Regulation of Cockroach Antigen-Induced Allergic Airway Hyperreactivity by the CXCR3 Ligand CXCL9

Molly S. Thomas, Steven L. Kunkel and Nicholas W. Lukacs

http://www.jimmunol.org/content/173/1/615

---

**References**

This article cites 63 articles, 33 of which you can access for free at:
http://www.jimmunol.org/content/173/1/615.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Regulation of Cockroach Antigen-Induced Allergic Airway Hyperreactivity by the CXCR3 Ligand CXCL9

Molly S. Thomas, Steven L. Kunkel, and Nicholas W. Lukacs

Allergic asthma is an increasing health concern, especially for children living in metropolitan areas (1, 2). Allergic asthma is characterized by a robust Th2-type inflammatory response arising from an intense lymphocytic infiltration of the bronchial mucosa and elaboration of Th2-type cytokines, such as IL-4, IL-5, and IL-13 (3–8). The selective chemokine-mediated recruitment of subsets of T cells and eosinophils into the lung allows for the elaboration of epithelial cell damage, mucus production, and increased airway hyperreactivity that are characteristic of the asthmatic response (9–13).

Chemokines are chemotactic cytokines that function in the recruitment of specific cellular populations to sites of injury and inflammation. CXCL9 (monokine induced by IFN-γ), CXCL10 (IFN-inducible protein, 10 kDa), and CXCL11 (IFN-inducible T cell α chemoattractant) are members of the CXC/ELR+ family of chemokines. These proteins are induced by IFN-γ in a variety of cells, including monocytes and epithelial cells (14). CXCL9, CXCL10, and CXCL11 are ligands for CXCR3 (15) that is predominantly expressed on CD45RO+CD4+ and CD8+ T cells as well as on NK cells, monocytes, and APCs (16–20). In addition, it is widely reported that CXCR3-expressing T lymphocytes are specifically recruited to sites of inflammation that exhibit a Th1-type phenotype, such as in rheumatoid arthritis and inflammatory bowel disease (21–24).

It has been established that Th1 and Th2 responses are antagonistic to each other (25–29). Administration of IFN-γ, a Th1-type cytokine, into the airways of allergic mice has been demonstrated to reduce the severity of the Th2-type response and attenuate asthma-like responses (30, 31). The CXCR3 ligands CXCL9 and CXCL10 are IFN-γ-inducible chemokines and generally correspond to Th1-type diseases. Several reports have described a role for CXCL9 in the modulation of allergic airway disease. It has been reported that asthmatics have increased levels of CXCL9 in their bronchoalveolar lavage (BAL) fluid (32). Overexpression of CXCL10 during sensitization to OVA has been shown to decrease levels of Th2-type cytokines and eosinophil accumulation in the airway after OVA challenge (33). CXCL10 has also been demonstrated to enhance pulmonary eosinophil accumulation and increase pulmonary levels of Th2-type cytokines after cockroach allergen challenge (34).

CXCL9 is almost exclusively induced by IFN-γ and is expressed by monocytes and epithelial cells as well as on endothelial cells of high endothelial venules and in the T cell:B cell junctions in draining lymph nodes (14, 35, 36). CXCL9 has been shown to play a role in viral and bacterial cell clearance and may also take part in the recruitment of APCs to draining lymph nodes and in facilitating the APC, T cell, and B cell interactions leading to maximal Ab production (35). However, although no role for CXCL9 in allergic airway disease has been described to date, we were interested in investigating the role that the CXCR3 ligand, CXCL9, has in the modulation of allergic inflammation.
Materials and Methods

Animals

BALB/c mice, 6–8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained under standard pathogen-free conditions. All experiments involving the use of animals were approved by the University of Michigan care and use of animals committee.

Sensitization and induction of the allergic airway response

Normal BALB/c mice were sensitized i.p. and s.c. with 1000 protein nitrogen units (PNU) of cockroach Ag (CRA; Holliser Stier, Toronto, Canada) 1/1 in IFA (Sigma-Aldrich, St. Louis, MO). Mice were challenged intranasally (i.n.) with 150 PNU of CRA on day 14 after initial sensitization to localize the response to the lung. Previous studies in our laboratory have demonstrated that i.n. CRA challenge on day 14 after initial sensitization induced little inflammation in the lung. The parameters of the allergic response in mice that only received an i.n. allergen challenge on day 14 were measured on day 21, corresponding to the 24 h after intratrachial (i.t.) challenge time point in the treatment groups, and are representative of baseline levels of those measured parameters. To neutralize CXCL9 in the airway 100 μg of purified Ab against murine CXCL9 or control IgG (generated in our laboratory as previously described (34)) along with 300 PNU of CRA were instilled directly into the lung via i.t. injection on day 20 after initial sensitization. In separate experiments 300 PNU of CRA in sterile PBS or 80, 200, or 400 ng of recombinant murine CXCL9 (PeproTech, Rocky Hill, NJ) along with 300 PNU of CRA were instilled into the lung via i.t. challenge on day 20 after initial sensitization. All described time points were measured after i.t. challenge on day 20. In all experiments mice were anesthetized with 150 μg/kg ketamine and 45 μg/kg xylazine in normal saline, and the trachea was aseptically exposed and prebored using a 21-gauge needle. Ab and CRA or CRA with or without recombinant CXCL9 was instilled into the trachea in a total volume of 40 μl using a Hamilton syringe (Reno, NV), and wounds were closed using surgical staples.

Measurement of airway hyperreactivity

At various time points after the day 20 intratrachial allergen challenge the mice were anesthetized with 3.3 mg of pentobarbital (Vortech Pharmaceuticals, Dearborn, MI), and airway hyperreactivity was measured using a Buxco mouse plethysmograph (Buxco, Troy, NY). The trachea was cannulated using an 18-gauge needle, and mice were ventilated using a Harvard pump ventilator (tidal volume, 0.4 ml; frequency, 120 breaths/min; positive end-inspiratory pressure, 2.5–3.0 cm H 2 O; Harvard Apparatus, Holliston, MA). Changes in lung volume, represented by changes in transpulmonary pressure (P tp), were measured by a differential transducer. A second transducer was used to measure pressure swings at the opening of the trachea (P box). The trachea transducer was calibrated at a constant pressure of 20 cm of H 2 O. Airway reactivity was measured as the increase in transpulmonary pressure (P tp – P box) that occurred when the inspiratory volume and corresponds to airway hyperreactivity. Baseline airway reactivity, the airway reactivity observed during mechanical ventilation before methacholine challenge, was recorded, and the change in airway reactivity (∆R) was measured after i.v. injection of 12.5 μg of methacholine (Sigma-Aldrich) as previously described (37).

Morphometric analysis of airway and peribronchial eosinophil accumulation

Lungs were lavaged with 0.9% saline (Abbott Laboratories, North Chicago, IL). Bronchoalveolar cells were cytopsin and stained with Diff-Quik (Dade International, Miami, FL), and the percentages of monocytes, lymphocytes, neutrophils, and eosinophils in the airway were determined. The left lobe of lung was excised, inflated with 10% formalin, and embedded in paraffin. Five-micron sections were cut and stained with H&E. The number of eosinophils was determined using Image J software (NIH) and calculated as the number per high power fields around the largest bronchioles in each lung section.

Cytokine quantification using specific ELISA

The lower right lobe of lung was excised, snap-frozen in liquid nitrogen, and homogenized on ice using a Tissue Tearor (Biospec Products, Racine, WI) for 30 s in 1 mL of PBS (BioWhittaker, Walkersville, MD) containing 0.05% Triton X-100 (Sigma-Aldrich) and one tablet of Complete protease inhibitor (Roche, Mannheim, Germany). Cytokine protein concentra-

trations in these lung homogenates and BAL supernatants were quantified using basic sandwich ELISAs as previously described (34).

Immunohistochemical detection of CXCL9 protein expression

The left lobes of lung from mice immunized and subsequently challenged with CRA were excised 24 h post i.t. CRA challenge, inflated with formalin, and embedded with paraffin. Sections (5 μm) were cut and mounted onto glass slides. Sections were deparaffinized, rinsed in fresh xylene, and rehydrated through graded alcohol (100, 95, 70, and 50%) and PBS for 3 min each. Endogenous peroxidase activity was quenched by incubating the rehydrated sections in 1/1 methanol and 3% hydrogen peroxide for 15 min. CXCL9 protein expression was visualized using an HRP-3-amino-9-ethylcarbazole cell and tissue staining kit (R&D Systems, Minneapolis, MN) along with primary rabbit anti-mouse Ab against CXCL9 generated and purified in our laboratory (see above; 50 μg/ml) or rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 50 μg/ml) as an isotype control.

Analysis of mRNA expression

Lymph node cells (3.0 × 10 5) isolated from the mediastinal and thoracic lymph nodes from mice 24 h after i.t. CRA challenge were restimulated with 50 ng of recombinant murine CXCL9 or CXCL10 with or without 300 PNU of CRA in DMEM containing 15% FCS and 15% penicillin-streptomycin-Fungizone (BioWhittaker) for 6 or 24 h. RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA (1.0 μg) was then reverse transcribed using an 18-μer oligo(dT) (Sigma-Aldrich) and Moloney murine leukemia virus reverse transcriptase at 37°C for 60 min; the reaction was stopped by incubating the cDNA at 90°C for 5 min. One microgram of cDNA was then amplified using predeveloped chemokine primer and probe pairs for murine IL-4, IL-12, and IFN-γ from Applied Biosystems (Foster City, CA) in a TaqMan 7700 thermocycler for 40 cycles of 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 h. The fold difference in mRNA expression between treatment groups was determined using software developed by Applied Biosystems. The fold difference is reported using the expression of the target gene in unstimulated lymph node cells as the reference baseline.

Analysis of in vitro cytokine expression

Lymph node cells (3.0 × 10 5) isolated from the mediastinal and thoracic lymph nodes from mice 24 h after i.t. CRA challenge were restimulated with 50 ng of recombinant murine CXCL9 or CXCL10 with or without 300 PNU of CRA in DMEM containing 15% FCS and 15% penicillin-streptomycin-Fungizone for 48 h. Lymph node cells were lysed in 200 μl of Nonident P-40 lysis buffer (1% Triton X-100, 50 mM NaF, 2 mM EDTA, 0.15M NaCl, and 0.1 M NaPO 4 ) on ice for 30 min, insoluble debris was removed via centrifugation, and lysates were subsequently frozen at −80°C until analysis. Endogenous production of IL-4 and IFN-γ after chemokine with or without allergen stimulation was quantitated using basic sandwich ELISAs as described above.

Flow cytometric analysis

Lungs were dispersed in 0.2% collagenase (Sigma-Aldrich) in RPMI 1640 (BioWhittaker) and 15% FCS (Atlas) at 37°C for 45 min. After lysing RBCs with ammonium chloride lysis buffer (4.01 g of NH 4 Cl, 0.42 g of NaHCO 3 , and 0.185 g of Tetra-sodium EDTA in 500 ml of deH 2 O), the cells were stained with either 0.5 mg/ml anti-murine CD8-FITC or anti-murine CD4-FITC (BD PharMingen, San Diego, CA) and anti-murine CXCR3 (Y-16; Santa Cruz Biotech, Santa Cruz, CA) at 4°C for 30 min, washed, and resuspended in 2% paraformaldehyde. The number of CD4+ T cells was measured by flow cytometry (Beckman Coulter, Fullerton, CA).

Isolation of murine eosinophils from Schistosome mansoni-infected mice

Swiss albino mice infected with the parasite S. mansoni were obtained from Dr. F. Lewis (Biomedical Research Institute, Bethesda, MD). At 7–8 wk
postinfection the mice were injected i.p. with 3000 *S. mansoni* eggs and 50 ng of recombinant murine IL-5 (R&D Systems) in 1 ml of 4.0% thioglycolate medium (Difco, Detroit, MI). Eosinophils were isolated via a peritoneal wash with 25 mM EDTA and 0.5% penicillin/streptomycin (BioWhittaker) 48 h after i.p. egg injection. The isolated eosinophils were incubated in RPMI 1640 medium containing 10% FCS, 1% L-glutamine, and 0.5% penicillin/streptomycin (BioWhittaker) for 1 h at 37°C before further purification. The isolated cells were then resuspended in 90 μl of sterile PBS (BioWhittaker) and 0.5% BSA (Sigma-Aldrich)/10^7 cells; incubated with 10 μl/10^7 cells of anti-CD90 (Thy 1.2), anti-CD45RO (B220), and anti-MHC II Abs coupled to magnetic beads (Miltenyi Biotec, Auburn, CA); and purified via negative selection by running the cell suspension through a magnetic column. The purified population was consistently >98% eosinophils, and these eosinophils were consistently >95% viable by trypan blue exclusion.

**Eosinophil chemotaxis**

Purified murine eosinophils were assayed for their ability to chemotax to the CCR3 ligand, CCL11, and the CXCR3 ligands CXCL9 and CXCL10 using 12-well Boyden chambers (NeuroProbe, Gaithersburg, MD). Varying concentrations of the recombinant murine CCL11, CXCL9, or CXCL10 in PBS and 0.5% BSA (medium) was placed in the lower wells of the chamber. Freshly purified eosinophils (2 × 10^5) in medium were placed in the upper wells of the chamber. The assembled chambers were then incubated at 37°C for 1 h. In separate experiments the purified eosinophils were pretreated with 10 or 100 ng/ml recombinant CXCL9 for 30 min at room temperature and then washed to remove exogenous chemokine before chemotactic analysis. Eosinophil chemotaxis through a 5-μm pore size polycarbonate, polyvinylpolyfluoride membrane (Osmonics, Minnetonka, MN) was determined by staining the membrane with DifQuik and counting the number of eosinophils in five consecutive ×1000 high power fields per well on the underside of the membrane.

**Results**

**CXCL9 expression is induced in airway bronchial epithelial cells after Ag challenge**

A number of chemokines, including CXCL10, CCL11, CCL2, and CCL3, have been previously shown to play important roles in CRA-induced allergic airway disease. However, allergen sensitization and challenge do not result in a global up-regulation of all cytokines in the lung. For example the cytokines IL-10, IL-12, and IFN-γ and the chemokines CCL17 and CCL22 were not significantly up-regulated in the lung after CRA challenge in the presented experiments. To demonstrate the relevance of CXCL9 in allergic airway disease, we determined the levels of pulmonary CXCL9 expression after allergen challenge. Bronchial epithelial cells and monocytes have been previously reported to express CXCL9 (14, 36). We have demonstrated that bronchial epithelial cells, but not pulmonary macrophages, express CXCL9 in allergen-sensitized and challenged lungs by immunohistochemical staining (Fig. 1A). CXCL9 expression in whole lung homogenates is significantly increased in a time-dependent manner after allergen challenge as assessed by ELISA (Fig. 1B). CXCL9 expression in the BAL fluid is also increased 10-fold 24 h after i.t. CRA challenge compared with control animals (0.28 vs 0.028 ng/ml respectively; data not shown). Although no role for CXCL9 in the allergic airway has been previously described, these results suggest that
CXCL9 may participate in the allergic inflammatory response in the lung.

Neutralization of CXCL9 in the airway at the time of allergen challenge exacerbates allergic airway hyperreactivity

Given that CXCL9 was localized immunohistochemically to bronchial epithelial cells, and therefore would primarily be secreted into the airway, the effect of neutralization of CXCL9 in the airway in our model of CRA-induced allergic airway disease was examined. BALB/c mice were sensitized and challenged with CRA as described. On day 20 after initial sensitization, 100 μg of control IgG or Ab against CXCL9 along with CRA were instilled directly into the airway via i.t. injection. Interestingly, neutralization of CXCL9 in the airway at the time of allergen challenge exacerbated allergic airway hyperreactivity (Fig. 2A) and pulmonary eosinophil accumulation at 24 h after allergen challenge (Fig. 2, B and C). Although CXCL9 neutralization did not have any significant effect on the percentage of monocytes or lymphocytes in the airway, the percentage of neutrophils was significantly decreased after neutralization and allergen challenge (data not shown).

Neutralization of CXCL9 in the airway was confirmed by the reduction in protein levels of CXCL9 in the BAL as measured by ELISA (control IgG, 0.33 ng/ml; anti-CXCL9, 0.15 ng/ml). CXCL9 levels in the lung were also reduced after Ab treatment, although not significantly. Airway neutralization of CXCL9 dramatically decreased protein levels of IL-12, whereas IL-4 levels were significantly increased in the BAL fluid (Fig. 3). Interestingly, CXCL9 neutralization had no significant effect on protein levels of IFN-γ or on other Th2 type mediators, such as IL-5, IL-13, IL-10, or CCL11, in either lung or airway. Flow cytometric analysis of the cellular populations in the airway from postallergen challenge BAL samples revealed a significant decrease in the number of CXCR3+CD4+ cells in the anti-CXCL9-treated group (Fig. 4). However, CXCL9 neutralization did not significantly affect total CD4+ lymphocyte recruitment into the airway (data not shown). No significant differences in the numbers of CD8+ or DX5+CD3+ (NKT) cells in the airway or in the number of CXCR3+CD4+ cells in the lung was observed (data not shown). These results suggest that neutralization of local levels of CXCL9 allow for the elaboration of Th2-type cytokines, leading to an exacerbation of the allergic response in the lung.

Local administration of recombinant CXCL9 at the time of allergen challenge reduces airway hyperreactivity and eosinophil accumulation

We next investigated how administration of exogenous CXCL9 may influence an established allergic response in the lung. Local administration of 80 ng of recombinant murine CXCL9 at the time of allergen challenge resulted in a significant reduction of airway hyperreactivity 24 h after CRA challenge (Fig. 5A). Peribronchial eosinophil accumulation in the lung was also greatly reduced after i.t. CXCL9 and CRA challenge (Fig. 5B). Furthermore, exogenous CXCL9 administration significantly reduced airway eosinophil accumulation 24 h after allergen challenge (data not shown). This effect on airway hyperreactivity and eosinophil accumulation was dose dependent, as instillation of up to 400 ng of recombinant CXCL9 resulted in an additive decrease in airway hyperreactivity and eosinophil accumulation (data not shown). The percentage of monocytes in the airway was significantly increased after CXCL9 and allergen challenge; however, no significant change in either lymphocyte or neutrophil populations was observed (data not shown).

Exogenous airway CXCL9 administration alters the phenotype of cytokine production in the lung

The administration of exogenous CXCL9 at the time of allergen challenge into the airway of allergic mice decreased levels of Th2-type cytokines. Protein levels of IL-4 and IL-5 in the lung were significantly reduced 24 h after CXCL9 and allergen challenge, whereas protein levels of IL-12 were significantly increased (Fig. 6). No significant changes in protein levels of IFN-γ or the type 2 mediators, IL-10, IL-13, or CCL11, in lung or airway were observed. Taken together these results suggest that CXCL9 may exert its effect on the allergic response in the lung by modulating the phenotype of cytokine expression in lung and airway.
CXCL9 inhibits CCL11 induced eosinophil chemotaxis

In addition to CXCL9’s effect on cytokine production, eosinophil recruitment into the lung was significantly altered. To better define the effect of CXCL9 on eosinophil recruitment, we investigated the ability of CXCL9 to influence eosinophil chemotaxis in vitro. Purified murine eosinophils were assessed for their ability to chemotax in response to a CCL11, CXCL9, or CXCL10 stimulus. Initial dose-response experiments using chemokine concentrations between 0 and 1000 ng/ml revealed that the most effective concentration range for chemotactic analysis of these purified eosinophils was between 1 and 100 ng/ml (data not shown). CCL11 and CXCL10 induced significant chemotaxis in purified eosinophils, but CXCL9 did not (Fig. 8A). Moreover, at 1 ng/ml, CXCL9 appeared to inhibit eosinophil chemotaxis. Additionally, preincubation of eosinophils with CXCL9 was able to significantly inhibit their chemotaxis toward CCL11 (Fig. 8B). Preincubation of eosinophils with CXCL10 had no effect on CCL11-induced chemotaxis (data not shown). Our results demonstrate that in addition to the ability of CXCL9 to modulate the phenotype of cytokine gene expression, CXCL9 may exert a regulatory effect on eosinophil migration.

Discussion

Allergic asthma is characterized by a robust Th2-type inflammatory response arising from an intense lymphocytic infiltration of the bronchial mucosa and elaboration of Th2-type cytokines, such as IL-4, IL-5, and IL-13 (4-8, 38, 39) leading to airway hyperreactivity, eosinophil accumulation, and mucus hypersecretion. It has been previously demonstrated that modulation of Th2 cytokines has a significant effect on an allergen-induced inflammatory response (29, 30, 37, 40) and that Th1- and Th2-type responses are antagonistic to each other. Introduction of Th1-type mediators, such as IL-12 and IFN-γ, into an allergic response has been shown to decrease the severity of that inflammatory response (25, 26, 28, 31). IL-12 is produced mainly by monocytes and APCs (41). It has been shown to inhibit allergen-induced airway hyperreactivity and eosinophil accumulation after allergen challenge as well as decrease levels of the Th2 cytokines, IL-4 and IL-5 (31, 42). In addition, IL-12 has been linked to eosinophil apoptosis (43). IL-12 induces the production of IFN-γ, which then induces the production of several type 1 mediators, including the CXCR3 ligands, CXCL9 and CXCL10 (44). In this report we demonstrate that the IFN-inducible CXCR3 ligand, CXCL9, is able to regulate Th2-type allergic inflammation.

CXCL9 inhibits CCL11 induced eosinophil chemotaxis

In addition to CXCL9’s effect on cytokine production, eosinophil recruitment into the lung was significantly altered. To better define the effect of CXCL9 on eosinophil recruitment, we investigated the ability of CXCL9 to influence eosinophil chemotaxis in vitro. Purified murine eosinophils were assessed for their ability to chemotax in response to a CCL11, CXCL9, or CXCL10 stimulus. Initial dose-response experiments using chemokine concentrations between 0 and 1000 ng/ml revealed that the most effective concentration range for chemotactic analysis of these purified eosinophils was between 1 and 100 ng/ml (data not shown). CCL11 and CXCL10 induced significant chemotaxis in purified eosinophils, but CXCL9 did not (Fig. 8A). Moreover, at 1 ng/ml, CXCL9 appeared to inhibit eosinophil chemotaxis. Additionally, preincubation of eosinophils with CXCL9 was able to significantly inhibit their chemotaxis toward CCL11 (Fig. 8B). Preincubation of eosinophils with CXCL10 had no effect on CCL11-induced chemotaxis (data not shown). Our results demonstrate that in addition to the ability of CXCL9 to modulate the phenotype of cytokine gene expression, CXCL9 may exert a regulatory effect on eosinophil migration.

Discussion

Allergic asthma is characterized by a robust Th2-type inflammatory response arising from an intense lymphocytic infiltration of the bronchial mucosa and elaboration of Th2-type cytokines, such as IL-4, IL-5, and IL-13 (4-8, 38, 39) leading to airway hyperreactivity, eosinophil accumulation, and mucus hypersecretion. It has been previously demonstrated that modulation of Th2 cytokines has a significant effect on an allergen-induced inflammatory response (29, 30, 37, 40) and that Th1- and Th2-type responses are antagonistic to each other. Introduction of Th1-type mediators, such as IL-12 and IFN-γ, into an allergic response has been shown to decrease the severity of that inflammatory response (25, 26, 28, 31). IL-12 is produced mainly by monocytes and APCs (41). It has been shown to inhibit allergen-induced airway hyperreactivity and eosinophil accumulation after allergen challenge as well as decrease levels of the Th2 cytokines, IL-4 and IL-5 (31, 42). In addition, IL-12 has been linked to eosinophil apoptosis (43). IL-12 induces the production of IFN-γ, which then induces the production of several type 1 mediators, including the CXCR3 ligands, CXCL9 and CXCL10 (44). In this report we demonstrate that the IFN-inducible CXCR3 ligand, CXCL9, is able to regulate Th2-type allergic inflammation.

CXCL9 inhibits CCL11 induced eosinophil chemotaxis

In addition to CXCL9’s effect on cytokine production, eosinophil recruitment into the lung was significantly altered. To better define the effect of CXCL9 on eosinophil recruitment, we investigated the ability of CXCL9 to influence eosinophil chemotaxis in vitro. Purified murine eosinophils were assessed for their ability to chemotax in response to a CCL11, CXCL9, or CXCL10 stimulus. Initial dose-response experiments using chemokine concentrations between 0 and 1000 ng/ml revealed that the most effective concentration range for chemotactic analysis of these purified eosinophils was between 1 and 100 ng/ml (data not shown). CCL11 and CXCL10 induced significant chemotaxis in purified eosinophils, but CXCL9 did not (Fig. 8A). Moreover, at 1 ng/ml, CXCL9 appeared to inhibit eosinophil chemotaxis. Additionally, preincubation of eosinophils with CXCL9 was able to significantly inhibit their chemotaxis toward CCL11 (Fig. 8B). Preincubation of eosinophils with CXCL10 had no effect on CCL11-induced chemotaxis (data not shown). Our results demonstrate that in addition to the ability of CXCL9 to modulate the phenotype of cytokine gene expression, CXCL9 may exert a regulatory effect on eosinophil migration.

Discussion

Allergic asthma is characterized by a robust Th2-type inflammatory response arising from an intense lymphocytic infiltration of the bronchial mucosa and elaboration of Th2-type cytokines, such as IL-4, IL-5, and IL-13 (4-8, 38, 39) leading to airway hyperreactivity, eosinophil accumulation, and mucus hypersecretion. It has been previously demonstrated that modulation of Th2 cytokines has a significant effect on an allergen-induced inflammatory response (29, 30, 37, 40) and that Th1- and Th2-type responses are antagonistic to each other. Introduction of Th1-type mediators, such as IL-12 and IFN-γ, into an allergic response has been shown to decrease the severity of that inflammatory response (25, 26, 28, 31). IL-12 is produced mainly by monocytes and APCs (41). It has been shown to inhibit allergen-induced airway hyperreactivity and eosinophil accumulation after allergen challenge as well as decrease levels of the Th2 cytokines, IL-4 and IL-5 (31, 42). In addition, IL-12 has been linked to eosinophil apoptosis (43). IL-12 induces the production of IFN-γ, which then induces the production of several type 1 mediators, including the CXCR3 ligands, CXCL9 and CXCL10 (44). In this report we demonstrate that the IFN-inducible CXCR3 ligand, CXCL9, is able to regulate Th2-type allergic inflammation.
CXCL9 has previously been shown to be important in mediating Th1-type antiviral responses (45, 46). CXCL9 seems to behave as a typical Th1-type chemokine and is able to antagonize Th2-type allergic inflammation. We have demonstrated that introduction of exogenous CXCL9 into an allergic lung greatly reduced airway hyperreactivity, eosinophil accumulation, and Th2 cytokine levels while significantly increasing levels of the type 1-associated cytokine IL-12. Interestingly, CXCL10 has been shown to play a role in both modulation and exacerbation of allergic inflammation and seems to be required during sensitization to adequately promote a Th1-type response. CXCL10−/− mice have defective effector T cell development and are unable to mount efficient T cell responses after Ag challenge (47). In addition, adenoviral overexpression of CXCL10 during sensitization to OVA decreased eosinophil accumulation and IL-4 levels after OVA challenge (33). However, CXCL10 transgenic mice displayed increased eosinophil accumulation, airway hyperreactivity, and IL-4 levels after OVA challenge (48). Similarly, introduction of exogenous CXCL10 into an allergic lung increased Th2-type cytokines, airway hyperreactivity, and eosinophil accumulation after allergen challenge (34). Thus, the role of CXCL10 is complex and probably depends on the location and timing of production. Our findings that administration of exogenous CXCL10 into an allergic lung had the opposite effect of exogenous CXCL10 suggest that these two CXCR3 ligands can differentially regulate allergic disease through their specific individual interactions with CXCR3.

In the paradigm of lymphocyte activation, IL-4 and IL-12 are two apical cytokines that determine the phenotype of the activated lymphocyte. Depending on whether IL-4 or IL-12 is present at the time of initial activation, the lymphocyte will be skewed to produce either Th2- or Th1-type cytokines. IL-4 stimulation induces the production of other Th2-type cytokines and inhibits the production of type 1 mediators. IL-12 stimulates the production of

**FIGURE 5.** Introduction of exogenous CXCL9 at the time of allergen challenge attenuates the allergic response in the lung. Mice were immunized and challenged with CRA as described. On day 20 after initial sensitization the mice were challenged i.t. with 80 ng of recombinant murine CXCL9 in 300 PNU of CRA (CXCL9) or with 300 PNU of allergen alone (CRA). Various parameters of the allergic response were measured at the indicated time points thereafter. The allergic response in mice that only received an i.n. allergen challenge on day 14 after initial sensitization (IN) was also measured and is indicative of baseline levels of the measured parameters. Airway reactivity (ΔR; A) and pulmonary eosinophil accumulation (B) were significantly decreased 24 h after CXCL9 and allergen challenge. Graphs are representative of at least four separate experiments in which n ≥ 5. *p < 0.05.

**FIGURE 6.** CXCL9 alters the cytokine phenotype in the lung after allergen challenge. Lungs from mice sensitized and challenged with allergen were harvested 24 h after i.t. challenge with 80 ng of CXCL9 and CRA (CXCL9) or with allergen alone (CRA) and pulmonary cytokine levels were quantitated by ELISA as described. Baseline pulmonary cytokine levels in the lungs of mice that only received an i.n. allergen challenge on day 14 after initial sensitization (IN) were also measured. Introduction of exogenous CXCL9 at the time of allergen challenge resulted in dramatically decreased levels of IL-4 and IL-5 and significantly increased levels of IL-12 at 24 h. Graphs are representative of at least four separate experiments in which n ≥ 5. *p < 0.05.
Th1-type cytokines, such as IFN-γ, and inhibits the production of type 2 mediators (49–51). The CXCR3 ligands, CXCL9 and CXCL10, seemed to have opposing effects on allergic inflammation in our in vivo model that manifested in differential lymphocyte cytokine production in lung and airway. We have shown that CXCL9 and CXCL10 can directly influence lymphocyte and/or APC activation, leading to differential cytokine production. CXCL9 promoted IL-12 and IFN-γ production, whereas CXCL10 promoted IL-4 production. This is one of the first reports demonstrating that two ligands that bind the same receptor can differentially affect the cell expressing that receptor. Studies have shown that CXCL9 and CXCL10 compete for binding to CXCR3, and that they each bind to separate binding sites on the receptor with distinct kinetics (52, 53). Therefore, it is possible that differences in the strength of signal resulting from CXCL9 vs CXCL10 bound to CXCR3 or that the receptor confirmation following ligation by CXCL9 or CXCL10 could stimulate the differential activation of signaling pathways within the same cell, resulting in the functional differences between these two chemokines that were observed in our model. A final possibility may be that either CXCL9 or CXCL10 binds to a second, as yet undiscovered, receptor. The mechanisms by which these two CXCR3 ligands may be differentially stimulating CXCR3-expressing cells are still unknown, but are currently under investigation in our laboratory.

The CXCR3+ CD4+ lymphocyte population is also increased in the lung after CXCL9 and allergen challenge (data not shown) and is significantly decreased in the airway after CXCL9 neutralization. It has been reported that CXCR3 is associated with Th1-type inflammatory diseases and may be a marker of IFN-γ-producing lymphocytes (18, 21). However, others have recently shown that CXCR3 expression does not necessarily correlate with IFN-γ expression (54) and that subsets of CXCR3+ CD4+ lymphocytes produce IL-4 (55). The observed effects on cytokine phenotype in the lung after CXCL9 and allergen challenge along with our in vitro observations suggest that CXCL9 may be directing the cytokine phenotype of infiltrating CXCR3+ CD4+ lymphocytes toward a more Th1-type profile, resulting in a diminished allergic response. The exact mechanism of this latter finding is presently unclear. Although CXCL9 probably has a direct effect on the preferential recruitment of CXCR3+ T cells, we must also consider its effects on local and recruited CXCR3+ APCs that would be reflected in the increased expression of IL-12 in the present studies.

In addition to lymphocytes, eosinophils are considered to be one of the major effector cells in allergic airway disease (7, 8). Eosinophil degranulation products, such as cysteinyl leukotrienes, eosinophil cationic protein, and eosinophil peroxidase, are known to mediate several characteristics of allergic airway disease, such as airway hyperreactivity and epithelial cell damage (56–58). Eosinophils highly express CCR3 and chemotax to the CCR3 ligand CCL11 (59–62). Eosinophils have also been shown to express CXCR3 (34, 63). We have shown that although CXCL10 is a sufficient chemotactic stimulus for eosinophils, CXCL9 inhibits eosinophil chemotaxis in vitro. Furthermore, we have demonstrated that preincubation of eosinophils with CXCL9 is able to

![Image](http://www.jimmunol.org/)
inhibit eosinophil chemotaxis toward CCL11 (data not shown). These in vitro experiments demonstrate that CXCL9 is not a chemotactic stimulus for eosinophils, and that CXCL9 is able to inhibit eosinophil migration toward a potent chemotactic stimulus, such as CCL11. Unpublished observations from calcium flux experiments using these purified murine eosinophils have indicated that CXCL9 stimulation does not inhibit calcium flux in response to a subsequent CCL11 stimulus. These results suggest that CXCL9 does not desensitize CCR3, and therefore, CXCL9 is probably sending an inhibitory signal toward CCL11. The mechanism underlying the ability of the CXCR3 ligands to regulate eosinophil chemotaxis is currently under investigation in our laboratory. The ability of CXCL9 to inhibit eosinophil chemotaxis together with its ability to alter cytokine phenotype after allergen challenge demonstrate the potential importance of this chemokine for local regulation of allergic responses in the airway.

Acknowledgments
We thank Allison Miller and Matt Schaller for their helpful discussions, and Aaron Berlin, Ted Martens, Holly Evanoff, and Pamela Lincoln for their invaluable technical assistance.

References
Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that plays a crucial role in the pathogenesis of allergic airway disease. TNF-α is produced by a variety of cell types, including macrophages, neutrophils, and eosinophils, and it can activate other cells through binding to its specific receptor, TNFR1.

In allergic airway disease, TNF-α is not only produced by these cell types but is also increased in the airway tissues and bronchoalveolar lavage fluid. This upregulation of TNF-α can lead to the recruitment of inflammatory cells and the production of pro-inflammatory mediators, which contribute to the development of asthma.

In general, TNF-α production in allergic airway disease is increased, and this increased production can be attributed to the activation of Th2 cells and the release of IL-4 and IL-13, which are known to upregulate TNF-α expression. TNF-α can then act in a paracrine manner to enhance the activation of Th2 cells and other immune cells, creating a positive feedback loop that perpetuates the allergic response.

Moreover, TNF-α has been shown to induce the expression of other pro-inflammatory cytokines, such as IL-6 and IL-8, which can contribute to the chronic inflammation seen in asthma. Therefore, the inhibition of TNF-α has been a target for therapeutic interventions in allergic airway disease.