/-}$ 129sv mice, and $CCR8^{+/+}$ 129Sv × C57BL/6 F2 mice obtained from The Jackson Laboratory. $CCR8^{-/-}$ mice displayed similar defects when compared with all control strains. *Schistosoma mansoni*–infected Swiss outbred mice were obtained from Biomedical Research Laboratories. All mice were maintained under specific pathogen-free conditions and provided with food and water ad libitum.

Sensitization and Granuloma Induction. For comparison of type 1 and 2 granulomas, secondary Ag-bead granulomas were generated as described previously (13). In brief, mice were sensitized by subcutaneous injection of 20 μg purified protein derivative (PPD; Department of Agriculture, Veterinary Division, Ames, IA) of *Mycobacteria bovis* incorporated into 0.25 ml CFA (product no. F-5881; Sigma-Aldrich) or 3,000 *S. mansoni* eggs suspended in 0.5 ml PBS. 14 to 16 d later, PPD and schistosome egg–sensitized mice were respectively challenged by tail vein with 6,000 Sepharose 4B beads (in 0.5 ml PBS) covalently coupled to PPD or to soluble schistosome egg antigens (SEA) obtained from the World Health Organization. Study parameters were evaluated on day 4 of granuloma formation.

Lung Aqueous Extracts. Snap-frozen lung lobes were suspended in 2 ml of PBS and homogenized for 20 s using a Tissue Tearor (Biospec Products, Inc.). Next, 0.1 ml of fetal bovine serum (FBS) was added as a protein stabilizer. The homogenate was centrifuged at 3,000 *g* for 20 min, and then the supernate was collected, aliquoted, and frozen at −80°C before cytokine assay. Total protein concentration was determined in experimental and control samples, then cytokine levels were normalized to milligrams of lung protein after subtraction of the FBS protein component.

Sensitization and Induction of the Allergic Airway Response. To induce a Th2 type response, control and knockout mice were immunized with cockroach allergen (Bayer Pharmaceuticals), as described previously (14). At the end of the sensitization phase, mice were then rechallenged once with allergen for cytokine analysis. Induction of the ovalbumin allergic response was performed in a similar manner as described previously (15). Cytokines and chemokines were quantified in homogenized (PBS) lung aqueous extracts and cell-free supernatants by specific ELISA.

Analysis of Leukocyte Subsets in Lungs of Allergic Mice. Leukocyte differentials and flow cytometric analyses of lymphocyte subsets were carried out in dispersed lung samples from $CCR8^{+/+}$ and $CCR8^{-/-}$ allergic mice. Mice immunized and challenged with cockroach allergen were killed and the lungs were inflated in situ with 1 ml ice-cold RPMI 1640, excised, and chopped into 2- to 3-mm pieces. Chopped lung tissue was resuspended in 3 volumes of sterile digestion medium consisting of RPMI 1640 containing 10 mM Hepes, 0.2% type IV collagenase, and 20 μg/ml tissue culture grade gentamicin. The mixture was agitated for 45 min at 37°C. The resulting suspension was strained through a no. 100 steel mesh and the cells centrifuged. The resuspended pellet was strained again through a 50-micron nylon sieve to remove any remaining clumps. The cells were then counted and total leukocyte numbers determined for each mouse. Next, cytospin slides were prepared, fixed, Wright stained, and the percentages of leukocyte populations were determined by standard 200-cell differential analysis. For flow cytometry, cells were then washed three times by centrifugation then resuspended in buffered saline with 2% FBS (16).

Cytokines, Abs, and Cytokine Assays. All cytokines and chemokines used in this study were obtained as purified carrier-free recombinant proteins from PeproTech and R&D Systems. Recombinant cytokines used for T cell culture were: mouse IL-4 (DNAX) and mouse IL-12 (BD PharMingen). Monoclonal anti-cytokine Abs used in culture were anti–IL-4 (clone 11B11; reference 17) and anti–IFN-γ (18). Anti–mouse CD3 and CD28 mAbs used for T cell stimulation were purchased from BD PharMingen. Interleukins 2, 4, 5, and 13, and IFN-γ were measured by standard ELISA using commercially available reagents (R&D Systems and BD PharMingen); sensitivities ranged from 15 to 50 pg/ml.

Morphometry. At designated intervals after tail vein challenges, lungs and draining lymph nodes were excised and prepared as de-

scribed below. In some experiments, lungs were inflated and fixed with 10% buffered formalin for morphometric analysis. Granuloma area was measured in a blinded fashion in hematoxylin and eosin–stained sections of paraffin-embedded lungs using a morphometer and software program (The Morphometer). A minimum of 20 lesions was measured per lung. Only granulomas with full cross-sections of the bead nidus were measured.

Differential Analyses of Granulomas, Blood, and Bone Marrow. Dispersed granulomas were prepared as described previously (19). A 200-cell differential analysis was performed on duplicate Wright-stained cytospin preparations of dispersed granulomas. At the time of sacrifice, samples of blood were obtained for total leukocyte counting and 100-cell differential. Bone marrow was obtained by perfusion of the femur, and then cytospin preparations were subjected to 200-cell differential.

Lymph Node Cell Culture. Mediastinal lymph nodes were collected aseptically at the time of lung harvest and teased into a single cell suspension. After washing, the cells were cultured in RPMI 1640 medium (JRH Biosciences) containing 10% FBS (Intergen), 10 mM glutamine, and 100 mg/ml streptomycin, and 100 U/ml penicillin (RPMI-FBS) at 5×10^6 cells/ml in 100-mm dishes with 5 μg/ml PPD or SEA for 24 h at 37°C in a humidified 5% CO_2 atmosphere. Supernates were collected by centrifugation and stored at −45°C before performing cytokine assays.

Eosinophil Assays. Eosinophils were obtained from either the spleen or blood of transgenic mice expressing the IL-5 gene in multiple tissues (unpublished observations). After lysing red blood cells in lysis buffer (Sigma-Aldrich), cells were spun and the pellets resuspended in RPMI supplemented with 10% FCS and 50 μM β-mercaptoethanol. Cells were cultured for 5 d and used for RNA and calcium flux experiments. Before RNA extraction, flow cytometry and hematoxylin/eosin staining was used to determine viability and cellular composition. Over 90% of the cells in culture were alive, had typical cytoplasmic eosinophilic granules, and were CCR3/Mac-1 positive. RNA was extracted by conventional methods from the thymus of both $CCR8^{+/+}$ and $CCR8^{-/-}$ mice (as control for CCR8 reaction) and from the cultured eosinophils using methods described below. cDNA was synthesized using SuperScript Preamplification System (GIBCO BRL) according to the manufacturer's recommendations. PCR reactions were done using the following primers for CCR8: TY248, 5′-ATGACCGACTACTACCCTGATTTCT-3′, and TY249, 5′-ACGCTGGCCGTCCTCACCTTGATGG-3′; for CCR3: TY254, 5′-AGACTGTGGTTGAAAGCTTTGAGAC-3′, and TY255, 5′-TTGTAGCTTCCTGTACTTTATGAGG-3′; for G3PDH: 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAAGGCCATGAGGTCCACCAC-3′.

The PCR conditions were: 94°C for 2 min, then 35 cycles of 94°C for 3 min, 55°C for 1 min, 72°C for 1 min, and 72°C for 5 min. The final PCR products were analyzed on a 2% agarose gel containing ethidium bromide. Intracellular calcium mobilization was examined in real time using fluorescence imaging plate reader (FLIPR; Molecular Devices). Eosinophils were loaded with Fluo-3-AM (Sigma-Aldrich) in RPMI containing 10% serum for 1 h at 37°C, then washed three times in flux buffer (HBSS, 20 mM Hepes, 0.1% BSA). Just before analysis, cells were aliquoted into polylysine-coated 96-well black-walled plates at a density of 5×10^5 cells/well and spun for 2 min at 300 *g* to settle cells. The data obtained were expressed as fluorescence units versus time. Chemokines were obtained from R&D Systems.

Lung RNA Extraction. Perfused lung lobes excluding major bronchi were snap frozen with liquid nitrogen and total cellular RNA was extracted by a modified method of Chirgwin et al. (20) and Jonas et al. (21). The frozen tissues were suspended in extraction buffer (25 mM Tris, pH 8.0, 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol), homogenized, then added to an equal volume of extraction buffer (100 mM Tris, pH 8.0, 10 mM EDTA, and 1% SDS). The mixture was then serially extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA is next precipitated at −70°C in ethyl alcohol, washed, and reprecipitated. The pellet was finally dissolved in DEPC water and RNA concentrations determined spectrophotometrically before storage at −70°C.

Cytokine Primers and Probes. Cytokine primers and probes (18–22 mer) were designed based on mRNA nucleotide sequences downloaded from the National Center for Biotechnology Information (NCBI) database and using primer design software (Premier Biosoft International). Designed primer and probe sequences for murine IFN-γ, IL-4, IL-5, IL-13, and endoplasmic reticulum–specific cyclophilin mRNA have been published previously (13). All primers and biotinylated probes were prepared by Genosys Biotechnologies Inc.

RT-PCR ELISA Detection of Cytokine mRNA. The isolated RNA was reverse transcribed to DNA and detection of PCR products was performed by semiquantitative colorimetric PCR-ELISA as described previously (13, 22). A 96-well plate ELISA reader was used to measure OD at 405 nm at 15 and 30 min. The mRNA ratio was calculated as follows: mRNA ratio = OD target gene/OD housekeeping gene (cyclophilin). Unlike simple gel detection, the PCR-ELISA method employs a hybridization step that specifically captures target amplicons. Consequently, it is highly specific and has proven to be 10–100-fold more sensitive than gel detection and allows amplification cycles to be kept to a minimum.

Th Cell Development In Vitro. Naive $CD4^+$ T cells were prepared as described previously (23). Cultures were established by stimulating cells with anti-CD3 and anti-CD28 in 24-well plates coated with anti-CD3 in PBS (10 μg/ml, 0.5 ml per well) for at least 2 h at 37°C in a humidified incubator pulsed with CO_2, and wells were washed twice with cRPMI before use; soluble anti-CD28 was added to the cultures at 10 μg/ml and cultures were at 10^6 cells/well, in the presence of medium alone, IL-4 (10 ng/ml) plus anti–IFN-γ mAb (10 μg/ml; Th2 conditions), or IL-12 (5 ng/ml) plus anti–IL-4 (10 μg/ml; Th1 conditions). After 3 d, cultures were split 1:3, and placed in fresh wells. After 6 d, T cells were harvested, counted, and restimulated for phenotype analysis. The method for the intracellular cytokine staining was as reported (24). In brief, T cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) at 10^6 cells/ml in 24-well plates, after 2 h brefeldin A (10 ng/ml) was added, and 2 h later the cells were harvested and fixed. Cells were permeabilized with 0.5% saponin and stained with anti–IFN-γ–FITC, anti–IL-4–PE, or isotype controls as described (24). The cells were analyzed using a FACScan™ flow cytometer (Becton Dickinson).

Statistics. The paired Student's *t* test was used to compare paired groups. Analysis of variance (ANOVA) was used for multigroup analysis. Values of $P > 0.05$ were considered to indicate lack of significance.

Results

$CCR8^{-/-}$ Mice Develop and Reproduce Normally. Mice lacking CCR8 (Fig. 1) developed normally to term and were fertile. Histologic and flow cytometric analysis of lymphoid and hematopoietic organs, including thymus, lymph nodes, spleen, blood, and bone marrow, did not reveal sig-

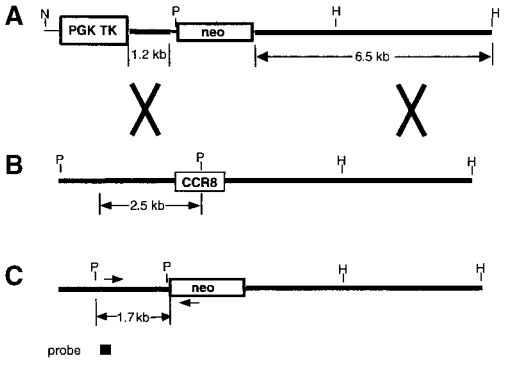

Figure 1. Generation of CCR8 knockout mice. (A) CCR8 targeting vector; (B) CCR8 genomic locus; (C) predicted recombined CCR8 locus; and (D) RT-PCR demonstration of disrupted CCR8 transcription in $CCR8^{-/-}$ mice. Messenger RNA was isolated from lungs of five mice with type 2 SEA granuloma formation and subjected to semiquantitative RT-PCR. No CCR8 mRNA was detected in knockout mice. *$P < 0.05$ comparing $CCR8^{+/+}$ to $CCR8^{-/-}$.

nificant developmental differences between $CCR8^{-/-}$ and control mice (not shown). The mutant animals had a normal life span and did not spontaneously develop disease.

CCR8 Deletion Impairs Local Th2 but Not Th1 Cytokine Responses. Anamnestic Th1 and Th2 responses can be studied in mice by sensitizing with protein Ags of *M. bovis* (PPD) or *S. mansoni* eggs (SEA) followed by an intravenous challenge of agarose beads covalently coupled to the respective sensitizing antigens. In these models, the antigen-coated beads embolize to the lung where they induce granulomatous responses that are maximal at day 4 (13, 19). To directly compare the effect of the CCR8 deletion on defined Th1 and Th2 memory responses, groups of $CCR8^{+/+}$ and $CCR8^{-/-}$ mice were sensitized with either *M. bovis* PPD in CFA or *S. mansoni* eggs. 2 wk after sensitization, lung granulomas were elicited in the respective groups by embolization of agarose beads covalently coupled to PPD or SEA (4 ng/bead). Multiple parameters were then assessed on day 4, the time of maximum lung granuloma formation.

Fig. 2, top left shows the effect of CCR8 deletion on day 4, type 1 (PPD), and type 2 (SEA) granuloma formation. Interestingly, CCR8 deletion had no significant effect on either type 1 or type 2 lesion cross sectional area. However, although type 1 lesions were histologically unchanged, the normally eosinophil-rich, type 2 lesions showed an apparent reduction in eosinophil content (Fig. 2, bottom). Quantitative analysis after enzymatic dispersal of lesions confirmed a significant abrogation of eosinophils (decreasing by 25 to 50% in three separate experiments). In contrast, type 1 lesion composition was unaffected by CCR8 deficiency (Fig. 2, top right).

To assess the effect of CCR8 deletion on cytokine profiles in the local inflammatory environment, we measured cytokine mRNA and protein levels in lungs with type 1

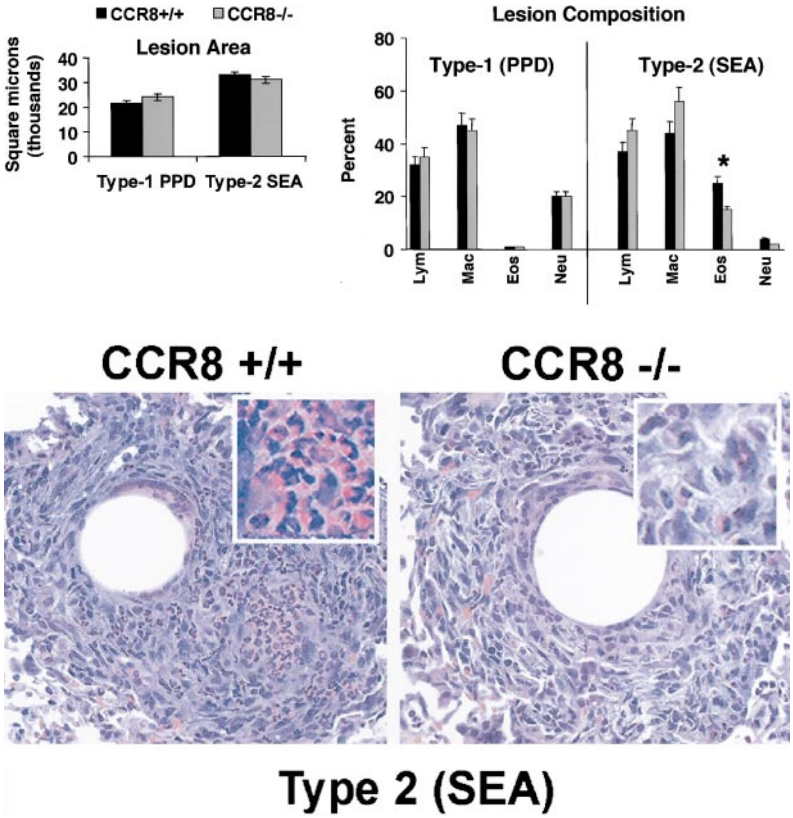

Figure 2. Effect of CCR8 deletion on secondary type 1 (PPD) and type 2 (SEA) granuloma formation. Groups of $CCR8^{+/+}$ (129 × B6) and $CCR8^{-/-}$ (129 × B6) mice were sensitized subcutaneously with *M. bovis* PPD in Freund's adjuvant or intraperitoneally with 3,000 *S. mansoni* eggs. After 14 d of sensitization, lung granulomas were elicited with 6,000 PPD or SEA Ag-coated beads. Granulomas were examined on day 4 after bead challenge. (Top left) Type 1 and type 2 granuloma cross-sectional areas. (Top right) Cellular composition of type 1 and type 2 lung granulomas. Five to six mice per group. (Bottom) Histologic appearances of type 2 (SEA) bead granulomas in $CCR8^{+/+}$ and $CCR8^{-/-}$ (original magnifications: ×400; inset, ×800). All observations were repeated in three separate experiments. Bars are means ± SEM. *$P < 0.05$ comparing $CCR8^{+/+}$ to $CCR8^{-/-}$. Findings were similar in separate studies using either 129sv or 129sv × C57BL/6 F2 background mice. Lym, lymphocytes; Mac, macrophages; Eos, eosinophils; Neu, neutrophils.

and type 2 granulomas. IFN-γ, IL-4, IL-5, and IL-13 transcripts were analyzed by RT-PCR ELISA. As shown in Fig. 3 A, cytokine transcript levels were not significantly affected by CCR8 deficiency during the type 1 response, which was characterized by a predominant increase in IFN-γ message compared with unchallenged lungs. In contrast, the type 2 responses were markedly reduced in the lungs of $CCR8^{-/-}$ mice. In particular, levels of IL-5 and IL-13 mRNA were reduced by ~30 and 40%, respectively (Fig. 3 B). In some experiments, the levels of IL-13 transcripts were similar to those expressed in unchallenged lungs. Despite reductions in IL-5 and IL-13 mRNA, IL-4 transcript levels were unchanged and interestingly, IFN-γ transcripts were increased two- to threefold. It should be noted that transcripts for CCR8 were detected in lungs of $CCR8^{+/+}$ mice with both type 1 and type 2 inflammation, but as expected these were absent in $CCR8^{-/-}$ mice (Fig. 1 D).

Cytokine transcript levels correlated well with direct measurement of cytokine protein levels in lung extracts. These likewise showed an unchanged type 1 pattern (Fig. 3 C). However, the type 2 pattern showed significant reductions in IL-5 and IL-13 (ranging from 30 to 80% in separate experiments), but there was no detectable change in IL-4 (Fig. 3 D). Levels of IFN-γ protein were unchanged despite the observed increase in message.

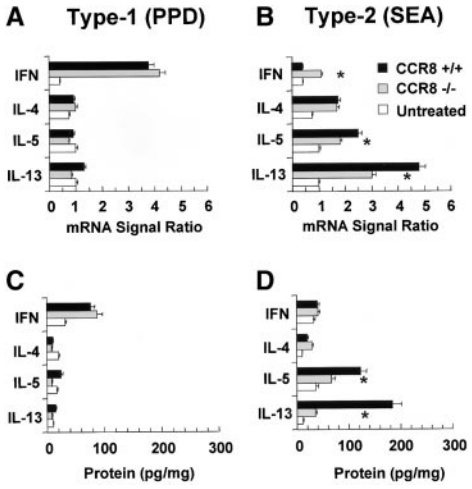

Figure 3. Effect of CCR8 deletion on cytokine mRNA and protein levels in lungs with secondary type 1 (PPD) and type 2 (SEA) granuloma formation. Lung granulomas were induced in $CCR8^{+/+}$(129 × B6) and $CCR8^{-/-}$(129 × B6), as described in Materials and Methods, then on day 4 upper and lower right lung lobes were snap frozen for parallel mRNA and protein analysis. Relative transcript levels were measured by RT-PCR-ELISA. Cytokine levels were determined by ELISA in aqueous extracts then normalized to total protein. Lungs of untreated mice served as baseline controls. Untreated $CCR8^{+/+}$ and $CCR8^{-/-}$ controls were statistically equivalent. $CCR8^{+/+}$ (black bars); $CCR8^{-/-}$ (shaded bars); untreated mice (white bars). (A and B) Cytokine mRNA ratios of type 1 and type 2 lungs, respectively; (C and D) corresponding cytokine protein levels. Values are means ± SEM derived from the lungs of five to six mice. $^*P < 0.05$ comparing $CCR8^{+/+}$ to $CCR8^{-/-}$ lungs by ANOVA. Findings were similar in separate studies using either 129sv or 129sv × C57BL/6 F2 background mice.

CCR8 Deletion Impairs Th2 but Not Th1 Cytokine Responses in Regional Lymphoid Tissue. To determine if the Th2 cytokine defect extended to lymphoid tissue, draining mediastinal lymph nodes were cultured and analyzed for cytokine profiles. Both type 1 and type 2 Ag-stimulated cultures from $CCR8^{-/-}$ mice displayed altered cytokine profiles as measured by protein ELISA. The IFN-γ production by type 1 cultures was augmented by nearly threefold in $CCR8^{-/-}$ mice and the low levels of IL-5 and IL-13 produced were reduced even further (Fig. 4, top). In type 2 node cultures, IL-5 decreased by 35 to 45% and IL-13 by 30 to 80% in three separate experiments. In addition, the normally low IFN-γ levels in type 2 cultures increased by 2- to 10-fold (Fig. 4, bottom). Interestingly, there was no consistent effect on IL-4, which was significantly reduced in two experiments and unchanged in two others.

In summary, both local and regional cytokine analyses indicated impaired IL-5 and IL-13 production in $CCR8^{-/-}$ mice during the type 2 response with no impairment or augmentation of type 1 cytokines.

Eosinophil Accumulation Is Attenuated in $CCR8^{-/-}$ Allergic Mice. We next examined the effect of CCR8 deletion in mouse models of allergic airway inflammation. In a well-characterized model of aerosolized ovalbumin-induced, allergic pulmonary inflammation (15), antigen-challenged $CCR8^{-/-}$ mice had fewer peribronchial eosinophils than challenged $CCR8^{+/+}$ mice, as demonstrated by histologic examination (Fig. 5 A). Analysis of the bronchoalveolar lavage (BAL) of challenged lungs likewise revealed a significant decrease in eosinophils in the $CCR8^{-/-}$ mice (Fig. 5 B). These results indicate that $CCR8^{-/-}$ mice have reduced pulmonary eosinophil accumulation after sensitization and inhalation of aerosolized ovalbumin.

To determine whether the impaired eosinophil recruitment in lungs of ovalbumin-challenged $CCR8^{-/-}$ mice was similarly impaired in another model of allergic pulmonary inflammation, we performed experiments using cockroach antigen (CRA). In humans, particularly in inner city children, allergic responses to CRAs are recognized as a

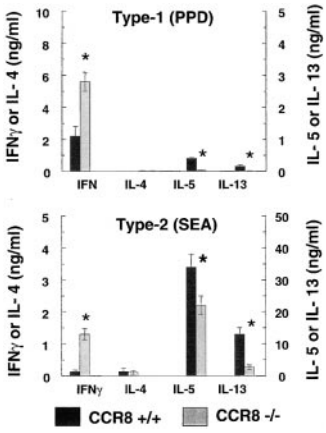

Figure 4. Effect of CCR8 deletion on cytokine profiles in draining lymph nodes cultures during secondary type 1 (PPD) and type 2 (SEA) granuloma formation. Lung granulomas were induced in $CCR8^{+/+}$ (129 × B6) and $CCR8^{-/-}$ (129 × B6), as described in Materials and Methods, then on day 4 draining lymph nodes were collected and cultured. Bars are means ± SEM of Ag-elicited cytokine levels from a representative experiment. (Top) Type 1 (PPD) response; (bottom) type 2 (SEA) response. Five to six animals per group. All observations were repeated in three separate experiments and findings were similar using either 129sv or 129sv × C57BL/6 F2 background mice. $^*P < 0.05$ comparing $CCR8^{+/+}$ to $CCR8^{-/-}$.

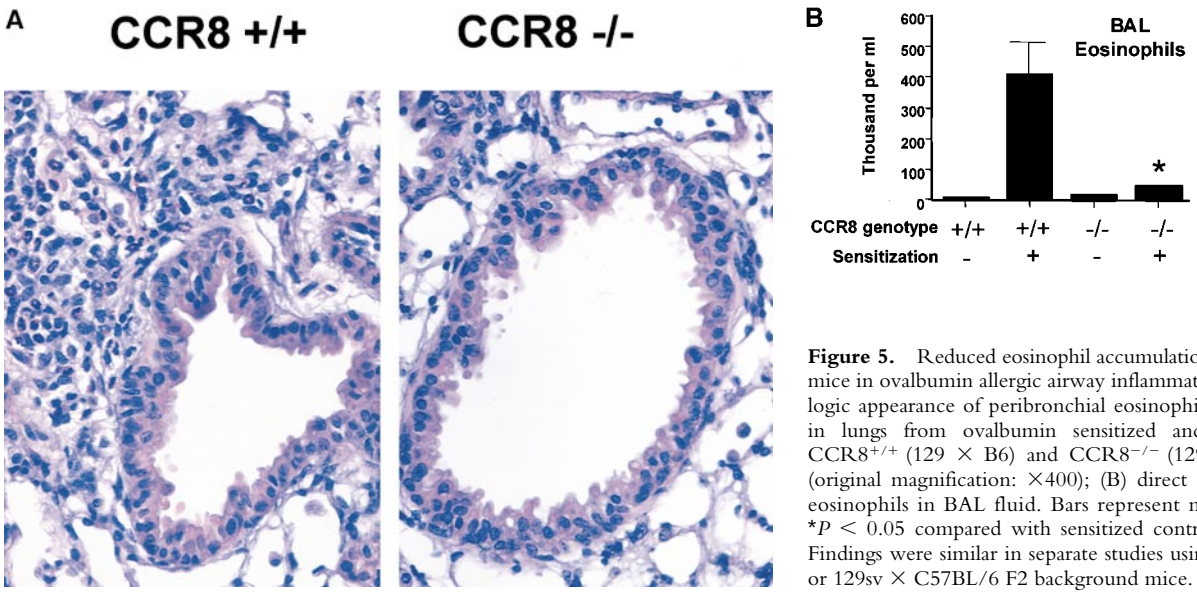

Figure 5. Reduced eosinophil accumulation in CCR8$^{-/-}$ mice in ovalbumin allergic airway inflammation. (A) Histologic appearance of peribronchial eosinophil accumulation in lungs from ovalbumin sensitized and rechallenged CCR8$^{+/+}$ (129 × B6) and CCR8$^{-/-}$ (129 × B6) mice (original magnification: ×400); (B) direct quantitation of eosinophils in BAL fluid. Bars represent means ± SEM. *$P < 0.05$ compared with sensitized control +/+ mice. Findings were similar in separate studies using either 129sv or 129sv × C57BL/6 F2 background mice.

major cause of asthma. The murine model of CRA-induced allergic airway inflammation recapitulates many aspects of human asthma and is considered a more clinically relevant experimental model of asthma (14). After CRA sensitization and challenge, CCR8$^{+/+}$ and CCR8$^{-/-}$ mice were assessed histologically for eosinophil accumulation within the lungs. As in the ovalbumin model, CCR8$^{-/-}$ mice had markedly fewer peribronchial eosinophils compared with CCR8$^{+/+}$ mice (Fig. 6 A). Morphometric enumeration of the peribronchial eosinophils revealed that challenged CCR8$^{-/-}$ mice had a 70% decrease compared with CCR8$^{+/+}$ mice (Fig. 6 B). However, a defect of lymphocyte recruitment could not be established. As shown in Table I, flow cytometric analysis of enzymatically dispersed lungs did not reveal significant differences between the two groups in absolute numbers of CD4$^+$ and CD8$^+$ T cells. This finding indicated that CCR8 deficiency did not affect gross lymphocyte infiltration, yet effects on minor populations of antigen-specific cells cannot be ruled out. Similarly, no defects in macrophage mobilization could be established. In fact, by flow cytometry the proportion of cells bearing the monocyte/macrophage marker, F4/80, was increased (CCR8$^{+/+}$, 25 ± 5% versus CCR8$^{-/-}$, 46 ± 5%; $n = 4$ per group). The latter was largely a relative change, because in ovalbumin-challenged mice, CRA-challenged CCR8$^{-/-}$ mice had an ~50% reduction in the number of

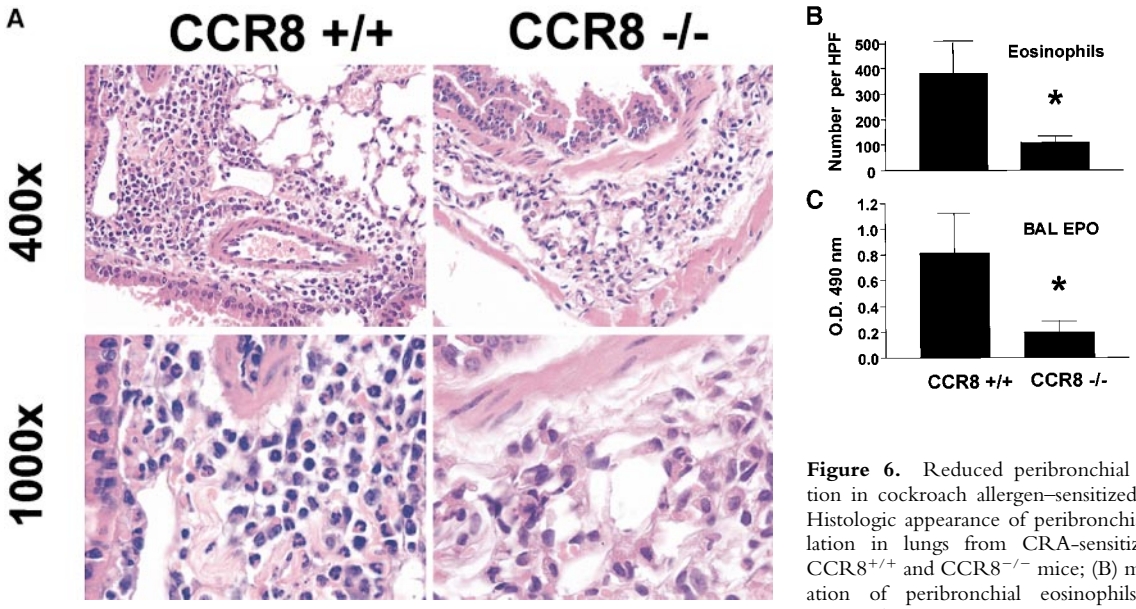

Figure 6. Reduced peribronchial eosinophil accumulation in cockroach allergen–sensitized CCR8$^{-/-}$ mice. (A) Histologic appearance of peribronchial eosinophil accumulation in lungs from CRA-sensitized and rechallenged CCR8$^{+/+}$ and CCR8$^{-/-}$ mice; (B) morphometric enumeration of peribronchial eosinophils in CCR8$^{+/+}$ and CCR8$^{-/-}$ mice; (C) parallel analysis of EPO in the BAL fluid of the CCR8$^{+/+}$ (129sv) and CCR8$^{-/-}$ (129sv) mice. Bars represent the means ± SEM of five mice. *$P < 0.05$. Findings were similar in separate studies using either 129sv or 129sv × C57BL/6 F2 background mice. HPF, high power field.

Table I. *Analysis of Lymphocyte Subsets in Cockroach Allergen-challenged Lungs of Control and CCR8$^{-/-}$ Mice*

Group	Total cells (10^7)	CD4$^+$ (10^6)	CD8$^+$ (10^6)	CD19$^+$ (10^6)
CCR8$^{+/+}$	2.2 ± 0.16	1.2 ± 0.8	0.50 ± 0.2	0.8 ± 0.2
CCR8$^{-/-}$	1.6 ± 0.18	1.8 ± 0.14	0.58 ± 0.3	1.3 ± 0.14
P	0.015	>0.05	>0.05	>0.05

Cockroach antigen sensitization and challenge was performed as described in Materials and Methods. Lungs were collected and dispersed 24 h after the final intratracheal challenge, and then subjected to flow cytometric analysis. Values are means ± SD derived from four individual mice. The reduction in total cell numbers in CCR8$^{-/-}$ was due to decreased eosinophil recruitment (CCR8$^{+/+}$, 25.5 ± 5% versus CCR8$^{-/-}$, 14 ± 4%). Control and knockout mice were on inbred 129 background.

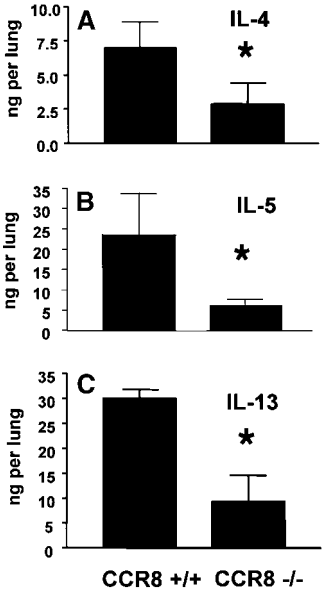

Figure 7. Reduced cytokine levels in the lungs of cockroach allergen challenged CCR8$^{-/-}$ mice. Aqueous extracts were prepared from whole snap frozen lungs of allergen-challenged CCR8$^{+/+}$ (129sv) and CCR8$^{-/-}$ (129sv) mice at 6 h after challenge, then assayed for cytokines with values expressed as amount per whole lung. (A) IL-4 levels; (B) IL-5 levels; and (C) IL-13 levels. Bars represent the mean ± SEM of individual lungs of four mice. *$P < 0.05$. Findings were similar in separate studies using either 129sv or 129sv × C57BL/6 F2 background mice.

eosinophils in the lung compared with CCR8$^{+/+}$ mice (data not shown). This was further demonstrated in the CRA model by quantitative analysis of eosinophil activity in the BAL using another parameter, eosinophil peroxidase (EPO) levels, which revealed that CCR8$^{-/-}$ mice had a 75% reduction of EPO levels in cell-free BAL compared with CCR8$^{+/+}$ mice (Fig. 6 C). Thus, in two different models of allergic pulmonary inflammation, CCR8$^{-/-}$ mice displayed relatively specific reduction in eosinophil recruitment in and around the airways compared with similarly challenged CCR8$^{+/+}$ mice.

To investigate the underlying causes of the reduced eosinophil recruitment in allergen-challenged CCR8$^{-/-}$ mice, we conducted a more detailed series of experiments using the CRA model. First, blood collected from unchallenged CCR8$^{+/+}$ and CCR8$^{-/-}$ mice was differentially stained for leukocytes. Before allergen challenge there was no difference between the groups in the number of circulating eosinophils. However, after antigen sensitization and rechallenge, a significant reduction was observed in the number of eosinophils in the blood of CCR8$^{-/-}$ mice compared with CCR8$^{+/+}$ mice; eosinophils averaged 10.3 ± 3.7% of the circulating cells in the CCR8$^{+/+}$ group and <2% in the CCR8$^{-/-}$ mice. These results suggest that the reduction in the numbers of eosinophils in the lungs might be related to a defect in eosinophil maturation or release into the circulation.

Th2 Type Cytokines Are Reduced in Lungs of CCR8$^{-/-}$ Allergic Mice. Next, we assessed cytokine levels in whole lung homogenates at different time points during the CRA-induced allergic response. Levels of IFN-γ and IL-12 were not altered within the CCR8$^{-/-}$ lungs compared with CCR8$^{+/+}$ lungs (data not shown). However, the levels of IL-4 (Fig. 7 A), IL-5 (Fig. 7 C), and IL-13 (Fig. 7 D) were significantly reduced in the CCR8$^{-/-}$ lungs. Thus, unlike the type 2 granuloma model, reduced IL-4 levels were demonstrable in the allergy model possibly due to the greater cellular complexity of the granulomatous response. It should be noted that despite reductions in cytokines, no consistent changes were observed in airway hyperreactivity (data not shown), supporting the notion that airway hyperreactivity can be established independently of IL-4 (25).

Circulating Eosinophils Do Not Express CCR8 or Respond to CCR8 Ligands. A potential explanation for the reduced eosinophil recruitment in CCR8$^{-/-}$ mice was that eosinophils use CCR8 for chemotaxis. To address this question, we examined CCR8 expression and functional responses (calcium flux and chemotaxis) in eosinophils from CCR8$^{+/+}$ mice. Although expression of CCR3 was readily detected by RT-PCR analysis (Fig. 8 A), no CCR8 mRNA was detected in these cells. These findings were confirmed by functional assays examining calcium flux in response to CCR3 and CCR8 ligands. As expected, both human and mouse eotaxin generated a significant calcium flux (Fig. 8 B) in mouse eosinophils, whereas no such response was observed with either TCA3 or its human homologue I-309. In vitro chemotaxis assays gave comparable results. The results of all assays were similar regardless of whether the eosinophils were isolated from blood or spleen (data not shown). These results indicate that CCR8 is not expressed by peripheral mouse eosinophils.

Eosinophil Production Is Impaired in CCR8$^{-/-}$ Mice. As CCR8 was not normally expressed by peripheral eosinophils, then impaired recruitment during the type 2 response was likely due to other factors. Circulating IL-5 is an important mediator for eosinophil recruitment, function, and the differentiation in bone marrow (26–28). Moreover, it is specifically required for eosinophil mobilization to schistosome egg granulomas (29). To determine if impaired eosinophil recruitment was possibly related to a reduction of systemic IL-5 release, we examined peripheral blood leukocytes, bone marrow eosinophilopoiesis, and serum IL-5 levels in mice with ongoing type 1 and type 2 granuloma formation. Differential analysis revealed no change in blood leukocyte populations during the type 1 response (data not shown). In contrast, distinct changes were observed in

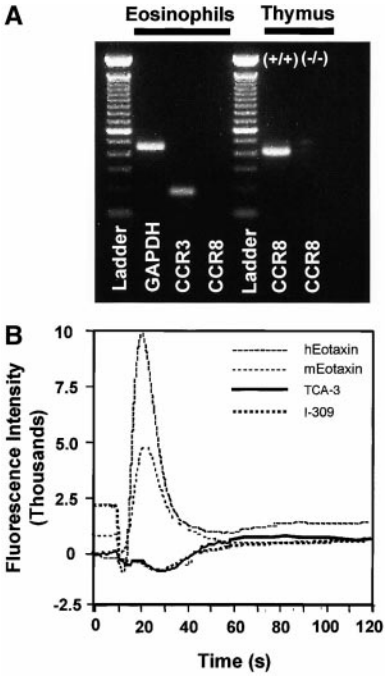

Figure 8. Circulating eosinophils lack CCR8 mRNA and are unresponsive to CCR8 ligands. (A) Eosinophils isolated from the blood of IL-5 transgenic mice were examined for expression of CCR3 and CCR8 by RT-PCR. CCR3 (184-bp band) expression was detected in mouse eosinophils (2×10^5 cells). CCR8 expression was not detected in circulating eosinophils. CCR8 transcript (411-bp band) was detected in wild-type thymocytes, but not in thymocytes from $CCR8^{-/-}$ mice (negative control). GAPDH (540-bp band) was included as a procedural control. (B) Eosinophils (blood) were also examined for their ability to flux calcium in response to CCR3 and CCR8 ligands. Human and mouse eotaxin (100 ng/ml) both generated a significant transient calcium flux, whereas TCA3 and I-309 (100 ng/ml, respectively) were ineffective in this assay.

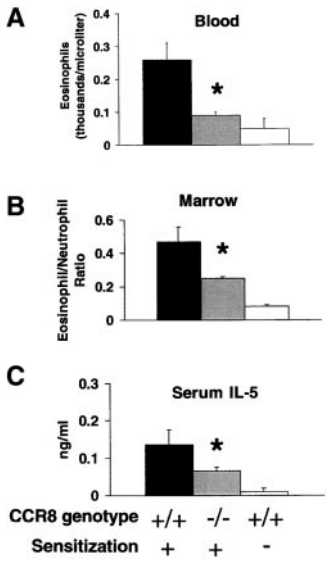

Figure 9. Blood and bone marrow eosinophil mobilization as related to serum IL-5 levels in $CCR8^{+/+}$ and $CCR8^{-/-}$ mice with secondary type 2 (SEA) granulomas. Lung granulomas were induced in $CCR8^{+/+}$ and $CCR8^{-/-}$ as described in Materials and Methods, then on day 4, blood, bone marrow, and serum was evaluated. (A) Peripheral blood eosinophil counts; (B) bone marrow eosinophil differentiation index; (C) serum IL-5 levels. $CCR8^{+/+}$ (129 × B6; black bars); $CCR8^{-/-}$ (129×B6; shaded bars); untreated mice (white bars). Untreated $CCR8^{+/+}$ and $CCR8^{-/-}$ controls were statistically equivalent. $^*P < 0.05$ comparing sensitized $CCR8^{+/+}$ by ANOVA. Findings were similar in separate studies using either 129sv or 129sv × C57BL/6 F2 background mice.

$CCR8^{-/-}$ mice with type 2 granulomas. As shown in Fig. 9, the type 2 response was associated with eosinophil mobilization in blood and bone marrow, and as observed in the CRA allergy model, knockout mice displayed an ~70% decrease in absolute numbers of circulating eosinophils (Fig. 9 A), suggesting a systemic impairment in eosinophil production. Indeed, $CCR8^{-/-}$ mice with type 2 lesions showed reduced eosinophil differentiation in bone marrow with the ratio of eosinophil to neutrophil differentiation decreasing by 50% (Fig. 9). In addition, the eosinophilopoietic cytokine, IL-5, was similarly reduced in serum (Fig. 9 C), suggesting that impaired eosinophil mobilization could be related to a systemic defect in IL-5 production.

CCR8 Is Not Required for In Vitro Development of Th2 Cells. A possible cause for the impaired Th2 cytokine response in $CCR8^{-/-}$ mice was that CCR8 was required for the normal development and expansion of Th2 cells. To test this possibility, naive $CD4^+$ T cells from wild-type or CCR8-deficient mice were cultured with anti-CD3 and anti-CD28 under Th1 (IL-12, anti–IL-4), neutral (no cytokine additions), and Th2 (IL-4, anti–IL-12) conditions. After 6 d, the T cells were harvested and restimulated at equal cell number with PMA and ionomycin and their cytokine profile was analyzed using flow cytometric analysis for intracellular cytokine production. As shown in Fig. 10, T cells cultured under Th1 conditions produced IFN-γ and IL-10 and no IL-4 or IL-5, as described previously (30). However, there was no difference in the number of IFN-γ^+ or IFN-γ^+IL-10^+-producing Th1 cells obtained from the wild-type (18%) and CCR8 (17%) mice. In addition, T cells cultured under Th2 conditions differentiated into cells producing IL-4, IL-5, and IL-10, but no IFN-γ, as described previously (30–33). Again, similar numbers of T cells produced IL-4, IL-5, and IL-10 when they were cultured from wild-type (20% IL-4^+, 17% IL-5^+, and 52% IL-10^+) or $CCR8^{-/-}$ (25% IL-4^+, 23% IL-5^+, and 46% IL-10^+) mice. T cells cultured under neutral conditions differentiated into Th2-type cells reflecting endogenous levels of IL-4 during primary culture, which can differ depending on the genetic background of the mouse as well as the number of $CD4^+$ T cells per well (30). Again there was no significant difference in the number of IL-4–, IL-5–, or IL-10–producing cells obtained using $CD4^+$ T cells from wild-type (48% IL-4^+, 16% IL-5^+, and 49% IL-10^+) or $CCR8^{-/-}$ (50% IL-4^+, 23% IL-5^+, and 34% IL-10^+). Low levels of IFN-γ were observed under these conditions but were no different in wild-type versus $CCR8^{-/-}$ mice. These data clearly show that although CCR8 is expressed on Th2 but not Th1 cells (11), it has no significant effect on the ability of naive $CD4^+$ T cells to differentiate into Th2 or Th1 cells. Of significance is the lack of difference in IL-4– or IL-5–producing cells obtained under the more limiting neutral conditions. It remains possible that this effect may only be observed under more limiting conditions of antigen dose.

Discussion

Human and murine CCR8 are homologous G protein–coupled receptors whose reported ligands include human

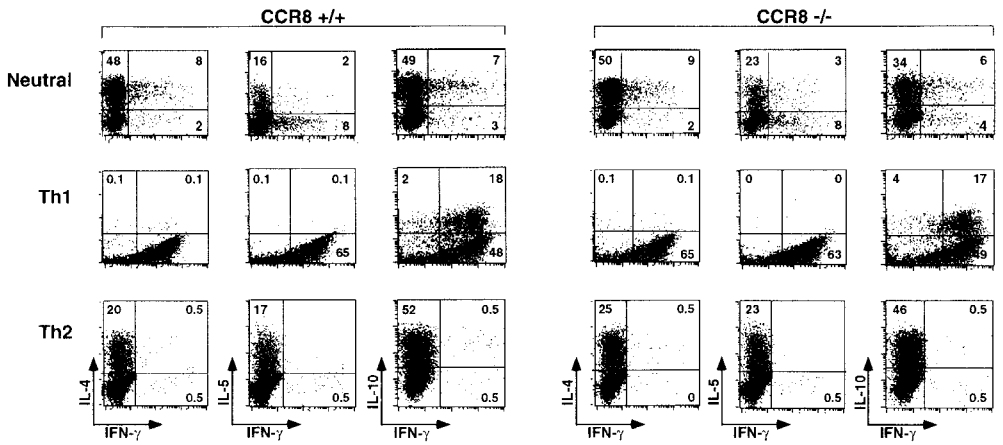

Figure 10. CCR8 is not required for in vitro development of Th2 cells. Naive $CD4^+$ T cells from $CCR8^{+/+}$ (129sv) or $CCR8^{-/-}$ (129sv) mice were stimulated with anti-CD3 and anti-CD28 mAbs, in the presence of medium alone (neutral conditions), IL-12 plus anti–IL-4 mAbs (Th1 conditions), or IL-4 plus anti–IFN-γ mAbs (Th2 conditions), and then harvested after 6 d and restimulated for analysis of intracellular cytokine production by flow cytometry (IL-4, IL-5, and IFN-γ).

I-309, thymus and activation-regulated chemokine (TARC), macrophage inflammatory protein (MIP)-1β, TCA3, and liver-expressed chemokine (LEC; references 34–37). In addition, the virally encoded chemokines, vMIP-I and vMIP-II, can also bind CCR8 (38–40). The selective expression of CCR8 in Th2 T cells (11, 12) suggests that it may have an important role in the function of these cells. However, there are currently no experimental data demonstrating such a role. To investigate this possibility, and to study other potential functions of CCR8, we have generated and analyzed CCR8-deficient mice. Using models of Th2 (schistosomal) cell-mediated immune responses as well as two models of Th2-mediated allergic airway disease, we show impairment of Th2 type cytokine expression and eosinophil mobilization in $CCR8^{-/-}$ mice. This defect was specific to the in vivo Th2 type response, as the Th1 response to mycobacterial Ags was not impaired by CCR8 deficiency. Although Th2 responses were reduced in CCR8-deficient mice in vivo compared with wild-type mice, there was no significant difference in the ability of naive $CD4^+$ T cells to differentiate into Th2 or Th1 cells. In addition, functional CCR8 expression was not detected in eosinophils, as these cells did not signal in response to the CCR8 ligands, TCA3 and I-309. In separate studies, using TNF or IL-4 stimulation of eosinophils, we have observed CCR8 mRNA expression by RT-PCR, but minimal chemotactic responses to CCR8 compared with CCR3 ligands (unpublished observations). Thus, reduced eosinophil mobilization seen in SEA-challenged $CCR8^{-/-}$ mice was not likely due to the absence of CCR8 on eosinophils. Rather, the observed defects in the $CCR8^{-/-}$ mice seemed more related to reduced Th2 cytokines indicating the CCR8 was important for normal Th2 cell function.

Several potential aspects of Th2 cell function may be impaired by CCR8 deficiency, including cell trafficking, regulation of T cell survival, and stimulation of cytokine production. Given the well-established role of chemokines in leukocyte trafficking, the most straightforward explanation for our observations would be failure of Th2 cells to efficiently migrate to lungs and lymph nodes and, indeed, the observed local impairment of Th2 cytokine production in these sites would support this notion. In addition, in vitro–generated $CCR8^{-/-}$ Th2 fail to show chemotactic responses to the CCR8 ligand TCA3 (data not shown). Although CCR8 likely does not contribute to trafficking of naive T cells or Th1 cells, it may have a central role in the trafficking of activated Th2 cells to sites of inflammation (11, 12). Direct counts of total $CD4^+$ T cells in lungs of allergen-challenged $CCR8^{-/-}$ mice did not reveal any gross evidence of a recruitment defect. However, ongoing studies in our laboratory indicate that the relevant Ag-specific, cytokine producing population represents 1% or less of the $CD4^+$ cells. Therefore, gross $CD4^+$ counting is likely not a sensitive parameter. Rather direct testing of migration in vivo will be required using labeled $CCR8^{-/-}$ Th2 cells or $CCR8^{-/-}$ mice with transgenic TCR expression.

Our results indicate a postdevelopmental functional impairment of Th2 cells in $CCR8^{-/-}$ mice, possibly a migration defect as noted above. However, alternative sites of compromise that could be considered include T cell activation or survival. For example, one explanation for the reduced Th2 cytokine production in SEA-induced granulomas and draining lymph node cultures of the $CCR8^{-/-}$ mice is that CCR8 ligands regulate Th2 cytokine production, either directly as costimulants, or indirectly by increasing Th2 cell survival. With regard to the latter, it has been reported that the CCR8 ligand, TCA3, a known product of T cells (41), can inhibit apoptosis of lymphoma cells (42). Thus, binding of TCA3 to CCR8 might protect Th2 cells from apoptosis and thereby allow prolonged survival of Th2 populations. However, our findings that $CCR8^{-/-}$ Th2 cells develop normally in vitro and that $CCR8^{-/-}$ mice harbor normal numbers of Ag-specific Th2 cells in lymphoid tissue (unpublished observations) would argue against this possibility. Other indirect T cell–related mechanisms might include impaired CCR8-mediated migration of Ag-presenting dendritic cells or macrophages to lymphoid tissues where accessory cells may be required for optimal Th2 cell function. However, activation requirements for memory T cells are reportedly less stringent than for naive T cells (43), and abundant potential accessory cells are present in granulomas. Alternatively, CCR8 ligands might directly stimulate Th2-type cytokine secretion at critical stages of activation. This notion would be in accord

with a growing body of evidence indicating that chemokines can regulate T cell maturation and function (44, 45). In this regard, several chemokine receptors, such as CCR3, CCR4, and CCR8 can potentially contribute to Th2 migration (11, 12, 46); therefore, it was surprising that CCR8 deletion was able to cause even a partial abrogation of the Th2 response. Current hypothetical models suggest stage-specific chemokine receptor expression by T cells (47); hence, it is reasonable to envision a stepwise expression of chemokine receptors that may govern both T cell migration and effector functions. It has recently been demonstrated that the Th2 cell–associated chemokine receptors CCR3 and CCR4 take dominance at different stages of hypersensitivity responses (48), and such may be the case with CCR8. A detailed explanation for different functional changes transduced by chemokine receptors will require a better understanding of the complexity of G protein–coupled receptor organization and regulation.

It was observed that CCR8$^{-/-}$ mice displayed similar defects in models of type 2 (SEA) bead granuloma formation and cockroach allergen challenge in all parameters except local IL-4 production by inflamed lungs. Although the precise explanation for this difference is unknown, it is likely related to the different experimental conditions. Parameters were measured at 6 h after challenge in the more acute allergy model but on day 4 in the granuloma model. Moreover, the type 2 granuloma is a complex, chronic inflammatory response that involves a variety of cell types that could serve as compensatory sources of IL-4. Among these are lymphoid populations such as double negative (CD4$^-$CD8$^-$) T cells, CD8$^+$ type 2 T cells, and non-T, non-B cells (49–51).

Finally, it should be noted that CCR8$^{-/-}$ mice had a phenotype similar to IL-4$^{-/-}$ mice under comparable experimental conditions (13). Both knockouts display reduced blood eosinophilia and impaired local eosinophil recruitment that is associated with attenuation but not elimination of Th2 cytokines. Similar to IL-4 knockout mice, both type 1 and type 2 lymph node cultures showed increased IFN-γ production consistent with alleviated Th2-mediated cytokine cross-regulation. Like IL-4 knockout mice, the secondary type 2 granuloma in CCR8$^{-/-}$ mice did not completely shift to a type 1 profile but established a compensated Th2-like granuloma of similar size despite reduced Th2 cytokine production. These findings are quite unlike those we reported for CCR2$^{-/-}$ mice, which have deficits in IFN-γ and IL-2 production and impaired monocyte recruitment with minor effects on Th2 cytokine production (52, 53). Thus, CCR8$^{-/-}$ mice like IL-4$^{-/-}$ mice have likely adapted to their deficiency, although in both mutants the eosinophil mobilization defect could not be completely overcome.

Taken together, our results demonstrate that CCR8 plays a role in Th2 responses in vivo and provide further support to the concept that chemokines and their receptors can potentially be targeted to modulate Th2-mediated immune responses.

We thank P. Zalamea, L. Hamilton, C. Young, D. Prosser, S. Abbondanzo, and D. Manfra for mouse generation and FACS® analysis, Aron Pollack and Stacey Haller for expert histologic support, and Dr. R. Coffman for critical advice.

This work was supported by grant AI43460 from National Institutes of Health-National Institute of Allergy and Infectious Diseases and in part by the Department of Veterans Affairs. Schistosome life stages or materials for this work were supplied through National Institutes of Health-National Institute of Allergy and Infectious Diseases contract NO1-AI55270.

Submitted: 8 September 2000
Revised: 16 January 2001
Accepted: 24 January 2001

References

1. Baggiolini, M. 1998. Chemokines and leukocyte traffic. *Nature*. 392:565–568.
2. Strieter, R.M., P.J. Polverini, D.A. Arenberg, and S.L. Kunkel. 1995. The role of CXC chemokines as regulators of angiogenesis. *Shock*. 4:155–160.
3. Taub, D.D., S.M. Turcovski-Corrales, M.L. Key, D.L. Longo, and W.J. Murphy. 1996. Chemokines and T lymphocyte activation: I. Beta chemokines costimulate human T lymphocyte activation in vitro. *J. Immunol*. 156:2095–2103.
4. Taub, D.D., J.R. Ortaldo, S.M. Turcovski-Corrales, M.L. Key, D.L. Longo, and W.J. Murphy. 1996. Beta chemokines costimulate lymphocyte cytolysis, proliferation, and lymphokine production. *J. Leuk. Biol*. 59:81–89.
5. Sallusto, F. 1999. The role of chemokines and chemokine receptors in T cell priming and Th1/Th2-mediated responses. *Haematologica*. 84:28–31.
6. Bonecchi, R., G. Bianchi, P.P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P.A. Gray, A. Mantovani, and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med*. 187:129–134.
7. Sallusto, F., D. Lenig, C.R. Mackay, and A. Lanzavecchia. 1998. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med*. 187:875–883.
8. Oppenheim, J.J., W.J. Murphy, O. Chertox, V. Schirrmacher, and J.M. Wang. 1997. Prospects for cytokine and chemokine biotherapy. *Clin. Cancer Res*. 3:2682–2686.
9. Gao, J.L., T.A. Wynn, Y. Chang, E.J. Lee, H.E. Broxmeyer, S. Cooper, H.L. Tiffany, H. Westphal, J. Kwon-Chung, and P.M. Murphy. 1997. Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J. Exp. Med*. 185:1959–1968.
10. Gerber, B.O., M.P. Zanni, M. Uguccioni, M. Loetscher, C.R. Mackay, W.J. Pichler, N. Yawalkar, M. Baggiolini, and B. Moser. 1997. Functional expression of the eotaxin receptor CCR3 in T lymphocytes co-localizing with eosinophils. *Curr. Biol*. 7:836–843.
11. Zingoni, A., H. Soto, J.A. Hedrick, A. Stoppacciaro, C.T. Storlazzi, F. Sinigaglia, D. D'Ambrosio, A. O'Garra, D. Robinson, M. Rocchi, et al. 1998. The chemokine receptor CCR8 is preferentially expressed in Th2 but not Th1 cells. *J. Immunol*. 161:547–551.
12. D'Ambrosio, D., A. Iellem, R. Bonecchi, D. Mazzeo, S. Sozzani, A. Mantovani, and F. Sinigaglia. 1998. Selective up-

regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. *J. Immunol.* 161:5111–5115.
13. Chensue, S.W., K. Warmington, J.H. Ruth, N. Lukacs, and S.L. Kunkel. 1997. Mycobacterial and schistosomal antigen-elicited granuloma formation in IFN-gamma and IL-4 knockout mice: analysis of local and regional cytokine and chemokine networks [published erratum at 162:3106]. *J. Immunol.* 159:3565–3573.
14. Campbell, E.M., I.F. Charo, S.L. Kunkel, R.M. Strieter, L. Boring, J. Gosling, and N.W. Lukacs. 1999. Monocyte chemoattractant protein-1 mediates cockroach allergen-induced bronchial hyperreactivity in normal but not CCR2$^{-/-}$ mice: the role of mast cells. *J. Immunol.* 163:2160–2167.
15. Kung, T.T., H. Jones, G.K. Adams, III, S.P. Umland, W. Kreutner, R.W. Egan, R.W. Chapman, and A.S. Watnick. 1994. Characterization of a murine model of allergic pulmonary inflammation. *Int. Arch. Allergy Immunol.* 105:83–90.
16. Lukacs, N.W., R.M. Strieter, K. Warmington, P. Lincoln, S.W. Chensue, and S.L. Kunkel. 1997. Differential recruitment of leukocyte populations and alteration of airway hyperreactivity by C-C family chemokines in allergic airway inflammation. *J. Immunol.* 158:4398–4404.
17. Ohara, J., and W.E. Paul. 1985. Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. *Nature.* 315:333–336.
18. Abrams, J. 1995. Immunometric assay of mouse and human cytokines using NIP-labelled anti-cytokine antibodies. *Curr. Protocols Immunol.* 13:1–15.
19. Chensue, S.W., K.S. Warmington, J.H. Ruth, P. Lincoln, and S.L. Kunkel. 1995. Cytokine function during mycobacterial and schistosomal antigen-induced pulmonary granuloma formation. Local and regional participation of IFN-gamma, IL-10, and TNF. *J. Immunol.* 154:5969–5976.
20. Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.
21. Jonas, E., T.D. Sargent, and I.B. Dawid. 1985. Epidermal keratin gene expressed in embryos of Xenopus laevis. *Proc. Natl. Acad. Sci. USA.* 82:5413–5417.
22. Hall, L.L., G.R. Bicknell, L. Primrose, J.H. Pringle, J.A. Shaw, and P.N. Furness. 1998. Reproducibility in the quantification of mRNA levels by RT-PCR-ELISA and RT competitive-PCR-ELISA. *BioTechniques.* 24:652–658.
23. Ferber, I.A., H.J. Lee, F. Zonin, V. Heath, A. Mui, N. Arai, and A. O'Garra. 1999. GATA-3 significantly downregulates IFN-gamma production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels. *Clin. Immunol.* 91:134–144.
24. Openshaw, P., E.E. Murphy, N.A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med.* 182:1357–1367.
25. Cohn, L., J.S. Tepper, and K. Bottomly. 1998. IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J. Immunol.* 161:3813–3816.
26. Wang, J., K. Palmer, J. Lotvall, S. Milan, X.F. Lei, K.I. Matthaei, J. Gauldie, M.D. Inman, M. Jordana, and Z. Xing. 1998. Circulating, but not local lung, IL-5 is required for the development of antigen-induced airways eosinophilia. *J. Clin. Invest.* 102:1132–1141.
27. Yamaguchi, Y., Y. Hayashi, Y. Sugama, Y. Miura, T. Kasahara, S. Kitamura, M. Torisu, S. Mita, A. Tominaga, and K. Takatsu. 1988. Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor. *J. Exp. Med.* 167: 1737–1742.
28. Yamaguchi, Y., T. Suda, J. Suda, M. Eguchi, Y. Miura, N. Harada, A. Tominaga, and K. Takatsu. 1988. Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J. Exp. Med.* 167:43–56.
29. Sher, A., R.L. Coffman, S. Hieny, P. Scott, and A.W. Cheever. 1990. Interleukin 5 is required for the blood and tissue eosinophilia but not granuloma formation induced by infection with *Schistosoma mansoni*. *Proc. Natl. Acad. Sci. USA.* 87:61–65.
30. Hsieh, C.S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of TH1 CD41 T cells through IL-12 produced by Listeria-induced macrophages. *Science.* 260:547–549.
31. Le Gros, G., S.Z. Ben-Sasson, R. Seder, F.D. Finkelman, and W.E. Paul. 1990. Generation of interleukin 4 (IL-4)–producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4–producing cells. *J. Exp. Med.* 172:921–929.
32. Hsieh, C.S., S.E. Macatonia, A. O'Garra, and K.M. Murphy. 1995. T cell genetic background determines default T helper phenotype development in vitro. *J. Exp. Med.* 181:713–721.
33. Swain, S.L., A.D. Weinberg, M. English, and G. Huston. 1990. IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* 145:3796–3806.
34. Roos, R.S., M. Loetscher, D.F. Legler, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1997. Identification of CCR8, the receptor for the human CC chemokine I-309. *J. Biol. Chem.* 272:17251–17254.
35. Goya, I., J. Gutierrez, R. Varona, L. Kremer, A. Zaballos, and G. Marquez. 1998. Identification of CCR8 as the specific receptor for the human beta-chemokine I-309: cloning and molecular characterization of murine CCR8 as the receptor for TCA-3. *J. Immunol.* 160:1975–1981.
36. Bernardini, G., J. Hedrick, S. Sozzani, W. Luini, G. Spinetti, M. Weiss, S. Menon, A. Zlotnik, A. Mantovani, A. Santoni, and M. Napolitano. 1998. Identification of the CC chemokines TARC and macrophage inflammatory protein-1 beta as novel functional ligands for the CCR8 receptor. *Eur. J. Immunol.* 28:582–588.
37. Howard, O.M., H.F. Dong, A.K. Shirakawa, and J.J. Oppenheim. 2000. LEC induces chemotaxis and adhesion by interacting with CCR1 and CCR8. *Blood.* 96:840–845.
38. Sozzani, S., W. Luini, G. Bianchi, P. Allavena, T.N. Wells, M. Napolitano, G. Bernardini, A. Vecchi, D. D'Ambrosio, D. Mazzeo, et al. 1998. The viral chemokine macrophage inflammatory protein-II is a selective Th2 chemoattractant. *Blood.* 92:4036–4039.
39. Endres, M.J., C.G. Garlisi, H. Xiao, L. Shan, and J.A. Hedrick. 1999. The Kaposi's sarcoma-related herpesvirus (KSHV)-encoded chemokine vMIP-I is a specific agonist for the CC chemokine receptor (CCR)8. *J. Exp. Med.* 189: 1993–1998.
40. Dairaghi, D.J., R.A. Fan, B.E. McMaster, M.R. Hanley, and T.J. Schall. 1999. HHV8-encoded vMIP-I selectively engages chemokine receptor CCR8. Agonist and antagonist profiles of viral chemokines. *J. Biol. Chem.* 274:21569–

21574.
41. Wilson, S.D., P.R. Burd, P.R. Billings, C.A. Martin, and M.E. Dorf. 1988. The expression and regulation of a potential lymphokine gene (TCA3) in CD4 and CD8 T cell clones. *J. Immunol.* 141:1563–1570.
42. Van Snick, J., F. Houssiau, P. Proost, J. Van Damme, and J.C. Renauld. 1996. I-309/T cell activation gene-3 chemokine protects murine T cell lymphomas against dexamethasone-induced apoptosis. *J. Immunol.* 157:2570–2576.
43. Metz, D.P., and K. Bottomly. 1999. Function and regulation of memory CD4 T cells. *Immunol. Res.* 19:127–141.
44. Taub, D.D., and J.J. Oppenheim. 1994. Chemokines, inflammation and the immune system. *Ther. Immunol.* 1:229–246.
45. Ward, S.G., K. Bacon, and J. Westwick. 1998. Chemokines and T lymphocytes: more than an attraction. *Immunity.* 9:1–11.
46. Sallusto, F., B. Palermo, A. Hoy, and A. Lanzavecchia. 1999. The role of chemokine receptors in directing traffic of naive, type 1 and type 2 T cells. *Curr. Top. Microbiol. Immunol.* 246: 123–128.
47. Sallusto, F., C.R. Mackay, and A. Lanzavecchia. 2000. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu. Rev. Immunol.* 18:593–620.
48. Lloyd, C.M., T. Delaney, T. Nguyen, J. Tian, A.C. Martinez, A.J. Coyle, and J.C. Gutierrez-Ramos. 2000. CC chemokine receptor (CCR)3/eotaxin is followed by CCR4/monocyte-derived chemokine in mediating pulmonary T helper lymphocyte type 2 recruitment after serial antigen challenge in vivo. *J. Exp. Med.* 191:265–274.
49. Cheng, L., S. Dejbakhsh-Jones, R. Liblau, D. Zeng, and S. Strober. 1996. Different patterns of TCR transgene expression in single-positive and double-negative T cells. Evidence for separate pathways of T cell maturation. *J. Immunol.* 156: 3591–3601.
50. Cerwenka, A., L.L. Carter, J.B. Reome, S.L. Swain, and R.W. Dutton. 1998. In vivo persistence of CD8 polarized T cell subsets producing type 1 or type 2 cytokines. *J. Immunol.* 161:97–105.
51. Brunet, L.R., E.A. Sabin, A.W. Cheever, M.A. Kopf, and E.J. Pearce. 1999. Interleukin 5 (IL-5) is not required for expression of a Th2 response or host resistance mechanisms during murine schistosomiasis mansoni but does play a role in development of IL-4-producing non-T, non-B cells. *Infect. Immun.* 67:3014–3018.
52. Boring, L., J. Gosling, S.W. Chensue, S.L. Kunkel, R.V. Farese, Jr., H.E. Broxmeyer, and I.F. Charo. 1997. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J. Clin. Invest.* 100:2552–2561.
53. Warmington, K.S., L. Boring, J.H. Ruth, J. Sonstein, C.M. Hogaboam, J.L. Curtis, S.L. Kunkel, I.R. Charo, and S.W. Chensue. 1999. Effect of C-C chemokine receptor 2 (CCR2) knockout on type-2 (schistosomal antigen-elicited) pulmonary granuloma formation: analysis of cellular recruitment and cytokine responses. *Am. J. Pathol.* 154:1407–1416.