

Matrix Metalloproteinase-8 Deficiency Promotes Granulocytic Allergen-Induced Airway Inflammation¹

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Matrix metalloproteinases (MMPs) are involved in inflammatory reaction, including asthma-related airway inflammation. MMP-8, mainly produced by neutrophils, has recently been reported to be increased in the bronchoalveolar lavage fluid (BALF) from asthmatic patients. To evaluate the role of MMP-8 in asthma, we measured MMP-8 expression in lung tissue in an OVA-sensitized mouse model of asthma and addressed the effect of MMP-8 deletion on allergen-induced bronchial inflammation. MMP-8 production was increased in lungs from C57BL/6 mice exposed to allergens. After allergen exposure, MMP-8^{-/-} mice developed an airway inflammation characterized by an increased neutrophilic inflammation in BALF and an increased neutrophilic and eosinophilic infiltration in the airway walls. MMP-8 deficiency was associated with increased levels of IL-4 and anti-OVA IgE and IgG1 in BALF and serum, respectively. Although allergen exposure induced an enhancement of LPS-induced CXC chemokine, KC, and MIP-2 levels in BALF and lung parenchyma, no difference was observed between the two genotypes. Inflammatory cell apoptosis was reduced in the lungs from MMP-8^{-/-} mice. For the first time, our study evidences an important role of MMP-8 in the control of neutrophilic and eosinophilic infiltration during allergen-induced lung inflammation, and demonstrates that the anti-inflammatory effect of MMP-8 is partly due to a regulation of inflammatory cell apoptosis. *The Journal of Immunology*, 2005, 175: 2589–2597.

Asthma is a complex inflammatory disease characterized by an eosinophilic inflammation of the bronchial walls and airway hyperresponsiveness. Neutrophils also seem to play a significant role in asthma as soon as the disease becomes severe (1, 2). A significant contribution of neutrophils to the bronchial hyperresponsiveness is suggested by in vitro studies (3) and by the increased migratory activity (4) observed in neutrophils from human asthmatics. The airways of human asthmatics display significant morphological changes referred to as bronchial remodeling (5). Among those changes, many are linked to an excessive degradation of the extracellular matrix and mainly of its collagen components. The main feature of remodeling in humans is the deposition of a dense layer made of different collagens and fi-

bronectin just beneath the basement membrane of the bronchial epithelium (6).

Matrix metalloproteinases (MMPs)³ are a family of zinc proteases that can degrade virtually all extracellular matrix components and whose activity is controlled among others by the tissue inhibitors of metalloproteinases (TIMPs) (7–10). Most MMPs are secreted from the cells as inactive precursors requiring the cleavage of an amino-terminal peptide of ~10 kDa to be activated. MMP-1 (fibroblast collagenase, collagenase-1), MMP-8 (neutrophil collagenase, collagenase-2), and MMP-13 (collagenase-3) belong to the interstitial collagenase subgroup of MMPs and display the potency to degrade fibrillar interstitial collagens (types I-III) (11). In addition, MMP-14, a membrane-type MMP, is able to degrade type I collagen and contributes to collagen matrix remodeling (12, 13). These proteases, in association with others including type IV collagen degrading gelatinases (MMP-2 and MMP-9), could potentially contribute to the bronchial remodeling observed in asthma. Accordingly, significant increases of various MMPs (MMP-1, MMP-2, MMP-8, and MMP-9) have been reported in the sputum or bronchoalveolar lavage (BAL) from asthmatics (14–16). MMPs produced by inflammatory cells such as MMP-8 may be of particular importance in the pathogenesis of inflammatory disorders. MMP-8 is expressed during the myelocyte stage of neutrophil development in the bone marrow. It is stored as a latent enzyme (pro-MMP-8) within specific granules and released upon neutrophil activation (17–20). After release from granules, a significant part of the MMP-8 is associated to the membrane of neutrophils and exerts a pericellular proteolysis (21). An excess of MMP-8 secretion has been reported in BAL fluids (BALF) of human patients with bronchiectasis and has been correlated with disease severity (22, 23). In asthmatics, increased expression of

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³ Abbreviations used in this paper: MMP, matrix metalloproteinase; BAL, bronchoalveolar lavage; BALF, BAL fluid; TIMP, tissue inhibitor of metalloproteinase.

Table I. Oligonucleotides used for RT-PCR

	Oligonucleotide Sense	Oligonucleotide Antisense	Number of PCR Cycles	Expected RNA Size (bp)
MMP-2	5'-AGATCTTCTTCTTCAAGGACCGGT-3'	5'-GGCTGGTCAGTGGCTTGGGGTA-3'	36	225
MMP-8	5'-CCAAGTGGGAACGCCTAAGTGA-3'	5'-TGGAGAATTGTCACCGTGATCTCTT-3'	38	200
MMP-9	5'-CCCACATTTGACGTCAGAGAAGAA-3'	5'-GTTTTTGATGCTATTGCTGAGATCCA-3'	36	208
MMP-12	5'-ACATTTTCGCCTCTCTGTGATGAC-3'	5'-CAGAAACCTTCAGCCAGAAGAACC-3'	38	201
TIMP-1	5'-GGCATCCTCTGTGCTATCACTG-3'	5'-GTCATCTTGATCTCATAACGCTGG-3'	35	168
MIP-2	5'-GACTTCAAGAACATCCAGAGCT-3'	5'-AGCGAGGCACATCAGGTACGAT-3'	38	280
28S	5'-GTTACCCACTAATAGGGAACGTGA-3'	5'-GATTCTGACTTAGAGCGGTTCACT-3'	19	212

MMP-8 mRNA has been detected in bronchial biopsies and was correlated with the intensity of the disease (24). MMP-8 could contribute to inflammatory cell trafficking and inflammation in different ways, through the cleavage of fibrillar collagens, or through the degradation or activation of protease inhibitors (α_1 -antitrypsin, α_2 -macroglobulin) and/or chemokines such as LPS-induced CXC chemokine, a neutrophil chemoattractant (25–28). However, the functions and mechanisms of action of MMP-8 in inflammatory disorders are not yet well established. Recently, unexpected inhibitory effects of MMP-8 in cancer progression have been evidenced (25, 29). Indeed, MMP-8 gene deletion in mice increased tumor development after carcinogen treatment (25), and genetic manipulation of metastatic cells to overproduce MMP-8 resulted in decreased metastatic dissemination (29). These observations suggest that MMP-8 may have multiple functions regulating protective immune functions and/or tissue infiltration.

In the present work, the key role played by MMP-8 in the processes leading to airway inflammation after allergen exposure is demonstrated, for the first time, in an experimental mouse model of asthma. Our data have therapeutic implications warning against the use of broad spectrum inhibitors in asthma therapy.

Materials and Methods

Sensitization and allergen exposure protocol

Wild-type (MMP-8^{+/+}) and MMP-8 knockout (MMP-8^{-/-}) mice were generated in a C57BL/6J129 genetic background, as previously described (25). In additional experiments, we used C57BL/6 mice from Denmark (Taconic Farms). Males of 6–8 wk old were sensitized by i.p. injection of 10 μ g of OVA (Sigma-Aldrich) emulsified in aluminum hydroxide (AlumInject; Perbio) on days 1 and 8. Mice were subsequently exposed to allergens by daily inhalation of an aerosol of 1% OVA, for 30 min, generated by ultrasonic nebulizer (Devilbiss 2000), from day 21 to 27. Mice sacrifice was performed on day 28, as previously reported (30). Results are those of three independent experiments ($n = 19$ –28 animals per experimental condition).

BALF

Mice were sacrificed, and a BAL was performed using 4 \times 1 ml of 0.05 mM PBS-EDTA (Calbiochem), as previously described (30). Cells were recovered by gentle manual aspiration. After centrifugation of BALF (1200 rpm for 10 min, at 4°C), the supernatant was frozen at –80°C for protein assessment and the cell pellet was resuspended in 1 ml of 0.05 mM PBS-EDTA. The differential cell counts were performed on cytocentrifuged preparations (Cytospin) after staining with Diff-Quick (Dade).

Pulmonary histology and tissue processing

After BAL, the thorax was opened and the left main bronchus was clamped. The left lung was excised and frozen immediately at –80°C for protein and mRNA extraction. The right lung was infused with 4% paraformaldehyde, embedded in paraffin, and used for histology. Sections of 5- μ m thickness were cut off from paraffin and were stained with H&E. The extent of peribronchial inflammation was estimated by a score calculated by quantification of peribronchial inflammatory cells, as previously described (30). A value of 0 was adjudged when no inflammation was detectable; a value of 1 when there were occasionally inflammatory cells; a value of 2 when most bronchi were surrounded by a thin layer (1–5 cells)

of inflammatory cells; and a value of 3 when most bronchi were surrounded by a thick layer (>5 cells) of inflammatory cells. Because 5–7 randomly selected tissue sections per mouse were scored, inflammation scores are expressed as a mean value per mouse and can be compared between groups. After Congo red staining, the eosinophilic infiltration in the airway walls was quantified by manual count and reported to the perimeter of epithelial basement membrane defining an eosinophilic inflammatory score. Immunohistochemistry was performed on paraffin sections to detect MMP-8 and neutrophils. Slides were heated in autoclave in citrate buffer (Dako Target Retrieval Solution; DakoCytomation) and incubated with primary Ab (rabbit anti-mouse MMP-8, Abcam; rat anti-mouse neutrophils, Serotec) and secondary Ab (swine anti-rabbit HRP and rabbit anti-rat HRP; DakoCytomation). Slides were then incubated with 3-amino-9-ethylcarbazole (DakoCytomation).

TUNEL (Roche) was used to study apoptosis. Sections were incubated in Xylo, dehydrated, and pretreated with 1% Triton X-100 and hydrogen peroxide (H₂O₂). Sections were incubated 1 h at 37°C with enzyme solution and nucleotide mixture (UTP-FITC). To use standard light microscope, slides were incubated with an anti-FITC/HRP Ab (converter POD, an anti-fluorescein antibody conjugated with horseradish peroxidase). Finally, sections were counterstained with hematoxylin and mounted. For each mouse, five different areas were analyzed in the whole lung. The percentage of neutrophils undergoing apoptosis was calculated for each mouse. Apoptosis was also studied by measuring the percentage of granulocytes displaying a caspase 3 activation. Caspase 3 was detected in situ by using a primary Ab (rabbit anti-human/mouse caspase 3 active; R&D Systems) and secondary Abs coupled with biotin (biotinylated goat anti-rabbit; DakoCytomation) and streptavidin (streptavidin/HRP; DakoCytomation). Slides were then incubated with 3-amino-9-ethylcarbazole (DakoCytomation). Five different areas were analyzed for each mouse, and

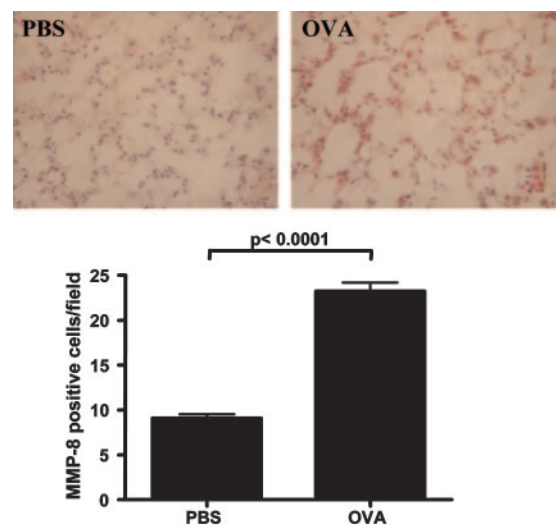


FIGURE 1. MMP-8 production in the lungs of 7-day allergen-challenged mice 24 h after the last allergen inhalation. Immunohistochemical staining of MMP-8 was performed on lung sections of sham (PBS)- or allergen-challenged (OVA) C57BL/6 mice. An increase in the number of MMP-8-positive cells was assessed by counting the cells on 10 areas for each mice ($n = 10$) ($p < 0.0001$).

Table II. Total cell numbers and cellular composition of BALF^a

	MMP-8 ^{+/+} PBS (n = 19)	MMP-8 ^{+/+} OVA (n = 24)	MMP-8 ^{-/-} PBS (n = 20)	MMP-8 ^{-/-} OVA (n = 28)
Epithelial cell ($\times 10^4$ /ml)	2.6 \pm 1	2.3 \pm 0.7	2.1 \pm 0.5	2.4 \pm 1.4
Eosinophils ($\times 10^4$ /ml)	1.1 \pm 0.6	26.7 \pm 8.3 [#]	2.8 \pm 1.1	33.6 \pm 4.9 [#]
Neutrophils ($\times 10^4$ /ml)	2.1 \pm 0.8	4.3 \pm 1.1	1.4 \pm 0.4	14.3 \pm 3.6* [#]
Lymphocytes ($\times 10^4$ /ml)	6.6 \pm 1.6	6.4 \pm 1.6	4.1 \pm 1.1	9.4 \pm 1.8
Macrophages ($\times 10^4$ /ml)	48.3 \pm 4.2	33.5 \pm 5.3	33.9 \pm 5.5	30.7 \pm 5.9
Total cells ($\times 10^4$ /ml)	64.2 \pm 6.6	71.1 \pm 13.8	42.1 \pm 6.1	90.4 \pm 11.4 [#]

^a Total cell numbers and cellular composition of BALF in sensitized MMP-8^{+/+} mice and MMP-8^{-/-} mice exposed to PBS or OVA aerosol. Numbers of cells were expressed as mean values \pm SEM. Three independent experiments have been performed.

[#], $p < 0.05$ vs PBS-exposed mice. *, $p < 0.05$ vs wild-type mice (exposed or not to OVA). n = total number of mice per experimental group.

the percentage of neutrophils undergoing apoptosis was calculated. Detection of cells bearing on their surface the orphan receptor T₁/ST₂ was performed by immunohistochemistry using a rat mAb to mouse T₁/ST₂ purchased from Morwell Diagnostics. T₁/ST₂-positive lymphocyte-shaped cells were counted in the peribronchial area of six bronchi per mouse.

The left lung of each animal was crushed using a Mikro-Dismembrator (Braun). For protein extraction, the crushed lung tissue was incubated overnight at 4°C in a solution containing 2 M urea, 1 M NaCl, and 50 mM Tris (pH 7.5), and subsequently centrifuged 15 min at 16,000 $\times g$. The supernatant was stored at -80°C for zymography. Total RNA was extracted with Rneasy Mini kit (Qiagen). RNA levels and purity were assessed using a SmartSpect 3000 spectrophotometer (Bio-Rad). To rule out the possibility of genomic DNA contamination, all of our RNA samples underwent a PCR with primers amplifying the 28S DNA. The 28S rRNA (ribosomal RNA), MMPs, and chemokine mRNAs were measured by RT-PCR using the GeneAmp ThermoStable rTth reverse-transcriptase RNA PCR kit (Roche Molecular Systems). Oligonucleotides used for RT-PCR are shown in Table I. Reverse transcription was performed at 70°C for 15 min, followed by 2-min incubation at 95°C for denaturation of RNA-DNA heteroduplexes. Amplification started at 94°C for 15 s, 68°C for 20 s, and 72°C for 10 s, and terminated by 2 min at 72°C. Amplification cycle was repeated 18 times for detection of 28S rRNA and 38 times for detection of MMP and TIMP mRNA. RT-PCR products were resolved on 10% acrylamide gels and analyzed with a Fluor-S MultiImager (Bio-Rad) after staining with Syber Green (Molecular Probes). Quantitative RT-PCR results are expressed as a ratio to 28S rRNA.

To perform zymography (14), protein extracts were mixed with the same amount of sample buffer. Electrophoresis was conducted on an SDS-10% polyacrylamide gel containing gelatin at a concentration of 1 mg/ml. Gels were incubated for 30 min in 2% Triton X-100. After incubation in an activation buffer containing 100 mM CaCl₂ and 100 mM NaCl at 37°C overnight, gels were rinsed and stained for 30 min in Coomassie blue. Gelatinase activity was detected as a white lysis band against a blue background. Quantitative evaluation of the gelatinolytic activity was performed by scanning gel using an imaging densitometer (Bio-Rad; Fluor-S MultiImager). On each gel, we used dilutions of culture medium conditioned by HT1080 cells, known to produce spontaneously high amounts of MMP-2 and MMP-9, as an internal standard. Gelatinolytic activity of the murine MMP-2 and MMP-9 was determined by the lysis band in the 72- and 95-kDa area, respectively.

Measurements of cytokines by ELISA and Western blots

IL-4, IL-5, IFN- γ , IL-17, and KC levels were assessed using commercial ELISAs (R&D Systems), while ELISAs for MIP-2 and LIX proteins were developed using Abs from R&D Systems and the R&D DuoSet ELISA development kit (R&D Systems). Western blots were performed to quantify LIX and KC levels and to give additional data on the state of activation of those proteins. Samples were electrophoretically separated in SDS-10% polyacrylamide gels in the presence of 5% 2-ME. Proteins were transferred to a polyvinylidene difluoride membrane (NEN Life Science Products), which was then incubated for 1 h at room temperature in TBS containing 5% skimmed milk. The blots were then incubated for 1 h at room temperature with a specific mouse anti-LIX or anti-KC mAb (R&D Systems) diluted in TBS, washed three times in TBS-Tween-20 (0.1%), and finally incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG (Dako-Cytomation) diluted 1/1000. The peroxidase activity was assessed using an ECL kit (NEN Life Science Products).

Measurement of allergen-specific serum IgE

At the end of the time of sacrifice, blood was drawn from the heart for measurement of OVA-specific serum IgE. Ninety-six-well microtiter plates

were coated with 300 μ l/well of an OVA solution (5 mg/L). Serum was added and, after incubation and rinsing, followed by a biotinylated polyclonal sheep anti-mouse IgE (Calbiochem) used at 1/1000. A serum pool from OVA-sensitized animals was used as internal laboratory standard; 1 U was arbitrarily defined as 1/100 dilution of this pool.

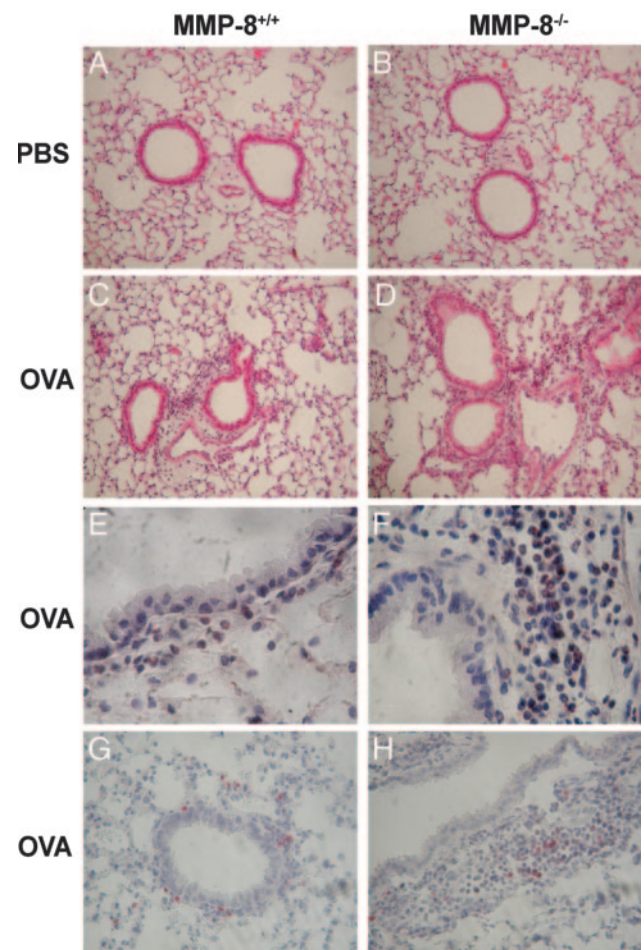


FIGURE 2. Histopathological analysis of lungs 24 h after a 7-day allergen exposure. *A–D*, Lung paraffin sections stained with H&E (magnification $\times 200$). Wild-type mice (MMP-8^{+/+}) (*A* and *C*) or deficient mice (MMP-8^{-/-}) (*B* and *D*) were exposed to PBS (*A*, MMP-8^{+/+} PBS; *B*, MMP-8^{-/-} PBS) or to OVA (*C*, MMP-8^{+/+} OVA; *D*, MMP-8^{-/-} OVA). *E–H*, Representative photomicrographs of lung sections of MMP-8^{+/+} mice exposed to OVA (*E* and *G*) and MMP-8^{-/-} mice exposed to OVA (*F* and *H*). Eosinophilic infiltration in the airway walls was studied by Congo red staining to evidence eosinophils in red/orange ($\times 200$) (*E* and *F*). Neutrophilic infiltration of the peribronchial area was evidenced by immunohistochemistry, as described in *Materials and Methods* (*G* and *H*).

Blood cell count assessment

Whole blood with heparin was obtained from mouse by cardiac puncture immediately after sacrifice. Differential cell counts were performed using an automatic hemocytometer (Bayer) and validated by performing blood smears.

Statistical analysis

Results of BAL cell count, pulmonary histology, cytokines, and mRNA levels were expressed as mean \pm SEM, and the comparison between the groups was performed using Mann-Whitney *U* test. Mann-Whitney *U* test was performed using GRAPHPAD INSTANT version 3.00 for WINDOWS 95 (GraphPad; (www.graphpad.com)). Values of $p < 0.05$ were considered as significant.

Results

MMP-8 expression in the lungs of allergen-challenged mice

To determine whether MMP-8 production is modulated in the OVA mice model of asthma, we performed an immunohistochemical analysis on lung sections from C57BL/6 mice exposed either to OVA or PBS. Mice exposure to allergen led to a large increase of the number of cells positive for MMP-8 in lung tissue ($p < 0.0001$) (Fig. 1).

BALF

Exposure of mice to aerosolized OVA induced a significant increase in total cell counts in MMP-8^{-/-} mice ($p < 0.05$). Eosinophil counts were significantly increased after allergen exposure in both MMP-8^{+/+} ($n = 43$) and MMP-8^{-/-} ($n = 48$) genotypes, as compared with their nonexposed counterparts ($p < 0.0001$). Interestingly, BALF of MMP-8^{-/-} mice were characterized by a 10-fold increase in neutrophil counts after allergen exposure when compared with their nonexposed counterpart (Table II). In sharp contrast, only a 2-fold enhancement of neutrophil number was induced by allergen treatment in MMP-8^{+/+} mice. Such an effect of MMP-8 deficiency on cell infiltration in BALF was specific to neutrophils, because other cell counts (epithelial cells, lymphocytes, or macrophages) were not affected.

Histopathology of the lungs

No developmental abnormalities were observed in the lungs of MMP-8^{-/-} mice (data not shown). The airways from MMP-8^{+/+} and MMP-8^{-/-} mice exposed to sham challenge (PBS) showed normal histology (Fig. 2, A and B). After sensitization and subsequent allergen exposure, both MMP-8^{-/-} and MMP-8^{+/+} displayed a significant peribronchial and perivascular inflammation (Fig. 2, C and D). Such an enhanced peribronchial inflammation in both genotypes was confirmed by the higher inflammation scores ($p < 0.0001$) (Fig. 3A).

Eosinophilic and neutrophilic tissue infiltration was studied on Congo red-stained slides (Fig. 2, E and F) and by immunohistochemistry (Fig. 2, G and H), respectively. Quantitative results were expressed as a number of cells/mm epithelial basement membrane (Fig. 3, B and C). Although the number of eosinophils was increased in both groups after allergen exposure ($p < 0.0001$), the enhancement was even higher in MMP-8^{-/-} mice than in MMP-8^{+/+} ($p < 0.05$) (Fig. 3B).

The neutrophilic infiltration was increased after allergen exposure in both genotypes. Again, the enhancement of neutrophil counts in the bronchial wall was 2 times higher in MMP-8^{-/-} when compared with MMP-8^{+/+} (Fig. 3C).

Blood cell counts in MMP-8^{+/+} and MMP-8^{-/-} mice

To address some potential differences regarding blood inflammatory cells between MMP-8^{+/+} and MMP-8^{-/-}, differential cell counts were performed, and no significant difference was found when comparing the counts of neutrophils, lymphocytes, monocytes, eosinophils, and basophils (Fig. 4A).

Assessment of sensitization to allergen

Serum levels of anti-OVA-specific IgE were detected by ELISA. Specific IgE levels were increased in the sera of allergen-exposed mice from both genotypes ($p < 0.0001$). However, an enhancement at higher extent was detected in the sera of MMP-8^{-/-} mice

FIGURE 3. Quantitative study of airway inflammation in histology 24 h after a 7-day allergen exposure. A, The airway inflammation has been quantified on H&E-stained slides, and results are expressed as a bronchial inflammation score, as described in *Materials and Methods*. B, The number of eosinophils was counted using a digitalized picture, and was reported to the length of the epithelial basement membrane. C, Neutrophils detected by immunohistochemistry were counted in the peribronchial area, and their number was reported to the length of the epithelial basement membrane. Results are expressed as means \pm SEM.

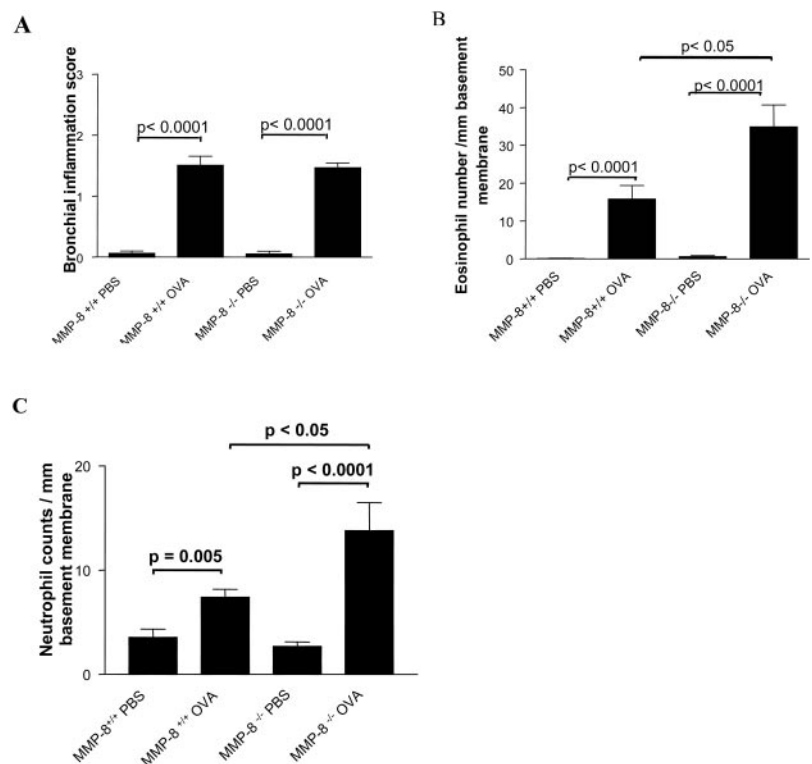
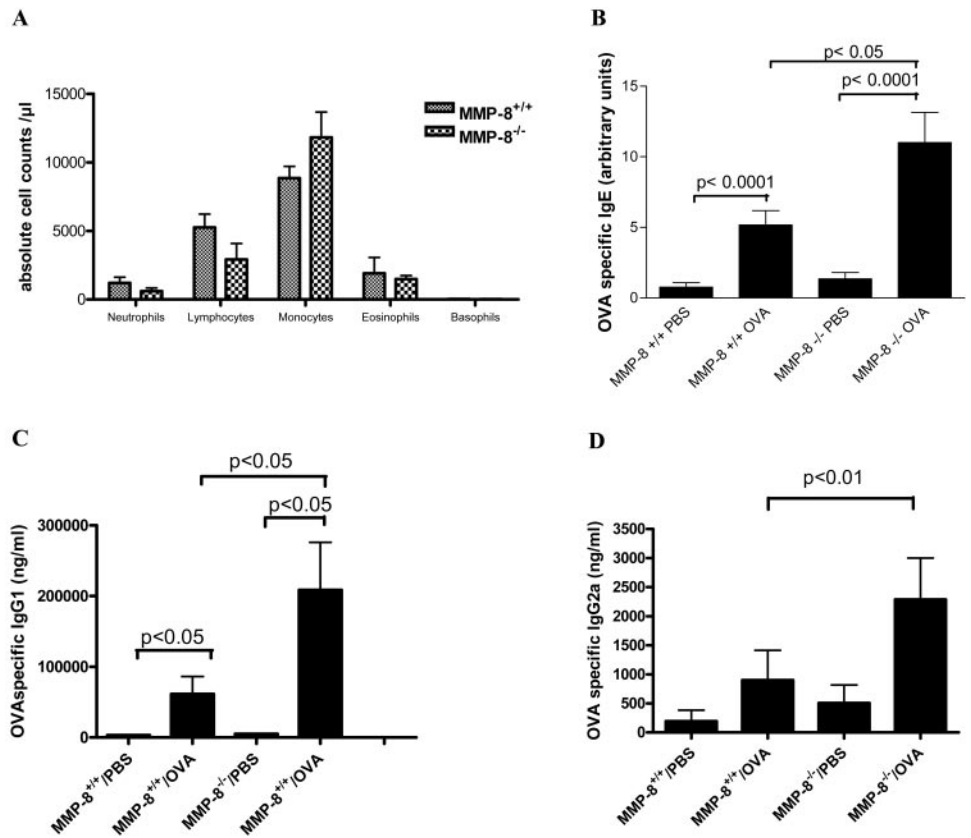


FIGURE 4. *A*, Blood differential cell counts in MMP-8^{+/+} and MMP-8^{-/-} mice. Results are expressed as number of cells/ μ l total blood. Measurements were performed in nonsensitized and unexposed mice. *B*, Quantitative analysis of IgE levels by ELISA. Significant differences in the anti-OVA IgE levels measured by ELISA were observed between mice exposed to OVA aerosol as compared with mice exposed to PBS. Results are expressed as means \pm SEM. *C*, Measurement of OVA-specific IgG1 by ELISA. Results are expressed as means \pm SEM. *D*, Measurement of OVA-specific IgG2a by ELISA. Results are expressed as means \pm SEM. All measurements shown in *B–D* were performed 24 h after a 7-day allergen exposure.



as compared with MMP-8^{+/+} ($p < 0.05$) (Fig. 4*B*). To investigate a potential Th1/Th2 imbalance in MMP-8^{-/-} mice, we measured the levels of anti-OVA-specific IgG1 and IgG2a. IgG1 levels were significantly increased after allergen exposure in both genotypes ($p < 0.05$) and even more elevated in MMP-8^{-/-} mice as compared with MMP-8^{+/+} ($p < 0.05$) (Fig. 4*C*). Measurements of OVA-specific IgG2a did not show any difference between allergen-challenged and unchallenged mice, but levels were higher in MMP-8^{-/-} after allergens as compared with MMP-8^{+/+} ($p < 0.01$) (Fig. 4*D*). Total IgE levels were also studied by ELISA, and their levels were found to be increased significantly in both knockout and wild-type mice after allergen exposure without difference between the genotypes (data not shown).

Cytokines in BALF and lung protein extracts

To further investigate the mechanisms leading to an increased inflammation and increased levels of OVA-specific IgE in MMP-8^{-/-} mice, we measured by ELISA, Western blots, and RT-PCR a panel of relevant ILs (IL-4, IL-5, and IFN- γ) and chemokines (KC, IL-17, MIP-2, LIX, eotaxin) that may be involved in inflammatory cell recruitment.

Although IL-4 levels assessed by ELISA were increased in BALF and lung protein extracts after allergen exposure in both genotypes ($p < 0.001$), statistical differences were only reached in MMP-8^{-/-} mice ($p < 0.001$) (Fig. 5, *A* and *B*). Allergen exposure induced a greater enhancement of IL-4 levels in BALF of MMP-8^{-/-} mice than in wild-type mice. IL-5 and IL-17 levels in BALF or lung protein extracts were not significantly different between the groups, and IFN- γ levels were only rarely detectable in BALF (Fig. 5, *C* and *H*, respectively). Eotaxin, an eosinophil chemoattractant, was measured in BALF and lung protein extracts. Its levels were significantly increased after allergen exposure in both

genotypes without any difference between MMP-8^{+/+} and MMP-8^{-/-} (Fig. 5*D*).

We also focused on neutrophil chemoattractants: KC, MIP-2, and LIX. Levels of KC measured by ELISA in lung protein extracts were similarly increased after allergen exposure in both genotypes ($p = 0.0001$) (Fig. 5*E*) and were rarely detectable in BALF. ELISA analysis of LIX levels in BALF revealed a tendency to increase after allergen exposure in both genotypes, but these differences did not reach statistical significance (data not shown). LIX production was also studied by Western blot analysis in BALF. Although significant increase of LIX was observed in mice exposed to allergens ($p < 0.05$), again no difference was detected between MMP-8^{-/-} and MMP-8^{+/+} mice (Fig. 5, *F* and *G*). Because MIP-2 levels were rarely detectable by ELISA, RT-PCR analysis was performed in lung tissue extracts. MIP-2 mRNA levels were increased after allergen challenge in both groups without any differences between MMP-8^{-/-} and MMP-8^{+/+} mice (Fig. 6).

Determination of Th2 recruitment in lung parenchyma

An anti-T_H1/ST₂ (orphan receptor expressed by Th2 cells) immunohistochemistry was performed on slides from lungs of MMP-8^{+/+} ($n = 10$) and MMP-8^{-/-} ($n = 12$) mice exposed to OVA. Numbers of lymphocyte-shaped cells positive for this staining were significantly increased in MMP-8^{-/-} mice as compared with MMP-8^{+/+} ($p < 0.05$), indicating that Th2 recruitment is increased in the lungs from MMP-8^{-/-} (Fig. 7).

Assessment of cell apoptosis in lung sections

TUNEL analysis and caspase 3 immunohistochemistry performed on paraffin-embedded lung sections revealed that the number of

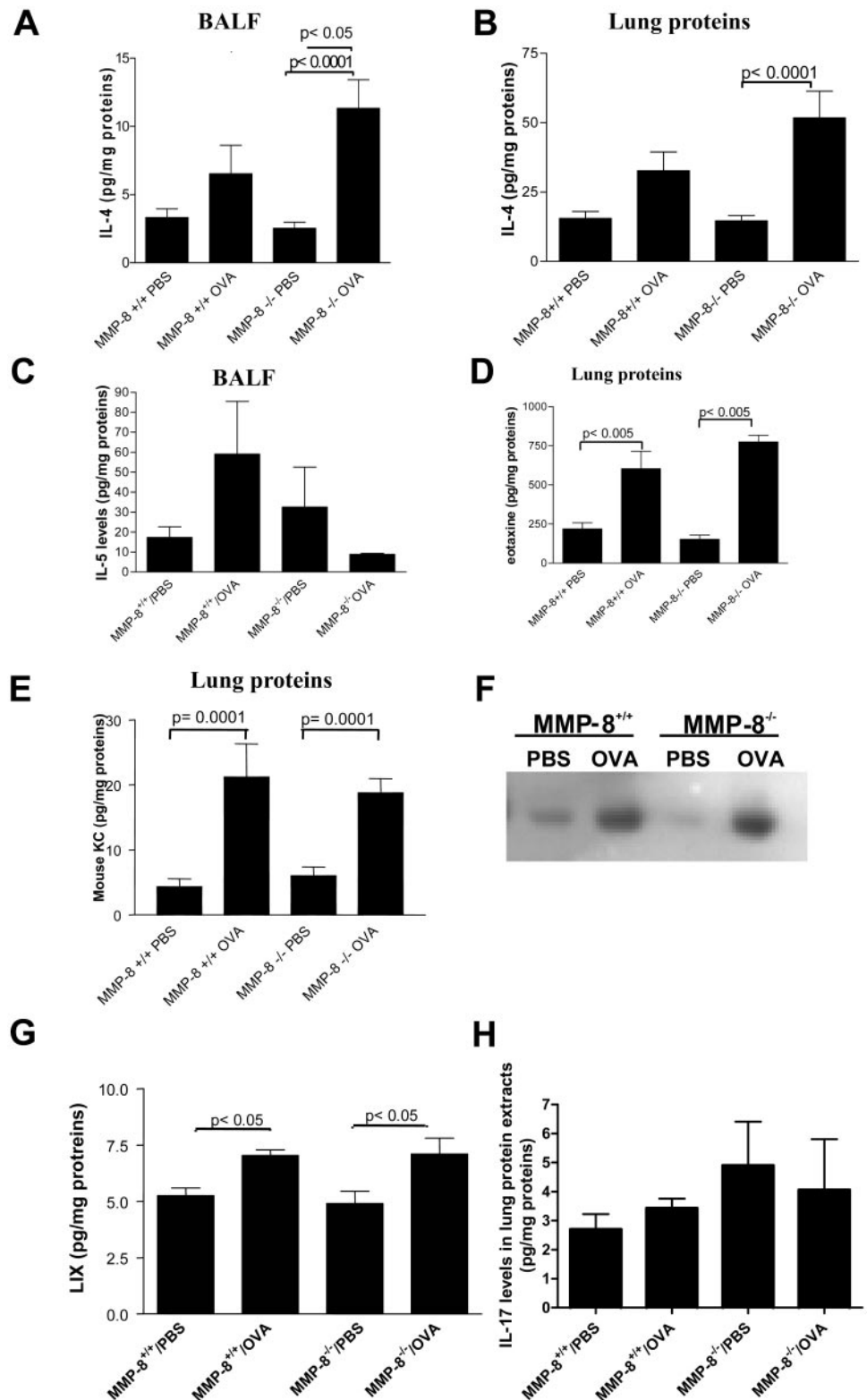


FIGURE 5. Measurements of chemokines and cytokines in the BAL and lung protein extracts 24 h after a 7-day allergen exposure. Levels of IL-4 measured in BALF (A) and lung protein extracts (B). Significant differences were observed between MMP-8^{-/-} mice exposed to OVA aerosol and mice exposed to PBS. IL-4 levels were higher after OVA exposure in BALF of MMP-8^{-/-} mice when compared with MMP-8^{+/+} in BALF. IL-5 levels measured in BALF by ELISA were not significantly different between the groups (C). Eotaxin levels measured in the lung protein extracts were increased after allergen exposure in both genotypes without difference between the groups (D). Levels of KC measured in lung protein extracts (E) were increased after OVA challenge in both groups ($p = 0.0001$). LIX expression was studied in BALF by Western blots (F) showing a significant increase in mice exposed to allergens ($p < 0.05$) without any obvious difference between MMP-8^{-/-} and MMP-8^{+/+} (G). IL-17 levels were measured in lung proteins extracts by ELISA, but no differences were found between the groups (H). Results are expressed as means \pm SEM.

apoptotic neutrophils was significantly increased after allergen exposure both in MMP-8^{+/+} and MMP-8^{-/-}. Interestingly, the extent of neutrophil apoptosis was significantly higher in MMP-8^{+/+} when compared with MMP-8-deficient mice (Fig. 8).

MMP measurements in the lung protein extracts and BALF

To determine whether MMP-8 deficiency could be associated with an up-regulation of other members of the MMP family, MMP ex-

pression, production, and activity were investigated by RT-PCR and zymographic analysis. In zymography, we confirmed that MMP-9 in pro- and activated forms was increased in the lung protein extracts after allergen exposure (Fig. 9B). MMP-2 was increased after allergen exposure, both at protein and mRNA levels, as assessed by zymographic (Fig. 9A) and RT-PCR analysis (data not shown). Intriguingly, MMP-12 expression measured by RT-PCR was increased after allergen in MMP-8^{-/-} and MMP-8^{+/+}

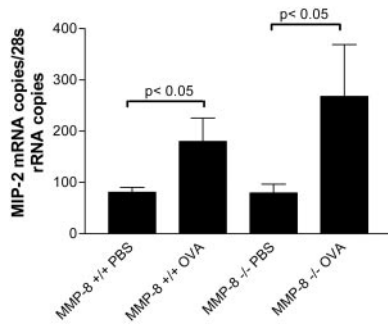


FIGURE 6. MIP-2 expression in lung tissue extracts 24 h after a 7-day allergen exposure. MIP-2 expression was analyzed by RT-PCR in lung tissue extracts, and its expression was increased after allergen in both groups without any differences between MMP-8^{-/-} and MMP-8^{+/+} mice. Results are expressed as means \pm SEM.

mice, but the extent of this increase was significantly higher in MMP-8^{-/-} than in MMP-8^{+/+} mice (Fig. 9C). MMP-8 mRNAs were rarely detectable in the samples, and levels of TIMP-1 did not display significant differences between the groups (Fig. 9D).

Discussion

Based on clinical data depicting increased levels of MMP-8 (neutrophil collagenase) in the sputum from asthmatics (24) and a potentially important role of neutrophils in asthma (31), we studied the MMP-8 expression after allergen exposure in mice and found MMP-8 to be significantly increased after allergens. Therefore, we decided to apply a mouse model of allergen-induced airway inflammation to recently generated MMP-8^{-/-} mice (25). In contrast to initial expectation, we demonstrate in this study that MMP-8 deletion in mice did not impair the development of allergen-induced airway inflammation. On the contrary, when compared with allergen-exposed MMP-8^{+/+} mice, MMP-8^{-/-} mice displayed: 1) an increased neutrophilic inflammation in the BALF and peribronchial area; 2) an enhanced eosinophilic infiltration in airway walls; and 3) increased levels of IgE and IgG1 in serum and of IL-4 in BALF. Allergen exposure was linked to increased levels of LIX, KC, and MIP-2 expression both in MMP-8^{-/-} and MMP-8^{+/+} mice. Using the TUNEL technique and caspase 3 immunohistochemistry, we demonstrated that neutrophil apoptosis was increased by allergens, but at a lower extent in MMP-8^{-/-} mice. Although MMP-2 and -9 activities and MMP-12 mRNA expression were increased after allergen exposure in both experimental groups, MMP-12 expression was higher in MMP-8^{-/-} than in MMP-8^{+/+} mice.

The lack of inhibition of the allergen-induced inflammation by MMP-8 deletion indicates that this protease is not essential for leukocytes and in particular neutrophil migration. MMP-8 is produced during maturation of neutrophils and is mainly located in the secondary granules that are secreted in response to a wide range of stimuli (32). Nevertheless, other cell types such as macrophages and structural cells (14, 33, 34) have also been reported to produce MMP-8, and this protease could therefore play other roles than degrading interstitial collagens, as suggested by the recent study of Balbin et al. (25), showing its capacity to process chemokines such as LIX.

The increased number of neutrophils in the BAL after allergen exposure is intriguing and of great interest, as it confirms in a different context the recent observation of an increased neutrophilic influx in chemically induced skin carcinomas (25) and the increased neutrophilic influx obtained in the lung tissue after acute LPS exposure in MMP-8^{-/-} mice (21). Our data suggest that in-

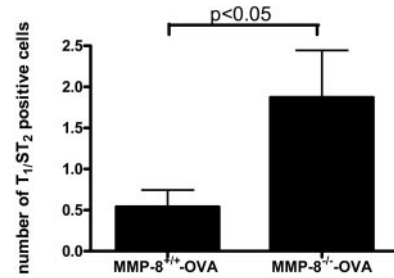
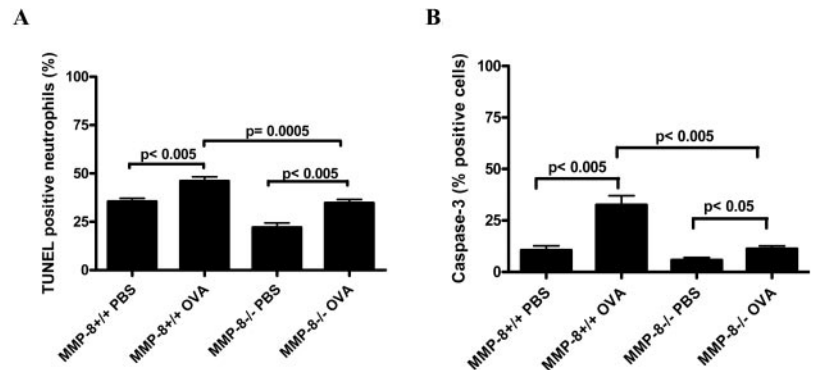


FIGURE 7. Counts of T₁/ST₂-positive cells in the peribronchial area from MMP-8^{+/+} ($n = 10$) and MMP-8^{-/-} ($n = 12$) mice exposed to OVA (six peribronchial areas/mouse). Results are expressed as mean \pm SEM. Measurements were performed 24 h after a 7-day allergen exposure.

creased neutrophil number in the lungs after allergen in MMP-8^{-/-} mice results at least partly from a reduced apoptosis and a delayed clearance of recruited neutrophils. Accordingly, the protective effect of MMP-8 toward inflammatory responses in a chemically induced cancer mice model could also be in part the consequence of a delayed apoptosis of neutrophils in MMP-8^{-/-} mice (25). The exact role of MMP-8 in the cascade of events leading to neutrophil apoptosis remains to be determined.

The complexity of the cytokine/chemokine receptor network puts difficulties in elucidating an eventual contribution of cytokine/chemokine modulation in the observed neutrophil accumulation. We report in this work for the first time an increased expression of LIX, KC, and MIP-2 in the BALF or lung parenchyma from allergen-exposed mice. Nevertheless, because their levels were similar in both genotypes, the enhancement of those chemoattractants for neutrophils cannot explain per se our finding of increased neutrophilic inflammation in the BALF of MMP-8^{-/-} mice after allergen exposure. We acknowledge that by measuring mediator levels by ELISA, we cannot exclude the hypothesis that one of these mediators would have been cleaved modulating its chemokine activity, as demonstrated recently (35). Such a mediator processing can only rarely be addressed by Ab-based methods and requires the use of other techniques such as mass spectrometry. Another parameter not addressed by the present study and of potential relevance in the regulation of the MMP-cytokine-chemokine network of reciprocal interactions is the regulation of chemokine receptors on neutrophils. Of interest is our finding that IL-4 levels are increased after allergen exposure in the BALF of MMP-8^{-/-} mice. IL-4 is a very potent inducer of many biological events and is one of the key cytokines in the development of Th2 inflammation. Interestingly, IL-4 has been reported to induce a delay of neutrophil apoptosis (36), suggesting that IL-4 by itself could play a role in the reduction of neutrophilic apoptosis observed in the lung and leading to neutrophil accumulation in the BALF. In addition, IL-4 is a multifunctional cytokine that can regulate the secretion of neutrophil-attracting chemokines such as growth-related oncogene- α and IL-8 (37), and control the expression of VCAM-1 on the surface of vascular endothelial cells (38), thereby modulating neutrophil recruitment and trafficking. In the present study, increased IL-4 levels are linked with anti-OVA-specific IgE levels that were strongly enhanced in the serum of MMP-8^{-/-}. Knowing that neutrophils bear on their plasma membrane a significant number of IgE receptors, these cells could therefore be activated directly by IgE (39). As IL-4 is a cytokine produced by lymphocytes, this suggests that the deletion of MMP-8 in mice could interfere with the function of those cells. In line with the increase of IL-4, specific anti-OVA IgE, and anti-OVA IgG1 levels in MMP-8^{-/-}, suggesting a Th2 profile in serum, we report in this work that the

FIGURE 8. Assessment of cell apoptosis 24 h after a 7-day allergen exposure. The percentage of neutrophils undergoing apoptosis was significantly increased after allergen exposure both in MMP-8^{+/+} and MMP-8^{-/-} ($p < 0.005$), as assessed by TUNEL method (A) and caspase 3 immunohistochemistry (B) on paraffin sections. The extent of neutrophil apoptosis was significantly higher in MMP-8^{+/+} when compared with MMP-8-deficient mice ($p = 0.0005$).



number of cells expressing the orphan receptor T₁/ST₂ is increased in lung parenchyma of MMP-8^{-/-} exposed to OVA, confirming that Th2 cell recruitment is increased in the lungs from MMP-8^{-/-} in the context of allergenic stimulation. Very interestingly, a recent study by McMillan et al. (40), although using an exposure protocol based on high concentrations of allergens different from ours, demonstrated such an increased Th2 cell recruitment in the BALF of mice knockout for the MMP-9 gene. Taken together, those data clearly suggest that there exists a mutual interplay between the occurrence of Th2 inflammation and MMPs, those latter playing a crucial role in its control.

The increased levels of pro- and activated MMP-9 found in the lung tissue from mice exposed to allergens when compared with sham-challenged mice confirm our previous observations in mice and humans (14, 15, 30, 41). Furthermore, these data suggest that the bronchial morphological changes observed in asthma could be at least in part explained by an increase of the net MMP load in the lung of individuals exposed to allergens. In line with MMP-9, we also describe in this work increased levels of MMP-2 to be present in the lung parenchyma of mice after allergen exposure. MMP-2 could therefore take part in the increased smooth muscle mass described in a model similar to ours (33), because this protease has been reported to be mitogenic for smooth muscle cells in cell cul-

ture (34). In accordance with our previous report (30), MMP-12 levels were increased after allergen in both MMP-8^{+/+} and MMP-8^{-/-}. However, interestingly, MMP-12 levels found in MMP-8^{-/-} were significantly higher than in MMP-8^{+/+}, suggesting an eventual compensatory mechanism of the MMP family following MMP-8 deletion.

In conclusion, our data demonstrate, in the context of allergic asthma, the anti-inflammatory effect of MMP-8 and suggest an implication of MMP-8 in the cascade of events leading to neutrophil apoptosis. As potential therapeutic issues have been proposed with MMP inhibitors, the present study describing an increased neutrophilic inflammation in MMP-8^{-/-} warns against the use of nonspecific MMP inhibition. In this context, other authors have reported that MMP-2^{-/-} mice display increased numbers of various inflammatory cells in the alveola due to a lack of egression of those cells (42). Taken together, these studies suggest that MMP inhibition should be selective and target specific MMPs, such as MMP-9 (30), for the therapy of asthma.

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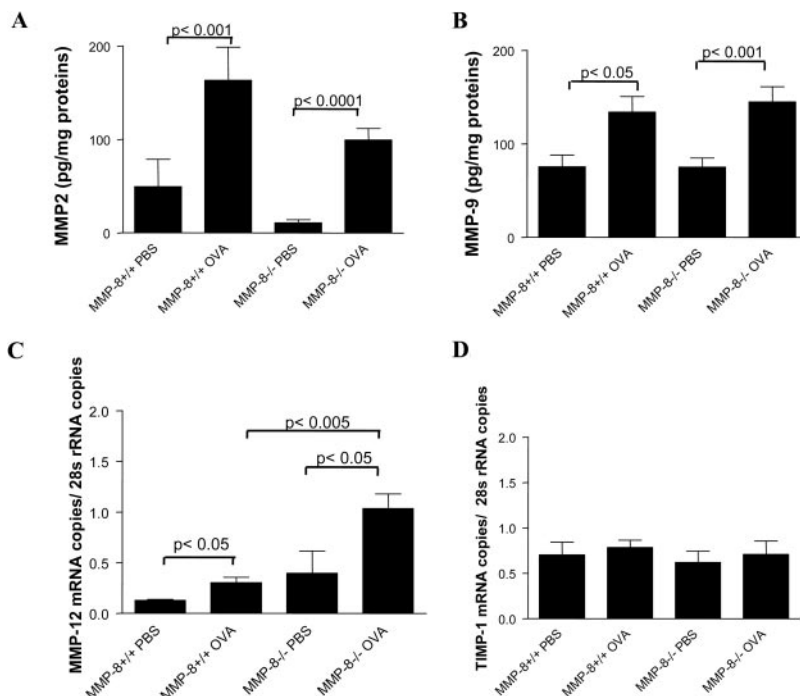


FIGURE 9. Analysis of MMP proteins and mRNA expression in lung extracts 24 h after a 7-day allergen exposure. Levels of MMP-2 and -9 were analyzed by zymography and quantified by densitometric scanning (A and B). MMP-12 and TIMP-1 mRNA levels were assessed by RT-PCR, as described in *Materials and Methods* (C and D, respectively). Results are expressed as means \pm SEM.

Disclosures

The authors have no financial conflict of interest.

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