

# Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling

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IL-13 potently stimulates eosinophilic and lymphocytic inflammation and alveolar remodeling in the lung, effects that depend on the induction of various matrix metalloproteinases (MMPs). Here, we compared the remodeling and inflammatory effects of an IL-13 transgene in lungs of wild-type, MMP-9-deficient, or MMP-12-deficient mice. IL-13-induced alveolar enlargement, lung enlargement, compliance alterations, and respiratory failure and death were markedly decreased in the absence of MMP-9 or MMP-12. Moreover, IL-13 potently induced MMPs-2, -12, -13, and -14 in the absence of MMP-9, while induction of MMPs-2, -9, -13, and -14 by IL-13 was diminished in the absence of MMP-12. A deficiency in MMP-9 did not alter eosinophil, macrophage, or lymphocyte recovery, but increased the recovery of total leukocytes and neutrophils in bronchoalveolar lavage (BAL) fluids from IL-13 transgenic mice. In contrast, a deficiency in MMP-12 decreased the recovery of leukocytes, eosinophils, and macrophages, but not lymphocytes or neutrophils. These studies demonstrate that IL-13 acts via MMPs-9 and -12 to induce alveolar remodeling, respiratory failure, and death and that IL-13 induction of MMPs-2, -9, -13, and -14 is mediated at least partially by an MMP-12-dependent pathway. The also demonstrate that MMPs-9 and -12 play different roles in the generation of IL-13-induced inflammation, with MMP-9 inhibiting neutrophil accumulation and MMP-12 contributing to the accumulation of eosinophils and macrophages.

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## Introduction

Tissue remodeling responses are prominent features of inflammatory disorders of the airway and parenchyma of the lung. These responses are readily apparent in chronic obstructive pulmonary disease (COPD), asthma, and interstitial lung diseases: in which remodeling

causes the alveolar septal destruction and changes in compliance that are characteristic of pulmonary emphysema (1), the subepithelial fibrosis, mucus metaplasia, and other structural alterations seen in asthmatic airway remodeling (2), and the pulmonary fibrosis that characterizes the interstitial disorders (3). Elevated levels of matrix metalloproteinases (MMPs) have been noted in patients with these disorders (4–12). In addition, studies from our laboratories and others demonstrated that MMP-12 plays an essential role in the pathogenesis of cigarette smoke-induced emphysema in mice (13) and that pretreatment with broad-spectrum MMP antagonists decreases the inflammation and airway hyperresponsiveness in murine models of asthma and the fibrosis in murine models of interstitial lung disorders (14, 15). Surprisingly, little else is known about the roles that individual MMPs play in the pathogenesis of these important pulmonary disorders.

IL-13 is a pleiotropic 12-kDa product of a gene on chromosome 5 at q31 that is produced in large quantities by Th2 cells and in lesser quantities by Th1 cells.

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**Nonstandard abbreviations used:** chronic obstructive pulmonary disease (COPD); matrix metalloproteinase (MMP); Clara cell 10-kDa protein (CC10); doxycycline (dox); bronchoalveolar lavage (BAL); hematoxylin and eosin (H&E); periodic acid-Schiff with diastase (D-PAS); histologic mucus index (HMI); macrophage inflammatory protein-2 (MIP-2); monocyte chemoattractant protein-1 (MCP-1); tissue inhibitor of metalloproteinases (TIMP).

Exaggerated IL-13 production has been documented in a variety of diseases characterized by inflammation and remodeling, including asthma, idiopathic pulmonary fibrosis, scleroderma, nodular sclerosing Hodgkin disease, and type II tissue granulomatous inflammation (16–24). The inflammatory and remodeling effector properties of IL-13 can be appreciated in studies from our laboratory that demonstrated that the constitutive and/or inducible transgenic overexpression of IL-13 in the murine lung generates a striking phenotype with eosinophil-, lymphocyte-, and macrophage-rich inflammation, airway remodeling with subepithelial fibrosis, parenchymal fibrosis, mucus metaplasia, and striking increases in alveolar size, lung size, and pulmonary compliance (25, 26). IL-13 is a potent inducer and activator of a variety of MMPs, including MMP-9 and MMP-12, in both of these transgenic modeling systems (25, 26). Previous studies from our laboratory demonstrated that MMP-9 plays a key role in the generation of IL-13-induced tissue fibrosis by contributing to the activation of latent TGF- $\beta$  (27). The contributions that MMP-9 and MMP-12 make to the pathogenesis of the inflammatory and alveolar remodeling effects of IL-13 have, however, not been defined.

We hypothesized that MMP-9 and MMP-12 play important and distinct roles in the generation of IL-13-induced tissue phenotypes. To test this hypothesis, we compared the inflammatory and remodeling responses induced by IL-13 in mice with wild-type (+/+) or null (-/-) MMP-9 or MMP-12 loci. These studies demonstrate that IL-13 induces alveolar enlargement, lung enlargement, compliance alterations, respiratory failure, and death via a, MMP-9- and MMP-12-dependent mechanism(s), and that IL-13 induction of MMPs-2, -9, -13, and -14 is mediated, in part, by an MMP-12-dependent pathway(s). They also demonstrate that MMP-9 and MMP-12 play different roles in the generation of IL-13-induced inflammation, with MMP-9 inhibiting neutrophil accumulation and MMP-12 making a crucial contribution to the accumulation of eosinophils and macrophages.

## Methods

**Transgenic mice.** Two types of 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 the CC10-IL-13 mice, the CC10 promoter drives the expression of murine IL-13 in a constitutive fashion. To allow IL-13 to be expressed in a temporally regulated fashion, CC10-rtTA-IL-13 mice were used. In these dual transgenic mice, the IL-13 transgene is activated by adding doxycycline (dox) to the animal's drinking water. In both transgenic systems, IL-13 caused a mononuclear cell- and eosinophil-rich tissue inflammatory response, alveolar enlargement, subepithelial and parenchymal fibrosis, and mucus metaplasia, as previously described (25, 26). In keeping with the chronic nature of the IL-13 production in the

CC10-IL-13 mice, the phenotype of these animals progressed most rapidly. These animals died prematurely from an inflammatory-fibrodestructive lung disorder.

The MMP-9<sup>-/-</sup> mice and the MMP-12<sup>-/-</sup> mice were generated as described previously by our laboratories (13, 28) and bred with C57BL/6 mice. CC10-IL-13 and CC10-rtTA-IL-13 mice with wild-type and null MMP loci were generated by breeding of the IL-13-overexpressing mice with the MMP<sup>-/-</sup> animals. As previously described (25, 26, 29), PCR was used to define the transgenic status of all offspring, using primers that detected rtTA and/or the junction region of our murine IL-13-human growth hormone construct. The MMP-9 loci were evaluated using PCR primers that determined whether exon 2 was intact and/or whether NEO inserts could be detected (28). The MMP-12 loci were evaluated by PCR using primers that were located in exon 1 (5'-CTG CCT GTG GGG CTG CTC CCAT-3') and exon 3 (5'-ATC CTC ACG CTT CAT GTC CG-3').

**Dox water administration.** In experiments performed with CC10-rtTA-IL-13 transgene<sup>+</sup> animals and their littermate controls, all animals were maintained on normal water until they were 1 month old. They were then randomized to receive either normal water or water with dox (500 mg/ml in 40% sucrose) for the duration of the experiment.

**Bronchoalveolar lavage and lung volume and compliance assessments.** Lung inflammation was assessed by bronchoalveolar lavage (BAL), and lung volume and compliance measurements were made using pressure fixation and volume displacement as previously described (25, 26, 30, 31).

**Histologic morphometric and mucus evaluations.** Mice were anesthetized, a sternotomy was performed, right heart perfusion with PBS was undertaken, and the heart and lungs were removed. The lungs were then inflated with Streck solution to a fixed pressure of 25 cm, embedded in paraffin, sectioned at 5  $\mu$ m, and stained. Hematoxylin and eosin (H&E), trichrome, and periodic acid-Schiff with diastase (D-PAS) stains were performed in the Research Histology Laboratory of the Department of Pathology at Yale University School of Medicine. Alveolar size was estimated from the mean chord length of the airspace as previously described by our laboratory (25, 31). The histologic mucus index (HMI), a measurement of the percentage of D-PAS<sup>+</sup> epithelial cells per unit of airway basement membrane, was calculated as previously described by our laboratory (25).

**mRNA analysis.** mRNA levels were evaluated by RT-PCR analysis, using whole-lung RNA and primer pairs previously described by our laboratory (25).  $\beta$ -Actin was used as an internal standard. Amplified PCR products were detected using ethidium bromide gel electrophoresis, quantitated electronically, and confirmed by nucleotide sequencing.

**Quantification of IL-13, chemokines, and TGF- $\beta$ <sub>1</sub>.** BAL IL-13, macrophage inflammatory protein-2 (MIP-2), KC, and monocyte chemoattractant protein-1 (MCP-1) were quantitated using commercial ELISA kits (R&D

Systems Inc., Minneapolis, Minnesota, USA) according to the manufacturer's instructions. Total and bioactive TGF- $\beta$ 1 were evaluated using mink lung epithelial cells permanently transfected with a construct containing the TGF- $\beta$ 1-responsive plasminogen activator inhibitor-1 promoter fused to the luciferase reporter gene (a gift from John Munger, New York University Medical Center, New York, New York, USA). Assays were performed with untreated and acid-treated BAL fluids incubated with control antibodies or antibodies that neutralize TGF- $\beta$ 1, as previously described (27).

**Collagen evaluation.** Lung collagen content was evaluated using trichrome evaluations, Sircol Collagen Assays (Biocolor Ltd., Belfast, United Kingdom), and Picosirius red staining as previously described (27).

**In situ hybridization.** To localize MMP-2 and MMP-14, in situ hybridization was used. The methods that were employed have been described previously by our laboratory (27).

**Western blot analysis.** Transgene<sup>-</sup> and transgene<sup>+</sup> CC10-rtTA-IL-13 mice with wild-type and null MMP loci were randomized to normal water or dox water at 1 month of age and maintained on this regimen for an additional month. Their lungs were then removed, and lysates were prepared using lysis buffer supplemented with antiproteases (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton, 2.5 mM Na phosphate, 50  $\mu$ M Na molybdate, 1 mM  $\beta$ -glycerolphosphate, 1  $\mu$ M Na vanadate, 1  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 1 mM PMSF). Equal concentrations (10  $\mu$ g) of protein from each lung were then loaded and run on 7% stacking and 12% resolving SDS-PAGE gels. Transfer to Immobilon (Millipore Inc., Bedford, Massachusetts, USA) PVDF membranes was accomplished as previously described (27). Protein detection was accomplished with a Super Signal West Femto Maximum kit (Pierce Chemical Co., Rockford, Illinois, USA) according to the manufacturer's instructions. Polyclonal goat antisera against MMP-2, MMP-9, MMP-13, and MMP-14 (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) were the primary antibodies that were employed. The secondary antibody was a mouse anti-goat IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology Inc.).

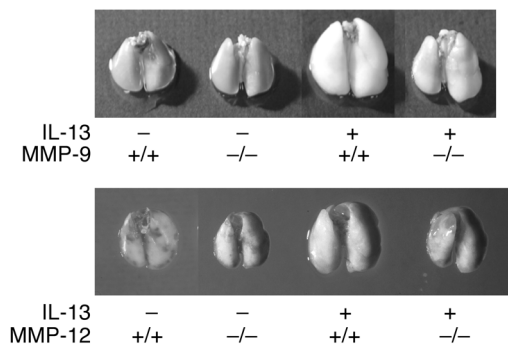
**Statistics.** Normally distributed data are expressed as means  $\pm$  SEM and were assessed for significance by Student's *t* test or ANOVA as appropriate. Data that were not normally distributed were assessed for significance using the Wilcoxon rank sum test.

## Results

**Role(s) of MMPs in IL-13-induced alterations in lung volume and alveolar size.** To determine whether MMP-9 or MMP-12 played an important role in the pathogenesis of IL-13-induced alterations in lung and alveolar size, we compared these parameters in CC10-rtTA-IL-13 transgene<sup>+</sup> mice with wild-type and null MMP-9 or MMP-12 loci. Lung size, lung volume, and alveolar size were similar in lungs from MMP-9<sup>-/-</sup> mice, MMP-12<sup>-/-</sup>

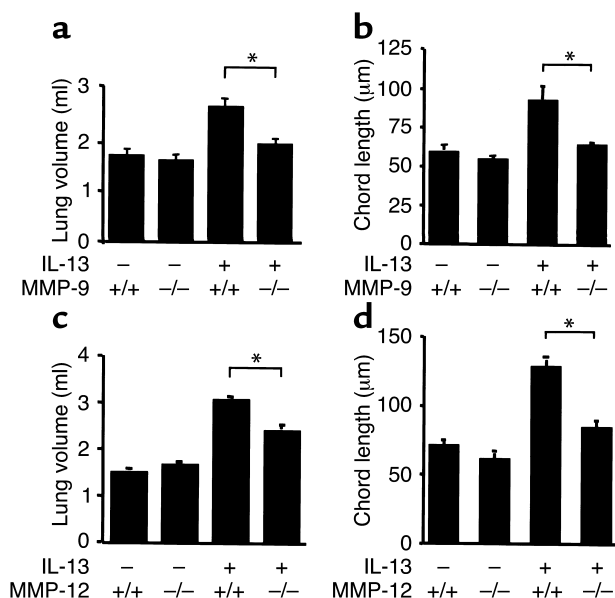
mice, and wild-type littermate controls (Figures 1 and 2). In accord with previous observations (25), these parameters were not altered by dox administration (data not shown). Dox induction of IL-13 production in transgene<sup>+</sup>/MMP<sup>+/+</sup> mice did, however, induce an impressive increase in all three of these parameters (Figures 1 and 2). In contrast, IL-13 did not have the same effect in mice that were deficient in either of these MMPs. After 1 or 2 months of dox administration, the size and volume of lungs from CC10-rtTA-IL-13<sup>+</sup>/MMP-9<sup>-/-</sup> mice and CC10-rtTA-IL-13<sup>+</sup>/MMP-12<sup>-/-</sup> mice were significantly smaller than those of lungs from CC10-rtTA-IL-13<sup>+</sup> animals with wild-type MMP loci (Figures 1 and 2 and data not shown). Alveolar size was also significantly decreased in dox-treated CC10-rtTA-IL-13 transgene<sup>+</sup> animals with null MMP-9 or MMP-12 loci. This was readily apparent morphometrically where the mean alveolar chord length in CC10-rtTA-IL-13<sup>+</sup>/MMP-9<sup>-/-</sup> mice and CC10-rtTA-IL-13<sup>+</sup>/MMP-12<sup>-/-</sup> mice was significantly smaller than the chord length in lungs from CC10-rtTA-IL-13<sup>+</sup>/MMP<sup>+/+</sup> animals (Figure 2, b and d). Similar decreases in lung volume and alveolar size were seen in comparisons of CC10-IL-13<sup>+</sup>/MMP<sup>+/+</sup> mice and CC10-IL-13<sup>+</sup> mice with null mutations of MMP-9 or MMP-12 (data not shown). With both transgenic systems, the decrease in alveolar size in IL-13-producing mice with null versus wild-type MMP-9 or MMP-12 loci was also readily apparent in comparisons of H&E-stained tissue sections (Figure 3). When viewed in combination, these studies demonstrate that MMP-9 and MMP-12 play similar critical roles in the pathogenesis of IL-13-induced alveolar and lung enlargement.

**Role(s) of MMPs in IL-13-induced alterations in lung compliance.** Enhanced pulmonary compliance with decreased elastic recoil is characteristic of COPD (1) and asthma (32) and prominent in lungs from IL-13-producing transgenic mice (25). To see whether MMP-9 or MMP-12 played a role in the generation of



**Figure 1**

Effect of MMP-9 and MMP-12 deficiency on IL-13-induced alterations in lung size. CC10-rtTA-IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice with wild-type and null MMP-9 (top) or MMP-12 (bottom) loci were generated and placed on dox water at 1 month of age. They were maintained on this regimen for an additional month, at which time their lungs were removed, fixed to pressure, and photographed.



**Figure 2** Effects of MMP deficiency on IL-13-induced alterations in lung volume and alveolar size. CC10-rtTA-IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice with wild-type and null MMP-9 or MMP-12 loci were generated and placed on dox water at 1 month of age. They were maintained on this regimen for an additional month, at which time their lungs were removed and fixed to pressure. Lung volume (a and c) and alveolar size (chord length; b and d) were assessed as described in Methods. The noted values represent the mean ± SEM of a minimum of five animals in each group (\**P* < 0.01).

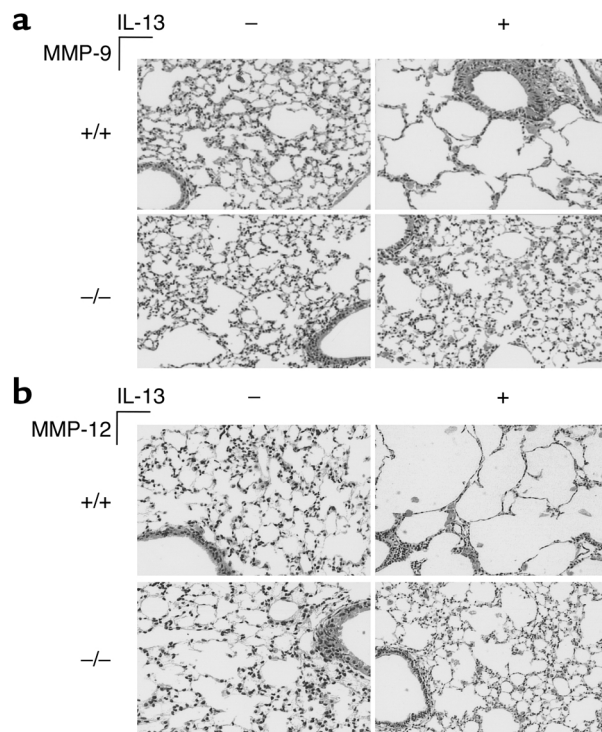
this abnormality, we compared lungs from littermate CC10-rtTA-IL-13 transgene<sup>+</sup> mice that were wild-type and null at these MMP loci. As previously reported (25), lungs from CC10-rtTA-IL-13<sup>+</sup>/MMP<sup>+/+</sup> mice were more compliant than lungs from transgene<sup>-</sup> controls (Table 1). Interestingly, lungs from CC10-rtTA-IL-13<sup>+</sup>/MMP-9<sup>-/-</sup> mice were less compliant than lungs from CC10-rtTA-IL-13<sup>+</sup>/MMP-9<sup>+/+</sup> animals (Table 1). Similarly, lungs from CC10-rtTA-IL-13<sup>+</sup>/MMP-12<sup>-/-</sup> mice were also less compliant than lungs from CC10-rtTA-IL-13<sup>+</sup>/MMP<sup>+/+</sup> controls (Table 1). These studies demonstrate that MMP-9 and MMP-12 play similar critical roles in the pathogenesis of the IL-13-induced pulmonary compliance alterations in these animals.

**Role(s) of MMPs in IL-13-induced mucus metaplasia.** Studies were next undertaken to determine whether MMP-9 or MMP-12 played an important role in the pathogenesis of IL-13-induced mucus metaplasia. As previously described (25), D-PAS-staining cells were not appreciated in airways from transgene<sup>-</sup> mice on normal or dox water, low-level mucus production was seen in CC10-rtTA-IL-13<sup>+</sup>/MMP<sup>+/+</sup> mice on normal water (HMI = 10.1 ± 3), and prominent mucus production was seen in CC10-rtTA-IL-13<sup>+</sup>/MMP<sup>+/+</sup> mice on dox water for 1 month (HMI = 53.1 ± 6). In addition, IL-13 induced epithelial hypertrophy and stimulated Muc5ac, Muc-4, and Muc-1 mRNA accumulation in lungs from these animals (data not shown). Importantly, similar levels of mucus metaplasia were seen in CC10-rtTA-IL-13

transgene<sup>+</sup> mice with null mutations of MMP-9 (HMI = 48.9 ± 9) or MMP-12 (HMI = 50.1 ± 6) (*P* > 0.5 vs. dox-treated CC10-rtTA-IL-13<sup>+</sup>/MMP<sup>+/+</sup> mice for both comparisons). In addition, IL-13 caused epithelial hypertrophy and stimulated mucin gene expression in the presence and absence of MMP-9 or MMP-12 (data not shown). Thus, neither MMP-9 nor MMP-12 played a critical role in the pathogenesis of IL-13-induced mucus metaplasia.

**Role(s) of MMP-12 in IL-13-induced tissue fibrosis.** Previous studies from our laboratory demonstrated that IL-13 is a potent stimulator of tissue fibrosis and that this tissue response is mediated, to a great extent, by the ability of IL-13 to stimulate the production of TGF-β<sub>1</sub> (27). Studies were thus undertaken to determine whether IL-13-induced TGF-β<sub>1</sub> elaboration and fibrosis were similarly altered in the absence of MMP-12. In these experiments, CC10-rtTA-IL-13 mice were used to evaluate TGF-β<sub>1</sub> production at time points before subepithelial fibrosis could be appreciated, and CC10-IL-13 mice were used to evaluate TGF-β levels at later time points when frank fibrosis was

**Figure 3** Alveolar effects of MMP-9 and MMP-12 deficiency. CC10-rtTA-IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice with wild-type and null MMP-9 (a) or MMP-12 (b) loci were generated and placed on dox water at 1 month of age. They were maintained on this regimen for an additional month, at which time their lungs were removed, fixed to pressure, and evaluated after H&E staining.



**Table 1**

IL-13-induced compliance alterations: role(s) of MMP-9 and MMP-12

Transgene	MMP	Volume (ml)	Compliance (ml/cm)
	<b>MMP-9</b>		
-	+/+	1.6	0.064 ± 0.001
-	-/-	1.55	0.062 ± 0.003
+	+/+	2.7	0.108 ± 0.040
+	-/-	1.85	0.074 ± 0.003 <sup>A</sup>
	<b>MMP-12</b>		
-	+/+	1.45	0.058 ± 0.001
-	-/-	1.60	0.065 ± 0.002
+	+/+	3.2	0.128 ± 0.050
+	-/-	2.2	0.088 ± 0.002 <sup>A</sup>

One-month-old CC10-rtTA-IL-13 mice with wild-type and null MMP-9 or MMP-12 loci were treated with dox water for 4 weeks. Lung volumes and static compliance were then assessed via volume displacement after pressure (25 cm) fixation. The noted values represent the mean ± SEM of a minimum of five animals (<sup>A</sup>*P* < 0.05 vs. transgene<sup>+</sup>/MMP<sup>+/+</sup> mice).

apparent. At the early (2- to 4-week) time points, IL-13 was a modest stimulator of TGF-β<sub>1</sub> elaboration in MMP-12<sup>+/+</sup> mice and this induction was decreased in IL-13<sup>+</sup>/MMP-12<sup>-/-</sup> animals (Figure 4). In contrast, at later time points (3.0–4 months), these differences could no longer be appreciated. At these time points, similar levels of tissue fibrosis and TGF-β<sub>1</sub> induction were noted in IL-13–overexpressing mice with wild-type and null MMP-12 loci (Figure 4).

**Roles of MMP-9 and MMP-12 in IL-13–induced respiratory failure and death.** As previously described (33), CC10-IL-13 mice experience progressive fibrodestructive lung alterations and die prematurely from respiratory failure. To determine whether MMP-9 or MMP-12 plays a role in these alterations, we compared the survival of CC10-IL-13 mice with wild-type and null MMP-9 or MMP-12 loci. The CC10-IL-13<sup>+</sup>/MMP<sup>+/+</sup> mice started to die when they were 90–100 days old, and 100% were dead by the time they were 144 days old. As can be seen in Figure 5, a deficiency of MMP-9 improved the survival of these animals. Overall, CC10-IL-13<sup>+</sup>/MMP-9<sup>-/-</sup> mice had a mean survival of 198 ± 14 days. In addition, 25% of these animals lived for more than 200 days (*P* < 0.01). Similarly, a deficiency of MMP-12 also improved the survival of the CC10-IL-13 animals, with 85% of CC10-IL-13<sup>+</sup>/MMP-12<sup>-/-</sup> animals living for more

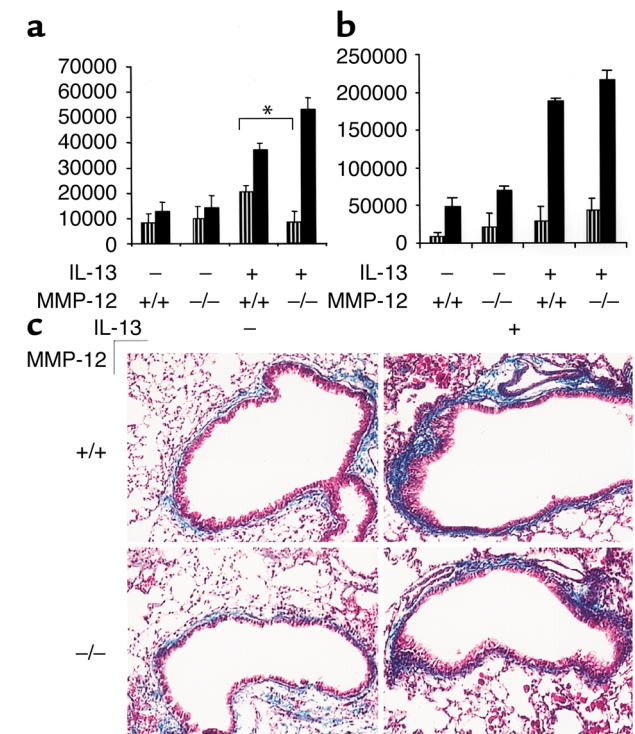
than 220 days (Figure 5). Interestingly, similar fibrotic, inflammatory, crystalline, and alveolar filling responses were noted in the terminal phases of the disease regardless of the status of the MMP-9 or the MMP-12 loci (Figure 5 and data not shown). Thus, MMP-9 and MMP-12 play important roles in the pathogenesis of IL-13–induced respiratory failure and death.

**Effect of MMP deficiency on IL-13 elaboration.** MMP deficiency could modify IL-13–induced phenotypes by altering IL-13 production or by modifying IL-13 effector function(s). To determine whether MMP-9 or MMP-12 regulated CC10 promoter–driven IL-13 elaboration, we compared the levels of BAL IL-13 in CC10-rtTA-IL-13 transgene<sup>+</sup> mice with wild-type and null MMP-9 or MMP-12 loci that had been on dox water for identical intervals. At all time points, similar levels of IL-13 were noted (data not shown). Thus, MMP-9 and MMP-12 alter IL-13–induced phenotypes by modifying IL-13 effector pathways.

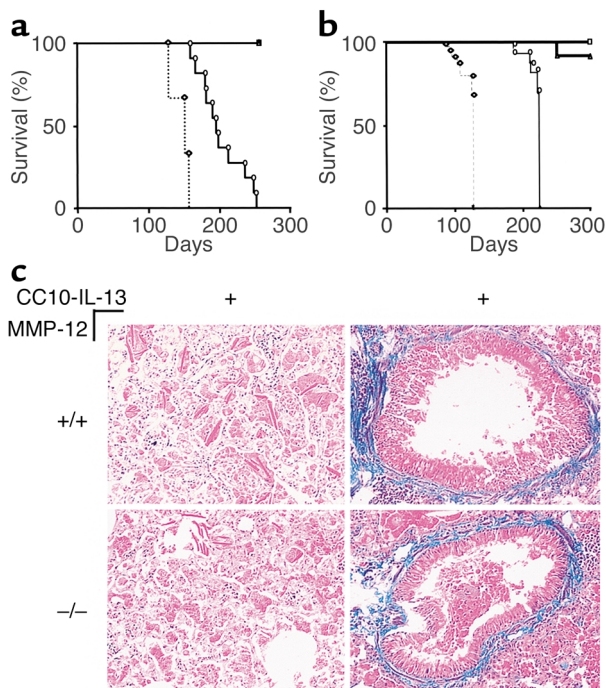
**Effects of MMP deficiency on IL-13 induction of lung proteases.** The studies noted above demonstrate that MMP-9 and MMP-12 play similar roles in the pathogenesis of IL-13–induced alveolar remodeling in the lung. We reasoned that this similarity could be due to the participation of both of these MMPs in the same MMP pathway(s) that mediates IL-13–induced lung proteolysis. To test this hypothesis, we compared the levels of MMP mRNA and protein in IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice with wild-type, MMP-9<sup>-/-</sup>, and MMP-12<sup>-/-</sup> loci. In transgene<sup>-</sup> mice, the levels of mRNA encoding MMP-2 and MMP-14 were slightly increased and the levels of mRNA encoding MMP-12 and MMP-13 were similar in MMP-9<sup>+/+</sup> and MMP-9<sup>-/-</sup> animals on normal water or

**Figure 4**

Effects of MMP-12 on IL-13–induced TGF-β<sub>1</sub> bioactivity and tissue fibrosis. (a) Transgene<sup>-</sup> and transgene<sup>+</sup> CC10-rtTA-IL-13 mice were placed on dox water for 4 weeks. BAL fluids were then obtained and the levels of bioactive (striped bars) and total (black bars) TGF-β<sub>1</sub> were assessed using mink lung epithelial cells as described in Methods. \**P* < 0.05. (b) BAL fluids were obtained from 3-month-old CC10-IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice, and the levels of bioactive and total TGF-β<sub>1</sub> were assessed using mink lung epithelial cells as described in Methods. (c) Trichrome stains are used to evaluate the subepithelial fibrosis in lungs from 3-month-old CC10-IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice with wild-type and null MMP-12 loci.







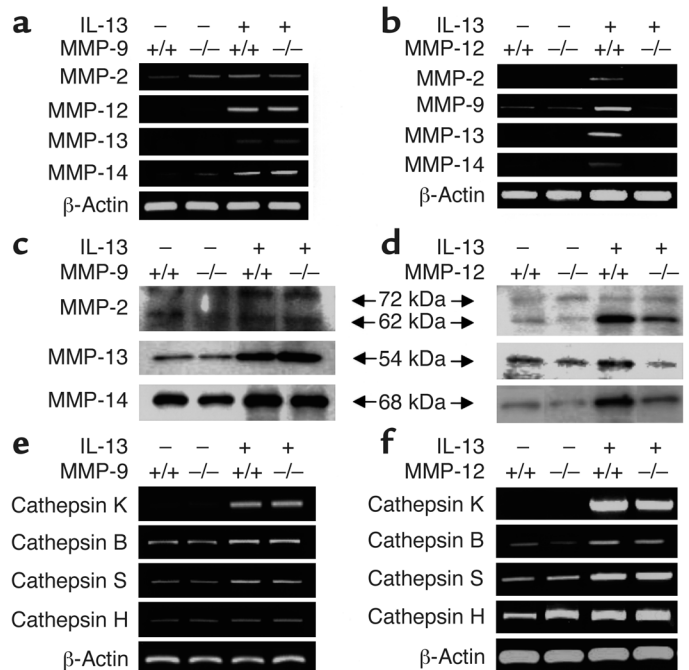
**Figure 5**

Survival and terminal pathology of CC10-IL-13 mice and littermate control mice with wild-type and null MMP loci. **(a)** The effects of MMP-9 deficiency on survival. **(b)** The effects of MMP-12 deficiency on survival. In **a** and **b** comparisons are made of the survival of transgene<sup>-</sup> mice with wild-type (squares) and null (triangles) MMP loci and transgene<sup>+</sup> mice with wild-type (diamonds) and null (circles) MMP loci. Each point represents the survival of a minimum of six animals. **(c)** The airway and parenchymal abnormalities in lungs from near-terminal 4-month-old IL-13<sup>+</sup>/MMP-12<sup>+/+</sup> mice and 6.5-month-old IL-13<sup>+</sup>/MMP-12<sup>-/-</sup> mice.

dox water (Figure 6a and data not shown). Similarly, the levels of mRNA encoding MMP-2, MMP-9, MMP-13, and MMP-14 were comparable in comparisons of MMP-12<sup>+/+</sup> and MMP-12<sup>-/-</sup> animals (Figure 6b and data not shown). In accord with previous studies from our laboratory (25), the levels of mRNA encoding MMPs-2, -9, -12, -13, and -14 were increased in dox-treated IL-13 transgene<sup>+</sup> mice with wild-type MMP loci (Figure 6, a and b). Similar levels of mRNA encoding MMPs-2, -12, -13, and -14 were seen in IL-13-producing mice with wild-type and null MMP-9 loci (Figure 6a). In contrast, the ability of IL-13 to stimulate MMP-2, MMP-9, MMP-13, and MMP-14 mRNA accumulation was decreased in the absence of MMP-12 (Figure 6b). This finding was most impressive for MMPs-2, -13, and -14. It was less impressive for MMP-9, whose mRNA induction was only partially abrogated in the absence of MMP-12 (Figure 6b). In all cases, comparable alterations in the levels of lung MMP proteins were noted on immunoblot evaluations (Figure 6, c and d). In addition, these findings were at least partially specific for these MMPs, as IL-13 induction of cathepsins H, K, L, and S were not altered in IL-13 transgene<sup>+</sup>/MMP-12<sup>-/-</sup> mice (Figure 6, e and f). Importantly, in situ hybridization demonstrated that MMP-2 and MMP-14 were made in the same cells in transgene<sup>+</sup> mice with wild-type and null mutant MMP loci. Specifically, MMP-2 mRNA was detected in airway and alveolar epithelial cells and inflammatory cells, and MMP-14 mRNA was detected in airway and alveolar epithelial cells and macrophages in IL-13 transgene<sup>+</sup> mice with

wild-type or null MMP loci (Figure 7 and data not shown). These studies demonstrate that a deficiency of MMP-9 is not associated with a decrease in the basal or IL-13-induced levels of expression of important respiratory MMPs. They also demonstrate that a deficiency of MMP-12 is associated with decreased MMP induction, because IL-13 stimulates the production of MMPs-2, -9, -13, and -14 via a pathway(s) that is, at least partially, MMP-12-dependent.

*Effects of MMP deficiency on IL-13-induced inflammation.* Studies were next undertaken to determine whether IL-13 induced similar inflammatory responses in mice with wild-type or null MMP loci. The number of cells that were recovered and their differentials were similar in BAL fluids from transgene<sup>-</sup> littermate controls, MMP-9<sup>-/-</sup> mice, and MMP-12<sup>-/-</sup> mice on normal water



**Figure 6**

IL-13 regulation of lung MMPs and cathepsins. CC10-rTA-IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice with wild-type and null MMP-9 loci (**a**, **c**, and **e**) or MMP-12 loci (**b**, **d**, and **f**) were placed on dox water at 1 month of age. After an additional month on this regimen, the levels of mRNA encoding the noted MMPs (**a** and **b**), lung lysate MMP protein levels (**c** and **d**), and the levels of mRNA encoding the noted cathepsins (**e** and **f**) were evaluated via RT-PCR and immunoblot analysis as described in Methods.

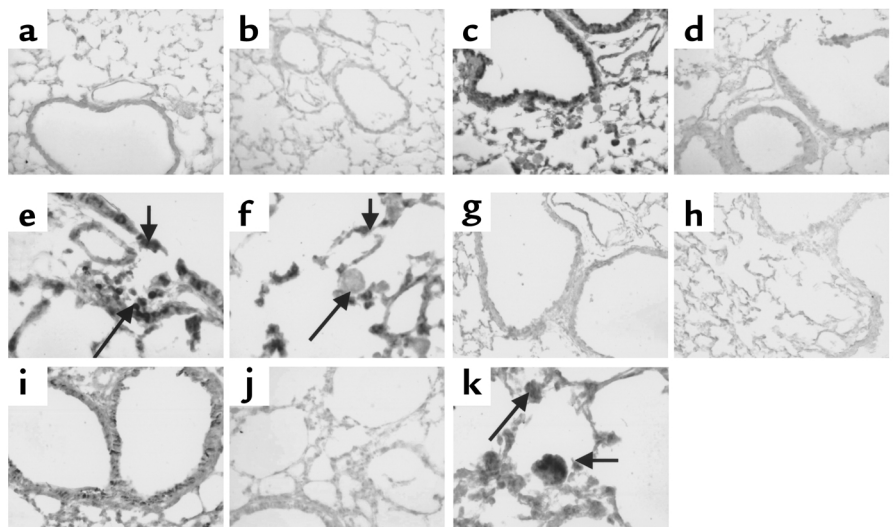
and dox water (Figures 8 and 9 and data not shown). IL-13 production in CC10-rtTA-IL-13 mice altered these parameters by increasing BAL cell recovery and the percentage and recovery of lymphocytes and eosinophils (Figures 8 and 9). MMP-9 deficiency did not decrease the number of cells that were recovered in BAL fluids from CC10-rtTA-IL-13 transgene<sup>+</sup> mice. In fact, significantly greater numbers of leukocytes were noted in BAL fluids from dox-treated CC10-rtTA-IL-13 transgene<sup>+</sup> mice with null versus wild-type MMP-9 loci (Figure 8a). Differential cell counts demonstrated that a deficiency of MMP-9 did not significantly alter IL-13-induced BAL macrophage, lymphocyte, and eosinophil recovery (Figure 8) but did increase the percentage and recovery of BAL neutrophils (Figure 8d and data not shown). Interestingly, these BAL alterations were associated with a modest decrease in tissue inflammation and scattered tissue neutrophils (Figure 10). In contrast, IL-13-induced BAL cellularity was markedly decreased in CC10-rtTA-IL-13<sup>+</sup> mice with null MMP-12 loci (Figure 9a). Differential cell counts demonstrated that a deficiency of MMP-12 did not alter neutrophil or lymphocyte recovery but did markedly diminish the recovery of macrophages and eosinophils (Figure 9, b–e). These findings were associated with a significant decrease in tissue inflammation (Figure 10). When viewed in combination, these studies demonstrate that MMP-9 and MMP-12 play different roles in the pathogenesis of IL-13-induced inflammation, with MMP-9 inhibiting IL-13-induced neutrophil accumulation and MMP-12 playing a critical role in IL-13-induced macrophage and eosinophil influx.

*MMP-9 regulation of neutrophil chemotactic cytokines.* To gain insight into mechanisms that might contribute to the enhanced BAL neutrophilia in IL-13-producing mice with null versus wild-type MMP-9 loci, we compared the levels of the neutrophil chemotactic chemokines KC and MIP-2 in BAL fluids from these animals. KC and MIP-2 were undetectable at all time points in BAL fluids from wild-type and MMP-9<sup>-/-</sup> mice that received normal or dox water (Figure 11 and data not shown). In contrast, IL-13 was a potent inducer of both of these

chemokines. This stimulation was seen after as few as 10 days and continued to rise during the 2-month course of dox administration. Importantly, the levels of both of these cytokines were significantly increased in CC10-rtTA-IL-13 transgene<sup>+</sup> mice with null MMP-9 loci. This increase was seen after 10 days and leveled off after 30 days of dox administration. It was also at least partially specific for these chemokine moieties, since a deficiency of MMP-9 decreased the levels of IL-13-stimulated MCP-1 in the same fluids (Figure 11). A similar decrease in MCP-1 levels was noted in BAL fluids from IL-13<sup>+</sup>/MMP-12<sup>-/-</sup> animals (data not shown). These studies demonstrate that MMP-9 is an important and selective inhibitor of the accumulation of neutrophil chemotactic chemokines.

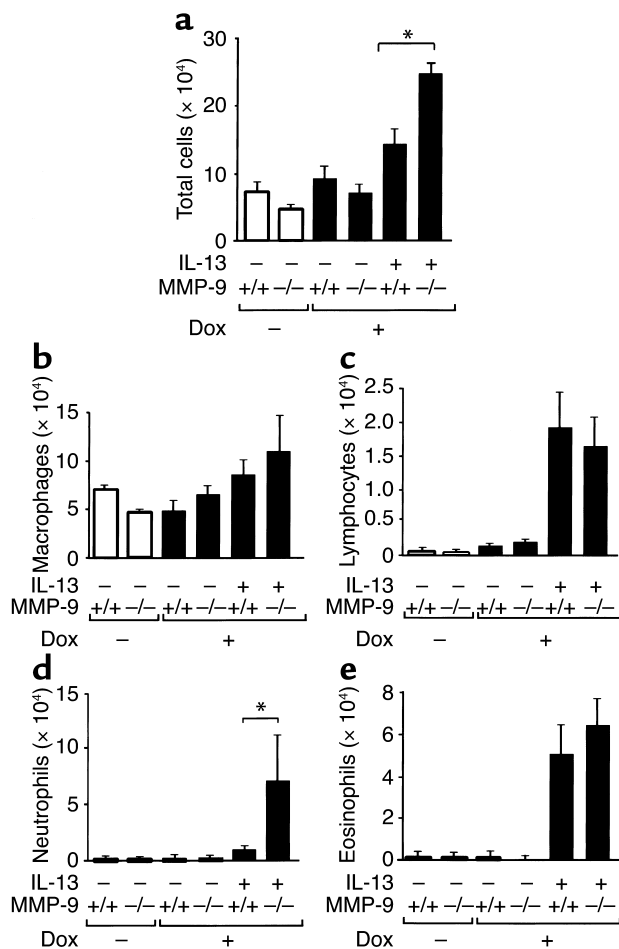
## Discussion

IL-13 was originally described as an IL-4-like cytokine with effector properties relevant to Th2 inflammation (16, 34–36). More recent studies, from our laboratory and others, demonstrated that IL-13 is also a powerful *in vivo* regulator of tissue remodeling with the ability to activate a variety of MMPs and cathepsins and generate destructive and fibrotic structural responses (22, 25–27, 37, 38). To gain additional insight into the mechanisms of IL-13-induced inflammation and remodeling, we took advantage of transgenic systems



**Figure 7**

Localization of MMP-2 and MMP-14 mRNA. (a–f) ISH was used to localize the MMP-2 mRNA in lungs from dox-treated CC10-rtTA-IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice. (a) A transgene<sup>-</sup> mouse incubated with antisense probe. (b) A transgene<sup>-</sup> mouse incubated with sense probe. (c) A transgene<sup>+</sup> mouse incubated with antisense probe. (d) A transgene<sup>+</sup> mouse incubated with sense probe. (e) A transgene<sup>+</sup> mouse incubated with antisense probe. The large and small arrows in e illustrate inflammatory cell and epithelial cell staining, respectively. (f) A transgene<sup>+</sup> mouse incubated with antisense probe. The large and small arrows in f illustrate negatively staining macrophages and positively staining alveolar epithelial cells, respectively. (g–k) ISH was used to localize the MMP-14 mRNA in lungs from dox-treated CC10-rtTA-IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice. (g) A transgene<sup>-</sup> mouse incubated with antisense probe. (h) A transgene<sup>-</sup> mouse incubated with sense probe. (i) A transgene<sup>+</sup> mouse incubated with antisense probe. (j) A transgene<sup>+</sup> mouse incubated with sense probe. (k) A transgene<sup>+</sup> mouse incubated with antisense probe. The large and small arrows in k highlight positively staining alveolar epithelial cells and macrophages, respectively.



**Figure 8** Effect of MMP-9 deficiency on BAL leukocyte recovery. BAL was performed on transgene<sup>-</sup> and transgene<sup>+</sup> CC10-rtTA-IL-13 mice with wild-type and null MMP-9 loci that had been randomized to normal water or dox water. Total cell recovery (a) and the recovery of macrophages (b), lymphocytes (c), neutrophils (d), and eosinophils (e) are illustrated. Each value represents the mean  $\pm$  SEM of a minimum of four animals (\* $P < 0.01$ ).

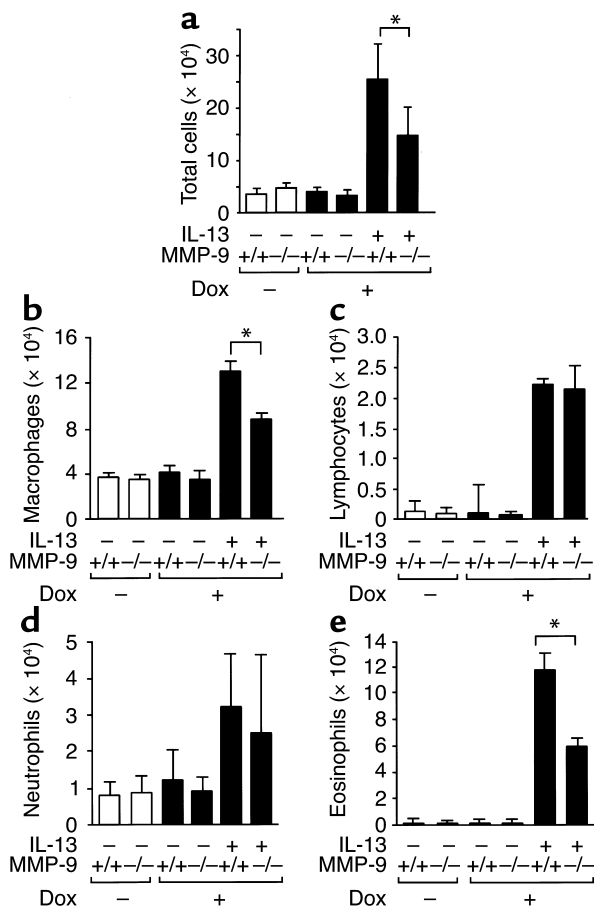
developed in our laboratory in which IL-13 overexpression causes eosinophil-, lymphocyte-, and mononuclear cell-rich lung inflammation, MMP and cathepsin induction, alveolar enlargement, lung enlargement, and enhanced pulmonary compliance (25, 26). By comparing the phenotypes induced by IL-13 in mice with wild-type and null MMP-9 or MMP-12 loci, we were able to define the contributions that these MMPs make in the generation of these responses. These studies demonstrate previously unappreciated relationships between these MMPs and IL-13, and between MMP-12 and other IL-13-regulated MMPs. Specifically, they demonstrate that IL-13-induced alveolar enlargement, lung enlargement, compliance alterations, respiratory failure, and death are mediated by MMP-9- and MMP-12-dependent pathways. They also demonstrate that MMP-9 and MMP-12 play important but distinct roles in the

pathogenesis of IL-13-induced inflammation, with MMP-9 having prominent inhibitory effects on BAL leukocyte accumulation and neutrophilia, and MMP-12 making a critical contribution to the accumulation of eosinophils and macrophages. Lastly, these studies demonstrate that, unlike MMP-9, MMP-12 plays an important role in MMP induction, since MMP-12 is required for optimal IL-13 stimulation of MMP-2, -9, -13, and -14 mRNA accumulation. When viewed in combination, these studies demonstrate that MMP-9 and MMP-12 are critical downstream mediators and regulators of IL-13-induced inflammatory and alveolar remodeling responses. They also provide crucial support for the concept that therapies directed against these MMPs can be used to control IL-13-induced tissue responses.

Our studies demonstrate that the ability of IL-13 to induce alveolar enlargement, lung enlargement, compliance alterations, respiratory failure, and death was decreased in mice with null MMP-9 or MMP-12 mutations when compared with wild-type controls. A variety of effector pathway alterations can be postulated to contribute to the similarity of these findings. These include the decrease in the degradation of common matrix substrates (such as type IV collagen, elastin, aggrecan, and laminin) that would be seen in these MMP<sup>-/-</sup> animals (39). Alternatively, a deficiency of one or both of these MMPs could enhance the production of antiproteases that regulate these responses. Credence for this possibility comes from studies that demonstrate that MMP-9 can inactivate the important pulmonary antiprotease  $\alpha_1$ -antitrypsin (40). Lastly, one or both of these MMPs could play a critical role in the production of other proteases that are involved in this response. The present studies support this concept by demonstrating, for the first time to our knowledge, that MMP-12 is required for the optimal induction of MMPs-2, -9, -13, and -14 by IL-13. This MMP-12 dependence was most pronounced for MMP-2 and MMP-14. Interestingly, in situ hybridization undertaken with lungs from transgene<sup>+</sup> MMP<sup>+/+</sup> and MMP<sup>-/-</sup> mice demonstrated that MMP-2 is produced by inflammatory cells and airway and alveolar epithelial cells while MMP-14 is produced by macrophages and epithelial cells in these tissues. This demonstrates that the decreased production of MMP-2 and MMP-14 in lungs from transgene<sup>+</sup> MMP-12-null mutant mice cannot be attributed solely to the decrease in tissue inflammation that is seen in comparisons of these mice and IL-13-overexpressing MMP-12<sup>+/+</sup> animals. Additional experimentation will be required, however, to fully define the mechanism(s) that is responsible for the decreased production of these MMPs in IL-13-stimulated MMP-12-deficient pulmonary tissues.

In prior studies we demonstrated that IL-13 is a potent stimulator of pulmonary inflammation and that treatment with broad-spectrum MMP antagonists inhibited IL-13-induced BAL leukocytosis and pulmonary inflammation (25, 26). Tissue inhibitors of metalloproteinases



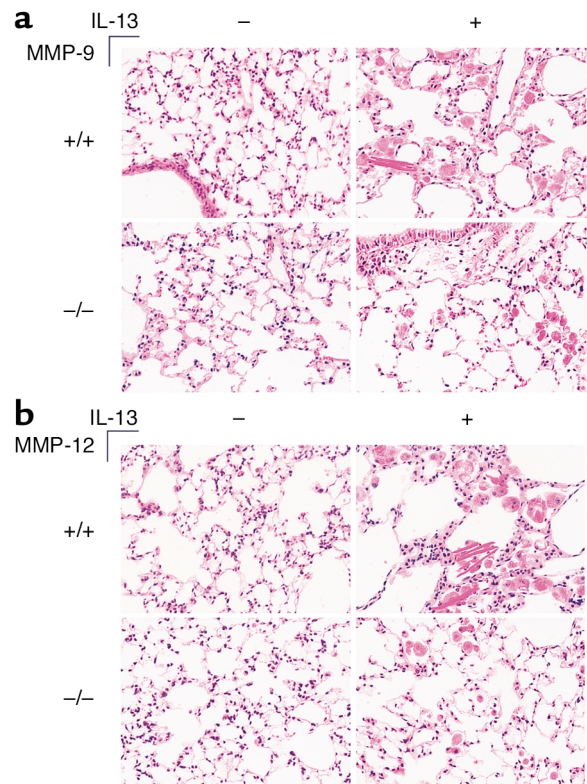


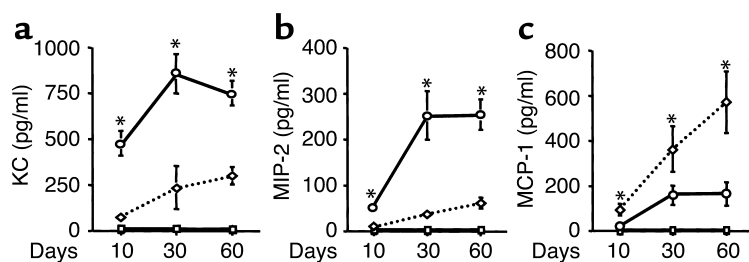
**Figure 9**  
Effect of MMP-12 deficiency on BAL leukocyte recovery. BAL was performed on transgene<sup>-</sup> and transgene<sup>+</sup> CC10-rtTA-IL-13 mice with wild-type and null MMP-12 loci that had been randomized to normal water or dox water. Total cell recovery (a) and the recovery of macrophages (b), lymphocytes (c), neutrophils (d), and eosinophils (e) are illustrated. Each value represents the mean ± SEM of a minimum of four animals (\**P* < 0.01).

metabolizes and inactivates neutrophilic chemokines, including GRO $\alpha$  (44) and mature IL-1 $\beta$  (43); elevated levels of tissue neutrophils have been noted in adult MMP-9<sup>-/-</sup> mice (42); and enhanced inflammatory cell infiltration and IL-1 $\alpha$  expression have been noted in epithelial wounds in MMP-9<sup>-/-</sup> animals (45). In accord with the documented ability of MMPs to regulate and degrade chemokines, we noted markedly elevated levels of the neutrophil chemotactic chemokines KC and MIP-2, but not the macrophage chemotactic chemokine MCP-1, in BAL fluids from IL-13 transgenic mice with null MMP-9 loci. We also noted that the enhanced levels of KC and MIP-2 were not associated with comparable increases in the mRNA encoding these moieties, demonstrating that these events are regulated by translational and/or posttranslational processes (S. Lanone and J.A. Elias, unpublished observations). When viewed in combination, these studies demonstrate that, in contrast to their similar roles in IL-13-induced alveolar remodeling, MMP-9 and MMP-12 play different roles in the pathogenesis of IL-13-induced inflammation. Specifically, they demonstrate that MMP-9 is a physiologic regulator of neutrophilic responses while MMP-12 plays a critical

(TIMPs), synthetic MMP antagonists, and a null mutation of MMP-12 have also been demonstrated to decrease inflammation in murine asthma, COPD, and immune complex models (13, 15, 41). As a result we hypothesized that MMP-9 and/or MMP-12 play key roles in the generation of IL-13-induced tissue inflammation. In accord with our hypothesis, IL-13-induced leukocyte, macrophage, and eosinophil BAL recovery and tissue inflammation were decreased significantly in MMP-12<sup>-/-</sup> animals. In contrast, a deficiency of MMP-9 did not diminish IL-13-induced eosinophil and lymphocyte recovery and increased IL-13-induced leukocyte and neutrophil accumulation. On superficial analysis this would appear to be counterintuitive in light of studies that demonstrate that MMP-9 enhances neutrophil chemotaxis in response to some chemokines (42), activates pro-IL-1 $\beta$  (43), increases the bioactivity of IL-8 (44), and contributes to neutrophil influx in early stages of glomerulonephritis (39). On the other hand, MMP-9

**Figure 10**  
Inflammation regulating effects of MMP-9 and MMP-12. CC10-rtTA-IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice with wild-type and null MMP-9 (a) or MMP-12 (b) loci were generated and placed on dox water at 1 month of age. One month later their lungs were removed, fixed to pressure, sectioned, stained with H&E, and evaluated via light microscopy (×20).





**Figure 11**

Effect of MMP-9 deficiency on BAL chemokine accumulation. CC10-rtTA-IL-13 transgene<sup>-</sup> and transgene<sup>+</sup> mice with wild-type and null MMP-9 loci were placed on dox water at 1 month of age and maintained on dox water for an additional 10–60 days as noted. The levels of BAL KC (a), MIP-2 (b), and MCP-1 (c) protein were assessed by ELISA. Circles, CC10-rtTA-IL-13<sup>+</sup>/MMP-9<sup>+/+</sup>; diamonds, CC10-rtTA-IL-13<sup>+</sup>/MMP-9<sup>-/-</sup>; squares, CC10-rtTA-IL-13<sup>-</sup>/MMP-9<sup>+/+</sup>. Note that the levels of chemokines in BAL fluids from CC10-rtTA-IL-13 transgene<sup>-</sup> mice with null and wild-type MMP-9 loci are superimposed in these graphs. The noted values represent the mean ± SEM of a minimum of six animals at each time point (\**P* < 0.01).

role in the generation of eosinophilic, and monocyte/macrophage responses in pulmonary tissues. It is important to point out, however, that MMPs can also regulate tissue inflammation via a variety of other mechanisms, including the generation of chemotactic matrix and complement fragments, regulation of leukocyte passage through the vascular basement membrane, regulation of cell surface cytokines and receptors, and the generation of chemokine fragments with receptor antagonist properties (13, 41, 46). The degree to which these mechanisms contribute to the effects of MMP-9 and MMP-12 in our models will need to be investigated.

In keeping with our prior observations (33), the present studies demonstrate that IL-13 causes a progressive fibrodestructive and alveolar filling lung disorder that eventuates in respiratory failure and premature death. They also demonstrate that survival is enhanced in transgene<sup>+</sup> animals with MMP-9<sup>-</sup> or MMP-12<sup>-</sup> null mutations. Interestingly, when transgene<sup>+</sup> MMP<sup>-/-</sup> animals died they had pulmonary pathologic alterations that could not be distinguished from those in transgene<sup>+</sup> MMP<sup>+/+</sup> animals. This demonstrates that a deficiency of a single MMP slows but does not abrogate the relentless progression of IL-13-induced disease in this model. It also suggests that, in the absence of MMP-9 or -12, other MMPs (or other proteolytic enzymes) are able to “fill in” to generate the proteolytic conditions and tissue destruction seen in the lungs from transgene<sup>+</sup> animals. These studies highlight the potential utility of therapeutic interventions that target MMP-9 and/or MMP-12 in the treatment of IL-13-induced fibrodestructive disorders. In addition, if the MMP or other proteolytic events that are operative in the absence of MMP-9 or -12 can be defined, these studies suggest that an intervention(s) that simultaneously targets MMP-9 and/or -12 and these permissive proteolytic events may be particularly useful.

Chronic inflammation is a characteristic feature of asthmatic airways. In most patients, eosinophils and lymphocytes are the major inflammatory cells.

However, in some patients, especially those with chronic refractory disease, impressive increases in neutrophils have been noted (47). Via poorly understood mechanisms, these alterations are believed to cause the airway obstruction and airway hyperresponsiveness that are the most commonly assessed physiologic abnormalities in this disorder. A significant decrease in lung elastic recoil has also been reported in asthmatics (32, 48). The mechanisms that are responsible for the different types of inflammation in asthmatic airways are poorly understood. In addition, the mechanisms that are responsible for the loss of elastic recoil are among the most elusive in asthma pathogenesis (32). A large body of data suggests that IL-13 is a crucial mediator in the pathogenesis of asthma (18–20).

Furthermore, numerous studies have demonstrated that MMP-9 is found in exaggerated quantities in BAL fluids, sputum, serum, and tissues from patients with asthma (5–7, 49), and elevated MMP-9/TIMP-1 ratios have been shown to correlate with therapeutic responses to steroids in these patients (50). Surprisingly, the role(s) of MMP-9 in the generation of the asthmatic phenotype and the relationships between MMP-9 and the anatomic, inflammatory, and physiologic alterations characteristic of asthma have not been elucidated. Our studies, however, provide insights into the roles that MMP-9 may be playing in this disorder. Specifically, our demonstration that MMP-9 plays an important role in IL-13-induced proteolytic responses, inflammatory responses, and compliance alterations in the murine lung suggests that MMP-9 may play similar roles in the human asthmatic respiratory system. Thus, the levels of MMP-9 could influence the intensity and nature of the asthmatic inflammatory response. In particular, elevated levels of MMP-9 might be seen in patients with steroid-responsive disease characterized by eosinophilic and lymphocytic inflammation. In contrast, decreased levels of MMP-9 (or MMP-9 function) might be seen in patients with steroid-unresponsive, refractory disease with neutrophil-rich tissue inflammation (47, 50). In addition, genetic polymorphisms or other alterations that cause individual-to-individual alterations in MMP-9 production or effector function could contribute to the varied natural history of this disorder. It is also tantalizing to hypothesize that MMP-9 is a critical mediator of the loss of elastic recoil seen in the asthmatic lung and that MMP-9 antagonists will eliminate this abnormality. This is a very exciting possibility, because this physiologic abnormality is responsible for 35–55% of the reduction in maximal expiratory flow in patients with chronic persistent asthma (32), and treatments that address this abnormality do not presently exist.

The protease/antiprotease hypothesis has dominated pathogenetic thinking in COPD for more than 35 years. It proposes that an antiprotease “shield” protects the normal lung from locally elaborated proteases and that emphysema is the result of an abnormal increase in proteases and/or reduction in pulmonary antiproteases (1). Inflammation has been proposed to contribute to the pathogenesis of COPD by altering this balance. In accord with this hypothesis, macrophage-, lymphocyte-, neutrophil-, and/or eosinophil-rich inflammation is a characteristic feature of lungs from patients with COPD, and epidemiologic and transgenic studies suggest that IL-13 and/or related cytokines may contribute in important ways to the pathogenesis of this disorder (51–56). The exaggerated production and/or expression of MMPs have been repeatedly documented in studies of tissues, sputum, BAL fluids, and BAL cells from patients with COPD (4, 8, 10, 57, 58). Our studies demonstrate that MMP-9 and MMP-12 play crucial roles in the alveolar enlargement, lung enlargement, enhanced compliance, and eventual respiratory failure and death seen in IL-13-overexpressing transgene<sup>+</sup> mice. They also demonstrate that MMP-9 and MMP-12 play unique roles in the generation of the neutrophilic and eosinophil/macrophage-rich inflammation seen in these animals. These observations suggest that these MMPs may play similar crucial roles in the pathogenesis of the alveolar remodeling, inflammation, and respiratory failure in patients with COPD. As a consequence, these MMPs are outstanding targets against which pharmacologic therapies can be directed. The validation of these targets is an important issue for COPD, since therapies that intercede in the pathogenetic pathways that destroy lung tissue in this disease do not exist and our present therapies (bronchodilators and corticosteroids) do not consistently decrease MMP expression (59). Therapeutic interventions focused on MMP-9 and/or MMP-12 may also be important in a wide variety of other pulmonary diseases characterized by exaggerated MMP and/or IL-13 production, including idiopathic pulmonary fibrosis, hypersensitivity pneumonitis, lung cancer, nonspecific interstitial pneumonitis/bronchiolitis obliterans organizing pneumonia (NSIP/BOOP), and silicosis (9, 11, 12, 60).

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