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The C10/CCL6 Chemokine and CCR1 Play Critical Roles in the Pathogenesis of IL-13-Induced Inflammation and Remodeling

Bing Ma,* Zhou Zhu,* Robert J. Homer,† Craig Gerard,‡ Robert Strieter,§ and Jack A. Elias*2

IL-13 is a potent stimulator of inflammation and tissue remodeling that plays a key role in the pathogenesis of a variety of diseases. To further understand these responses, studies were undertaken to define the role(s) of the chemokine C10/CCL6 in the pathogenesis of IL-13-induced alterations in the murine lung. IL-13 was a very potent stimulator of C10/CCL6 mRNA and protein, and IL-13-induced inflammation, alveolar remodeling, and compliance alterations were markedly ameliorated after C10/CCL6 neutralization. Treatment with anti-C10/CCL6 decreased the levels of mRNA encoding matrix metalloproteinase-2 (MMP-2), MMP-9, and tissue inhibitor of metalloproteinase-4 (TIMP-4) in lungs from wild-type mice. C10/CCL6 neutralization also decreased the ability of IL-13 to stimulate the production of monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, MMP-2, MMP-9, and cathepsins-K, -L, and -S and the ability of IL-13 to inhibit α1-antitrypsin. In accord with these findings, a targeted null mutation of CCR1, a putative C10/CCL6 receptor, also decreased IL-13-induced inflammation and alveolar remodeling and caused alterations in chemokines, proteases, and antiproteases comparable to those seen after C10/CCL6 neutralization. These C10/CCL6 and CCR1 manipulations did not alter the production of transgenic IL-13. These studies demonstrate that IL-13 is a potent stimulator of C10/CCL6 and signaling via CCR1 in the pathogenesis of the IL-13-induced pulmonary phenotype. They also describe a C10/CCL6 target gene cascade in which C10/CCL6 induction is required for optimal IL-13 stimulation of selected chemokines (monocyte chemoattractant protein-1 and MIP-1α) and proteases (MMP-2, MMP-9, and cathepsins-K, -L, -S) and the inhibition of α1-antitrypsin. The Journal of Immunology, 2004, 172: 1872–1881.

Interleukin-13 is a pleiotropic 12-kDa product of a gene on chromosome 5 at q31 that is produced in large quantities by Th2 cells. Early studies highlighted shared effector properties with IL-4. It was subsequently appreciated that IL-4 and IL-13 play different roles in Th2 inflammation where IL-4 contributes predominantly to Th2 cell development and response generation, whereas IL-13 contributes in a major way, to effector pathway activation (1, 2). Studies from our laboratory and others have used overexpression transgenic modeling and other approaches to define the effector properties of IL-13. These studies demonstrated that IL-13 is a potent stimulator of eosinophil-, lymphocyte-, and macrophage-rich inflammation; mucus metaplasia; tissue fibrosis; and parenchymal proteolysis (2–6). They also demonstrated that in macrophage-rich inflammation; mucus metaplasia; tissue fibrosis; and anti-proteases comparable to those seen after C10/CCL6 neutralization. These C10/CCL6 and CCR1 manipulations did not alter the production of transgenic IL-13. These studies demonstrate that IL-13 is a potent stimulator of C10/CCL6 and signaling via CCR1 in the pathogenesis of the IL-13-induced pulmonary phenotype. They also describe a C10/CCL6 target gene cascade in which C10/CCL6 induction is required for optimal IL-13 stimulation of selected chemokines (monocyte chemoattractant protein-1 and MIP-1α) and proteases (MMP-2, MMP-9, and cathepsins-K, -L, -S) and the inhibition of α1-antitrypsin. The Journal of Immunology, 2004, 172: 1872–1881.

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Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; BAL, bronchoalveolar lavage; dox, doxycycline; D-PAS, periodic acid-Schiff with diastase; HMI, histologic mucus index; ISH, in situ hybridization; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; rTA, reverse tetracycline trans-activator; TIMP, tissue inhibitor of metalloproteinase; α1-AT, α1-antitrypsin; HCC, hemofiltrate CC chemokine, CCF18, CC chemokine F-18.
bleomycin lung, and chronic peritonitis (14, 17–21). In these tissues, CC10/CCL6 is a chemotactic agent for monocytes and macrophages and, to a lesser extent, CD4 T cells and eosinophils (14, 17, 18). C10/CCL6 shares significant homology with other chemokines, including human MIP-1y, CC chemokine F-18 (CCF-18), hemofiltrate CC chemokine (HCC-1), and HCC-2. These moi eties have an unusual genomic structure, with a unique second exon and the ability to bind to and activate CCR1 (14, 18, 22–25).

Previous studies from our laboratories demonstrated that IL-13 is a potent stimulator of C10/CCL6 in vivo (14, 15). The contribu tions of C10/CCL6 and CCR1 signaling to the generation of the IL-13 phenotype have not been investigated, however.

We hypothesized that C10/CCL6 and signaling via CCR1 play important roles in the pathogenesis of IL-13-induced tissue alterations in vivo. To test this hypothesis, we characterized the expression of C10/CCL6 in transgenic mice in which IL-13 was overexpressed in a lung-specific fashion and defined the effects of C10/CCL6 neutralization and a null (−/−) mutation of CCR1 on the IL-13-induced phenotype in these animals. These studies demonstran t that IL-13 is a potent stimulator of macrophage C10/CCL6 production. They also demonstrate that C10/CCL6 neutralization or a deficiency of CCR1 markedly ameliorate IL-13-induced inflammation and parenchymal remodeling. Lastly, they provide mechanistic insights by demonstrating that IL-13 stimulates the production of monocyte chemotactic protein-1 (MCP-1), MIP-1α, matrix metalloproteinase-2 (MMP-2), MMP-9, and cathepsins-K, -L, and -S and inhibits 1-antitrypsin via C10/CCL6- and CCR1-dependent mechanisms.

Materials and Methods

Transgenic mice

Two types of overexpression transgenic mice were generated in our laboratories and used in these studies. Both use the Clara cell 10-kDa protein (CC10) promoter to target transgene expression to the lung. In CC10-IL-13 mice, the CC10 promoter drives the expression of murine IL-13 in a constitutive fashion. The methods used to generate and characterize these mice were described previously (3). To allow IL-13 to be expressed in a temporally regulated fashion, CC10-reverse tetracycline activator (rtTA)-IL-13 mice were used. These are dual-transgenic mice that use rtTA and doxycycline (dox) to activate transgene expression. The IL-13 trans- rtTA of rtTA-IL-13 mice with wild-type (+/+) and null (−/−) CCR1 loci were generated by breeding the IL-13-overexpressing mice with the CCR1−/− animals. Genotyping was accomplished as previously described (3, 4, 26, 27).

Dox water administration

In experiments performed with CC10-rtTA-IL-13 transgene-positive animals and their littermate controls, all animals were maintained on normal water until they were 1 mo of age. They were then randomized to receive either normal water or water with dox for the duration of the experiment. Dox was administered at 500 mg/liter in 4% sucrose and kept in dark brown bottles to prevent light-induced degradation.

Treatment with anti-C10/CCL6 Abs

To define the role(s) of C10/CCL6 in the IL-13 phenotype, we compared the phenotypes of CC10-rtTA-IL-13 transgene-positive mice that had been randomized to normal water or dox water at 1 mo of age and treated with a polyclonal rabbit anti-murine C10/CCL6 (R&D Systems, Minneapolis MN; anti-C10/CCL6) or control antiserum. The specificity and neutralizing capacity of this antiserum have been previously defined by our laboratories (14). The test and control antisera (0.5 ml) were administered every other day via the i.p. route starting the day before dox administration.

Bronchoalveolar lavage (BAL)

Lung inflammation was assessed by BAL as previously described (3, 4). The BAL samples from each animal were then pooled and centrifuged. The numbers and types of cells in the cell pellet were determined as previously described (3, 4). The supernatants were stored at −20°C until used.

Lung volume and compliance assessments

Lung volume and compliance were assessed as previously described (4). In brief, animals were anesthetized, the trachea was cannulated, and the lungs were removed and inflated with PBS at 25 cm. The size of the lung was evaluated by volume displacement.

Histologic evaluation

H&E and periodic acid-Schiff with diastase (D-PAS) stains were performed after pressure fixation with Streck solution (Streck Laboratories, St. La Vista, NE) in the Research Histology Laboratory of Department of Pathology, Yale University School of Medicine, as previously described (4).

Morphometric analysis

Alveolar size was estimated from the mean chord length of the airspace as previously described by our laboratory (4). When CC10-IL-13 mice were being evaluated, at least five animals were studied at each time point. When CC10-rtTA-IL-13 mice were being evaluated, at least six animals that had received dox water were studied at each time point. Chord length increases with alveolar enlargement.

Calculation of histologic mucus index (HMI)

Mucus metaplasia was assessed by calculating HMI values. The HMI is a measurement of the percentage of epithelial cells that are D-PAS positive. The data were expressed as the mean ± SE. Statistical analysis was performed with the Student t test. Differences were considered significant at p < 0.05.

Messenger RNA analysis

Messenger RNA levels were evaluated by RT-PCR analysis using whole lung RNA as previously described (3, 4). The primers employed for the

<table>
<thead>
<tr>
<th>Moiety</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Annealing Temperature (°C)</th>
<th>Product Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>ACCACCCCAACTCTGACTGAAAC</td>
<td>CAGAATTGCTGAGGTTGTTGG</td>
<td>60</td>
<td>463</td>
</tr>
<tr>
<td>MCP-2</td>
<td>AGTGCCTTTCGTTCCTGCCTCATAG</td>
<td>ATGAGAAGACACCGACCCACGACC</td>
<td>58</td>
<td>389</td>
</tr>
<tr>
<td>MCP-3</td>
<td>AGCGTCTGTGCTGCTGCTCGTAT</td>
<td>GGTTAAATGGGGAAAAAGGGGAGAAT</td>
<td>57</td>
<td>564</td>
</tr>
<tr>
<td>MCP-5</td>
<td>CGTACCGCTCTCTGCATAG</td>
<td>CTCAAAACACTTCCTCTTGG</td>
<td>54</td>
<td>389</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>CCACTGCTTTCCCTTCCACCATG</td>
<td>ATGCCCACTATCCCTTATGGC</td>
<td>56</td>
<td>546</td>
</tr>
<tr>
<td>C-10</td>
<td>AGGACTGGAGAGGCGCGAGG</td>
<td>TCCACCAGAGGCGCCATTTCCA</td>
<td>58</td>
<td>577</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>TAGTCTCTTCGTGCTGATG</td>
<td>CAGTGTCACACCAGGGCTAT</td>
<td>60</td>
<td>271</td>
</tr>
<tr>
<td>MIP-2</td>
<td>CTGCTGTCATCGGTGGAGG</td>
<td>CTTGCTTCTTCGAGGTTAG</td>
<td>60</td>
<td>259</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GTGGGGCGCTCCTAGCCACCAA</td>
<td>CTCTCTGATGCACGGCAGATTC</td>
<td>60</td>
<td>540</td>
</tr>
</tbody>
</table>
MMP and cathepsin evaluations have been previously described (3, 4). The primers used for the chemokine and β-actin evaluations are illustrated in Table I. Amplified PCR products were detected using ethidium bromide gel electrophoresis, quantitated electronically, and confirmed by nucleotide sequencing.

**Quantification of IL-13 and chemokines**

BAL IL-13 and chemokine levels were quantitated using commercial ELISA kits (R&D Systems) according to the manufacturer’s instructions. Polyclonal neutralizing anti-C10/CCL6 Abs were purchased from R&D Systems.

**In situ hybridization (ISH)**

ISH was undertaken as previously described (3, 16). Formaldehyde fixation and a cDNA encoding the portion of C10/CCL6 between nt 89 and 1123 were employed. The C10/CCL6 probe was generated by cloning the cDNA into pBS II KS that contains T3 and T7 primer sequences flanking a multiple cloning site (Stratagene, La Jolla, CA). Sense and antisense RNA probes were generated, labeled with a digoxigenin RNA labeling kit (Roche, Indianapolis, IN), denatured at 65°C, and added to commercially available hybridization buffer (Ambion, Austin, TX) at 6 ng/μl, and the hybridization mixture was incubated with tissue overnight at 52°C. The tissues were then washed twice with 4× SSC for 5 min each time at room temperature and twice with 2× SSC for 10 min each time at 37°C, and incubated with RNase A (10 μg/ml) for 45 min at 37°C. This was followed by two 10-min washes in 2× SSC at room temperature and three 20-min washes in 0.2× SSC at 50°C. Probe was detected by overnight incubation with sheep Abs to digoxigenin-labeled with alkaline phosphatase (Roche), followed by 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate as described by the manufacturer.

**Statistics**

Normally distributed data are expressed as the mean ± SEM and were assessed for significance by Student’s t test or ANOVA as appropriate. Data that were not normally distributed were assessed for significance using the Wilcoxon rank-sum test.

**Results**

**Effect of IL-13 on C10/CCL6**

Studies were undertaken to define the effects of IL-13 on C10/CCL6 in the murine lung. These studies demonstrated that transgenic IL-13 was a potent stimulator of C10/CCL6 mRNA accumulation. This effect was readily apparent in lungs from inducible and constitutive transgenic mice. In the former, C10/CCL6 induction was seen after as little as 7 days of dox administration and continued throughout the 3-mo assessment interval (Fig. 1A and data not shown). At all time points, the dose-dependent nature of this response was readily appreciated because the low level of transgene expression that is characteristic of transgene-positive mice receiving normal water also caused modest levels of C10/CCL6 induction (Fig. 1A data and not shown). In the latter, C10/CCL6 mRNA induction was readily apparent at all time points (1–3 mo) assessed (Fig. 1B). In all cases, alterations in C10/CCL6 mRNA accumulation were associated with comparable increases in BAL C10/CCL6 protein elaboration. C10/CCL6 protein was not readily apparent in BAL fluids from transgene-negative animals. In contrast, levels of C10/CCL6 ≥50 ng/ml were noted in BAL fluids from 3-mo-old transgene-positive CC10-IL-13 mice (Fig. 1C). Interestingly, these levels greatly exceeded the levels of BAL MCP-1 and MIP-1α, which were 1000–1500 and 300–500 pg/ml, respectively (data not shown). These studies demonstrate that IL-13 is a potent stimulator of C10/CCL6 elaboration in the murine lung and highlight the impressive magnitude of this inductive response.

**Localization of C10/CCL6 in lungs from IL-13 transgenic mice**

ISH was used to define the sites of C10/CCL6 mRNA accumulation in lungs from transgene-negative and -positive animals. C10/CCL6 mRNA was not readily apparent in lungs from transgene-negative animals. In contrast, the antisense probe revealed impressive levels of C10/CCL6 mRNA in macrophages from transgene-positive animals (Fig. 2). Significant staining with the sense probe was not detected (Fig. 2). These studies demonstrate that IL-13 stimulates alveolar macrophages to produce C10/CCL6 in the murine lung.

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Effects of transgenic IL-13 on C10. A, CC10-rTAT-IL-13 mice and littermate controls were randomized to dox water and normal water for 3 mo, and C10 mRNA levels were evaluated by RT-PCR. B, Levels of C10/CCL6 mRNA in lungs from 1- to 3-mo-old CC10-IL-13 transgene-positive mice and transgene-negative littermate controls were evaluated. A and B are representative of n = 6. C, Levels of BAL C10/CCL6 were evaluated in 3-mo-old CC10-IL-13 mice and littermate controls. The values are the mean ± SEM of a minimum of six animals from each group. *p < 0.01 vs controls.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.**ISH localization of C10/CCL6 mRNA. Comparisons were undertaken with lungs from 3-mo-old CC10-IL-13 transgene-positive and -negative mice incubated with sense (S) and antisense (AS) probes.
Effects of C10/CCL6 neutralization on IL-13-induced inflammation

To understand the role(s) of C10/CCL6 in the generation of IL-13-induced inflammation, we compared the inflammation in CC10-rtTA-IL-13 transgene-positive mice that had been randomized to normal water or dox water at 1 mo of age and treated with antiserum against C10/CCL6 (anti-C10/CCL6) or control antiserum. Compared with that in transgene-negative controls, IL-13 increased BAL cell recovery by ~8-fold and significantly increased the percentage of cells that were lymphocytes and eosinophils (Fig. 3). Treatment with anti-C10/CCL6 did not alter the number or differential of the cells recovered in BAL fluids from transgene-negative mice (Fig. 3). In contrast, neutralization of C10/CCL6 significantly decreased the total number of cells recovered in BAL fluids from CC10-rtTA-IL-13 transgene-positive mice (Fig. 3). Anti-C10/CCL6 did not, however, alter the differential of the cells that were recovered, as comparable decreases in macrophage, eosinophil, and lymphocyte recoveries were noted (Fig. 3). Treatment of constitutive CC10-IL-13 mice at 1 mo of age with anti-C10/CCL6 for 2 wk diminished BAL cell recovery in a similar fashion (data not shown). In all cases, the ability of anti-C10/CCL6 treatment to diminish BAL cellular influx was associated with a comparable decrease in tissue inflammation (data not shown). These studies demonstrate that C10/CCL6 plays a crucial role in defining the intensity, but not the nature, of IL-13-induced pulmonary inflammation.

Effects of C10/CCL6 neutralization on IL-13-induced alterations in lung volume, alveolar size, and pulmonary compliance

To define the role(s) of C10/CCL6 in the pathogenesis of IL-13-induced alveolar remodeling, we compared the alterations in lung size, lung volume, alveolar size, and lung compliance in CC10-rtTA-IL-13 transgene-positive mice treated with control antiserum or anti-C10/CCL6. In accord with previous observations (4), dox induction of IL-13 caused an impressive increase in all these parameters. Treatment with anti-C10/CCL6 did not alter these parameters in lungs from wild-type mice (Fig. 4 and Table II). In contrast, the effects of IL-13 were markedly diminished in mice treated with anti-C10/CCL6. After as little as 2 wk of dox administration, lungs from CC10-rtTA-IL-13 transgene-positive mice treated with anti-C10/CCL6 were significantly smaller and less compliant than the lungs from transgene-positive mice treated with the control serum (Fig. 4A and Table II). Alveolar size was similarly decreased when assessed with morphometric (Fig. 4B) or light microscopic (Fig. 4C) approaches. IL-13-induced mucus metaplasia was not significantly altered (data not shown). When viewed in combination, these studies demonstrate that C10/CCL6 plays a critical role in the pathogenesis of selected aspects of the IL-13-induced remodeling response in the murine lung.

Effect of C10/CCL6 neutralization on IL-13 elaboration

A deficiency of C10/CCL6 could modify the IL-13-induced phenotype by altering IL-13 production or modifying IL-13 effector
functions. To determine whether C10/CCL6 neutralization regulated the production of transgenic IL-13, we compared the levels of BAL IL-13 in CC10-rtTA-IL-13 transgene-positive and -negative mice treated with control serum or anti-C10/CCL6. IL-13 was not readily apparent in BAL fluids from transgene-negative mice regardless of the type of serum to which they were exposed (Fig. 5). In contrast, significant levels of BAL IL-13 were noted in dox-treated, transgene-positive animals. Neutralization of C10/CCL6 by treating transgene-positive mice with anti-C10/CCL6, however, did not alter the levels of BAL IL-13 (Fig. 5). This demonstrates that C10/CCL6 neutralization alters the IL-13 phenotype by modifying IL-13-induced effector pathway activation.

Importance of C10/CCL6 in IL-13-induced chemokine elaboration

To investigate the mechanism(s) by which C10/CCL6 deficiency inhibited IL-13-induced inflammation, we compared the expression of selected chemokines in CC10-rtTA-IL-13 transgene-positive and -negative mice treated with control serum or anti-C10/CCL6. In transgene-negative mice treated with control serum or anti-C10/CCL6, the levels of mRNA encoding MCP-1/CCL2, MCP-2/CCL8, MCP-3/CCL7, MCP-5/CCL12, MIP-1a/CCL3, MIP-2/CXCL2/3 and eotaxin/CCL11 were comparable and were near or below the limits of detection of our assays (Fig. 6). As previously reported (4), IL-13 caused a marked increase in the levels of mRNA encoding the chemokines.

Table II. Effect of C10/CCL6 neutralization on lung compliance

<table>
<thead>
<tr>
<th>Transgene Status</th>
<th>Dox</th>
<th>Antiserum</th>
<th>Compliance (ml/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.048 ± 0.002</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>Control</td>
<td>0.050 ± 0.004</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>C10/CCL6</td>
<td>0.049 ± 0.005</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>Control</td>
<td>0.056 ± 0.003</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>C10/CCL6</td>
<td>0.108 ± 0.006</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>C10/CCL6/6</td>
<td>0.068 ± 0.003b</td>
</tr>
</tbody>
</table>

* One-month-old transgene (–) and (+) mice were placed on normal water (dox –) or dox water (dox +) and treated with saline (antiserum –), control antisera, or anti-C10/CCL6 as noted. Two weeks later, lungs were harvested and fixed to pressure, and static compliance was assessed. The noted values represent the mean ± SEM of a minimum of six animals in each group.

b p < 0.05 vs transgene + mice given dox water and control antiserum or saline.
these chemokine moieties in transgene-positive mice (Fig. 6A). C10/CCL6 neutralization markedly diminished the ability of IL-13 to stimulate the accumulation of MCP-1/CCL2 and MIP-1α/CCL3 mRNA and protein (Fig. 6). In contrast, C10/CCL6 neutralization did not alter the ability of IL-13 to stimulate the accumulation of mRNA encoding MCP-2/CCL8, MCP-3/CCL7, MCP-5/CCL12, MIP-2/CXCL2/3, or eotaxin/CCL11 (Fig. 6A). These studies demonstrate that IL-13 stimulates CC chemokines in the lung via C10/CCL6-dependent and -independent pathways, with MCP-1/CCL2 and MIP-1α/CCL3 being induced by the former and MCP-2/CCL8, MCP-3/CCL7, MCP-5/CCL12, MIP-2/CXCL2/3, and eotaxin/CCL11 being induced by the latter.

Importance of C10/CCL6 in IL-13-induced protease alterations

We reasoned that a deficiency of C10/CCL6 could modulate IL-13-induced inflammatory and alveolar phenotypes by decreasing the production of respiratory proteases (4, 28). To test this hypothesis we compared the levels of mRNA encoding lung-relevant MMPs and cathepsins in transgene-negative and -positive CC10-rtTA-IL-13 mice treated with control serum or anti-C10/CCL6. Comparable levels of mRNA encoding MMP-12, MMP-14, and cathepsins-B, -H, -K, -L, and -S were noted in lungs from transgene-negative mice treated with preimmune serum or anti-C10/CCL6 (Fig. 7). Interestingly, treatment with anti-C10/CCL6 decreased the levels of mRNA encoding MMP-2 and MMP-9 in these transgene-negative littermate controls (Fig. 7A). In accord with previous studies from our laboratory (4), dox induction of IL-13 increased the levels of expression of these MMPs and cathepsins (Fig. 7A). These mRNA alterations were associated with proportionate changes in protease protein elaboration (Fig. 7B and data not shown). Interestingly, C10/CCL6 neutralization decreased the ability of IL-13 to stimulate the accumulation of mRNA encoding MMP-2 and MMP-9 and, to a lesser extent, cathepsins-K, -L, and -S. (Fig. 7). In contrast, C10/CCL6 neutralization did not alter the ability of IL-13 to stimulate the accumulation of mRNA encoding MMP-12, MMP-14, or cathepsins-H and -B (Fig. 7). When viewed in combination, these studies demonstrate that IL-13 selectively induces MMP-2 and MMP-9 and cathepsins-K, -L, and -S via a C10/CCL6-dependent activation pathway.

Importance of C10/CCL6 in IL-13-induced antiprotease alterations

To determine whether the alterations in IL-13 effector function noted in the absence of C10/CCL6 were due to alterations in antiproteases, the levels of mRNA encoding selected moieties were evaluated in lungs from CC10-rtTA-IL-13 transgene-negative and -positive mice treated with control serum or anti-C10/CCL6. In
transgene-negative animals, these treatments did not alter the levels of expression of α1-anti-trypsin (α1-AT) tissue inhibitor of metalloproteinase-1 (TIMP-1), or TIMP-2 (Fig. 8). Interestingly, treatment of transgene-negative mice with anti-C10/CCL6 did, however, decrease the levels of mRNA encoding TIMP-4 (Fig. 8). In accord with previous studies from our laboratory (4), IL-13 inhibited the expression of α1-AT and enhanced the expression of TIMP-1 (Fig. 8). Neutralization of C10/CCL6 markedly diminished the ability of IL-13 to inhibit lung α1-AT (Fig. 8). After C10/CCL6 neutralization, the levels of mRNA encoding α1-AT in lungs from dox-treated transgene-positive animals were equal to or greater than those in wild-type animals (Fig. 8). Anti-C10/CCL6 did not cause similar alterations in the IL-13-stimulated expression of the other antiproteases (Fig. 8). These studies demonstrate that C10/CCL6 stimulates the production of TIMP-4 in normal lung. They also demonstrate that at sites of IL-13-induced pathology, IL-13 selectively inhibits lung α1-AT production via a C10/CCL6-dependent mechanism.

Effects of CCR1 deficiency on the IL-13 phenotype
To address the possibility that the effects of C10/CCL6 are mediated via CCR1, we compared the alterations in the IL-13 phenotype that were induced by the absence of CCR1 and by C10/CCL6 neutralization. The contributions of CCR1 to the IL-13 phenotype were defined by comparing the phenotype of CCL10-IL-13 transgene-positive mice with wild-type (+/+?) and null mutant (−/−) CCR1 loci. As noted above, IL-13 caused a significant increase in lung size, lung volume, alveolar size, and total cell, eosinophil, and lymphocyte recoveries in BAL fluids from CCR1+/− animals. In CCR1-deficient, CCL10-IL-13 transgene-positive mice, each of these IL-13 effects was markedly ameliorated (Fig. 9 and data not shown). CCL10-IL-13 transgene-positive/CCR1+/− mice had an intermediate phenotype compared with CCR1-sufficient and -deficient transgenic animals in many of these parameters (Fig. 9 and data not shown). In addition, IL-13 stimulation of MCP-1/CCL2, MIP-1α/CCL3, MMP-2, MMP-9, and, to a lesser extent, cathepsins-K, -L, and -S and IL-13 inhibition of α1-AT were diminished in CCL10-IL-13 transgene-positive mice with a null mutation of CCR1 (Fig. 10). A similar decrease in IL-13-induced inflammation and alveolar remodeling and similar alterations in the ability of IL-13 to regulate the production of chemokines, proteases, and antiproteases were also found in comparisons of CCL10-rTA-IL-13 transgene-positive mice with wild-type and null mutant CCR1 loci (data not shown). In all cases null mutations of CCR1 did not alter the levels of transgenic IL-13 (Fig. 10C and data not shown). These studies demonstrate that similar alterations in the IL-13 phenotype are induced by interventions that neutralize C10/CCL6 and interventions that delete its putative receptor, CCR1.

Discussion
To further understand the cellular and molecular events involved in IL-13-induced phenotype generation, we took advantage of transgenic systems developed in our laboratory in which IL-13 effector pathways can be selectively assessed in vivo and used these systems to characterize the role(s) of C10/CCL6 in the pathogenesis of IL-13-induced alterations in the lung. These studies demonstrate that IL-13 is a potent stimulator of C10/CCL6, with BAL C10/CCL6 levels that were >30-fold greater than the levels of MCP-1/CCL2 and other key IL-13-induced chemokines. They also demonstrate that C10/CCL6 plays a central role in the pathogenesis of the IL-13 phenotype, because IL-13-induced inflammation and alveolar remodeling were markedly decreased by C10/CCL6 neutralization. Insights into the mechanisms of these responses were provided by the demonstration that C10/CCL6 is an integral component of the chemokine, protease, and antiprotease cascade that IL-13 uses to stimulate MCP-1/CCL2, MIP-1α/CCL3, MMP-9, MMP-2, and cathepsins-K, -L, and -S and inhibit α1-AT in the lung. Lastly, these studies demonstrate that a null mutation of CCR1 causes similar alterations in IL-13-induced inflammation, alveolar remodeling, and target gene activation. This supports the contention that C10/CCL6 mediates its effects in the lung at least in part by binding to and activating CCR1.

IL-13 was originally described as an IL-4-like cytokine and was noted to have effector properties relevant to Th2 inflammation.
However, recent studies from our laboratory and others demonstrated that IL-13 is also a powerful regulator of tissue remodeling in vivo and in vitro (4, 13). In accord with these findings, dysregulated IL-13 elaboration has been implicated in the pathogenesis of a variety of disorders characterized by chronic inflammation and remodeling, including asthma, COPD, pulmonary fibrosis, scleroderma, hepatic fibrosis, and nodular sclerosing Hodgkin’s disease (1, 6, 8–14). Unfortunately, the processes that generate these responses and the mechanisms of their chronicity have not been adequately defined. Our studies addressed this deficiency by demonstrating that IL-13 is a potent stimulator of C10/CCL6 production and that this inductive event plays a critical role in IL-13-induced inflammation and remodeling in vivo in the murine lung. C10/CCL6 can also induce IL-13 elaboration (18) and is present in (24) and stimulates (18, 20) eosinophil chemotaxis. When viewed in combination, these observations allow for the speculation that an IL-13-C10/CCL6 positive feedback/amplification loop exists in IL-13-mediated tissue responses. In these responses, IL-13 would induce macrophage C10/CCL6 elaboration; C10/CCL6 would, in turn, induce tissue eosinophilia and enhance eosinophil C10/CCL6 release. The macrophage- and eosinophil-derived C10/CCL6 would then feed back to further stimulate IL-13 elaboration. This amplification response could contribute to the intensity and chronicity of Th2 responses in a variety of disorders. It also provides a tempting explanation for the life-long nature of the inflammatory and remodeling seen in asthma, COPD, idiopathic pulmonary fibrosis, and other disorders characterized by IL-13 dysregulation.

Chemokines are small, 8- to 10-kDa cytokines that have been subdivided into four supergene families (CXC, CC, C, and CXXXXC) based on the position of either one of the two cysteine residues located near the N terminus of each protein. The CC and CXC chemokine groups are large and contain >50 identified ligands. Although in vitro characterizations would suggest that there is impressive redundancy in this system, examinations of a limited number of ligands in vivo have demonstrated that a deficiency of an individual ligand or its receptor can cause striking alterations in tissue phenotype (15, 29, 30). This is due in part to the organized nature of the chemokine response, which allows the effector functions of a specific moiety to be restricted to a specific stage of disease development and/or site of pathology (29, 30). It has been proposed that the inflammatory and physiologic response characteristics of the Th2 respiratory response are the result of the coordinated interactions of a variety of chemokines, including eotaxin/CCL11, MCP-1/CCL2, RANTES/CCL5, MIP-1α/CCL3, and macrophage-derived chemokine/CCL22 (29, 30). Our studies add to our knowledge of the coordination of this response by demonstrating for the first time that IL-13, a major effector at sites of Th2 inflammation, induces MCP-1/CCL2 and MIP-1α/CCL3 via a C10/CCL6-dependent mechanism. This is the first demonstration of a cytokine cascade contributing to the pathogenesis of the IL-13 phenotype and the first demonstration that C10/CCL6 is an obligatory intermediate in MCP-1/CCL2 production. MCP-1/CCL2 neutralization dramatically diminishes airway hyperresponsiveness and inflammation in the Ag-sensitized and -challenged airway (29). In addition, MCP-1/CCL2 signaling via CCR2 plays a critical role in the pathogenesis of IL-13-induced tissue responses (15). In accord with these observations, the present studies demonstrate that neutralization of C10/CCL6 and a null mutation of CCR1 markedly diminished IL-13-induced inflammation and tissue remodeling. It is tempting to speculate that these effects are due at least in part to the decreased ability of IL-13 to stimulate MCP-1/CCL2 in this setting. Additional investigation will be required, however, to determine the extent to which MCP-1/CCL2-dependent and -independent effects of C10/CCL6 contribute to these responses.

C10/CCL6 shares a significant degree of homology with a number of human chemokines, including MIP-1α/CCL15, CCF 18/CCL9/10, HCC-1/CCL14, and HCC-2/CCL15 (18, 22, 25). In
contrast to the standard genomic structure of CC chemokines, these moieties have four exons, with a novel second exon that is an important contributor to their biologic effector profiles (31). The human chemokines all signal via CCR1 (18, 25). Like C10/CCL6, they are also chemotactic for T cells and monocytes (18). Our studies demonstrate that CCR1 signaling is a critical event in the generation of IL-13-induced inflammation and remodeling. This is the first study to demonstrate that CCR1 plays an important role in the pathogenesis of Th2 responses and the first to suggest that CCR1 may play an important role in Th2-dominated diseases, such as asthma, COPD, and pulmonary fibrosis. Interestingly, the alterations in the IL-13 tissue phenotype seen after C10/CCL6 neutralization were very similar to those found in the absence of CCR1. These observations suggest that C10/CCL6, like the other members of its chemokine group, signals in part via CCR1.

The ability of IL-13 to induce alveolar enlargement, lung enlargement, and compliance alterations was decreased in mice treated with anti-C10/CCL6 and in mice with a null mutation of CCR1. These findings could be caused by a decrease in IL-13 production or an alteration in IL-13 effector function(s). Our studies demonstrate that effector mechanisms were altered because there were similar levels of IL-13 in BAL fluids from transgene-positive mice treated with anti-C10/CCL6 or the control Ab and mice with wild-type or null mutant CCR1 loci. A variety of effector pathway alterations could be postulated to contribute to these findings, including the diminished production of important proteases or the enhanced production of antiproteases. Our studies demonstrate that C10/CCL6 plays an important role in the regulation of both these groups of moieties. C10/CCL6 neutralization in wild-type mice diminished basal levels of MMP-2, MMP-9, and TIMP-4. This demonstrates that the levels of C10/CCL6 in normal lung stimulate the production of these moieties. Our studies also demonstrate that IL-13 stimulation of MMP-2, MMP-9, and cathepsins-K, -L and -S and IL-13 inhibition of α1-AT are mediated via C10/CCL6-dependent mechanisms. These are the first studies to demonstrate a relationship between C10/CCL6 and tissue protease or antiprotease responses. Previous studies from our laboratory demonstrated that IL-13 causes lung and alveolar enlargement and compliance changes at least in part via its ability to induce the production of MMPs and regulate the production of α1-AT (4, 15, 28). It is easy to see how amelioration of the inflammatory and remodeling effects of IL-13 after C10/CCL6 neutralization could be mediated by C10/CCL6-dependent protease and antiprotease alterations.

In summary, our studies demonstrate that IL-13 is a potent stimulator of C10/CCL6, and that C10/CCL6 production and signaling via its putative receptor, CCR1, are critical events in the pathogenesis of IL-13-induced inflammation and remodeling. They demonstrate that C10/CCL6 production and CCR1 signaling are also essential events in IL-13 induction of chemokines (MCP-1/CCL2 and MIP-1α/CCL3) and proteases (MMP-2, MMP-9, and cathepsin-K, -L, and -S) and IL-13 inhibition of α1-AT. Exaggerated IL-13 production has been implicated in the pathogenesis of a variety of disorders, including asthma, COPD, pulmonary fibrosis,
scleroderma, hepatic fibrosis, and nodular sclerosing Hodgkin’s disease. The present studies suggest that the effects of IL-13 in these disorders may be beneficially controlled via interventions that neutralize the human homologue of C10/CCL6 (presumably MIP-1α/H9254 or by interventions that regulate the function or signaling of CCR1. This establishes C10/CCL6 and the CCR1 pathway as worthwhile sites for future investigations designed to identify therapeutic agents that can be used to treat these and other IL-13-mediated disorders.

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References