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Article

Pharmacological blockade of protease-Activated Receptor 2 improves airway remodeling and lung inflammation in experimental allergic asthma

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Protease-activated receptors (PARs) are metabotropic G-protein-coupled receptors that are activated via proteolytic cleavage of a specific sequence of amino acids in their N-terminal region. PAR2 has been implicated in mediating allergic airway inflammation. This study aims to study the effect of PAR2 antagonist ENMD1068in lung inflammation and airway remodeling in experimental asthma. Allergic lung inflammation was induced in sensitized BALB/c mice through intranasal instillations of ovalbumin (OVA), and mice were pretreated with ENMD1068 1 hour before each OVA challenge. Bronchoalveolar lavage fluid (BALF) was collected, and the lungs were removed at different time intervals after OVA challenge to analyze inflammation, airway remodeling and airway hyperresponsiveness. Ovalbumin promoted leukocyte infiltration into BALF in a PAR2-dependent manner. ENMD1068 impaired eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activity in the lung parenchyma into BALF and reduced the loss of dynamic pulmonary compliance, lung resistance in response to methacholine, mucus production, collagen deposition and chemokine (C-C motif) ligand 5 expression compared to those in OVA-challenged mice. We propose that proteases released after an allergen challenge may be crucial to the development of allergic asthma in mice, and PAR2 blockade may be useful as a new pharmacological approach for the treatment of airway allergic diseases.

Keywords: Protease-activated receptor 2. Lung inflammation. Airway remodeling. Allergic asthma.

INTRODUCTION

Protease-activated receptors (PARs) are a G-protein-coupled receptors (GPCRs) that includes four PARs that exhibit a unique mechanism of activation that distinguishes them from other GPCRs; their unique mechanism involves proteolytic and irreversible cleavage of a specific fragment of an amino group in the extracellular N-terminal domain of the receptor itself, exposing a novel anchored fragment that acts as a ligand for the receptor, which triggers downstream cellular signaling events (Adams *et al.*, 2011). PAR2 is a member of the PAR family and is expressed in a wide variety of airway cells, including vascular smooth muscle cells, airway endothelial and epithelial cells, fibroblasts, mast cells, eosinophils recruited from the microcirculation to the airways after allergen challenge, alveolar macrophages, and the bronchial epithelium in asthmatics(Cocks, Moffat,2001; Schmidlin *et al.*, 2001). PAR2 activation triggers cellular responses, such as tissue injury, angiogenesis, and pain (Coughlin, Camerer, 2003),

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and importantly, the anatomic distribution of PAR2 supports its role in allergic and immune surveillance in lung and airway inflammatory responses. In fact, it is well established that PAR2 plays a critical role in the pathophysiology of experimental airway allergic inflammation, mainly by mediating the allergen-induced bronchial hyperresponsiveness, and the activation of inflammatory effector cells including mast cells, macrophages, eosinophils and neutrophils (Schmidlin *et al.*, 2002; Matos *et al.*, 2013; Reed, Kita, 2004).

Many endogenous PAR ligands are serine proteases that constitute a class of proteolytic enzymes that are characterized by having a unique catalytic domain consisting of a triad of serine, histidine and aspartate, which are residues capable of hydrolyzing peptide bonds (Rinderknecht, 1986). PAR2 is activated by trypsin and several other serine proteases released in the allergic environment after antigen exposure, including mast cell (MC) tryptase, leukocyte proteinase-3, cathepsin G, bacterial and allergen-derived proteases and coagulation regulating proteases, leading to pro-inflammatory events, including vasodilation, extravasation of plasma proteins, and secretion of pro-inflammatory cytokines (Arizmendi *et al.*, 2011; Coughlin, 2000; Steinhoff *et al.*, 2005).

PAR2 blockade may be useful for impairing allergic inflammation once PAR2-activating proteases are released in the inflammatory microenvironment following allergen exposure playing a role in the pathogenesis of allergic asthma (Yu, Chen, 2018). Blockade of PAR2 can be achieved through the use of selective antagonists, such as ENMD1068 (N1-3-methlybutyryl-N4-6-aminohexanoyl-piperazine), which is highly selective for PAR2 (Ramachandran, Hollenberg, 2008).Studies carrying out PAR2 blockade indicate a role for PAR2 in the pathophysiology of allergic asthma and airway inflammation (Davidson *et al.*, 2013; de Boer *et al.*, 2014), however, the mechanisms involved in the impairment of airway remodeling following PAR2 antagonist (ENMD1068) treatment are still not well elucidated. Here, we investigated the role of PAR2 blockade on airway remodeling and lung inflammation in a murine model of allergic asthma induced by ovalbumin.

MATERIAL AND METHODS

Animals

Female BALB/c mice (18–25 g) obtained from the Bioterism Center of the Biological Sciences Institute of the Federal University of Minas Gerais (CEBIO/UFMG) were housed under controlled conditions and provided food and water *ad libitum*. All the experimental procedures were approved by the local animal ethics committee (certificate number 348/2014).

Drugs and Reagents

Ovalbumin (OVA, grade V) and aluminum hydroxide gel were obtained from Sigma-Aldrich (St. Louis, MO). The PAR2 antagonist ENMD1068 was purchased from Enzo Life Sciences (San Diego, CA, USA), and methacholine was purchased from Sigma-Aldrich (St. Louis, MO, USA). The PAR2 antagonist ENMD1068 was dissolved in phosphate-buffered saline (PBS, pH 7. 4), and the samples were stored (stock solution: 1 mg / mL) at −20°C until use. Anti-CC chemokine ligand 5 (CCL5) was obtained from R&D Systems (clone 53405.111, Minneapolis, MN) and diluted in antibody diluent (Dako, Carpinteria, CA, USA).

Sensitization and antigen challenging

Mice were immunized with OVA (100 μg) adsorbed on aluminum hydroxide gel as previously described (Kurowska-Stolarska *et al.*, 2008). Briefly, 0.2 mL of a solution of OVA and aluminum hydroxide gel were intraperitoneally (*i.p.*) injected on day 1. On day 9, mice were anesthetized *i.p.* with 130 mg/kg ketamine (Vetnil™, Louveira, Brazil) and 8.5 mg/kg xylazine (Kensol™, König, Brazil) in a saline solution and challenged by an intranasal (*i.n.*) instillation of OVA (10 μg/20 μL) or PBS on days 9, 10, 11, 13, 15 and 17.

Effects of PAR2 antagonist on leukocyte recruitment induced by OVA

The PAR2 antagonist ENMD1068 (0.5 mg/kg) was administered *i.p.* 1 hour prior to the *i.n.* instillation of OVA or

PBS on days9, 10, 11, 13, 15 and 17 in sensitized mice. After 4, 24 or 48 h after the last challenge, mice were submitted to terminal anesthesia following *i.p.* administration of a ketamine/xylazine solution (20 µL/kg), and then, the trachea was cannulated and lungs washed two times with 1 mL of PBS for collection of BALF. BALF was centrifuged at 100 g for 5 minutes at 4°C, and the pellets were used for total cell and differential cell counts as previously described (Matos *et al*., 2014), and lungs were removed for further eosinophil peroxidase and myeloperoxidase assays, histopathological and immunohistochemical analysis.

Assessment of respiratory mechanics

Another group of sensitized mice was utilized to investigate the effects of PAR2-blockade on the respiratory mechanics. ENMD1068 was administered as previously described and 48 h after the last challenge, mice were anesthetized via a subcutaneous injection of ketamine (130 mg/kg) and xylazine (8.5 mg/kg) to maintain spontaneous breathing under anesthesia, tracheostomized, placed in a body plethysmograph and connected to a computer-controlled ventilator (Forced Pulmonary Maneuver System©, Buxco Research Systems©, Wilmington, North Carolina, USA).This spirometer provides, semi-automatically, three different maneuvers, Boyle's Law FRC, quasi-static pressure-volume and a fast-flow volume maneuver, as previously described (Nogueira *et al*., 2016). First, an average breathing frequency of 160 breaths/min was imposed on anesthetized mice by pressure-controlled ventilation until a regular breathing pattern and complete expiration at each breathing cycle were obtained, considering Rinx (index of rejection) = 0. Under mechanical respiration the Dynamic Compliance (Cdyn) and Lung Resistance (Rl) were determined by Resistance and Compliance RC test. To measure the Total Lung Capacity (TLC), the quasi-static Pressure-Volume maneuver was performed, which inflates the lungs to a standard pressure of + 30 cm H_2O and then slowly exhales until a negative pressure of -30 cm H_2O is reached. With the Fast-Flow Volume maneuver, lungs were first inflated to +30 cm H_2O and immediately after wards connected to a highly negative pressure in order to enforce expiration until -30 cm H_2O. The Flow-Volume curve was recorded during this maneuver. To evaluate airway hyperresponsiveness, the same mice used in previous maneuvers (basal level) received methacholine (1 mg/kg, intravenously), and 20 seconds later, a new set of maneuvers were conducted to obtain the Lung Resistance (Rl). Suboptimal maneuvers were rejected, and for each test in each mouse, at least three acceptable maneuvers were conducted to obtain a reliable mean for all the numeric parameters (Russo *et al*., 2018).

Eosinophil peroxidase and neutrophil myeloperoxidase assays

After BALF collection, the right lung of each mouse was perfused with 5 mL of saline, collected, and stored for measurement of the activities of eosinophil peroxidase (EPO) and neutrophil myeloperoxidase (MPO). The enzymes present in lung homogenates were measured as previously described (Nogueira *et al*., 2016).

Histopathological analysis

The left lungs were fixed in a 10% neutral solution of formalin and buffered with monobasic and dibasic sodium phosphate. The lungs were processed and embedded in paraffin, and 4-µm thick sections were obtained and stained with hematoxylin and eosin (H&E) or periodic acid of Schiff (PAS) for the detection of mucous secreted or with Gomori Trichrome for collagen detection. Histochemical analyses were performed for evaluation of the average of 10 images per mouse composed of areas containing bronchioles, vessels, and alveoli. Data from each animal were archived, analyzed, and expressed as the integrated density (intensity) in pixels. The area and intensity of the staining in pixels were determined using the software WCIF ImageJ (Media Cybernetics Manufacturing, Rockville, MD, USA). Inflammation scoring was performed from qualitative analysis of H&E slides (Horvat *et al*., 2007; Garcia *et al*., 2010) to evaluate airway vascular and parenchymal inflammation and neutrophilic and eosinophilic infiltration (0, absent; 1, minimal; 2, slight; 3, moderate; 4, marked; and 5, severe). The scoring was performed by two different and independent investigators (Supplemental Methods Section, Supplementary Table SI).

Immunohistochemical analysis of CCL5

Four-micron sections of the left lungs were obtained from paraffin blocks, transferred to gelatinized slides, and then deparaffinized and rehydrated. Antigen retrieval was performed using antigen retrieval solution (Dako, USA). Peroxidase blockade was performed with 10% hydrogen peroxide diluted in methyl alcohol (3 times, 5 min each) and protein block solution (Dako, USA). The primary antibody CCL5 was diluted (1:500) and added to the incubation sections in a humid chamber overnight at 4°C. The signal amplification and development were performed with the NovoLink™ polymer-based system (Leica Biosystems, Newcastle, UK) and developed with a chromogenic solution of 3,3'-diaminobenzidine (DAB, Leica Biosystems, UK). Negative controls were obtained by the omission of primary antibodies, and immunohistochemical analysis performed under a light microscope by two different pathologists who were blinded to the experiment. CCL5 scoring was performed from qualitative analysis of the slides to evaluated epithelium of the airways, inflammatory cells, parenchyma and overall expression(Supplemental Methods Section, Supplementary Table SII).

Statistical analysis

The results were analyzed using GraphPad Prism 5.0 and are expressed as the mean ± SEM. Statistically significant differences among groups were calculated by analysis of variance (ANOVA), followed by the Student-Newman-Keuls post test, with the level of significance set at $p < 0.05$. To analyze the inflammation score, data were analyzed using one-way ANOVA, followed by the Kruskal-Wallis test and Dunn's multiple comparisons posttest; $p < 0.05$ was considered significant. To analyze the immunohistochemistry score, the comparison between the groups was analyzed using Mann-Whitney test, $p < 0.05$ was considered significant.

RESULTS

The initial experiments were designed to test the impact of the PAR2 antagonist ENDM1068 on leukocyte recruitment into BALF and on inflammation in a mice model of allergic asthma induced by ovalbumin. Multiple challenges with OVA instillation induced the recruitment of neutrophils and eosinophils 4 or 24 h after intranasal challenge, peaking at 4 h for neutrophils and 48 h for eosinophils and mononuclear cells compared to PBS-instilled mice. The PAR2 antagonist ENMD1068 inhibited recruitment of eosinophils in BALF of OVA-instilled mice 4, 24 or 48 h after the last OVA challenge; however, ENMD1068 treatment inhibited neutrophil recruitment only 48 h after antigen challenge. Indeed, ENMD1068 also impaired the accumulation of total cells in BALF (Table I, Figure 1A).

TABLE I - Total and differential cell counts (x 10 5 cells) in BALF 4h or 24 h after i.n. instillation of OVA in PAR-2 antagonist ENMD1068-treated sensitized mice

		Treatment groups		
		PBS i.p.	**PBS i.p.**	**ENMD1068 i.p.**
		PBS i.n.	**OVA 10 µg i.n.**	
4 h	Eosinophils	0.00 ± 0.00	0.2 ± 0.05***	0.1 ± 0.03$^{\#}$
	Neutrophils	0.02 ± 0.01	1.8 ± 0.2***	1.5 ± 0.3
	Mononuclear cells	4.0 ± 0.2	2.7 ± 0.2**	2.8 ± 0.3**
	Total	4.1 ± 0.2	4.84 ± 0.4	4.4 ± 0.4

TABLE I - Total and differential cell counts (x 10 5 cells) in BALF 4h or 24 h after i.n. instillation of OVA in PAR-2 antagonist ENMD1068-treated sensitized mice

		Treatment groups		
		PBS i.p.	**PBS i.p.**	**ENMD1068 i.p.**
		PBS i.n.	**OVA 10 µg i.n.**	
24 h	Eosinophils	0.02 ± 0.01	2.4 ± 0.3***	1.0 ± 0.1$^{###}$
	Neutrophils	0.01 ± 0.01	0.4 ± 0.1**	0.4 ± 0.05
	Mononuclear cells	4.4 ± 0.2	3.2 ± 0.3*	3.8 ± 0.4
	Total	4.4 ± 0.2	6.0 ± 0.4*	5.2 ± 0.5

Ovalbumin-sensitized mice were instilled with PBS, OVA or pretreated with ENMD1068 1 h before intranasal instillation of OVA, and cells were harvested 4 or 24 h after the last intranasal allergen challenge. The results are expressed as the mean ± SEM of n= 5-7 mice/group; *p<0.05 **p<0.01 ***p<0.001 represents a significant difference between OVA or ENMD1068 group when compared to control group (PBS i.n.); $^{#}$p<0.05,$^{###}$p<0.001 represent a significant difference between OVA group when compared to ENMD1068.

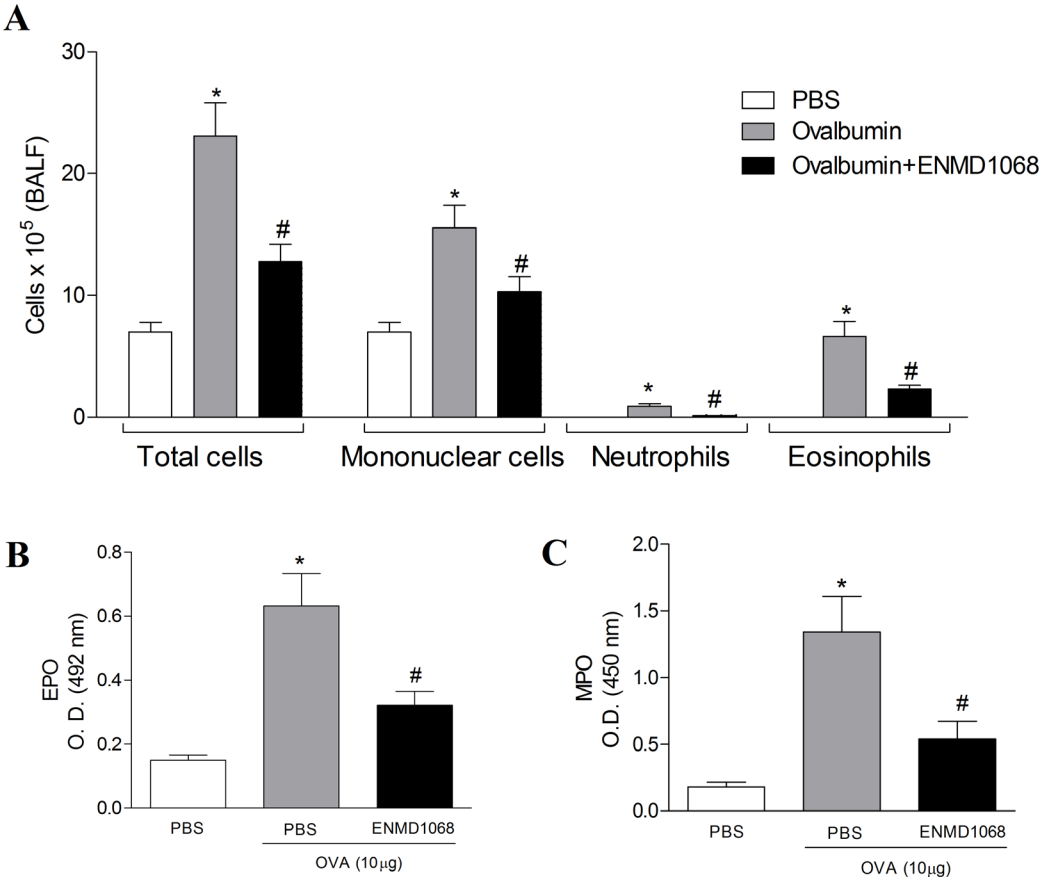

FIGURE 1 - PAR2 antagonist ENMD1068 inhibits antigen-induced cellular recruitment and eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activity in the lungs of sensitized mice after multiple exposures to an antigen. Infiltrating leukocytes (A), EPO (B) and MPO (C) measurement in BALF 48 hours after the last intranasal allergen challenge in mice. The results are representative of 2 independent experiments and are expressed as the mean ± S.E.M. of n= 6-8 mice/group. *p<0.001 compared to the PBS group (white bar), #p<0.05:ENMD1068 group when compared to OVA group (ANOVA followed by the Student-Newman-Keuls test).

Eosinophils and neutrophils are polymorphonuclear which are characterized by the presence of enzymes in the cytoplasm such as EPO and MPO successively, and an excess of enzymes release can lead to tissue damage resulting in chronic inflammation. As shown in Figures 1B and 1C, the activities of EPO and MPO significantly increased in lung homogenates 48 h after *i.n.* OVA challenge, whereas PAR2 antagonist treatment strongly reduces this activity. In relation to the histopathological analysis, we observed absence of inflammation in the lungs obtained from PBS-instilled mice (Figure 2A), while the lungs obtained from OVA-instilled mice had perivascular and peribronchiolar inflammation with leukocyte infiltration (Figure 2B). However, 48 h after antigen challenge the treatment with the PAR2 antagonist ENMD1068 did not change the histopathological parameters, as assessed by the inflammatory score (Figure 2D).

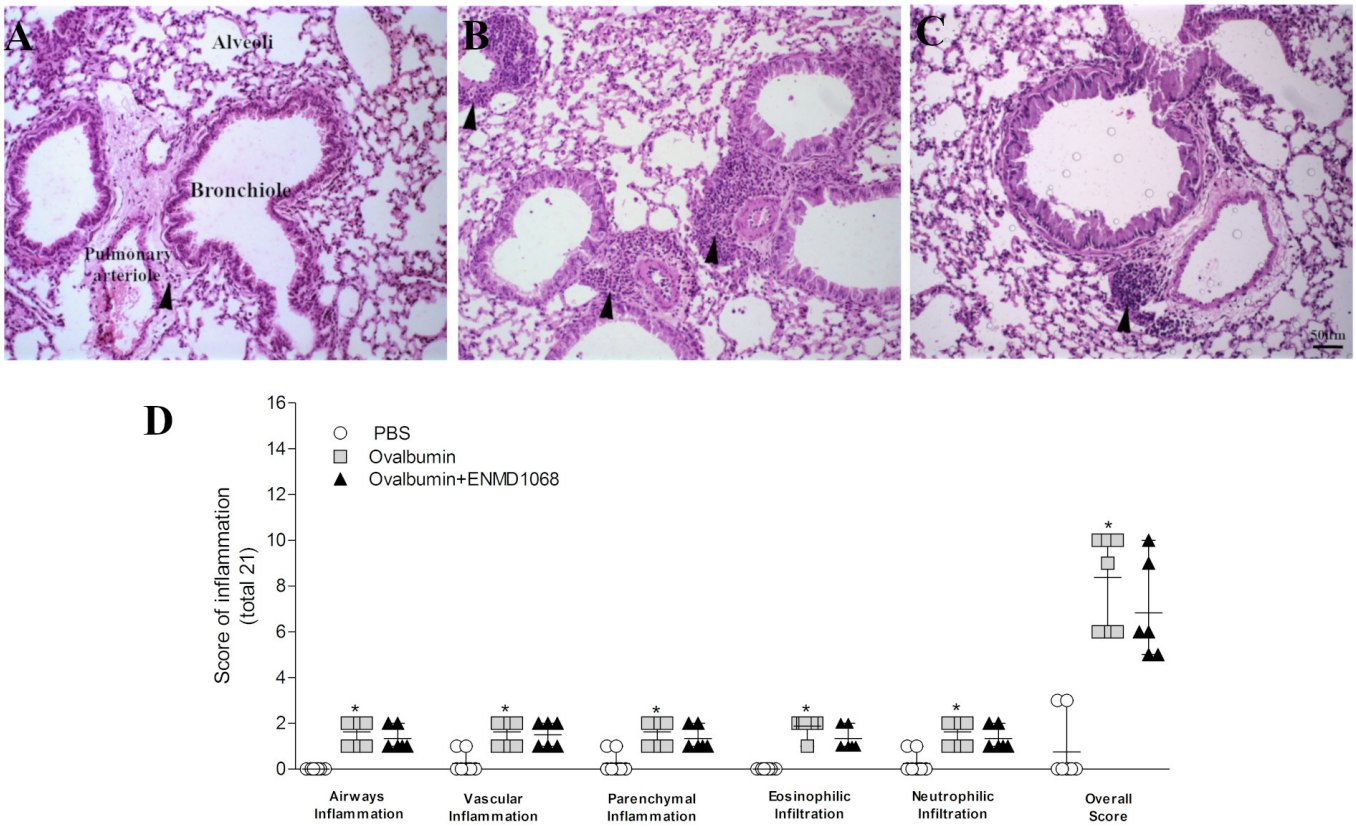

FIGURE 2 - Effects of PAR2 peptide antagonist ENMD1068 treatment on the histological changes in the lungs of sensitized mice by multiple exposures to OVA. Representative histological sections of lungs of mice 48 hours after the last intranasal allergen challenge and stained by hematoxylin and eosin. The black point arrows indicate inflammatory cell infiltration around bronchioles and vessels in ovalbumin-sensitized mice instilled with PBS (A), OVA (B) or pretreated with ENMD1068 1 h before intranasal instillation of OVA (C). Histopathological score of inflammation (D). The results are representative of 2 independent experiments and are expressed as the mean ± SEM of 6-8 mice/group. * $p<0.05$ compared to the PBS group (Kruskal-Wallis test followed by Dunn's multiple comparison test).

Pulmonary mechanics has been performed 48 hours after the last allergen *i.n.* challenge. In this experimental setting, pulmonary dysfunction was evident after OVA challenge in sensitized mice and it was demonstrated by progressive reduction of lung elasticity, as seen by progressive reduction in Dynamic compliance (Figure 3A), marked reduction of total lung capacity (Figure 3B), gain of lung resistance in OVA-instilled mice (Figure 3C), and the reduced airflow was aggravated in response to muscarinic agonist methacholine (Figure 3D). Overall, allergen challenge promoted a significant reduction in lung

volumes and airway flow (Figure 3E) and all these mechanic functional changes were less intense in PAR2 antagonist ENMD1068-treated mice, suggesting a protective role against changes in respiratory patterns.

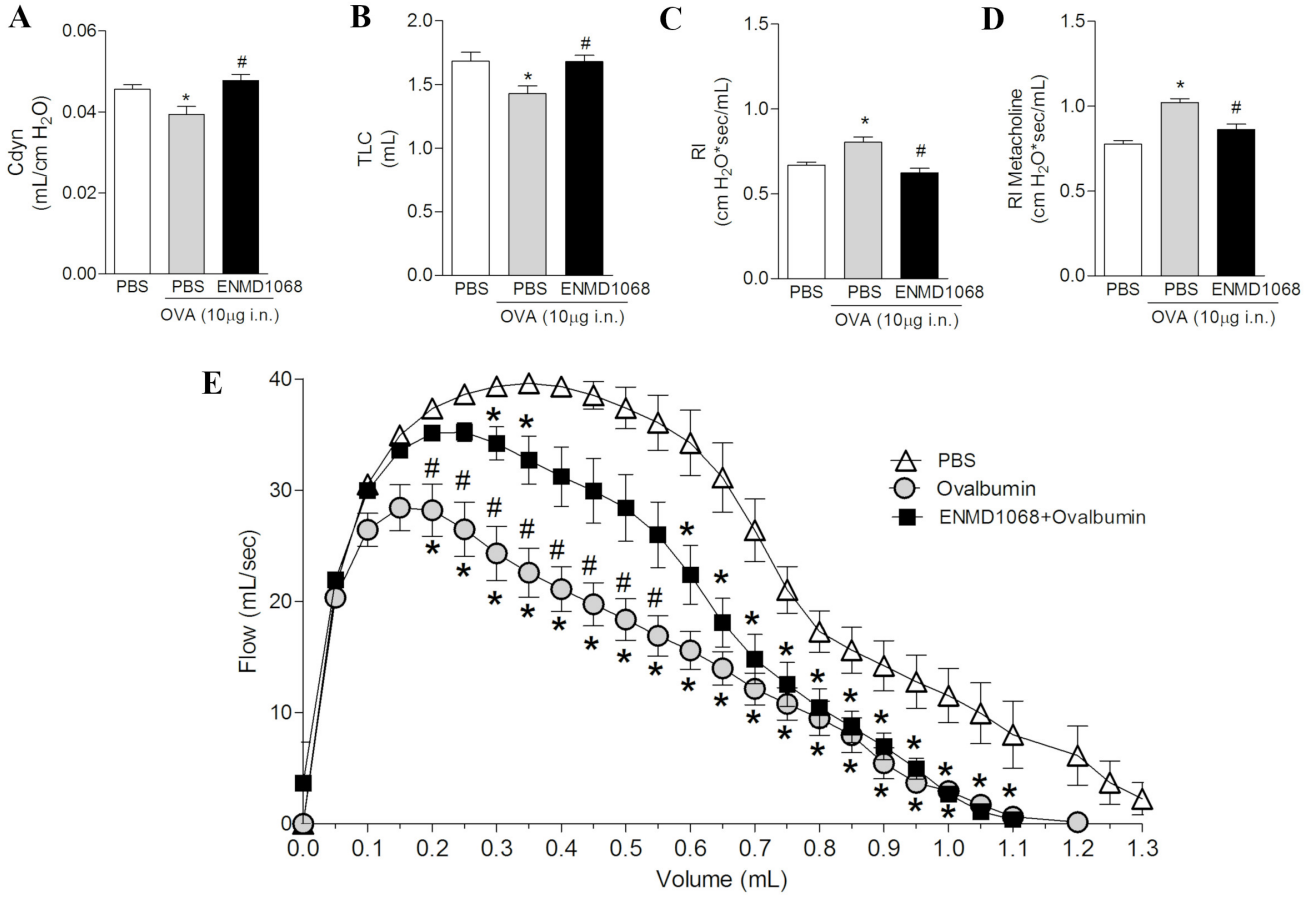

FIGURE 3 - PAR2 blockade modulates pulmonary function in mice. Effects of PAR2 antagonist ENMD1068 (0.5 mg/kg) treatment on the parameters of respiratory mechanics 48 hours after the last intranasal allergen challenge. (A) Cdyn: dynamic compliance; (B) TLC: Total lung capacity; (C) RI: lung resistance; (D) RI methacholine: Bronchoprovocation's test using methacholine (1 mg/kg); (E) flow-volume curve. The results are representative of 2 independent experiments and are expressed as the mean ± SEM of 6-8 mice/group. *$p<0.05$ compared to the PBS group and $^{\#}p<0.05$ ENMD1068 group when compared to OVA group (ANOVA followed by the Student-Newman-Keuls test).

The impact of PAR2 blockade on tissue remodeling following lung inflammation was determined following lung histopathological analysis. We investigated the effects of PAR2 blockade on mucous and collagen production by staining lungs with PAS to detect mucous substances, such as glycoproteins, glycolipids and mucins, in tissues or by staining lungs with Gomori trichrome to detect areas that contained collagen. Lungs obtained from PBS-instilled mice were not reactive to PAS (Figure 4A) and were weakly reactive to Gomori trichome, exhibiting mild staining in the perivascular region due to the fibrous connective tissue present there (Figure 4E). On the other hand, lungs removed from OVA-instilled mice were highly reactive to PAS, with staining of the bronchial epithelium a purple-magenta color (Figure 4B), and a slight increase in collagen fibers was observed to Gomori trichome, with staining of regions with collagen fibers, most of which were interspersed in the pulmonary parenchyma in perivascular and peribronchial regions (Figure 4F). Lungs obtained from sensitized mice pretreated with ENMD1068 before *i.n.* instillation of OVA showed reduced production of mucopolysaccharide (Figure 4C)

and few regions stained by Gomori's trichrome, which were partially interspersed in perivascular areas (Figure 4G). Importantly, ENMD1068 reduced the intensity of the PAS-stained mucus (Figure 4D) and Gomori trichrome stain to identify the collagen fibers (Figure 4H) when compared to OVA-instilled mice.

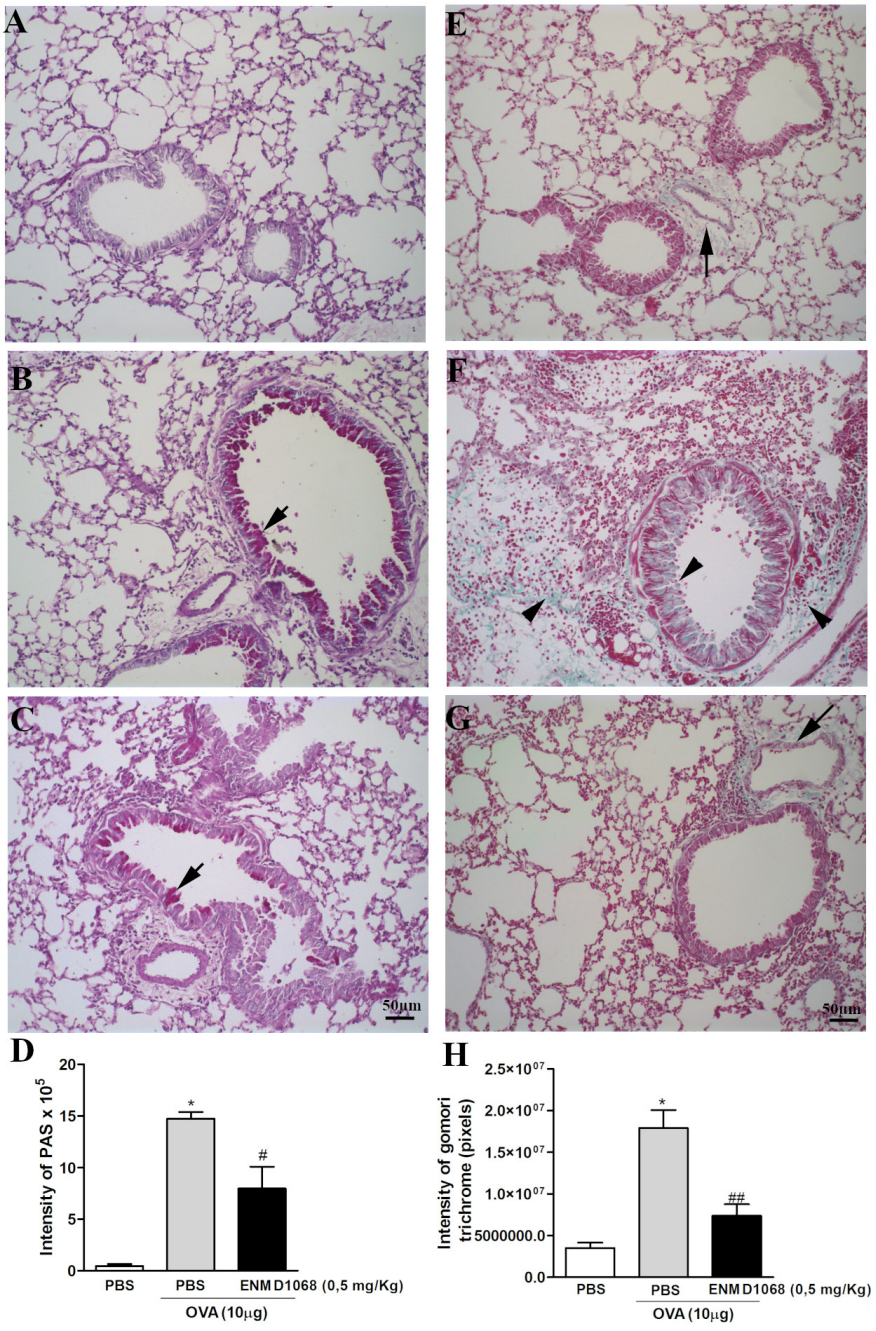

FIGURE 4 - Effects of PAR2 peptide antagonist ENMD1068 treatment on mucus and collagen production in the lungs of sensitized mice by multiple exposures to OVA. Representative histological sections of lungs of mice 48 hours after the last intranasal allergen challenge. Ovalbumin-sensitized mice were instilled with PBS (A, E), OVA (B, F) or pretreated with ENMD1068 1 h before intranasal instillation of OVA (C, G).Lungs stained by PAS, black arrows show vesicles releasing mucous and mucous accumulation in the lung parenchyma (A-C).Lungs stained by Gomori Trichrome, black arrow points show collagen deposition in the bronchial epithelium and lung parenchyma (E-G).PAS (D) and Gomori Trichrome (H) staining in lung tissue. The results are representative of 2 independent experiments and are expressed as the mean ± SEM of 6-8 mice/group. *$p<0.001$ compared to the PBS group, $^{\#}p<0.05$ $^{\#\#}p<0.001$ ENMD1068 group when compared to OVA group (one-way ANOVA / Newman-Keuls).

Because the chemokine CCL5 is well known to be an eosinophil chemoattractant and plays a key role in epithelial biology and mucosal immunity, we investigated the effects of the PAR2 antagonist ENMD1068 on its production in lungs of mice with OVA-mediated allergic inflammation. Lungs from PBS-instilled sensitized mice were negative for CCL5 in the bronchial epithelium and lung parenchyma, while lungs from OVA-challenged allergic mice were highly positive for CCL5 (Figure 5A, 5C). PAR2 antagonist-treated mice had low CCL5 expression levels in the monocyte cytoplasm and perivascular and peribronchiolar regions (Figure 5B, 5D). Histological scores demonstrated that ENMD1068-treated mice exhibited less CCL5 expression in the epithelium of the airways and inflammatory cells than OVA-challenged mice, however no difference was observed in the parenchyma (Figure 5E). In general, CCL5 expression was significantly lower in ENMD1068-treated mice when compared to OVA-challenged mice (Figure 5E, total score).

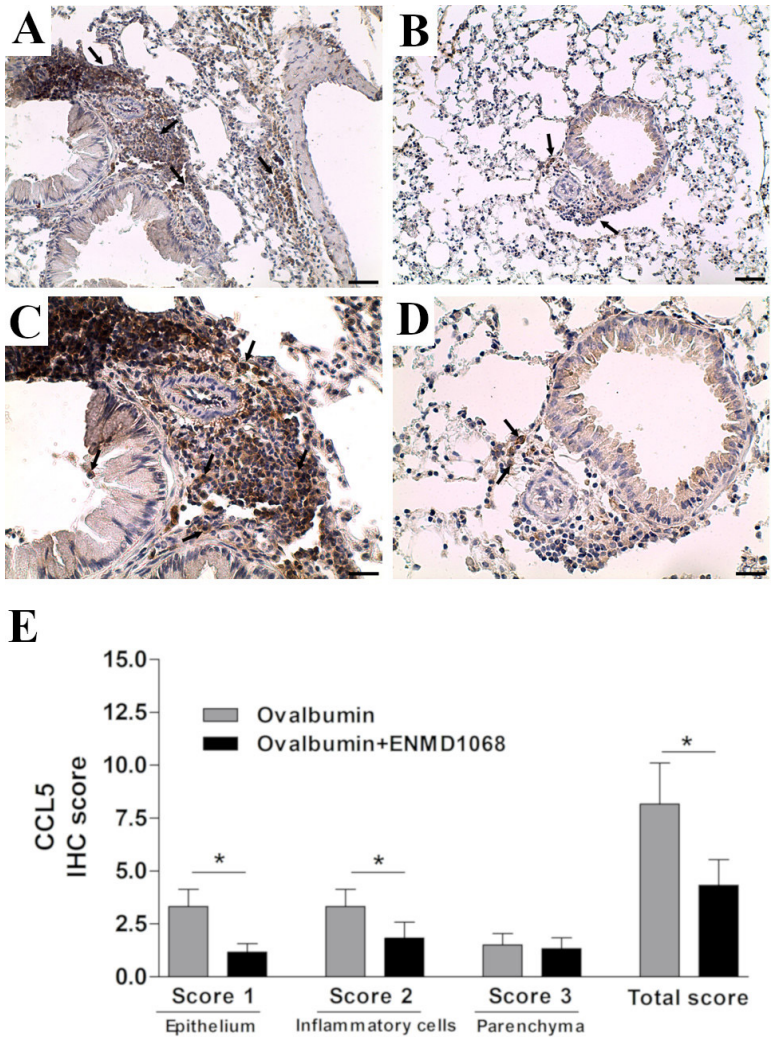

FIGURE 5 - CCL5 localization in lung following PAR2 peptide antagonist ENMD1068 administration after multiple exposures to OVA in lungs of sensitized mice. Representative histological sections of lungs of mice immunostained for CCL5 expression 48 hours after the last intranasal allergen challenge. Ovalbumin-sensitized mice were instilled with OVA (A, C) or pretreated with ENMD1068 1 h before intranasal instillation of OVA (B, D). Pictures were taken under a 20X objective (A, B) and insertion was evaluated under 40X objective (C, D), scale bar = 50 µm. The black arrows indicate CCL5 expression in peribronchial regions, and black head arrows indicate CCL5 expression on inflammatory cells. Immunohistochemistry (ICH) score (E). The results are representative of two independent experiments with similar results, and were expressed as the mean ± standard deviation, n= 6 mice/group. *$p<0.05$ based on Mann-Whitney test.

DISCUSSION

Allergic airway inflammation is triggered by a broad spectrum of allergens, including pollens, house dust mite allergens and cockroach allergens. Inhaled allergens that act on epithelial cells, lymphocytes, mast cells and alveolar macrophages promote a microenvironment with PAR2 activation by allergen-associated proteases (e.g., neutrophil elastase, MC tryptase), which are released by resident and infiltrating cells, such as mast cells, macrophages, neutrophils and eosinophils, and contribute to the establishment and progression of allergic diseases. Although it has been well established that PAR2plays a role in allergen-mediated airway inflammation and hyperresponsiveness (Asaduzzaman *et al.*, 2015), the efficacy of PAR2 antagonists in allergen-induced airway remodeling in asthma is still not fully understood. In the present study, we described a role for PAR2 in allergen-mediated lung inflammation and airway remodeling in a mice model of allergic asthma induced by ovalbumin.

We have previously shown that the endogenous PAR2 agonist MC tryptase mediates eosinophil recruitment and that PAR2 blockade impairs the eosinophil recruitment induced by the chemokine CCL-11 or OVA to the pleural cavity of sensitized mice in a short-term model of allergic inflammation (Matos *et al.*, 2013, Matos *et al.*, 2014).In the present study, a model of six intranasal allergen challenges was developed in consideration of generating airway inflammation through continuous allergen exposure as well as chronic treatment with the PAR2 antagonist ENMD1068 administered before each allergen challenge. In this model, intranasal instillation of OVA in sensitized mice induced eosinophil recruitment recovered in BALF peaked 48 h after the OVA challenge compared to that in PBS-instilled mice. PAR2 activation mediates these inflammatory events after allergen challenge because PAR2 blockade byENMD1068 reduced the number of eosinophils recovered in BALF by 50%, 58%, and 65% at 4, 24 or 48 h after the last challenge with OVA compared to those in OVA-instilled mice.

Neutrophils are the first blood leukocytes found in inflamed tissue and remain there for a few hours after exposure to inflammatory stimuli; however, in our study, neutrophils were found in BALF 48 h after the last allergen challenge and were reduced in PAR2 antagonist ENMD1068-treated mice. A possible hypothesis to the maintenance of neutrophils for 48 h is that the inflammatory milieu was triggered by continuous exposure to allergens in the airways of mice, leading to a continuous production of inflammatory mediators and neutrophil chemoattractants by alveolar macrophages and mononuclear, epithelial and mast cells, which in turn contributed to the maintenance of the inflammatory process, including inflammatory edema and leukocyte infiltration of the tissue. PAR2-activating proteases increase the expression of ICAM-1 on the endothelium enhancing the adhesion of lymphocytes to arteries and the production of reactive oxygen species (Lim *et al.*, 2006) activate both calcium and MAPKinase pathway (Polley *et al.*, 2017) as well as a G-protein-independent mechanism mediated by the β-arrestin pathway leading to leukocyte migration in the airway (Nichols *et al.*, 2012).We hypothesized that PAR2 blockade in the allergic milieu impairs both PAR2 pathways, impairing leukocyte infiltration and plasma protein extravasation in allergen-mediated asthma, although its effects on the expression of adhesion molecules in endothelial cells in microcirculation and on the production of chemoattractants should also be considered.

In support of our BALF results, blockade of PAR2 also inhibited the pulmonary enzymatic activities of EPO and MPO 48 h after OVA challenge in the lungs of mice, indicating impaired activity of eosinophils and neutrophils in the lungs of ENMD1068-treated mice. Indeed, there are evidence suggesting increased EPO and MPO activity, and neutrophilic inflammation maybe associated with the severe persistent asthma (Pritam *et al.*, 2021; Ray, Kolls, 2017). Once in the airway, infiltrating leukocytes contribute to the development of inflammation and airway remodeling, leading to a persistent airflow limitation, decreased lung function, and airway hyperresponsiveness, all of which contribute to the pathogenesis of asthma and may lead to failure in corticosteroid therapy (Mogensen *et al.*, 2019; Al-Muhsen, Johnson, Hamid, 2011). Thus, we investigated the impact of PAR2 blockade on the histological changes, pulmonary mechanics, mucous and collagen deposition in the lungs of OVA-challenged mice. When analyzed 48 h

after the last intranasal allergen challenge and compared to PBS-instilled mice, OVA-challenged mice had impaired total lung volume and lung extension, and the reduced airflow was aggravated in response to methacholine. PAR2 blockade restored these parameters to PBS-instilled mice levels. Interestingly, Koga *et al.* (2013) showed that pretreatment of allergen-induced asthma in mice with a neutrophil elastase inhibitor significantly reduced methacholine-induced hyperresponsiveness in the airway and leukocyte infiltration of the airway in OVA-challenged mice. Mucous deposition in the airway is one of the pathological conditions that contribute to the high airflow resistance and obstruction, as well as changes of the bronchial smooth muscle observed in asthmatics (Dunican *et al.*, 2018), and collagen deposition in the airway is associated with fibrosis in the peribronchiolar and perivascular regions, which ranges from mild to moderate in intensity according to the amount of collagen deposition. Indeed, fibrosis significantly contributes to chronic asthma, leading to pulmonary insufficiency (Royce *et al.*, 2012). Here, we demonstrated that blockade of PAR2 significantly decreased the synthesis of mucopolysaccharides by bronchial epithelial cells and mucous deposition in the pulmonary parenchyma and reduced collagen production in the bronchial epithelium and bronchial and inflammatory cells in a in a mice model of allergic asthma.

Although bronchodilators and corticosteroids have been used to alleviate the respiratory allergic symptoms, clinical studies have not shown an effective treatment to repair all structural changes and improve the lung function (Ito *et al.*, 2019). However, studies have demonstrated that PAR2 plays a role in the progression of pulmonary fibrosis; for example, a soy isoflavone, reduced bleomycin-induced PAR2 expression in an experimental model of pulmonary fibrosis in rats as well as the expression of the pro-fibrotic factor TGF-β (Soumyakrishnan *et al.*, 2014).Evidences suggest that PAR2 plays an important role in collagen deposition, and ENMD1068, contributing to a reduction in hepatic collagen content and histological fibrosis accompanied by decreasing hepatic cells activation in culture of primary mouse (Sun *et al.*, 2019). More recently, Asaduzzaman *et al.* (2018) demonstrated that an anti-PAR2 antibody prevented collagen accumulation in lung tissue in a long-term model of cockroach-dependent allergic airway inflammation. It is known that excessive collagen deposition and mucus hypersecretion are evident in airway remodeling in many chronic asthmatics (Kenyon *et al.*, 2003) and may impact the worsening of lung function and decrease the lung elasticity (Ito *et al.*, 2019). In the present study, PAR2 blockade impaired collagen deposition and leads to a reduction of airway remodeling, as observed in our mechanics results.

Among the genes identified as candidates for susceptibility to the development of asthma, the eosinophil chemoattractant chemokine CCL5 has been directly associated with epithelial biology, and mucosal immunity plays a role in the maintenance of tissue injury and scar formation (Vercelli, 2008). In this allergic model of asthma, there is high labeling of CCL5 in allergen-challenged mice in the bronchial epithelium, perivascular and peribronchiolar infiltrates, and the cytoplasm of mononuclear cells, specifically macrophages and lymphocytes. Because PAR2 blockade impairs CCL5 labeling in macrophages and lymphocytes and CCL5 mediates eosinophil recruitment to inflamed airways (Venge *et al.*, 1996) we suggest that PAR2 plays a role in the pathophysiology of allergen airway inflammation, at least in part through CCL5 released from these inflammatory cells (Kawabata *et al.*, 1998), contributing to the pathophysiology of allergen airway inflammation.

CONCLUSION

Together, our data favor the hypothesis that the local release of PAR2 activating proteases following allergen challenge may activate PAR2 in lung and airway cells, including mast cells and macrophages, leading to IgE-mediated release of inflammatory mediators in the airways of mice. The mediators released, in turn, drive the recruitment of leukocytes, airway remodeling and bronchial hyperresponsiveness. BecausePAR2 blockade may reduce the migration of leukocytes to the airway, airway vascular permeability, mucus hypersecretion, collagen deposition and CCL5 expression, a PAR2-based therapy may be useful as a new pharmacological approach for the treatment of airway allergic diseases.

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Declaration of interest statement/Conflict of interest

The authors declare that they have no of conflict of interest.

Authors' contributions

NAM planned and carried out the experiments, wrote the manuscript, and analyzed the data. DCR, LKR, and MSM carried out the experiments. RCR, GDC and ACP carried out the experiments and analyzed the data. AK conceived and planned the experiments, analyzed the data and wrote the manuscript.

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SUPPLEMENTAL METHODS SECTION

SUPPL. TABLE SI - Histopathological Scoring System for Mouse Lungs (maximum of 21) to evaluate airways, vascular and parenchymal inflammation, eosinophil and neutrophil infiltrating

Histopathological Scoring System for Mouse Lungs						
Points	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Total**
	Airway inflammation	**Vascular inflammation**	**Parenchymal inflammation**	**Eosinophil infiltrating**	**Neutrophil infiltrating**	
0	Lack of inflammatory cells around airways – Absent	Lack of inflammatory cells around vessels – Absent	< 1% affected	No eosinophilic inflammation around airways	No neutrophilic inflammation around airways	
1	Some airways have small numbers of cells – Mild	Some vessels have small numbers of cells - Mild	1-9% affected	Few eosinophils around airways	Few neutrophils around airways	
2	Some airways have significant inflammation – Moderate	Some vessels have significant inflammation - Moderate	10-29% affected	Some eosinophils in airways	Some neutrophils in airways	
3	Majority of airways have some inflammation – Marked	Majority of vessels have some inflammation – Marked	30-49% affected	Many eosinophils in airways	Many neutrophils in airways	
4	Majority of airways are significantly inflamed– Severe	Majority of vessels are significantly inflamed - Severe	50-69% affected	Airways are significantly inflamed	Airways are significantly inflamed	
5	-	-	> 70% affected	-	-	
Total	**4**	**4**	**5**	**4**	**4**	**21**

SUPPL. TABLE SII - Immunohistochemistry Scoring System for Mouse Lungs (maximum of 13) to evaluate airways, vascular, parenchymal and overall inflammation

Immunohistochemistry Scoring System for Mouse Lungs				
Points	**Score 1**	**Score 2**	**Score 3**	**Total**
	Epithelium of the airways	**Inflammatory cells**	**Parenchyma**	
0	Absence of expression in the airway epithelium – Absent	Absence of inflammatory cells with expression – Absent	< 1% affected	
1	Low expression in the airway epithelium – Mild	Few cells marked – Mild	1-9% affected	
2	Significant marking of airway epithelium – Moderate	Significant expression – Moderate	10-29% affected	
3	Most airways are marked - Marked	Most cells express – Marked	30-49% affected	
4	Airway epithelium is significantly reactive – Severe	Very intense expression – Severe	50-69% affected	
5	-	-	> 70% affected	
Total	**4**	**4**	**5**	**13**

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