University of Groningen

Pim1 kinase: a double-edged sword

de Vries, Maaike

Publication date:
2015

Chapter 5

Pim1 kinase activity preserves airway epithelial integrity upon house dust mite exposure.

M. de Vries, L. Hesse, M. van den Berge, A.J.M. van Oosterhout, I.H. Heijink and M.C. Nawijn

Abstract

Most patients with allergic asthma are sensitized to house dust mite (HDM). The allergenicity of HDM largely depends on disruption of the integrity and pro-inflammatory activation of the airway epithelium. In this study, we hypothesized that Pim1 kinase activity attenuates HDM-induced asthma by preserving airway epithelial integrity. The effects of Pim1 kinase activity on barrier function and release of the pro-inflammatory mediators IL-1α and CCL20 were studied *in vitro* in 16HBE and primary bronchial epithelial cells (PBECs). *Pim1*-proficient and deficient mice were exposed to a HDM-driven model of allergic asthma, and airway hyper-responsiveness (AHR) was measured upon metacholine challenge. Airway inflammation and pro-inflammatory mediators in lung tissue and BAL fluid were determined. We observed that inhibition of Pim1 kinase prolongs the HDM-induced loss of barrier function in 16HBE cells and sensitizes PBECs to HDM-induced barrier dysfunction. Additionally, inhibition of Pim1 kinase increased the HDM-induced pro-inflammatory activity of 16HBE cells as measured by IL-1α secretion. In line herewith, HDM exposure induced an enhanced production of the pro-inflammatory chemokines CCL17 and CCL20 in *Pim1*-deficient mice compared to wild-type controls. While we observed a marked increase in eosinophilic and neutrophilic granulocytes as well as mucus cell metaplasia and AHR to metacholine in mice exposed to HDM, these parameters were independent of Pim1 kinase activity. In contrast, levels of the Th2-cytokines IL-5 and IL-10 were significantly augmented in HDM-treated *Pim1*-deficient mice. Taken together, our study shows that Pim1 kinase activity maintains airway epithelial integrity and protects against HDM-induced pro-inflammatory activation of the airway epithelium.

Keywords

Introduction

Asthma is a chronic inflammatory airways disease characterized by variable airflow obstruction, symptoms of cough and dyspnea, and airway hyper-responsiveness (AHR) and remodeling [1]. With a prevalence of approximately 300 million people worldwide, asthma related healthcare costs are substantial [2][3]. The most common sub-phenotype of the disease, allergic asthma, can be distinguished by infiltration of eosinophils and CD4^{+}T Helper (Th) type 2 cells into the airway sub-mucosa as a consequence of sensitization and continued exposure to aero-allergens [4][5]. While several aero-allergens are known to be involved in the inception and exacerbation of allergic asthma including cockroach, pollen, mold and pets, the most commonly observed sensitization is to the aero-allergen house dust mite (HDM) [4][6].

The allergenicity of HDM is caused by the complex composition of the mite bodies and their fecal pellets, containing allergenic Dermatophagoides pteronyssinus (Der P) proteases, β-glucans and the glucosamine-based polymer chitins as well as microbial compounds like lipopolysaccharide (LPS) [4]. Upon inhalation, HDM precipitates on the airway epithelial cells of the conducting airways. The airway epithelium forms a continuous physical and immunological barrier between the external environment of the respiratory tract and the inner sub-mucosal tissue [7]. HDM impairs the integrity of the airway epithelial barrier through delocalization of the epithelial junctional molecules E-cadherin, ZO-1 and occludin, which subsequently facilitates uptake of HDM by and activation of immature dendritic cells (DCs) [8][9][10]. We have previously shown that disruption of epithelial barrier function also leads to increased production of pro-inflammatory chemokines from the airway epithelial cells - including the Th2 cell-attracting chemokine CCL17 - and further augments the inflammatory immune response [11]. In addition, we have shown that HDM-induced allergic sensitization and AHR *in vivo* correlates with its capacity to induce loss of epithelial barrier function as well as production of the DC and T cell-attractant CCL20 *in vitro* [9]. Moreover, HDM has been

shown to induce the release of IL-1α from airway epithelial cells *in vivo* and *in vitro*, which subsequently triggers the release of DC-attracting chemokines in an autocrine manner [12]. Therefore, we and others have proposed an important role for the airway epithelium in the inception of allergic asthma and factors influencing the integrity of the airway epithelium could be important in the pathogenesis of allergic asthma [13] [14].

Pim1 kinase is a constitutively active serine/threonine kinase, highly expressed in the airway epithelium. By phosphorylating serine and/ or threonine residues of its protein targets, Pim1 kinase is involved in a wide variety of cellular processes like cell growth and differentiation, and is most well-known for its anti-apoptotic properties [15]. A study by Shin *et al* showed that pharmacological inhibition of Pim1 kinase activity in an ovalbumin-induced asthma model reduces eosinophilic airway inflammation and subsequent AHR to metacholine, indicating that Pim1 kinase is involved in the pathogenesis of allergic asthma [16]. Based on the protective effect of Pim1 kinase on CS-induced damage of the airway epithelial cells [17], we hypothesize that Pim1 kinase activity also protects against the development of HDM-induced allergic asthma by preserving airway epithelial integrity. To investigate this, we assessed the role of Pim1 kinase both on HDM-induced airway epithelial barrier dysfunction and pro-inflammatory responses as well as in a mouse model of HDM-induced allergic asthma.

Material and methods

Cell culture

The human bronchial epithelial cell line 16HBE was kindly provided by Dr DC Gruenert (University of California, San Francisco, CA, USA). The cells were cultured in 30 µg/ml collagen (Purecol, Advanced BioMatrix Inc, San Diego, CA, USA) and 10 µg/ml BSA (Sigma Aldrich, Zwijndrecht, The Netherlands) coated flasks in EMEM (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Thermo Scientific, Cramlington, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies Europe, Bleiswijk, The Netherlands) as previously described [11].

Primary bronchial epithelial cells (PBECs) from healthy individuals were obtained by bronchial brushings according to standard guidelines, and approved by the Medical Ethics Committee of the University Hospital of Groningen (Groningen, The Netherlands). All participants signed informed consent, and subject characteristics can be found in table 1. PBECs were cultured in tissue culture flasks coated with 30 µg/ml collagen (Purecol, Advanced BioMatrix Inc), 30 µg/ml fibronectin (Sigma- Aldrich) and 10 µg/ml BSA (Sigma-Aldrich) in hormonally-supplemented bronchial epithelial growth medium (BEGM, Lonza) containing 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies Europe) as previously described [11].

Table 1: Summary of clinical and physiological characteristics of healthy subjects used to study the effects of inhibition of Pim1 kinase activity on airway epithelial barrier function upon HDM exposure

Age (y)	Sex, male/female	FEV_1 (% predicted)	Inhaled corticosteroids yes/no
45 (21 - 70)	1/3	103 (98 - 109)	0/4

All subjects were recruited after ethical approval and informed consent.

Resistance measurements

The airway epithelial resistance of cultures of 16HBE cells and PBECs was measured using the electric cell-substrate impedance sensing (ECIS, Applied Biophysics Inc, Troy, NY, USA) technique as described by Heijink *et al* [11]. Gold-film electrode arrays were coated with collagen, BSA and/or fibronectin as described for cell culture and cells were seeded at a density of 100,000 cells/well for 16HBE cells and 75,000 cells/well for PBECs in the cell-specific culture medium. The resistance of the airway epithelial cells was measured real-time. Once resistance had stabilized, 16HBE or PBECs were placed for 24 h in EMEM without serum or hormonally-deprived bronchial epithelium basal medium (BEBM) containing 10 μg/ml transferrin (Sigma-Aldrich), 5 μg/ml insulin (Sigma-Aldrich), 50 μg/ml Gentamycin (Sigma- Aldrich) and 50 ng/ml Amphotericin (Sigma-Aldrich), respectively. Subsequently, cells were pre-treated with 5 μM Pim1 kinase inhibitor K00135 (PKI) [18] or DMSO as vehicle control for 1 h and stimulated with 50 or 100 μg/ml HDM for 16HBE cells and PBECs respectively. Normalized resistance was calculated relative to the resistance of the cultures at the last measurement prior to addition of PKI or HDM.

Release of IL-1α and CCL20 from 16HBE cells

16HBE cells were cultured at a density of 100,000 cells/well in 24 wells plates until confluence as previously described [11], and serum starved overnight in the presence of 5 μM PKI or DMSO as vehicle control. Subsequently, cells were stimulated with 50 μg/ml HDM or medium control for 24 h. Supernatant was collected and the levels of IL-1α and CCL20 were measured in cell-free supernatant by ELISA, according to manufacturer's instructions (R&D Systems).

Mouse studies

Female *Pim1*-deficient and -proficient FVB/Nrcl mice (10-18 wk) were bred and genotyped at the animal facility of the University of Groningen, The Netherlands. Mice were kept under specific pathogen-free conditions in individually ventilated cages, and maintained on a 12:12-h light-dark cycle, with food and water ad libitum. Animal housing, breeding, and experiments were performed after ethical review by and written approval of the Institutional Animal Care and Use Committee (IACUC-RUG) of the University of Groningen, The Netherlands.

House dust mite sensitization protocol

Twenty µl HDM extract (Greer Laboratories, Lenoir, NC, USA) dissolved in sterile PBS (2.5 mg total weight/ml) or PBS as vehicle control was administered intranasally twice a week for 5 weeks using isoflurane/oxygen anesthesia, as schematically depicted in figure 3A. Airway resistance upon intravenous metacholine challenge was measured by FlexiVent (SCIREQ, Montreal, Canada) 24 h after the last HDM challenge as described before [9], and bronchoalveolar lavage (BAL) fluid, blood and lungs were collected. The left lung lobe was inflated with TissueTek OCT Compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) for histological analysis 24 h after the last HDM challenge.

Collection of BAL fluid

BAL fluid was collected as previously described(5). Briefly, lungs were lavaged through a tracheal cannula with 1 ml PBS containing 3% BSA (Sigma Aldrich, Zwijndrecht, The Netherlands) and Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland). Cell-free supernatant was stored at -80°C until further analysis. Lavage was repeated 4 times with 1-ml aliquots of PBS and after pooling the

cells, total BAL cell numbers were counted with a Z2 coulter particle count and size analyzer (Beckman Coulter, Woerden, The Netherlands), and cytospins were prepared.

For the preparation of single cell suspensions, lungs were collected in PBS containing 1% BSA, and sliced into a homogenous suspension. The cell suspension was incubated in RPMI containing 1% BSA, 4 mg/ml collagenase A (Roche Diagnostics) and 0.1 mg/ml DNase I (Roche Diagnostics) for 1 h at 37 °C, and after filtering through a 70-µM Falcon cell strainer (BD Biosciences, San Jose, CA, USA) pelleted by centrifugation. Red blood cells were lysed in lysis buffer (Hospital Pharmacy UMCG, Groningen, The Netherlands) and resuspended in 200 µl PBS containing 1% BSA. Total cell numbers were determined as described above, and cytospin preparation were made.

Analysis of single cell suspension of BAL fluid and lung tissue

Cytospin preparations of the cellular components of BAL fluid and lung tissue were stained with Diff-Quick (Merz & Dade, Dudingen, Switzerland), and evaluated in a blinded fashion. Cells were identified and differentiated into mononuclear cells, neutrophils and eosinophils by standard morphology upon counting of at least 300 cells per preparation.

Preparation of lung tissue homogenates

Mouse lungs were homogenized on ice using a T10 basic Ultra TURRAX (IKA®-Werke GmBH & Co. KG, Staufen, Germany) in 4 µl Luminex buffer containing 50 mM Tris-HCl (Biorad Laboratories, Hercules, CA, USA), 150 mM NaCl (Sigma-Aldrich), 0.002% Tween (Sigma-Aldrich) and Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics) per mg lung tissue. Total protein levels in cell debris-free supernatant were determined with the PierceTM BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Cytokine analysis of BAL fluid and lung tissue homogenates

The levels of IL-5 and IL-10 in BAL fluid were determined using individual ELISA components from BD Biosciences optimized in our laboratory (1.0 µg/ml capture and detection antibody, and a standard curve starting at 7500 pg/ml). The levels of IL-1α, CCL17 and CCL20 in lung tissue homogenates were determined by ELISA, according to manufacturer's instructions (R&D Systems, Abingdon, UK) and corrected for the amount of total protein. Outliers were identified with the ROUT method (with Q set to 1%) and removed upon identification.

Preparation and staining of lung tissue sections

Inflated lungs were fixed in 10% Formalin for 24 h, and embedded in paraffin. 3 µM-thick sections were stained with haemotoxylin/eosin (HE) and Periodic acid-Schiff (PAS). Representative images were made using Hamamatsu Nanozoomer 2.0 HT (Hamamatsu Corporation, Bridgewater, MA, USA), and analyzed with Aperio ImagaScope (Leica Biosystems, Nussloch, Germany).

Statistical analysis

The Mann-Whitney U test was used to test for statistical significance between groups in the *in vivo* experiments, except for the statistical analysis of the AHR, which was conducted with the generalized estimating equations method [19]. ECIS data was analyzed with the 2-way ANOVA, and differences in the release of IL-1α and CCL20 were tested using the Student's t-test for paired observations.

Results

Inhibition of Pim1 kinase activity enhances HDM-induced epithelial barrier dysfunction and release of pro-inflammatory chemokines in 16HBE cells

To test our hypothesis that Pim1 kinase affects the sensitivity to HDM by protecting the airway epithelium against loss of integrity, we first assessed whether inhibition of Pim1 kinase activity had an effect on the airway epithelial barrier function. We treated a confluent layer of the human airway epithelial cell line 16HBE with a highly specific pharmacological Pim1 kinase inhibitor [18] and determined the effects on barrier function by measuring low frequency electrical resistance across the epithelial layer, the most sensitive parameter to monitor changes in cell-cell contacts [20]. As shown in fig. 1A, the addition of PKI resulted in a transient loss of epithelial resistance, while treatment with the solvent alone (DMSO) did not affect airway epithelial resistance (data not shown). The fact that PKI did not affect capacitance – a highly sensitive parameter for cell-matrix contacts [20] – indicates that inhibition of Pim1 kinase specifically acts on cell-cell contacts in 16HBE cells.

We previously showed that HDM induces a transient loss of airway epithelial barrier function in 16HBE cells, with subsequent release of CCL20 into the supernatant [9]. Therefore, we tested whether pharmacological inhibition of Pim1 kinase activity augmented the loss of airway epithelial resistance observed upon exposure to HDM. A confluent layer of 16HBE cells was pre-treated with PKI, where after HDM was added. As shown in fig. 1B, exposure to HDM resulted in a rapid decrease of 16HBE monolayer resistance, that returned to baseline within 1 h after the start of the exposure, in line with our previous observations [9]. Interestingly, pre-treatment with PKI significantly prolonged the loss of epithelial barrier function upon HDM exposure, without affecting the magnitude of the response (fig. 1B).
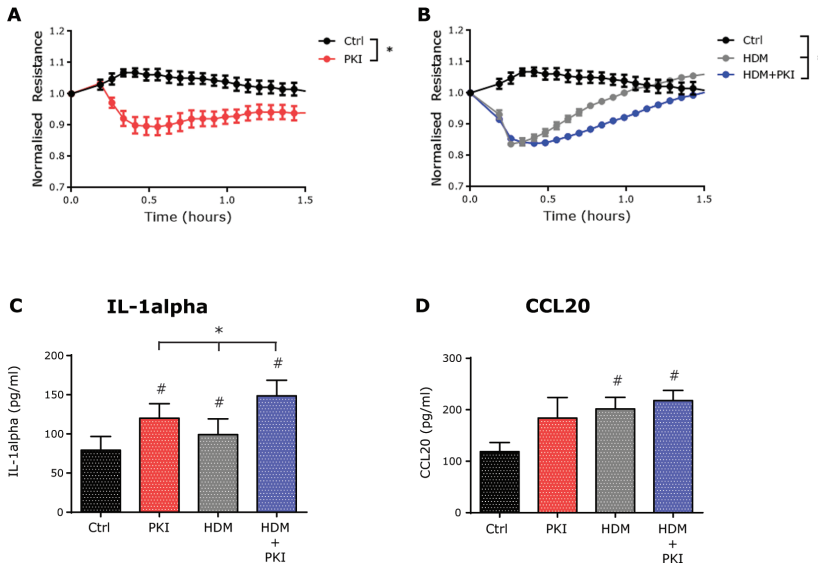
106

Figure 1: Inhibition of Pim1 kinase activity enhances HDM-induced epithelial barrier dysfunction and release of pro-inflammatory chemokines in 16HBE cells
*Low frequency electrical resistance across a monolayer of 16HBE cells was measured for up to 90 minutes using ECIS upon treatment with 5 µM Pim1 kinase inhibitor (PKI) (A) and after stimulation with 50 µg/ml HDM with or without pre-treatment with PKI for 1 h (B). Normalized resistance relative to the resistance of the monolayer cultures at the last measurement prior to addition of PKI or HDM is shown. The release of IL-1α (C) and CCL20 (D) into the tissue culture supernatant of a confluent monolayer of 16HBE cells, 24 h after treatment with PKI, stimulation with HDM or stimulation with HDM after pre-treatment with PKI was determined with ELISA. Independent experiments were performed 4 times for the measurement of resistance and 5 times for the measurement of cytokine release. Mean and SEM across the independent experiments are shown. * P < 0,05.*

To assess if concurrent inhibition of Pim1 kinase also augments the HDM-induced release of the pro-inflammatory mediators IL-1α and CCL20 *in vitro*, we pre-treated 16HBE cells with PKI, and measured the release of both mediators upon stimulation with HDM. Inhibition of Pim1 kinase increased both baseline and HDM-induced release of IL-1α (fig. 1C). HDM also significantly enhanced the release of

CCL20, however, inhibition of Pim1 kinase activity did not enhance the release of CCL20 either at baseline or upon exposure to HDM (fig. 1D).

In summary, these results show that inhibition of Pim1 kinase itself induces a transient loss of epithelial barrier function, which is accompanied by an increased release of the pro-inflammatory cytokine IL-1α. Moreover, inhibition of Pim1 kinase activity prolongs the HDM-induced loss of airway epithelial barrier function and release of IL-1α.

Inhibition of Pim1 kinase impairs barrier function in the presence of HDM in primary bronchial epithelial cells

To increase the translational relevance of our findings, we used PBECs from healthy subjects to confirm our findings in 16HBE cells. As observed before [21], exposure of PBECs from healthy controls to HDM did not significantly affect barrier function as measured by low-frequency resistance (fig. 2A). Furthermore, inhibition of Pim1 kinase itself did not induce significant changes in airway epithelial resistance in PBECs (fig. 2B). However, pre-treatment with PKI results in significantly decreased electrical resistance upon subsequent HDM exposure compared to the control treated PBECs (fig. 2C), to a similar extent as previously observed in PBECs derived from asthma patients [21]. Since no significant effect of PKI or HDM on high-frequency capacitance was observed (data not shown), the decrease in airway epithelial resistance is likely to be specific for cell-cell contacts. These data indicate that inhibition of Pim1 sensitizes PBECs to HDM-induced loss of epithelial barrier function, identifying Pim1 as a relevant regulatory node in maintenance of airway epithelial integrity.
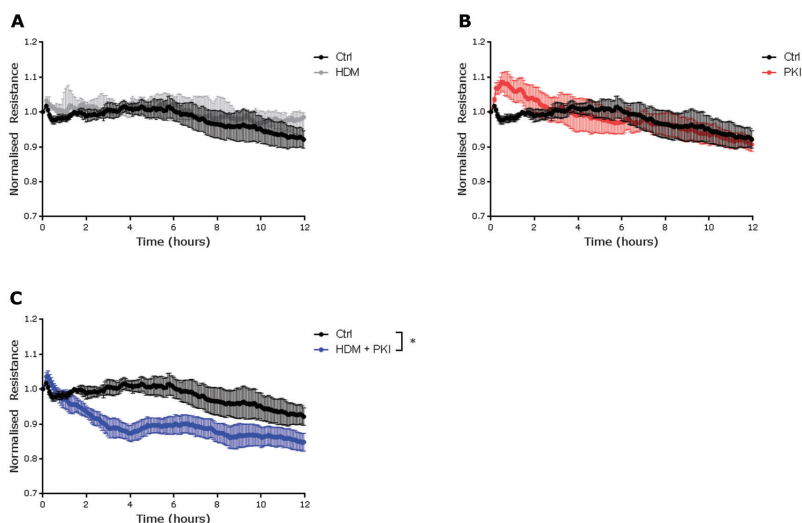
Figure 2: Inhibition of Pim1 kinase impairs barrier function in the presence of HDM in primary bronchial epithelial cells

*Primary bronchial epithelial cells (PBECs) derived from bronchial brushes of healthy control subjects were cultured and the low frequency electrical resistance across a monolayer of PBECS was measured by ECIS for up to 12 h. PBECs were stimulated with 100 μg/ml HDM (A), treated with 5 μM Pim1 kinase inhibitor (PKI) (B), and stimulated with HDM upon pre-treatment for 1 h with PKI (C). Normalized resistance relative to the resistance of the monolayer cultures at the last measurement prior to stimulation is shown. Experiments were repeated 4 times with PBECs from independent donors and mean and SEM are shown. * P < 0,05.*

HDM-induced release of pro-inflammatory cytokines is augmented in *Pim1*-deficient mice

Pro-inflammatory cytokine and chemokine release by damaged airway epithelium is thought to play an important role in airway inflammation in HDM-driven experimental mouse models of allergic asthma, leading to activation of innate cell subpopulations such as DCs and innate lymphoid cells [12]. Specifically, it has been shown that HDM exposure increases the production of the pro- inflammatory mediators CCL17, CCL20 and IL-1α *in vivo* [9][12]. Therefore, we assessed whether Pim1 kinase

activity affects the induction of pro-inflammatory chemokines and cytokines upon repeated HDM challenges in a HDM-driven mouse model of allergic asthma. To this end, we subjected *Pim1*-deficient FVB/Nrcl mice and wild-type controls intranasally to HDM twice a week for 5 weeks and determined the levels of pro-inflammatory mediators in lung tissue (fig. 3A).

In line with our *in vitro* data, the levels of IL-1α in lung homogenates of wild-type FVB/Nrcl mice challenged with HDM were significantly enhanced compared to mice treated with PBS, with a stronger increase in *Pim1*-deficient mice, although this failed to reach statistical significance (fig. 3B). Next we analyzed release of the epithelial-derived chemokines CCL17 and CCL20, which contribute to activation of DCs and Th2 cells upon HDM exposure [11][22].
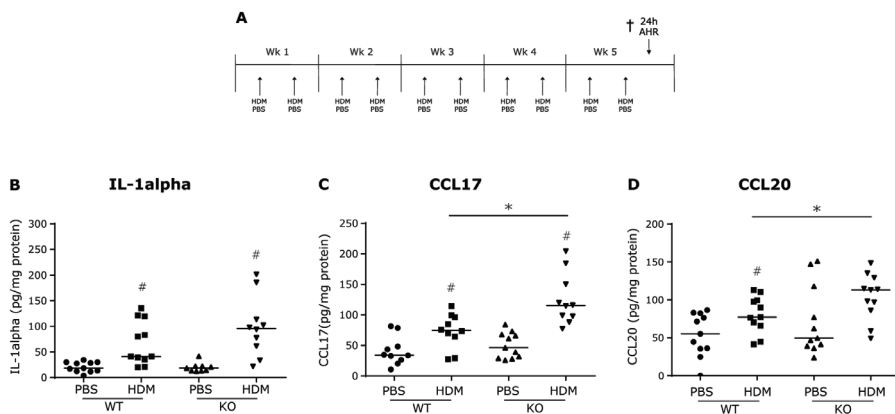

Figure 3: HDM-induced release of pro-inflammatory cytokines is augmented in Pim1-deficient mice
*Female FVB/Nrcl mice deficient (KO) or proficient (WT) for Pim1 kinase were treated intranasally with 20 µl HDM extract (2.5 mg total weight/ml) or PBS twice a week for 5 weeks, and mice were sacrificed for analysis 24 h after the last HDM exposure (A). The levels of IL-1α (B), CCL17 (C) and CCL20 (D) in lung homogenates were determined by ELISA, and expressed as pg/mg total protein in lung homogenate. Each experimental group consists of 10-11 mice and the median of each group is presented. * P < 0,05.*

By analyzing the levels of CCL17 in lung homogenates of *Pim1*-deficient and wild-type mice, we observed significantly higher levels of CCL17 in the HDM-challenged groups compared to the PBS-challenged groups (fig. 3C). Interestingly, the levels of CCL17 after HDM treatment were also significantly elevated in lung tissue of *Pim1*-deficient mice over wild-type controls. A similar effect was observed for CCL20 (fig. 3D), where levels were significantly higher upon HDM treatment in the *Pim1*-deficient compared to the wild-type mice. Of note, only a trend (p=0.09) towards increased levels of CCL20 upon HDM challenge could be observed in *Pim1*-deficient mice.

HDM-induced local airway inflammation is independent of Pim1 kinase activity

Next, we evaluated whether *Pim1*-deficiency predisposes towards enhanced HDM-induced local airway inflammation by analyzing the cellular composition of the BAL fluid. As shown in figure 4A, exposure to HDM significantly enhanced the total number of cells present in the BAL fluid of wild-type mice. Differential cell counts revealed a significant increase in mononuclear cells, eosinophils and neutrophils in BAL fluid upon challenge with HDM in the wild-type mice (fig. 4B, C and D, respectively). A comparable effect was observed in *Pim1*-deficient mice with significant higher total cell numbers and BAL counts of mononuclear cells, eosinophils and neutrophils in the HDM-challenged compared to PBS-treated mice. Thus, the increase in inflammatory cells in BAL fluid induced by HDM exposure is not affected by *Pim1* deficiency. Similar results were observed for the differential cell count in lung tissue, with an increased fraction of eosinophils and neutrophils in the mice challenged with HDM, and no differences between the *Pim1*-deficient and proficient mice (data not shown).
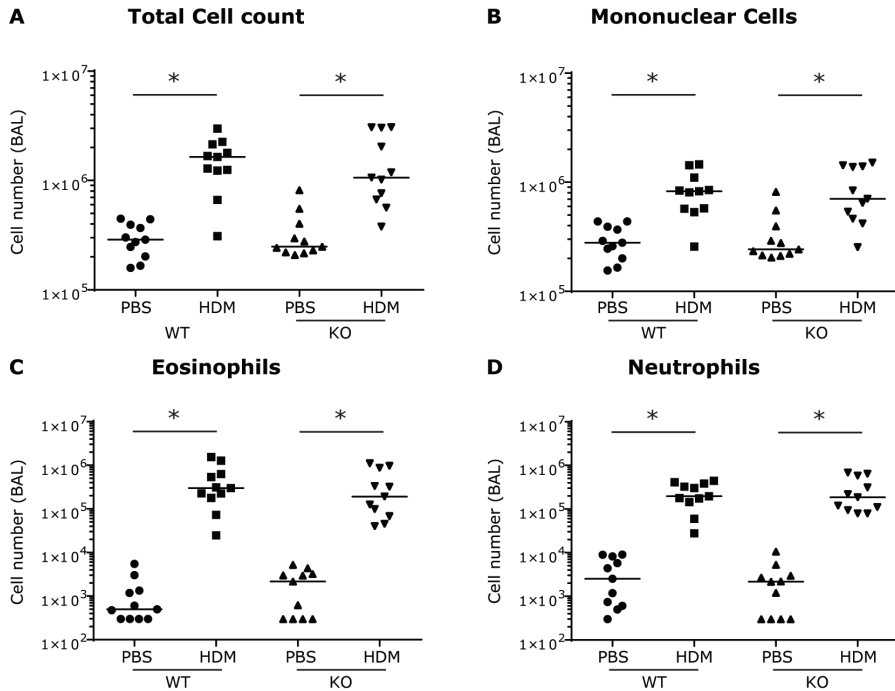
Figure 4: HDM-induced inflammatory cells infiltrate in BAL fluid is independent of Pim1 kinase activity

The BAL fluid of the mice described in figure 3 was analyzed for total cell numbers (A), the number of mononuclear cells (B), eosinophils (C) and neutrophils (D). The lower limit of detection was 300 cells. Each experimental groups consists of 11 mice and the median of each group is presented. * P < 0,05.

In addition to inflammatory cell infiltrates, we assessed whether HDM could induce airway inflammation and mucus secretion, and whether these manifestations of asthma were increased in the absence of Pim1 kinase activity. As shown by HE staining, there was no inflammation around the airways in mice challenged with PBS (fig. 5A and 5C). HDM challenge, on the other hand, resulted in marked inflammation of the airways (fig. 5B and 5D). Comparison of the inflammation between wild-type (fig. 5B) and *Pim1*-deficient mice (fig. 5D) revealed no differences between the two groups.

Next to airway inflammation, HDM challenge induced marked goblet cell metaplasia compared to PBS treated controls (fig. 5E-H). Again, no differences could be observed when comparing the PAS staining in wild-type (fig. 5F) and *Pim1* knock out mice (fig. 5H).
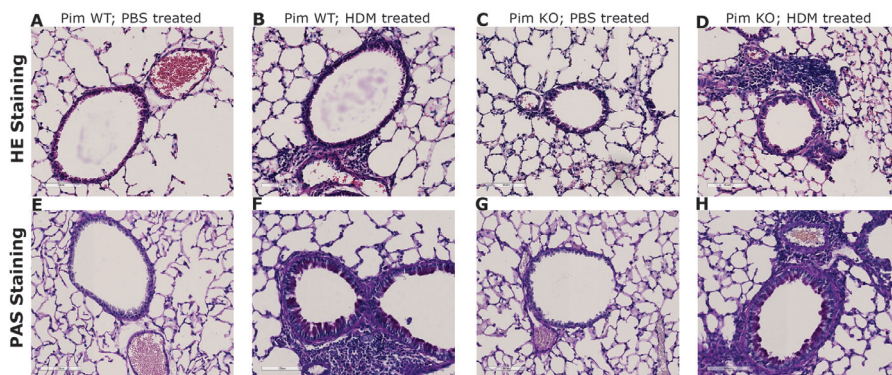

Figure 5: Pim1 kinase activity does not affects HDM-induced local airway inflammation
Female FVB/Nrcl mice deficient (KO) or proficient (WT) for Pim1 kinase were treated intranasally with 20 µl HDM extract (2.5 mg total weight/ml) or PBS twice a week for 5 weeks and 24 h after the last HDM exposure lungs were collected for histological analysis. HE staining was performed on lung sections of PBS-treated WT mice (A), HDM-treated WT mice (B), PBS-treated Pim1 KO mice (C) and HDM-treated Pim1 KO mice (D). PAS staining was performed on lung sections of PBS-treated WT mice (E), HDM-treated WT mice (F), PBS-treated Pim1 KO mice (G) and HDM-treated Pim1 KO mice (H). Representative staining of 3-5 mice in each experimental groups are shown.

Pim1-deficiency does not affect HDM-induced AHR

AHR is an important clinical feature of allergic asthma, and we previously showed AHR to be induced in this experimental mouse model of HDM-induced allergic airway inflammation in BALB/cByJ mice [9]. Therefore, we measured airway resistance in *Pim1*-deficient and proficient FVB/Nrcl mice exposed to HDM or PBS upon challenge with increasing doses of metacholine. As shown in figure 6A, exposure of mice to HDM for 5 weeks resulted in increased airway resistance in response to metacholine compared to mice exposed to PBS. However, the within-group variation in airway resistance is considerable, and significant differences were not found between wild-type mice exposed to HDM or PBS. Nevertheless, in contrast to the wild-type mice, HDM significantly induced AHR compared to the PBS-challenged littermates in Pim1-deficient mice. Furthermore, we observed a trend (p=0.08) towards a higher AHR in the *Pim1*-deficient mice compared to the wild-type controls challenged with HDM.
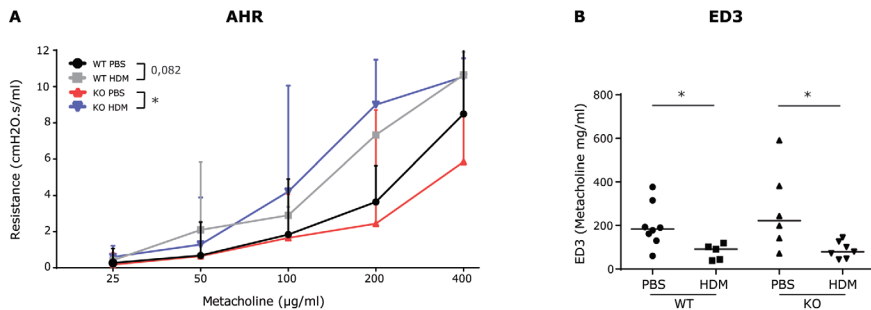

Figure 6: HDM induced AHR is not affected by Pim1 kinase activity
*Female FVB/Nrcl mice deficient (KO) or proficient (WT) for Pim1 kinase were treated intranasally with 20 µl HDM extract (2.5 mg total weight/ml) or PBS twice a week for 5 weeks and 24 h after the last HDM exposure AHR to metacholine challenge was determined by invasive measurement of airway resistance (Flexivent). AHR upon increasing doses of metacholine was measured (A) and the metacholine dose required to obtain a resistance of 3 cmH2O.s/ml was calculated (B).The number of mice in each experimental group varies between 3 and 9 mice. The median and range are shown for each group. * P < 0,05.*

To assess whether this trend reflected a by-chance finding, we evaluated the dose of metacholine needed to achieve an airway resistance of 3 cmH2O.s/ml (ED3). The ED3 was significantly lower in the mice challenged with HDM compared to the PBS-challenged group (Fig. 6B), but without significant difference between the *Pim1*-deficient mice and wild-type controls, suggesting that metacholine-induced AHR is not affected by *Pim1*-deficiency in this mouse model of HDM-induced allergic asthma.

Th2-mediated inflammatory immune response upon HDM exposure is augmented in the absence of Pim1 kinase activity

Notwithstanding the fact that the HDM-induced local airway inflammation and AHR was not critically regulated by Pim1 kinase activity, we next investigated whether the augmented increase in CCL17 and CCL20 was associated with increased Th2 activity in the lungs by assessing the production of Th2-type cytokines [14].
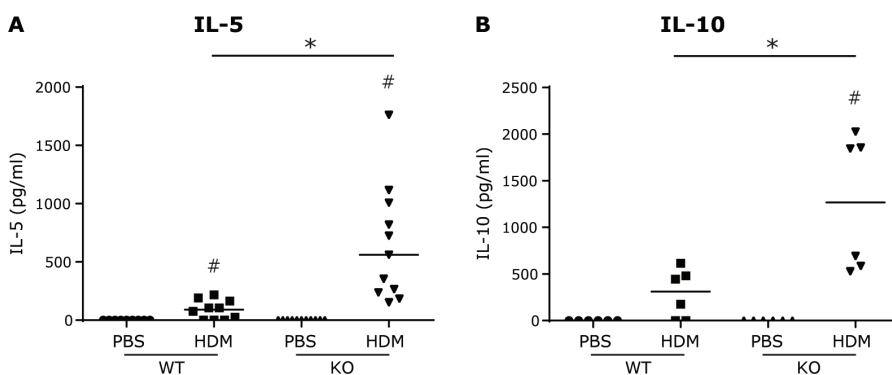

Figure 7: Th2-mediated inflammatory immune response upon HDM exposure is augmented in the absence of Pim1 kinase activity
*The levels of IL-5 (A) and IL-10 (B) in BAL fluid of the mice previous described in figure 3 were determined by ELISA. Each experimental group consists of 11 and 6 mice for the measurement of IL-5 and IL-10, respectively. Median of each group is presented. * P < 0,05.*

We observed that levels of IL-5 and IL-10 in BAL fluid were significantly enhanced upon HDM exposure compared to PBS-treated mice in both Pim1-deficient mice and wild-type controls (fig. 7A and 7B, respectively), while IL-13 was not detectable (data not shown). Of note, the levels of IL-10 were only measured in a subset of mice (n=6). In line with the increased levels of CCL17 and CCL20 in the absence of Pim1, we observed significantly higher levels of IL-5 (fig. 7A) and IL-10 (fig. 7B) in the BAL fluid of HDM-treated *Pim1*-deficient mice compared to the *wild-type* controls.

In conclusion, our results show that pharmacological inhibition of Pim1 kinase activity provokes a stronger effect of HDM on epithelial barrier function in PBECs and prolongs the HDM-induced loss of barrier function in 16HBE cells, which is accompanied by increased release of the pro-inflammatory cytokine IL-1α. In vivo, the absence of Pim1 kinase increases the release of pro-inflammatory mediators by the airway epithelium upon allergic sensitization with HDM as well as the activity of Th2 cells in the lungs, while airway inflammation and AHR are not affected.

Discussion

In this study, we showed that Pim1 protects against HDM-induced barrier dysfunction and the associated release of pro-inflammatory cytokine IL-1α by airway epithelial cells *in vitro*. Interestingly, the levels of the pro-inflammatory mediator CCL17 and CCL20 - and to a lesser extent IL-1α - were augmented in *Pim1*-deficient FVB/Nrcl mice exposed to HDM. While we could not observe a direct effect of *Pim1* deficiency on influx of eosinophilic and neutrophilic granulocytes into the airways or on AHR upon metacholine challenge in the HDM-treated mice, the release of the Th2-cytokines IL-5 and IL-10 was augmented in the lungs Pim1-deficient compared to wild-type mice upon exposure to HDM. Taken together, our results suggest that Pim1 protects against HDM-induced barrier dysfunction *in vitro*, and that loss of Pim1 activity sensitizes the airway epithelium to HDM-induced pro-inflammatory activity, leading to enhanced Th2 cell activity and associated release of Th2-cytokines upon allergic sensitization *in vivo*.

The allergenicity of HDM is exerted through a combined effect on the innate and adaptive immune response, and this particular combination makes HDM a highly potent allergen [23]. A pivotal role in the sensitization to HDM is reserved for the airway epithelium. Specifically, disruption of the airway epithelial barrier upon HDM exposure has been suggested to be important in the allergenic effects of HDM [9]. *In vitro* studies of our group and others have shown that HDM exposure induces disruption of airway epithelial integrity and increased release of pro-inflammatory cytokines and chemokines [8][10][21][24][25]. By transcriptional knock-down of E-cadherin using siRNA in bronchial epithelial cells, we previously showed that loss of epithelial integrity in the absence of any further allergens is sufficient to induce CCL17 expression, underscoring the relevance of loss of epithelial integrity for the pro-inflammatory response to allergens [11]. Furthermore, *in vivo* studies of HDM-driven mouse models of allergic asthma showed that the ability of HDM extract to induce loss of epithelial

barrier function was associated with the release of the pro-inflammatory chemokines CCL20 and CCL17 and induction of a type-2 inflammatory immune response [9]. This further highlights the importance of the airway epithelium in allergic asthma.

Our current study reveals that while the HDM-induced influx of eosinophilic and neutrophilic granulocytes and mucus cell metaplasia is not affected by Pim1 kinase activity, the innate epithelial immune response as measured by the release of pro-inflammatory chemokines CCL17 and CCL20 is enhanced in its absence. In line with the increased levels of these chemotactic mediators, *Pim1*-deficiency resulted in augmented BAL levels of IL-5 and IL-10 upon HDM challenge, reflecting enhanced Th2 activity. While IL-5 is traditionally postulated as a typical Th2 cytokine, the increased levels of IL-5 observed in *Pim1*-deficient mice may also reflect increased activation of group 2 innate lymphoid cells (ILC2s). ILC2s, which resemble Th2 cells but lack the antigen-specific receptors, have been shown to be a major producer of IL-5 in HDM-induced allergic airway inflammation in mice [26][27]. Moreover, a recent study by Wang *et al* found that pharmacological inhibition of Pim1 kinase suppresses Th2 differentiation, arguing against a prominent role for Th2 cell activity in the HDM-induced IL-5 response observed in *Pim1*-deficient mice[28]. ILC2 activation is dependent on the release of pro-inflammatory mediators such as IL-25, IL-33 and Thymic Stromal Lymphopoietin (TSLP) by airway epithelial cells. Therefore, we anticipate that augmented loss of epithelial integrity - which we have previously shown to lead to increased TSLP expression [11] - upon HDM exposure in *Pim1*-deficient mice may result in increased release of IL-5 by ILC2s [14][26]. In support of this notion, our *in vitro* data using 16HBE cells and PBECs clearly indicate that loss of airway epithelial barrier function and release of pro-inflammatory mediators in response to HDM is enhanced in the absence of Pim1 kinase activity.

An explanation for the discrepancy between absence of an enhanced eosinophilic airway inflammation and AHR on the one hand and the exaggerated innate epithelial inflammatory response observed in *Pim1*-deficient mice upon exposure to HDM on the other, might be found in the involvement of Pim1 in eosinophil survival [29]. Based on the enhanced levels of IL-5 observed in *Pim1*-deficient mice exposed to HDM and the well-known role of IL-5 in driving eosinophilia [14], one would expect an increased recruitment of eosinophils to the lungs in these mice. The lack of a difference in eosinophil numbers between HDM-treated *Pim1*-deficient mice and wild-type controls might therefore reflect the net result of increased migration of eosinophils to lung tissue and reduced survival of these cells in the lungs of *Pim1*-deficient mice.

Interestingly, a previous study by Shin *et al* showed reduced AHR, eosinophilic airway inflammation and Th2 cytokine concentrations in the BAL fluid of ovalbumin (OVA) sensitized and challenged BALB/c mice upon pharmacological inhibition of Pim1 kinase activity [16]. However, the experimental set-up of these two studies are rather different and a number of explanations for the inconsistencies between both studies can be postulated.

First of all, Shin *et al* used a specific pharmacological inhibitor for Pim1 kinase activity, which was administered in OVA sensitized mice only during OVA challenges. In our study, we used mice with a germline deficiency for Pim1 on a FVB/Nrcl background, where Pim1 kinase activity was absent throughout the complete HDM exposure protocol. Pim1 kinase is known to be crucial for the survival of eosinophils and it has been shown that the viability of eosinophils is markedly reduced in the absence of Pim1 kinase activity [29]. As described by Shin *et al*, pharmacological inhibition of Pim1 kinase activity during OVA challenges directly affected the eosinophil numbers. Here, the reduced eosinophilic airway inflammation observed upon inhibition of Pim1 kinase activity may be caused by increased apoptosis of eosinophils, resulting in a decreased allergic response compared to vehicle-treated mice [29]. Our experiments

using *Pim1*-deficient mice show that Pim1 activity is not critically required for the differentiation of eosinophilic granulocytes and migration of these cells to the lung tissue. The presence of eosinophils in *Pim1*-deficient mice may also indicate that these eosinophils likely adapted to the loss of Pim1 by compensatory mechanisms.

Secondly, while we used a HDM-driven mouse model of allergic asthma, Shin *et al* used an OVA-driven mouse model of allergic asthma [16]. In OVA-driven models, the development of allergic asthma is induced by intraperitoneal ovalbumin sensitization combined with the adjuvant aluminium hydroxide, resulting in a strong, peripherally initiated Th2-driven eosinophilic response [4]. In HDM-driven models, allergic sensitization is dependent on airway epithelial activation upon inhalation of allergens [4], thereby mimicking the route of allergen sensitization observed in humans. Hence, the main difference between the OVA- and HDM-induced mouse models of allergic asthma is the dependence on airway epithelial activation for sensitization to the allergen. We showed previously that the damaging effects of HDM on airway epithelial barrier function and the subsequent release of pro-inflammatory chemokines are crucial for allergic sensitization [9] and we now demonstrate with our *in vitro* experiments that Pim1 kinase is important for the integrity of the airway epithelial barrier. In contrast to the HDM-induced mouse model, the OVA-induced mouse model does not inflict epithelial damage directly, but requires involvement of inflammatory cells, e.g. eosinophils. Hence, the effect of *Pim1* deficiency on airway epithelial function would not readily be revealed in an OVA-driven mouse model of allergic asthma.

In conclusion, this study shows for the first time that Pim1 kinase preserves the integrity of the airway epithelial barrier and attenuates HDM-induced allergic asthma. Since factors influencing the integrity of the airway epithelial barrier are important for the pathogenesis and potential future therapeutic treatment of allergic asthma, it is of interest to study the role of Pim1 kinase in allergic asthma into further detail.

References

1. From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2014. Available from: http://www.ginasthma.org.

2. Croisant S. Epidemiology of asthma: prevalence and burden of disease. *Adv Exp Med Biol 2014; 795: 17-29.*

3. Martinez FD, Vercelli D. Asthma. *Lancet 2013; 382: 1360-1372.*

4. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol 2011; 32: 402-411.*

5. Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med 2012; 18: 673-683.*

6. Gaffin JM, Phipatanakul W. The role of indoor allergens in the development of asthma. *Curr Opin Allergy Clin Immunol 2009; 9: 128-135.*

7. Nawijn MC, Hackett TL, Postma DS, van Oosterhout AJ, Heijink IH. E-cadherin: gatekeeper of airway mucosa and allergic sensitization. *Trends Immunol 2011; 32: 248-255.*

8. Heijink IH, van Oosterhout A, Kapus A. Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction. *Eur Respir J 2010; 36: 1016-1026.*

9. Post S, Nawijn MC, Hackett TL, Baranowska M, Gras R, van Oosterhout AJ, Heijink IH. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax 2012; 67: 488-495.*

10. Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB, Robinson C. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest 1999; 104: 123-133.*

11. Heijink IH, Kies PM, Kauffman HF, Postma DS, van Oosterhout AJ, Vellenga E. Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. *J Immunol 2007; 178: 7678-7685.*

12. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, Hammad H. Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J Exp Med 2012; 209: 1505-1517.*

13. Heijink IH, Nawijn MC, Hackett TL. Airway epithelial barrier function regulates the pathogenesis of allergic asthma. *Clin Exp Allergy 2014; 44: 620-630.*

14. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol 2014; 16: 45-56.*

15. Nawijn MC, Alendar A, Berns A. For better or for worse: the role of Pim oncogenes in tumorigenesis. *Nat Rev Cancer 2011; 11: 23-34.*

16. Shin YS, Takeda K, Shiraishi Y, Jia Y, Wang M, Jackson L, Wright AD, Carter L, Robinson J, Hicken E, Gelfand EW. Inhibition of Pim1 kinase activation attenuates allergen-induced airway hyperresponsiveness and inflammation. *Am J Respir Cell Mol Biol 2012; 46: 488-497.*

17. de Vries M, Heijink IH, Gras R, den Boef LE, Reinders-Luinge M, Pouwels SD, Hylkema MN, van der Toorn M, Brouwer U, van Oosterhout AJ, Nawijn MC. Pim1 kinase protects

airway epithelial cells from cigarette smoke- induced damage and airway inflammation. *Am J Physiol Lung Cell Mol Physiol 2014; 307: L240-51*.

18. Pogacic V, Bullock AN, Fedorov O, Filippakopoulos P, Gasser C, Biondi A, Meyer-Monard S, Knapp S, Schwaller J. Structural analysis identifies imidazo[1,2-b]pyridazines as PIM kinase inhibitors with in vitro antileukemic activity. *Cancer Res 2007; 67: 6916-6924.*

19. Twisk JW. Longitudinal data analysis. A comparison between generalized estimating equations and random coefficient analysis. *Eur J Epidemiol 2004; 19: 769-776.*

20. Heijink IH, Brandenburg SM, Noordhoek JA, Postma DS, Slebos DJ, van Oosterhout AJ. Characterisation of cell adhesion in airway epithelial cell types using electric cell-substrate impedance sensing. *Eur Respir J 2010; 35: 894-903.*

21. Post S, Nawijn MC, Jonker MR, Kliphuis N, van den Berge M, van Oosterhout AJ, Heijink IH. House dust mite- induced calcium signaling instigates epithelial barrier dysfunction and CCL20 production. *Allergy 2013; 68: 1117-1125.*

22. Reibman J, Hsu Y, Chen LC, Bleck B, Gordon T. Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. *Am J Respir Cell Mol Biol 2003; 28: 648-654.*

23. Jacquet A. The role of innate immunity activation in house dust mite allergy. *Trends Mol Med 2011; 17: 604-611.*

24. Asokananthan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ, Stewart GA. 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: 4572-4578.*

25. Kauffman HF, Tamm M, Timmerman JA, Borger P. House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells by protease-dependent and protease-independent mechanisms. *Clin Mol Allergy 2006; 4: 5.*

26. Gold MJ, Antignano F, Halim TY, Hirota JA, Blanchet MR, Zaph C, Takei F, McNagny KM. Group 2 innate lymphoid cells facilitate sensitization to local, but not systemic, TH2-inducing allergen exposures. *J Allergy Clin Immunol 2014; 133: 1142-1148.*

27. Klein Wolterink RG, Kleinjan A, van Nimwegen M, Bergen I, de Bruijn M, Levani Y, Hendriks RW. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol 2012; 42: 1106-1116.*

28. Wang M, Okamoto M, Domenico J, Han J, Ashino S, Shin YS, Gelfand EW. Inhibition of Pim1 kinase prevents peanut allergy by enhancing Runx3 expression and suppressing T(H)2 and T(H)17 T-cell differentiation. *J Allergy Clin Immunol 2012; 130: 932-44.e12.*

29. Andina N, Didichenko S, Schmidt-Mende J, Dahinden CA, Simon HU. Proviral integration site for Moloney murine leukemia virus 1, but not phosphatidylinositol-3 kinase, is essential in the antiapoptotic signaling cascade initiated by IL-5 in eosinophils. *J Allergy Clin Immunol 2009; 123: 603-611.*