

Functional inhibition of PAR₂ alleviates allergen-induced airway hyperresponsiveness and inflammation

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Summary

Background Proteinase-activated receptor 2 (PAR₂) is a G protein-coupled receptor activated by trypsin-like serine proteinases. PAR₂ activation has been associated with inflammation including allergic airway inflammation. We have also shown that PAR₂ activation in the airways leads to allergic sensitization. The exact contribution of PAR₂ in the development of eosinophilic inflammation and airway hyperresponsiveness (AHR) in sensitized individuals is not clear.

Objective To investigate whether functional inhibition of PAR₂ during allergen challenge of allergic mice would inhibit allergen-induced AHR and inflammation in mouse models of asthma.

Methods Mice were sensitized and challenged with ovalbumin (OVA) or cockroach extract (CE). To investigate the role of PAR₂ in the development of AHR and airway inflammation, we administered blocking anti-PAR₂ antibodies, or a cell permeable peptide inhibitor of PAR₂ signalling, pepducin, i.n. before allergen challenges and then assessed AHR and airway inflammation.

Results Administration of anti-PAR₂ antibodies significantly inhibited OVA- and CE-induced AHR and airway inflammation. In particular, two anti-PAR₂ antibodies, the monoclonal SAM-11 and polyclonal B5, inhibited AHR, airway eosinophilia, the increase of cytokines in the lung tissue and antigen-specific T cell proliferation, but had no effect on antigen-specific IgG and IgE levels. Pepducin was also effective in inhibiting AHR and airway inflammation in an OVA model of allergic airway inflammation.

Conclusions and Clinical Relevance Functional blockade of PAR₂ in the airways during allergen challenge improves allergen-induced AHR and inflammation in mice. Therefore, topical PAR₂ blockade in the airways, through anti-PAR₂ antibodies or molecules that interrupt PAR₂ signalling, has the potential to be used as a therapeutic option in allergic asthma.

Keywords proteinase-activated receptor 2, asthma, mouse, airway inflammation, ovalbumin, cockroach

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Introduction

Allergic asthma is characterized by reversible airway obstruction and eosinophilic airway inflammation [1]. Despite new advances in asthma therapeutics, there are still a significant number of patients with uncontrolled disease [2]. Most available treatments target inflammation and/or bronchoconstriction, but do not

directly target the pathways leading to the development of allergic airway inflammation [3, 4]. This deficiency is being addressed with the introduction of biologics [5, 6]. There are many biologics under investigation and they offer much promise for treatment of asthma. However, new therapeutic options targeting other inflammatory pathways are still needed.

Proteinases play an important role in asthma pathogenesis [7]. Many of the proteinases involved in asthma are serine proteinases and are either constituents of inhaled allergens or released by inflammatory and/or tissue resident cells. Serine proteinases mediate some of their effects through activation of a family of G protein-coupled receptors called proteinase-activated receptors (PARs) [8]. One of the members of the family, PAR₂, can be activated by a variety of allergens [9–11] and endogenous serine proteinases [12, 13]. PAR₂ is expressed by a variety of cells in the lung [14–17] and is involved in inflammation [18–20]. In addition, PAR₂ is upregulated in the airways of asthmatics [21]. These data indicate that PAR₂ is a candidate for sensing the presence of serine proteinases in the airways and mediating allergic airway inflammation. In addition, a recent paper showed that PAR₂ polymorphisms are associated with the development of atopy in Korean children [22] underscoring the importance of PAR₂ for the development of allergic inflammation.

PAR₂ has been implicated in the development of allergic airway inflammation in animal models. PAR₂ knock-out mice develop decreased airway inflammation and AHR in response to sensitization and challenge with OVA [23] and cockroach allergens [9]. We have also shown that PAR₂ activation in the airways enhances allergen-induced AHR and inflammation [24].

These findings indicate that PAR₂ is important for the development of allergic airway inflammation. We, therefore, asked whether PAR₂ blockade may be a viable therapeutic approach to treat asthma. To answer this question, we administered blocking anti-PAR₂ antibodies to mice during the challenge phase of cockroach and OVA models of allergic airway inflammation. We also administered a new peptide inhibitor of PAR₂ signalling, pepducin, that has been shown to inhibit PAR₂ effects in other *in vivo* systems. In this manuscript, we show that PAR₂ blockade prevented AHR and allergic airway inflammation and also decreased the immune response to the inhaled allergen. These findings suggest a significant role for PAR₂ in allergic airway inflammation and identify PAR₂ as an important therapeutic target.

Methods

Confocal microscopy

FITC-conjugated SAM-11 antibody was administered i.n. to WT and PAR₂^{-/-} mice, and mice were euthanized 24 h later. Lung sections were photographed with a LEICASP2 confocal microscope.

CE and OVA sensitization and challenge

Male BALB/c mice (6–8 weeks of age) were used. Experiments were approved by the University of Alberta

Health Sciences Laboratory Animal Ethics Committee. Mice were sensitized and challenged using whole-body German cockroach (*B. germanica*) extract (Greer Laboratories, Lenoir, NC, USA) or OVA (Profos AG, Regensburg, Germany) according to the protocols shown in Fig. 3(a), 4(a) and 9(a). To study the role of PAR₂ in these models, mice were administered anti-PAR₂ antibodies, the mouse monoclonal anti-PAR₂ antibody SAM-11 (10 ng per administration per mouse), or the rabbit polyclonal antibody B5 (25 µL of a 1/500 dilution of rabbit antiserum per administration per mouse), or a peptide PAR₂ inhibitor, P2pal-18S pepducin (pepducin) (25 µL of a 4 mM solution per administration per mouse), i.n. before every challenge with CE or OVA. The concentrations of antibodies and inhibitors were selected based on *in vitro* studies we performed using airway epithelial cells (data not shown). Pepducin was synthesized by the University of Calgary Peptide Synthesis Facility (peplab@ucalgary.ca). The peptide was verified to be > 95% pure by HPLC and mass spectral analysis.

Evaluation of AHR

Twenty-four hours after the final challenge, mice were anaesthetised, mechanically ventilated, and then respiratory system resistance (R) and elastance (E) were measured after nebulisation of increasing doses of aerosolized methacholine [25].

Allergic airway inflammation

Mice were euthanized 24 or 48 h after the last allergen challenge. Lungs were cannulated and then lavaged with PBS (pH 7.4), to collect bronchoalveolar lavage (BAL) fluid. Total cells were counted, and cytopins were prepared and stained with Diff-Quick (Fisher Scientific, Kalamazoo, MI, USA). Airway inflammation was assessed by the number of total cells in BAL and the differential counts of cells on the cytopins as described previously [26].

Detection of allergen-specific immunoglobulins

OVA-specific IgG1 and IgE [27] and cockroach-specific IgG1 [9] were measured in the serum of OVA and CE-challenged mice, respectively, by ELISA as previously described.

Cytokine quantification

Lungs were removed immediately after measuring AHR and kept at –70°C until analysis. Measurement of cytokines and chemokines in the lung lysates was performed by Eve Technologies Corporation (Calgary,

Canada) using a multiplex bead assay (described in <https://www.evetechologies.com/technology.php>).

Ex vivo T cell proliferation

Spleens were collected after measuring AHR and homogenized to obtain a single-cell suspension. Splenocytes were added to 96-well U-bottom plates (BD Falcon, Franklin Lakes, NJ, USA) and treated with media, concanavalin A (2.5 µg/mL) or OVA (100 µg/mL) in triplicate. After 72-h incubation, actively proliferating cells were evaluated with a BrdU ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's protocol using a Gen5 Lumimeter (BioTek Synergy HT, Winooski, USA).

Statistical analysis

Values are expressed as mean ± SEM. Statistical differences in the mean values among treatment groups were determined using a one-way ANOVA and Fisher's least significant difference test. The paired Student *t*-test was used to compare statistical differences in the mean values between two groups. In all of the cases, a *P* value < 0.05 was considered statistically significant.

Results

Functional PAR₂ blockade prevents the development of AHR and allergic airway inflammation in response to cockroach allergens in sensitized mice

Cockroach allergens have serine proteinase activity and can activate PAR₂ [9]. To determine whether blocking PAR₂ during allergen challenge prevents allergic inflammation, we used a blocking anti-PAR₂ antibody, SAM-11, as performed previously [9, 28]. To validate that i.n. administration of this antibody can target the airways, we administered fluorescently labelled SAM-11 to mice. Intranasally administered SAM-11 reached the lower airways and shows staining in WT animals, but not in PAR₂^{-/-} mice where only very low levels of non-specific staining is seen (Fig. 1).

We have previously shown that the proteinase activity of CE is important for development of allergic inflammation. Challenge of animals sensitized to native CE with heat-treated CE with very low proteinase activity leads to recruitment of eosinophils in the airways, something that is not evident in mice challenged with saline, but at a level greatly reduced compared to challenge with native CE (Fig. 2). The model used for this experiment is shown in Fig. 3(a). We then used the model shown in Fig. 3(a) to study specifically the role

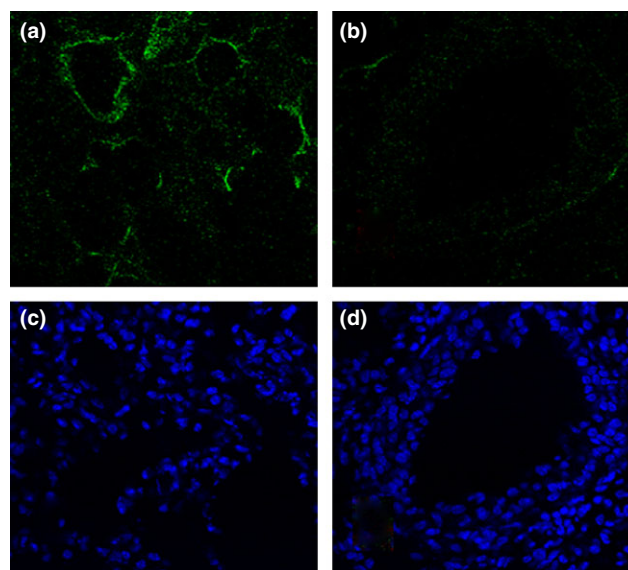


Fig. 1. Intranasally administered fluorescently labelled anti-PAR₂ antibody, stains structures around the airways in WT animals, but not in PAR₂^{-/-} mice. Confocal microscopy of murine lungs 24 h after the intranasal administration of SAM-11. Staining for PAR₂ (a and b) and nuclear staining with TO-PRO-3 (c and d) of lung sections from wild-type (a and c) and PAR₂ knockout (PAR₂^{-/-}) mice (b and d) are shown. Representative photomicrographs (20X) from 2 mice/group are shown.

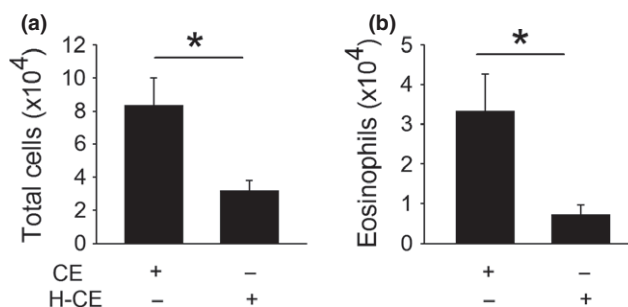


Fig. 2. Heat-treated CE extract causes low levels of eosinophilic inflammation compared to intact CE. Numbers of total cells (a) and eosinophils (b) in the BAL of CE-sensitized mice challenged with intact or heat-treated CE. *n* = 5/group. *, *P* < 0.05. Data are presented as mean ± SEM.

of PAR₂ in the development of CE-induced allergic airway inflammation in sensitized animals. Administration of SAM-11 antibody before every challenge with CE significantly decreased methacholine-induced airway resistance and elastance (Fig. 3b and c, respectively, *P* < 0.05), while an isotype control antibody had no effect. Administration of SAM-11 before every challenge also significantly reduced total cells (Fig. 3d), eosinophils (Fig. 3e), lymphocytes (Fig. 3f), neutrophils (Fig. 3g) and macrophages (Fig. 3h) in the BAL compared to mice treated with isotype control antibody. PAR₂ blockade also decreased the levels of eotaxin

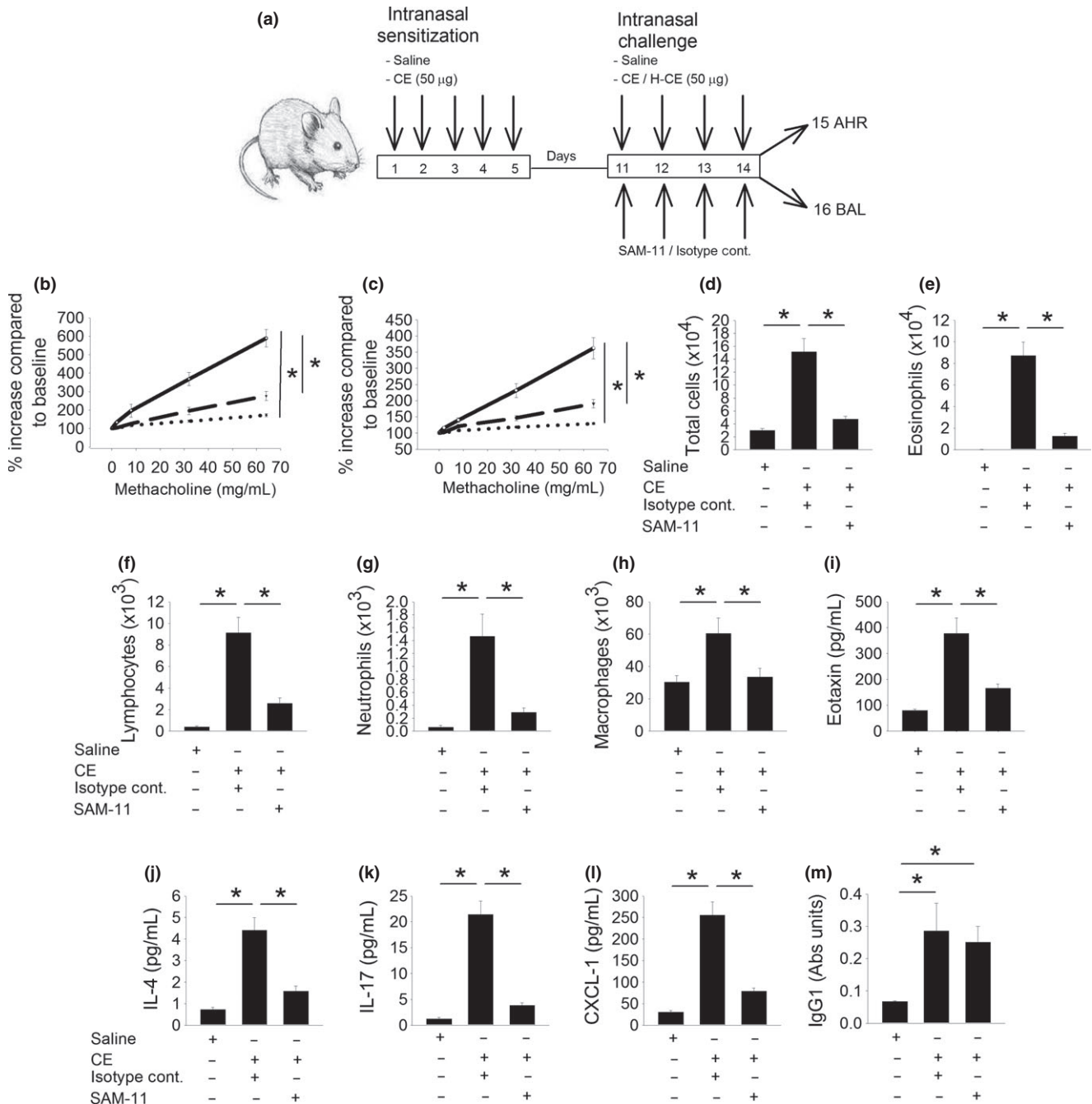


Fig. 3. Local PAR₂ blockade in the airways of allergen-sensitized mice reduces CE-induced AHR and allergic airway inflammation. (a) CE model of allergic airway inflammation. CE-challenged mice were administered SAM-11 or isotype control antibody before every challenge. (b–c) After sensitization, mice were challenged with CE in the presence of isotype control antibody (solid line) or SAM-11 (dashed line), or with saline only (dotted line). Airway resistance (b) and elastance (c) was measured 24 h after final CE challenge by FlexiVent ($n = 10/\text{group}$). (d–h) Airway inflammation was assessed 48 h after final CE challenge. Total cells (d), eosinophils (e), lymphocytes (f), neutrophils (g) and macrophages (h) were counted in BAL fluid ($n = 10/\text{group}$). (i–l) Level of eotaxin (i), IL-4 (j), IL-17 (k) and CXCL-1 (l) in the lungs of sensitized and challenged mice 48 h after the last challenge with CE ($n = 4\text{--}5/\text{group}$). (m) Serum levels of cockroach-specific IgG1 antibody were measured 48 h after final allergen challenge. $n = 5/\text{group}$. *, $P < 0.05$. Data are presented as mean $\pm$ SEM.

(Fig. 3i), IL-4 (Fig. 3j), IL-17 (Fig. 3k) and CXCL1 (Fig. 3l) in the lung tissue of CE-challenged mice compared to mice treated with isotype antibody. The effect of PAR₂ blockade on the levels of 32 different

inflammatory mediators is shown in Table 1. In contrast, functional PAR₂ blocking during allergen challenge had no effect on serum levels of CE-specific IgG1 (Fig. 3m).

Table 1. Cytokine/chemokine levels in mouse lung tissue (model shown in Fig 3(a))

	Saline	CE + Isotype Cont.	CE + SAM-11
IL-1 α	69.1 $\pm$ 15.7	74.4 $\pm$ 14.5	47.5 $\pm$ 4.5
IL-1 β	29.1 $\pm$ 6.3	29.2 $\pm$ 2.8	26.1 $\pm$ 2.5
IL-2	30.8 $\pm$ 9.3	21.2 $\pm$ 4.5	14.6 $\pm$ 1.7
IL-3	0.02 $\pm$ 0.03	1.0 $\pm$ 0.6	0.7 $\pm$ 0.3
IL-4	0.7 $\pm$ 0.1	4.4 $\pm$ 0.6*	1.6 $\pm$ 0.3 [†]
IL-5	3.1 $\pm$ 1.0	7.4 $\pm$ 0.8*	3.9 $\pm$ 0.5 [†]
IL-6	8.7 $\pm$ 2.1	15.0 $\pm$ 2.1*	8.2 $\pm$ 0.5 [†]
IL-7	14.3 $\pm$ 2.9	10.4 $\pm$ 0.5	10.8 $\pm$ 0.8
IL-9	96.3 $\pm$ 28.3	50.0 $\pm$ 6.8	56.8 $\pm$ 6.2
IL-10	15.3 $\pm$ 3.2	10.1 $\pm$ 1.5	9.1 $\pm$ 2.1
IL-12p40	39.6 $\pm$ 18.7	15.9 $\pm$ 5.0	9.1 $\pm$ 2.1
IL-12p70	22.3 $\pm$ 5.8	13.3 $\pm$ 3.2	10.6 $\pm$ 2.5
IL-13	0.9 $\pm$ 0.6	2.1 $\pm$ 1.4	0.0 $\pm$ 0.0
IL-15	47.2 $\pm$ 8.9	42.5 $\pm$ 6.5	26.6 $\pm$ 3.8
IL-17	1.2 $\pm$ 0.4	21.4 $\pm$ 2.7*	3.8 $\pm$ 0.5 [†]
CCL2	72.3 $\pm$ 5.8	154.1 $\pm$ 13.8*	95.6 $\pm$ 5.8 [†]
CCL3	31.5 $\pm$ 5.5	48.0 $\pm$ 4.6	32.6 $\pm$ 4.7
CCL4	21.1 $\pm$ 3.6	59.1 $\pm$ 4.3*	28.0 $\pm$ 5.3 [†]
CCL5	62.7 $\pm$ 6.2	96.6 $\pm$ 10.0*	61.0 $\pm$ 4.5 [†]
CCL11	80.1 $\pm$ 4.7	378.1 $\pm$ 58.5*	165.8 $\pm$ 16.2 [†]
CXCL1	30.4 $\pm$ 3.3	255.3 $\pm$ 30.8*	79.3 $\pm$ 7.6 [†]
CXCL2	8.9 $\pm$ 1.5	18.4 $\pm$ 2.6*	10.5 $\pm$ 0.9 [†]
CXCL5	0.0 $\pm$ 0.0	0.5 $\pm$ 0.5	0.1 $\pm$ 0.1
CXCL9	51.6 $\pm$ 6.4	129.6 $\pm$ 4.9*	68.2 $\pm$ 15.0 [†]
CXCL10	15.0 $\pm$ 3.8	33.6 $\pm$ 5.0*	14.2 $\pm$ 2.5 [†]
G-CSF	2.7 $\pm$ 0.3	22.3 $\pm$ 2.2*	13.5 $\pm$ 1.7 [†]
GM-CSF	6.7 $\pm$ 5.1	18.8 $\pm$ 5.4	7.2 $\pm$ 5.6
IFN- γ	7.8 $\pm$ 3.1	7.2 $\pm$ 0.5	2.4 $\pm$ 1.8
LIF	1.4 $\pm$ 0.2	4.9 $\pm$ 1.1*	2.2 $\pm$ 0.3 [†]
M-CSF	6.5 $\pm$ 0.3	15.5 $\pm$ 2.3*	7.3 $\pm$ 0.5 [†]
TNF	0.2 $\pm$ 0.2	2.8 $\pm$ 0.7*	1.8 $\pm$ 0.2
VEGF	278.1 $\pm$ 84.4	126.7 $\pm$ 8.4	114.7 $\pm$ 6.4

Data are presented as GM $\pm$ SEM, $n = 5$ /group.

*Significant difference from saline, [†]significant difference from CE + isotype control.

Functional PAR₂ blockade prevents the development of AHR and allergic airway inflammation in response to an allergen without serine proteinase activity in sensitized animals

As CE contains PAR₂-activating proteinases, we cannot conclude whether SAM-11 blocked the effects of CE proteinases or endogenous proteinases released following allergen challenge. To investigate the effects of PAR₂ blockade in a model of allergic airway inflammation in response to an allergen without serine proteinase activity, we administered SAM-11 or an isotype control antibody to OVA-sensitized mice before each i.n. OVA challenge (Fig. 4a). Aerosolized methacholine dose dependently increased dynamic whole-lung resistance, (Fig. 4b) and elastance (Fig. 4c) in OVA-sensitized animals challenged with OVA in the presence of isotype control antibody ($P < 0.05$ vs. saline-

treated controls). SAM-11 administration significantly attenuated both resistance and elastance increases by more than 95% (Fig. 4b and c, $P < 0.05$ vs. animals receiving isotype control antibody). These results suggest that PAR₂ activation plays an important role in OVA-induced AHR, similar to its role in CE-induced AHR.

Mice sensitized and challenged with OVA also showed increased numbers of total cells (Fig. 4d, $P < 0.05$) and eosinophils (Fig. 4e, $P < 0.05$) in the BAL fluid compared to mice that were sensitized and challenged with saline. This increase was not affected by the administration of isotype control antibody. Administration of SAM-11, however, significantly reduced total cell and eosinophil BAL counts compared to isotype control antibody-treated animals (Fig. 4d and e, respectively, $P < 0.05$). Dose-response experiments showed that the dose of SAM-11 used for the experiments presented in Fig. 4 was optimal to decrease AHR (Fig. 5a) and also to decrease airway inflammation (Fig. 5b and c).

SAM-11 also prevented OVA-induced increases in eotaxin (Fig. 4f), IL-13 (Fig. 4g), GM-CSF (Fig. 4h) and CXCL-1 (Fig. 4i) levels in lung tissue. Among the other two cytokines shown in Fig. 2, IL-4 was also increased significantly in the airways after OVA challenge, but administration of anti-PAR₂ monoclonal antibody did not prevent this increase, while IL-17 was not increased in the ovalbumin model (Table 2). The effect of PAR₂ blockade on the levels of 32 different inflammatory mediators is shown in Table 2.

Effects of PAR₂ blockade on markers of allergic sensitization to OVA

We next asked whether PAR₂ blockade alters the immune response to OVA. OVA-sensitized and challenged mice exhibited increased levels of OVA-specific IgG1 (Fig. 6a, $P < 0.05$) and IgE (Fig. 6b, $P < 0.05$) in serum compared to saline-treated mice. Functional PAR₂ blockade or isotype control antibody treatment prior to allergen challenge had no effect on the serum levels of antigen-specific antibodies (Fig. 6a and b).

We then isolated splenocytes and cultured them *ex vivo* in the presence of OVA. *Ex vivo* OVA stimulation-induced proliferation of splenocytes collected from mice sensitized and challenged with OVA in the presence of an isotype control antibody ($P < 0.05$, for the comparison between cells alone vs. cells + OVA), but no proliferative response to OVA was observed in cells collected from the animals sensitized and challenged with saline only (Fig. 6c). The *ex vivo* OVA-stimulated leucocyte proliferation was significantly reduced for splenocytes isolated from mice administered anti-PAR₂ monoclonal antibody before OVA treatment as compared to the isotype control-treated group (Fig. 6c,

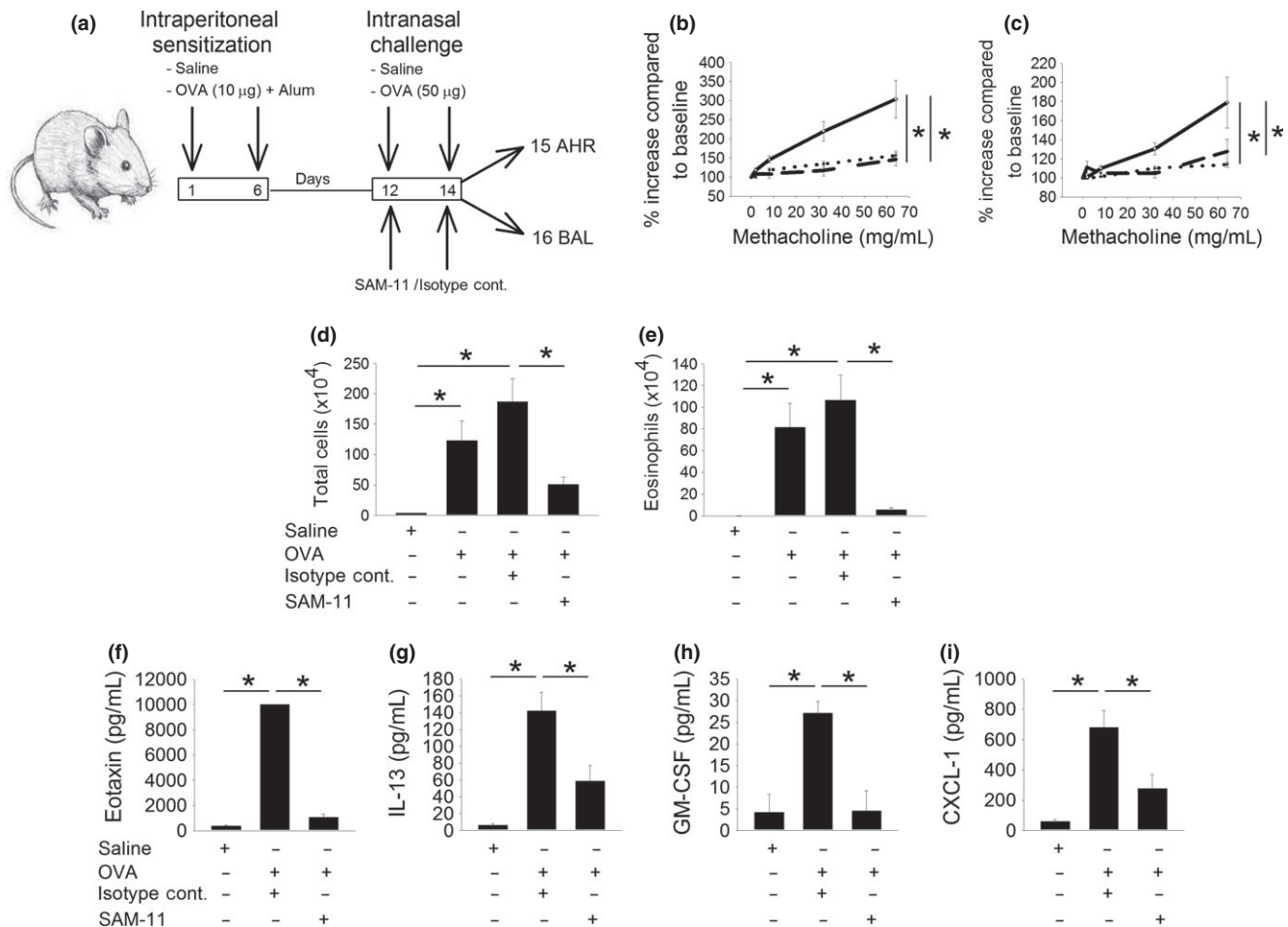


Fig. 4. Local PAR₂ blockade in the airways of sensitized mice prevents OVA-induced AHR and airway inflammation. (a) OVA model of allergic airway inflammation. (b–c) After sensitization, mice were challenged with OVA in the presence of isotype control antibody (solid line) or SAM-11 (dashed line), or, with saline only (dotted line). Airway resistance (b) and elastance (c) were measured 24 h after final OVA challenge by FlexiVent ($n = 7$ –8/group). (d–e) Airway inflammation was assessed 48 h after final OVA challenge. Total cells (d) and number of eosinophils (e) were counted in BAL fluid ($n = 8$ –10/group). (f–i) Level of eotaxin (f), IL-13 (g), GM-CSF (h) and CXCL1 (i) in the lungs of sensitized and challenged mice 48 h after the last challenge with OVA ($n = 4$ –5/group). *, $P < 0.05$. Data are presented as mean $\pm$ SEM.

$P < 0.05$). These results indicate that OVA-specific splenocyte proliferation was significantly inhibited by *in vivo* PAR₂ blockade, while B cell responses did not appear to be affected. As above for AHR and inflammation, this dose of SAM-11 was optimal for inhibiting lymphocyte proliferation (Fig. 5d).

To validate that PAR₂ blockade was the mode of action of SAM-11, we also used another blocking anti-PAR₂ antibody in the same model. Use of B5, a rabbit polyclonal anti-PAR₂ antibody, had effects similar to SAM-11 and decreased airway inflammation (Fig. 7a and b) and spleen cell proliferation (Fig. 7c).

Recently, a peptide PAR₂ antagonist, pepducin, has been described [29], which can inhibit PAR₂-dependent neutrophil chemotaxis *in vitro* and mouse paw oedema *in vivo*. Sevigny et al. [29] and Michael et al. [30] described in detail the specificity of this polypeptide in

inhibiting PAR₂ signalling both *in vitro* and *in vivo*. We synthesized pepducin and used it in the model shown in Fig. 4(a) except that we administered pepducin instead of SAM-11. Pepducin administered intranasally before every challenge inhibited AHR and airway inflammation in response to OVA (Fig. 8a–d) in a way similar to SAM-11.

PAR₂ blockade decreases established airway inflammation

So far we have shown that blocking PAR₂ prevents the development of AHR and airway inflammation in CE and OVA model of allergic airway inflammation. The next question was whether the same approach can be effective in cases where allergic airway inflammation has already developed. To address this, we challenged

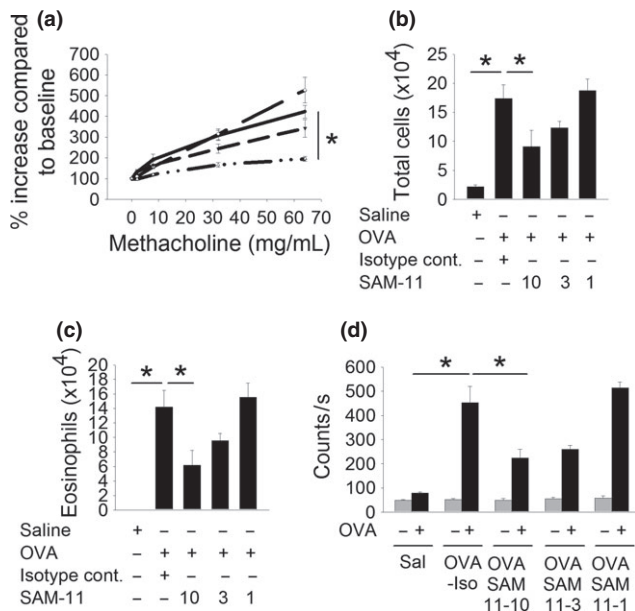


Fig. 5. SAM-11 dose dependently reduces OVA-induced AHR and airway inflammation. (a) After sensitization, mice were challenged with OVA in the presence of isotype control antibody (solid line) or SAM-11 10 ng (long dashed line), 3 ng (short dashed line) or 1 ng (dash dotted line). Airway resistance was measured by FlexiVent 24 h after final OVA challenge ($n = 4$ /group). Airway inflammation was assessed 48 h after final OVA challenge. (b–c) Total cells (b) and number of eosinophils (c) were counted in BAL fluid 48 h after last OVA challenge ($n = 4$ –5/group). (d) *Ex vivo* OVA-specific splenocyte proliferation was performed 48 h after final challenge with OVA. Spleens were collected from mice challenged with OVA in the presence of isotype control antibody and OVA in the presence of SAM-11 10, 3 and 1 ng/mouse ($n = 3$ /group). *, $P < 0.05$. Data are presented as mean $\pm$ SEM.

OVA-sensitized mice once with OVA to develop inflammation and then treated them with SAM-11 before each one of the next two challenges (model can be seen in Fig. 9a – treatment protocol).

Mice sensitized and challenged with OVA showed increased number of total cells (Fig. 9b, $P < 0.05$) and eosinophils (Fig. 9c, $P < 0.05$) in the airways compared with mice that received saline. Administration of anti-PAR₂ antibody after the development of allergic inflammation significantly reduced total cells (Fig. 9b) and eosinophils (Fig. 9c) compared to isotype control antibody-treated animals. OVA-sensitized and challenged mice exhibited increased levels of OVA-specific IgG1 (Fig. 9d, $P < 0.05$ vs. saline) and IgE antibodies (Fig. 9e, $P < 0.05$ vs. saline) in the serum, and functional PAR₂ blockade after the development of inflammation had no effect on serum levels of antigen-specific antibodies.

Discussion

In this study, we show that in sensitized animals, PAR₂ blockade during the encounter with the sensitizing

Table 2. Cytokine/chemokine levels in lung tissue (model is shown in Fig. 4(a))

	Saline	OVA + Isotype Cont.	OVA + SAM-11
IL-1 α	88.7 $\pm$ 19.3	104.8 $\pm$ 13.6	46.6 $\pm$ 8.7
IL-1 β	22.6 $\pm$ 2.0	58.2 $\pm$ 19.1	29.4 $\pm$ 2.4
IL-2	6.7 $\pm$ 1.6	16.4 $\pm$ 3.0*	7.1 $\pm$ 1.5 [†]
IL-3	0.5 $\pm$ 0.2	6.5 $\pm$ 1.6*	1.6 $\pm$ 0.5 [†]
IL-4	0.4 $\pm$ 0.1	140.8 $\pm$ 26.4*	72.8 $\pm$ 19.6
IL-5	0.6 $\pm$ 0.1	300.9 $\pm$ 37.8*	192.7 $\pm$ 48.1
IL-6	1.7 $\pm$ 0.4	262.0 $\pm$ 66.2*	86.9 $\pm$ 25.5 [†]
IL-7	4.8 $\pm$ 0.1	10.3 $\pm$ 1.8*	6.2 $\pm$ 0.2 [†]
IL-9	18.1 $\pm$ 3.0	45.9 $\pm$ 20.8	17.5 $\pm$ 3.1
IL-10	2.1 $\pm$ 0.6	7.2 $\pm$ 2.2	2.5 $\pm$ 0.4
IL-12p40	9.3 $\pm$ 1.8	33.3 $\pm$ 17.3	7.7 $\pm$ 2.0
IL-12p70	8.7 $\pm$ 2.3	27.3 $\pm$ 10.1	10.5 $\pm$ 2.7
IL-13	6.0 $\pm$ 1.9	142.1 $\pm$ 22.0*	58.7 $\pm$ 18.5 [†]
IL-15	7.5 $\pm$ 1.5	40.5 $\pm$ 13.5*	16.4 $\pm$ 4.3
IL-17	2.4 $\pm$ 0.7	4.5 $\pm$ 1.3	2.3 $\pm$ 0.6
CCL2	75.6 $\pm$ 10.0	1175.0 $\pm$ 215.8*	618.2 $\pm$ 96.6
CCL3	24.4 $\pm$ 2.5	1129.3 $\pm$ 240.0*	450.0 $\pm$ 107.0 [†]
CCL4	4.6 $\pm$ 0.7	591.2 $\pm$ 146.9*	268.0 $\pm$ 56.7 [†]
CCL5	159.4 $\pm$ 52.0	4423.9 $\pm$ 2179.2	193.7 $\pm$ 61.3
CCL11	394.3 $\pm$ 53.0	10000.0 $\pm$ 0.0*	1068.8 $\pm$ 234.1 [†]
CXCL1	60.6 $\pm$ 10.6	680.8 $\pm$ 112.3*	276.9 $\pm$ 93.7 [†]
CXCL2	11.7 $\pm$ 3.9	157.4 $\pm$ 33.9*	33.6 $\pm$ 9.4 [†]
CXCL5	1187.9 $\pm$ 364.1	2451.1 $\pm$ 747.1	1660.6 $\pm$ 462.1
CXCL9	80.2 $\pm$ 10.7	3805.2 $\pm$ 2175.4*	1214.3 $\pm$ 184.5
CXCL10	34.8 $\pm$ 7.7	383.3 $\pm$ 41.8*	178.5 $\pm$ 36.5 [†]
G-CSF	3.4 $\pm$ 1.0	43.7 $\pm$ 12.1*	15.6 $\pm$ 1.3 [†]
GM-CSF	4.2 $\pm$ 4.2	27.2 $\pm$ 2.7*	4.6 $\pm$ 4.6 [†]
IFN- γ	23.9 $\pm$ 12.9	23.7 $\pm$ 6.0	12.5 $\pm$ 2.5
LIF	1.7 $\pm$ 0.6	105.3 $\pm$ 15.3*	28.8 $\pm$ 7.7 [†]
M-CSF	23.8 $\pm$ 5.1	531.4 $\pm$ 73.7*	123.1 $\pm$ 24.7 [†]
TNF	1.2 $\pm$ 0.2	5.9 $\pm$ 1.0*	3.4 $\pm$ 0.8 [†]
VEGF	250.8 $\pm$ 20.8	254.7 $\pm$ 35.8	184.4 $\pm$ 7.6

Data are presented as GM $\pm$ SEM, $n = 5$ /group.

*Significant difference from saline, [†]significant difference from CE + isotype control.

allergen prevents allergen-induced AHR and airway inflammation; PAR₂ blockade is effective even if instituted after inflammation has already developed. We further show that PAR₂ blockade is effective not only for allergens with proteinase activity, such as cockroach allergens, but also for allergens such as OVA that have no intrinsic proteinase activity. This observation suggests that there are endogenous proteinases released in sensitized animals upon mucosal encounter with the sensitizing allergen that mediate both AHR and airway inflammation in a PAR₂-dependent fashion. The observations presented here indicate that we may be able to treat allergic airway inflammation and asthma in general using PAR₂ inhibitors. The quest for effective PAR₂ inhibitors has been long with many failures on the way. Promising inhibitors have been described recently and one of them, pepducin, interferes with the ability

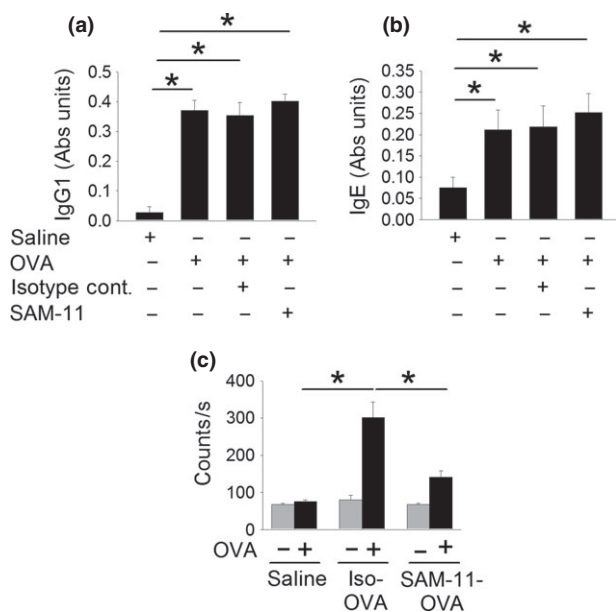


Fig. 6. Local PAR₂ blockade in the airways decreases OVA-induced *ex vivo* splenocyte proliferation, but has no effect on antigen-specific serum immunoglobulin labels. (a–b) OVA-specific serum IgG1 (a) and IgE (b) were measured by ELISA 48 h after the last OVA challenge ($n = 9$ –12/group). (c) *Ex vivo* OVA-specific splenocyte proliferation was performed 24 h after final challenge with OVA. Splens were collected from mice challenged with saline, OVA in the presence of isotype control antibody and OVA in the presence of SAM-11 ($n = 6$ /group). *, $P < 0.05$. Data are presented as mean $\pm$ SEM.

of PAR₂ to interact with G-proteins and, therefore, prevents PAR₂ signalling [29, 30]. We have shown here that intranasal administration of pepducin has effects similar to the effects of anti-PAR₂ antibodies in preventing AHR and airway inflammation.

As we administered the PAR₂ antibodies and the PAR₂ inhibitor i.n., we suggest that their effects were due to local PAR₂ blockade within the airways. We have shown that PAR₂ is expressed on epithelial cells, smooth muscle cells and alveolar macrophages in the airways of mice [24], and it is known that it is also expressed on other immune cells. Previous reports suggest that the expression of PAR₂ is increased in the bronchial epithelium of asthmatics [21], which makes the epithelium the primary candidate to mediate the pro-inflammatory effects of proteinases in asthmatic individuals. We also showed here that i.n. administered PAR₂ blocking antibody reaches the lungs and binds to cells lining the airways, which we postulate are airway epithelial cells. PAR₂-mediated activation of epithelial cells releases cytokines that may mediate asthma [17, 31, 32] and preventing the release of these cytokines may dampen allergic airway inflammation. We have shown here that pro-inflammatory factors that can be produced by the airway epithelium, such as eotaxin, GM-CSF and CXCL1, are decreased following

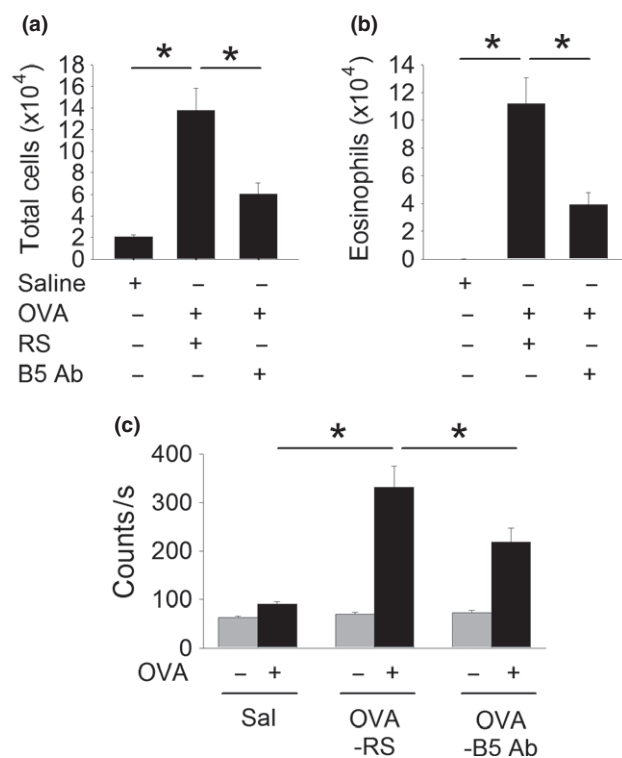


Fig. 7. Local PAR₂ blockade using B5 polyclonal antibody prevents OVA-induced airway inflammation. (a–b) Total cells (a) and number of eosinophils (b) were counted in BAL fluid 48 h after final OVA challenge ($n = 10$ /group). (c) *Ex vivo* OVA-specific splenocyte proliferation was performed after collecting spleens from the animals treated with OVA in the presence of rabbit serum (RS) and OVA in the presence of B5 antibody (1 : 500 dilution) ($n = 8$ /group). *, $P < 0.05$. Data are presented as mean $\pm$ SEM.

PAR₂ blockade. We therefore propose that blocking PAR₂-mediated inflammatory mediator release from the epithelium may be the mechanism of the effects we describe, but further work is needed to prove the involvement of the epithelium. Alternatively, the presence in the airways of PAR₂ antibodies or inhibitors may allow anti-inflammatory pathways, pathways that are normally inhibited by homeostatic PAR₂ activation on epithelial or immune cells, to dominate and prevent the development of inflammation. All airway epithelial cells, from those found in the upper airways to alveolar epithelial cells, express PAR₂. The relative contribution of each of these epithelial cells on PAR₂-mediated airway inflammation is not known. Studies comparing the effects of PAR₂-mediated activation of different types of airway epithelial cells will be invaluable to answer this question.

The importance of blocking PAR₂ activation on other immune cells, such as dendritic cells, alveolar macrophages or mast cells, cannot be excluded at this point. Identifying the PAR₂-expressing cell that mediates the pro-inflammatory effects of endogenous proteinases

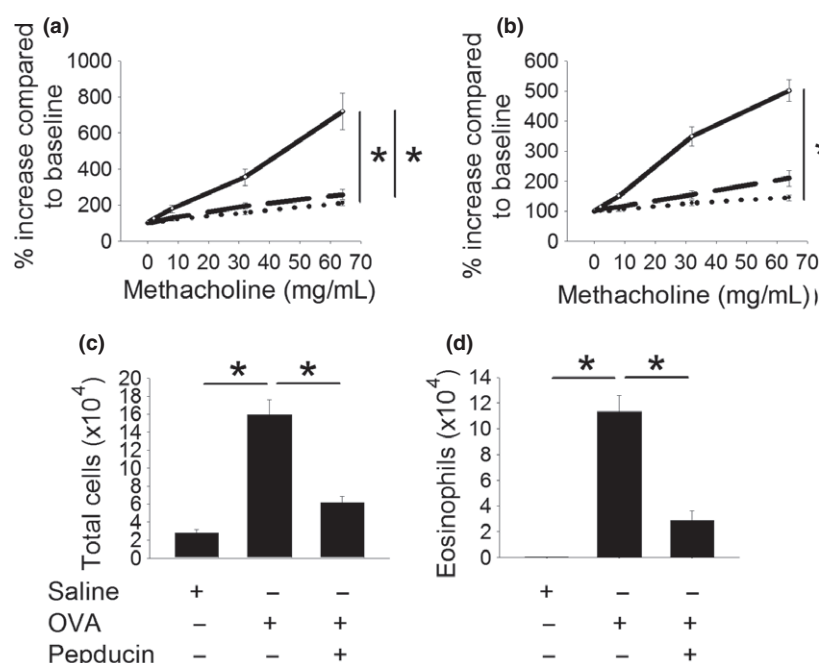


Fig. 8. Pepducin prevents OVA-induced AHR and airway inflammation. (a–b) After sensitization, mice were challenged with OVA in the presence of vehicle (solid line) or pepducin 9 mg/kg (dashed line), or, with saline only (dotted line). Airway resistance (a) and elastance (b) measurements were performed using FlexiVent 48 h after final allergen challenge ($n = 8/\text{group}$). (c–d) Airway inflammation was also assessed at the same time. Total cells (c) and number of eosinophils (d) were counted in BAL fluid ($n = 8/\text{group}$). *, $P < 0.05$. Data are presented as mean $\pm$ SEM.

will be a difficult endeavour, as there are no conditional or tissue-specific PAR₂ knockout mice available.

Accumulation of inflammatory cells in the airways, especially eosinophils, is a cardinal characteristic of allergic inflammation in animal models [33] and in human asthma [34]. Functional blockade of PAR₂ before allergen challenge attenuated eosinophil accumulation in the airways in both the CE and the OVA models. We have previously shown that the BAL of non-sensitized mice challenged with CE contains < 30% of the eosinophils found in the BAL of mice sensitized and challenged with CE [9]. As PAR₂ blockade in the experiments presented here blocks almost completely CE-induced BAL eosinophilia in sensitized and challenged animals, we suggest that PAR₂ blockade inhibits both innate and adaptive immune responses to CE. PAR₂ blockade was equally effective in preventing eosinophil accumulation even when it was instituted after the first challenge with OVA. In addition, functional inhibition of PAR₂ before allergen challenge significantly decreased allergen-induced cytokine levels in the lung tissue, including eotaxin and CXCL1 in both CE and OVA models. Other cytokines and chemokines were also decreased in the presence of PAR₂ blockade. Decreased eotaxin release may be responsible for reduced eosinophil recruitment into the lungs in the presence of PAR₂ blockade. CXCL1 increases in induced sputum 24 h after allergen challenge [35] and correlates

with endothelial progenitor recruitment in human disease and also in allergen challenged mice [36]. Decreased CXCL1 levels following PAR₂ blockade may alter local vascular remodelling and prevent other long-term consequences of allergic inflammation.

AHR is a major feature of asthma [37]. PAR₂ blockade prevented the increase in airway resistance and elastance induced by allergen challenge in both the CE and OVA models of asthma. In particular in the latter model, PAR₂ blockade prevented OVA-induced IL-13 upregulation in lung tissue. Decreased IL-13 may explain, at least in part, the diminished AHR, because IL-13 is required for the development of AHR in mouse models of asthma [38], although this observation would not explain decreased AHR in our CE model where there was no induction of IL-13 by CE. Other mediators and possibly direct effects on smooth muscle must also be responsible for the decreased AHR we observed following PAR₂ blockade.

We also studied the effect of PAR₂ blockade in the airways on various aspects of the immune response to allergens. As expected, sensitization and challenge with CE or with OVA increased serum antigen-specific antibody levels, but this increase was not affected by PAR₂ blockade during the challenge phase. This result may indicate that allergen-induced increase in antigen-specific immunoglobulin levels is PAR₂ independent, but may also be the result of the timing of PAR₂ blockade in our model. Anti-PAR₂ antibodies were administered

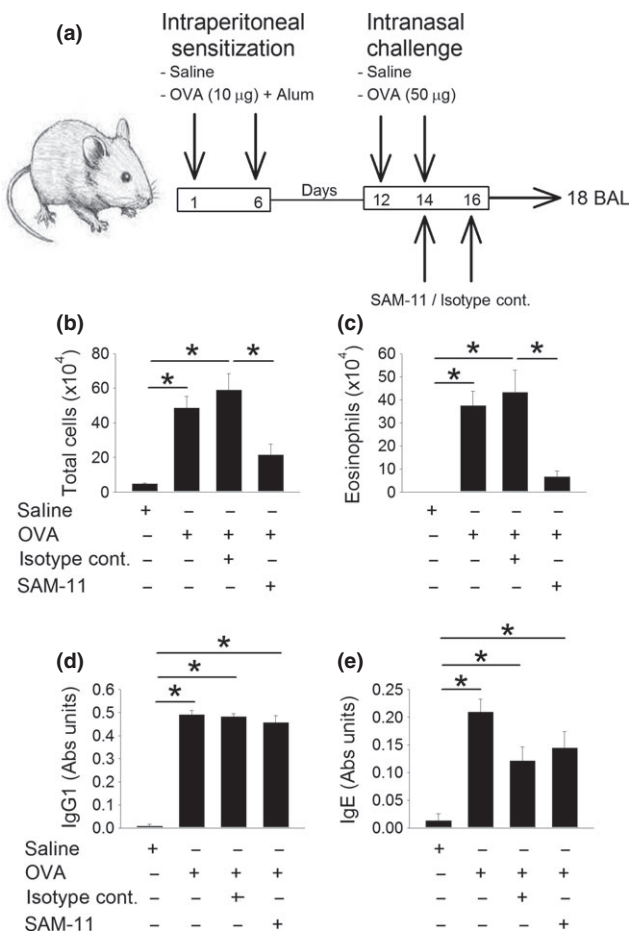


Fig. 9. Local PAR₂ blockade in the airways reduces established OVA-induced allergic airway inflammation. (a) OVA model of allergic airway inflammation. (b–e) OVA-challenged mice were administered SAM-11 or isotype control antibody. Airway inflammation was assessed 96 h after final challenge with OVA. Total cell numbers (b) and number of eosinophils (c) were counted in BAL fluid ($n = 4$ –8/group). Serum was analysed by ELISA for the presence of OVA-specific IgG1 (d) and IgE (e) antibodies ($n = 4$ –8/group). *, $P < 0.05$. Data are presented as mean $\pm$ SEM.

when the mice were already sensitized and PAR₂ blockade may not have been able to decrease the number of antigen-specific B cells and therefore production of antigen-specific immunoglobulins. In contrast, we found that *ex vivo* OVA-induced splenocyte proliferation was lower in mice that received PAR₂ blockade during the challenge phase compared to mice that received isotype control antibody. The mechanism of this effect needs to be studied further. This result may indicate that there is a decreased expansion of antigen-specific CD4 cells during the challenge phase in the presence of PAR₂ blockade due to decreased levels of circulating cytokines.

The importance of proteinases in the development of AHR and airway inflammation has been shown [39–41].

Those studies, however, did not address the mechanism of the proteinase effects. We have previously shown that PAR₂ activation at the time of OVA challenge potentiates AHR and allergic airway inflammation [24], and we have proposed that PAR₂ activation may be the dominant mechanism of proteinase-mediated allergic airway inflammation [42]. This idea is also supported by the fact that PAR₂^{-/-} mice show decreased airway inflammation and AHR compared to wild-type mice in response to ovalbumin [23] and cockroach [9]. However, knockout mice do not permit differentiation between the role of PAR₂ in allergic sensitization vs. its role in the development of AHR and inflammation in sensitized mice. For this reason and to better understand the therapeutic potential of PAR₂ blockade in sensitized individuals, we elected to inhibit PAR₂ function during the challenge phase of our models and showed that this approach prevents the development of AHR and airway inflammation.

We also do not know the identity of the proteinase(s) mediating the PAR₂ effects we prevented using PAR₂ blockade with blocking antibodies or cell permeable peptides. Mast cell tryptase activates PAR₂ [43] and is present in BAL fluid of asthmatic patients [44]. Other proteinases present in the airways in asthma, such as neutrophil elastase [45], HAT [13, 46] and kallikreins [47], also have the ability to activate PAR₂. Using activity-based probes, as we have performed before [48], we have identified several serine proteinases in the BAL of mice sensitized and challenged with OVA (unpublished observation). However, we have not yet characterized these proteinases in order to study their ability to activate PAR₂ and/or their role in the development of allergic airway inflammation.

Our data so far indicate that PAR₂ blockade may be a viable therapeutic option in asthma. The next question that has to be addressed is whether this could have any side-effects. PAR₂ activation has also been shown to have bronchodilatory and bronchoprotective effects and it may regulate ion secretion in the airway mucosa [49, 50] and therefore increase fluidity of mucus, and may also inhibit pro-inflammatory effects of cytokines [51]. It will have to be studied whether inhibiting PAR₂ will interfere with any of these mechanisms. Furthermore, PAR₂ activation may also have homeostatic effects in the airways. For example, PAR₂ activation initiates pro-survival pathways that rescue gut epithelial cells from inflammation-induced apoptosis [52]. We do not know whether the same happens for airway epithelial cells in general, but a recent study has shown that PAR₂ activation mediates airway epithelial basal cell survival [53]. Loss of these signals could have detrimental effects in the airways. Our data presenting here did not show any adverse events following PAR₂ blockade. We have extended the administration of PAR₂ antibodies up to 12 weeks and still did

not see any adverse effects in our mice (manuscript in preparation).

In conclusion, the present study demonstrates the important role of PAR₂ in OVA and CE mediated allergic inflammation in mice. We have shown that PAR₂ is involved in the development of the characteristics of asthma in these mice and therefore PAR₂ blockade may be used to prevent the development of airway inflammation as well as treat established airway inflammation. Thus, our novel findings suggest that PAR₂ activation is a key signalling event that coordinates OVA- and CE-induced AHR and inflammation. Understanding the role of PAR₂ during the development of allergic inflammation, following sensitization, will allow us to develop therapeutic options for asthma.

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Conflicts of interest

The authors have no financial conflicts of interest.

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