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Control of Allergen-Induced Inflammation and Hyperresponsiveness by the Metalloproteinase ADAMTS-12

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A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) constitute a family of endopeptidases related to matrix metalloproteinases. These proteinases have been largely implicated in tissue remodeling associated with pathological processes. Among them, *ADAMTS12* was identified as an asthma-associated gene in a human genome screening program. However, its functional implication in asthma is not yet documented. The present study aims at investigating potential ADAMTS-12 functions in experimental models of allergic airways disease. Two different in vivo protocols of allergen-induced airways disease were applied to the recently generated *Adamts12*-deficient mice and corresponding wild-type mice. In this study, we provide evidence for a protective effect of ADAMTS-12 against bronchial inflammation and hyperresponsiveness. In the absence of *Adamts12*, challenge with different allergens (OVA and house dust mite) led to exacerbated eosinophilic inflammation in the bronchoalveolar lavage fluid and in lung tissue, along with airway dysfunction assessed by increased airway responsiveness following methacholine exposure. Furthermore, mast cell counts and ST2 receptor and IL-33 levels were higher in the lungs of allergen-challenged *Adamts12*-deficient mice. The present study provides, to our knowledge, the first experimental evidence for a contribution of ADAMTS-12 as a key mediator in airways disease, interfering with immunological processes leading to inflammation and airway hyperresponsiveness. ***The Journal of Immunology*****, 2012, 189: 000–000.**

Despite intense ongoing research, the worldwide prevalence of asthma is still rising (1). Asthma is characterized by a specific pattern of inflammation and airway hyperresponsiveness (AHR). A correlation between eosinophilic inflammation (2), bronchial hyperresponsiveness, and disease severity (3, 4) has been established. Asthma is also associated with structural changes in the airways that include epithelium desquamation, subepithelial fibrosis, mucous metaplasia, an increase in airway smooth muscle mass, and exaggerated deposition of extracellular matrix proteins (5).

Many mediators such as cytokines and chemokines have been implicated in a complex molecular network leading to asthma phenotype (6–8). Additionally, proteinases emerge as potential mediators in this airway disease associated with important tissue remodeling. Among them, several individual matrix metalloproteinases (MMPs) have been studied in asthma (9, 10) and have been reported to play either a protective or an inducer role in the generation of experimentally induced asthma in murine models (9–14). Three members of the related a disintegrin and metalloproteinases (ADAM) family, ADAM-8, ADAM-10 and ADAM-33, have also been linked to asthma (15–17). These membrane-associated enzymes bear multifunctional domains, including a catalytic domain, a disintegrin domain, and a cysteine-rich domain (18). Their expression is increased in asthmatic patients (19, 20) and in murine asthma models (21, 22). *ADAM33* is the first asthma susceptibility gene that has been recognized by positional cloning (16). More recently, a second asthma-associated gene, *ADAMTS12*, has been identified (23). It belongs to a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS) family, which shares a similar structure with ADAMs except for the presence of at least an additional thrombospondin (TSP)-1 in the C-terminal domain (24–26). ADAMTS-1, -2, and -12 are known to be expressed in human lung tissues (27). *ADAMTS12* expression in fetal lung tissues suggests that this enzyme might participate in essential tissue modeling processes during lung development. However, at the present time, no experimental data are available on ADAMTS-12 implication in asthma.

To address this issue, we applied two different experimental allergic airways disease models to the recently generated *Adamts12* knockout (KO) mouse strain (28). These murine experimental models, based on allergen challenge with either OVA (10) or house dust mite (HDM) (29), mimic the main features of human asthma,

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Abbreviations used in this article: ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; HDM, house dust mite; KO, knockout; MMP, matrix metalloproteinase; TSP, thrombospondin; WT, wild-type.

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including airway inflammation. In this study, we provide, to our knowledge, the first experimental evidence that *Adamts12* deficiency exacerbates allergen-induced inflammation and airway responsiveness by inducing a Th2 inflammation.

Materials and Methods

Experimental asthma protocol

Mice genetically deficient for *Adamts12* (KO; *Adamts12*$^{-/-}$) were generated as previously described (28). Homozygous mutant mice and the corresponding wild-type (WT) animals (*Adamts12*$^{+/+}$) were littermates deriving from interbreeding of heterozygotes with a mixed C57BL/6–129/Ola background. Mice were then been bred with C57BL/6 mice to generate *Adamts12* KO mice in a C57BL/6 genetic background (28). Briefly, to establish a mutant mouse strain deficient for the *Adamts12* gene, the targeting vector was designed to replace exons 6 and 7 (corresponding to the N-terminal part of the catalytic domain) by a neomycin-phosphoglycerate kinase cassette and to introduce a frame shift. Embryonic stem clones generated by homologous recombination were injected into C57BL/6J blastocysts to generate chimeric males. Heterozygous mice from the F_1 generation were intercrossed to generate *Adamts12*$^{-/-}$ mice that were obtained in the expected Mendelian ratio. For all assays, homozygous *Adamts12*$^{-/-}$ mice and their corresponding WT with a mixed background of 87.5% C57BL/6 and 12.5% 129/Ola were used. Mice were housed under specific pathogen–free conditions. Eight-week-old males were used for further experiments approved by the Animal Ethical Committee of the University of Liège (Belgium) in accordance with the institutional guidelines for animal care.

In the OVA-induced inflammation model, mice were immunized by i.p. injection of OVA (10 μg; Sigma-Aldrich, Schnelldorf, Germany) and aluminum hydroxide on days 0 and 7. From days 21 to 25, mice were exposed to inhalation of 1% OVA or PBS for 30 min. Mice challenged with PBS were used as controls (sham mice). AHR was measured on day 26 before sacrifice.

In the HDM-induced inflammation model, mice were lightly anesthetized with inhaled isoflurane and challenged intranasally with 100 μg HDM (Greer Laboratories, Lenoir, NC) or NaCl (0.9%). Mice challenged with NaCl were use as controls (sham mice). Mice were challenged with three doses of intranasal HDM or NaCl on days 0, 7, and 14. On day 17, mice were sacrificed after AHR measurement.

Determination of AHR

Mice were anesthetized by an i.p. injection of a mixture of ketamine (10 mg/ml; Merial, Brussels, Belgium) and xylazine (1 mg/ml; VMD, Arendonk, Belgium). After insertion of a 20-gauge polyethylene catheter into the trachea, mice were connected to a flexiVent small animal ventilator (Scireq, Montreal, PQ, Canada) at a frequency of 150 breaths per minute, with a tidal volume of 10 ml/kg. A positive end-expiratory pressure was set at 2 hPa. Measurements started 2 min after mechanical ventilation. A sinusoidal 1-Hz oscillation was applied to the tracheal tube and allowed a calculation of dynamic resistance, elastance, and compliance of the airway by multiple linear regressions. A second maneuver consisting in an 8-s forced oscillatory signal with frequencies between 0.5 and 19.6 Hz allowed impedance measurement to evaluate tissue damping, elastance, and hysteresivity (30). After baseline measurement, mice were challenged with a saline aerosol (PBS) followed by aerosols containing increasing doses of methacholine (3, 6, 9, and 12 g/l or 3, 6, 12, 24, and 48 g/l) (ICN Biomedicals, Asse Relegem, Belgium). Aerosols were generated by ultrasonic nebulizer (Scireq) and delivered to the inspiratory line of flexiVent using a bias flow of medical air following the manufacturer's instructions. Each aerosol was delivered for 2 min and periods of measurement as described above were assessed at a 1-min interval following each aerosol. Mean airway resistance after methacholine exposure was the main parameter measured during the challenge.

Bronchoalveolar lavage fluid and cell counting

After AHR assessment, a canula was inserted in mouse trachea to rinse the lungs with 4 aliquots of 1 ml of PBS-EDTA 0.05 mM (Calbiochem, Darmstadt, Germany). The bronchoalveolar lavage fluid (BALF) was subjected to centrifugation for 10 min at 4°C and supernatants were stored at −80°C for further assessments while cell pellets were resuspended in 1 ml PBS-EDTA 0.05 mM to proceed with total and differential cell counts. Total cell number was measured by using a Z2 Coulter particle count and size analyzer (Beckman Coulter/Analis, Namur, Belgium), and differential cell count was assessed by a skilled observer blinded to experimental details, based on morphological criteria. For this purpose, cells were centrifuged (cytospin) on a slide and stained with Diff-Quick (Dade, Brussels, Belgium). A total of 300 cells were counted.

Lung tissue sampling and histology

After BAL, the thorax was opened and the right lungs were excised and snap frozen in liquid nitrogen for protein extraction. The left lung was insufflated at constant pressure with 4% paraformaldehyde and embedded in paraffin for further histological analysis. A peribronchial inflammation score was applied on each H&E-stained slide as previously reported (12). A value from 0 to 2 was adjudged to each bronchus. A score of 0 corresponded to bronchi without inflammation, a score 1 corresponded to occasional mononuclear cells observed around bronchi, and a score of 2 to one to five layers of inflammatory cells around bronchi. Six bronchi per mouse were counted, and statistical analysis was performed by using a GraphPad program. Congo red staining was performed on lung sections to detect peribronchial eosinophilic infiltration. Peribronchial eosinophil counts were determined on six bronchi per mouse and reported to the basal membrane epithelium perimeter measured with the ImageJ program. Mast cells were detected on lung sections through toluidine blue staining. Peribronchial mast cell number was determined for six bronchi per mouse, and these counts were reported to the perimeter of basal membrane epithelium. For the IL-33 immunodetection, slides were deparaffinized and, after treatment with target retrieval buffer (Dako, Glostrup, Denmark), endogenous peroxidases were blocked with 3% H_2O_2 (Merck, Darmstadt, Germany). Slides were incubated with primary Ab for 1 h (goat anti–IL-33, 1:2000; R&D Systems, Minneapolis, MN), followed by rabbit anti-goat biotin-coupled secondary Ab and by incubation with a streptavidin/HRP complex (Dako). Peroxidase activity of these proteins was revealed using the 3,3′-diaminobenzidine hydrochloride kit (Dako).

Protein extraction and analysis

Lung tissues were crushed and total protein extracts were prepared by incubating crushed lung tissues in a 2 M urea solution. Tissue lysates were centrifuged for 15 min at 16,100 × *g*. ELISA for CCL11, IFN-γ, IL-4, IL-5, IL-10, IL-13, IL-33, ST2, RANTES, and MIP-1γ in lung protein extracts and/or in supernatants from BALF were assessed using Abs from R&D Systems (Minneapolis, MN) and the R&D Systems DuoSet ELISA development kit.

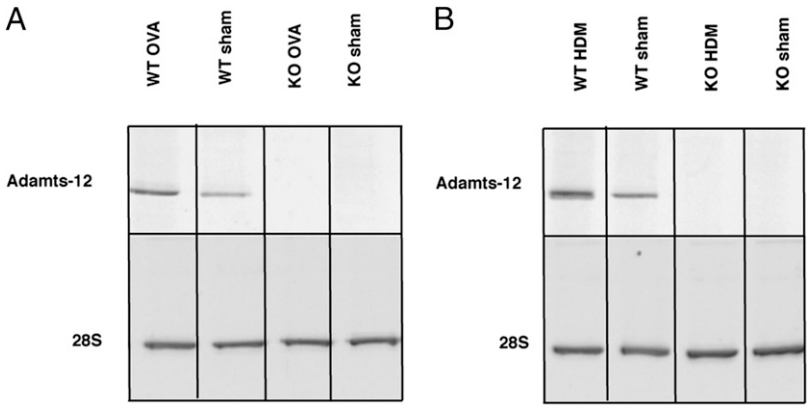

FIGURE 1. Expression of Adamts-12 in lungs of mice. A representative example of Adamts-12 mRNA expression in whole lung homogenates in (**A**) OVA- or (**B**) HDM-exposed acute inflammation models (*n* = 4/group).

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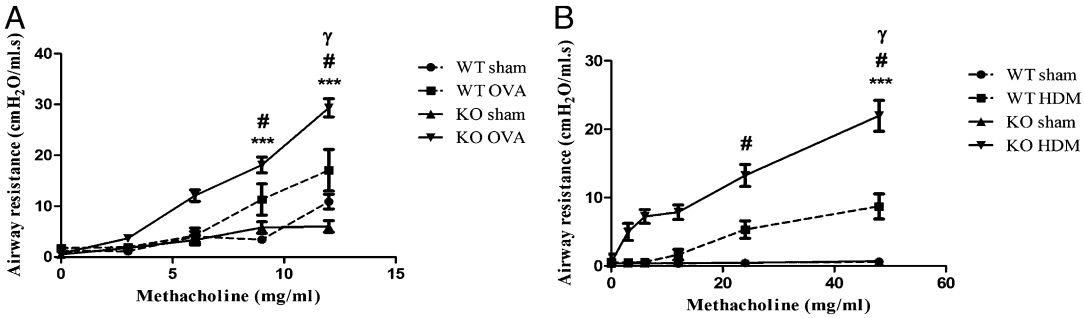

FIGURE 2. Effect of *Adamts12* deficiency on AHR. Airway reactivity was measured at day 26 in mice exposed to OVA (**A**) and at day 17 in mice exposed to HDM (**B**) (n = 4/group/experiment). The results presented are those of one experiment out of two. Similar results were obtained in two independent experiments. $^{***}p < 0.001$, WT sham versus WT allergen; $^{\#}p < 0.01$, KO sham versus KO allergen; $^{\gamma}p < 0.001$, WT allergen versus KO allergen, ANOVA test.

Determining total IgE, allergen-specific IgE, and allergen-specific IgG

Before performing BAL, blood was recovered for measurement of different Igs. Total IgE serum levels were measured by means of ELISA (Bethyl Laboratories, Montgomery, TX), according to the manufacturer's protocol. For the measure of allergen-specific IgE, IgG1, and IgG2a, we used a previously reported protocol (31). Briefly, 96-well plates were coated overnight at 4°C with allergen (HDM or OVA) or unlabeled anti-IgE, IgG1, and IgG2a (SouthernBiotech, Birmingham, AL) of corresponding isotype for standard wells. Nonspecific binding sites were blocked with BSA (Sigma-Aldrich) diluted in PBS before addition of serum or standards. Wells were then incubated with a HRP-conjugated goat anti-mouse IgE, IgG1, or IgG2a (SouthernBiotech). Plates were developed using tetramethylbenzidine substrate, and stopped. OD was determined at 450 nm.

RNA extraction and analysis

Lung tissues from *Adamts12*$^{-/-}$ and WT mice were disrupted and completely homogenized, forming a powder by a combination of turbulence and mechanical shearing. Total RNAs were extracted using a High Pure RNA isolation kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Adamts-12 mRNA expression levels were assessed by semiquantitative RT-PCR using the GeneAmp thermostable RNA RT-PCR kit (Applied Biosystems, Foster City, CA). The design of oligonucleotides for Adamts-12 was based on the sequence available in GenBank: 5′-GGA-GTT-GGG-TCG-TAC-TGA-ATC-T-3′ (antisense) and 5′-GTG-GCT-GTC-CTT-ATC-ACC-AGA-A-3′ (sense). The specificity of the selected sequences was verified using the National Center for Biotechnology Information BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/), and oligonucleotides were obtained from Eurogentec (Seraing, Belgium). Reverse transcription was performed on 4 ng total RNA at 70°C during 15 min. PCR amplification conditions were optimized so that PCR products did not reach any saturation levels. Amplification started at 94°C for 15 s, 60.5°C for 20 s, and 72° for 10 s for 30 cycles, followed by 2 min at 72°C. Products were then resolved on polyacrylamide gels (10%) and stained with GelStar (BioWhittaker, Rockland, MD). Analysis of the intensity of band was realized using Quantity One software (Bio-Rad, Hercules, CA). 28S rRNA was used as an internal control.

Statistical analysis

Results were expressed as means ± SEM. Statistical test was assessed on experimental groups using ANOVA or Mann–Whitney U test. These tests were performed using GraphPad InStat software (http://www.graphpad.com/instat). The p values are noted as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Results

Adamts-12 expression is induced in lungs of mice exposed to allergens

To investigate the extent of Adamts-12 expression during the development of an asthmatic phenotype, WT mice and *Adamts12*$^{-/-}$ mice were subjected to two different protocols of allergen challenges: 1) OVA sensitization by 2 i.p. injections followed by allergen challenge with aerosolized OVA (1%), and 2) intranasal instillation of HDM once a week for 3 wk. Lungs of mice exposed to allergens displayed higher levels of mRNA encoding Adamts-12 when compared with corresponding control mice (Fig. 1). As expected, no expression of Adamts-12 mRNA was detected in *Adamts12* KO mice.

Adamts12 *deficiency is associated with increased allergen-induced AHR*

To determine the potential role of Adamts-12 in airway dysfunction, AHR was assessed by measuring the extent of methacholine-induced bronchoconstriction by using the flexiVent system in mice, 24 h after

Table I. Cellular composition of BALF ($\times 10^3$/ml)

	WT Sham	WT OVA	KO Sham	KO OVA
Total cells	27.022 ± 5.406	26.376 ± 4.094	18.328 ± 4.683	37.756 ± 5.582**
Epithelial cells	2.705 ± 0.522	0.652 ± 0.274**	1.659 ± 0.340	0.781 ± 0.172*
Eosinophils	1.355 ± 0.760	11.765 ± 3.341**	0.484 ± 0.180	22.726 ± 4.667***,****
Neutrophils	0.023 ± 0.016	0.150 ± 0.058	0.010 ± 0.007	0.132 ± 0.051
Lymphocytes	0.028 ± 0.018	0.035 ± 0.027	0.001 ± 0.001	0.112 ± 0.054
Macrophages	26.205 ± 3.948	13.728 ± 1.991**	16.137 ± 4.238	13.946 ± 2.065
	WT Sham	**WT HDM**	**KO Sham**	**KO HDM**
Total cells	12.125 ± 0.394	26.195 ± 4.389**	22.825 ± 0.679	62.773 ± 6.544**
Epithelial cells	0.863 ± 0.270	0.481 ± 0.124	1.658 ± 0.188	0.176 ± 0.058
Eosinophils	0.025 ± 0.016	9.260 ± 4.284***	0.686 ± 0.159	43.613 ± 8.882***,****
Neutrophils	0.012 ± 0.008	0.080 ± 0.024	0.004 ± 0.017	0.001 ± 0.001
Lymphocytes	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.040 ± 0.026
Macrophages	11.586 ± 0.505	16.352 ± 1.255*	20.406 ± 0.726	18.866 ± 3.149

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus corresponding sham-exposed mice; $^{****}p < 0.001$ versus allergen-exposed WT mice.

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the final OVA challenge (Fig. 2A) or 3 d after the last dose of intranasal HDM (Fig. 2B). Similar responses to methacholine were observed in sham WT mice and sham mutant mice, confirming that *Adamts12*$^{-/-}$ mice do not display intrinsic functional respiratory tract abnormalities. Mice of both genotypes challenged with allergens showed increased AHR following methacholine exposure. However, in both experimental models, allergen-challenged *Adamts12*$^{-/-}$ mice displayed enhanced bronchial responsiveness to methacholine as compared with WT mice exposed to the allergen ($p < 0.001$) (Fig. 2).

Adamts12 *deficiency is associated with increased eosinophilic inflammation in allergen-challenged mice*

In both experimental models (OVA- and HDM-induced allergic airways disease), inflammatory cell recruitment into the airway walls was analyzed by studying BALF and through histological lung examination. Differential BALF cell counts revealed that after allergen exposure, eosinophil counts were drastically increased in both *Adamts12*$^{+/+}$ and *Adamts12*$^{-/-}$ mice. Moreover, after allergen challenge, higher eosinophil counts were observed in *Adamts12*$^{-/-}$ mice as compared with corresponding WT littermates (Table I).

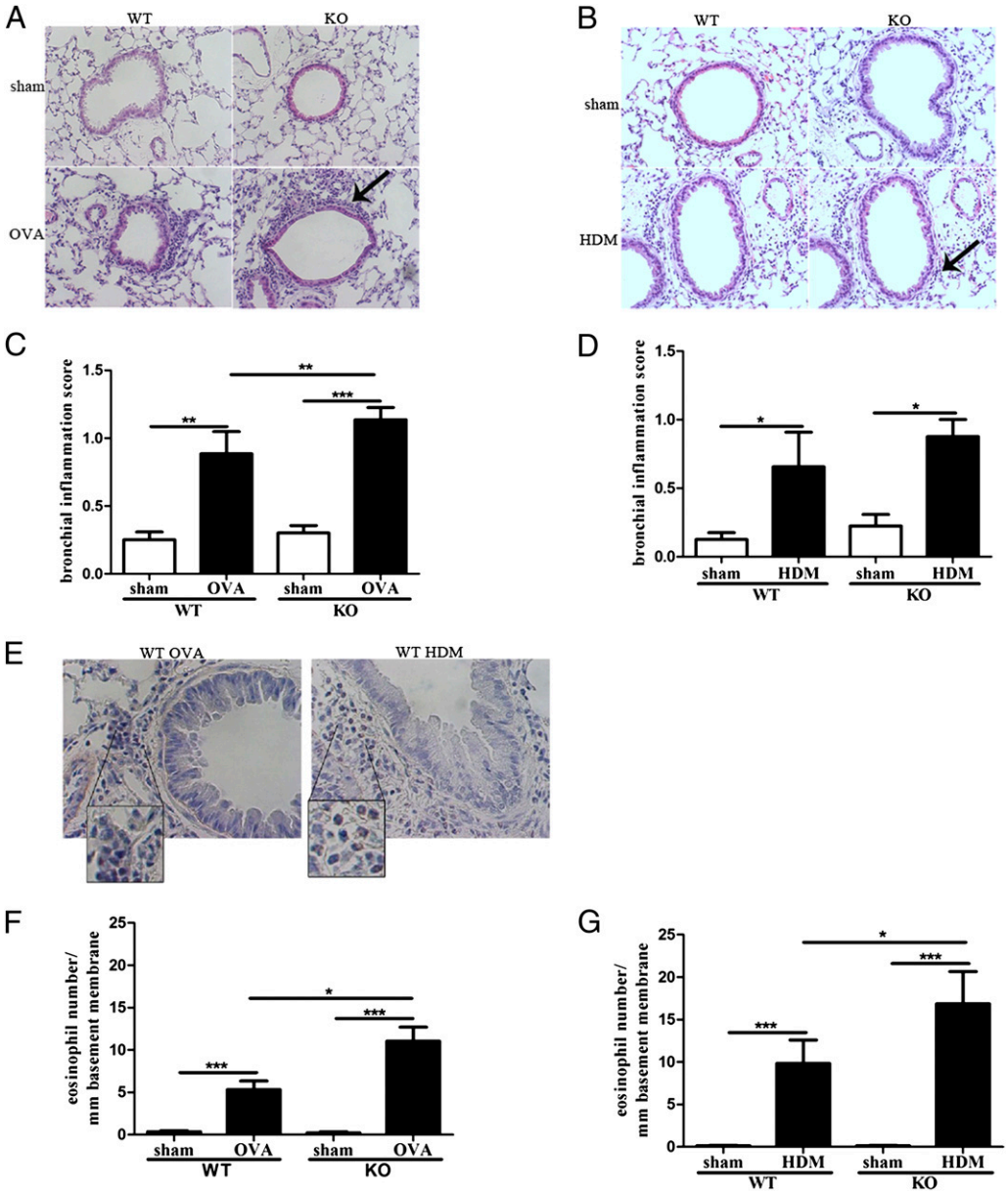

FIGURE 3. Histological analysis of lung tissue after exposure. (**A** and **B**) Lung sections stained with H&E. Original magnification ×200. No inflammation was detected in peribronchial tissue of sham mice. After allergen exposure, WT mice and *Adamts12*$^{-/-}$ mice displayed a bronchial inflammation. Each image is representative of at least 10 animals. (**C**) Scoring of peribronchial inflammation after H&E staining in OVA-exposed inflammation model. $^{**}p < 0.01$, $^{***}p < 0.001$, ANOVA test (n = 5–8/group). Similar results were obtained in two independent experiments. (**D**) Scoring of peribronchial inflammation after H&E staining in HDM-exposed inflammation model. $^{*}p < 0.05$, ANOVA test (n = 5–8/group). Similar results were obtained in two independent experiments. (**E**) Eosinophil infiltration evidenced by Congo red staining. Original magnification ×400. Each image is representative of at least 10 animals. (**F**) Quantitative analysis of eosinophils in bronchial walls in OVA-exposed inflammation model. Six bronchi per mouse were quantified and results were expressed as number of eosinophils per millimeter of epithelial basement membrane. $^{*}p < 0.05$, $^{***}p < 0.001$, ANOVA test (n = 5–8/group). Similar results were obtained in two independent experiments. (**G**) Quantitative analysis of eosinophils in bronchial walls in HDM-exposed inflammation model. Six bronchi per mouse were quantified and results were expressed as number of eosinophils per millimeter of epithelial basement membrane. $^{*}p < 0.05$, $^{***}p < 0.001$, ANOVA test (n = 5–8/group). Similar results were obtained in two independent experiments.

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Allergen-induced airway inflammation was determined by applying a score to peribronchial inflammatory cell infiltrates assessed by histological examination of lung tissue sections. As expected, mice displayed a characteristic huge peribronchial inflammation after exposure to OVA (Fig. 3A), as well as HDM (Fig. 3B). Interestingly, for both allergens, the induced peribronchial inflammation was increased in *Adamts12*$^{-/-}$ mice as compared with *Adamts12*$^{+/+}$ mice (Fig. 3C, 3D). However, the difference between *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice did not reach statistical significance after HDM exposure.

Eosinophil infiltration was evidenced by Congo red staining and quantified on tissue sections (Fig. 3E). After allergen exposure, eosinophil counts in airway walls were higher in mice exposed to OVA (Fig. 3F) or HDM (Fig. 3G) as compared with corresponding control mice. Moreover, the allergen-induced eosinophilic infiltration was increased more in *Adamts12*$^{-/-}$ lungs than in *Adamts12*$^{+/+}$ ones (Fig. 3F, 3G).

Adamts12 *deficiency is associated with increased allergen-specific IgE and IgG1 levels in allergen-challenged mice*

Total IgE levels in serum were increased after allergen exposure in *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice in the OVA-induced model (319.81 ± 39.67 in sham-exposed *Adamts12*$^{+/+}$ mice versus 488.20 ± 55.85 in OVA-exposed *Adamts12*$^{+/+}$ mice, $p < 0.05$; 258.77 ± 24.64 in sham-exposed *Adamts12*$^{-/-}$ mice versus 458.09 ± 78.66 in OVA-exposed *Adamts12*$^{-/-}$ mice, $p < 0.01$) and HDM-induced model (588.80 ± 32.51 in sham-exposed *Adamts12*$^{+/+}$ mice versus 1016.53 ± 271.77 in HDM-exposed *Adamts12*$^{+/+}$ mice; 579.94 ± 78.32 in sham-exposed *Adamts12*$^{-/-}$ mice versus 973.00 ± 116.49 in HDM-exposed *Adamts12*$^{-/-}$ mice, $p < 0.05$). Note that for both allergens, no significant difference was seen between *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice (data not shown). Moreover, we also assessed Ag-specific IgE and Ag-specific IgG1 as a marker of Th2-skewed inflammation and IgG2a as a marker of Th1-skewed inflammation. As expected, allergen exposure induced an increase in specific anti-OVA or anti-HDM IgE levels in the sera of mice from both genotypes (Fig. 4A, 4B). Allergen-specific IgG1 levels, a marker of a Th2-prone milieu, were increased in allergen-exposed animals versus sham-exposed mice in both experimental models (Fig. 4C, 4D). After allergen exposure, levels of IgG1 were significantly increased for both allergens in the sera of *Adamts12*$^{-/-}$ mice as compared with corresponding *Adamts12*$^{+/+}$ mice (Fig. 4C, 4D). In contrast, levels of IgG2a, a Th1-associated Ig, were not different among all groups (data not shown).

Adamts12 *deficiency is associated with higher mast cell recruitment and increased ST2/IL-33 pathway activation*

Mast cell numbers measured by toluidine blue staining were increased in allergen-exposed *Adamts12*$^{-/-}$ mice as compared with *Adamts12*$^{+/+}$ mice (Fig. 5). Because the ST2 receptor is expressed by mast cells, we assessed ST2 levels by ELISA in lung protein extracts of *Adamts12*$^{-/-}$ mice and WT mice. These levels were significantly higher in *Adamts12*$^{-/-}$ mice as compared with *Adamts12*$^{+/+}$ mice (Fig. 6A, 6B). Moreover, upon allergen exposure, the levels of IL-33, the ligand for ST2, were found higher in *Adamts12*$^{-/-}$ lungs as compared with *Adamts12*$^{+/+}$ ones (Fig. 6C, 6D). Immunohistochemical stainings revealed that IL-33 was mainly produced by epithelial and inflammatory cells (Fig. 6E).

Cytokine measurement in BALF and lung protein extracts in allergen-induced inflammation

We hypothesized that perturbation of the cytokine/chemokine network could account for the eosinophilia observed in mutant mice. Based on a prescreening performed through a cytokine array, several factors involved in inflammatory responses have been measured by ELISA. RANTES and MIP-1γ levels were increased upon OVA (Fig. 7A, 7C) or HDM (Fig. 7B, 7D) exposure in both genotypes, and these levels were significantly higher in *Adamts12*$^{-/-}$ mice as compared with WT mice. Additionally, IL-4 and IL-13 (protypical Th2 cytokines) levels were increased upon OVA (Fig. 7E, 7G) or HDM (Fig. 7F, 7H) exposure in both genotypes, and these levels were significantly higher in *Adamts12*$^{-/-}$ mice as compared with WT mice upon HDM exposure. Moreover, allergen exposure induced a similar IL-5 level enhancement in the lungs of *Adamts12*$^{-/-}$ mice and their WT counterpart (Fig. 7I, 7J). Levels of the chemokine CCL11 were enhanced upon allergen exposure in both genotypes (Fig. 7K, 7L). Note that levels of Th1 cytokines (IL-10 and IFN-γ) did not exhibit significant changes upon *Adamts12* depletion (Fig. 7M–P).

Discussion

The identification of *ADAMTS12* as an asthma susceptibility gene by positional cloning (23) prompted us to explore its putative

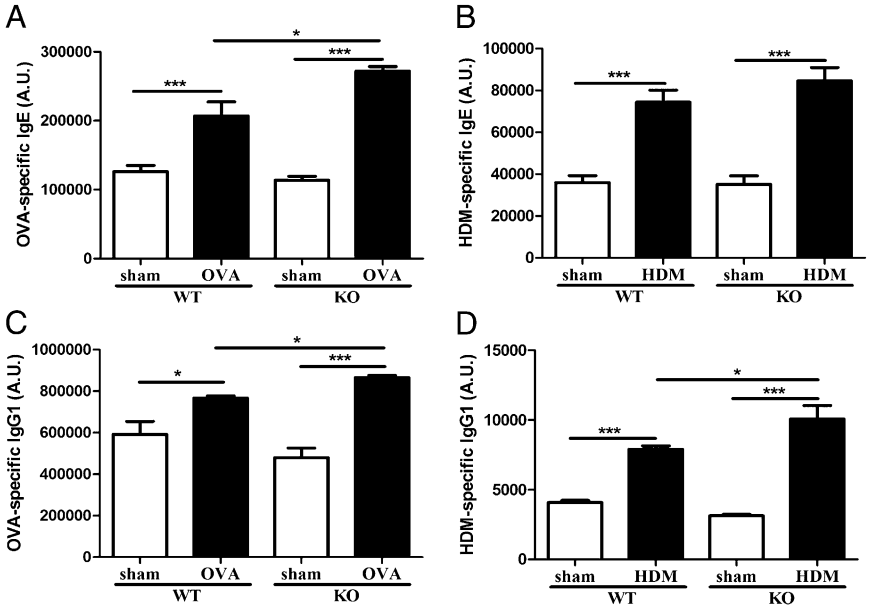

FIGURE 4. Measurement of allergen-specific IgE and IgG1 levels. IgE levels measured by ELISA in sera in (**A**) OVA- and (**B**) HDM-exposed mice (n = 5–8/group). Similar results were obtained in two independent experiments. *$p < 0.05$, ***$p < 0.001$, ANOVA test. IgG1 levels were measured by ELISA in sera in (**C**) OVA- and (**D**) HDM-exposed mice (n = 5–8/group). Similar results were obtained in two independent experiments. *$p < 0.05$, ***$p < 0.001$, ANOVA test. A.U., absorbance unit.

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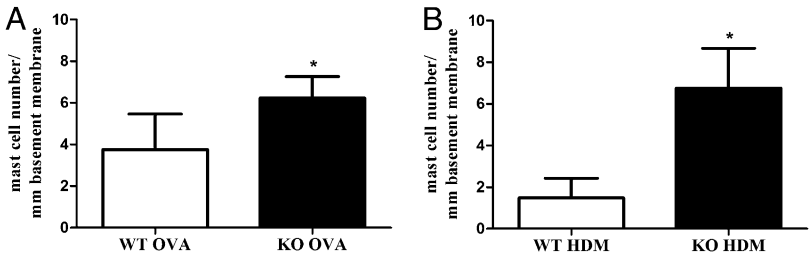
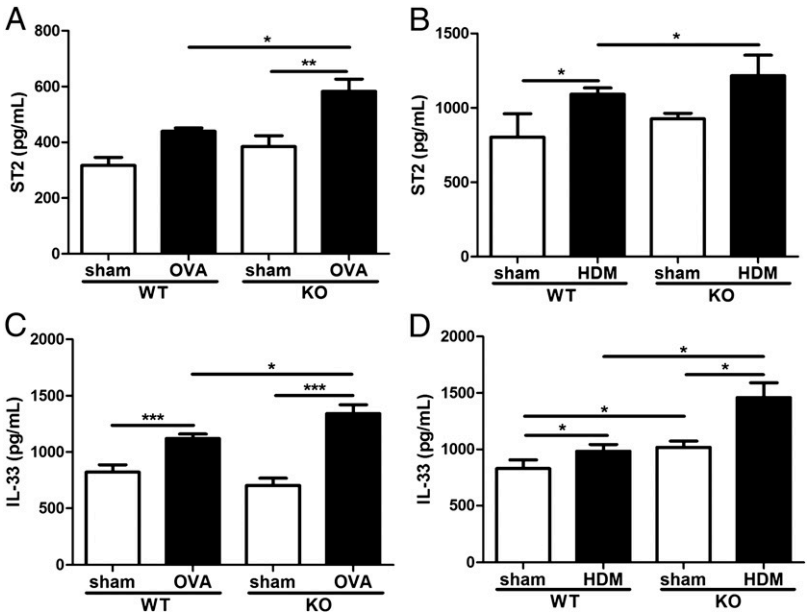


FIGURE 5. Mast cells recruitment in (**A**) OVA- or (**B**) HDM-exposed acute inflammation models. $*p < 0.05$, Mann–Whitney U test (n = 5–8/group). Similar results were obtained in two independent experiments.

functions in two experimental models of allergen-induced airways disease. To our knowledge, the present study provides the first experimental evidence through a genetic approach that ADAMTS-12 is involved in asthma. Our data clearly indicate that Adamts-12 plays protective roles acting against the development of asthma phenotype upon experimental allergen exposure. This is supported by the exacerbated inflammation and eosinophilia in BALF and pulmonary tissue observed in *Adamts12*-deficient mice. Increased mast cell recruitment observed in lung parenchyma of mutant mice is in line with the anti-inflammatory effect of Adamts-12. The exacerbated inflammation observed in *Adamts12*-deficient animals is associated with measurable airway dysfunction as assessed by methacholine challenge. The deleterious effects of *Adamts12* deficiency on the development of the asthmatic phenotype were similarly observed in two different allergic airways disease models induced by OVA or HDM exposure.

Investigations performed on experimental models of allergic airways disease have been often hampered by differences in the AHR and the distribution of proinflammatory cytokines and inflammatory cells depending on the allergen used, the induction protocol, as well as on the genetic background of mice (10, 14). In this context, OVA-challenged *Mmp9*-deficient mice displayed lower eosinophilia and hyperresponsiveness (9), whereas other authors using a different experimental protocol (far higher doses of allergen) reported increased eosinophilia in *Mmp9* KO mice (13). The importance of the experimental protocol used to assess a phenotype in mice is further highlighted by various reports stating that there are important differences when considering various strains of mice (32, 33). Notably, the present study provides evidence for similar exacerbated eosinophilia and increased AHR in *Adamts12*$^{-/-}$ mice of similar background, sensitized and challenged with two different allergens, the "classical" OVA and the very significant and widespread allergen for human asthmatics, HDM.

The exacerbated inflammation observed in *Adamts12*$^{-/-}$ mice is in line with the increased or reduced inflammation reported in



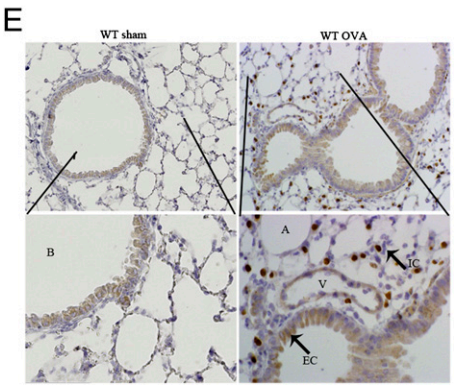


FIGURE 6. Measurement of IL-33 and ST2. IL-33 levels measured by ELISA in lung protein extracts in (**A**) OVA- and (**B**) HDM-exposed mice (n = 5–8/group). Similar results were obtained in two independent experiments. $*p < 0.05$, $**p < 0.01$, ANOVA test. ST2 levels were measured by ELISA in lung protein extracts in (**C**) OVA- and (**D**) HDM-exposed mice (n = 5–8/group). Similar results were obtained in two independent experiments. $*p < 0.05$, $***p < 0.001$, ANOVA test. (**E**) Production of IL-33 in lungs of mice in allergic airways disease model. Immunohistochemistry staining with an anti–IL-33 Ab in sham-exposed mice (*left panel*) and in allergen-exposed mice (*right panel*). Slides are counterstained with hematoxylin. *Upper panel*, original magnification ×200; *lower panel*, original magnification ×400. The brown staining shows epithelial cells (EC) and inflammatory cells (IC) in lung parenchyma. The complete procedure is described in the *Materials and Methods* in the paragraph headed *Lung tissue sampling and histology.* A, Alveolar space; B, lumen of bronchi; V, vascular space.

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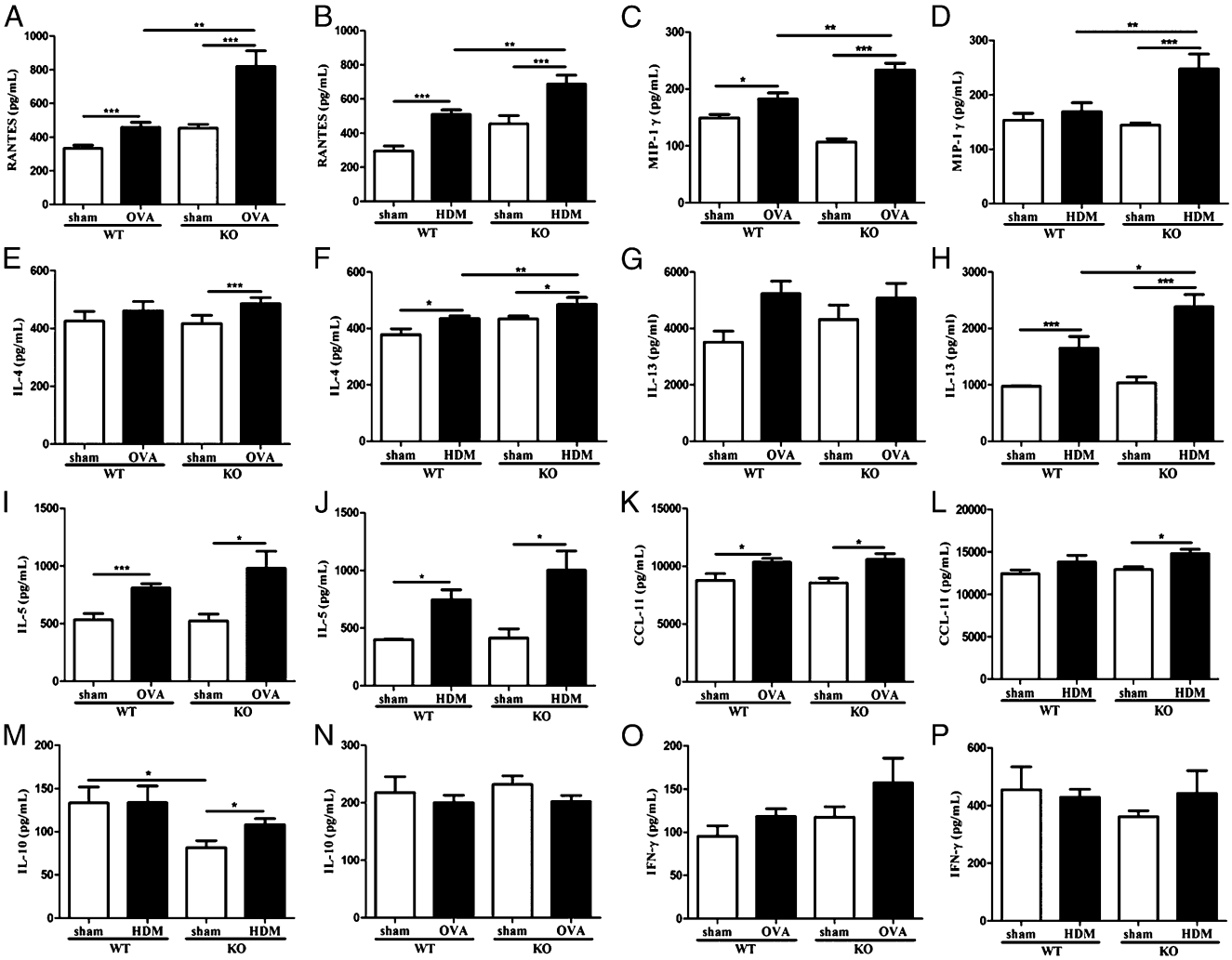

FIGURE 7. Measurement of cytokine/chemokine levels by ELISA in both inflammation models. Protein levels of (**A**, **B**) RANTES (CCL5), (**C**, **D**) MIP-1γ, (**E**, **F**) IL-4, and (**G**, **H**) IL-13 were measured in lung protein extracts and the levels of (**I**, **J**) IL-5 were measured in BALF in mice exposed to OVA or HDM. (**K** and **L**) Protein levels of CCL11 were measured in lung protein extracts in mice exposed to OVA or HDM, respectively. Protein levels of Th1 cytokines (**M**, **N**) IL-10 and (**O**, **P**) IFN-γ were measured in the two experimental models (OVA and HDM) (n = 5–8/group). Similar results were obtained in two independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ANOVA test.

several *Mmp*-deficient mice (9, 10, 12, 13, 34). Abnormal lymphocyte and neutrophil recruitments in the lungs have been shown for *Mmp*9- and *Mmp8*-deficient mice (9, 12). In terms of eosinophilia, we previously reported a dramatic eosinophilic inflammation in *Mmp19* mice sensitized and challenged with OVA (10). Thus, the present study extends the involvement of MMP-related enzymes to eosinophilia, which is an important feature of the asthma pathology. The observed phenotype in *Adamts12*$^{-/-}$ mice could not be related to a profoundly modified sensitization process as assessed by similarly increased IgE levels measured upon allergen exposure in both genotypes. Nevertheless, *Adamts12*$^{-/-}$ mice display intrinsically a Th2-skewed inflammatory process as strongly suggested by the increased levels of serum IgG1 in *Adamts12*-deficient animals. Interestingly, in both models (OVA and HDM), the dramatic eosinophilia observed in sensitized mutant mice was associated with increased levels of RANTES (CCL5), a major eosinophil chemoattractant, and of IL-5, which regulates eosinophil differentiation and survival (35). MIP-1γ levels were also found higher in mutant mice challenged with OVA and HDM. We can therefore hypothesize that these chemokines might contribute in a coordinated manner to eosinophil trafficking into the airways in allergen-induced inflammation models (36, 37). Additionally, the increased IL-33 levels detected in mutant mice could further contribute to exacerbated eosinophilia (38, 39), as well as to increased mast cell recruitment through ST2 receptor activation (40, 41). The present study unveils a strong modulation of potent pathways involving IL-33, RANTES, and MIP-1γ in *Adamts12*-deficient mice that could participate in the inflammatory cascade leading to the asthma phenotype (42). Our data clearly demonstrate that *Adamts12* deficiency interferes with the complex interactive network of cytokines/chemokines, leading to an imbalance in regulators of inflammation and especially of eosinophil and mast cell recruitment/survival.

Mechanisms that can explain the effects of deletion of a single MMP or ADAM/ADAMTS on the asthma phenotype are probably diverse. For instance, *Mmp8*$^{-/-}$ mice display higher neutrophilic inflammation, being the consequence of a lack of LIX processing that delays apoptosis of these cells (12, 43–45). *Adam8*$^{-/-}$ mice display less eosinophilic inflammation (22) and impaired dendritic cell recruitment (15, 22). *Adamts12* deletion leads to hyperresponsiveness associated with enhanced eosinophil and mast cell infiltration. However, the exact molecular mechanisms operating in the protective effects of ADAMTS-12 toward allergen-induced

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inflammation are not known. We have also stained the bronchial smooth muscles by immunohistochemistry in OVA-exposed *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$mice and we found increased smooth muscle mass in *Adamts12*-deficient animals, suggesting that this protease could be of importance also for smooth muscle homeostasis (data not shown). ADAMTS-12 is a complex molecule displaying multiple functions, with each related to specific domains of the protein. Identifying the precise molecular mechanisms that intervene to circumvent allergen-induced inflammation will imply deciphering the specific functions of different ADAMTS-12 domains, as well as identifying major peptidic mediators that drive the recruitment and/or survival of inflammatory cells on the inflammatory site. Further complex proteomic studies are needed to address these issues (46, 47). The proteolytic functions of ADAMTS-12 remain enigmatic. This enzyme has been involved in arthritis initiation and progression through the degradation of cartilage oligomeric matrix protein, a prominent noncollagenous matrix component of cartilage (48). On the contrary, the antiangiogenic properties of ADAMTS-12 evidenced in cancer experimental models do not rely on the catalytic domain (28), but more likely on its TSP-1 domains. The hepatocyte growth factor-induced scattering effect of ADAMTS-12 on tumor cells appeared to be also TSP domain-dependent (49). These TSP domains characterizing ADAMTS family members have the ability to sequester growth factors such as vascular endothelial growth factor (50). Additionally, they could exert a proapoptotic effect (51), illustrating the complexity of potential interactions with the different domains of ADAMTS-12. Accumulating evidence has shed light on an important functional link existing between the complex chemokine/cytokine network and MMP-related enzymes (43, 52, 53). To decipher the precise mechanism allowing ADAMTS-12 to prevent inflammation, animals with specific depletion of the catalytic site would be useful. Provided that the catalytic activity is implicated in the protective effect against allergen-induced inflammation, one can hypothesize that ADAMTS-12 might control inflammatory mediator activity through the proteolytic elimination of a few terminal amino acid residues as previously described for other proteases (54). Collectively, these data highlight the multiple and complex functions of ADAMTS-12 involving its catalytic domain among other molecular determinants. To our knowledge, the present study provides the first demonstration of a functional role of *ADAMTS12* in asthma. It supports its identification as a gene of asthma susceptibility in human (23). The first ADAM/ADAMTS family member identified as an asthma susceptibility gene was *ADAM33* (16). This association between *ADAM33* and asthma-associated hyperresponsiveness was reported in several studies (55–59), but not in others (59, 60). In humans, the bronchial expression of ADAM-33 has been correlated with disease severity (19). However, surprisingly, the *Adam33* gene deficiency in mice did not affect OVA-induced airway responsiveness and related inflammation (61). This could be related to an influence of genetic background or to some discrepancies between molecular mechanisms in humans and mice. In sharp contrast, the increased inflammation observed in the absence of *Adamts12* in mice demonstrates the importance of *Adamts12* as a key asthma susceptibility gene in vivo. From our results, ADAMTS-12 appears as an anti-target if therapeutic strategies with proteinase inhibitors are developed. The recently generated *Adamts12*$^{-/-}$ mice thus represent a valuable tool for further studies for unraveling the mechanisms of ADAMTS-12 action in asthma. In the near future, the study of ADAMTS-12 activity will be assessed by using interactomics to identify potential interactions with asthma-relevant mediators.

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Disclosures

The authors have no financial conflicts of interest.

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