AJRCMB Articles in Press. Published on October 20, 2009 as doi:10.1165/rcmb.2008-0426OC

MMP-19 deficiency promotes tenascin-C accumulation and allergen-induced airway inflammation.

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Running title: MMP-19 in allergen-induced asthma

This work was supported by grants of the Fonds National de la Recherche Scientifique (FNRS, Brussels, Belgium), the Walloon Region Government (DGTRE project #114/702), the Fondation Leon Fredericq (University of Liege), the CHU (Liege, Belgium), Action de Recherches Concertées, IAP6/35 network (funded by the Interuniversity Attraction Poles Programme, initiated by the Belgian State, Science Policy Office), European Union (FP6,-Cancerdegradome and FP7-Microenvimet), the Centre Anticancéreux près de l'Université de Liège, the ARCir Theradam project supported by the Conseil Regional du Nord-Pas de Calais and CICYT-Spain. DC is a scientific research worker of the FNRS and CG is a Research Associate from the FNRS (Belgium).

Abstract

Matrix metalloproteinases (MMPs) recently appeared as key regulators of inflammation, allowing recruitment and clearance of inflammatory cells and modifying the biological activity of many peptidic mediators by cleavage. MMP-19 is a newly described MMP and preferentially cleaves matrix proteins such as collagens and tenascin-C. The role of MMP-19 in asthma has not been described to date. The purpose of the present study was to assess MMP-19 expression in a murine asthma model and to address biological effects of MMP-19 deficiency in mice. Allergen-exposed wild-type (WT) mice displayed an increased expression of MMP-19 mRNA and an increased number of MMP-19-positive cells in the lungs detected by immunohistochemistry. After allergen challenge of MMP-19 knockout (MMP-$19^{-/-}$) mice, an exacerbated eosinophilic inflammation was detected in bronchoalveolar lavage fluid and bronchial tissue along with an increased airway responsiveness to methacholine. A shift towards increased Th_2-driven inflammation in MMP-$19^{-/-}$ mice was demonstrated by 1) increased numbers of cells expressing the IL-33 receptor T_1/ST_2 in lung parenchyma, 2) increased IgG_1 levels in serum and 3) higher levels of IL-13 and CCL11 in lung extracts. Tenascin-C was found accumulated in peribronchial areas of MMP-$19^{-/-}$ after allergen challenges as assessed by Western blot and immunohistochemistry analysis. We conclude that MMP-19 is a new mediator in asthma, preventing tenascin-C accumulation and directly or indirectly controlling Th_2-driven airway eosinophilia and airway hyperreactivity. Our data suggest that MMP-19 might act on Th_2 inflammation homeostasis through preventing tenascin protein accumulation.

Keywords: eosinophils, inflammation, lung, MMP-19, knockout mice.

Abbreviations: MMPs (Matrix Metalloproteinases); WT (Wild-Type); IL (Interleukin); BALF (Bronchoalveolaer Lavage Fluid); OVA (Ovalbumin).

Introduction

Asthma is a complex chronic inflammatory disease characterized by: (a) reversible airway obstruction, (b) airway hyperresponsiveness, (c) bronchial wall infiltration by inflammatory cells, and (d) bronchial remodeling. Eosinophilic inflammation, an important hallmark of asthma (1), correlates with bronchial hyperresponsiveness and disease severity (2,3). Biological events leading to eosinophil accumulation in the airway wall and airway lumen are complex and require the secretion of various soluble mediators responsible for their recruitment and survival. In asthmatic lungs, elevated levels of interleukin (IL)-13 are produced by CD_4^+ T helper-2 lymphocytes (Th_2 cells). Prominent biological effects of IL-13, include increased IgE production, release of CCL11 (eotaxin-1), mucus hypersecretion, airway eosinophilia and airway hyperreactivity (4).

Matrix metalloproteinases (MMPs) are able to degrade matrix components (5-7) and to cleave peptidic mediators leading either to their activation or inhibition (8-10). During the last decade, many studies have identified possible roles for several members of the MMP family in asthma. Indeed, several human studies have identified specific MMPs as being overexpressed in the bronchial tree from asthmatics (MMP-1, -2, -8, -9) (11-16). Animal models have been useful in demonstrating that deficiency of some MMPs can be either beneficial (e.g. MMP-9) (11) or deleterious (MMP-2 or MMP-8) in the context of allergen-induced inflammation (7,17-18). Interestingly, physiological inhibitors of MMPs (tissue inhibitors of MMPs or TIMPs) or synthetic MMP inhibitors have been suggested as potential new therapeutic agents for asthma (5,19-20). As new MMPs and MMPs-related enzymes (ADAM (A Disintegrin and Metalloprotease) and ADAMTS (ADAM with ThromboSpondin-like motifs) of unknown functions have been described recently, the present study focuses on the potential role of the recently described MMP-19 in an experimental mouse model of asthma. MMP-19 displays

unique structural features and a wide spectrum of proteolytic activities (21-22). Among extracellular matrix components, MMP-19 has the ability to cleave tenascin-C, gelatin, collagen IV, fibronectin, nidogen, aggrecan and collagen I (23-25). Tenascin-C accumulation has been detected in bronchial walls after allergen challenge and in chronic asthmatics (26-28). MMP-19 also displays the ability to cleave carriers proteins such as insulin-like growth factor binding protein-3 (IGFBP-3) that have been implicated in airway inflammation and tissue remodeling in asthma (29,30). MMP-19 is expressed in normal adult tissue, including the lung, supporting its role in normal tissue homeostasis (21,22). Initially identified as an autoantigen in patients with rheumatoid arthritis (31), MMP-19 is a putative new molecular mediator of inflammation. The recently generated knock-out (KO) mice for MMP-19 (32,33) provide a suitable tool to decipher the potential implication of MMP-19 in allergen-induced asthma.

In the present study, the role of MMP-19 in lung inflammation is demonstrated by applying a mouse model of allergen-induced airway inflammation and hyperresponsiveness to MMP-19 deficient-mice. We report that allergen exposure in MMP-19 deficient animals causes tenascin-C accumulation in airway walls, linked with a Th_2 cell-related inflammatory lung response. In a translational setting, we also demonstrate that MMP-19 is overexpressed in airway smooth muscle obtained from human asthmatics. Our findings support a protective role for MMP-19 in asthma which has potential implications in the perspective of designing MMP inhibitors-based therapeutic strategies for inflammatory disorders.

Material and methods

Sensitization and allergen exposure protocol

Care and use of experimental animals were performed following "principles of laboratory animal care" formulated by the National Society for Medical Research (USA) and experimental protocols approved by the University of Liege Animal Ethics' Committee. Wild type (WT) and MMP-19 knock-out (MMP-19$^{-/-}$) mice were generated as previously described (18,32). Six to eight weeks-old males were sensitized by intraperitoneal injection of ovalbumin (OVA) (Sigma Aldrich, Schnelldorf, Germany) emulsified in aluminum hydroxyde (AlumInject; Perbio, Erembodegem, Belgium) on days 1 and 8. From day 21 to 27, mice were exposed daily to OVA by inhalation of an aerosol generated by an ultrasonic nebulizer (Devilbiss 2000). On day 28, mice were sacrificed as previously reported (18). Results presented are representative of 3 independent experiments (5-12 mice per experimental conditions in each assay).

Measurement of bronchial responsiveness

Mice were anesthetized by intraperitoneal injection (200 µl) of a mixture of ketamine (10 mg/ml, Merial, Brussel, Belgium) and xylazine (1 mg/ml, VMD, Arendonk, Belgium). A tracheotomy was performed by inserting a 20 gauge polyethylene catheter into the trachea and ligating it around the catheter to avoid leaks and disconnections. Mice were ventilated with a flexiVent® small animal ventilator (SCIREQ, Montreal, Canada) at a frequency of 250 breaths per minute and a tidal volume of 10 ml/kg. A positive endexpiratory pressure was set at 2 hPa and lung function measures obtained after 2 minutes of mechanical ventilation. A sinusoidal 1-Hz oscillation was then applied to the tracheal tube to allow calculation of dynamic airways resistance, elasticity, and compliance using a multiple linear regression methods. A second manoeuvre consisting in an 8-s forced oscillatory signal ranging frequencies between 0.5 and

19.6 Hz allowed assessment of impedance to evaluate tissue damping, tissue elastance, and tissue hysteresivity (34). Following baseline lung function measurements, mice were exposed to a saline aerosol (PBS) followed by aerosols containing increasing doses (3, 6, 9, 12g/l) of methacholine (ICN Biomedicals, Asse Relegem, Belgium). Aerosols were generated by ultrasonic nebuliser (SYST'AM, LS 2000, Dupont Medical, Rhode-Saint-Genèse, Belgium) and delivered to the inspiratory line of the flexiVent® using a bias flow of medical air following the manufacturer's instructions. Each aerosol was delivered for 2 minutes and lung function measurements as described above were assessed at one-minute intervals following each aerosol. Mean airway resistance after methacholine exposure was the major parameter measured during the challenge.

Bronchoalveolar lavage fluid (BALF)

Immediately after mice sacrifice, a bronchoalveolar lavage using 4x1ml PBS-EDTA 0.05mM (Calbiochem, Darmstadt, Germany) was performed as previously described (18). Cells were recovered by gentle manual aspiration. The supernatant obtained after centrifugation of bronchoalveolar lavage fluid (BALF) (at 1200 rpm for 10 minutes, at 4°C) was frozen at –80°C for protein assessment. Cell pellets were used for cytocentrifuged preparations (Cytospin) in which cells on slides were stained with Diff-Quick (Dade, Belgium) to obtain differential cell counts from BALF (eosinophils, neutrophils, epithelial cells, lymphocytes and macrophages).

Pulmonary histology and tissue processing

After bronchoalveolar lavage, the thorax was opened and the left lung was excised and frozen immediately at –80°C for protein and RNA extractions. The right lung was inflated by gentle instillation with 4% paraformaldehyde by a continuous-release pump for 10 minutes under

constant pressure, embedded in paraffin and used for histology. Peribronchial inflammation scores were obtained as previously described (18) and expressed as a mean value of 8 randomly selected tissue sections per mouse (n=15 mice per group).

Congo red stains the two lobes of eosinophils nucleus in blue and allows the specific detection of cytoplasmic amyloid deposit in orange. Paraffin sections of 5µm were deparaffinized, hydrated in water and subsequently stain in Congo Red solution for 1 hour. Slides were rinsed in distilled and tap water and subsequently counterstained with hematoxylin eosin. These steps were followed by the dehydration through 70%, 95%, 100% alcohol and xylene. At least 5-7 randomly selected tissue sections per mouse were assessed by one experimented observer blinded to experimental details. Eosinophilic infiltration in airway walls was quantified by manually counting eosinophils in bronchi. Main bronchi (trachea and very proximal tree) were not considered. To normalize results with epithelial basement membrane length, eosinophils numbers were reported to the perimeter of basement membrane measured by using ImageJ software, (http:/rsb.info.nih.gov/nih-image/). These results were thus defined as number of cells/mm of epithelial basement membrane (n=15 mice per group).

For antigen recovery for immunodetection of MMP-19 and tenascin-C, slides were heated in autoclave in citrate buffer (Dako Target Retrival Solution, Dako, Glostrup, Denmark) and incubated with primary antibody during 1 hour to detect MMP-19 (rabbit anti-MMP-19, 1/1000) (Sigma, Saint-Louis, Missouri, USA) and overnight to detect tenascin-C (goat anti-tenascin-C, 1/200) (Santa-Cruz Biotechnology, CA, USA). Slides were washed in PBS Tween 0.05% and then incubated with corresponding secondary antibodies. A goat anti-rabbit HRP (1/400) (Dako, Glostrup, Denmark) was used to detect MMP-19 and number of MMP-19 positive cells by field was counted for each slide (5 fields/slide). A biotinylated rabbit anti-goat (1/400) (Dako,

Glostrup, Denmark) followed by incubation with a streptavidin/HRP (1/500) (Dako, Glostrup, Denmark) was used to detect tenascin-C expression. Apoptosis was studied by terminal deoxynucleotidyl transferase (TDT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) (Roche, Penzberg, Germany). Sections were incubated in Xylol, dehydrated, and pretreated with Triton X100 1% and hydrogen peroxide (H_2O_2). Sections were incubated 1 hour at 37°C with enzyme solution and nucleotide mixture (UTP-FITC). In order to use standard light microscope, slides were incubated with an anti FITC/HRP antibody (converter POD). Finally, sections were counterstained with haematoxylin and mounted. For each mouse, 5 different areas were analyzed in the whole lung. The percentage of eosinophils undergoing apoptosis was calculated for each mouse.

Detection of cells bearing the IL-33 receptor T_1/ST_2 on their surface was performed by immunohistochemistry using a rat monoclonal antibody to mouse T_1/ST_2 purchased from Morwell diagnostics (Zurich, Switzerland) (18,35-36). T_1/ST_2 positive-cells were counted in the peribronchial area of 6 bronchi per mouse.

The left lung was crushed using a Mikro-Dismembrator (Braun Biotech International, Gmbh Melsungen, Germany). Crushed lung tissue was incubated overnight at 4°C in a solution containing urea for proteins extraction. The supernatant was stored at –80°C for ELISA tests and for Western-blot analysis. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany).

Measurement of MMP-19 and tenascin-C mRNA expression by Real Time PCR

Total RNA was extracted from crushed lung tissue using Qiagen RNeasy Mini Kit (Qiagen, Venlo, Netherlands). RNA levels and purity were assessed using a smartspect 3000

spectrophotometer (BioRad, Hercules, CA, USA). Complementary DNA synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche Molecular Systems, Branchburg, New Jersey, USA) and PCR amplification was performed using the QuantiFast SYBR Green RT-PCR Kit (Qiagen, Venlo, Netherlands). The adapted amplification primers used for MMP-19, Tenascin-C and ribosomal RNA 18S are the Mus Musculus QuantiTect Primer (Mm_Mmp19_1_SG; Mm_Rn18s_2_SG; Mm_Tnc_1_SG) selected and purchased from Qiagen. Each sample was analyzed in duplicate and a calibration curve was run in parallel in each analysis. The levels of transcripts of the constitutive housekeeping gene product ribosomal RNA 18S were quantitatively measured in each sample to control for sample to sample differences in RNA concentration and quality. Three tissue samples were pooled for each determination. The results are expressed as the mean ± SEM of the two different experiments and were analyzed using the LightCycler® 480 Software from Roche.

Measurements of cytokines by ELISA

Mouse IL-5, IL-13, CCL5 (RANTES) and CCL11 (eotaxin-1) levels were assessed using commercial ELISAs following manufacturer's instructions (R&D systems, Abingdon, UK). ELISA assay detection limits were: 15.6 pg/ml (IL-5), 7.8 pg/ml (IL-13), 7.8 pg/ml (CCL-5), 15.6 pg/ml (CCL-11), respectively.

Measurement of allergen specific serum IgE and IgG

At sacrifice, measurements of OVA specific serum IgE levels were assessed by ELISA as previously described (18). OVA-specific IgG_1 and IgG_{2a} were detected using peroxidase-labeled goat anti-IgG_1 and anti-IgG_{2a} Abs (affinity purified Abs; Southern Biotechnology Associates, Birmingham, AL).

Isolation and culture of Human Airway Smooth Muscle Cells

Airway smooth muscle cells from four healthy subjects (methacholine PC_{20} >16 mg/ml, FEV_1 101±4%, age 29±5yr, 3 male, 1 female) and four glucocorticoid-naïve atopic asthmatics (methacholine PC_{20} 0.18±0.04 mg/ml, FEV_1 82±7%, age 25±2yr, 2 male, 2 female) were obtained in accordance with procedures approved by the Research Ethics Committees of King's College Hospital (study #11-03-209) and Guy's & St. Thomas' Hospitals (study #05/Q0704/72) by deep endobronchial biopsy from right middle or lower lobe bronchi. Smooth muscle bundles were visualized using a dissecting microscope and dissected free of surrounding tissue using fine needles. Cells were grown by explant culture from airway smooth muscle bundle fragments in 12-cm^2 flasks using methods described previously (37). Fluorescent immuno-cytochemistry routinely confirmed that near-confluent, fetal bovine serum (FBS)-deprived cells (passage 2) stained (>95%) for smooth muscle-specific α-actin, desmin and calponin (37). Cell passages 3-7 were used in all experiments.

Cell Culture and Collection of Cell-conditioned Medium

Cells ($2x10^4$cells/well) were grown in 24-well plates for 4 days in DMEM. Sub-confluent cells were incubated in serum-free RPMI 1640 (Gibco, Invitrogen, Paisley, United Kingdom) containing 25 mM HEPES, 2 mM L-glutamine, 100 U/ml:100 µg/ml penicillin/streptomycin (supplemented RPMI) with the addition of 1 µM insulin, 5 µg/ml transferrin, 100 µM ascorbate, and 1 mg/ml bovine serum albumin (BSA). After 72 hours, cells were stimulated with recombinant human IL-13 for 24 hours (R&D Systems, Abingdon, UK) in supplemented RPMI 1640 containing 1 mg/ml BSA. Cell-conditioned medium was collected and cell-free supernatants were stored at -70° C until measurement of MMP-19 levels by Western Blot analysis.

Western blot analysis

Samples (cell-free supernatant) were electrophoretically separated in SDS-10% polyacrylamide gels in the presence of 5% β-mercaptoethanol. Proteins were transferred onto a PVDF membrane (NEN Life Science Products) which was then incubated for 1 hour at room temperature in Phosphate Buffered Saline (PBS) containing 5% skim milk. Blots were incubated overnight at 4°C with a rabbit anti-human MMP-19 polyclonal antibody (Affinity BioReagents, Zhandhoven, Belgium) or with a polyclonal rabbit anti-human/mouse tenascin-C (Chemicon International, Würzburg, Germany) both diluted in PBS (1/1000), washed three times in PBS-tween (0.1%) and finally incubated for one hour respectively with peroxidase-conjugated goat anti-rabbit IgG diluted 1:1000 for MMP-19 staining or with a peroxidase-conjugated swine anti-rabbit diluted 1:1000 for tenascin-C detection (Dako, Glostrup, Denmark). Peroxidase activity was assessed using an enhanced chemiluminescence kit (NEN life science products). In order to normalize Western Blot data, beta-actin or GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) were detected in all samples with a rabbit anti-mouse/HRP diluted in PBS at 1:1000 (Sigma, Saint-Louis, Missouri, USA) or with a swine anti-rabbit/HRP antibody diluted in PBS at 1:10 000 (Chemicon International, Würzburg, Germany).

Statistical analysis

Results are expressed as mean +/- SEM and the comparison between the groups was performed using Mann-Whitney test, One-way ANOVA with post test or Unpaired t test. These tests were performed using GRAPHPAD INSTAT version 3.00 for WINDOWS 95 (GRAPHPAD SOFTWARE, San Diego, CA, USA, WWW.GRAPHPAD.Com). P values < 0.05 were considered as significant.

Results

MMP-19 expression is induced in lungs of mice exposed to allergens

In order to investigate MMP-19 expression after allergen challenge, C57/BL6 mice were sensitized and subsequently exposed to aerosolized ovalbumin (OVA) for 7 days. As previously described (18), this treatment led to an asthma-like phenotype characterized by an eosinophilic and neutrophilic inflammation in the bronchoalveolar lavage (BAL) (Table 1) and peribronchial infiltrates of eosinophils (data not shown). Real Time-PCR analysis revealed a significant increase of MMP-19 mRNA levels in lungs tissue extracts of animals exposed to allergen as compared to PBS-exposed mice (figure 1A). This finding was confirmed at the protein level by immunohistochemistry on lung sections showing a two fold increase of MMP-19 positive cells after allergen exposure (figure 1B-C).

MMP-19 deficiency is associated with higher allergen-induced airway responsiveness and increased eosinophilic inflammation

We subsequently applied the allergen-induced asthma model to MMP-19 deficient mice and their wild-type (WT) counterparts. Airway resistance was recorded by direct measurement following exposure to inhaled methacholine (3g/l to 12g/l) by using the Flexivent® system. The dose response curve presented in figure 2A shows that allergen-induced airway responsiveness was significantly higher in MMP-19$^{-/-}$ as compared to WT mice for each tested dose of methacholine (3, 6, 9, 12g/l) ($p<0.05$) (figure 2A). MMP-19 deficiency in mice was not associated with any pulmonary developmental abnormality as assessed by histology (data not shown). After allergen challenge, MMP-19$^{-/-}$ mice exposed to allergen showed a huge increase of total cell counts in BALF (13.62 ±3,97 for Wild-Type mice *vs* 53.7 ± 8,65 for MMP-19$^{-/-}$ mice; $p<0.001$). Eosinophils represented more than 85% of total cells and their mean number was 5 times higher

in BALF of allergen-exposed MMP-19$^{-/-}$ mice as compared to WT counterparts ($p<0.0005$) (Figure 2B). Neutrophil counts were also increased in challenged MMP-19$^{-/-}$ mice ($p<0.001$) but represented less than 5% of total cells in BALF from challenged MMP-19$^{-/-}$ mice (Figure 2C). No significant difference was observed between the experimental groups when considering lymphocytes, epithelial cells and macrophages (data not shown).

Histological observations correlated with results obtained on BALF. Indeed, no inflammation was evidenced in bronchial walls of wild-type (WT) or MMP-19$^{-/-}$ OVA-sensitized and exposed to PBS (figure 3A). In sharp contrast, an obvious peribronchial and perivascular inflammation was observed in both wild-type and MMP-19$^{-/-}$ mice after allergen exposure (figure 3A-B). A seven-fold increased eosinophilic infiltration in the peribronchial area was also evidenced by Congo Red staining in MMP-19$^{-/-}$ as compared with WT (figure 3A and C). As assessed by TUNEL labeling, no difference of apoptosis index for eosinophils was observed between experimental groups (figure 3D). This suggests that the eosinophilic inflammation detected in sensitized and challenged MMP-19$^{-/-}$ mice involved increased cellular recruitment rather than modification of cell survival.

MMP-19 deficiency leads to an increased Th_2 inflammation upon allergen challenge

Levels of OVA specific IgE measured by ELISA were increased in sera of mice sensitized and subsequently exposed to OVA aerosolization as compared to sham-exposed mice ($p<0.05$). OVA specific IgE production reflecting sensitization to this experimental allergen was similar in both genotypes and was not affected by MMP-19 deficiency (figure 4A). Levels of OVA specific IgG_1 and IgG_{2A} were also assessed in order to determine the Th_1/Th_2 profile. After allergen challenge, levels of IgG_1, a marker of Th_2-prone milieu, were significantly increased after allergen exposure in the sera from MMP-19$^{-/-}$ and corresponding WT mice but were drastically higher in serum

from MMP-19$^{-/-}$ mice as compared to WT ($p<0.05$) (figure 4B). In contrast, levels of IgG_{2A}, a putative Th_1marker, were not different between all experimental groups (data not shown).

To further characterize the immunological profile of lung parenchyma, we analyzed airway infiltration by Th_2 cells through immunohistochemical staining with an antibody raised against T_1/ST_2, the IL-33 receptor expressed on Th_2 cell surface. MMP-19$^{-/-}$ mice displayed a significantly higher number of T_1/ST_2 positive lymphocyte-shaped cells in the airways as compared to WT mice ($p<0.05$). These experiments indicate that Th_2 cells recruitment in the lung was increased in the absence of MMP-19 (figure 4C).

Cytokine measurements in BALF and lung protein extracts

To elucidate possible mechanisms underlying increased eosinophilic inflammation and airway responsiveness to methacholine observed in MMP-19$^{-/-}$ mice, levels of the key Th_2 cytokine IL-13 were quantified in BALF by ELISA. In both genotypes, allergen exposure induced at least a five time increase of IL-13 levels. Moreover, in MMP-19$^{-/-}$ mice, IL-13 levels in BALF and lung protein extracts were three times higher as compared to WT ($p<0.005$ and $p<0.0001$ respectively) (figure 5A and data not shown). In addition, CCL11 (eotaxin-1) levels measured in lung protein extracts were increased upon allergen exposure in both genotypes but levels were significantly higher in MMP-19$^{-/-}$ as compared to WT ($p<0.005$) (figure 5B). In sharp contrast, levels of CCL5 (RANTES) were increased in PBS-exposed MMP-19$^{-/-}$ mice ($p<0.005$) and allergen exposure affected CCL5 levels only in WT mice and not in MMP-19$^{-/-}$ mice ($p<0.05$) (figure 5C). No difference in IL-5, IL-10 and IFN-γ levels was observed between the experimental groups (data not shown).

Expression and deposition in the airways of MMP-19 substrates potentially involved in airway inflammation and remodeling

We next analyzed some key substrates of MMP-19 that might play a role in asthma reaction. Tenascin-C mRNA expression quantified by Real Time-PCR analysis in extracts from whole lungs was increased after allergen challenge in both MMP-19$^{-/-}$ and wild type mice when compared to naïve mice without any significant influence of the MMP-19 depletion (Figure 6A). When measuring protein levels of tenascin-C by western-blot analysis, we found that allergen exposure induced a significant increase of tenascin-C levels in MMP-19$^{-/-}$ and in wild-type mice ($p<0.05$) with significantly higher levels in MMP-19$^{-/-}$ mice ($p<0.05$) (Figure 6B). Immunohistochemical analysis confirmed tenascin-C deposition in lung tissue induced by allergen exposure both in wild-type and MMP-19$^{-/-}$ mice. However, a strong tenascin-C immunoreactivity appeared as an intense continuous band located beneath the epithelium basement membrane in mutant mice while tenascin-C labeling was less distinct, appearing as a thin interrupted line in WT counterparts (Figure 6C). Measurements of IGFBP-3 protein, another MMP-19 substrate, were also performed on lung extracts by Western blot analysis. However, no obvious difference was evidenced between MMP-19 wild-type and MMP-19$^{-/-}$ mice (data not shown).

Regulation of MMP-19 expression in human cultured primary airway smooth muscle cells

Since non respiratory smooth muscle cells have been reported to produce MMP-19 (38), we assessed MMP-19 production by cultured human airway smooth muscle cells derived from subjects with asthma and healthy controls (figure 7A). Under non-stimulated conditions, airway smooth muscle cells from asthmatics released significantly higher levels of MMP-19 protein than cells from healthy subjects ($p<0.05$). Incubation of these cells with IL-13 (0.1ng/ml to 10ng/ml)

led to a concentration-related increase of MMP-19 production in asthmatics ($p<0.05$) (figure 7B). Considering non asthmatic cells, after appropriate statistical analysis using One-way ANOVA with post test or Unpaired t test, we determined that there is a significant difference between unstimulated cells (0.4965 +/- 0.14) and cells stimulated with 0.1 ng/ml IL-13 (1.474 +/- 0.0559) ($p<0.01$) regarding MMP-19 production. A significant decrease of MMP-19 production by non asthmatic cells was found when these cells were stimulated with 0.1 ng/ml IL-13 compared with 10 ng/ml IL-13 (0.7615 +/- 0.196) ($p<0.05$). However, when comparing normal cells stimulated with 10 ng/ml IL-13 with unstimulated cells, no significant difference was evidenced.

Discussion

Allergic inflammation implies a complex interplay between diverse mediators including cytokines/chemokines, extracellular matrix components and proteases. The present study provides novel evidences concerning the function of MMP-19, suggesting a protective effect of this protease in allergen-driven inflammatory reaction. Our results indicate that MMP-19 could prevent tenascin-C deposition and therefore play a role in the driving of inflammatory processes. In this work, we identify MMP-19 as a gene overexpressed in allergen-exposed animals suggesting its implication in inflammatory cell recruitment. Through a gene deletion strategy, we demonstrate for the first time that MMP-19 deletion preeminently exacerbates Th_2-associated eosinophilic inflammation and airway hyperresponsiveness. The marked eosinophilia found in the airway walls of MMP-19 deficient mice was accompanied by a shift towards a Th_2 profile as assessed in lung tissue by T_1/ST_2 positive lymphocytes counts, Th_2 cytokine (IL-13) and chemokines (CCL5, CCL11) but also by serum IgG_1 measurements. A link between MMP-19 and IL-13 regulatory pathway is further evidenced by the demonstration of increased MMP-19 production in IL-13-stimulated human smooth muscle cells. Moreover, we demonstrate a peribronchial accumulation of tenascin-C protein after allergen challenge in MMP-$19^{-/-}$ mice only.

The observation of an exacerbated eosinophilic inflammation in MMP-$19^{-/-}$ mice is in line with the finding of increased eosinophilic inflammation in MMP-$2^{-/-}$ mice (17) and neutrophilic inflammation in MMP-$8^{-/-}$ mice (18), and broadens the involvement of MMPs in pulmonary inflammatory response (6). Our results are the first to associate MMP-19 with a Th_2-mediated immune response providing evidence that Th_2-related cytokines (IL-13, CCL11, and CCL5) are increased in lung tissues of MMP-$19^{-/-}$ mice. The differences in CCL11 levels could *per se* explain the increased eosinophilic inflammation in MMP-$19^{-/-}$ mice since CCL11 is a potent

eosinophil chemoattractant and activator (39). CCL5, which is a key regulator of eosinophil locomotion and activation, is produced by lymphocytes but also by epithelial, endothelial cells (40) and airway smooth muscle (41). Interestingly, MMP-19 expression was induced by CCL5 in human monocytes (41) and we show that baseline CCL5 levels were increased in MMP-19$^{-/-}$ mice as compared to WT mice, suggesting a crosstalk between CCL5 and MMP-19 with a putative negative feedback loop on CCL5 production when MMP-19 is secreted. Alternatively, increased baseline levels of CCL5 might reflect some profound alterations of cellular responses in MMP-19$^{-/-}$ mice.

IL-13 is a prototypic Th_2 cytokine that induces eosinophilic inflammation in the airways and airway hyperresponsiveness (11,42). Thus, the increased levels of IL-13 could be directly responsible for the increased airway hyperresponsiveness observed in MMP-19$^{-/-}$ mice. IL-13 levels after allergen exposure are significantly higher in MMP-19$^{-/-}$ mice while baseline levels were not affected by MMP-19 deficiency. Other experimental arguments strongly suggest that MMP-19 deletion induces a deregulation towards a Th_2 response. Indeed, we describe increased numbers of cells bearing the IL-33 receptor T_1/ST_2, which is specific for Th_2 lymphocytes in the lung parenchyma, and increased IgG_1 serum levels in MMP-19$^{-/-}$ mice (43). The sensitization process is assuredly not affected in MMP-19$^{-/-}$ mice since specific IgE levels were increased by allergen exposure in both deficient mice and wild type mice without differences. Taken together, these data strongly support the assumption that MMP-19 deficiency is directly or indirectly responsible for a shift towards Th_2-driven inflammation without affecting the sensitization process.

The involvement of MMP-19 in murine asthma model displaying Th_2 inflammation and airway hyperresponsiveness prompted us to evaluate the production of MMP-19 by cultured human airway smooth muscle cells in a translational setting. Although expression of MMP-19 by

smooth muscle cells outside the airways has been reported previously (38), this is the first report of MMP-19 expression by smooth muscle from the airways. Of great interest is the finding that smooth muscle cells derived from human asthmatics produced markedly greater levels of MMP-19 at baseline and were hyperreactive to IL-13 stimulation. The finding that cells obtained from asthmatics overproduced MMP-19 both in baseline conditions and when stimulated by IL-13 was unexpected and suggests that MMP-19 could be implicated in a cross-talk between airway inflammatory cells, extracellular matrix and smooth muscle cells (44). Moreover, increased MMP-19 expression observed in cells from asthmatic patients might reflect a profound deregulation of IL-13 pathway and could suggest that it exists a loop of reciprocal interactions between IL-13 (which might stimulate MMP-19 production *in vivo*) and MMP-19 (which seems to directly or indirectly restrain IL-13 production). When considering these results, we can speculate that a putative molecular cross talk could exist between MMP-19 and IL-13. Such a cross talk could be one of the key regulatory mechanisms of airway inflammation in the context of Th_2 inflammation. In our model, one can speculate that smooth muscle-derived MMP-19 could protect against allergen-induced inflammation by counteracting IL-13 production and by cleaving newly deposited tenascin-C, thereby regulating bronchial responsiveness and inflammation.

Interestingly, MMP-19 deficient animals displayed tenascin-C accumulation in the bronchial walls. Tenascin-C is a substrate for MMP-19 (25) and was recently reported to acutely accumulate in the bronchial walls from asthmatics after allergen challenges (26,27). In atopic asthmatics, thickness of tenascin deposition correlated with T-lymphocyte, eosinophil and macrophage cell counts, suggesting that tenascin may have a regulatory role on inflammatory cells accumulation in asthma (45). Many studies have investigated the association of genetic polymorphisms with asthma. Among those, tenascin-C was identified as a novel asthma

susceptibility gene (46). Most importantly, by using a mouse model of allergic asthma applied on tenascin-C deficient mice, Nakahara et al (47) described that depletion of this glycoprotein significantly decrease the asthmatic phenotype in mice. Tenascin-C deficient animals are therefore protected against allergen-induced airway inflammation indicating that tenascin-C displays some pro-inflammatory properties. Based on our experimental results, we suggest that MMP-19 could prevent tenascin-C accumulation in the airway walls by cleaving this molecule, therefore participating in the control of Th_2 inflammation. Since it was recently shown that the presence of tenascin-C promotes a motile cell phenotype (48), we could also speculate that the presence of higher amount of tenascin-C in the lung of MMP-$19^{-/-}$ mice might promote inflammatory cell adhesion or accumulation trough its capacity to interact with integrins (49,50). This MMP-19 related tenascin-C regulatory process could also be of importance in humans since this glycoprotein accumulates at 24h after an allergen challenge and diminishes significantly after 7 days (27). Tenascin-C deposition was also decreased by anti-IL-5 monoclonal antibody in atopic asthmatics (51). Moreover, we speculate that tenascin-C could play a key role in bronchial hyperresponsiveness since we found that exaggerated accumulation correlates with higher responsiveness in animals. In line with this finding, Kariyawasam et al (28) have shown recently that tenascin-C accumulation display the same time-course as bronchial hyperresponsiveness installation. As stated above, tenascin-C appears as of particular importance since the expression of this glycoprotein of the extracellular matrix is increased in several lung inflammatory diseases, including bronchial asthma (26,27). Moreover, IFN-γ and TNF-α, two pro-inflammatory cytokines, are able to induce the production of tenascin-C by cultured human bronchial epithelial cells (52). Taken together these data suggest that in our model, higher amounts of tenascin-C in MMP-$19^{-/-}$ animals might play a key role in the shift towards exaggerated Th2-related inflammation and responsiveness.

We conclude that MMP-19 is an allergen-induced gene and that MMP-19 deletion in mice induces tenascin-C accumulation in airway walls. This is thought to modify the pulmonary inflammatory process towards an excess of Th_2 response leading to much more severe eosinophilic inflammation and airway responsiveness.

Acknowledgements

The authors thank Fabienne PERIN, Christine FINK and Fabrice OLIVIER for the invaluable technical help. Aurore BRASSEUR did also take part to the preliminary experiments of this work.

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Figure legends

Figure 1: MMP-19 expression in lungs. (A) Real Time-PCR measurement of MMP-19 mRNA levels in lungs of allergen-challenged (OVA) (n=11) and sham-exposed (PBS) (n=6) mice. (B) Immunohistochemistry with an anti-MMP-19 antibody on lung sections from mice exposed to PBS (left panel) or OVA (right panel) (magnification 200x). (C) Quantification of MMP-19 positive cells in lung tissue of allergen-exposed mice (OVA) (n=7) and sham-exposed mice (PBS) (n=6).

Figure 2: Airway responsiveness assessment in wild-type and MMP-19$^{-/-}$ mice. After methacholine inhalation, allergen-exposed mice (OVA) displayed increased airway resistances as assessed by FlexiVent®. This increase was significantly higher in MMP-19$^{-/-}$ (n=8) as compared to WT mice (n=8) (A). Number of eosinophils (B) and neutrophils (C) in bronchoalveolar lavage. Allergen exposure induce an increase of eosinophil ($p<0.001$) and neutrophil number ($p<0.05$) both in wild-type and MMP-19$^{-/-}$ mice. A significant increase of eosinophils ($p<0.005$) and neutrophils ($p<0.001$) number was observed in MMP-19$^{-/-}$ mice when compared to wild-type mice after ovalbumin inhalation.

Figure 3: Histological analysis of lung sections. (A) Lung paraffin sections stained with haematoxylin-eosine (HE) (100x) or Congo Red (CR - lower panels) (400x) (n=14 per experimental group). No inflammation was evidenced in bronchial walls of sham-exposed WT (PBS) or MMP-19$^{-/-}$ mice. After allergen exposure, an obvious peribronchial and perivascular inflammation (arrow) was observed in both MMP-19$^{-/-}$ mice and WT. (B) Peribronchial

inflammation was scored on HE stained sections as described in Material and Methods. Eosinophilic infiltration of peribronchial areas was detected by CR staining in allergen-exposed MMP-19$^{-/-}$ and WT (C). Eosinophilic infiltration was quantified on CR stained sections as described in Material and Methods (D). Assessment of cell apoptosis by TUNEL method on paraffin sections (E).

Figure 4: Measurement of allergen specific antibodies and Th_2 inflammation. (A) The amounts of OVA specific IgE were increased in sera of allergen-exposed mice (OVA) ($p>0.05$) without any difference between MMP-19$^{-/-}$ and WT (n= 14 per group) (B) IgG_1 serum levels were significantly increased after allergen exposure in both genotypes. After allergen challenge (OVA), the increase in IgG_1 levels was higher in serum from MMP-19$^{-/-}$ mice. Results in figure 4A and 4B were expressed in arbitrary units (A.U.) (n= 14 per group). (C) Allergen exposure significantly increased the number of Th_2 lymphocytes in both genotypes. Allergen-exposed MMP-19$^{-/-}$ mice displayed a significantly higher number of Th_2 lymphocytes in the airways as compared to WT mice (n= 14 per group).

Figure 5: Cytokine levels in lung protein extracts. Amounts of IL-13 (A), CCL11 (B) and CCL5 (C) were measured by ELISA in lavage fluid or lung protein extracts (n=14 per experimental condition in all these experiments).

Figure 6: Tenascin-C expression in bronchial walls. (A) Real Time-PCR measurements of tenascin-C mRNA levels in lungs of MMP-19$^{-/-}$ and wild-type mice after allergen (OVA). Tenascin-C mRNA levels were significantly increased in lungs after allergen exposure and without any effect of MMP-19 depletion (OVA, n=11). (B) Representative results of Western-

blot performed with an anti-tenascin-C antibody and quantitation. Results are expressed as a ratio between density of tenascin-C bands and density of GAPDH bands, used as internal control. Levels of tenascin-C were significantly increased in MMP-19$^{-/-}$ mice when compared to WT mice. (C) Tenascin-C accumulation was detected by immunohistochemistry on lung sections from mice exposed to OVA (magnification 200x). Deposition of tenascin-C in lung tissue was higher in MMP-19$^{-/-}$ mice when compared to WT mice exposed to OVA.

Figure 7: Measurement of MMP-19 expression by Western blot analysis in human airway smooth muscle cells. (A) Representative Western blot showing MMP-19 production by IL-13 in cultured airway smooth muscle cells from healthy subjects (non asthmatics, NA) or those with asthma (A). (B) Western blot results are expressed as a ratio between density of MMP-19 bands and density of actin bands, used as internal control.

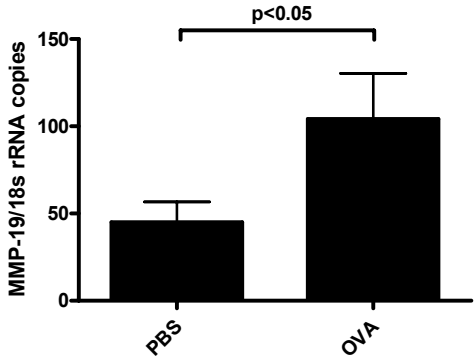
Table 1: Total cell number and cellular composition of Bronchoalveolar Lavage Fluid

Genotype	Wild-Type	Wild-Type	MMP-19$^{-/-}$	MMP-19$^{-/-}$
Exposure	PBS (n= 13)	OVA (n= 15)	PBS (n= 15)	OVA (n= 14)
Epithelial cells (x 10^4/ml)	3,9 +/- 1,46	0,83 +/- 0,23 (*)	0,18 +/- 0,06	0,59 +/- 0,2
Eosinophils (x 10^4/ml)	0 +/- 0	8,78 +/- 3,21 (†)	0,43 +/- 0,19	45,71 +/- 6,98 (†,‡)
Neutrophils (x 10^4/ml)	0 +/- 0	0,04 +/- 0,01	0,32 +/-0,15	2,65 +/- 0,72 (*, §)
Lymphocytes (x 10^4/ml)	0,1 +/- 0,06	0,03 +/- 0,01	0,06 +/- 0,03	0,25 +/- 0,09
Macrophages (x 10^4/ml)	22,7 +/- 0,74	39,2 +/- 0,72	79,7 +/- 1,54	4,46 +/- 1,4
Total cells (x 10^4/ml)	26,7 +/- 1	48,88 +/- 3,97 (*)	80,69 +/- 1,74	53,66 +/- 8,65 (*, †, ‡)

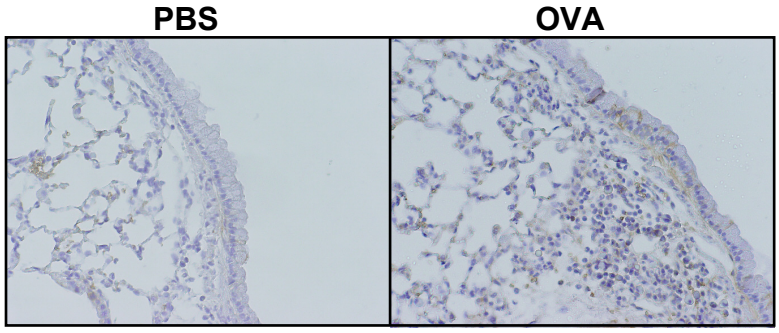
Table I: Total cell number and cellular composition of Bronchoalveolar Lavage Fluid (BALF) of WT or MMP-19$^{-/-}$ challenged with ovalbumin (OVA) or PBS. (*): $p< 0.05$ *vs* PBS mice, (†): $p< 0.001$ *vs* PBS mice, (‡): $p< 0.0005$ vs WT mice, (§): $p<0.001$ *vs* OVA-exposed WT mice.

Figure 1:

A. Real-Time PCR



B. Immunostaining



C.

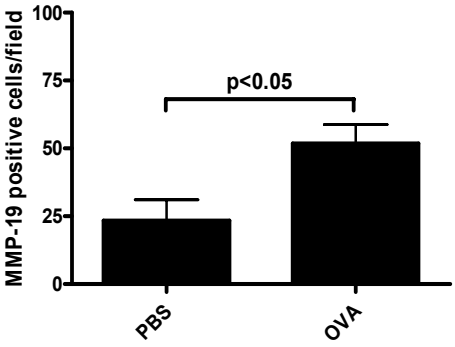
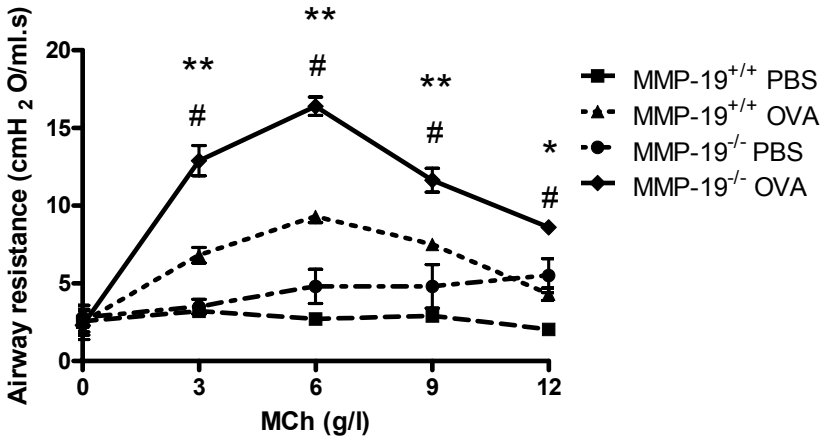
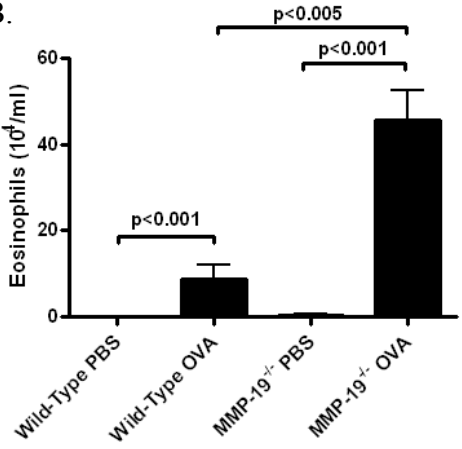


Figure 2:

A.


Airway resistance (cmH 2 O/ml.s)
MCh (g/l)
0
3
6
9
12
0
5
10
15
20
**
#
**
#
**
#
*
#
MMP-19+/+ PBS
MMP-19+/+ OVA
MMP-19-/- PBS
MMP-19-/- OVA

B.


Eosinophils (10⁴/ml)
0
20
40
60
p<0.005
p<0.001
p<0.001
Wild-Type PBS
Wild-Type OVA
MMP-19-/- PBS
MMP-19-/- OVA

C.

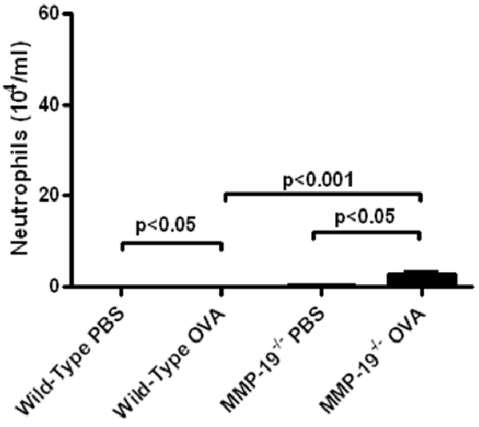

Neutrophils (10⁴/ml)
0
20
40
60
p<0.001
p<0.05
p<0.05
Wild-Type PBS
Wild-Type OVA
MMP-19-/- PBS
MMP-19-/- OVA

Figure 3:

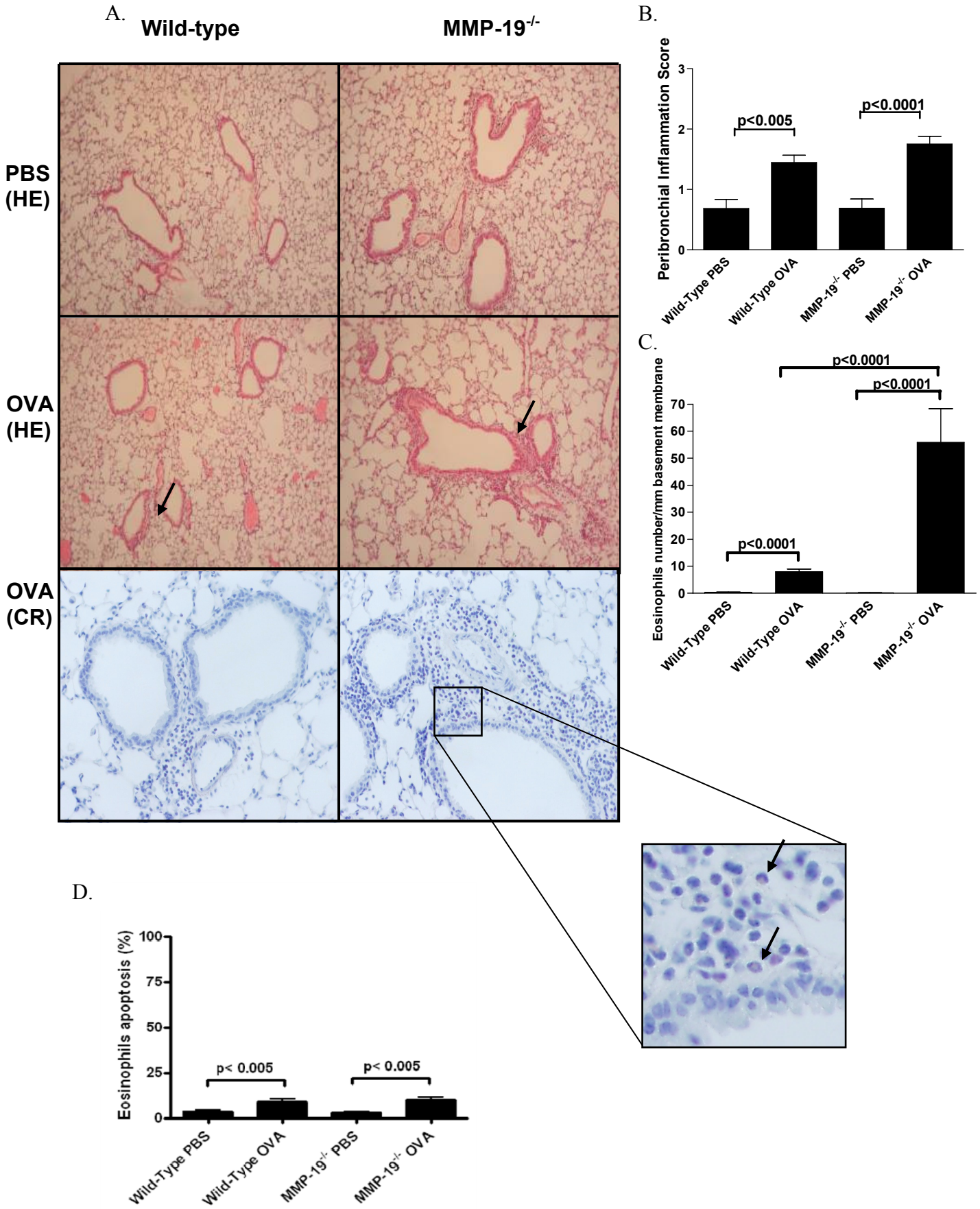
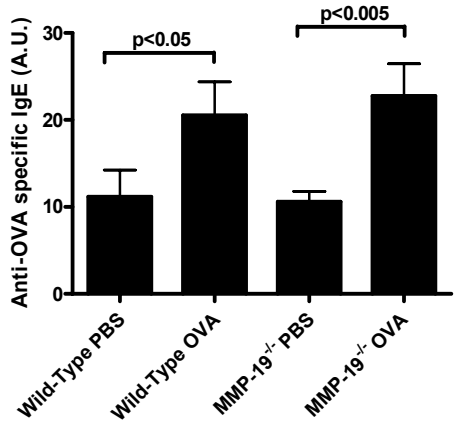
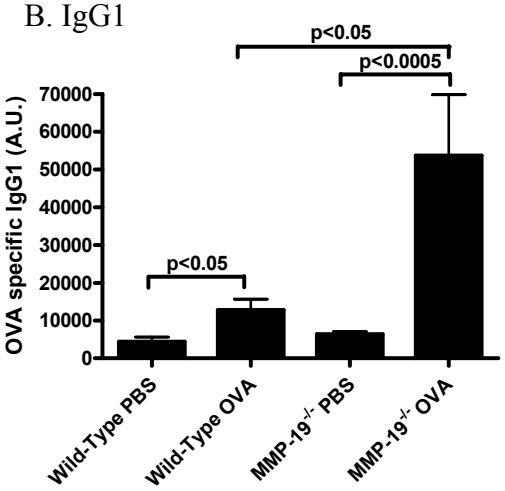

A.
Wild-type
MMP-19-/-
PBS (HE)
OVA (HE)
OVA (CR)
B.
Peribronchial Inflammation Score
p<0.005
p<0.0001
Wild-Type PBS
Wild-Type OVA
MMP-19-/- PBS
MMP-19-/- OVA
C.
Eosinophils number/mm basement membrane
p<0.0001
p<0.0001
p<0.0001
D.
Eosinophils apoptosis (%)
p< 0.005
p< 0.005

Figure 4 :

A. IgE



B. IgG1



C. Th_2 lymphocytes

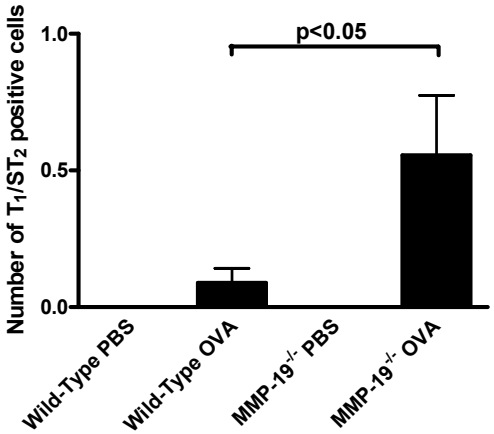
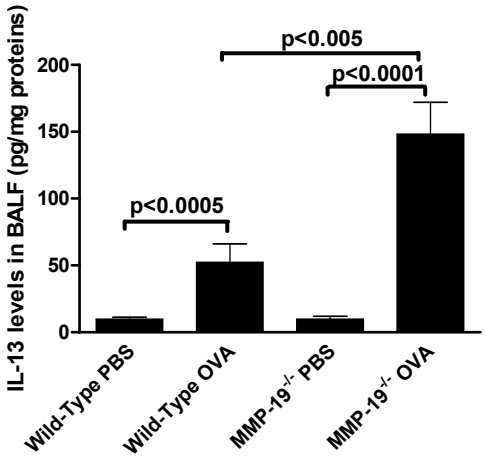
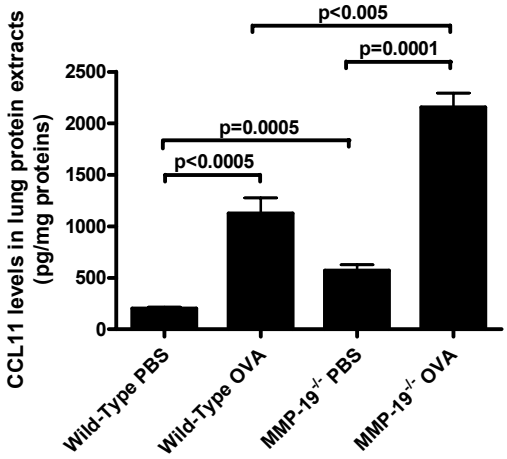


Figure 5 :

A. IL-13



B. CCL11



C. CCL5

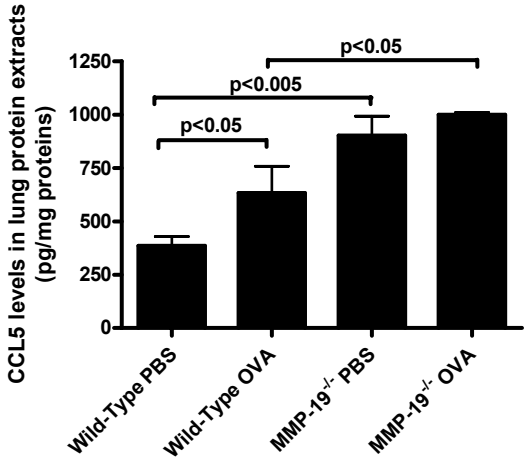
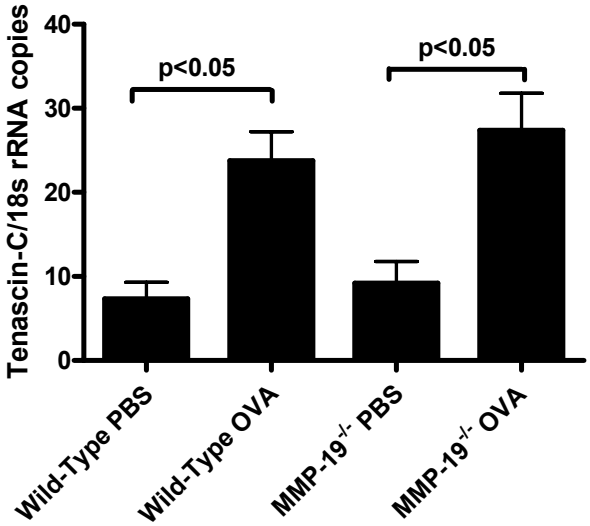
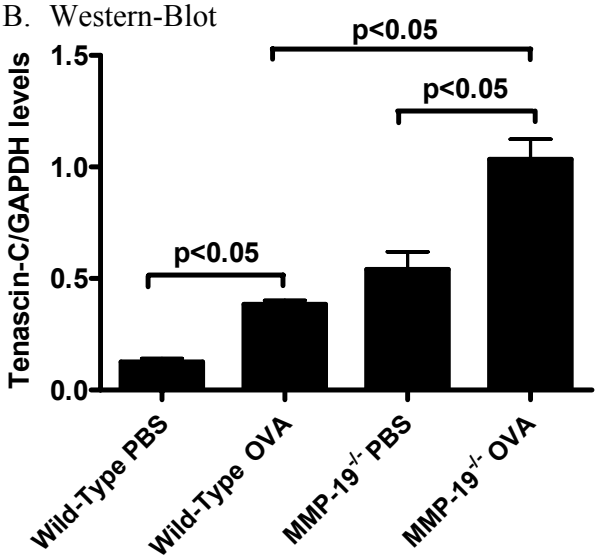


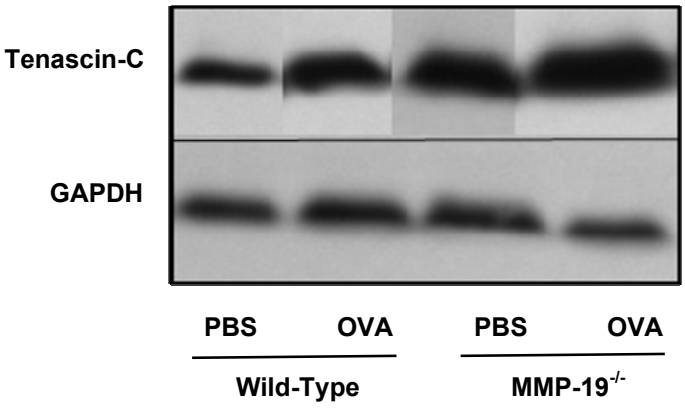
Figure 6:

A. Real-Time PCR



B. Western-Blot





C. Immunostaining

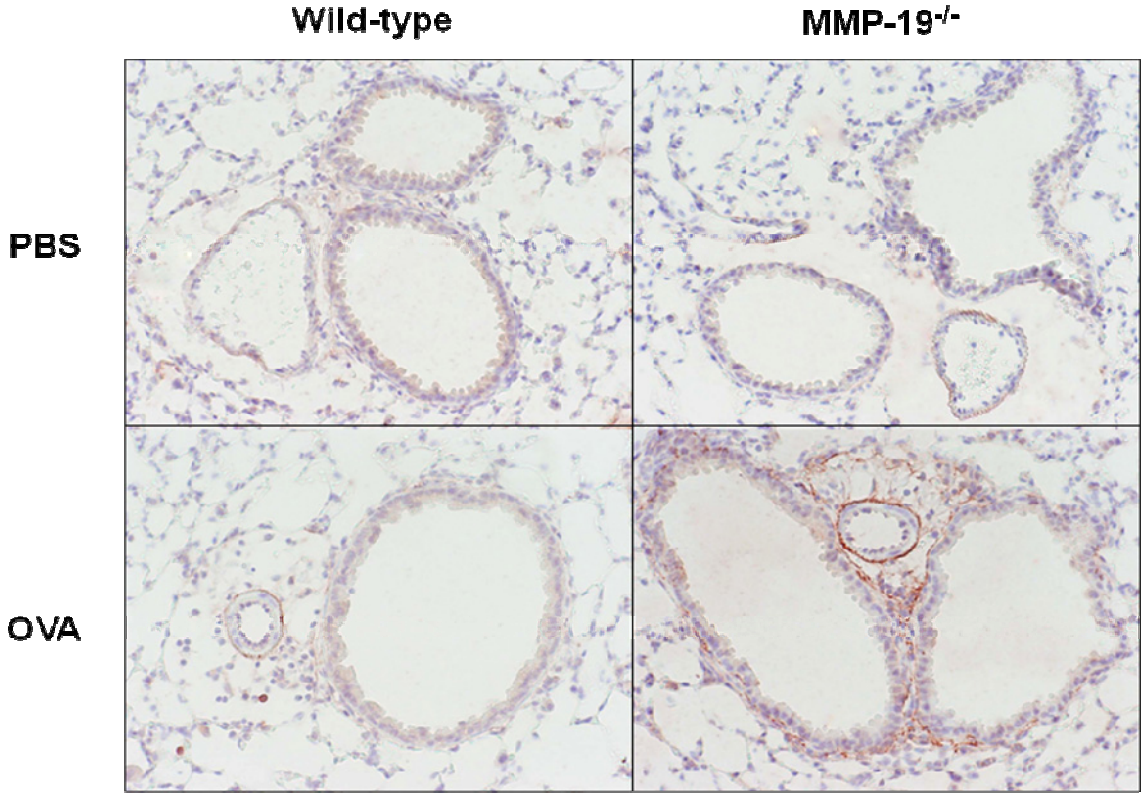
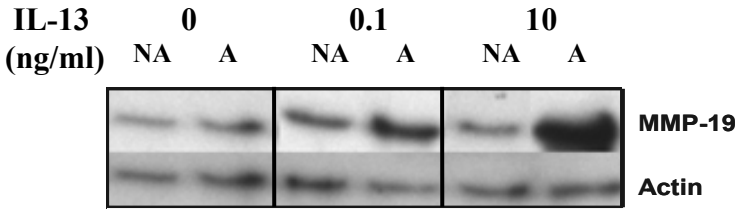
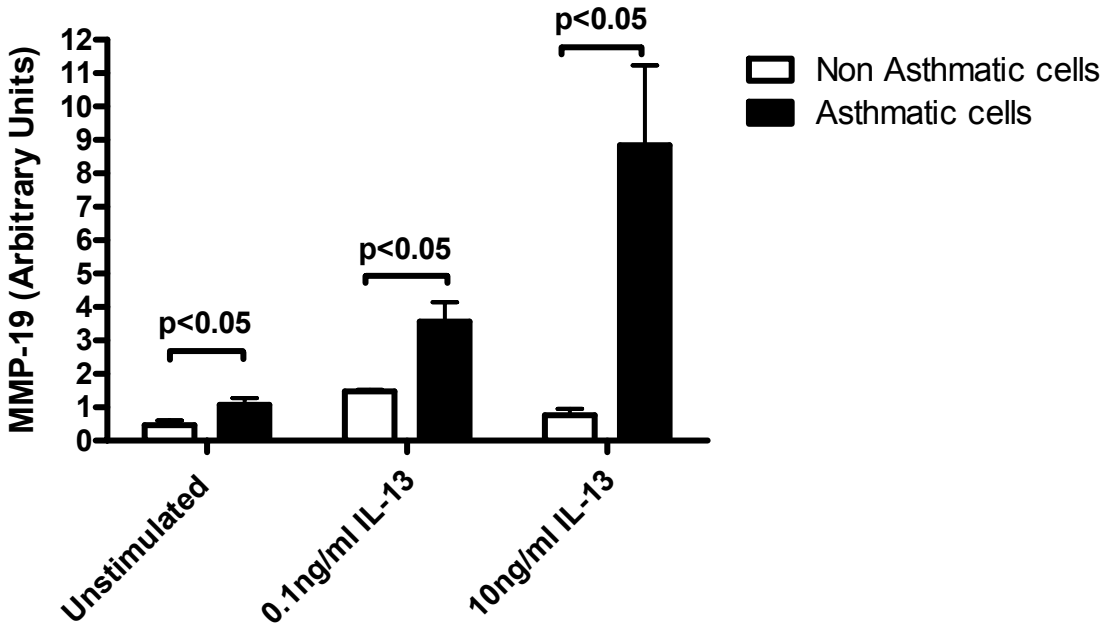


Figure 7 :

A.


IL-13
(ng/ml)
0
0.1
10
NA
A
NA
A
NA
A
MMP-19
Actin

B.


MMP-19 (Arbitrary Units)
12
11
10
9
8
7
6
5
4
3
2
1
0
p<0.05
p<0.05
p<0.05
Unstimulated
0.1ng/ml IL-13
10ng/ml IL-13
Non Asthmatic cells
Asthmatic cells