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Reduced peribronchial fibrosis in allergen-challenged MMP-9-deficient mice

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Lim, Dae Hyun, Jae Youn Cho, Marina Miller, Kirsti McElwain, Shauna McElwain, and David H. Broide. Reduced peribronchial fibrosis in allergen-challenged MMP-9-deficient mice. *Am J Physiol Lung Cell Mol Physiol* 291: L265–L271, 2006; doi:10.1152/ajplung.00305.2005.—Matrix metalloproteinases (MMPs) are a family of extracellular proteases that are responsible for the degradation of the extracellular matrix during tissue remodeling. We have used a mouse model of allergen-induced airway remodeling to determine whether MMP-9 plays a role in airway remodeling. MMP-9-deficient and wild-type (WT) mice were repetitively challenged intranasally with ovalbumin (OVA) antigen to develop features of airway remodeling including peribronchial fibrosis and increased thickness of the peribronchial smooth muscle layer. OVA-challenged MMP-9-deficient mice had less peribronchial fibrosis and total lung collagen compared with OVA-challenged WT mice. There was no reduction in mucus expression, smooth muscle thickness, or airway responsiveness in OVA-challenged MMP-9-deficient compared with OVA-challenged WT mice. OVA-challenged MMP-9-deficient mice had reduced levels of bronchoalveolar lavage (BAL) regulated on activation, normal T cell expressed, and secreted (RANTES), as well as reduced numbers of BAL and peribronchial eosinophils compared with OVA-challenged WT mice. There were no significant difference in levels of BAL eotaxin, thymus- and activation-regulated chemokine (TARC), or macrophage-derived chemokine (MDC) in OVA-challenged WT compared with MMP-9-deficient mice. Overall, this study demonstrates that MMP-9 may play a role in mediating selected aspects of allergen-induced airway remodeling (i.e., modest reduction in levels of peribronchial fibrosis) but does not play a significant role in mucus expression, smooth muscle thickness, or airway responsiveness.

eosinophil; airway remodeling; smooth muscle; transforming growth factor- β 1; matrix metalloproteinase-9

MATRIX METALLOPROTEINASE-9 (MMP-9) belongs to a family of extracellular proteases that are responsible for the degradation of the extracellular matrix during tissue remodeling (2). Levels of MMP-9 (gelatinase B) are significantly increased in bronchoalveolar lavage (BAL) fluid (BALF), blood, and sputum from allergic asthmatic patients (11, 13, 15). In addition, allergen challenge in asthmatics induces expression of MMP-9 in the airway (11). The functional significance of the detection of increased levels of expression of MMP-9 in the airway of human asthmatics is at present unknown. MMPs are thought to contribute to the pathogenesis of asthma by influencing the migration of inflammatory cells such as eosinophils across the basement membranes of blood vessels, as well as by influencing extracellular matrix deposition and matrix degradation. Thus theoretically MMP-9 could exert effects both on airway inflammation and/or airway remodeling in asthma.

In terms of the potential effects of MMP-9 on airway inflammation in asthma, in vitro studies have demonstrated that MMP-9 may be important in mediating migration of eosinophils across the basement membrane of blood vessels (21). In contrast, studies with neutrophils have shown no defect in MMP-9-deficient neutrophil transendothelial migration under conditions of flow in vitro (1). To explore the potential in vivo role of MMP-9 in mediating eosinophilic airway inflammation and airway responsiveness, prior studies have utilized MMP-9-deficient mice in mouse models of acute allergen-induced asthma (4, 9, 16, 22). These studies have produced differing results regarding the role of MMP-9 in mediating eosinophilic airway inflammation and airway responsiveness (4, 9, 16, 22). The majority of studies have demonstrated that MMP-9-deficient mice have reduced levels of eosinophilic airway inflammation and reduced levels of airway responsiveness following acute allergen challenge (4, 9, 22). In contrast, a study has also demonstrated that MMP-9-deficient mice had enhanced allergen-induced airway inflammation and enhanced airway responsiveness following acute allergen challenge (16). These studies came to different conclusions regarding the role of MMP-9 in mediating eosinophilic airway inflammation and airway responsiveness following acute antigen challenge; at present no studies in MMP-9-deficient mice have addressed whether MMP-9 plays a role in allergen-induced airway remodeling following repetitive allergen challenge, which was the focus of this study.

MATERIALS AND METHODS

Induction of Allergen-Induced Airway Remodeling

The methods we have utilized to administer ovalbumin (OVA) allergen to induce airway remodeling in mice have previously been described (5–7). The protocol utilizes a low dose of OVA antigen, which favors T helper (Th) 2 responses (8) and is administered intranasally twice a week for 3 mo (chronic protocol) as opposed to the standard acute OVA challenge protocol performed in previous studies with MMP-9-deficient mice (4, 9, 16, 22). In brief, MMP-9-deficient and wild-type (WT) C57/Bl control mice (16 mice/group in all experiments described; The Jackson Laboratory, Bar Harbor, ME) were used when they reached 8–10 wk of age. Mice were immunized subcutaneously on days 0, 7, 14, and 21 with 25 μ g of OVA (grade V; Sigma, St. Louis, MO) adsorbed to 1 mg of alum (Aldrich) in 200 μ l of normal saline. Intranasal OVA challenges (20 ng/50 μ l in PBS) were administered on days 27, 29, and 31 under isoflurane (Vedco, St. Joseph, MO) anesthesia. Intranasal OVA challenges were then repeated twice a week for 3 mo. Age- and sex-matched control mice were sensitized but not challenged with OVA during the 3-mo study. Mice were killed 24 h after the final OVA challenge, and BALF and

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lungs were analyzed. All animal experimental protocols were approved by the University of California, San Diego Animal Subjects Committee.

Quantitation of Peribronchial Fibrosis

Peribronchial trichrome staining. Lungs in the different groups of mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma) to preserve the pulmonary architecture. The area of peribronchial trichrome staining in paraffin-embedded lung was outlined and quantified under a light microscope (Leica DMLS, Leica Microsystems) attached to an image analysis system (Image-Pro plus, Media Cybernetics) as previously described (5–7). Results are expressed as the area of trichrome staining per micrometer of length of basement membrane of bronchioles 150–200 μm in internal diameter.

Lung collagen assay. The amount of lung collagen was measured in lung homogenates as previously described in this laboratory (5–7) with a collagen assay kit that uses a dye reagent that selectively binds to the [Gly-X-Y]_n tripeptide sequence of mammalian collagens (Bio-color, Newtownabbey, UK). In all experiments a collagen standard was used to calibrate the assay.

Lung TGF- β 1

As TGF- β 1 has been implicated in mediating peribronchial fibrosis in asthma (10, 20), as well as in mouse models of airway remodeling induced by repetitive allergen challenge (7), we measured lung levels of transforming growth factor (TGF)- β 1. The concentrations of bioactive TGF- β 1 in lung were assayed by ELISA (R&D Systems) as previously described (6). Lungs homogenized in lysis buffer (0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl_2 , 1 mM MgCl_2) were centrifuged at 10,000 g for 20 min. The lung supernatant was then passed through a 0.8- μm pore-size filter and assayed for TGF- β 1 (assay sensitivity 61 pg/ml). Before the TGF- β 1 assay, the lung supernatant samples were treated with 2.5 N acetic acid to activate any latent TGF- β 1 as previously described (6).

MMP-9 Detection

Gelatin zymography. Gelatin zymography (12) was performed to detect MMP-9 in BALF in the different groups of mice. Samples of BALF were added to loading buffer and separated in 10% SDS-polyacrylamide gels that contained 0.1% gelatin. SDS was removed by two 30-min washes with 2.5% Triton-X 100 before incubation of gels for 24 h at 37°C in developing buffer (Bio-Rad, Hercules, CA). Gelatin gels were stained with 0.25% Coomassie blue. An MMP-9 standard (Chemicon, Temecula, CA) was used as a positive control.

Immunohistochemistry. A primary MAb directed against MMP-9 (Chemicon) was utilized for immunohistochemical detection of MMP-9 in the lung sections using the immunoperoxidase method as previously described by this laboratory (5–7).

Quantitation of Airway Mucus Expression

To quantitate the level of mucus expression in the airway the number of periodic acid-Schiff (PAS)-positive and PAS-negative epithelial cells in individual bronchioles were counted as previously described by this laboratory (5–7). Results are expressed as the percentage of PAS-positive cells/bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells of each bronchiole.

Quantitation of Airway Smooth Muscle Thickness

The thickness of the airway smooth muscle layer was measured using an image analysis system as previously described (5–7). Lungs that had been fixed in 3% glutaraldehyde and 1% osmium tetroxide were stained with basic fuchsin-toluidine blue, which allowed the best visualization of the smooth muscle layer. The thickness of the

smooth muscle layer (the transverse diameter) was measured from the innermost aspect to the outermost aspect of the smooth muscle layer. The smooth muscle layer thickness in at least 10 bronchioles of similar size (150–200 μm) were counted on each slide.

Determination of Airway Responsiveness to Methacholine In Vivo

Airway responsiveness to methacholine (MCh) was assessed 24 h after the final OVA challenge (after 3 mo of repetitive OVA challenges) in intubated and ventilated mice (flexiVent ventilator; Scireq, Montreal, PQ, Canada) anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. The frequency-independent airway resistance (Raw) was determined using Scireq software in mice exposed to nebulized PBS and MCh (3, 24, 48 mg/ml). The following ventilator settings were used: tidal volume (10 ml/kg), frequency (150/min), positive end-expiratory pressure (3 cmH_2O).

Eosinophilic Inflammation

BALF. The killed mice had their tracheae surgically exposed and cannulated with 27-gauge silicon tubing attached to a 23-gauge needle on a 1-ml tuberculin syringe. After instillation of 800 μl of sterile saline through the trachea into the lung, BALF was withdrawn and cytopun (3 min at 500 rpm) onto microscope slides. Eosinophil counts were performed as previously described (5–7).

Bone marrow. The number of eosinophils in bone marrow was determined as previously described in this laboratory (3).

Peribronchial eosinophils. The number of peribronchial eosinophils was determined as previously described in this laboratory using an anti-mouse major basic protein (MBP) antibody (Ab) kindly provided by Dr. J. Lee (Mayo Clinic, Scottsdale, AZ) (5–7).

BAL Chemokines

Levels of BAL chemokines that mediate eosinophil recruitment directly [eotaxin, regulated on activation, normal T cell expressed, and secreted (RANTES)], or levels of chemokines [thymus- and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC)] that mediate recruitment of Th2 cells and thus may indirectly mediate recruitment of eosinophils were measured by ELISA (R&D, Minneapolis, MN) as previously described in this laboratory (6).

Statistical Analysis

Results in the different groups of mice were compared by ANOVA using the nonparametric Kruskal-Wallis test followed by posttesting using Dunn's multiple comparison of means. All results are presented as means $\pm$ SE. A statistical software package (Graph Pad Prism, San Diego, CA) was used for the analysis. *P* values of <0.05 were considered statistically significant.

RESULTS

Peribronchial Fibrosis in WT vs. MMP-9-Deficient Mice

We used two methods to quantitate peribronchial fibrosis, namely the area of peribronchial trichrome staining (expressed as the stained area in $\mu\text{m}^2/\mu\text{m}$ circumference of bronchiole) and total lung collagen. The area of peribronchial trichrome staining in WT mice that were repetitively challenged with OVA was significantly greater than in control non-OVA-challenged WT mice (1.64 ± 0.08 vs. 0.68 ± 0.04 $\mu\text{m}^2/\mu\text{m}$, $P = 0.001$; Figs. 1A and 2). Baseline levels of peribronchial trichrome staining in non-OVA-challenged mice were similar in MMP-9-deficient and WT mice. However, MMP-9-deficient mice repetitively challenged with OVA had a significant reduction in levels of peribronchial trichrome staining compared

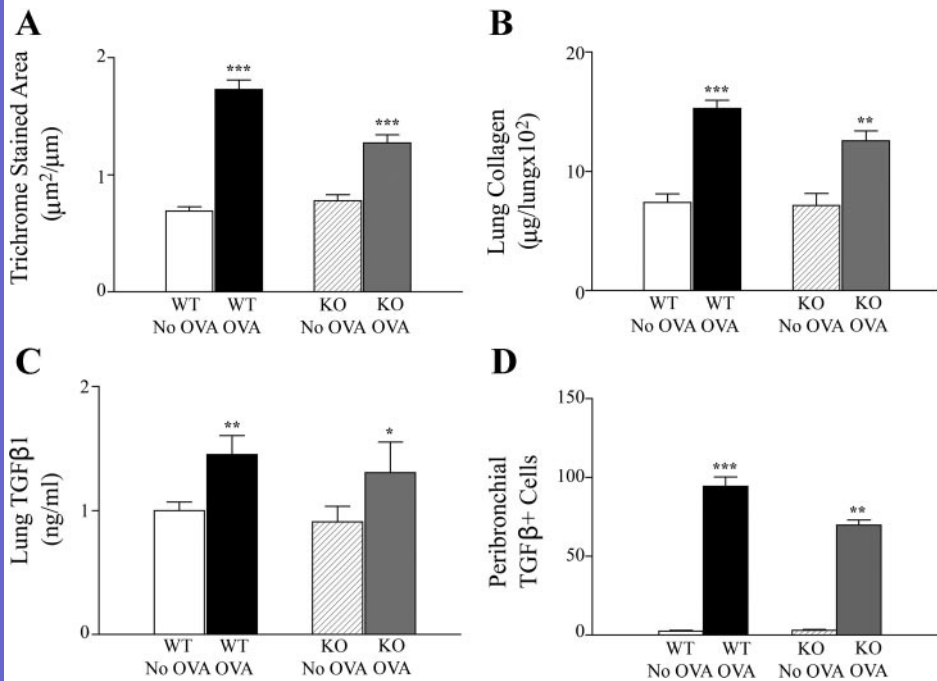


Fig. 1. Peribronchial fibrosis and transforming growth factor (TGF)- β 1 levels in wild-type (WT) vs. matrix metalloproteinase (MMP)-9-deficient (KO) mice. Levels of peribronchial fibrosis were quantitated using both trichrome staining (A) and total lung collagen measurements (B) in WT and MMP-9-deficient mice subjected to repetitive ovalbumin (OVA) challenge for 3 mo. Levels of bioactive lung TGF- β 1 were assessed by ELISA (C), and the number of TGF- β 1+ cells by immunohistochemistry (D) * P = 0.05, ** P = 0.01, *** P = 0.001.

with WT mice (MMP-9-deficient OVA vs. WT OVA, P = 0.001; Figs. 1A and 2).

In addition to increased levels of peribronchial trichrome staining, levels of lung collagen were also significantly increased in WT mice that were repetitively challenged with OVA (15.3 ± 0.7 vs. $7.4 \pm 0.7 \times 10^2$ μ g/lung, WT OVA vs. WT no-OVA; P = 0.001; Fig. 1B). In contrast, levels of lung collagen in OVA-challenged MMP-9-deficient mice were significantly reduced compared with levels noted in OVA-challenged WT mice (MMP-9-deficient OVA vs. WT OVA, P = 0.01; Fig. 1B).

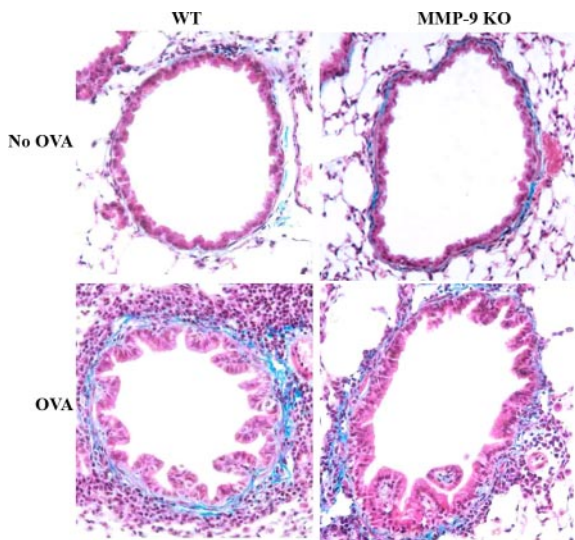


Fig. 2. Trichrome staining of lungs in WT vs. MMP-9-deficient mice. Levels of peribronchial fibrosis were assessed using trichrome staining (blue color) in WT and MMP-9-deficient mice subjected to repetitive OVA challenge for 3 mo.

Lung TGF- β 1 in WT vs. MMP-9-Deficient Mice

As TGF- β 1 is able to induce peribronchial fibrosis, we determined whether reduced levels of expression of TGF- β 1 in the lung could account for reduced levels of peribronchial fibrosis noted in MMP-9-deficient mice. Levels of lung TGF- β 1 were significantly increased in WT mice exposed to repetitive OVA challenge (1.45 ± 0.16 vs. 1.00 ± 0.07 ng/ml, P = 0.05, WT OVA vs. WT no OVA; Fig. 1C). Repetitive OVA challenge in MMP-9-deficient mice also induced a significant increase in levels of lung TGF- β 1 (1.31 ± 0.25 vs. 0.91 ± 0.13 ng/ml, MMP-9-deficient OVA vs. MMP-9-deficient no OVA; P = 0.05; Fig. 1C). There was no significant difference in the levels of lung TGF- β 1 in MMP-9-deficient compared with WT mice repetitively challenged with OVA (1.31 ± 0.25 vs. 1.45 ± 0.16 ng/ml, MMP-9-deficient OVA vs. WT OVA; Fig. 1C). Although there was no difference in the levels of lung TGF- β 1 in WT vs. MMP-9-deficient mice, the number of peribronchial TGF- β 1+ cells (eosinophils and mononuclear cells) was reduced by ~25% in MMP-9-deficient mice compared with WT mice (WT OVA vs. MMP-9-deficient OVA, P = 0.05; Fig. 1D).

Detection of MMP-9 by Zymography in BAL and by Immunostaining in Lung

MMP-9 was detected by zymography in BAL in WT mice following OVA challenge for 3 mo, but not in non-OVA-challenged WT mice (Fig. 3). Immunohistochemical staining of lung sections also demonstrated significantly increased levels of peribronchial expression of MMP-9 in WT mice following OVA challenge compared with non-OVA-challenged WT mice (Fig. 4). In contrast, MMP-9-deficient mice had no detectable expression of MMP-9 as assessed by zymography (Fig. 3) or by immunohistochemistry (Fig. 4) before or after OVA challenge.

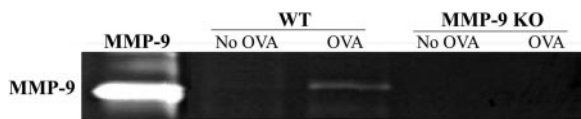


Fig. 3. Expression of MMP-9 detected by zymography. Levels of MMP-9 were assessed by zymography in bronchoalveolar lavage (BAL) fluid derived from WT and MMP-9-deficient mice. *Lane 1* is the MMP-9 standard. In *lanes 2–5* are BAL fluids derived from WT mice (*lane 2* is WT no OVA, *lane 3* is WT OVA) and BAL fluids derived from MMP-9-deficient mice (*lane 4* is MMP-9-deficient/no-OVA, and *lane 5* is MMP-9-deficient/OVA).

Airway Mucus Expression in WT vs. MMP-9-Deficient Mice

The percentage of airway epithelium that stained positively with PAS was significantly increased in WT mice repetitively challenged with OVA (34.6 ± 2.0 vs. $6.0 \pm 1.0\%$, WT OVA vs. WT no OVA; $P = 0.001$; Fig. 5A). There was no significant difference in the percentage of airway epithelium staining positively with PAS in MMP-9-deficient mice compared with WT mice repetitively challenged with OVA [33.2 ± 2.0 vs. $34.6 \pm 2.0\%$, MMP-9-deficient OVA vs. WT OVA; $P =$ not significant (ns); Fig. 5A]. Although there was a trend for non-OVA-challenged MMP-9-deficient mice to have greater levels of PAS+ epithelial cells compared with non-OVA-challenged WT mice this was not statistically significant.

Peribronchial Smooth Muscle Layer Thickness in WT vs. MMP-9-Deficient Mice

The thickness of the peribronchial smooth muscle layer in WT mice repetitively challenged with OVA was significantly greater than in control non-OVA-challenged mice (3.69 ± 0.12 vs. 2.32 ± 0.09 μm , WT OVA vs. WT no-OVA; $P = 0.001$; Fig. 5B). Similarly, the thickness of the peribronchial smooth muscle layer in MMP-9-deficient mice repetitively challenged with OVA was significantly increased (3.56 ± 0.10 vs. 2.47 ± 0.09 μm , MMP-9-deficient OVA vs. MMP-9-deficient no-OVA; $P = 0.001$; Fig. 5B). There was no significant reduction in the thickness of the peribronchial smooth muscle layer in MMP-9-deficient compared with WT mice repetitively challenged with OVA (3.56 ± 0.10 vs. 3.69 ± 0.12 μm , MMP-9-deficient OVA vs. WT OVA; $P =$ ns; Fig. 5B).

Airway Responsiveness in WT vs. MMP-9-Deficient Mice

WT mice challenged with repetitive administration of OVA developed increased airway responsiveness to MCh (WT OVA vs. WT no OVA, $P = 0.001$, MCh 48 mg/ml; Fig. 5C). Similarly, MMP-9-deficient mice repetitively challenged with OVA developed significant increased airway responsiveness to MCh (MMP-9-deficient OVA vs. MMP-9-deficient no OVA; $P = 0.001$, MCh 48 mg/ml; Fig. 5D). The percent increase in Raw following repetitive OVA challenge was similar in WT and MMP-9-deficient mice (WT OVA vs. MMP-9-deficient OVA; $P =$ ns, MCh 48 mg/ml; Fig. 5, C and D).

Levels of BAL and Peribronchial Eosinophils in WT vs. MMP-9-Deficient Mice

Repetitive OVA challenge in WT mice significantly increased the absolute number of BAL eosinophils ($287.3 \pm 39.0 \times 10^2$ vs. $28.3 \pm 7.4 \times 10^2$ BAL eosinophils, WT OVA vs. WT no-OVA; $P = 0.001$; Fig. 6A) and peribronchial eosinophils (33.8 ± 1.4 vs. 1.0 ± 0.1 MBP+ eosinophils/

bronchus, WT OVA vs. WT no-OVA; $P = 0.001$; Fig. 6B). The number of BAL eosinophils in MMP-9-deficient mice were significantly decreased compared with WT mice challenged with OVA ($187.0 \pm 24.3 \times 10^2$ vs. $287.3 \pm 39.0 \times 10^2$ BAL eosinophils, MMP-9-deficient OVA vs. WT OVA; $P = 0.05$; Fig. 6A). Similarly, the number of peribronchial eosinophils were significantly reduced in MMP-9-deficient mice compared with WT mice repetitively challenged with OVA (20.6 ± 1.2 vs. 33.8 ± 1.4 MBP+ eosinophils/bronchus; $P = 0.001$; MMP-9-deficient OVA vs. WT OVA; Fig. 6B). There was no difference in the percentage of bone marrow eosinophils in WT compared with MMP-9-deficient mice following repetitive challenge with OVA (7.4 ± 0.8 vs. $7.3 \pm 0.6\%$ eosinophils, MMP-9-deficient OVA vs. WT OVA; $P =$ ns).

Levels of BAL Chemokines in WT vs. MMP-9-Deficient Mice

As following acute OVA challenge levels of BAL chemokines in MMP-9-deficient mice have been reported to be reduced in some studies (e.g., RANTES see Ref. 22; eotaxin, TARC, see Ref. 9), or increased in other studies (e.g., eotaxin, MDC) (16), we investigated whether changes in levels of BAL chemokines were present following repetitive as opposed to acute OVA challenge in MMP-9-deficient mice. Repetitive OVA challenge in WT mice induced a significant increase in BAL levels of RANTES ($P = 0.05$ vs. non-OVA WT), eotaxin ($P = 0.05$ vs. non-OVA WT), as well as increased levels of TARC ($P = 0.05$ vs. non-OVA WT) and MDC ($P = 0.05$ vs. non-OVA WT) (Fig. 7, A–D). Levels of BAL RANTES were significantly reduced in MMP-9-deficient mice repetitively challenged with OVA (MMP-9-deficient OVA vs. WT OVA, $P = 0.05$; Fig. 7A). There was a trend for reduced BAL levels of eotaxin (Fig. 7B), TARC (Fig. 7C), and MDC (Fig. 7D) in MMP-9-deficient compared with WT mice repetitively challenged with OVA, but none of these trends were statistically significant. Although there was a trend for non-OVA-challenged MMP-9-deficient mice to have greater levels of

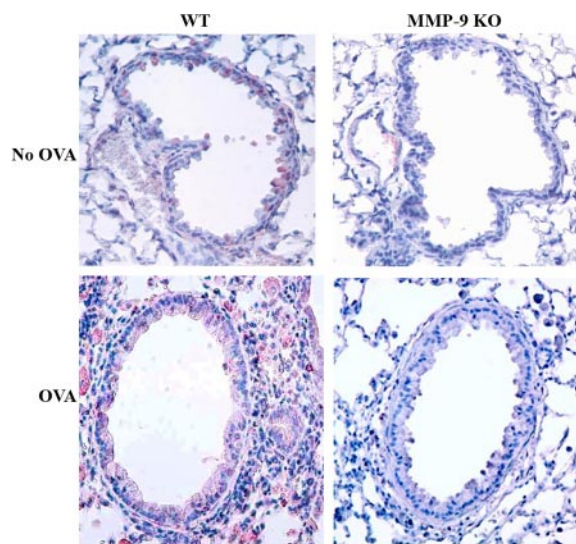


Fig. 4. Immunohistochemistry detection of expression of MMP-9. Lung sections from WT mice (non-OVA-challenged; OVA-challenged) as well as MMP-9-deficient mice (non-OVA-challenged; OVA-challenged) were immunostained with an anti-MMP-9 antibody (Ab) (red color) using the immunoperoxidase method.

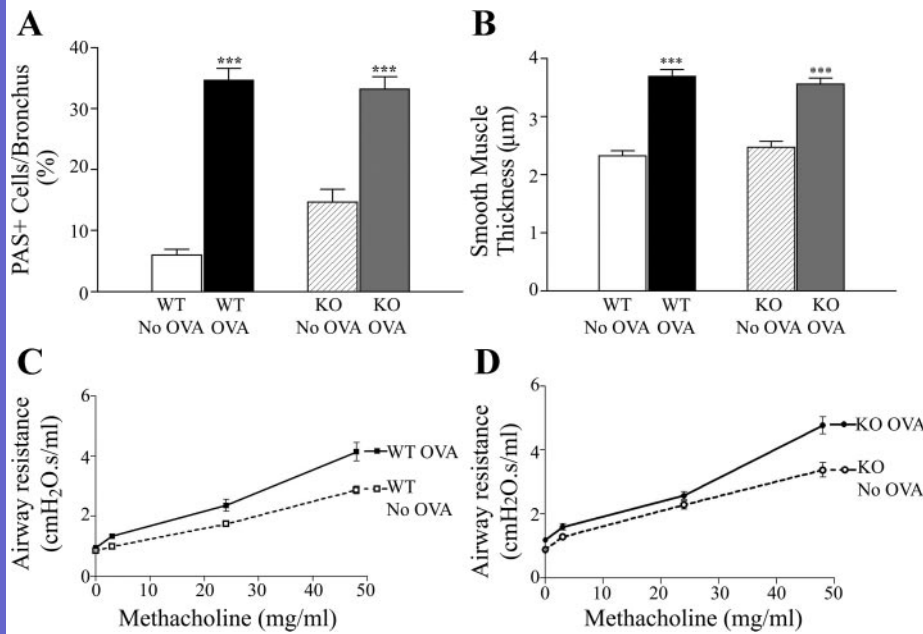


Fig. 5. Mucus, smooth muscle layer thickness, and airway hyperreactivity in WT vs. MMP-9-deficient mice. Levels of mucus were assessed by periodic acid-Schiff (PAS) staining (A), smooth muscle layer thickness by image analysis (B), and airway resistance in intubated and ventilated mice (C and D) in studies comparing WT with MMP-9-deficient mice repetitively challenged with OVA for 3 mo. *** $P = 0.001$.

RANTES and TARC compared with non-OVA-challenged WT mice this was also not statistically significant.

DISCUSSION

Although several studies have demonstrated that MMP-9 is expressed in asthma (11, 13, 15), the biological significance of this association of MMP-9 expression in subjects with asthma to airway remodeling has not previously been investigated. In

this study we have utilized MMP-9-deficient mice to demonstrate that allergen-induced MMP-9 expression plays a role in mediating certain aspects of allergen-induced airway remodeling (e.g., modulates levels of peribronchial fibrosis) without playing a significant role in mediating other aspects of airway remodeling (e.g., thickness of the smooth muscle layer or mucus expression) or in mediating airway responsiveness. MMP-9-deficient mice challenged repetitively with allergen exhibited a reduction in levels of peribronchial fibrosis as assessed by both peribronchial trichrome staining and total lung collagen levels. Although levels of peribronchial fibrosis were partially reduced in allergen-challenged MMP-9-deficient mice, MMP-9-deficient mice still had significant levels of peribronchial fibrosis, suggesting that mechanisms independent of MMP-9 were also important in mediating peribronchial fibrosis. Further studies are needed to determine whether the mechanism(s) by which MMP-9 promotes fibrosis include direct effects of MMP-9 on fibroblasts, myofibroblasts, and/or the extracellular matrix. Alternatively MMP-9 could have indirect effects on fibroblasts, myofibroblasts, and/or the extracellular matrix through effects on resident or recruited lung cells (e.g., eosinophils) or mediators derived from these cells (e.g., TGF- β 1).

MMP-9-deficient mice have normal levels of total lung TGF- β 1 but a reduced number of peribronchial cells expressing TGF- β 1 following allergen challenge. As double-labeling experiments with an anti-MBP Ab demonstrate that eosinophils are $\sim 2/3$ of the peribronchial cells expressing TGF- β 1 and mononuclear cells $\sim 1/3$, MMP-9-deficient mice have an $\sim 16\%$ reduction in eosinophils expressing TGF- β 1. Thus MMP-9 could also play a role in recruitment of TGF- β 1-positive eosinophils. In vitro studies of eosinophil transmigration across basement membranes have used a pore membrane coated with basement membrane components (21). These studies demonstrated that an Ab to MMP-9 inhibited eosinophil migration across the pore membrane coated with basement membrane components. Eosinophil transmigration was associated with

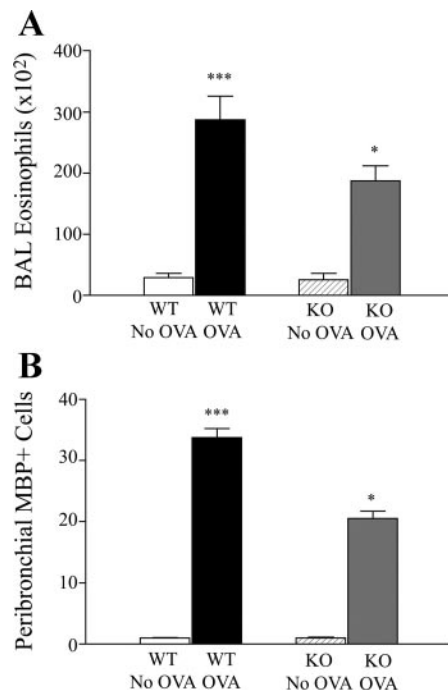
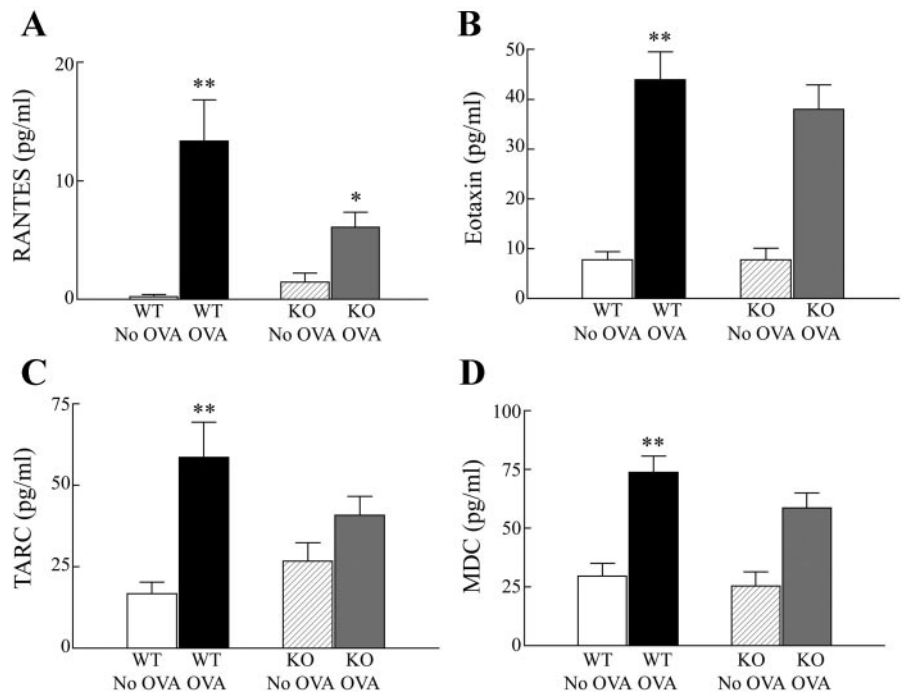


Fig. 6. Eosinophil levels in WT vs. MMP-9-deficient mice. Levels of BAL eosinophils were assessed by Wright-Giemsa staining (A) and peribronchial eosinophils with an anti-MBP antibody (B) in studies comparing WT with MMP-9-deficient mice repetitively challenged with OVA for 3 mo. * $P = 0.05$, *** $P = 0.001$.



Fig. 7. BAL chemokine levels in WT vs. MMP-9-deficient mice. Levels of BAL regulated on activation, normal T cell expressed, and secreted (RANTES, A), BAL eotaxin (B), BAL thymus- and activation-regulated chemokine (TARC, C), and BAL MDC (D) were assessed by ELISA in studies comparing WT with MMP-9-deficient mice repetitively challenged with OVA for 3 mo. * $P = 0.05$, ** $P = 0.01$.



degradation of basement membrane components, as assessed by electron microscopy, and eosinophil generation of MMP-9 (21). In addition to studies of eosinophilic inflammation in MMP-9-deficient mice (4, 9, 16, 22), administration of tissue inhibitor of metalloproteinase-1 (an MMP inhibitor) significantly reduced eosinophilic inflammation in WT mice in response to allergen challenge (12). In this study we have examined the chemokine and eosinophil response in the airway following repetitive allergen challenge for 3 mo as opposed to previous studies examining the eosinophil response in the airway following acute challenge (<1 wk) (4, 9, 16, 22). Whereas previous studies in MMP-9-deficient mice have examined levels of eosinophils in BAL and lung, we have also measured levels of eosinophils in the bone marrow to determine whether the bone marrow generation or bone marrow release of eosinophils could also account for differences in levels of lung eosinophils following allergen challenge. Consistent with the majority of previous studies using acute allergen challenge in MMP-9-deficient mice (4, 9, 22), we have noted impaired eosinophil infiltration into BALF in MMP-9-deficient mice subjected to chronic as opposed to acute allergen challenge. In contrast to studies demonstrating impaired eosinophil migration across the airway epithelium following acute OVA challenge (9), the transmigration of eosinophils from the lung into the airway lumen in MMP-9-deficient mice repetitively challenged with allergen was not impaired, as MMP-9-deficient mice had a similar reduction in peribronchial eosinophils (39%) and BAL eosinophils (34%). We also demonstrated that the reduced eosinophil response in the peribronchial space in the lung in MMP-9-deficient mice was not due to reduced bone marrow production and release of eosinophils in MMP-9-deficient mice. However, we did note that levels of the eosinophil chemottractant RANTES was significantly reduced in BALF in MMP-9-deficient mice repetitively challenged with OVA and that there was a trend for

reductions in levels of TARC and eotaxin as has been noted following acute OVA challenge (9, 22), suggesting that reduced lung expression of these chemokines could contribute to reduced eosinophil migration into the lung in MMP-9-deficient mice following repetitive OVA challenge. The mechanism by which a deficiency in MMP-9 could reduce levels of BAL chemokines is at present unknown. In vitro studies have demonstrated that MMPs can cleave chemokines (17–19), with the resultant formation of natural CC chemokine receptor antagonists. However, if MMP-9 cleaved chemokines in vivo one would anticipate that MMP-9-deficient mice would not be able to cleave BAL chemokines, which would result in higher levels of BAL chemokines, rather than the lower levels of BAL chemokines that we and others (4, 9, 22) have reported in MMP-9-deficient mice. An alternative hypothesis is that MMP-9 regulates chemokine gradients in vivo through MMP-mediated proteolysis of proteoglycans associated with the extracellular matrix that are known to bind and regulate levels of chemokines (14). At present the mechanism accounting for reduced BAL chemokine levels in MMP-9-deficient mice is unknown. In addition to impaired chemotaxis of eosinophils from the blood stream into the lung in MMP-9-deficient mice, impaired transmigration of MMP-9-deficient eosinophils across pulmonary blood vessels may also account for the reduced numbers of lung eosinophils in MMP-9-deficient mice. Unfortunately, the number of peripheral blood eosinophils in MMP-9-deficient mice is too small to purify, fluorescently label, and visualize with intravital microscopy to directly determine whether MMP-9-deficient eosinophils have impaired transmigration across basement membranes of blood vessels in vivo.

In contrast to the effect of MMP-9 deficiency on the levels of peribronchial fibrosis and eosinophil accumulation, MMP-9 did not play a role in the development of increased smooth muscle layer thickness, airway hyperreactivity, or mucus ex-



pression. Previous studies of acute OVA challenge in MMP-9-deficient mice have produced conflicting results in relation to effects on airway responsiveness (4, 9, 16, 22) and have not examined the role of MMP-9 in smooth muscle layer thickness. It should be noted that our model of chronic OVA challenge in mice differs from the standard acute OVA challenge model in that mice are subjected to repetitive OVA challenge for up to 3 mo and exhibit features of airway remodeling not noted in the acute OVA challenge models. The differences in results may also relate to the different methods used for measuring airway responsiveness in the previous studies (i.e., pulmonary resistance in intubated mice vs. noninvasive enhanced pause) (4, 9, 16, 22). In this study we measured pulmonary resistance in intubated MMP-9-deficient mice following chronic OVA challenge. Similar to results obtained in intubated MMP-9-deficient mice following acute OVA challenge (4, 9, 22), we noted no difference in airway resistance in MMP-9-deficient compared with WT mice following chronic OVA challenge.

In summary, in this study we demonstrate a role for MMP-9 in mediating peribronchial fibrosis and eosinophilic inflammation in a mouse model of airway remodeling. Reduced BAL levels of chemokines such as RANTES, as well as reduced transmigration of eosinophils across the basement membrane of blood vessels, are likely to contribute to reduced peribronchial and BAL eosinophil levels in MMP-9-deficient mice. Although MMP-9-deficient mice had reduced levels of peribronchial fibrosis, they did not exhibit reduced thickness of the smooth muscle layer, reduced airway responsiveness, or reduced mucus expression. Thus therapies targeting MMP-9 to reduce airway remodeling in asthma may result in modest reductions in the levels of peribronchial fibrosis but not influence levels of mucus expression, thickness of the smooth muscle layer, or airway responsiveness.

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