

Attenuation of Allergen-Induced Responses in CCR6^{-/-} Mice Is Dependent upon Altered Pulmonary T Lymphocyte Activation

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We have established a defect in CCR6^{-/-} mice in response to a cockroach allergen airway challenge characterized by decreased IL-5 production, reduced CD4⁺ T and B cells as well as decreased eosinophil accumulation. To determine the nature of the defect in CCR6^{-/-} mice T lymphocyte populations from allergen-sensitized wild-type mice were transferred into sensitized CCR6^{-/-} mice. The reconstituted response was characterized by an increase in IL-5 levels, eosinophil accumulation, and serum IgE levels in recipient CCR6^{-/-} mice. Analysis of lymphocytes from draining lymph nodes of CCR6^{+/+} and CCR6^{-/-} sensitized or challenged mice demonstrated a significant decrease in IL-5 and IL-13 production in CCR6^{-/-} mice. In contrast, the systemic response in allergen-rechallenged spleen cells demonstrated no significant alteration in allergen-induced cytokine production. Transfer of isolated splenic T lymphocytes from sensitized CCR6^{+/+} mice induced airway hyperresponsiveness in wild-type but not CCR6^{-/-} naive mice, suggesting that T cells alone were not sufficient to induce airway hyperresponsiveness in CCR6^{-/-} mice. Additional analysis demonstrated decreased CD11c⁺, CD11b⁺ and CD11c, and B220 subsets of dendritic cells in the lungs of CCR6^{-/-} mice after allergen challenge. Using in vitro cell mixing studies with isolated pulmonary CD4⁺ T cells and CD11c⁺ cells from CCR6^{+/+} or CCR6^{-/-} mice, we demonstrate alterations in both CCR6^{-/-} T cells and CCR6^{-/-} pulmonary APCs to elicit IL-5 responses. Altogether, the defect in CCR6^{-/-} mice appears to be primarily due to an alteration in T cell activation, but also appears to include local pulmonary APC defects. *The Journal of Immunology*, 2005, 174: 2054–2060.

The role of chemokine receptors in the development of allergen-specific responses has been an active area of research over the past several years. The identification of particular chemokine receptors and their role in immunologic responses has been difficult to assess due to the lack of proper reagents and the complexity of the promiscuous chemokine systems (1, 2). Presently there are 16 CXCLs (CXCL1–CXCL16) and 28 CCLs (CCL1–CCL28) that have been identified (3). There are also two minor groups, C and CX3C chemokines, both having only a single identified member. These chemokine ligands bind to G protein-coupled seven transmembrane receptors, but the function and cellular expression patterns still require classification. Chemokines have diverse functions during asthmatic responses, which relate to recruitment, cellular activation/degranulation, differentiation, as well as directly altering the immune response (1). The identification of chemokines in the airways of asthma patients after allergen provocation initially suggested that these molecules might have a significant role in the accumulation of leukocytes (4). Furthermore, the expression of distinct chemokine receptors on infiltrating cell populations, especially lymphocytes and eosinophils, provides an attractive opportunity to attenuate the influx of these cell populations.

Another interesting aspect of the diverse role of chemokines in immunologic responses has been that several of the chemokines can be divided into compartmentalized functions depending upon their role in homeostatic vs inflammatory recirculation (5). Recent research has established delineations in chemokine receptors that are involved in homeostatic recirculation of lymphocytes and APCs vs those receptors involved in migration during immune activation (6, 7). One receptor, CCR6, is expressed by immature dendritic cells (DC)² and down-regulated during the maturation process as the DC migrates to the lymph node to participate in its APC function (8–10). In addition to its apparent role in APC function, CCR6 has also been identified on eosinophils and memory T lymphocytes, as well as on most B lymphocytes (11–17). Using CCR6-deficient mice, studies have demonstrated defects in DC positioning in the gut mucosa as well as defects in delayed-type hypersensitivity responses centered on T lymphocyte accumulation (18). Thus, CCR6 has the potential to participate in a vast range of immunologic responses and therefore may not be segregated to only homeostatic function. Our initial studies indicated that CCR6 is important in the generation of an allergic airway response using a cockroach allergen-induced model that recapitulates many aspects of asthma in humans. The data in the present studies extend those earlier results (19) and further demonstrate that CCR6⁺ T lymphocytes are important in the progression of the airway inflammatory responses generated in our animal model. However, these studies also identify that other cell populations are also required, especially the pulmonary DC.

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² Abbreviation used in this paper: DC, dendritic cell.

Materials and Methods

Mice

Male C57BL/6 mice were purchased from The Jackson Laboratory. Male CCR6^{-/-} mice were obtained as previously described and subsequently backcrossed for eight generations onto the C57BL/6 background (19, 20).

Cockroach Ag challenge

C57BL/6 mice were immunized with cockroach allergen (Holister Steir Laboratories) as previously described (19, 21, 22). Briefly, animals were immunized by i.p. injection with cockroach allergen (10 μ g) emulsified in IFA. After 14 days the mice were lightly anesthetized and given an intranasal administration of cockroach allergen (1 μ g) to localize the systemic response to the airway. Subsequently, 7 days later the animals were given an intratracheal injection of allergen (4 μ g in 40 μ l) followed by a second intratracheal administration 48 h later. This procedure has demonstrated a strong Th2-mediated, eosinophil-rich response.

Measurement of airway hyperreactivity

Airway hyperreactivity in anesthetized mice was measured as previously described (19, 21, 22) with a mouse plethysmograph (Buxco Electronics) using a direct ventilation method specifically designed for low tidal volumes. Briefly, mice were anesthetized with sodium pentobarbital, intubated via cannulation of the trachea with an 18-gauge metal tube, and ventilated with a Harvard pump ventilator (0.3 ml tidal volume; 120 breaths/min). Mice were sealed within the plethysmograph for 5 min before baseline resistance readings were taken (via the division of tracheal pressure by the change in box volume). A dose-response curve to i.v. methacholine was performed to determine the optimal dose required to induce airway hyperresponsiveness. Mice were subsequently injected with 150 μ g/kg methacholine and peak airway resistance recorded. The change in airway resistance was assessed by subtracting the baseline resistance from the peak methacholine-induced airway resistance.

RNA isolation and analysis

Lungs were harvested at various times postchallenge and RNA was prepared from them using TRIzol according to the manufacturer's specifications. Complementary DNA was prepared from individual lungs using the SuperScript Reverse Transcriptase kit (Invitrogen Life Technologies) with the addition of oligo(dT) primers. cDNA was analyzed by quantitative, real-time PCR using a TaqMan 7700 instrument (Applied Biosystems). TaqMan PCR consisted of 25 ng of cDNA, 0.9 μ M each diagnostic primer, 0.25 μ M diagnostic probe, 1 $\times$ final of rRNA predeveloped assay reagent (Applied Biosystems), and 1 $\times$ final of TaqMan Universal PCR Mastermix (Applied Biosystems). The default 7700 thermocycler parameters were used. Spectral Data from TaqMan runs were analyzed using the Sequence Detection Systems software, version 1.6.3 (Applied Biosystems). Raw data were normalized to GAPDH control standards within each well.

ELISA

Whole lungs were homogenized in 1 ml of high salt lysis buffer containing protease inhibitors. Debris-free supernatants were isolated and the cytokines measured by ELISA as described (19, 21, 22). Ab pairs from R&D Systems were used for analysis in the ELISA. The sensitivity of the analyses was $\sim$ 10 pg/ml. No cross-reactivity to any other chemokine or cytokine was detected.

Flow cytometric analysis

Lungs were dispersed using 0.2% collagenase (Sigma-Aldrich) in RPMI 1640 plus 15% FCS at 37°C for 45 min. After lysing RBCs, an FcR-blocking step was used to limit nonspecific staining. Subsequently, the cells were stained with either 5 μ g/ml CD4-FITC, CD8-FITC, B220-FITC, F4/80-FITC, CD11b-FITC, or isotype control FITC Ab for 30 min. For the DC subset determination we used a CD11c-PE or isotype control PE along with an appropriate FITC-labeled Ab previously listed for two-color analysis to define the subsets. Cells were fixed in 1% paraformaldehyde and kept in the dark at 4°C until analysis. Cell populations were analyzed using a Coulter XL flow cytometer (Beckman Coulter). Total number of cells for each population in individual lungs was calculated using gating percentage multiplied by total number of cells in each lung preparation.

MACS isolation of lymphocyte and DC populations

Spleens from sensitized CCR6^{+/+} or CCR6^{-/-} mice were dispersed through a wire mesh to a single cell suspension. Adherent cell populations were removed using plastic tissue culture plated adherence over 1 h incubation.

The nonadherent cells populations were washed and resuspended in PBS/BSA and lymphocytes were isolated using negative selection with immunomagnetic bead-coupled Abs to exclude contaminating immune cells by MACS system (Miltenyi Biotec). The Abs used for B cell isolation included anti-Thy1 (for T cells), anti-CD11b (for APC), and anti-NK1.1 (for NK cells). For isolation of T cells, anti-B220 (for B cells), anti-class II, and anti-NK1.1 were used. The T lymphocyte isolation routinely gave us 90–95% pure CD3⁺ T cells, whereas the B cell isolation procedure gave 80–85% purity. For CD11c cell isolation, the dispersed pulmonary cell populations were labeled with immunomagnetic bead-coupled anti-CD11c and the DC were isolated to >90% by positive selection.

Statistics

Statistical significance was determined by ANOVA and unpaired Student's *t* test. Values of *p* < 0.05 were considered significant.

Results

Deletion of CCR6 results in decreased accumulation of T and B lymphocytes into the lung

Our published observations indicated that there was an alteration of responses within the CCR6^{-/-} mice that related to attenuation of airway hyperreactivity, IL-5 production, eosinophilia, and a reduction in IgE responses (19). In the present studies we have further characterized these responses. The production of CCL20, the only CCR6 ligand, in the lungs of allergen-challenged mice was significantly increased at both the 8 and 24 h time points postallergen challenges (Fig. 1). Histologically, a clear difference can be observed in the lungs of CCR6^{+/+} vs CCR6^{-/-} mice (Fig. 2) related to both total inflammation including both eosinophil and mononuclear cell accumulation as previously reported (17). To further specifically examine the lymphocyte subset accumulation, lungs of wild-type and CCR6^{-/-} mice were dispersed by collagenase digestion and the number of individual lymphocyte subsets identified. Flow cytometric analysis revealed a significant decrease in both CD4⁺ T lymphocytes and B220⁺ B lymphocytes within the lungs of allergen challenged CCR6^{-/-} mice compared with the wild-type controls (Table I). Although CD8⁺ lymphocytes were reduced, they were not statistically different in the CCR6^{-/-} mice. Thus, it appeared that in addition to the previously reported reduction in eosinophil accumulation, CD4⁺ T cells and B cells were also reduced in the lungs of CCR6^{-/-} animals.

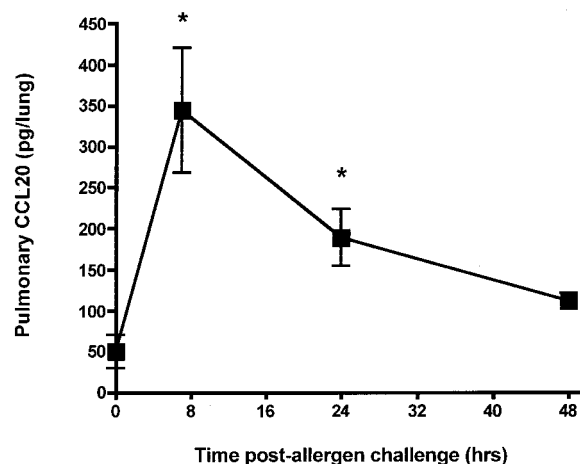


FIGURE 1. Increased temporal expression of CCL20 in the lungs of allergen-challenged mice. Lungs of allergic mice at specific time points were harvested and homogenized in buffer (PBS + antiprotease + 0.05% Triton X-100) and centrifuged to collect the supernatant. CCL20-specific ELISA was performed on the debris-free supernatant. Data represent mean $\pm$ SE of four to five mice per group. *, *p* < 0.05 compared with control.

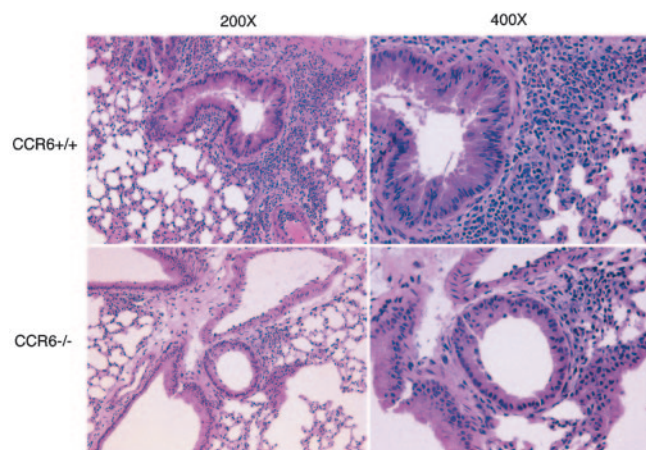


FIGURE 2. Histologic examination of CCR6^{+/+} and CCR6^{-/-} mice after allergen challenge demonstrates reduced inflammation in CCR6^{-/-} mice. Mouse lungs were harvested from allergic mice 24 h after final allergen challenge, inflated with 10% buffered formalin, fixed overnight, and processed for histologic analysis. Lower magnification ($\times 200$) depicts an overall decreased inflammation. Higher magnification ($\times 400$) indicates a reduced peribronchial eosinophil and mononuclear cell accumulation within the CCR6^{-/-} mice.

Differential alteration of cytokine production in spleen and lymph nodes of CCR6^{-/-} mice

Our initial analysis has been focused on the recruitment of cells to the lungs of allergen-challenged mice. However, it may be important to examine the draining lymph nodes, as migration into the nodes might also be altered. For this purpose, we have initially characterized the expression of CCR6 and its ligand in the draining nodes over time to ascertain whether this particular receptor is up-regulated. The data in Fig. 3 indicate that both CCR6 and its only ligand CCL20 are up-regulated by 2–4 h, maximal at 6 h and the expression maintained at least until 24 h postallergen challenge in draining thoracic lymph nodes. To better understand the role of CCR6 in local vs systemic responses we isolated spleen cells and thoracic lymph node cells from sensitized and challenged mice. Cells (5×10^6 /ml) were then rechallenged with cockroach allergen (200 protein nitrogen unit/ml) for 24 h and cell-free culture supernatant was harvested for cytokine analyses. The data in Fig. 4 illustrate that although the animals had similar cytokine responses in restimulated cells from spleens, the lymph node cells demonstrated a significant decrease in Th2-type cytokines in the CCR6^{-/-} mice compared with cells from CCR6^{+/+} mice. These data correlate well with the reduced pathophysiologic responses observed in the lung, including decreased airway hyperreactivity and eosinophilia previously reported (17). Interestingly, not only was IL-5 reduced as in the lungs, but a significant reduction in IL-13 expression was also observed. Thus, a more severe alteration of the cockroach-specific response may be evident in the draining lymph nodes of CCR6^{-/-} mice than in the lungs. There was no alteration of IFN- γ (Fig. 4) or IL-4 production (data not shown).

Transfer of T lymphocytes but not B lymphocytes partially reconstitutes the allergic response in CCR6^{-/-} animals

Because there were significant changes in the lymphocyte populations that migrated to the lung we wanted to examine whether the response in sensitized and challenged CCR6^{-/-} mice could be reconstituted using T or B lymphocytes from wild-type CCR6^{+/+} mice. Lymphocyte populations, CD3⁺ or B220⁺ cells, were isolated from spleens of cockroach allergen-sensitized animals (21 days) using negative selection MACS columns (see *Materials and*

Table I. Leukocyte subsets in allergic mice^a

Group	CD4 ⁺ ^b	CD8 ⁺	B220 ⁺ ^b
CCR6 ^{+/+}	11 $\pm$ 0.3	3.3 $\pm$ 0.9	5.5 $\pm$ 1.7
CCR6 ^{-/-}	6.4 $\pm$ 0.2	2.6 $\pm$ 0.7	2.8 $\pm$ 1.6

^a All values are ($\times 10^5$)/lung.

^b Significant difference between the two groups.

Methods). The isolated lymphocyte populations were greatly enriched for the particular cell populations; $>90\%$ T lymphocytes or $>80\%$ B lymphocytes, respectively. Cockroach allergen-sensitized CCR6^{-/-} mice were then used for tail vein transfer of the isolated wild-type cell populations (5×10^6 cells/mouse). After 24 h posttransfer the CCR6^{-/-} mice were challenged intratracheally with allergen two times at 48 h apart as previously described. When pulmonary IL-5 levels were examined a significant increase in IL-5 was observed in the animals that received T lymphocytes from wild-type CCR6^{+/+} animals (Fig. 5A). In addition, there were increased peribronchial eosinophils (Fig. 5B) and a reconstituted serum IgE response (Fig. 5C) in the animals that received splenic T cells from sensitized CCR6^{+/+} animals. Thus, the transfer of T cells appeared to reconstitute the local inflammatory and cytokine responses as well as the systemic levels of IgE. In contrast, when we examined the consequences of transferring B lymphocytes from CCR6^{+/+} sensitized animals into the CCR6^{-/-} animals in a similar manner as with the T lymphocytes there was no alteration observed in the pulmonary responses (data not shown). Furthermore, in animals that received enriched B lymphocytes from spleens of sensitized CCR6^{+/+} mice no alteration in IgE production was observed (data not shown). These data together indicate that the defect in the CCR6^{-/-} mice appeared to be centered on altered T cell activity.

Transfer of airway responses to naive mice by CCR6^{+/+} but not CCR6^{-/-} T lymphocytes

To determine whether T lymphocytes from CCR6^{-/-} mice were functional, isolated T cell populations from spleens of sensitized CCR6^{+/+} and CCR6^{-/-} mice were used for transfer studies into naive mice. After isolation the T lymphocytes (5×10^6 cells/mouse) were injected via tail vein into naive wild-type and CCR6^{-/-} mice. Twenty-four hours later the animals were challenged with two intratracheal allergen challenges 48 h apart. Airway hyperreactivity was then examined 24 h after the final allergen challenge. The data in Fig. 6 illustrate that T lymphocytes from wild-type mice but not CCR6^{-/-} mice transferred airway hyperreactivity to the naive CCR6^{+/+} mice. Using the same isolated cell populations we also injected naive CCR6^{-/-} mice to transfer the airway response. In these latter animals neither spleen cells from the wild-type nor the CCR6^{-/-} mice could transfer the response to the CCR6^{-/-} mice. Together these studies suggest two important aspects: first, the T lymphocytes from CCR6^{-/-} mice have a significant defect in their ability to transfer disease. Second, the inability of sensitized CCR6^{+/+} T lymphocytes to transfer disease to CCR6^{-/-} mice suggests that there may be a generalized defect in the pulmonary immune response in the CCR6^{-/-} involving other cell populations, such as DC, that could only be uncovered in a nonsensitized environment.

Differential DC numbers and cytokine production in pulmonary cells from CCR6^{-/-} mice

Our data just described on the inability of wild-type T cells from sensitized mice to transfer airway hyperreactivity into naive

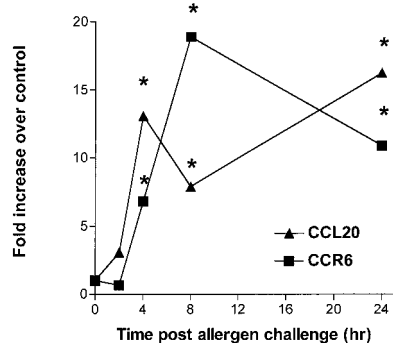


FIGURE 3. CCR6 and its only ligand, CCL20, are significantly up-regulated during allergen challenge in lung draining thoracic lymph nodes. Lymph nodes of four to five mice were harvested from the thoracic cavity of cockroach-sensitized mice at various times postchallenge and subjected to quantitative real-time PCR analysis for the expression of CCR6 and CCL20. Fold expression increases were calculated as increases over expression in lymph nodes taken from control mice. Data represent the mean of four to five mice per group. *, $p < 0.05$ compared with expression from lymph nodes from unchallenged control mice.

CCR6^{-/-} mice suggested that other cell populations may be defective within the CCR6^{-/-} mice. A cell population that is central to developing immune response and expresses CCR6 is DC. We therefore investigated the total number of CD11c⁺, MHC class II⁺ cells in the lungs of wild-type and CCR6^{-/-} mice and found no difference in the total numbers of these cells in the lungs of allergen-challenged mice (data not shown). However, when we examined the CD11c⁺ subsets, we found a decrease in CD11c⁺, CD11b⁺ (myeloid DC), and CD11c⁺, B220⁺ (plasmacytoid DC), but not CD11c⁺, CD8⁺ cells in the lungs of CCR6^{-/-} vs CCR6^{+/+} mice after allergen challenge (Table II). We next wanted to determine whether there were any functional differences in the CD11c⁺ cells from the lung. Pulmonary CD4⁺ T cells (>85%) and CD11c⁺ (>95%) cell populations were isolated from wild-type or CCR6^{-/-} sensitized mice 24 h after allergen challenge using MACS (see *Materials and Methods*) and subjected to allergen recall response in vitro using cell mixing experiments. In studies depicted in Fig. 7, we determined that when we combined CD11c⁺ and CD4⁺ T cells from wild-type mice they produced significant increases in IL-5 expression in response to allergen re-

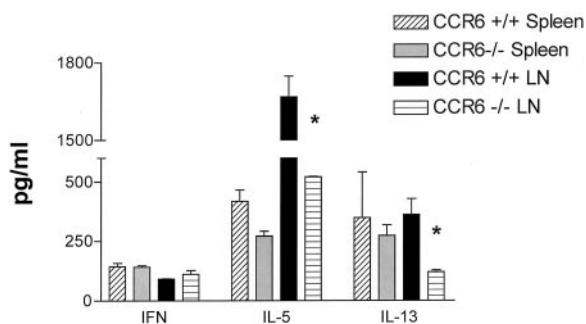


FIGURE 4. Alteration of cytokine responses in CCR6^{-/-} lymph node but not spleen cells compared with CCR6^{+/+} mice. Cells were harvested from cockroach allergen sensitized and allergen rechallenged mice. Single cell suspensions (5×10^6 /ml) were rechallenged in vitro with 200 protein nitrogen unit/ml ($\sim 2 \mu\text{g}/\text{ml}$) of cockroach allergen. After 24 h cell-free supernatants were harvested and cytokine expression levels analyzed by specific ELISA. Data represent the mean $\pm$ SE of four to five mice per group. *, $p < 0.05$. Data from 4 or 48 h cultures showed no significant increase in cytokines in either group.

challenge. In contrast, when CD11c⁺ cells from CCR6^{-/-} mice were mixed with CD4⁺ T cells from CCR6^{+/+} mice no increase of IL-5 was induced, suggesting that there may be a defect in the ability of the CCR6^{-/-}, CD11c⁺ cells to induce the activation of the T cells. When CD11c⁺ cells and T cells from CCR6^{-/-} mice or when wild-type CD11c⁺ cells were mixed with CCR6^{-/-} T cells there was no significant increase in IL-5 expression. No differences were found when IL-4 and IL-13 expression was examined. These results suggested a defect in allergen-induced response by CCR6^{-/-} T cells even in the context of CD11c⁺ cells from CCR6^{+/+} mice related to IL-5 production. Altogether, these mixing studies further suggest that the observed altered responses may be controlled by both the CD11c⁺ cells and infiltrating CD4⁺ T cell populations in the challenged mice during allergic responses

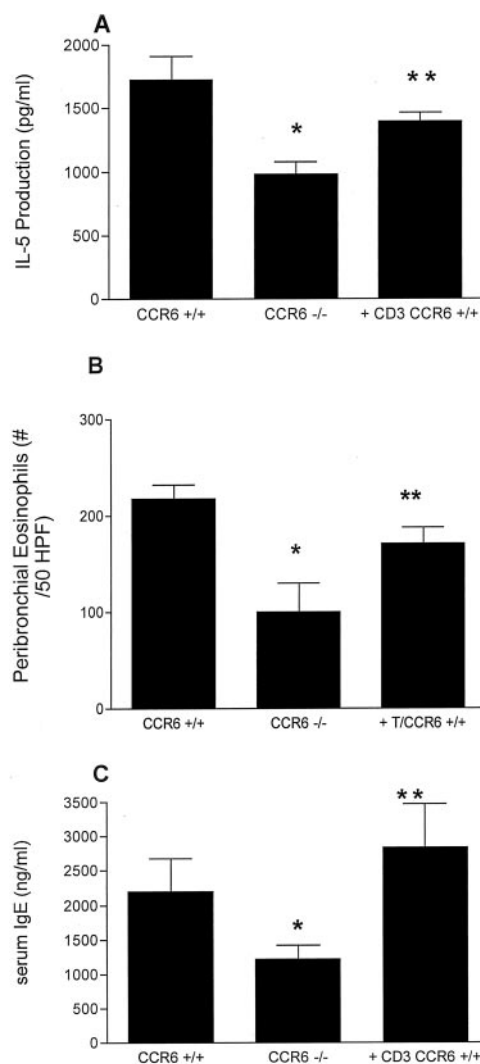
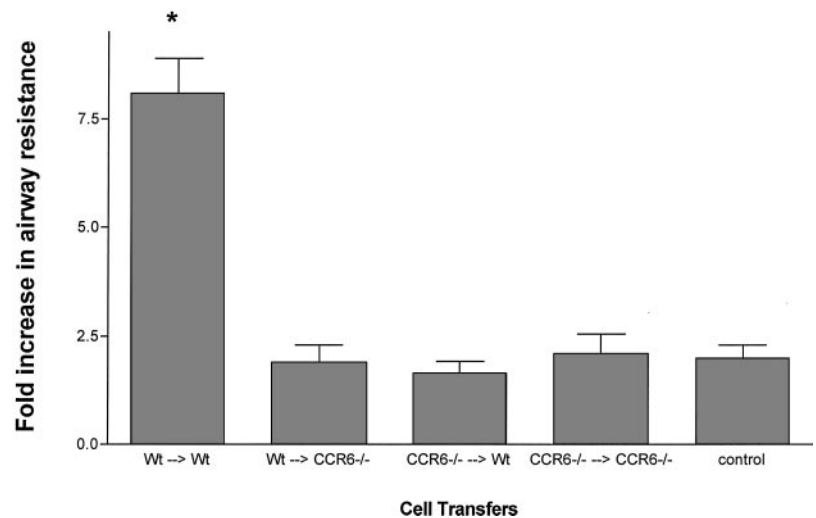


FIGURE 5. Reconstitution of pulmonary cytokines, peribronchial eosinophils, and serum IgE levels by transfer of splenic T cells from CCR6^{+/+} to CCR6^{-/-} mice. MACS isolated splenic T cell populations (5×10^6 /mouse) were transferred from sensitized CCR6^{+/+} mice into sensitized CCR6^{-/-} mice via tail vein 24 h before final allergen challenges (+CD3 CCR6^{+/+} group). Lungs and serum, harvested 24 h postfinal intratracheal challenge, were assessed for IL-5 (A), peribronchial eosinophilia (B), and serum IgE levels (C). The data represent the mean $\pm$ SE of four to five mice per group. A repeat experiment demonstrated similar responses. Airway hyperreactivity was also assessed but no reconstitution change was observed after the transfer in the CCR6^{-/-} mice (data not shown). *, $p < 0.05$ compared with CCR6^{+/+}; **, $p < 0.05$ compared with CCR6^{-/-} with no transfer.

FIGURE 6. Transfer of isolated splenic T cells from sensitized CCR6^{+/+} but not CCR6^{-/-} mice transfer airway hyperreactivity into naive CCR6^{+/+} mice. Isolated T cells (5×10^6 /mouse) from sensitized mice were transferred via tail vein into either CCR6^{+/+} (Wt) or CCR6^{-/-} naive mice. Twenty-four hours after transfer the naive mice were subjected to two intratracheal cockroach allergen challenges separated by 48 h. The airway hyperresponsiveness was assessed by box plethysmography at 24 h postfinal allergen challenge. Normal mice or mice given naive spleen cells displayed similar change in airway resistance (ranging from 0.9 to 1.8 fold increase). Data represent the mean $\pm$ SE of five mice per group.



and correlate to the observed differences identified in the earlier experiments.

Discussion

A number of chemokines and receptors have been implicated in pulmonary specific immune responses during allergic airway disease (4, 23–27). The present study highlights how a specific chemokine receptor-ligand interaction has a role in a complex immune response and involves multiple cell populations. Our initial data in these studies indicated a defective accumulation of CD4⁺ T and B lymphocytes within the allergen challenged lungs of CCR6^{-/-} mice, which led to the defective production of IL-5 and eosinophilia. CCL20 and CCR6 were increased in the lung and draining lymph node during allergen activation, which may be important for recruitment of multiple populations, including immature or maturing DC as well as B and T lymphocytes. Previous studies have established that subsets of CD4⁺ and CD8⁺ lymphocytes are CCR6⁺ (28), whereas activated T lymphocytes up-regulate CCR6 on their surface and are able to migrate to CCL20 (11, 12, 29). Thus, CCR6 may be used by T lymphocytes to migrate into both the allergen-challenged lung and into the draining lymph node. These latter concepts were supported by transfer of T lymphocytes from CCR6^{+/+} mice into CCR6^{-/-} animals, which allowed reconstitution of the IL-5 and eosinophil responses within the lungs of CCR6^{-/-} mice. In addition, transferred T lymphocytes from CCR6^{+/+} mice allowed reconstitution of the humoral response and outlined a requirement for CCR6⁺ T lymphocytes for B cells to produce IgE, perhaps by allowing optimal localization and interaction between the two cell populations (30). Studies to examine this latter aspect are presently ongoing with focus on cognate interaction of B and T lymphocytes in the lymph node.

Although we could reconstitute the inflammatory response (IL-5, eosinophils) within the airway by simply transferring T lymphocytes from CCR6^{+/+} sensitized mice, there was no reconsti-

tution of the altered physiologic responses. The mechanism for this observation is not presently clear. However, these data would be consistent with the results in naive animals in which we could not transfer airway hyperreactivity into naive CCR6^{-/-} mice with cells from sensitized wild-type mice. A likely scenario is that there is not only a defect in T lymphocyte migration but also a defect in another cell population, likely DC (8, 20, 31). Within the mouse lung both DC and alveolar macrophages can express CD11c and both populations likely contribute to the immune responses at this mucosal surface (32–35). The CCR6^{-/-} mice have a similar number of total CD11c⁺ cells in the lungs in both control and sensitized/challenged mice. However, the examination of CD11c subsets indicated that there was a significant decrease in myeloid and plasmacytoid type DC (CD11c⁺, CD11b⁺, and CD11c⁺, B220⁺) in the CCR6^{-/-} mice. It is the myeloid DC subset that has been implicated in establishing and promoting Th2-type responses in the lungs of mice, whereas the plasmacytoid have been implicated in promoting a suppressive response in allergic lungs (36–38). Previous studies in these same CCR6^{-/-} mice have demonstrated that there is a positioning problem of CD11c⁺ cells in the intestines of CCR6^{-/-} mice (20), which is consistent with a more recent study that found CCR6 and its ligand are important for positioning of the CD11c⁺ cells in epithelial and lymphoid tissues (28). A similar defect might be operative with the current study in the lungs. However, the mixing experiments demonstrated that the cells from the CCR6^{-/-} mice had little ability to generate IL-5 expression. We hypothesize that the CCR6^{-/-} mice have a distinct defect in the pulmonary DC population within the lung during the allergen-induced responses. This concept corresponds directly with data from the in vivo model after allergen challenge where alterations in cytokines correspond to decreases in pulmonary pathophysiology. Our future studies will further examine DC populations within the CCR6^{-/-} mice to determine whether 1) there is an altered topological distribution of DC in lungs and lymph

Table II. Differences in CD11c subsets in the lungs of CCR6^{-/-} mice

Subsets	Group	% Total Lung	No. of Cells/Lung ($\times 10^5$)	p Value
CD11c/CD11b	Wild-type	1.76 $\pm$ 0.28	3.80 $\pm$ 0.45	0.049
	CCR6 ^{-/-}	0.98 $\pm$ 0.13	2.25 $\pm$ 0.28	
CD11c/B220	Wild-type	2.61 $\pm$ 0.28	5.82 $\pm$ 0.35	0.002
	CCR6 ^{-/-}	1.55 $\pm$ 0.12	3.60 $\pm$ 0.34	
CD11c/CD8	Wild-type	0.77 $\pm$ 0.14	1.60 $\pm$ 0.32	0.36
	CCR6 ^{-/-}	0.81 $\pm$ 0.34	1.14 $\pm$ 0.38	

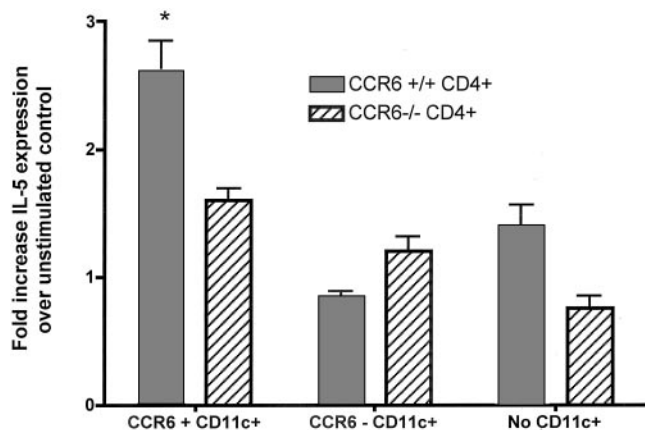


FIGURE 7. Differential expression of cytokines by pulmonary T cells incubated with wild-type or CCR6^{-/-} CD11c cells. Pulmonary CD4⁺ T cells and DC were isolated from lungs of allergen sensitized and challenged CCR6^{+/+} and CCR6^{-/-} mice using MACS. Pulmonary CD4⁺ T cells were enriched by negative selection using Abs to CD11c, MHC class II, CD19, CD8, and NK cells, whereas DC were positively selected using CD11c Abs. Combinations of T cells (4×10^5) and DC (2×10^5) from the different mice were subjected to cockroach allergen challenge and mRNA isolated and assessed for cytokine expression by real-time PCR. Fold increases were calculated over media control of the same cell combination in two repeat assays in cells harvested at 24 h postchallenge (time of peak inflammation).

nodes, 2) whether there is a defect in migration of the DC within the lung and/or to the lymph node, or 3) whether the DC from CCR6^{-/-} have a functional defect that might be dependent on CCR6 signaling. Previous observations have detailed how DC move into the airway upon allergen challenge (39). Because CCL20 has been shown to be produced by airway epithelial cells (40, 41), CCR6 might be required for localization of DC and/or T cells to the airway. CCL20 production in the airway may be a crucial step for immature CCR6⁺ DC to track to the airway, become activated, and acquire Ag for transport back to the draining lymph node. Furthermore, there may be specific defects in defined subsets of DC within the airway and/or decreased accumulation within the draining lymph nodes attributed to the CCR6 deficiency. This latter concept would be consistent with studies demonstrating that CCR6 is preferentially displayed on myeloid DC populations and allergen challenge invokes an influx of circulating myeloid DC into the lung (28). A defect in this latter process might also significantly alter subsequent allergen responses. Although the data are consistent with the concept that the pulmonary mucosal environment appears to be altered in these animals, the present studies do not specifically address the role of CCR6 in this migratory process within the CCR6^{-/-} mice and further experiments will need to be performed.

Altogether, these studies further extend our observations in CCR6^{-/-} mice and identify a specific defect in T lymphocyte function in the lungs during allergen challenge. These changes may stem completely from a potential defect in APC function, but likely are dependent upon both T cell-mediated and DC-mediated responses. Although we have further defined some of the defects in the CCR6^{-/-} animals we will need to investigate how CCR6 specifically contributes to the migration and/or activation of specific populations within the airways and draining lymph nodes of allergic animals.

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