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Blockade of protease-activated receptor 2 attenuates allergen-mediated acute lung inflammation and leukocyte recruitment in mice

Natália Alves de Matos[1,6], Onésia Cristina Oliveira Lima[2,3], Josiane Fernandes da Silva[2,4], Annie Rocio Piñeros[4,5], Juliana Carvalho Tavares[2], Virgínia Soares Lemos[2], José Carlos Alves-Filho[4] and André Klein[6]*

[1]*Department of Biological Sciences, Institute of Exact and Biological Sciences, Federal University of Ouro Preto (UFOP), Ouro Preto, Minas Gerais, Brazil*

[2]*Department of Physiology and Biophysics, Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil*

[3]*Neuropharmacology and Neurochemistry Laboratory, Department of Pharmacology, Federal University of Goiás (UFG), Goiânia, Goiás, Brazil*

[4]*Department of Pharmacology, Ribeirão Preto Medical School, University of Sao Paulo, São Paulo, Brazil*

[5]*Department of Pedriatics, Indiana University School of Medicine, Indianapolis, IN 46202, USA*

[6]*Department of Pharmacology, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil*

**Corresponding author (Email, klein@ufmg.br)*

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Protease-activated receptor (PAR)2 has been implicated in mediating allergic airway inflammation. We investigate the role of PAR2 in lung inflammation and neutrophil and eosinophil recruitment into the lungs in a mouse model of short-term acute allergic inflammation. Allergic lung inflammation was induced in sensitized BALB/c mice through intranasal instillations of ovalbumin (OVA), and mice were pretreated with the PAR2 antagonist ENMD1068 or with the PAR2-activating peptide (PAR2-AP) 1 hour before each OVA challenge. Bronchoalveolar lavage fluid (BALF) was collected, and the lungs, trachea and lymph nodes were removed after the last challenge to analyze the airway inflammation. PAR2 blockade reduced OVA-induced eosinophil and neutrophil counts, CXCL1, CCL5, amphiregulin, and interleukin (IL)-6 and 13 levels. Moreover, PAR2 blockade reduced OVA-induced PAR2 expression in cells present in BALF 2 hour after OVA challenge, and PAR2-AP acted synergistically with OVA promoting eosinophil recruitment into BALF and increased IL-4 and IL-13 levels in lymph nodes. Conversely, PAR2 blockade increased IL-10 levels when compared with OVA-treated mice. Our results provide evidence for a mechanism by which PAR2 meditates acute lung inflammation triggered by multiple exposures to allergen through a modulatory role on cytokine production and vascular permeability implicated in the lung diseases such as asthma.

Keywords. Protease-activated receptor 2; PAR2; lung inflammation; leukocyte recruitment; allergic inflammation

Abbreviations: PAR2, Protease-activated receptor 2; PAR2-AP, PAR2-activating peptide; OVA, Ovalbumin; BALF, Bronchoalveolar lavage fluid; AREG, Amphiregulin protein; CCL5, Chemokine (C-C motif) ligand 5; CXCL1, Chemokine (C-X-C motif) ligand 1; ENMD1068 (PAR2 antagonist), N1-3-methylbutyryl-N4-6-aminohexanoyl-piperazine); SLIGRL–NH_2 (PAR2-activating peptide), Ser-Leu-Ile-Gly-Arg-Leu-NH_2; LRGILS-NH_2 (peptide control), Leu-Arg-Gly-Ile-Leu-Ser-NH_2; i.n., Intranasal; i.p., Intraperitoneal; IL, Interleukin; ELISA, Enzyme-linked immunosorbent assay; *Par2*$^{-/-}$, PAR2 knockout mice.

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1. Introduction

Eosinophils appear to play an important role in the pathogenesis of allergic diseases. Typically, eosinophils are tissue dwelling cells, and in allergic disorders, an increased number of activated eosinophils is found in the submucosa and mucosa of affected tissues (Vu *et al.* 1991). These cells are recruited from blood vessels into the allergy inflamed tissue via the local production of eotaxin-1/C-C motif chemokine 11 (CCL11), leukotriene (LT)B_4, and interleukin (IL)-5 (Faccioli *et al.* 1996, 1991; Pope *et al.* 2005), produced by airway epithelial cells, macrophages, eosinophils, and mast cells (Cook *et al.* 1998; Martin *et al.* 1984). Neutrophils are prominent in acute inflammation, recruited from blood vessels into tissues in response to chemoattractants released at local sites of injury which directly act on their surface (Kobayashi 2008). In the inflammatory environment, neutrophil and eosinophil accumulation play a role in the progression of tissue damage through the release of proteases contained in their azurophil granules, lysosomal enzymes, reactive oxygen species, and cationic proteins, promoting lesions in surrounding tissues and contributing to the severity of inflammatory diseases, as well as playing an important role in the pathogenesis and severity of allergic and inflammatory diseases (Hosoki *et al.* 2016; Korkmaz *et al.* 2010; Rothenberg and Hogan 2006; Weiss 1989). Thus, elucidating mechanisms underlying eosinophil and neutrophil recruitment *in vivo* may aid in the development of novel strategies for the treatment of inflammatory and allergic diseases.

Protease-activated receptors (PARs) are a unique family of four G protein-coupled receptors (PAR 1-4), first described by Coughlin *et al.* in the early 1990s as the proteolytic receptor for thrombin (Vu *et al.* 1991), and activated through the proteolytic cleavage of a specific site in the N-terminus of the receptor, revealing a tethered ligand that binds to and activates the receptor (Hollenberg and Compton 2002). PARs can also may be activated independently of proteolytic unmasking of the tethered ligand sequence, through small synthetic peptides corresponding to the first five or six amino acids of the tethered ligand sequence (activator ligand), such as for PAR2 the human sequence SLIGKV-NH_2, and the more potent murine variant, SLIGRL-NH_2 (Hollenberg and Compton 2002). As opposite, blockade of PAR2 at activator ligand site, can be achieved through the use of selective antagonists, such as ENMD1068, a piperazine derivative, resulting in the inhibition of PAR2-induced calcium signaling on human and murine cells (Kelso *et al.* 2006).The role of proteinases and PAR activation in the development of inflammation has been widely investigated, and their activation has been implicated in the regulation of inflammatory events, including vascular endothelial cell activity (Hollenberg and Compton 2002; Vergnolle *et al.* 2002), *in vivo* leukocyte recruitment (Gomides *et al.* 2012) and cytokine release (Asokananthan *et al.* 2002).

PAR2 is widely expressed in airway cells such as endothelial and vascular smooth muscle cells, bronchial smooth muscle cells, airway epithelial cells and others (Adams *et al.* 2011; Bolton *et al.* 2003; Cocks and Moffatt 2001; Compton *et al.* 2000; Schmidlin *et al.* 2001), playing a major role in the pathophysiology of allergic airway inflammation (Ebeling *et al.* 2007). In this study, we investigated the effects of PAR2 blockade on lung inflammation and neutrophil and eosinophil recruitment into the lungs of mice in a mouse model of short-term acute allergic lung inflammation.

2. Materials and methods

2.1 *Animals*

Female BALB/c mice (8–10 weeks old, 18–25 g) were obtained from the Animal Care Facilities of the UFMG and housed in a temperature-controlled room with free access to commercial chow and water. All experimental procedures were subject to evaluation and were approved by the local Animal Ethics Committee (CETEA, certificate number 348 /2014).

2.2 *Drugs and reagents*

Ovalbumin (OVA, grade V) and aluminum hydroxide gel were obtained from Sigma-Aldrich (St. Louis, MO). The anti-PAR2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The PAR2 antagonist ENMD1068 (N1-3-methylbutyryl-N4-6-aminohexanoyl-piperazine), was purchased from Enzo Life Sciences (San Diego, CA, USA). The PAR2-activating peptide SLIGRL–NH_2 (H–Ser–Leu–Ile–Gly–Arg–Leu–NH_2), and PAR2 peptide control, LRGILS-NH_2, were purchased from Tocris Bioscience (Bristol, UK). ENMD1068, SLIGRL–NH_2, and LRGILS-NH_2 were dissolved in phosphate-buffered saline (PBS, pH 7.4) and all samples were stored at −20°C until use. Ketamine (Vetnil^{TM}) was purchased from Louveira

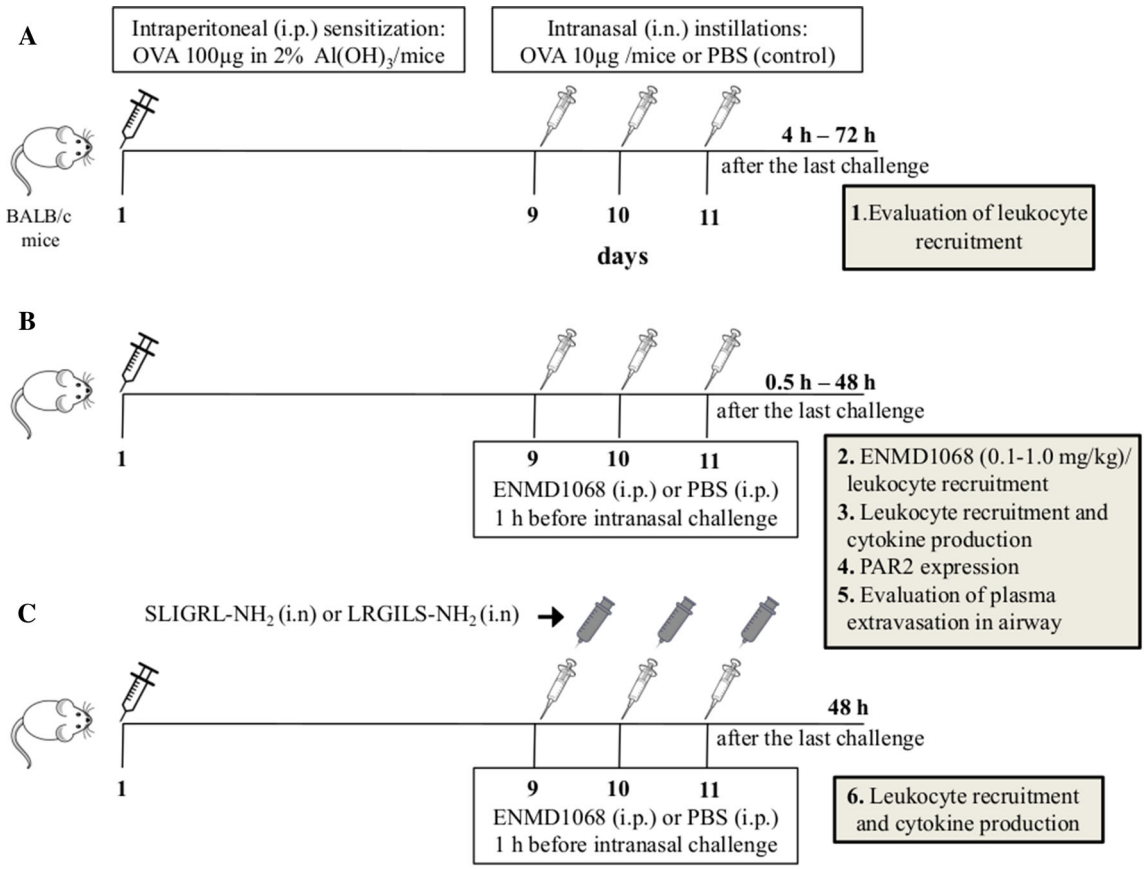

Figure 1. Immunization scheme and experimental protocols (1–6). Sensitization was performed by one intraperitoneal (i.p.) injection of OVA (100 µg) in 2% aluminum hydroxide gel $Al(OH)_3$. (**A**) On days 9, 10, and 11 post-sensitization, mice were challenged with intranasal instillation of PBS (20 µL/control) or OVA (10 µg/20 µL); 4–72 h after the last OVA challenge bronchoalveolar lavage fluid (1) was collected. (**B**) On days 9, 10, and 11 post-sensitization, mice were pretreated or not with the PAR2 antagonist ENMD1068 (0.1–1.0 mg/kg, i.p.) or PBS (i.p.) 11h before the intranasal instillation (i.n.) of PBS (20 µL), OVA (10 µg/20µL), 0.5–48 h after the last OVA challenge (according to each research protocol) leukocyte recruitment (2), inflammatory mediators (3), PAR2 expression (4) or airway vascular permeability (5) were evaluated. (**C**) On days 9, 10, and 11 post-sensitization, mice were pretreated or not with the PAR2-activating peptide SLIGRL–NH_2 (PAR2-AP, 30 µg/20 µL, i.n.), or PAR2 peptide control LRGILS-NH_2 (30 µg/20 µL, i.n.), or SLIGRL–NH_2 + OVA, leukocyte recruitment and cytokine production were evaluated 48 h after the last treatment (6).

(Brazil), and xylazine (Kensol™) from König (*Santana de Parnaíba*, Brazil). Enzyme-linked immunosorbent assay (ELISA) kits for detection of the murine chemokines (C-X-C motif) ligand 1 (CXCL1) and (C-C motif) ligand 5 (CCL5), as well as the amphiregulin protein (AREG), were obtained from R&D Systems (Rock Hill, NJ, USA). ELISA kits for interleukin (IL)-4, IL-10, and IL-13 were obtained from and eBioscience (Thermo Fisher Scientific, USA), and the IL-6 ELISA kit was obtained from BD Biosciences (NJ, USA).

2.3 *Sensitization*

Mice were sensitized with an intraperitoneal (i.p.) injection (200 µL) of OVA (100 µg) in 2% aluminum hydroxide gel as previously described (Kurowska-Stolarska *et al.* 2008). Control mice were injected (200 µL, i.p.) 2% aluminum hydroxide gel alone (non-sensitized/naïve mice). The protocol used in this study is shown in figure 1.

2.4 *Leukocyte migration induced by OVA or PAR2-activating peptide to the lungs of sensitized mice*

On days 9, 10, and 11 after sensitization, mice were anesthetized with i.p. ketamine (130 mg/kg) and xylazine (8.5 mg/kg) in a saline solution and challenged with intranasal instillation of PBS (20 µL), OVA (10 µg/20 µL), PAR2-activating peptide

SLIGRL–NH_2 (PAR2-AP, 30 μg/20 μL), or PAR2 peptide control LRGILS-NH_2 (30 μg/20 μL). On day 11, at 4, 24, 48, or 72 h after the last challenge with PBS or OVA, mice were anesthetized by i.p. administration of a solution of ketamine and xylazine, and a catheter was inserted into the trachea of each animal to collect the bronchoalveolar lavage fluid (BALF), and the lung was washed with 2 mL PBS (2 × 1000 μL). A Neubauer chamber was used to determine thetotal leukocyte counts using Turk's reagent, and differential cell counts were performed on cytospin preparations stained with May-Grünwald solution, using standard morphological criteria to identify cell types, with 100 cells were counted per slide as previously described (Klein *et al.* 2001). In some experiments, OVA-sensitized mice were challenged with the intranasal instillation of PAR2-AP (30 μg/30 μL) 1 h before intranasal OVA instillation and leukocytes present in the BALF harvested 48 h after the last challenge.

2.5 *Effects of PAR2 antagonist pretreatment on neutrophil and eosinophil recruitment induced by OVA*

To investigate the contribution of PAR2 in OVA-induced leukocyte recruitment, the PAR2 antagonist, ENMD1068 (0.1–1.0 mg/kg) or PBS (100 μL), were administered i.p. 1 h before the intranasal instillation of OVA (10 μg/20 μL) or PBS on days 9–11 in sensitized and naïve mice, respectively, and leukocytes were assessed 48 h after the last challenge. In BALF, neutrophils and eosinophils were harvested at 0.5, 2, 4, 8, and 12 h after the last challenge.

2.6 *Enzyme-linked immunosorbent assay (ELISA) for inflammatory mediators*

Frozen supernatants obtained from lymph node and lung homogenates were used to detect IL-4 and IL-13 inflammatory mediators 48 h after the last OVA challenge. Briefly, axillary lymph nodes were collected in the RPMI culture medium, and the supernatant was used for cytokine quantification by ELISA following the manufacturer recommendations for each kit (Alves-Filho *et al.* 2006). In other experiments, BALF was used to determine the concentrations of CXCL1, CCL5, AREG, IL-6, and IL-10, at 0.5, 2, 4, 8, and 12 h after the last OVA challenge. To collect samples, the mice were anesthetized, BALF was collected and centrifuged at 400 × g for 10 min, and the supernatant was recovered and used for cytokine quantification by ELISA.

2.7 *Western blot analysis of leukocyte PAR2 expression in OVA-injected mice*

PAR2 expression was evaluated in leukocytes recovered from collected BALF obtained at 2 or 4 h after intranasal instillation of OVA. The fluid recovered from the animals was pooled and centrifuged at 1300×g. Next, 0.3 mL of the lysis buffer containing 1% Triton X-100 and 0.5% sodium dodecyl sulfate, plus a cocktail of protease inhibitors (SigmaFAST), was added to the cell pellets. The lysate was centrifuged at 8000 g at room temperature, and the protein concentration was spectrophotometrically determined using the Bradford method. 30 μg of protein was transferred to a nitrocellulose membrane. The blots were blocked with 3% bovine serum albumin in TBS and 0.1% Tween-20 at 4°C overnight. The following primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used: goat anti-PAR2 polyclonal immunoglobulin (Ig)G (1:1000) and mouse monoclonal anti-β-actin (1:3000), and goat anti-mouse IgG-horseradish peroxidase (HRP) (1:8000) and rabbit anti-goat IgG-HRP (1:8000)were used as secondary antibodies. Immunocomplexes were detected using a chemiluminescent reaction (Luminata Western HRP Substrates, Millipore™), followed by densitometric analyses using the Image Quant software package. β-actin was used as a housekeeping control.

2.8 *Effects of PAR2 antagonist ENMD1068 on airway vascular permeability in OVA-injected mice*

To determine the impact of PAR2 blockade on OVA-induced vascular permeability, we measured the vascular extravasation of Evans blue dye into the lung and trachea parenchyma as a direct estimate of vascular permeability, as previously described (Lundberg *et al.* 1983), and the results are presented as the amount of Evans blue (pg) present in 100 mg of tissue.

2.9 *Statistical analysis*

Results are presented as the mean ± standard deviation (SD). Statistical analyses were performed using SigmaStat® 3.5 software (San Diego, CA, USA) and

GraphPad Prism version 5.0 (Windows 7, GraphPad Software, San Diego, CA, USA). Comparisons between groups were performed using One-way ANOVA, and differences between groups were assessed using Newman–Keuls post-test. $P < 0.05$ was considered significant.

3. Results

3.1 *PAR2 mediates allergen-induced eosinophil recruitment and cytokine production*

Intranasal instillation of OVA induced time-dependent eosinophil recruitment to the lungs of OVA-sensitized mice, peaking 48 h after the intranasal OVA challenge (figures 1A [1] and 2A). In contrast, eosinophil recruitment was not detected in mice injected with the adjuvant aluminum hydroxide alone, followed by the intranasal challenge of PBS or OVA (figure 2A). OVA instillation in the sensitized mice induced the recruitment of neutrophils and eosinophils at 4 h after intranasal challenge in BAL fluid (figure 1A [1]; table 1), and a significant increase in total cells was observed at 48 and 72 h after the last challenge when compared to PBS-instilled mice (sensitized and naive mice). Eosinophil recruitment after OVA challenge was indistinguishable at all the evaluated times, which peaked after 48 h, and pretreatment with the PAR2 antagonist ENMD1068 significantly inhibited eosinophil recruitment and total cells recovered in BALF at 48 h after intranasal instillation of OVA (figures 1B [2] and 2B; table 2).

Once ENMD1068 inhibited eosinophil recruitment induced by OVA in the lungs of sensitized mice, we examined whether PAR2-AP promoted the eosinophil recruitment (figure 1C). As shown in Figs. 3A–D, SLIGRL-NH_2 promoted leukocyte and neutrophil recruitment, acted synergistically with OVA to promote eosinophil recruitment into the lungs of sensitized mice at 48 h after allergen challenge, as well as increased IL-4 and IL-13 levels as measured in lymph nodes, when compared with the control group (figure 3E and F). Higher IL-13 level was observed in lung homogenates at 48 h after i.n. OVA challenge, whereas PAR2 antagonist treatment strongly reduced these levels (figure 3H). For IL-4, no significant differences were observed between the experimental groups (figure 3G).

3.2 *PAR2 mediates allergen-induced neutrophil recruitment*

Given that neutrophils contribute to the pathophysiology of inflammatory diseases and have demonstrated a role for PAR2 in OVA-induced lung inflammation in sensitized mice, we investigated whether the blockade of PAR2 would interfere with the recruitment of BAL neutrophils in OVA-instilled mice (figure 1B [3]). OVA induced time-dependent neutrophil and eosinophil

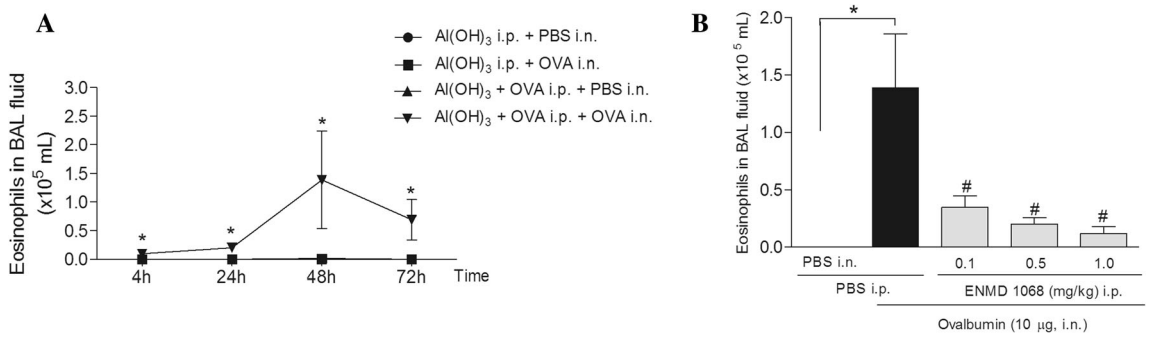

Figure 2. Effects of PAR2 antagonist ENMD1068 on eosinophil recruitment by OVA. OVA-sensitized and non-sensitized/naïve mice were challenged by intranasal (i.n.) instillations of PBS or OVA (10 µg), and cells present in BAL fluid were harvested at 4, 24, 48 or 72 h after last challenge. Data are expressed as mean ± standard deviation (SD) of 4–6 mice/group (**A**). Ovalbumin-sensitized mice were instilled with PBS, OVA, or pretreated with ENMD1068 (0.1–1.0mg/kg) 1 h before i.n. instillation of OVA. Infiltrating eosinophils were measured in BALF 48h after the last intranasal allergen challenge in mice. Data are expressed as mean ± SD of 5–6 mice/group (**B**). One-way ANOVA followed by Newman–Keuls post hoc test was used for comparison of eosinophil recruitment among the groups. In all analyses, statistical differences were considered significant at $p<0.05$. (A) *$p<0.001$ OVA-treated mice and immunized with $Al(OH)_3$ instilled with OVA (▼) versus others groups. (B) *$p<0.001$ versus PBS-treated mice, #$p<0.001$ versus OVA-treated mice.

Table 1. Total and differential count of bronchoalveolar lavage fluid ($\times 10^5$mL) of experimental groups.

		Naïve mice $Al(OH)_3$ i.p.		Sensitized mice $Al(OH)_3$ + OVA i.p.	
Time		PBS i.n.	OVA i.n.	PBS i.n.	OVA i.n.
4 h	Eosinophils	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.04[a,b,c]
	Neutrophils	0.04±0.1	0.1±0.1	0.1±0.06	2.2±0.9[a,b,c]
	Mononuclear cells	8.5±4.1	9.5±6.6	5.0±1.5	4.1±1.2
	Total cells	8.6±4.1	9.6±6.4	5.1±1.6	6.4±1.8
24 h	Eosinophils	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.08[a,b,c]
	Neutrophils	0.04±0.06	0.07±0.07	0.03±0.05	0.3±0.3
	Mononuclear cells	6.7±1.7	5.7±2.0	5.0±1.6	6.1±0.9
	Total cells	6.8±1.7	5.8±2.0	5.0±1.6	6.6±0.9
48 h	Eosinophils	0.01±0.02	0.01±0.03	0.02±0.04	1.4±0.8[a,b,c]
	Neutrophils	0.05±0.07	0.06±0.06	0.2±0.2	0.2±0.1
	Mononuclear cells	6.4±2.2	5.9±1.8	6.2±3.1	12.2±6.7[c]
	Total cells	6.4±2.2	6.0±1.8	6.4±3.4	13.8±7.3[a,b,c]
72 h	Eosinophils	0.0±0.0	0.0±0.0	0.0±0.0	0.7±0.3[a,b,c]
	Neutrophils	0.01±0.03	0.03±0.07	0.0±0.0	0.08±0.1
	Mononuclear cells	4.7±1.8	5.3±2.1	4.7±0.9	12.0±2.4[a,b,c]
	Total cells	4.7±1.8	5.3±2.1	4.7±0.9	12.8±2.7[a,b,c]

Control mice (non-sensitized/naïve mice) and OVA-sensitized mice were challenged by i.n. multiple instillations of PBS or OVA (10 μg), and cells present in BAL fluid were harvested at 4, 24, 48, or 72 h after the last challenge. (a) Significant difference between the groups compared to naïve mice treated with PBS (b) Significant difference between the groups compared to naïve mice treated with OVA, and (c) Significant difference between the groups compared to sensitized mice instilled with OVA. Data are expressed as mean ± standard deviation and were analyzed by one-way ANOVA followed by Newman-Keuls post-test ($p<0.05$, n = 4–6 mice/group).

Table 2. Effects of PAR2 antagonist on total and differential count of bronchoalveolar lavage fluid ($\times 10^5$ mL) in sensitized mice.

	Sensitized mice: $Al(OH)_3$ + OVA i.p.				
		OVA i.n. (10 μg)			
			ENMD1068 i.p. (mg/kg)		
	PBS i.n. + PBS i.p	PBS i.p.	0.1	0.5	1
Eosinophils	0.0±0.0	1.4±0.2*	0.3±0.04[#]	0.2±0.02[#]	0.1±0.02[#]
Neutrophils	0.0±0.0	0.01±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Mononuclear cells	3.9±0.9	4.4±1.1	3.5±0.6	3.4±0.7	3.4±0.8
Total cells	3.9±0.9	5.8±1.3*	3.8±0.7[#]	3.6±0.7[#]	3.5±0.8[#]

OVA-sensitized cells were challenged by i.n. multiple instillations of PBS or OVA (10 μg), and cells present in BAL fluid were harvested at 48h after the last challenge.*$p<0.05$, compared to control group (PBS i.p. + PBS i.n.), #$p<0.05$, compared to OVA-treated mice (OVA i.n. + PBS i.p.). Data are expressed as mean ± standard deviation and were analyzed by one-way ANOVA followed by Newman-Keuls post-test ($p<0.05$, n = 5–6 mice/group).

recruitment into the lung of sensitized mice after the intranasal OVA challenge, and concerning neutrophils were dropped to baseline levels after 12 h. Pretreatment of OVA-sensitized mice with ENMD1068, before OVA instillation, potently inhibited neutrophil and eosinophil recruitment at 2, 4, 8 and 12 h following the intranasal OVA challenge (figure 4A and B).

3.3 *PAR2 modulates pro- and anti-inflammatory cytokine production in BALF*

Considering that the PAR2 antagonist ENMD1068 inhibited neutrophil and eosinophil recruitment in BALF of sensitized mice following the OVA challenge, and chemokines and pro-inflammatory cytokines

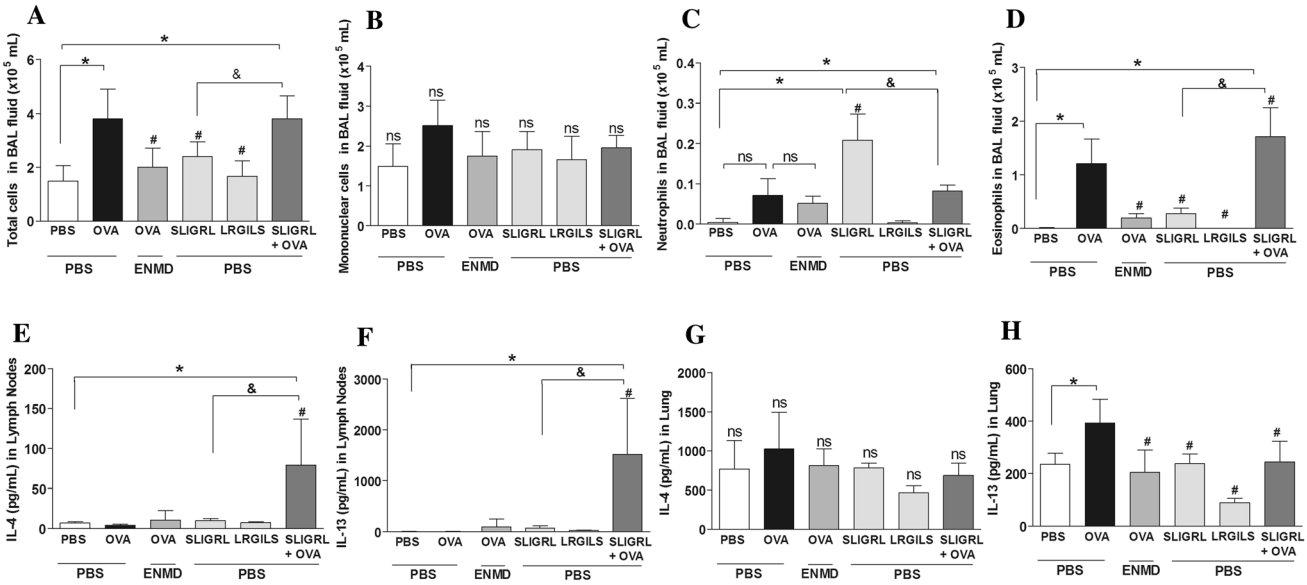

Figure 3. Effects of PAR2 antagonist ENMD1068 or PAR2-activating peptide (PAR2-AP) on the leukocyte recruitment and cytokine production induced by OVA. On days 9, 10, and 11 post-sensitization, OVA-sensitized mice were pretreated with PBS (i.p.) or PAR2 antagonist ENMD1068 (0.5 mg/kg, i.p.) 1 h before i.n. instillation with PBS (20 μL), or OVA (10 μg/20 μL), or PAR2-activating peptide SLIGRL–NH_2 (PAR2-AP, 30 μg/20 μL), or PAR2 peptide control LRGILS-NH_2 (30 μg/20 μL), or SLIGRL–NH_2 + OVA. 48 h after the last i.n. instillation BAL fluid was collected and leukocytes were harvested: (**A**) total cells, (**B**) mononuclear cells, (**C**) neutrophils and (D) eosinophils. Lymph nodes (**E** and **F**) and lungs (**G** and **H**) also were removed and used for determination of IL-4 and IL-13 using a commercially available ELISA kit, the cytokine levels correspond to an average obtained from triplicates for each animal representative of 2 independent experiments. (A–H) Data are expressed as the mean ± SD of 3–5 mice/group. One-way ANOVA followed by Newman–Keuls post hoc test was used for comparison among the groups. In all analyses, statistical differences were considered significant at $p<0.05$. *ns* indicates not significant ($p > 0.05$) whereas *$p<0.001$ versus the PBS-treated mice, #$p<0.001$ versus the OVA-treated mice, and &$p<0.001$ versus the SLIGRL-NH_2 treated mice.

CXCL1, CCL5, AREG and IL-6 play a major role in mediating the recruitment of neutrophils and eosinophils following an allergen challenge, we examined whether PAR2 plays a role in pulmonary allergic inflammation by modulating the release of cytokines and chemokines (figure 1B [3]). As shown in figure 4, in OVA-sensitized animals, the OVA challenge induced a significant increase in the levels of CXCL1, CCL5, AREG, and IL-6 in BALF, with maximal levels detected at 2 and 4 h, when compared with the PBS control, and ENMD1068 significantly inhibited this effect, specifically 2 h after the last OVA challenge (figure 4C–F). Interestingly, CXCL1 production, as well as their inhibition by the PAR2 antagonist ENMD1068, persisted for 12 h, while CCL5 and AREG returned to baseline levels by 8 h. Consistent with the ability of ENMD1068 to inhibit chemokines and pro-inflammatory cytokine release, PAR2 blockade by ENMD1068 induced an increase in the levels of the anti-inflammatory cytokine IL-10, detected in BALF of sensitized mice 4 h after intranasal OVA (figure 4G).

3.4 *PAR2 protein expression increases after antigen challenge*

PAR2 was highly and significantly expressed in mice 2 and 4 h after the intranasal OVA challenge when compared with PBS-instilled mice (figures 1B [4] and 5). Furthermore, the PAR2 antagonist ENMD1068 abrogated PAR2 expression at 2 h, but not 4 h after OVA challenge (figure 5).

3.5 *PAR2 blockade impairs OVA-induced airway inflammation*

To evaluate the impact of the pharmacological blockade of PAR2 on OVA-induced airway inflammation, we measured the vascular leakage of Evans blue dye into the lung and tracheal tissues 2 h after the last OVA challenge (figure 1B [5]). Pretreatment of sensitized mice with ENDM1068 (0.5 mg/kg) 1 h before OVA instillation, inhibited the OVA-induced increase in

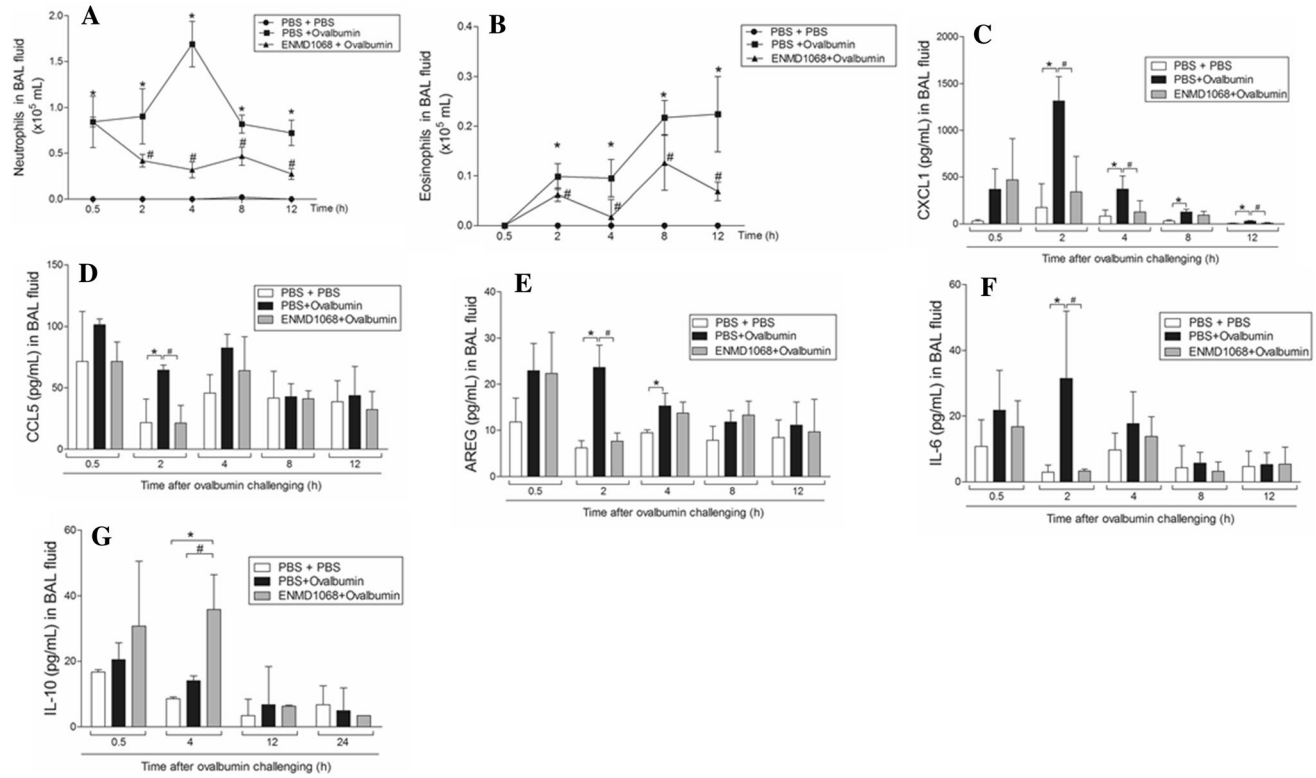

Figure 4. Time-course effects on the recruitment of neutrophils and eosinophils, and cytokine/chemokine production in lungs of OVA-instilled mice treated with PAR2 antagonist ENMD1068. Mice were immunized with OVA and pretreated with ENMD1068 (0.5 mg/kg) 1 h before the intranasal instillation of OVA (10 μg/20 μL) or PBS9, 10, 11 days after, and BAL fluid was collected and neutrophil (**A**) and eosinophil (**B**) recruitment assessed at 0.5, 2, 4, 8 and 12 h after last challenge. Data are expressed as the mean ± SD of 4–5 mice/group. The cells were removed by centrifugation, and the supernatants were used for determination of cytokines and chemokines using a commercially available ELISA kit. The cytokine levels correspond to an average obtained from triplicates for each animal representative of 2 independent experiments expressed as the mean ± SD of 3–4 mice/group (**C–G**). One-way ANOVA followed by Newman–Keuls post hoc test was used to assess comparisons among the groups. In all analyses, statistical differences were considered significant at $p<0.05$. *$p<0.05$ versus the PBS + PBS group #$p<0.05$ versus the PBS + OVA-treated mice (**A–G**).

vascular permeability as demonstrated by Evans blue extravasation into the lung parenchyma (figure 6A), and confirmed with pulmonary macroscopic analysis (figure 6B-D). Conversely, PAR2 blockade failed to reduce the OVA-induced increased permeability in the trachea, as shown in figure 6E and confirmed at the macroscopic level (figure 6F–H).

4. Discussion

It is well established that lung diseases are characterized by initial alterations induced by allergens, typically involving eosinophil and neutrophil accumulation that leads to an inflammatory environment. These cells play a pivotal role in the progression of airway inflammatory diseases, through the release of granule proteases, among other effects that amplify the inflammatory response and the underlying tissue damage (Rothenberg and Hogan 2006; Weiss 1989). Thus, it is hypothesized that drugs that block neutrophil or eosinophil recruitment to the inflamed tissues and/or their activation may be important as new therapeutic strategies for the treatment of inflammatory diseases. Several experimental studies support the role of PAR2 in the development of allergen-induced airway inflammation, specifically on the mechanisms of leukocyte recruitment or activation in experimental airway inflammation and in human asthma (Asaduzzaman *et al.* 2015; Cocks and Moffatt 2001; Davidson *et al.* 2013; de Boer *et al.* 2014; Schmidlin *et al.* 2001). However, to date, the role of PAR2 in mediating OVA-induced leukocyte recruitment in airway acute inflammation and the underlying mechanisms are not well

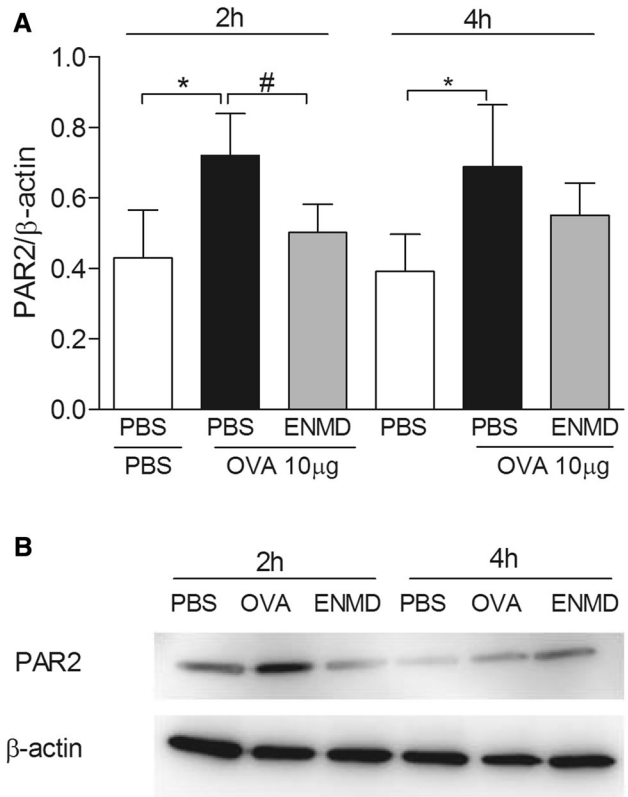

Figure 5. Western blot analysis of PAR2 protein expression in BALF of OVA-instilled mice treated with the PAR2 antagonist ENMD1068. Mice were immunized with OVA and pretreated with ENMD1068 (0.5 mg/kg) 1 h before i.n. instillation of OVA (10 μg/20 μL) or PBS 9, 10, 11 days after, and BALF was collected at 2 or 4 h after last challenge. The cells were centrifuged and pooled, and protein expression was determined by Western Blot. (**A**) Quantitative analysis and (**B**) representative gel of PAR2 and β-actin proteins. This gel is representative of three experiments with similar outcomes. The results were normalized by the β-actin contained in each sample and expressed as the mean ± SD of 5 mice/group. One-way ANOVA followed by Newman–Keuls post hoc test was used to assess comparison among the groups. In all analyses, statistical differences were considered significant at $p<0.05$. *$p<0.05$ when compared to PBS group, #$p<0.05$ when compared to OVA-treated mice.

understood. In this study, we investigated the relevance of PAR2 on neutrophil and eosinophil recruitment into the lungs of mice in a mouse model of short-term acute allergic inflammation.

We demonstrated that pharmacological blockade of PAR2 abolished neutrophil and eosinophil migration evoked by i.n. instillation of OVA in sensitized mice and impaired the development of allergen-induced acute lung inflammation. These effects occurred at least partially through a modulatory role on cytokine production in cells present in the BALF and vascular permeability in the lung. PAR2 has been extensively studied with a focus on understanding its role in mechanisms of recruitment and activation of leukocytes in allergic inflammation (Asaduzzaman *et al.* 2015; Davidson *et al.* 2013; de Boer *et al.* 2014; Matos *et al.* 2014), and previous studies have demonstrated that PAR2 and endogenous serine proteases play a role in neutrophil and eosinophil recruitment in allergic inflammation (de Boer *et al.* 2014; Matos *et al.* 2014, 2013; Saw *et al.* 2012). However, to the best of our knowledge, this is the first study demonstrating that PAR2 plays a key role in the initial hours of airway inflammation following acute allergen exposure, with a synergic effect in eosinophil migration in BALF as well IL-4 and IL-13 production by lymph nodes, played between PAR2 activation and the inflammatory mechanisms triggered by allergen challenge.

Several allergens have previously been used to investigate the effects of PAR2 in an allergic environment (Arizmendi *et al.* 2011; de Boer *et al.* 2014). Our study focused on the importance of PAR2 activation in the production of inflammatory mediators and the recruitment of neutrophils and eosinophils in an experimental model of OVA-induced acute asthma following multiple exposures to allergens. It is well documented that neutrophil accumulation in the airways is correlated with severe forms of chronic asthma, exhibiting exacerbation of the acute phase of the disease (Dejager *et al.* 2015; Fahy 2009) and characterized by the release of cytokines, proteases, oxygen metabolites, and lipid mediators. CXCL1 and CCL5 chemokines play a major role in OVA-induced allergic asthma models, and CXCL1 is increased in OVA-induced allergic asthma models (Chiu *et al.* 2021; Hanashiro *et al.* 2019; Hou *et al.* 2021; Yi *et al.* 2020). Thus, it has been hypothesized that drugs that inhibit the release of these chemokines may be useful in the treatment of allergic asthma. Importantly, other studies suggest that PAR2 activation on vascular smooth muscle cells increases the expression of CXCL1, while PAR2 deficiency significantly reduced levels of this chemokine in the circulation and macrophage content in atherosclerotic lesions (Jones *et al.* 2018), Furthermore, we recently demonstrated that pharmacological blockade of PAR2 impairs CXCL1 chemokine release in the BAL fluid of LPS-instilled mice (Almeida *et al.* 2020). Here, we demonstrated that PAR2 blockade impaired the release of AREG, an epidermal growth factor produced and released by eosinophils and mast cells in response to various inflammatory stimuli (Matsumoto *et al.* 2009; Wang *et al.* 2005), in the lungs

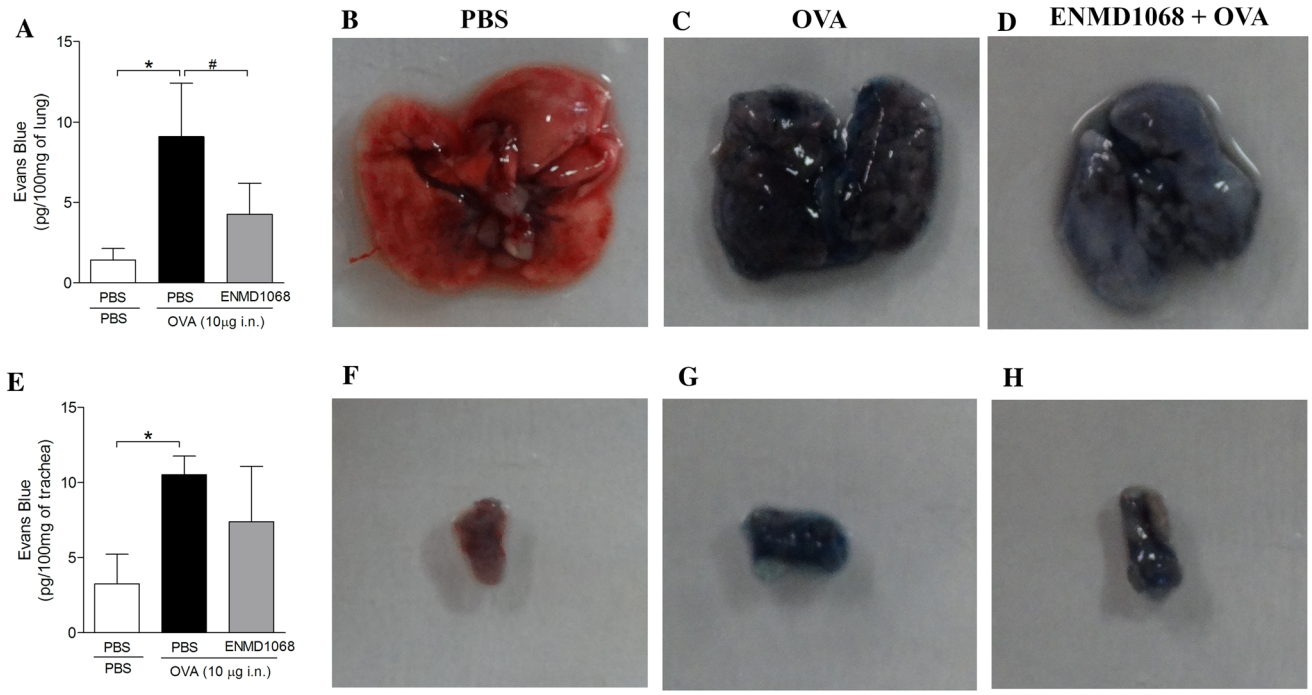

Figure 6. Effects of PAR2 antagonist ENMD1068 on vascular permeability in airways of OVA-instilled mice. Mice were immunized with OVA and pretreated with ENMD1068 (0.5 mg/kg) 1 h before the i.n. instillation of OVA (10µg) or PBS 9, 10, 11 days after. Mice were intravenously injected with 0.2 mL of 2% Evans blue dye in saline, 1h before the last OVA instillation and 2 h after, lungs and trachea were removed, weighed, dried at 37°C for 48 h and Evans blue extracted by formamide. The amount of Evans blue was colorimetrically measured using spectrometry at 620 nm. (**A** and **E**) The results are presented as the amount (pg) of Evans blue present in 100 mg of tissue and are expressed as the mean ± SD of 3–5 mice/group. One-way ANOVA followed by Newman–Keuls post hoc test was used to assess comparison among groups. In all analyses, statistical differences were considered significant at $p<0.05$. *$p<0.05$ when compared to PBS group, and #$p<0.05$ when compared to OVA-treated mice. (**B**, **C**, **D**; **F**, **G**, **H**) Photomicrographs of lungs and trachea were captured before weighed and dried.

of OVA-instilled mice, and increased levels of the anti-inflammatory cytokine IL-10 in the BALF of OVA-instilled mice. AREG plays a role in pulmonary remodeling in allergic diseases, resulting in the proliferation of primary human pulmonary fibroblasts (Wang *et al.* 2005), and has previously been associated with airway remodeling in chronic allergic disorders (Isgro *et al.* 2013; Morimoto *et al.* 2018).

Some of the major factors controlling allergy are T helper (Th) 2 polarization (Leung *et al.* 2015) and the commitment of immune tolerance by immune regulatory cells, such as regulatory B cells and their cytokines, such as IL-10 (Braza *et al.* 2014; Lykken *et al.* 2015). In this study, a significant increase in neutrophils and eosinophils was observed in BALF after the last allergen challenge, and PAR2 blockade partially inhibited neutrophil and eosinophil recruitment, impairing the release of the pro-inflammatory cytokines CXCL1, IL-6, and CCL5 in BALF 2 h after the last OVA challenge, as well as IL-13 in lung homogenate 48 h after the last challenge. We speculate that PAR2 blockade may play a role in both neutrophil recruitment and Th2 inflammation in allergic airway disease, increasing the release of the anti-inflammatory cytokine IL-10, suggesting that PAR2 blockade may be useful in airway inflammatory diseases acting on the onset of acute lung inflammation, at least in part due to their ability to inhibit the production of pro-inflammatory cytokines, such as the chemokine CXCL1, triggered after allergen exposure. Our results are consistent with previous studies in short-term models of allergic airway inflammation, demonstrating an impairment in eosinophil infiltration after exposure to allergens in *Par2*$^{-/-}$ mice as compared to wild-type mice and in mice intranasally pretreated with anti-PAR2 monoclonal antibody (Asaduzzaman *et al.* 2015; Davidson *et al.* 2013; de Boer *et al.* 2014). A recent study by Nadeem *et al.* demonstrated that the number of neutrophils recovered from BALF as well as pro-inflammatory cytokines and chemokines, followed by dust mite

extract instillation, is attenuated by ENMD1068 (5 mg/kg) treatment before allergen administration (Nadeem *et al.* 2019). Our results are in line with recent evidence suggesting that human peripheral B cells express PAR2, and activation of PAR2 inhibits the expression of IL-10 in B cells (Xue *et al.* 2017), suggesting that an increase in this cytokine may play a regulatory role in acute airway allergic inflammation following pharmacological PAR2 blockade. Interestingly, allergic patients produce lower levels of IL-10 following allergen exposure, and restoring IL-10 secretion from dendritic cells is one of the objectives of allergen-specific immunotherapy (Schülke 2018). Furthermore, PAR2 blockade partially inhibited PAR2 expression in cells collected from BALF, and partially restored microvascular leakage in lungs obtained from OVA-instilled mice.

Taken together, our results suggested that the influx of neutrophils and eosinophils into the lung in OVA-instilled mice, plasma exudation, and cytokine/chemokine production in the lung, may be at least partially dependent on the local release of PAR2-agonist proteases such as mast cell tryptase and neutrophil elastase by leukocytes. Given that these effects are essential to reduce the tissue damage induced by allergen exposure, PAR2-based therapy may be a potential pharmacological approach for the treatment of allergic lung diseases such as asthma.

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