

Proteinase-activated receptor-2 activation participates in allergic sensitization to house dust mite allergens in a murine model

C. E. Davidson¹, M. Asaduzzaman¹, N. G. Arizmendi¹, D. Polley², Y. Wu¹, J. R. Gordon³, M. D. Hollenberg², L. Cameron¹ and H. Vliagoftis¹

¹Pulmonary Research Group, Department of Medicine, University of Alberta, Edmonton, AB, Canada, ²Department of Physiology & Pharmacology, University of Calgary, Calgary, AB, Canada and ³Division of Respiriology, Critical Care and Sleep Medicine, Department of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Clinical & Experimental Allergy

Summary

Background Many aeroallergens contain proteinase activity and are able to induce allergic sensitization when presented to mucosal surfaces. Some of these allergens activate proteinase-activated receptor-2 (PAR₂).

Objective To determine the role of PAR₂ activation in a murine house dust mite (HDM) allergy model.

Methods We sensitized and challenged PAR₂-deficient mice with HDM, and examined allergic outcomes compared to wild-type animals. To focus on the role of PAR₂ in allergic sensitization, we administered a PAR₂ blocking antibody to wild-type animals during the sensitization phase and examined the outcomes immediately after sensitization or following subsequent allergen challenge.

Results We found PAR₂-deficient mice sensitized and challenged with HDM failed to develop airway inflammation, did not produce HDM-specific IgG1 and had less IL-4 mRNA in the lungs than wild-type animals. Prevention of PAR₂ activation during sensitization in wild-type mice diminished the levels of Th2 mediators, including IL-4, IL-5 and IL-13, in the lungs. Blocking PAR₂ during the sensitization phase also led to decreased manifestations of allergic disease, including airway hyperresponsiveness (AHR) and airway inflammation following subsequent allergen challenge. HDM-induced proliferation of splenocytes obtained from animals sensitized in the presence of PAR₂ antibody was reduced relative to those that did not receive antibody. The effect of PAR₂ blockade could be transferred to naïve mice through splenic CD4⁺ T cells from sensitized mice.

Conclusions and Clinical Relevance PAR₂ activation plays a key role during the sensitization phase of our HDM allergy model, leading to increased lung cytokine production and augmented lung reactivity. PAR₂ activation is a common mechanism for sensitization to a wide variety of allergens and is therefore a potential pharmacological target to prevent allergy.

Keywords allergic airway inflammation, allergic sensitization, CD4 lymphocytes, house dust mites, proteinase-activated receptor-2

Submitted 05 April 2013; revised 03 July 2013; accepted 09 August 2013

Correspondence:

Dr. Harissios Vliagoftis, Pulmonary Research Group, Department of Medicine, 550 Heritage Medical Research Centre, University of Alberta, Edmonton, AB T6G 2S2, Canada.
E-mail: harissios.vliagoftis@ualberta.ca
Cite this as: C. E. Davidson, M. Asaduzzaman, N. G. Arizmendi, D. Polley, Y. Wu, J. R. Gordon, M. D. Hollenberg, L. Cameron and H. Vliagoftis, *Clinical & Experimental Allergy*, 2013 (43) 1274–1285.

Introduction

While most inhaled environmental proteins are innocuous, some have the ability to facilitate a Th2 immune response and initiate allergic sensitization. A number of these common inhaled allergens, including house dust mite (HDM), cockroach and fungal allergens, contain

proteinase activity, a critical factor in promoting allergic sensitization and IgE production [1–3]. Inhalation of a proteinase can lead to mucosal sensitization to the proteinase [4] or other antigen(s) inhaled along with the enzyme [2, 5]. Mice given cockroach extract develop airway hyperresponsiveness (AHR) and allergic airway inflammation dependent on proteinase activity in the

extract [6]. This cockroach proteinase effect is due in part to direct activation of proteinase-activated receptor-2 (PAR₂), a member of a family of G-coupled receptors that are activated by serine proteinases.

PAR₂ is expressed by structural [7–9] and immune cells [10–12] in the airways. PAR₂ is increased in asthmatic lung epithelia, suggesting a role in human asthma [13]. A recent study linked atopy in Korean children with a synonymous single nucleotide polymorphism in PAR₂ which increases PAR₂ expression [14]. *In vitro*, PAR₂-mediated human airway epithelial activation leads to the release of several inflammatory mediators including GM-CSF [15], thymic stromal lymphopoietin (TSLP) [16] and CCL11 [15]. Similar mediators may be released by PAR₂ activation *in vivo* with cockroach extract [17]. PAR₂ knockout mice show decreased airway eosinophilia and AHR following ovalbumin sensitization [18]. We have shown that PAR₂ activation can mediate sensitization to antigens presented to the airway mucosa, indicating that this may be the mechanism used by proteolytic antigens [19]. These findings indicate a role for PAR₂ in allergic sensitization.

We have previously established a role for PAR₂ in a model of allergic sensitization to inhaled cockroach extracts, using both PAR₂ knockout mice and administration of PAR₂ blocking antibodies during the sensitization phase [6]. However, the precise immunological mechanisms underlying the involvement of PAR₂ in sensitization and its importance in regards to other proteinase allergens, such as HDM, remain to be determined.

HDM is the most common allergen associated with asthma development and severity world-wide [20]. Several HDM allergens possess proteinase activity [21, 22] and can activate epithelial cells via PAR₂-dependent mechanisms [23].

In this study, we examined the role of PAR₂ in sensitization to HDM. We found that blocking PAR₂ activation in the airways during the sensitization phase prevents many of the characteristics of the allergic response, including Th2 cell cytokine production. Similarly, a complete lack of PAR₂, as in the PAR₂ knockout mice, prevented the development of allergic airway inflammation. These effects are mediated, at least in part, through altered function of CD4⁺ T cells. Collectively, these data indicate PAR₂ may play an important role in HDM sensitization and subsequent development of allergic disease.

Materials and methods

Animals

Male BALB/c mice (18–20 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were 6 to 8 weeks old when experiments were

performed. Mice were housed in virus- and Ab-free conditions and maintained on a 12-h light-dark cycle. The University of Alberta Health Sciences Laboratory Animal Ethics Committee (Edmonton, AB, Canada) approved all experiments described. Male C57BL/6 mice, both wild-type and PAR₂^{−/−} were bred at the University of Calgary.

Intranasal administration of HDM extract and anti-PAR₂ Ab

Lyophilized, whole body extracts of HDM (*Dermatophagoides pteronyssinus*) were purchased from Greer Laboratories, (Lenoir, NC, USA) and re-suspended at a concentration of 6 mg/mL of total protein in 0.9% sterile saline. The lyophilized HDM extract contained 39.6 EU (endotoxin units)/mg protein as reported by the manufacturer. Mice were lightly anesthetized with 75 mg/kg ketamine and 2.5 mg/kg acepromazine and given 25 µg of HDM extract in 25 µL of 0.9% sterile saline intranasally, along with either a rabbit polyclonal anti-PAR₂ blocking antibody (B5) or normal rabbit serum (RS) as a control (Fig. 1a). In each experiment, a group of mice was given 25 µL 0.9% saline without HDM on each day of allergen administration.

Measurement of AHR and airway inflammation

AHR was measured 24 h after the final i.n. challenge using one of two methods. In the first, conscious mice were placed in whole body plethysmographic chambers (Buxco Electronics, Wilmington, NC, USA) and, following a 10 min stabilization period, given increasing doses of aerosolized methacholine (2–32 mg/mL; Sigma, Saint Louis, MO, USA) for 3 min each; mean enhanced respiratory pause values were recorded for 5 min and dose-response curves to aerosolized methacholine were generated. In the second method, mice were anesthetized with ketamine (75 mg/kg) and acepromazine (2.5 mg/kg) and then paralyzed with pancuronium bromide (1 mg/kg). The trachea was cannulated and mechanical ventilation was performed [FlexiVent; SCIREQ Inc., Montreal, Canada; tidal volume = 5.6 mL/kg; respiratory rate of 150 breaths/min; 3 cm H₂O positive end-expiratory pressure (PEEP)]. Resistance (R) and elastance (E) were calculated after nebulisation of increasing doses of methacholine (0–64 mg/mL in saline solution). Data with a coefficient of determination greater than 0.9 were used for analysis. Peak responses for each dose were selected and data expressed as percent increase over the baseline response to nebulized saline.

Blood collection and bronchoalveolar lavage (BAL) were performed either 24 or 48 h after the final i.n. allergen challenge to assess serum immunoglobulin levels and airway inflammation respectively. Mice

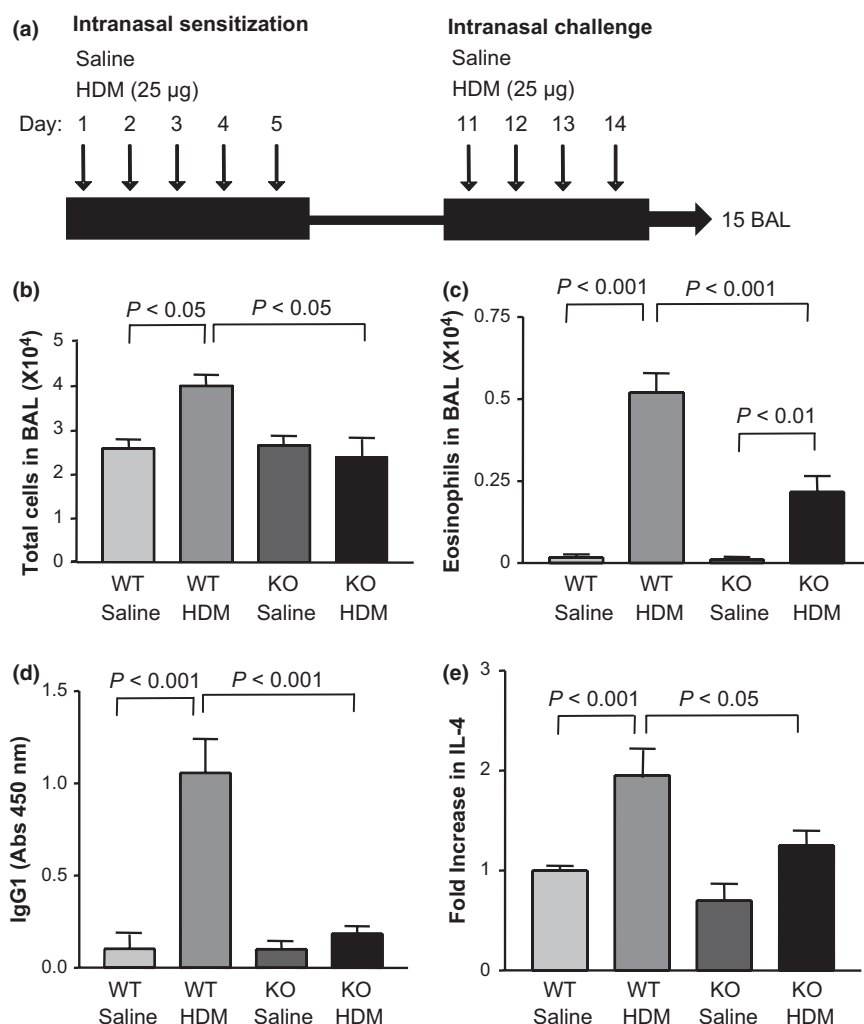


Fig. 1. House dust mite (HDM) sensitization and challenge in a proteinase-activated receptor-2 (PAR₂) knockout mouse model. (a) General model of mucosal HDM sensitization and challenge. C57BL/6 wild-type or PAR₂ KO mice were given either 25 µg HDM or saline i.n. for 5 days, then left untreated for 5 days, and finally they were given i.n. HDM or saline for 4 additional days ($n = 5$ per group, 1 independent experiment). (b and c) Total cell infiltration of the airways 24 h after the final challenge was assessed by bronchoalveolar lavage (BAL) (b), as was the number of eosinophils (c). (d) HDM-specific IgG1 was measured in the serum by ELISA. (e) IL-4 mRNA levels were assessed in whole lung tissue using quantitative RT-PCR. IL-4 levels are expressed as fold increase over the mean value of the saline group.

were killed with an i.p. injection of 2 mg sodium pentobarbital. Cardiac puncture was used to collect blood, followed by tracheal intubation with polyethylene tubing. The lungs were washed twice, with 1 mL of phosphate buffered saline (PBS), pH 7.4, and the 2 mL of lavage fluid was collected. The BAL fluid (BALF) was spun at 300 *g* for 5 min, and cytopspins were prepared with 5000 cells. The slides were stained with Diff Quick (Fisher Scientific, Co. Kalamazoo, MI, USA), and the inflammatory cells were counted to assess airway inflammation.

HDM-specific immunoglobulin measurement

HDM-specific IgG1 and IgE levels were measured by ELISA, using previously reported techniques [24].

For HDM-specific IgG1, HDM allergen was used as the capture reagent and biotinylated anti-IgG1 as the detection reagent. HDM-specific IgE was measured using anti-IgE for the capture and biotinylated HDM allergen for detection. The results are expressed in absorbance units (405 nm).

Expression analysis of inflammatory mediators in the lung tissue

Lung tissue was collected on either day 6 or 15, 24 h after the last allergen administration. Lung lysates were analyzed for the levels of 32 cytokines and chemokines using a multiplex bead-based assay (Eve Technology, Calgary, AB, Canada) or quantitative PCR. Samples were either stored at -80°C or in RNeasy lysis buffer at -20°C until

use for multiplex cytokine analysis or quantitative RT-PCR respectively. Lung tissue for the multiplex cytokine assay was thawed and lysed in 1 mL of PBS with 0.5% NP-40 and a protease inhibitor cocktail (Sigma) per 100 mg of tissue.

Lung tissue for qRT-PCR was homogenized and total RNA was extracted using an RNeasy mini kit and Qia-shredders (Qiagen, Venlo, Limburg, the Netherlands). Reverse transcription was performed on 1 µg of RNA per reaction using oligo dT primers and M-MLV (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was done in an Eppendorf realplex4 using Taqman Gene Expression Master mix and Taqman Gene Expression Assays from Applied Biosystems (Carlsbad, CA, USA). The PCR protocol consisted of 10 min at 95°C followed by 40 cycles of 30 s at 95°C and 60 s at 60°C. All samples were run in triplicate and Hprt1 was used as a reference gene to correct for the cDNA input. The relative expression of the target genes was determined using the $\Delta\Delta C_t$ method, for which the average ΔC_t for the saline samples was set at 1 and all samples were compared to this value.

Splenocyte proliferation assay

Spleens were collected on day 9 from mice that received saline, B5 antibody + HDM or RS + HDM from days 1 through 5. The spleens were disrupted in RPMI 1640 media (Gibco, Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS), 1% Penicillin-streptomycin, 1% L-Glutamine, 1% Non-essential amino acids, 0.01% β -Mercaptoethanol, and a dispersed suspension was obtained by passing the cell suspension through a 100 µm cell strainer. Red blood cells were lysed by adding 20 mM Tris and 140 mM ammonium chloride, re-suspended in complete RPMI 1640 media at a concentration of 1.25×10^6 /mL. Cells were cultured in 96-well U-bottom plates in 100 µL of media at 37°C and 5% CO₂. Spleen cells were treated with media alone, Con A (2.5 µg/mL), or HDM extract (100 µg/mL). After 72 h the proliferating cells were counted using a BrdU ELISA kit (Roche, Basel, Switzerland). Chemiluminescence was measured with a luminometer.

CD4⁺ T cell transfer

Spleens were collected on day 10 from mice that received saline, B5 antibody + HDM or RS + HDM from days 1 through 5. Spleens were pooled and disrupted to create single cell suspensions, and CD4⁺ T cells were purified using an EasySep Mouse CD4⁺ T Cell isolation negative selection kit, that yielded a population with ~85% purity (StemCell Technologies, Vancouver, BC, Canada). Naïve BALB/c mice were given 3 million of these T cells intravenously by tail vein injection. These mice were then challenged twice with HDM intrana-

sally, 6 h following the CD4⁺ cell transfer, and again 2 days later. BAL and flexivent were performed 24 h after the final challenge (Fig. 6a).

Statistical analysis

Statistics were performed using Prism 3 (GraphPad Software, La Jolla, CA, USA). Data are presented as means $\pm$ SEM and were analyzed for significance using a one-way ANOVA and Tukey's multiple comparison tests. AHR data were analyzed using an *F*-test, which allows comparisons of the dose-response curves between groups. A *P*-value < 0.05 was considered statistically significant.

Results

PAR₂ KO mice have decreased lung inflammation, Th2 cytokines and serum IgG1 following HDM sensitization and challenge

To examine whether PAR₂ is essential for sensitization to HDM, we sensitized and challenged PAR₂ KO and wild-type C57BL/6 mice with intranasal HDM as shown in Fig. 1a. Mice were evaluated 24 h after the last i.n. challenge. HDM administration increased the total number of cells in the BALF of wild-type, but not PAR₂ KO mice (Fig. 1b). HDM increased eosinophil numbers in the BALF in both the wild-type and PAR₂ KO animals, but the increase in the wild-type group was more than twice that of the PAR₂ KO group (Fig. 1c). We saw no differences in the number of lymphocytes, neutrophils or macrophages in the BALF between any groups (data not shown). HDM-specific serum IgG1 was increased in wild-type mice that received HDM compared to those that received saline, but this increase was not seen in the PAR₂ KO mice (Fig. 1d). HDM-specific IgE levels were below our detection limit for all treatment groups. Wild-type mice also had increased IL-4 mRNA levels in the lung following HDM sensitization and challenge, relative to saline-treated mice (Fig. 1e), indicating a Th2 immune response, but HDM treatment did not increase the IL-4 levels significantly in PAR₂ KO mice.

Preventing PAR₂ activation during HDM sensitization decreases Th2 cytokine expression in the lungs

To determine PAR₂-dependent and PAR₂-independent effects of allergic sensitization in the lung, before extensive inflammation develops, we treated BALB/c mice with either control RS or an B5, 30 min prior to intranasal administration of HDM for 5 days and collected lungs and serum on day 6. A third control group was given only saline. IL-4 and IL-13 mRNA up-regulation

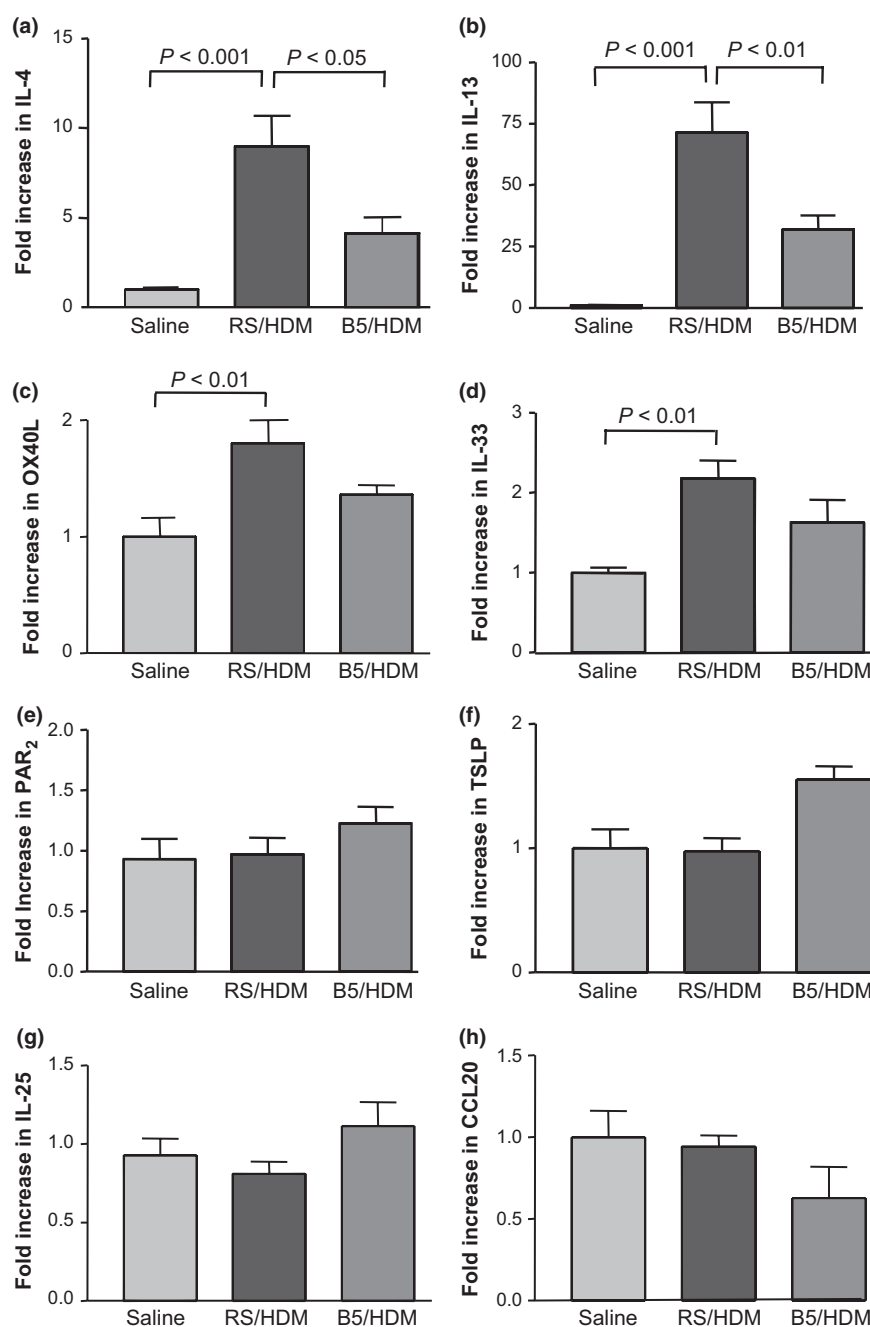


Fig. 2. Expression of Th2 cytokines, OX40L and proteinase-activated receptor-2 (PAR₂) in the lung following a 5-day sensitization to house dust mite (HDM). Mice were given i.n. saline, i.n. rabbit serum (RS), followed by HDM, or i.n. anti-PAR₂ antibody (B5), followed by HDM for 5 days, and whole lung tissue was collected on day 6 for quantitative PCR analysis ($n = 10$ per group, two independent experiments). RNA was extracted, and reverse transcribed to make cDNA, and quantitative PCR with FAM-labelled probes was used to assess the levels of IL-4 (a), IL-13 (b), OX40L (c), IL-33 (d), PAR₂ (e) TSLP (f), IL-25 (g) and CCL20 (h). All transcript levels are expressed as fold increase over the mean value of the saline group.

occurred in mice given RS with HDM but their expression was significantly reduced in the B5-treated mice (Fig. 2a and b). In contrast, HDM upregulation of OX40L and IL-33 was not attenuated by blocking PAR₂ activation (Fig. 2c and d). PAR₂ mRNA levels in the lungs were similar for all groups (Fig. 2e). TSLP and IL-25, two more innate immunity molecules that have been implicated in

allergic sensitization, were also not increased in our model following HDM administration and did not change with PAR₂ blockade (Fig. 2f and g). Finally, CCL20, a chemokine which can recruit and activate dendritic cells, was not increased in our model (Fig. 2h).

To further understand the role of PAR₂ on the mucosal response to HDM, we tested whole lung tissue for

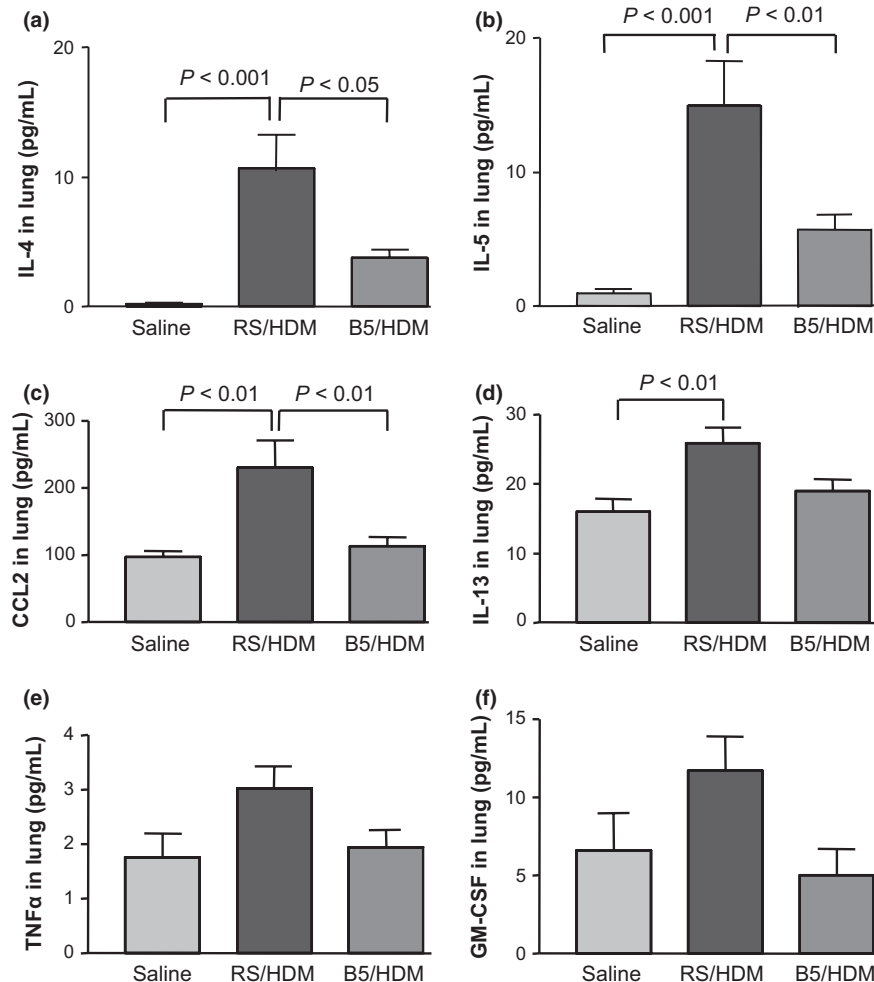


Fig. 3. Impact of proteinase-activated receptor-2 (PAR₂) blockade on cytokine expression in lung tissue following house dust mite (HDM) sensitization. Mice were given i.n. saline, i.n. rabbit serum (RS), followed by HDM, or i.n. B5 Ab followed by HDM for 5 days, and whole lung tissue was collected on day 6 for a multiplex bead-based cytokine array ($n = 10$ per group, two independent experiments). The three major Th2-associated cytokines IL-4 (a), IL-5 (b) and IL-13 (d) were increased in the RS-HDM group as compared to the saline group. This up-regulation of IL-4 and IL-5 was inhibited by preventing PAR₂ activation. The chemokine CCL2 was also increased in the RS-HDM group as compared to the saline, and its upregulation was also inhibited by the B5 antibody treatment (c). A similar trend was seen for TNF- α (e) and GM-CSF (f), although in this case the inhibition was not statistically significant.

the presence of cytokines and chemokines using a multiplex bead-based assay. There was a significant increase in 13 of the 32 cytokines between saline-treated and RS-HDM-treated mice. Of these, IL-4, IL-5 and CCL2 were significantly lower in the B5-HDM, as compared to RS-HDM-treated mice (Fig. 3a–c), while IL-13, GM-CSF and TNF- α showed a trend towards decreased levels in the B5-HDM group, compared to the RS-HDM group (Fig. 3d–f).

Of the 13 cytokines that were significantly increased by HDM, several were not affected by PAR₂ blockade, including IL-17, CXCL9, M-CSF, IL-3, LIF, CXCL10, CXCL1, CCL3 and CCL4 (data not shown). Finally, many of the mediators tested were not affected by HDM exposure in this model, including CCL11 and IL-10 as well as IL-9, IFN- γ , IL-7, IL-15 and CXCL5 (data not shown).

Blocking PAR₂ activation during sensitization to HDM is sufficient to prevent airway hyperreactivity and airway inflammation following allergen challenge.

Once we determined that PAR₂ plays a role in the development of allergic sensitization to HDM, we assessed whether blocking PAR₂ activation during the sensitization phase would prevent the development of airway inflammation and AHR following allergen challenge. We used the same sensitization and challenge model as with the PAR₂ KO mice, except that B5 or RS were given on days 1 through 5 (Fig. 4a).

Upon challenge, the mice that received HDM extract with RS during sensitization, showed increases in AHR (Fig. 4b), as well as in total cells (Fig. 4c) and eosinophils (Fig. 4d) in the airways, as compared to B5-HDM or saline controls. RS-HDM-treated mice also had increased

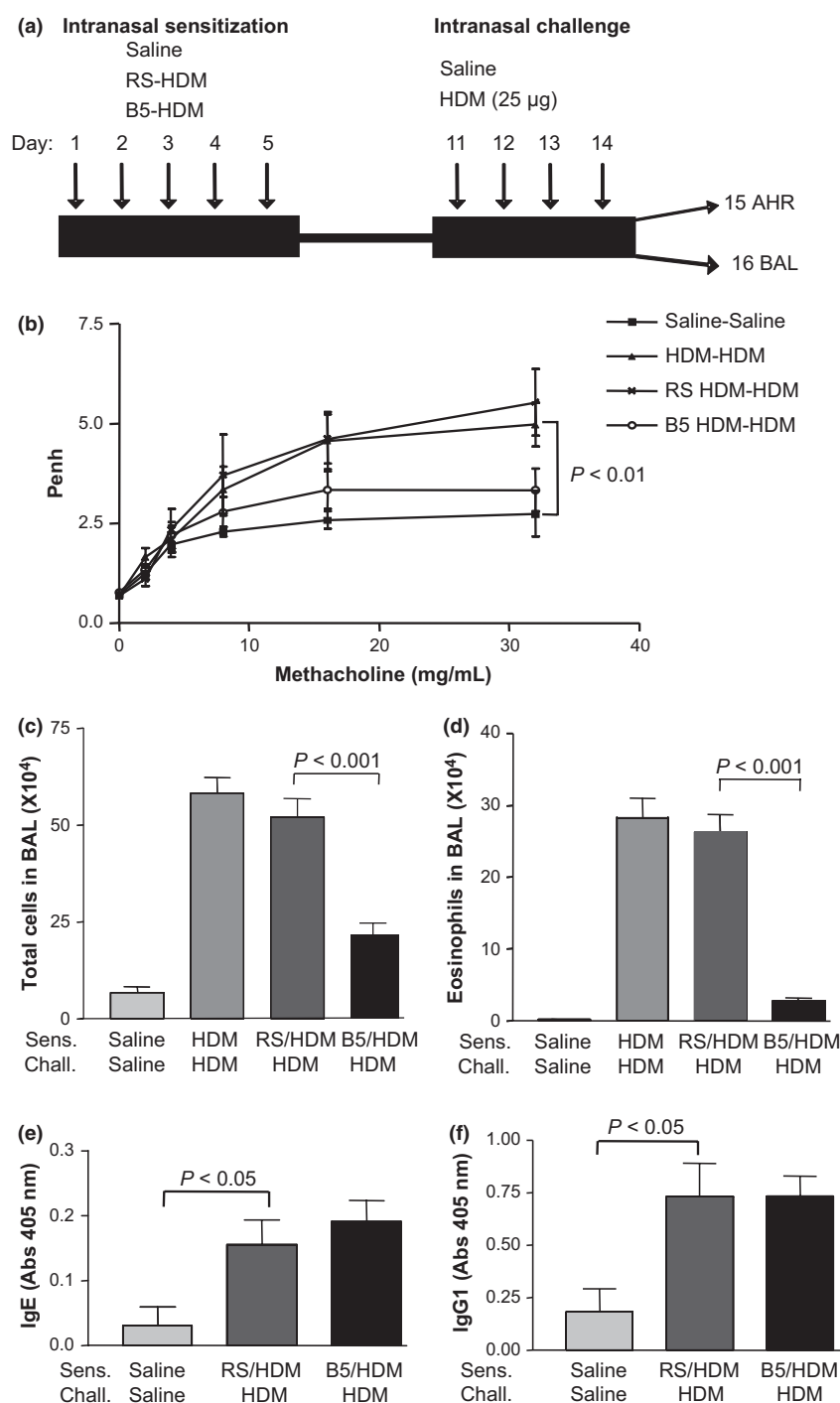


Fig. 4. Blocking proteinase-activated receptor-2 (PAR₂) activation during sensitization to house dust mite (HDM) prevents airway hyperresponsiveness (AHR) and airway inflammation following allergen challenge. Mice were sensitized for 5 days with i.n. saline, or with HDM, with or without an anti-PAR₂ blocking antibody or an rabbit serum (RS) control, and after 5 days of rest they were challenged on four consecutive days with i.n. saline or HDM (Saline-Saline, $n = 7$; HDM-HDM, $n = 8$; RS/HDM-HDM, $n = 8$; B5/HDM-HDM, $n = 9$; two independent experiments) (a). AHR measurements were taken using whole body plethysmography 24 h after the final challenge. Enhanced pause (Penh) in response to various doses of methacholine is presented (b). Bronchoalveolar lavage (BAL) was used to assess the total number of inflammatory cells (c) and the number of eosinophils (d) present in the lungs 48 h after the final challenge (Saline-Saline, $n = 13$; HDM-HDM, $n = 9$; RS/HDM-HDM, $n = 14$; B5/HDM-HDM, $n = 15$). Serum HDM-specific IgE (e) and IgG1 (f) were measured by ELISA. Values are presented as mean $\pm$ SEM.

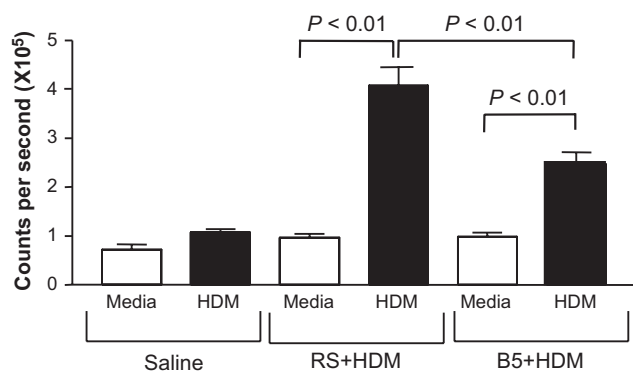


Fig. 5. *In vitro* house dust mite (HDM)-induced splenocyte proliferation in animals exposed to HDM, with or without pre-treatment with anti-proteinase-activated receptor-2 (PAR₂) antibody during the sensitization phase. Mice were given i.n. saline, i.n. rabbit serum (RS) followed by HDM, or i.n. B5 antibody followed by HDM for 5 days, and their spleen cells were isolated on day 9, 4 days following the final HDM exposure ($n = 6$ per group, two independent experiments). Splenocytes were cultured with media alone, or with HDM extract (100 μ g/mL). B5 antibody pre-treatment showed a 50% decrease in the T cell proliferation compared to the RS pre-treatment in the HDM restimulation.

serum HDM-specific IgE (Fig. 4e) and IgG1 levels (Fig. 4f) following allergen challenge, as compared to the saline-treated group; however, B5 pre-treatment did not prevent this response.

Intranasal blockade of PAR₂ during HDM sensitization decreases HDM-specific splenic cell proliferation

Since PAR₂ blockade during the sensitization phase affected the development of allergic airway inflammation following allergen challenge, we investigated whether this was dependent on an altered T cell phenotype. Mice were again treated with either control RS or an B5, 30 min prior to intranasal administration of HDM for 5 days and spleens were collected on day 9. Spleen cells were cultured for 3 days with either media alone, the direct T cell stimulant Concanavalin A, or HDM extract. Splenocytes from mice treated *in vivo* with HDM and RS proliferated in response to HDM, unlike cells harvested from saline-treated mice. Mice treated *in vivo* with B5-HDM yielded splenocytes with a ~50% decreased proliferative response to HDM compared to those from mice treated with RS-HDM (Fig. 5). In contrast, Concanavalin A induced similar levels of proliferation in all three groups (data not shown).

The effect of PAR₂ blockade on outcomes of allergic disease is mediated through CD4⁺ T cells

Since the T cells seem to respond differently to antigen, we tested whether these T cells are deficient in disease transfer. CD4⁺ T cells have been shown to be able to

transfer disease from HDM sensitized mice to naïve mice [25]. For this reason, we isolated spleen CD4⁺ T cells after 5 HDM sensitizations in the presence of RS or B5, and transferred them to naïve mice. Mice were challenged twice with HDM following CD4⁺ T cell transfer (Fig. 6a). The mice that received CD4⁺ T cells from RS-HDM treated mice had increased airway resistance (Fig. 6b) and elastance (Fig. 6c), as compared to those that received CD4 cells from saline treated mice. However, mice that received CD4⁺ T cells from B5-HDM sensitized mice did not show this increase. In addition, the mice that received CD4⁺ T cells from RS-HDM treated mice had an increase in total cells (Fig. 6d) and eosinophils (Fig. 6e) in the airways compared to those that received cells from saline treated mice. This effect was not evident in mice that received cells from B5-HDM sensitized mice.

Discussion

The main finding of our study is that full mucosal sensitization to intranasally administered HDM allergens depends on PAR₂ activation. PAR₂ KO mice failed to develop HDM-specific IgG1 and developed less HDM-induced airway inflammation than wild-type controls. Blocking PAR₂ activation in wild-type mice prior to sensitization with HDM results in decreased Th2 cytokines (IL-4, IL-5 and IL-13) and CCL2 in the lung tissue, as well as decreased splenocyte proliferation in response to HDM *ex vivo*. Importantly, blocking PAR₂ activation during sensitization to HDM was also sufficient to prevent AHR and airway inflammation following subsequent allergen challenge. Inhibition of Th2 cytokine induction was the most pronounced effect of PAR₂ blockade during the sensitization phase, indicating that T cells are directly or indirectly, affected by PAR₂ activation. Our data further confirmed this by showing that adoptive transfer of CD4⁺ cells from spleens of mice sensitized to HDM following PAR₂ blockade, which, unlike CD4⁺ cells from mice sensitized to HDM in the absence of the blocking antibody, was not able to induce AHR and airway inflammation in naïve mice. Cockroach allergen sensitization [6] and sensitization to ovalbumin, which lacks proteinase activity [18, 26], are also PAR₂ dependent. Thus, these findings indicate PAR₂ activation represents a common pathway that mediates allergic responses to aeroallergens.

We have previously proposed that PAR₂ activation acts as an adjuvant in the airways and can mediate allergic sensitization to otherwise innocuous antigens that are presented at the same time to the mucosal immune system [19]. Since Toll-like receptor 4 (TLR4) activation also acts as an adjuvant during mucosal allergic sensitization [27], HDM allergens [28] along with lipopolysaccharide in the HDM preparation we used may

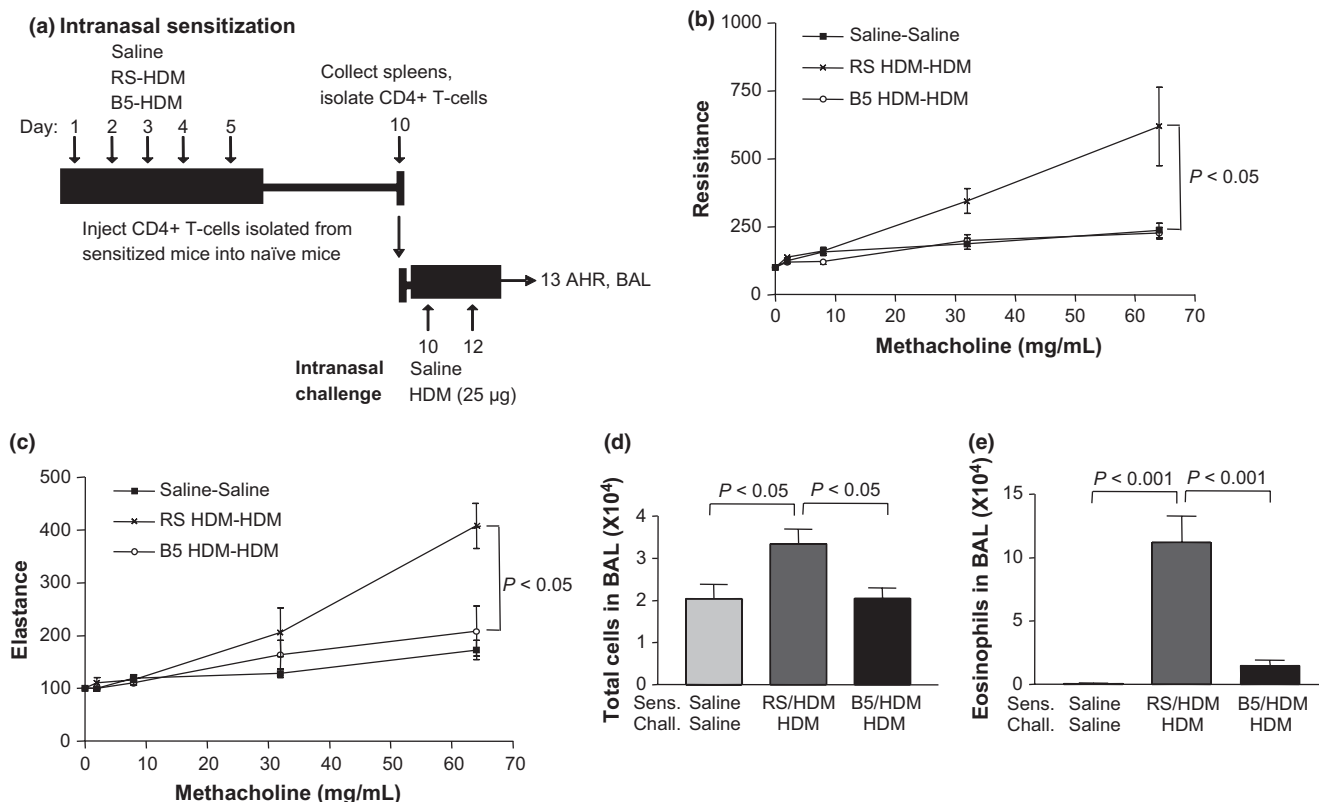


Fig. 6. The effects of proteinase-activated receptor-2 (PAR₂) blockade during sensitization can be mediated through CD4⁺ T cells. Mice were sensitized for 5 days with i.n. saline, or with house dust mite (HDM) with either an anti-PAR₂ blocking antibody or an rabbit serum (RS) control, and after an additional 5 days, CD4⁺ T cells from the spleens of these animals were transferred to naïve mice, which were challenged twice with HDM and assessed for airway hyperresponsiveness (AHR) and airway inflammation (a). AHR measurements were taken using FlexiVent, from which the lung resistance (b) and elastance (c) were determined (Saline-Saline, $n = 4$; RS/HDM-HDM, $n = 4$; B5/HDM-HDM, $n = 4$; one independent experiment). Both resistance and elastance are presented as the percent increase over baseline (the response to saline solution). Bronchoalveolar lavage (BAL) was used to assess the total number of inflammatory cells (d) and the number of eosinophils (e) present in the lungs 24 h after the final challenge (Saline-Saline, $n = 5$; RS/HDM-HDM, $n = 5$; B5/HDM-HDM, $n = 5$).

activate TLR4, resulting in some of the PAR₂-independent effects observed in our model. TLR4 on structural cells in the airway has been shown to mediate some of the effects of HDM on allergic sensitization [29].

This study focuses on the role of PAR₂ activation in the airways in mediating allergic immunological responses. We have previously shown that PAR₂ is involved in the development of allergic sensitization to cockroach allergens [6], an observation that has also been shown by other groups [30]. The data presented in this manuscript indicate that this is a general role of PAR₂ which participates in allergic sensitization to all allergens with serine proteinase activity. More importantly, this study also explores the mechanisms of this effect and shows that it is mediated through altered T cell responses. The decreased *ex vivo* splenocyte proliferation in mice exposed to HDM extract in the context of PAR₂ blockade indicates a dependence of systemic sensitization to inhaled HDM allergens on PAR₂ activation in the airways. This finding is in agreement with

the decreased IL-4, IL-5 and IL-13 production in the lung, likely due to lower numbers of antigen-specific Th2 cells.

The cell transfer experiments also support this, as transfer of CD4⁺ T cells from the spleens of HDM sensitized mice into naïve mice induces AHR and airway inflammation [25]. In our study, we reproduced those results and showed that PAR₂ blockade abrogated the ability of CD4⁺ T cells to transfer disease. It has also been shown that ovalbumin-specific [31] or HDM-allergen-specific [32], Th2 polarized CD4⁺ T cell lines induce lung eosinophilia in adoptive transfer models, whereas ovalbumin-specific Th1 cells do not. This indicates that it is the Th2 population that is transferring the allergic phenotype and orchestrating the immune response in the naïve animals upon allergen challenge. These data support the hypothesis that PAR₂ activation may play a role in Th2 polarization of the immune response observed in asthma and PAR₂ blockade may decrease Th2 polarization. Whether this is a direct effect of PAR₂

activation on T cells or an indirect effect is not clear from our data. One study has suggested that direct PAR₂-mediated activation of T cells may be responsible for allergic airway inflammation [33]. However, an indirect effect through the release of cytokines and chemokines from PAR₂-activated epithelial cells, which then alter the activation of dendritic cells and/or T cells cannot be excluded.

The exact mechanisms leading from PAR₂ activation to altered T cell responses are not clear at this point. Our data implicate changes in various cytokines and chemokines in altered T cell differentiation and/or activation, and lead to some hypotheses discussed below, but more work needs to be done to fully understand these effects.

CCL2 is required for generation of Th2 immune responses in an ovalbumin allergy model [34]. HDM can induce CCL2, which mediates DC accumulation [35]. Decreased CCL2 levels following PAR₂ blockade may lead to decreased DC accumulation in the lungs in our model and decreased allergic sensitization.

Allergic sensitization can be partially blocked by neutralizing GM-CSF [36] or TNF- α [19]. Our previous work has shown that in an ovalbumin model, PAR₂-mediated allergic sensitization is TNF- α -dependent [19]. Although the decreases in TNF- α and GM-CSF resulting from PAR₂ blockade in our model did not reach significance, we may have missed the optimal time-point in which to observe this difference.

We observed a clear up-regulation of both IL-33 and OX40L in our HDM model. IL-33 is released from airway epithelial cells in a HDM model of allergic airway inflammation and plays an important role in the development of disease [37]. IL-33 is also of interest, as the binding of IL-33 to ST2 on Th2 cells has been shown to produce IL-5 and IL-10 [38], and this pathway has been implicated in the activation and migration of Th2 cells [39]. Increased levels of IL-33 have also been associated with human asthma [40]. OX40L is known to be up-regulated on antigen presenting cells, like dendritic cells and B cells, following antigen exposure. OX40L plays an important role in allergic sensitization in mouse models [41, 42]. We have previously shown, using an ovalbumin model of allergic sensitization, that PAR₂ activation in the airway increases antigen uptake by CD11c cells and increases dendritic cell trafficking to the spleen [19]. However, based on our results, it is very likely that the upregulation of IL-33 or OX40L expression seen in our mouse asthma model is PAR₂-independent.

The initial stimulus that initiates allergic sensitization to HDM is not very clearly defined. IL-33 plays an important role in allergic sensitization to HDM in a couple of slightly different models [37, 43]. It has also been shown that although HDM administration induces IL-25 and TSLP in the lung tissue, these two cytokines are not required for allergic sensitization [43]. On the other hand,

it has been shown that TSLP [44] and IL-25 [45] inhibition can both prevent airway remodelling in more long-term HDM models of allergic sensitization. Interestingly, there was no up-regulation of IL-25 or TSLP in our HDM model, so it seems unlikely that these cytokines are responsible for the initiation of the Th2 response. We also observed that CCL20, which has been shown to be increased in the airways of mice exposed to cockroach frass in a PAR₂-dependent manner and to recruit myeloid DC to the lungs, was not increased in our model [17].

The allergen-specific IgG1 and IgE responses to HDM challenge were not diminished when HDM exposure during the sensitization phase was accompanied by PAR₂ blockade (Fig. 4e and f). HDM-specific IgG1 was decreased in the PAR₂ knockout mouse (Fig. 1d); therefore, this may be the result of incomplete PAR₂ blockade by the antibody.

Our results also identified a number of cytokines and chemokines induced by HDM exposure that were not affected by PAR₂ blockade, such as IL-17 and MIG. These results indicate that although HDM induced Th2-, Th1- and Th17-related cytokines/chemokines, a blockade of PAR₂ activation prevented primarily the increase of Th2-associated cytokines/chemokines, which was sufficient to abrogate the response to challenge/development of disease.

Interestingly, we found that CCL11 and CCL5 are not increased following HDM sensitization. In a previous study, we found that administration of the same PAR₂ blocking antibody during the sensitization phase in a cockroach model decreased CCL11 induction by the allergen [46]. It is possible that CCL11 is only induced after challenge with an antigen and that is the reason we did not detect it in our model where we analyzed lungs immediately after sensitization.

We showed no increase in PAR₂ mRNA in the lung following sensitization with HDM. PAR₂ is increased on the epithelium of asthmatic individuals [13], but this may indicate a predisposition to asthma, not an up-regulation secondary to allergic sensitization, or may result from chronic allergen exposure.

In conclusion, we have shown that PAR₂ activation mediates some of the immune changes seen following sensitization to HDM allergens. These data support the observation that PAR₂ polymorphisms in Korean children are associated with allergy development [14]. Therefore, blockade of PAR₂ activation may represent a therapeutic strategy for attenuating the allergenic response not only to HDM but in general for allergens that contain PAR₂-activating proteinases.

Acknowledgements

We thank Xiaobei Zhang for the measurement of HDM-specific immunoglobulins in mouse serum samples. This

study was supported by grants from the Canadian Institutes of Health Research, Alberta Innovates – Health Solutions, and the Lung Association of Alberta and Northwest Territories.

Conflict of interest statement

No conflict of interest declared for any authors.

References

- Kikuchi Y, Takai T, Kuhara T *et al*. Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. *J Immunol* 2006; **177**:1609–17.
- Kheradmand F, Kiss A, Xu J, Lee SH, Kolattukudy PE, Corry DB. A protease-activated pathway underlying Th cell type 2 activation and allergic lung disease. *J Immunol* 2002; **169**:5904–11.
- Gough L, Schulz O, Sewell HF, Shakib F. The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J Exp Med* 1999; **190**:1897–902.
- Comoy EE, Pestel J, Duez C *et al*. The house dust mite allergen, Dermatophagoides pteronyssinus, promotes type 2 responses by modulating the balance between IL-4 and IFN- γ . *J Immunol* 1998; **160**:2456–62.
- Fattouh R, Pouladi MA, Alvarez D *et al*. House dust mite facilitates ovalbumin-specific allergic sensitization and airway inflammation. *Am J Respir Crit Care Med* 2005; **172**:314–21.
- Arizmendi NG, Abel M, Mihara K *et al*. Mucosal allergic sensitization to cockroach allergens is dependent on proteinase activity and proteinase-activated receptor-2 activation. *J Immunol* 2011; **186**:3164–72.
- Danahay H, Withey L, Poll CT, van de Graaf SF, Bridges RJ. Protease-activated receptor-2-mediated inhibition of ion transport in human bronchial epithelial cells. *Am J Physiol Cell Physiol* 2001; **280**:C1455–64.
- Hwa JJ, Ghibaudi L, Williams P *et al*. Evidence for the presence of a proteinase-activated receptor distinct from the thrombin receptor in vascular endothelial cells. *Circ Res* 1996; **78**:581–8.
- Schmidlin F, Amadesi S, Vidil R *et al*. Expression and function of proteinase-activated receptor 2 in human bronchial smooth muscle. *Am J Respir Crit Care Med* 2001; **164**:1276–81.
- Roche N, Stirling RG, Lim S *et al*. Effect of acute and chronic inflammatory stimuli on expression of protease-activated receptors 1 and 2 in alveolar macrophages. *J Allergy Clin Immunol* 2003; **111**:367–73.
- Howells GL, Macey MG, Chinni C *et al*. Proteinase-activated receptor-2: expression by human neutrophils. *J Cell Sci* 1997; **110**(Pt 7):881–7.
- Stenton GR, Nohara O, Dery RE *et al*. Proteinase-activated receptor (PAR)-1 and -2 agonists induce mediator release from mast cells by pathways distinct from PAR-1 and PAR-2. *J Pharmacol Exp Ther* 2002; **302**:466–74.
- Knight DA, Lim S, Scaffidi AK *et al*. Protease-activated receptors in human airways: upregulation of PAR-2 in respiratory epithelium from patients with asthma. *J Allergy Clin Immunol* 2001; **108**:797–803.
- Lee JH, Kim KW, Gee HY *et al*. A synonymous variation in protease-activated receptor-2 is associated with atopy in Korean children. *J Allergy Clin Immunol* 2011; **128**:1326–1334.e3.
- Vliagoftis H, Befus AD, Hollenberg MD, Moqbel R. Airway epithelial cells release eosinophil survival-promoting factors (GM-CSF) after stimulation of proteinase-activated receptor 2. *J Allergy Clin Immunol* 2001; **107**:679–85.
- Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteinases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol* 2009; **183**:1427–34.
- Day SB, Ledford JR, Zhou P, Lewkowich IP, Page K. German cockroach proteases and protease-activated receptor-2 regulate chemokine production and dendritic cell recruitment. *J Innate Immun* 2012; **4**:100–10.
- Schmidlin F, Amadesi S, Dabbagh K *et al*. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. *J Immunol* 2002; **169**:5315–21.
- Ebeling C, Lam T, Gordon JR, Hollenberg MD, Vliagoftis H. Proteinase-activated receptor-2 promotes allergic sensitization to an inhaled antigen through a TNF-mediated pathway. *J Immunol* 2007; **179**:2910–7.
- Arlian LG, Platts-Mills TA. The biology of dust mites and the remediation of mite allergens in allergic disease. *J Allergy Clin Immunol* 2001; **107**:S406–13.
- Asokanathan N, Graham PT, Stewart DJ *et al*. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J Immunol* 2002; **169**:4572–8.
- Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol* 2001; **167**:1014–21.
- Adam E, Hansen KK, Astudillo Fernandez O, Coulon L, Bex F, Duhant X, Jaumotte E, Hollenberg MD, Jacquet A. The house dust mite allergen Der p 1, unlike Der p 3, stimulates the expression of interleukin-8 in human airway epithelial cells via a proteinase-activated receptor-2-independent mechanism. *J Biol Chem* 2006; **281**:6910–23.
- Schneider AM, Li F, Zhang X, Gordon JR. Induction of pulmonary allergen-specific IgA responses or airway hyperresponsiveness in the absence of allergic lung disease following sensitization with limiting doses of ovalbumin-alum. *Cell Immunol* 2001; **212**:101–9.
- Katoh S, Kaminuma O, Hiroi T *et al*. CD44 is critical for airway accumulation of antigen-specific Th2, but not Th1, cells induced by antigen challenge in mice. *Eur J Immunol* 2011; **41**:3198–207.
- Ebeling C, Forsythe P, Ng J, Gordon JR, Hollenberg M, Vliagoftis H. Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway

- hyperresponsiveness through different pathways. *J Allergy Clin Immunol* 2005; **115**:623–30.
- 27 Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002; **196**:1645–51.
 - 28 Trompette A, Divanovic S, Visintin A *et al.* Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009; **457**:585–8.
 - 29 Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009; **15**:410–6.
 - 30 Lewkowich IP, Day SB, Ledford JR *et al.* Protease-activated receptor 2 activation of myeloid dendritic cells regulates allergic airway inflammation. *Respir Res* 2011; **12**: 122.
 - 31 Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K. Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp Med* 1997; **186**:1737–47.
 - 32 Huang CH, Loo EX, Kuo IC *et al.* Airway inflammation and IgE production induced by dust mite allergen-specific memory/effector Th2 cell line can be effectively attenuated by IL-35. *J Immunol* 2011; **187**:462–71.
 - 33 Liang G, Barker T, Xie Z, Charles N, Rivera J, Druey KM. Naive T cells sense the cysteine protease allergen papain through protease-activated receptor 2 and propel TH2 immunity. *J Allergy Clin Immunol* 2012; **129**: 1377–1386.e13.
 - 34 Gu L, Tseng S, Horner RM, Tam C, Loda M, Rollins BJ. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* 2000; **404**:407–11.
 - 35 Pichavant M, Charbonnier AS, Taront S *et al.* Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment. *J Allergy Clin Immunol* 2005; **115**:771–8.
 - 36 Cates EC, Fattouh R, Wattie J *et al.* Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J Immunol* 2004; **173**:6384–92.
 - 37 Willart MA, Deswarte K, Pouliot P *et al.* Interleukin-1 α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J Exp Med* 2012; **209**:1505–17.
 - 38 Lohning M, Stroehmann A, Coyle AJ *et al.* T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc Natl Acad Sci USA* 1998; **95**:6930–5.
 - 39 Komai-Koma M, Xu D, Li Y, McKenzie AN, McInnes IB, Liew FY. IL-33 is a chemoattractant for human Th2 cells. *Eur J Immunol* 2007; **37**:2779–86.
 - 40 Sakashita M, Yoshimoto T, Hirota T *et al.* Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis. *Clin Exp Allergy* 2008; **38**:1875–81.
 - 41 Damayanti T, Kikuchi T, Zaini J *et al.* Serial OX40 engagement on CD4 + T cells and natural killer T cells causes allergic airway inflammation. *Am J Respir Crit Care Med* 2010; **181**:688–98.
 - 42 Hoshino A, Tanaka Y, Akiba H *et al.* Critical role for OX40 ligand in the development of pathogenic Th2 cells in a murine model of asthma. *Eur J Immunol* 2003; **33**:861–9.
 - 43 Chu DK, Llop-Guevara A, Walker TD *et al.* IL-33, but not thymic stromal lymphopoietin or IL-25, is central to mite and peanut allergic sensitization. *J Allergy Clin Immunol* 2013; **131**: 187–200.e1–8.
 - 44 Chen ZG, Zhang TT, Li HT *et al.* Neutralization of TSLP inhibits airway remodeling in a murine model of allergic asthma induced by chronic exposure to house dust mite. *PLoS ONE* 2013; **8**:e51268.
 - 45 Gregory LG, Jones CP, Walker SA *et al.* IL-25 drives remodelling in allergic airways disease induced by house dust mite. *Thorax* 2013; **68**:82–90.
 - 46 Arizmendi NG, Abel M, Puttagunta L *et al.* Mucosal exposure to cockroach extract induces allergic sensitization and allergic airway inflammation. *Allergy Asthma Clin Immunol* 2011; **7**:22.