

Interleukin-13–Dependent Expression of Matrix Metalloproteinase-12 Is Required for the Development of Airway Eosinophilia in Mice

Mahmoud A. Pouladi, Clinton S. Robbins, Filip K. Swirski, Meghan Cundall, Andrew N.J. McKenzie, Manel Jordana, Steven D. Shapiro, and Martin R. Stämpfli

Department of Pathology and Molecular Medicine, Centre for Gene Therapeutics, McMaster University, Hamilton, Ontario, Canada; MRC Laboratory of Molecular Biology, Cambridge, United Kingdom; and Department of Respiratory Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

We investigated the expression and function of matrix metalloproteinase-12 (MMP-12) in a model of allergic airway inflammation. Mice were sensitized mucosally by exposure to aerosolized ovalbumin (OVA) daily over a period of 10 d in the context of adenovirus-mediated granulocyte macrophage colony-stimulating factor (GM-CSF) expression. The ensuing inflammatory response is characterized by a Th2 cytokine profile, OVA-specific IgE, and airway eosinophilia. Using real-time, quantitative reverse transcriptase–polymerase chain reaction we assessed MMP-12 mRNA expression in whole lung tissue. We observed a 12- and 70-fold increase in expression at Days 7 and 11, respectively, in OVA-exposed mice when compared with naive controls. Immunoblot analysis revealed an increase in MMP-12 protein in the bronchoalveolar lavage fluid of mice exposed to OVA in the context of GM-CSF. No such elevation was observed in mice exposed to saline only in the context of GM-CSF. To assess functional role of MMP-12, MMP-12 knockout (KO) mice were subjected to the aforementioned protocol. We observed an 80% reduction in eosinophils in the bronchoalveolar lavage fluid of KO mice compared with their wild-type littermates. Using interleukin-13 KO mice, we demonstrated that expression of MMP-12 is interleukin-13–dependent. Collectively, our data indicate a novel function for MMP-12 in the process of airway eosinophil accumulation.

Matrix metalloproteinases (MMPs) are a family of extracellular proteinases that are responsible for the degradation of the extracellular matrix during tissue remodeling events. The enzymes share common structural and functional domains as well as activation mechanisms (1). MMPs are synthesized as secreted or transmembrane proenzymes and processed to an active form by the removal of an amino terminal propeptide. Their enzymatic activity is Zn^{2+} -dependent and is active at neutral pH. Collectively, MMPs are responsible for the degradation of all components of the extracellular matrix, and they are classified based on

their catalytic/degradative ability into six groups: interstitial collagenases, stromelysins, gelatinases or type IV collagenases, matrilysin, metalloelastase or macrophage elastase, and membrane-type MMPs (2). Abnormal MMP production has been implicated in a number of chronic inflammatory disorders (3), including several lung diseases (4).

In asthma, a possible involvement of MMPs is suggested by morphologic studies showing features of matrix remodeling, lung tissue damage, or abnormal repair (5–8). Moreover, clinical studies have demonstrated elevated levels of MMPs in the bronchoalveolar lavage fluid (BAL) and the sputum of patients with asthma (9–11). MMP involvement in asthma is further supported by studies in animal models. Specifically, a study in mice overexpressing interleukin (IL)-13, a prototypical Th2 cytokine, has documented elevated levels of proteolytic enzymes, including MMP-2, -9, and -12, in the lung (12). Furthermore, in a murine model of allergen-induced airway inflammation, administration of tissue inhibitor of matrix metalloproteinase (TIMP)-1 and -2 resulted in reduced airway eosinophilia and airway hyper-responsiveness (13). The authors conclude that MMPs, especially MMP-2 and -9, are crucial for the infiltration of inflammatory cells. This notion is supported by studies utilizing murine models of antigen-induced airways inflammation in MMP-deficient animals, where a deficiency in MMP-2 led to reduced influx of cells into the BAL and an accumulation of inflammatory cells in the lung parenchyma (14). Additionally, animals deficient in MMP-9 had significantly reduced peribronchial mononuclear cell infiltration of the airways and reduced lymphocytes in the BAL, as compared with wild-type (WT) mice (15). In this study, we wished to investigate the role of MMP-12, a macrophage metalloelastase, in a murine model of allergic mucosal sensitization (16). Mice are exposed to ovalbumin (OVA) in the context of a granulocyte macrophage colony-stimulating factor (GM-CSF)–enriched airway microenvironment. GM-CSF expression is crucial, because exposure to OVA alone leads to the induction of inhalation tolerance (17). Previously, we have reported on this model in several studies, documenting robust airway eosinophilia, high levels of IgE production, and expression of Th2 cytokines such as IL-4, IL-5, and IL-13, all hallmarks of asthma (16, 18, 19).

We document increased expression of MMP-12 mRNA and protein in the lungs of mice subjected to a protocol of mucosal allergic sensitization. We find that the accompanying airway eosinophilia is dramatically reduced when MMP-12 knockout (KO) mice are subjected to this protocol, despite the presence of serum immunoglobulins and Th2 cytokines.

(Received in original form February 14, 2003 and in revised form June 26, 2003)

Address correspondence to: Martin R. Stämpfli, Ph.D., McMaster University, Department of Pathology and Molecular Medicine, Health Sciences Centre, Room 4H21A, 1200 Main Street West, Hamilton, ON, L8N 3Z5 Canada. E-mail: stampfli@mcmaster.ca

Abbreviations: alveolar macrophage, AM; bronchoalveolar lavage, BAL; enzyme-linked immunosorbent assay, ELISA; granulocyte macrophage colony-stimulating factor, GM-CSF; interleukin, IL; knockout, KO; matrix metalloproteinase, MMP; ovalbumin, OVA; phosphate-buffered saline, PBS; recombinant GM-CSF, rGM-CSF; recombinant IL, rIL; saline, SAL; tissue inhibitor of matrix metalloproteinase, TIMP; wild-type, WT.

Am. J. Respir. Cell Mol. Biol. Vol. 30, pp. 84–90, 2004
Originally Published in Press as DOI: 10.1165/rcmb.2003-0051OC on July 3, 2003
Internet address: www.atsjournals.org

Studies in IL-13 KO mice demonstrate that expression of MMP-12 was IL-13-dependent and required for the development of airway eosinophilia. This study demonstrates a novel function of MMP-12 in the process of airway eosinophil accumulation and sheds light on the diverse functions of matrix metalloproteinases in inflammatory processes.

Materials and Methods

Animals

Female Balb/c mice (6–8 wk old) were purchased from Charles River Laboratories (Montreal, PQ, Canada). Mice deficient in IL-13 (IL-13 KO) were generated as previously described (20) and backcrossed six generations onto a Balb/c background. MMP-12 KO mice were generated by targeted disruption of the MMP-12 gene as described previously (21) and backcrossed 10 generations onto a C57BL/6J background. Mice were housed under specific pathogen-free conditions in a 12-h light-dark cycle. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

Administration of Adenovirus Constructs

Prolonged expression of GM-CSF in the airway was achieved using an adenovirus-mediated gene transfer approach. Replication-deficient human type 5 adenoviral (Ad) constructs carrying the transgene for GM-CSF (22) in the E1 region of the viral genome were delivered intranasally. Adenoviral constructs were delivered in a total volume of 30 μ l of phosphate-buffered saline (PBS) vehicle (two 15- μ l administrations 5 min apart) into anesthetized animals. We used a dose of 3×10^7 pfu Ad/GM-CSF.

Mucosal Sensitization Protocol

We reported the details of the experimental protocol previously (16). Briefly, Ad/GM-CSF was delivered intranasally at Day –1. Subsequently, animals were exposed to aerosolized OVA (1% in saline; Sigma Chemicals, St. Louis, MO) in a plexiglass chamber for 20 min daily for 10 d (Days 0–9) unless otherwise stated. The OVA aerosol was produced by a Bennet/Twin nebulizer at a flow rate of 10 liters/min.

Collection and Measurement of Specimens

Mice were killed 48 h after the last OVA aerosolization, and bronchoalveolar lavage was performed as described previously (23). Briefly, the trachea of isolated lungs were cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD). The lungs were lavaged with two volumes of PBS, 250 μ l then 200 μ l, and gently agitated; 250–300 μ l were consistently recovered. Total cell counts were determined using a hemocytometer. After centrifugation, supernatants were stored at -74°C for cytokine measurements, cell pellets were resuspended in PBS, and smears for differential cell counting were prepared by cytocentrifugation (Shandon, Inc., Pittsburgh, PA) at 300 rpm for 2 min. The smears were then stained with HEMA 3 Stain Set (Biochemical Sciences Inc., Swedesboro, NJ) and differential cell counts of BAL cells were determined from at least 500 leukocytes using standard hemocytologic criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells (MNC). Additionally, peripheral blood was obtained using heparin-coated capillaries (Fisher Scientific, Pittsburgh, PA). Total white blood cell counts were determined after lysing red blood cells and cell differentials were assessed on smears stained with

the HEMA 3 Stain set. For serum, animals were bled with nonheparinized capillary tubes. Blood was incubated at 37°C for 30 min, and then centrifuged twice for 10 min at 4°C . Serum was then collected and stored at -74°C for further analysis. Finally, lungs were first inflated with 10% formalin using a constant pressure method (20 cm H_2O) and then placed in 10% formalin. Fixed tissue was embedded in paraffin and 3- μm -thick sections were stained with hematoxylin and eosin. Tissue eosinophils were quantified by scanning entire lung sections using a Nikon Coolscan III slide scanner (Toronto, ON, Canada). A grid (0.40 mm^2 of lung tissue/square) was then superimposed over the scanned images and eosinophils were counted in ten random squares under the light microscope.

Splenocyte Culture

Spleens were harvested on Day 11 of the aerosolization protocol. Tissue was triturated between the ends of sterile frosted slides and the resulting cell suspension was filtered through nylon mesh (BSH Thompson, Scarborough, ON, Canada). Red blood cells were lysed with ACK lysis buffer (0.5 M NH_4Cl , 10 mM KHCO_3 , and 0.1 mM Na_2EDTA at pH 7.2–7.4), and the splenocytes were washed twice with Hanks' balanced saline solution and resuspended in RPMI supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada), 1% L-glutamine, and 1% penicillin/streptomycin. Cells were cultured in medium alone or with 40 μg OVA/well at 8×10^5 cells/well in a flat-bottom, 96-well plate (Becton Dickinson, Franklin Lakes, NJ). After 5 d of culture, supernatants were harvested for cytokine measurements.

Cytokine and Immunoglobulin Measurement

Cytokine levels were measured using enzyme-linked immunosorbent assay (ELISA). The ELISA kit for IL-13 was purchased from R&D Systems (Minneapolis, MN) with a threshold of detection of 1.5 pg/ml, and the kit for IL-5 was obtained from Amersham (Buckinghamshire, UK) with a threshold of detection of 5 pg/ml. Levels of OVA-specific IgE and IgG1 were detected using ELISAs that have been described in detail previously (19). Briefly, OVA-specific IgE is measured using an antigen capture ELISA with anti-IgE monoclonal antibodies (University of Louvain, Brussels, Belgium) on the solid phase and biotinylated OVA as developing agent. OVA-specific IgG1 was measured using a conventional ELISA with OVA on the solid phase and biotinylated anti-IgG1 (Southern Biotechnology Associates, Birmingham, AL) as the developing agent. Ig levels are expressed in units per milliliter relative to standard sera. The threshold of detection of IgE and IgG1 was < 2 U/ml.

Alveolar Macrophage Isolation and Stimulation

Mice were killed and the tracheae of isolated lungs were cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD). The lungs were lavaged five times with 300 μ l of PBS. Cell counts were determined using a hemocytometer and $\sim 1 \times 10^6$ cells were consistently recovered. The cells were washed twice in RPMI supplemented with 10% fetal bovine serum (Invitrogen), 1% L-glutamine, and 1% penicillin/streptomycin, and then incubated at 1×10^6 cells/ml for 2 h at 37°C to allow alveolar macrophages (AMs) to adhere. The adherent AMs were then washed and any nonadherent cells were removed by replacing the media twice. Recombinant murine IL-13 (rIL-13) and GM-CSF (rGM-CSF) were purchased from R&D Systems. AMs were incubated in medium alone, rIL-13 (10 ng/ml), and/or rGM-CSF (10 ng/ml) for 24 h and culture supernatants were collected and stored at -20°C for immunoblotting.

Gel Electrophoresis and Immunoblotting

Thirty-microliter aliquots of lung lavage fluid or culture supernatant were separated on a 12% sodium dodecyl sulfate polyacrylamide gel under reducing conditions and then transferred to a nitrocellulose membrane using a Mini Trans-Blot blotting apparatus (Bio-Rad Laboratories, Hercules, CA). The blot was then blocked for 1 h at room temperature with 5% (wt/vol) nonfat milk in T-TBS (Tween-Tris buffered saline) and then probed with a polyclonal goat anti-mouse MMP-12 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) for 2 h. The blot was washed three times in T-TBS for 10 min and then incubated with a donkey anti-goat IgG antibody coupled to a horse-radish peroxidase (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h. Bands were detected using enhance chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Baie D'Urfe, PQ, Canada).

Real-Time, Quantitative Reverse Transcriptase–Polymerase Chain Reaction (*TaqMan*)

The expression of MMP-12 mRNA relative to GAPDH mRNA was measured using real-time quantitative polymerase chain reaction (PCR) analysis (*TaqMan*, Applied Biosystems, Foster City, CA) as described previously (24). The primers and probes were kindly provided by Dr. A. J. Coyle (Millennium Pharmaceuticals Inc., Cambridge, MA). The sequences of primers and the probe used are: forward, 3'-GCTTGCTGGTTTTCAGTTTATAAGT5'; reverse, 3'-CACCTCCCCTTACCTGTTACA5'; probe, 3'-TGCCAC-ATAGTTACACCTGAGCATAGAGTGA5'. Data were normalized as fold increases over expression in naive lungs.

Data Analysis

Data are expressed as means $\pm$ SEM. Whenever suitable, results were interpreted using ANOVA with a Fisher LSD *post hoc* test, unless otherwise noted. Differences were considered statistically significant when $P < 0.05$.

Results

Airway Eosinophilia Is Associated with IL-13 Production and MMP-12 Expression in the Lungs

To elicit airway eosinophilia, mice were subjected to a protocol of antigen-induced airway inflammation (16). In mice exposed to OVA, but not saline, in the context of GM-CSF, BAL total cell numbers were significantly increased at Day 7 and 48 h after the last OVA exposure (Day 11), when compared with naive controls (Figure 1A). Although we observed eosinophils in the BAL at Day 7 in these mice, the numbers were highest at Day 11 (Figure 1B). Expression of IL-5 and IL-13 in the BAL of OVA-exposed mice peaked at Day 7 and returned to naive levels by Day 11 (Figures 1C and 1D, respectively).

Real-time, quantitative reverse transcriptase (RT)-PCR analysis (*TaqMan*) of mRNA isolated from whole lung tissue of mice exposed to OVA, but not saline, in the context of GM-CSF showed that MMP-12 mRNA expression was elevated at Day 7 (12-fold) but highest at Day 11 (70-fold), when compared with naive controls (Figure 2A).

In addition, immunoblot analysis of Day 11 BAL showed that the increase in MMP-12 mRNA in mice exposed to OVA, compared with mice exposed to saline, in the context of GM-CSF was associated with elevated MMP-12 protein

levels; both proenzyme (54 kD) and activated forms of MMP-12 (29 kD) were detected (Figure 2B). To assess the relative impact of GM-CSF and IL-13 on MMP-12 protein expression, AMs were isolated from naive Balb/c mice and exposed in culture to rGM-CSF and/or rIL-13 for 24 h and culture supernatants were collected and analyzed by immunoblotting. Figure 2C shows that rGM-CSF alone induced low levels of MMP-12 protein expression. In contrast, AMs treated with rIL-13 expressed considerable amounts of MMP-12 protein. Finally, strongest induction of MMP-12 protein expression was observed in AMs treated with rIL-13 and rGM-CSF. No MMP-12 protein was detected in supernatants from AMs cultured in medium alone.

MMP-12 KO Mice Have Impaired Eosinophilic Inflammation

To assess whether deficiency in MMP-12 had an impact on eosinophilic airway inflammation, MMP-12 KO mice were subjected to our protocol of allergic mucosal sensitization. Whereas we observed high levels of eosinophils in the BAL of WT control littermates exposed to OVA in the context of GM-CSF, eosinophilia in MMP-12 KO mice was significantly reduced (Figure 3C). No eosinophils were observed in the BAL of MMP-12 KO animals and their control littermates that were exposed to saline only in the context of GM-CSF (data not shown). Histologic assessment of the

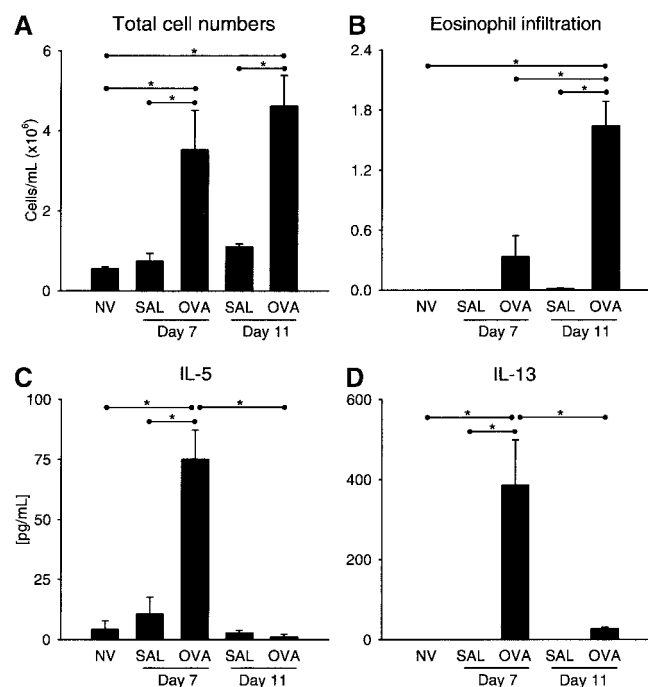


Figure 1. Eosinophil accumulation in the airway is preceded by IL-5 and IL-13 production. Balb/c mice were infected with Ad/GM-CSF intranasally at Day -1 and exposed to OVA or vehicle only (SAL). The mice were killed at Days 7 and 11, and BAL total cell and eosinophil numbers were assessed (A and B, respectively). The levels of IL-5 (C) and IL-13 in the BAL (D) were also determined. NV, naive. Data are expressed as means $\pm$ SEM and represent $n = 3-9$. * $P < 0.05$.

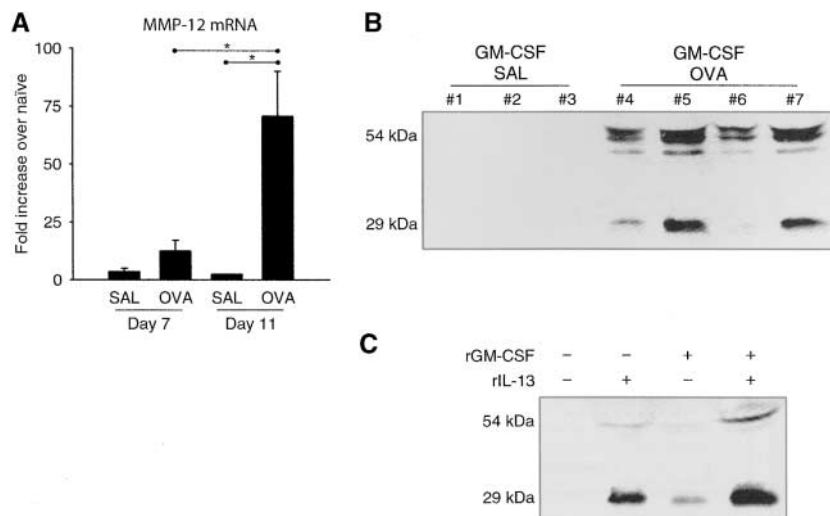


Figure 2. MMP-12 is expressed in lungs of mice exposed to OVA in the context of GM-CSF. Mice were infected with Ad/GM-CSF at Day -1 and exposed to OVA or vehicle only (SAL). MMP-12 mRNA levels were measured using real-time, quantitative RT-PCR (*TaqMan*, Applied Biosystems) at Days 7 and 11 (A). MMP-12 protein levels in the BAL of saline-exposed (#1-3) and OVA-exposed (#4-7) mice were detected by immunoblotting (B). AMs were isolated from naive Balb/c mice, stimulated in culture with rIL-13 (10 ng/ml) and/or rGM-CSF (10 ng/ml), and culture supernatants were harvested after 24 h. MMP-12 protein levels in culture supernatants were detected by immunoblotting (C). Data are expressed as means $\pm$ SEM and represent $n = 3-4$. * $P < 0.05$.

number of tissue eosinophils in MMP-12 KO animals exposed to OVA in the context of GM-CSF expression showed no difference compared with their WT littermates ($n = 6$, t test, $P = 0.244$; Figure 3D). Similar levels of eosinophils were observed in peripheral blood between MMP-12 KO animals and WT littermate controls exposed to OVA in the context of GM-CSF expression: $(4.30 \pm 1.87) \times 10^4$ cells/ml versus $(5.03 \pm 1.95) \times 10^4$ cells/ml, respectively; $n = 4-6$, t test, $P = 0.801$. Finally, no difference in the levels of BAL neutrophils between MMP-12 KO animals and WT littermates exposed to OVA in the context of GM-CSF was observed: WT littermates $(9.6 \pm 5.2) \times$

10^4 , and MMP-12 KO animals $(3.7 \pm 0.89) \times 10^4$ cells/ml ($n = 10$, t test, $P = 0.566$).

MMP-12 KO Mice Are Sensitized to OVA

To assess whether these animals were sensitized, splenocytes from MMP-12 KO and WT mice exposed to OVA in the context of GM-CSF were cultured for 5 d in the presence or absence of OVA. We found that, in the presence of OVA, there were no significant differences in the levels of IL-5 and IL-13 produced by MMP-12 KO splenocytes compared with their WT littermates (Figure 4). In addition, we observed significantly elevated levels of OVA-specific IgE and IgG1 in both MMP-12 KO and WT mice exposed to OVA in the context of GM-CSF, compared with their respective GM-CSF-only and naive controls (Table 1).

Expression of MMP-12 Is IL-13-Dependent

Given that we observed high levels of IL-13 and that IL-13 has previously been shown to induce proteolytic enzyme

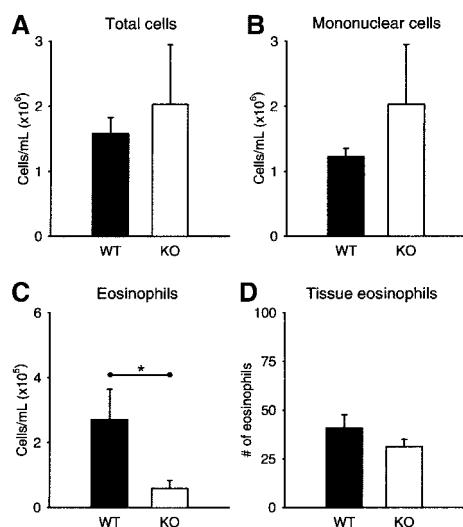


Figure 3. Impaired eosinophil accumulation in airway of MMP-12 KO mice. MMP-12 KO mice (KO) and their wild-type littermates (WT) infected with Ad/GM-CSF were killed after 10 exposures to OVA, and the cellular profile in the BAL (A-C) and lung tissues (D) was assessed. Data are expressed as means $\pm$ SEM and represent $n = 6-10$. Student's t test was used to assess statistical significance. * $P < 0.05$.

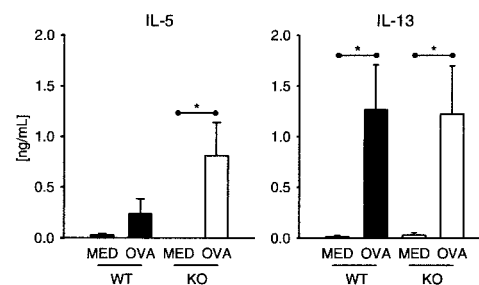


Figure 4. *In vitro* cytokine production in MMP-12 KO mice. Ad/GM-CSF-treated, OVA-exposed MMP-12 KO mice (KO) and their wild-type littermates (WT) were killed at Day 11. Their spleens were obtained, and isolated splenocytes incubated with OVA or medium (MED) alone were cultured for 5 d. The culture supernatants were then collected and the levels of IL-5 and IL-13 were measured by ELISA. Data are expressed as means $\pm$ SEM and represent $n = 3$. Similar results were obtained in an independent experiment where spleens from several animals were pooled before culture. * $P < 0.05$.

expression in a transgenic mouse model (12), we investigated MMP-12 expression in IL-13-deficient mice subjected to our protocol of allergic mucosal sensitization. At Day 11, no differences in the induction of MMP-12 were observed in IL-13 KO mice exposed to either OVA (17-fold) or saline (13-fold) in the context of GM-CSF. The increases in MMP-12 expression in both OVA and saline-exposed IL-13 KO mice were markedly lower than those observed in the OVA-exposed WT mice and in the same order of magnitude as those seen in saline-exposed WT animals. Decreased MMP-12 expression was associated with reduced number of eosinophils in the BAL of IL-13 KO animals compared with Balb/c animals (Figure 5). For comparison, we included in the figure the data for eosinophils and MMP-12 mRNA expression presented in Figures 1 and 2. Note that Ad/GM-CSF-infected, OVA-exposed IL-13 KO mice had significantly increased total cell number and mononuclear cells compared with naive animals. Hence the decrease in the number of eosinophils in the BAL was not due to a general decrease in inflammation in IL-13 KO mice but rather to a decrease in the percentage of eosinophils. Whereas we observed $\sim 35\%$ eosinophils in WT control mice, IL-13 KO animals showed $\sim 5\%$ eosinophils. Furthermore, we observed a significant decrease in neutrophils in IL-13 KO mice compared with WT animals: $(1.17 \pm 1.01) \times 10^4$ cells/ml versus $(4.79 \pm 1.24) \times 10^5$ cells/ml, respectively; $n = 5-6$, t test, $P = 0.011$.

Discussion

The accumulation of eosinophils in the BAL is the result of complex cellular and molecular interactions involving a variety of different cell types and the production of a wide range of effector molecules. Although the eosinopoietic and chemotactic mediators involved in eosinophil recruitment have been studied extensively (25–30), comparatively little is known with regard to the role of MMPs in this process.

We employed a murine model of allergic mucosal sensitization to assess the role of MMPs in eosinophilic airway inflammation (16). An adenovirus vector was used to transiently express GM-CSF in the lungs of Balb/c mice that were then exposed daily to aerosolized OVA over a period of 7–10 d. The ensuing inflammatory response is characterized by airway eosinophilia, expression of Th2 cytokines, and antigen-specific IgE synthesis. That sensitization occurs

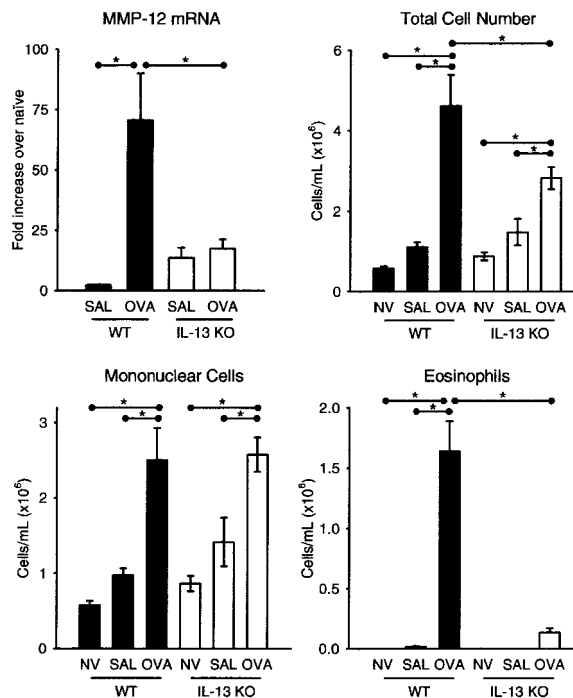


Figure 5. Reduced MMP-12 expression and airway eosinophilia in IL-13-deficient mice. IL-13 KO mice and the WT controls were infected with Ad/GM-CSF at Day -1 and exposed to OVA or vehicle only (SAL). The mice were then killed at Day 11 and the levels of MMP-12 mRNA expression in the lung and the cellular profile in the airway were assessed. Data are expressed as means $\pm$ SEM and represent $n = 3-9$. * $P < 0.05$.

mucosally, and not peritoneally, renders the model a better reflection of the route of sensitization in humans. Furthermore, that GM-CSF is expressed in humans with asthma (31–33) may make the adjuvant more relevant than the conventionally used aluminum hydroxide.

In accordance with our previous findings, we observed an increase in the number of eosinophils in BAL at Day 7, peaking at Day 11. Assessment of MMP-12 mRNA expression in whole lung tissue has shown a significant induction in the OVA-exposed mice compared with the saline-exposed controls. Specifically, we observed a 12-fold

TABLE 1
OVA-specific IgE and IgG1 levels in the serum of sensitized MMP-12 KO mice and their WT littermates

	OVA-Specific IgE		OVA-Specific IgG1	
	WT	KO	WT	KO
Naive	3.8 $\pm$ 1.5	9.5 $\pm$ 2.9	0.09 $\pm$ 0.09	0.00 $\pm$ 0.00
GM-CSF	16.1 $\pm$ 4.7	53.2 $\pm$ 20.7	0.01 $\pm$ 0.01	0.01 $\pm$ 0.01
GM-CSF/OVA	265 $\pm$ 92*	219 $\pm$ 54*	2.82 $\pm$ 0.82*	3.53 $\pm$ 1.47*

Definition of abbreviations: GM-CSF, granulocyte macrophage colony-stimulating factor; KO, knockout; MMP, matrix metalloproteinase; OVA, ovalbumin; WT, wild-type.

MMP-12 WT and MMP-12 KO mice were killed on Day 11 of the protocol and serum OVA-specific Ig levels were measured by ELISA. IgG1 levels are $\times 10^5$. Data are expressed as U/ml and represent the mean $\pm$ SEM; $n = 3-6$ /treatment group.

* $P < 0.05$ compared with corresponding naive and GM-CSF only controls.

and a 70-fold increase at Days 7 and 11, respectively, in OVA-exposed mice compared with a 3-fold increase in saline-exposed controls. Immunoblot analysis showed that MMP-12 protein levels in the BAL of OVA- and saline-exposed mice at Day 11 correlated with the MMP-12 mRNA levels measured. Specifically, although no MMP-12 protein was detected in the BAL of saline-exposed mice, both pro-enzyme (54 kD) and activated (29 kD) forms of MMP-12 were detected in the BAL of OVA-exposed mice. This, to our knowledge, is the first time that expression of MMP-12 on the protein level has been demonstrated in a model of allergic airway inflammation.

Next, we used MMP-12 KO mice to investigate whether expression of MMP-12 was functionally associated with the accumulation of eosinophils in the airway. These studies demonstrated that expression of MMP-12 was required for the development of airway eosinophilia. Although we observed no significant difference in the numbers of total and mononuclear cells between sensitized MMP-12 KO mice and the wild type controls, BAL eosinophilia in MMP-12 KO mice was significantly reduced compared with control littermates. This difference in the number of eosinophils recruited was not a result of an inability of MMP-12 KO mice to be sensitized (as indicated by the levels of serum OVA-specific IgE and IgG1 measured, as well as the levels of IL-5 and IL-13 in culture supernatants of OVA-stimulated splenocytes), or an inability to produce eosinophils as indicated by peripheral blood data. Also, unlike what has been reported recently for the inflammatory process in MMP-2 KO mice (14), histologic assessment did not show enhanced accumulation of eosinophils in the lungs of MMP-12 KO mice, indicating that the reduction in BAL eosinophil numbers in these mice was not due to decreased eosinophil egression into the lumen of the airways.

In this model of antigen-induced airway inflammation, development of airway eosinophilia is preceded by Th2 cytokine expression in the BAL. Of particular interest are the high levels of IL-13, a cytokine that has previously been shown to induce expression of proteolytic enzymes, including MMPs, in a transgenic mouse model (12). Thus, to investigate whether MMP-12 expression was IL-13-dependent in this model of allergic mucosal sensitization, we used IL-13 KO mice. Our data demonstrate that MMP-12 expression is, indeed, IL-13-dependent. In addition to the decrease in MMP-12 expression, we also observed reduced airway eosinophilia in IL-13 KO mice compared with WT. That we observe decreased airway eosinophilia is in agreement with previous reports using IL-13-neutralizing antibodies (34). Taken together, these data demonstrate a role for MMP-12 in eosinophil recruitment.

An association between MMPs and airway inflammation has been shown in clinical studies demonstrating increased levels of MMP-2 and -9 in subjects with asthma (9, 10, 35, 36). A functional role of these proteinases in the development of airway eosinophilia was first shown in a study by Kumagai and coworkers in a murine model of antigen-induced airway inflammation (13). By administering endogenous inhibitors of MMPs, TIMP-1 and TIMP-2, and a synthetic inhibitor (R-94138), the authors demonstrated decreased BAL eosinophilia. Because TIMPs have a broad

specificity and inhibit not only MMP-2 and -9, but also MMP-12, they cannot be used to dissect the individual contributions of specific MMPs. In a model of allergen-induced airways inflammation, Corry and colleagues demonstrated that MMP-2 KO mice had a reduced influx of cells into the BAL. This reduction was associated with an accumulation of inflammatory cells in the lung parenchyma (14). In a study by Cataldo and associates, the authors demonstrated that MMP-9 KO mice subjected to a protocol of antigen-induced airways inflammation had significantly reduced lymphocytes in the BAL as compared with WT mice. However, no such reduction in eosinophil numbers was observed (15). We show that MMP-12 KO mice subjected to a mucosal model of allergic sensitization have reduced BAL eosinophilia. This reduction is not associated with reduced production of eosinophils, or enhanced accumulation of eosinophils in lung tissue. That the phenotypes of MMP-2, -9, and -12 KO animals are different underscores the distinct roles of MMPs in airway inflammation. Taken together, the present study informs our understanding of the diverse roles of MMPs in allergic inflammation and suggests that MMP-12 is another critical factor in the development of eosinophilic airways inflammation.

There are several mechanisms by which MMP-12 may affect BAL eosinophilia. MMP-12 may be directly involved in eosinophil recruitment through tissue remodeling and eosinophil transmigration through basement membranes, as has previously been shown for MMP-9 (37). Alternatively, MMP-12 may generate proteolytic fragments of matrix components that are chemotactic for eosinophils. Although it has been demonstrated that elastin and collagen fragments are chemotactic for monocytes and neutrophils, no such evidence is currently available for eosinophils. MMP-12 may also modulate the activity of chemokines involved in eosinophil recruitment into the airway. In a model of cigarette smoke-induced emphysema, macrophage accumulation in MMP-12 KO animals was associated with reductions in the macrophage chemoattractant monocyte chemoattractant protein-1 (38). Also, MMP-12 may indirectly alter the survival of eosinophils. Indeed, a recent report by Churg and coworkers has implicated MMP-12 in the liberation of membrane-bound tumor necrosis factor- α , a cytokine shown to increase the survival of eosinophils (39–41). Finally, MMP-12 may impact retention of eosinophils in the BAL and a lack of this molecule may lead to decreased retention. We are currently pursuing experiments in an attempt to detail the mechanisms that underlie decreased eosinophil numbers in the BAL of MMP-12-deficient mice.

MMPs, specifically MMP-2 and MMP-9, have previously been implicated in eosinophilic airway inflammation. In this study, we demonstrate expression of MMP-12 in eosinophilic airway inflammation and elucidate a novel function for this MMP in the process of airway eosinophil accumulation.

Acknowledgments: The authors thank Anna Drannik and David Dawe for critical review of the manuscript. They also thank Susanna Goncharova and Ramzi Fattouh for their excellent technical help. The secretarial assistance of Mary Kiriakopoulos is gratefully acknowledged. This study was supported in part by the Ontario Thoracic Society, the Hamilton Health Sciences Corporation, and St. Joseph's Hospital. M.R.S. holds a Parker B. Francis Fellowship.

References

- Sternlicht, M., L. M. Coussens, T. H. Vu, and Z. Werb. 2000. Biology and regulation of the matrix metalloproteinases. In K. Appelt, editor. *Cancer Drug Discovery and Development: Matrix Metalloproteinase Inhibitors in Cancer Therapy*. Humana Press Inc., Totowa, NJ. 1–37.
- Mecham, R. P., and W. C. Parks. 1998. Matrix Metalloproteinases. (Biology of Extracellular Matrix Series.) Academic Press, San Diego.
- DiBattista, J. A., J. P. Pelletier, M. Zafarullah, N. Fujimoto, K. Obata, and J. Martel-Pelletier. 1995. Coordinate regulation of matrix metalloproteinases and tissue inhibitor of metalloproteinase expression in human synovial fibroblasts. *J. Rheumatol. Suppl.* 43:123–128.
- O'Connor, C. M., and M. X. FitzGerald. 1994. Matrix metalloproteinases and lung disease. *Thorax* 49:602–609.
- Roche, W. R., R. Beasley, J. H. Williams, and S. T. Holgate. 1989. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1:520–524.
- Brewster, C. E., P. H. Howarth, R. Djukanovic, J. Wilson, S. T. Holgate, and W. R. Roche. 1990. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am. J. Respir. Cell Mol. Biol.* 3:507–511.
- Bousquet, J., P. Chané, J. Y. Lacoste, R. White, P. Vic, P. Godard, and F. B. Michel. 1992. Asthma: a disease remodeling the airways. *Allergy* 47:3–11.
- Bousquet, J., J. Y. Lacoste, P. Chané, P. Vic, P. Godard, and F. B. Michel. 1996. Bronchial elastic fibers in normal subjects and asthmatic patients. *Am. J. Respir. Crit. Care Med.* 153:1648–1654.
- Mautino, G., N. Oliver, P. Chané, J. Bousquet, and F. Capony. 1997. Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *Am. J. Respir. Cell Mol. Biol.* 17:583–591.
- Cataldo, D., C. Munaut, A. Noel, F. Frankenke, P. Bartsch, J. M. Foidart, and R. Louis. 2000. MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. *Int. Arch. Allergy Immunol.* 123:259–267.
- Tanaka, H., N. Miyazaki, K. Oashi, S. Tanaka, M. Ohmichi, and S. Abe. 2000. Sputum matrix metalloproteinase-9: tissue inhibitor of metalloproteinase-1 ratio in acute asthma. *J. Allergy Clin. Immunol.* 105:900–905.
- Zheng, T., Z. Zhu, Z. Wang, R. J. Homer, B. Ma, R. J. Riese, Jr., H. A. Chapman, Jr., S. D. Shapiro, and J. A. Elias. 2000. Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema. *J. Clin. Invest.* 106:1081–1093.
- Kumagai, K., I. Ohno, S. Okada, Y. Ohkawara, K. Suzuki, T. Shinya, H. Nagase, K. Iwata, and K. Shirato. 1999. Inhibition of matrix metalloproteinases prevents allergen-induced airway inflammation in a murine model of asthma. *J. Immunol.* 162:4212–4219.
- Corry, D. B., K. Rishi, J. Kanellis, A. Kiss, L. Z. Song, L. Z. Xu, L. Feng, Z. Werb, and F. Kheradmand. 2002. Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency. *Nat. Immunol.* 3:347–353.
- Cataldo, D. D., K. G. Tournay, K. Vermaelen, C. Munaut, J. M. Foidart, R. Louis, A. Noel, and R. A. Pauwels. 2002. Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation. *Am. J. Pathol.* 161:491–498.
- Stampfli, M. R., R. E. Wiley, G. S. Neigh, B. U. Gajewska, X. F. Lei, D. P. Snider, Z. Xing, and M. Jordana. 1998. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J. Clin. Invest.* 102:1704–1714.
- Swirski, F. K., B. U. Gajewska, D. Alvarez, S. A. Ritz, M. J. Cundall, E. C. Cates, A. J. Coyle, J. C. Gutierrez-Ramos, M. D. Inman, M. Jordana, and M. R. Stampfli. 2002. Inhalation of a harmless antigen (ovalbumin) elicits immune activation but divergent immunoglobulin and cytokine activities in mice. *Clin. Exp. Allergy* 32:411–421.
- Stampfli, M. R., M. Cwiartka, B. U. Gajewska, D. Alvarez, S. A. Ritz, M. D. Inman, Z. Xing, and M. Jordana. 1999. Interleukin-10 gene transfer to the airway regulates allergic mucosal sensitization in mice. *Am. J. Respir. Cell Mol. Biol.* 21:586–596.
- Stampfli, M. R., G. Scott Neigh, R. E. Wiley, M. Cwiartka, S. A. Ritz, M. M. Hitt, Z. Xing, and M. Jordana. 1999. Regulation of allergic mucosal sensitization by interleukin-12 gene transfer to the airway. *Am. J. Respir. Cell Mol. Biol.* 21:317–326.
- McKenzie, G. J., A. Bancroft, R. K. Grencis, and A. N. McKenzie. 1998. A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr. Biol.* 8:339–342.
- Shipley, J. M., R. L. Wesselschmidt, D. K. Kobayashi, T. J. Ley, and S. D. Shapiro. 1996. Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. *Proc. Natl. Acad. Sci. USA* 93:3942–3946.
- Xing, Z., Y. Ohkawara, M. Jordana, F. Graham, and J. Gaudie. 1996. Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J. Clin. Invest.* 97:1102–1110.
- Ohkawara, Y., X. F. Lei, M. R. Stampfli, J. S. Marshall, Z. Xing, and M. Jordana. 1997. Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airways inflammation. *Am. J. Respir. Cell Mol. Biol.* 16:510–520.
- Coyle, A. J., S. Lehar, C. Lloyd, J. Tian, T. Delaney, S. Manning, T. Nguyen, T. Burwell, H. Schneider, J. A. Gonzalo, M. Gosselin, L. R. Owen, C. E. Rudd, and J. C. Gutierrez-Ramos. 2000. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity* 13:95–105.
- Clutterbuck, E., J. G. Shields, J. Gordon, S. H. Smith, A. Boyd, R. E. Callard, H. D. Campbell, I. G. Young, and C. J. Sanderson. 1987. Recombinant human interleukin 5 is an eosinophil differentiation factor but has no activity in standard human B cell growth factor assays. *Eur. J. Immunol.* 17:1743–1750.
- Clutterbuck, E. J., E. M. Hirst, and C. J. Sanderson. 1989. Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6, and GM-CSF. *Blood* 73:1504–1512.
- Clutterbuck, E. J., and C. J. Sanderson. 1990. Regulation of human eosinophil precursor production by cytokines: a comparison of recombinant human interleukin-1 (rhIL-1), rhIL-3, rhIL-5, rhIL-6, and rh granulocyte-macrophage colony-stimulating factor. *Blood* 75:1774–1779.
- McKenzie, A. N., B. Ely, and C. J. Sanderson. 1991. Mutated interleukin-5 monomers are biologically inactive. *Mol. Immunol.* 28:155–158.
- Griffiths-Johnson, D. A., P. D. Collins, A. G. Rossi, P. J. Jose, and T. J. Williams. 1993. The chemokine, eotaxin, activates guinea-pig eosinophils in vitro and causes their accumulation into the lung in vivo. *Biochem. Biophys. Res. Commun.* 197:1167–1172.
- Jose, P. J., D. A. Griffiths-Johnson, P. D. Collins, D. T. Walsh, R. Moqbel, N. F. Totty, O. Truong, J. J. Hsuan, and T. J. Williams. 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J. Exp. Med.* 179:881–887.
- Marini, M., E. Vittori, J. Hollemberg, and S. Mattoli. 1992. Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J. Allergy Clin. Immunol.* 89:1001–1009.
- Adachi, T., S. Motojima, A. Hirata, T. Fukuda, and S. Makino. 1995. Eosinophil viability-enhancing activity in sputum from patients with bronchial asthma: contributions of interleukin-5 and granulocyte/macrophage colony-stimulating factor. *Am. J. Respir. Crit. Care Med.* 151:618–623.
- Woolley, K. L., E. Adelroth, M. J. Woolley, R. Ellis, M. Jordana, and P. M. O'Byrne. 1995. Effects of allergen challenge on eosinophils, eosinophil cationic protein, and granulocyte-macrophage colony-stimulating factor in mild asthma. *Am. J. Respir. Crit. Care Med.* 151:1915–1924.
- Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282:2258–2261.
- Okada, Y., K. Naka, K. Kawamura, T. Matsumoto, I. Nakanishi, N. Fujimoto, H. Sato, and M. Seiki. 1995. Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications for bone resorption. *Lab. Invest.* 72:311–322.
- Hoshino, M., Y. Nakamura, J. Sim, J. Shimojo, and S. Isogai. 1998. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J. Allergy Clin. Immunol.* 102:783–788.
- Okada, S., H. Kita, T. J. George, G. J. Gleich, and K. M. Leiferman. 1997. Migration of eosinophils through basement membrane components in vitro: role of matrix metalloproteinase-9. *Am. J. Respir. Cell Mol. Biol.* 17:519–528.
- Hautamaki, R. D., D. K. Kobayashi, R. M. Senior, and S. D. Shapiro. 1997. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 277:2002–2004.
- Chung, A., R. D. Wang, H. Tai, X. Wang, C. Xie, J. Dai, S. D. Shapiro, and J. L. Wright. 2003. Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor- α release. *Am. J. Respir. Crit. Care Med.* 167:1083–1089.
- Valerius, T., R. Repp, J. R. Kalden, and E. Platzer. 1990. Effects of IFN on human eosinophils in comparison with other cytokines: a novel class of eosinophil activators with delayed onset of action. *J. Immunol.* 145:2950–2958.
- Temkin, V., and F. Levi-Schaffer. 2001. Mechanism of tumour necrosis factor α mediated eosinophil survival. *Cytokine* 15:20–26.