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

M. de Vries¹,³, L. Hesse¹,³, M.R. Jonker¹,³, M. van den Berge²,³, A.J.M. van Oosterhout¹,³, I.H. Heijink¹,³ and M.C. Nawijn¹,³

¹ University of Groningen, University Medical Center Groningen, Experimental Pulmonology and Inflammation Research, Department of Pathology and Medical Biology, Groningen, The Netherlands
² University of Groningen, University Medical Center Groningen, Department of Pulmonology, Groningen, The Netherlands
³ University of Groningen, University Medical Center Groningen, GRIAC Research Institute, Groningen, The Netherlands

Correspondence to:
M. de Vries, Msc
Experimental Pulmonology and Inflammation Research
Department of Pathology and Medical Biology
University Medical Center Groningen, The Netherlands
Hanzeplein 1, 9713 GZ, The Netherlands
m.de.vries04@umcg.nl
Tel: +0031 (0)50 361 1284
Fax: +0031 (0)50 361 9911

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Author’s contributions
M.d.V, I.H.H, M.v.d.B, A.J.v.O. and M.C.N. were involved in the conception, design and/or supervision of research; M.d.V., L.H. and M.R.J. performed experiments; M.d.V., I.H.H., L.H. and M.C.N. analyzed data; M.d.V., I.H.H. and M.C.N. interpreted results of experiments; M.d.V. prepared figures; M.d.V. drafted manuscript; I.H.H. and M.C.N. critically reviewed and revised manuscript; all authors read and approved the final version of manuscript.
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

Th2-cytokines; Epithelial barrier function; Allergen; Innate immune response; Eosinophils
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 (4) (19). 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 submucosa as a consequence of sensitization and continued exposure to aero-allergens (8) (14). 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) (6) (8).

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) (8). 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 (21). 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) (13) (23) (30). 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 (10). 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 (23). 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 (32). 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(11)(18).

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(20). 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(27). Based on the protective effect of Pim1 kinase on cigarette smoke-induced damage of the airway epithelial cells(5), 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(10).

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(10).

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

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Sex</th>
<th>FEV₁ (% predicted)</th>
<th>Inhaled corticosteroids,</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>male/female</td>
<td></td>
<td>yes/no</td>
</tr>
<tr>
<td>45</td>
<td>1 / 3</td>
<td>103</td>
<td>0 / 4</td>
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<tr>
<td>(21 – 70)</td>
<td></td>
<td>(98 – 109)</td>
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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* (10). 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)(22) 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(10), and serum starved overnight in the presence of 5 µM PKI or DMSO as vehicle control. Subsequently, cells were stimulated with 50 or 100 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).
Luciferase assay

The Pim1 cDNA plasmids, Lef-1/TCF reporter plasmid TopFLASH, Renilla reporter plasmid, Dishevelled2 plasmid and empty vector pcDNA3 were a kind gift of prof. A. Berns and Dr. R. van Amerongen (Netherlands Cancer Institute, Amsterdam) (29). The Pim1 WT DNA CS41 and its kinase-dead mutant were subcloned in our laboratory from Pim1 cDNA plasmids originally described by Saris et al (26). BEAS-2B cells (ATCC, Manassas, VA, USA) were seeded on collagen and BSA-coated 24 wells plates as previously described for 16HBE cells. 90 Percent confluent cells were transfected with 200 ng TopFLASH, 50 ng Renilla, 125 ng DVL2, 125 ng Pim1 WT and/or 125 ng Pim1 KD using Lipofectamine 2000 Transfection Reagent (Thermo Scientific, Rockford, IL, USA) in Opti-MEM Reduced Serum Medium (Thermo Scientific). To ensure an equal amount of DNA in all experimental conditions, the total amount of DNA was supplemented with pcDNA3 to 500 ng DNA per well. After 4 hours of transfection, the transfection reagents was removed and replaced with RPMI with L-glutamin and 25 mM HEPES (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone). After 16 hours, the cells were washed and serum deprived in RPMI for another 24 hours, where after the cells were lysed after washing with PBS in 100 µl Passive Lysis buffer (Dual-Luciferase Reporter Assay Kit, Promega Benelux, Leiden, The Netherlands). Firefly luciferase activity was determined using the Dual-Luciferase Reporter Assay Kit (Promega) and the Luminoskan Ascent (Thermo Scientific).

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(23), 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 for Immunodetection

Frozen mouse lungs were homogenized on ice using a T10 basic Ultra TURRAX (IKA®-Werke GmBH & Co. KG, Staufen, Germany) in 5 µl lysis buffer containing 1% Triton-X 100 (Sigma-Aldrich), 150 mM NaCl (Sigma-Aldrich), 5 mM MgCl₂ (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 1:50 Protease Inhibitor Cocktail (Roche Diagnostics) and 1:10 Phosphatase Inhibitor Cocktail (Roche Diagnostics) per mg lung tissue. Subsequently, lung homogenates were boiled for 10 minutes with 2x laemmlli buffer containing 2% β-mercaptoethanol and stored at -20 °C. Total protein levels in cell debris-free supernatant were determined with the Pierce™ BCA Protein Assay Kit (Thermo Scientific).

Preparation of lung tissue homogenates for ELISA

Mouse lungs were homogenized on ice using a T10 basic Ultra TURRAX (IKA®-Werke GmBH & Co. KG,) 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 Pierce™ BCA Protein Assay Kit (Thermo Scientific).

**Immunodetection**

Lung tissue homogenates (approximately 17.5 µg total protein per sample) were separated on 12.5% acrylamide SDS-page gels. Immunoblotting was performed for Pim1 kinase (1:1500, OriGene Technologies, Rockville, MD, USA) and β-actin (1:20,000, Sigma-Aldrich) conform standard procedures.

**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 haematoxylin/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).
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 (28). 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 (22) 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 (9). 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 (9) – 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 (23). 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 (23). 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).

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). Both PKI and HDM also significantly enhanced the release of CCL20, while concurrent inhibition of Pim1 kinase activity resulted in a trend (p=0.08) towards increased HDM-induced release of CCL20 (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 increased release of the pro-inflammatory cytokines IL-1α and CCL20. Moreover, inhibition of Pim1 kinase activity prolongs the HDM-induced loss of airway epithelial barrier function and increases the HDM-induced release of IL-1α and CCL20.

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 (24), 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 (24). 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.

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Overexpression of Pim1 kinase enhances airway epithelial integrity in vitro

To gain insight in the potential mechanism through which Pim1 kinase regulates the maintenance of the airway epithelial integrity, we measured the transcriptionally activity of β-catenin with a TopFLASH luciferase assay. Since β-catenin is a binding partner of the adherens junctional molecule E-cadherin, disruption of E-cadherin-mediated cell-cell contacts will result in the liberation of intracellular β-catenin and nuclear relocalization and transcriptional activity in the presence of active canonical Wnt signalling (12). Therefore, transcriptional activity of β-catenin can be used as an indirect measurement of airway epithelial integrity.

We studied the effect of Pim1 kinase on Dishevelled 2 (DVL2)-induced TopFLASH activity to prevent the degradation of β-catenin by the degradation complex. As shown in figure 3, overexpression of DVL2 resulted in induction of TopFLASH activity, while Pim1 WT and KD only marginally affect baseline TopFLASH activity. Interestingly, Pim1 KD did not affect DVL2-induced TopFLASH activity, while Pim1 WT significantly suppressed the DVL2-induced TopFLASH activity. These results suggest that Pim1 kinase indeed functions to reinforce barrier function, stabilizing adherens junctions and thereby preventing the liberation and transcriptional activity of β-catenin.

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 (32). Specifically, it has been shown that HDM exposure increases the production of the pro-inflammatory mediators CCL17, CCL20 and IL-1α in vivo (23)(32). 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. 4A).

We first assessed if exposure to HDM affected *Pim1* expression in the wild-type FVB/Nrcl mice. As shown in Figure 4B, we could not detect differences in the protein levels of *Pim1* upon HDM exposure. We continued with the measurement of the levels of IL-1α in lung tissue homogenates. 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. 4C).

Next we analyzed release of the epithelial-derived chemokines CCL17 and CCL20, which contribute to activation of DCs and Th2 cells upon HDM exposure (10)(25). 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. 4D). 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. 4E), 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.
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 5A, 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. 5B, 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).

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. 6A and 6C). HDM challenge, on the other hand, resulted in marked inflammation of the airways (fig. 6B and 6D). Comparison of the inflammation between wild-type (fig. 6B) and Pim1-deficient mice (fig. 6D) revealed no differences between the two groups.

Next to airway inflammation, HDM challenge induced marked goblet cell metaplasia compared to PBS treated controls (fig. 6E-H). Again, no differences could be observed when comparing the PAS staining in wild-type (fig. 6F) and Pim1 knock out mice (fig. 6H).
**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 (23). 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 7A, 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. To assess whether this trend reflected a by-chance finding, we evaluated the dose of metacholine needed to achieve an airway resistance of 3 cmH$_2$O.s/ml (ED3). The ED3 was significantly lower in the mice challenged with HDM compared to the PBS-challenged groups for both the Pim1-proficient and-deficient mice (Fig. 7B), indicating that exposure to HDM does enhance the sensitivity to metacholine-induced AHR. Nevertheless, we did not observe 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 (18). 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. 8A and 8B, 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. 8A) and IL-10 (fig. 8B) 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 by stabilization of epithelial adherens junctions and the associated release of pro-inflammatory cytokine such as 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 (15). 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 (23). 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 (3)(13)(16)(24)(30). 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 (10). 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 (23). 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 (7)(17). 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(31). 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 (10) - upon HDM exposure in Pim1-deficient mice may result in increased release of IL-5 by ILC2s (7)(18). 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. Furthermore, we show that in vitro overexpression of Pim1 kinase enhances the airway epithelial integrity most likely by stabilizing the adherens junction complex, preventing nuclear relocalization and transcriptional activity of β-catenin.
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 (2). 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 (18), 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 (27). 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 (2). 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 (2). 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 (27). 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 (8). In HDM-driven models, allergic sensitization is dependent on airway epithelial activation upon inhalation of allergens (8), thereby mimicking the route of allergen sensitization observed in humans. Since eosinophils are known to produce high levels of Pim1 kinase and ovalbumin evokes a stronger eosinophilic response compared to HDM (2)(8), the different mouse models of allergic asthma may explain the differences in expression of Pim1 kinase upon exposure to allergen between our study and the study of Shin *et al*. Furthermore, 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 (23) 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.
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References


Figure legends

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, 5 times for the measurement of the release of IL-1α and 8 times for the measurement of the release of CCL20. Mean and SEM across the independent experiments are shown. * P < 0.05. Statistically significant differences P < 0.05 compared to control are indicated by #.

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.
Figure 3: Overexpression of Pim1 kinase enhances airway epithelial integrity in vitro

BEAS-2B cells were transfected with Dishevelled 2 (DVL2), Pim1 WT and/or Pim KD and the luciferase activity was measured using the TopFLASH β-catenin driven Lef-1/TCF reporter plasmid. The mean and SEM of 4 independent experiments are shown. Significant statistical differences were tested with the Student’s paired t-test and * indicates P < 0.05. # Indicates significant differences compared to control.

Figure 4: 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). Protein levels of Pim1 kinase were assessed with immunodetection (B). The levels of IL-1α (C), CCL17 (D) and CCL20 (E) 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. # Indicates significant differences compared to control.

Figure 5: HDM-induced inflammatory cells infiltrate in BAL fluid is independent of Pim1 kinase activity

The BAL fluid of the mice described in figure 4 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.
Figure 6: Pim1 kinase activity does not affect 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.

Figure 7: 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.

Figure 8: 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 4 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. # Indicates significant differences compared to control.
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A) Total Cell count

B) Mononuclear Cells

C) Eosinophils

D) Neutrophils
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**Resistance (cmH2O.s/ml)**

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