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Title:

Nasal Epithelial Barrier Dysfunction Increases Sensitization and Mast Cell Degranulation in the Absence of Allergic Inflammation

Short title:

Barrier Dysfunction Facilitates Allergic Pathology

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/all.14132](#)

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Acknowledgements

We would like to thank Jonathan Cremer, Lieve Coorevits and Ellen Dilissen for their excellent technical assistance; Matthias Van Woensel for preparing the chitosan; Jens Brimnes, Jens Holm and Eva Diemar for sharing the method for passive sensitization with recombinant murine IgE antibodies; Jens Holm, Lars Harder Christensen and Jette Skovsgaard for making the recombinant murine and human antibodies. This research was supported by grants from IWT (TBM project 130260), the research council of the KU Leuven (GOA 14/011), and the Belgian Federal Government (IUAP P6/28). S.F.S. is supported by the research council of KU Leuven (PDMK/14/189). A.C.J. is supported by FWO-SB fellowship (1S20418N). B.S. is a Postdoctoral Fellow of the Fund for Scientific Research Flanders (FWO). P.W.H. is recipient of a senior researcher fellowship from FWO.

Conflicts of Interest

G.L. is employed at ALK Abello, Hørsholm, Denmark. The authors have no conflicts of interest to disclose.

Author contributions

I.K.K., J.L.C., S.F.S. and P.W.H. designed the study. I.K.K., A.C.J. and I.D.C. performed the experiments. I.K.K., S.F.S., G.L., J.L.C. and P.W.H. contributed to the data analysis, interpretation of the data and statistics. I.K.K. drafted the article, which was critically revised and edited by B.S., J.L.C. and P.W.H. All authors revised the manuscript and approved the final version.

Abbreviations

ALI: Air-liquid interface, AR: Allergic rhinitis, BAL: Bronchoalveolar lavage, rDer p: recombinant *Dermatophagoides pteronyssinus*, FP: Fluticasone propionate, HDM: house dust mite, IQR: interquartile ranges, i.v.: intravenous, MC: Mast cell, OD: optical density, OVA: Ovalbumin, pNEC: primary nasal epithelial cells, TEER: Trans-epithelial electrical resistance, TJ: Tight junctions

Abstract

Background: Increased epithelial permeability has been reported in allergic rhinitis, with histamine and type-2 inflammation being responsible for tight junction dysfunction. The impact of an epithelial barrier defect on allergic sensitization and mast cell (MC) degranulation remains speculative. **Methods:** Transepithelial passage of allergens was evaluated on primary human nasal epithelial cell cultures. Active sensitization was attempted by repeated intranasal ovalbumin (OVA) applications in naive mice. In a passive sensitization model, mice were injected with IgE to *Dermatophagoides pteronyssinus* (rDer p)2 and then exposed intranasally to the allergen. Chitosan was used to disrupt nasal epithelial integrity *in vitro* and *in vivo*. **Results:** Chitosan strongly reduced trans-epithelial electrical resistance and facilitated trans-epithelial allergen passage in cultured primary nasal epithelial cells. *In vivo*, intranasal chitosan affected occludin expression and facilitated allergen passage. After epithelial barrier disruption, intranasal OVA application induced higher OVA-specific IgG1 and total IgE in serum, increased eosinophilia and interleukin-5 in bronchoalveolar lavage (BAL) compared to sham-OVA mice. Chitosan exposure, prior to rDer p2 allergen challenge in passively sensitized mice, resulted in increased β -hexosaminidase levels in serum and BAL compared to sham-rDer p2 mice. Intranasal treatment with the synthetic glucocorticoid fluticasone propionate prevented chitosan-induced barrier dysfunction, allergic sensitization and MC degranulation. **Conclusion:** Epithelial barrier dysfunction facilitates trans-epithelial allergen passage, allergic sensitization and allergen-induced MC degranulation even in the absence of inflammatory environment. These results emphasize the crucial role of an intact epithelial barrier in prevention of allergy.

Key words

Allergic rhinitis, allergic sensitization, epithelial barrier, fluticasone propionate, mast cell degranulation

Introduction

Allergic rhinitis (AR) affects approximately 30% of the western population and causes symptoms such as nasal obstruction, rhinorrhea, sneezing and itchy nose. It results from a symptomatic inflammation of the nasal mucosa initiated by IgE dependent mast cell (MC) degranulation and a Th2-driven allergic response on re-exposure to inhaled allergens in sensitized individuals.^{1–3} The airway epithelium contributes to tissue surveillance, by forming a rigid barrier preventing the access of foreign molecules, including allergens, to the mucosa, which is supposed to be a requirement for allergic sensitisation. Some environmental allergens, such as *Dermatophagoides pteronyssinus* (*Der p* 1), have proteolytic activity and cleave tight junctions (TJs) to gain access to the host^{4,5}, which might explain a high sensitizing capacity. Allergens without proteolytic activity, e.g. *Der p*2, cannot easily cross an intact epithelial barrier but they might profit from cooperation with other proteolytic allergens, or they might penetrate as a result of ongoing inflammation. Ovalbumin (OVA) lacks proteolytic activity and hence fails to induce sensitization in mice despite repeated intranasal exposure.^{6,7}

Several studies have documented a defective airway epithelial barrier in AR^{8,9} and asthma.¹⁰ The impact of this epithelial barrier dysfunction as a facilitating factor for allergic sensitization and MC degranulation remains speculative. Currently, it remains unclear whether respiratory tract epithelial barrier dysfunction could be a primary defect, whether it is caused by external triggers that impair barrier function, or is a consequence of allergic inflammation. External triggers, such as allergens with proteolytic activity, air pollution or pathogens, may temporarily translocate TJ proteins or alter their expression leading to increased permeability. We demonstrated a correlation between the degree of barrier disruption and symptom severity in patients with AR⁸, and identified histamine and Th2-cytokines IL-4 and IL-13 as key players in disrupting TJ function.¹¹

In the current study, we documented the consequences of a barrier defect on allergic sensitization and on MC activation. Chitosan was used to induce reversible disruption of the epithelial barrier.^{12,13} Chitosan is a natural occurring polysaccharide, and has strong mucoadhesive capacities.¹⁴ Chitosan decreases trans-epithelial electrical resistance (TEER) in cultures of bronchial epithelial (16HBE cells)¹⁵ and of intestinal epithelial (Caco-2 cells)¹³ cell lines by transient translocation of TJ proteins, with minimal induction of immune responses.

We hypothesized that chitosan could also be used to disrupt the barrier function of the nasal mucosa enabling us to further document effects of a defective epithelial barrier on allergen passage, allergic sensitization and MC degranulation. Previously, our group showed that treatment with fluticasone

propionate (FP) prevents HDM-induced barrier dysfunction *in vivo*.^{8,16} FP is a synthetic glucocorticoid with anti-inflammatory and anti-allergic effects. FP is used intranasally as first-line treatment of allergic rhinitis. FP inhibits signaling pathways or the production of inflammatory mediators. Whether protection of epithelial barrier function contributes to the beneficial effects of FP has not been evaluated, a question which was also addressed in this study.

Finally, we evaluated whether increased allergen passage in already sensitized subjects can facilitate MC degranulation and thus enhance allergic disease. As allergic inflammation by itself affects barrier integrity,^{8,11,17–19} we designed a novel murine model with passive sensitization to *rDer p2* to circumvent the effect of allergic inflammation on the airway mucosal barrier. We here demonstrate that chitosan-induced barrier dysfunction increases allergen passage and not only facilitates allergic sensitization but also allergen-induced MC degranulation.

Methods

Primary human nasal epithelial cells

The use of primary human nasal epithelial cells (pNECs) from 3 healthy non-allergic controls was approved by the local committee of ethics at the University Hospitals Leuven. pNECs were isolated from inferior turbinates obtained during aesthetic and/or functional rhinoplasty as described previously.²⁰ Isolated pNECs (110,000 cells) were seeded on transwell inserts (pore size 1 μ M, Greiner Bio-One, Kremsmünster, Austria). Cells were cultured submerged for 5-7 days until a confluent monolayer was formed. Afterwards, pNECs were placed in air-liquid interface (ALI) for 2 weeks to obtain a fully differentiated epithelial monolayer using DMEM-F12 culture medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2% Ultrosor G (Pall Life Sciences, Belgium).

Trans-epithelial electrical resistance

Epithelial integrity of pNEC cultures was evaluated by TEER (Ω cm²) measurement with a voltmeter (EVOM/Endohm, WPI, Sarasota, Fla) as described previously.⁸

Trans-epithelial allergen passage

Aqueous acetic acid (0.01 M) was used as solvent for chitosan (0.06%) and as sham control. pNEC cultures were stimulated with 50 μ L of chitosan or sham and TEER was measured at baseline, every hour (h) for 5h and after 24h of incubation. 50 μ L of OVA (2 mg/mL, Grade V) in PBS was applied apically on the pNEC cultures together with 0.06% chitosan or sham control. To evaluate allergen passage, the concentration of OVA was measured in medium of the basolateral compartment at baseline, 3h and 5h. In a second series of experiments, pNEC monolayers were incubated for 24h with chitosan or sham and with rDer p2 (10 μ g/mL, ALK Abello, Hørsholm, Denmark), rDer p1 (10 μ g/mL, ALK Abello), heat-inactivated rDer p1 (60 minutes at 95°C) or HDM-extract (100 μ g/mL, Greer labs, USA). TEER was measured for 6h and after 24h of incubation. Medium samples from the basolateral compartment were collected at 0, 3, 5 and 24h and stored at -20°C for later use. To avoid interference of serum, serum-free culture medium was used during the experiments. All experiments were performed in duplicates.

Primary human mast cells

Buffy coats were purchased from the local blood bank. Primary human MCs were differentiated *in vitro* by culturing isolated CD133⁺ precursor cells with recombinant human (rh) stem cell factor (100 ng/mL, Miltenyi Biotec), rhIL-6 (50 ng/mL, Gibco, Thermo Fisher Scientific) and rhIL-3 (2 ng/mL, R&D systems Inc,

Minneapolis, MN USA) as previously described.^{21,22} More information is provided in the *Supplementary Materials*.

Bio-assay for rDer p1 and rDer p2 quantification

Mature MCs were sensitized by incubation with 10 kU/L IgE consisting of 80% human myeloma IgE (Calbiochem, Merck Chemicals, Nottingham, UK) and 20% chimeric mouse-human monoclonal IgE, either against rDer p2 (clones H10 and P4E, ALK Abello), or against rDer p1 (clones NH3 and NH5, ALK Abello) or a mix of all 4 clones in serum free medium at 37°C for 48h. Optimal concentrations of IgE were determined in preliminary experiments (**Fig. S1**). The model was based on previously described studies^{23,24} and modified for semi-quantitative evaluation of allergen concentrations. After washing, sensitized MCs (50,000 cells/tube) were incubated with the different conditioned culture medium samples from pNECs for 30 minutes at 37°C. To investigate indirect MC degranulation as a result of contact with allergen, levels of β -hexosaminidase in the supernatant and percentage of CD63⁺ MCs were determined. Maximum activation of MCs in the positive control sample (goat-anti-mouse IgE, 1 μ g/mL, Sigma Aldrich, St Louis, Missouri, USA) was set as highest outcome (100%), and results were expressed as a proportion of the highest value.

Murine models

All animal experiments were approved by the local Ethical Committee and were carried out in accordance with national and European Community Council guidelines for the care and use of laboratory animals (P103/2013). Male, 8-12 weeks old mice were purchased from Envigo (Horst, the Netherlands). Upon intranasal applications, inhalant anesthesia (4% isoflurane and 96% oxygen in a chamber) was used for 2 minutes.

First, to study the effects of nasal application of chitosan and FP on nasal allergen passage to the blood circulation, naïve BALB/c mice were treated daily intranasally with 6 μ g of FP (in saline with 0.6% DMSO) or sham (saline/DMSO 0.6 %) for 3 days. Afterwards, mice were intranasally exposed once to 30 μ L of chitosan (Heppe Medical Chitosan, Germany) in 1%/0.01 M acetic acid (based on a previous protocol²⁵ or sham (0.01 M acetic acid) followed by 50 μ L of OVA (6 mg/mL, grade V, Seramun Diagnostica, Heidesee, Germany) in PBS. Blood was collected 30 minutes later to determine serum OVA concentrations. To study the effect of chitosan on sensitization to intranasally applied allergens, male naïve C57BL/6J mice were intranasally instilled with 30 μ L of chitosan or sham, followed 10 minutes later by 50 μ L of OVA. This was repeated every second day for 12 days. Mice also received intranasally 6 μ g of FP or sham daily starting two days before the first application of chitosan and then before each application of chitosan or sham. At

day 14, bronchoalveolar lavage (BAL) fluid and blood was collected to measure levels of OVA-specific IgG1 and total IgE in serum. In BAL fluid, we counted the percentage of eosinophils in cytopins and measured IL-5 by enzyme-linked immunosorbent assay (ELISA, *Supplementary Materials*). Giemsa staining was performed on 5 μ m paraffin sections of the nasal mucosa to evaluate the influx of eosinophils.

Second, we developed a novel murine model to measure MC reactivity to *rDer p2* using passive sensitization with *rDer p2* specific IgE antibodies. At day -1, naïve male BALB/c mice were passively sensitized by intravenous (i.v.) injection of murine IgE from hybridoma clones H10, H12 and P4E producing IgE specific for 3 different epitopes of *rDer p2* (10 μ g in 200 μ L, ratio 1:1:1, ALK-Abello) or of PBS. Mice were treated intranasally with 6 μ g of FP or sham (saline/DMSO 0.6 %) on day -2, -1 and 0. At day 0, 30 μ L of chitosan or sham was applied intranasally, followed by *rDer p2* (5 μ g) or saline. Blood and BAL fluid were collected 40 minutes after allergen application to measure β -hexosaminidase in serum and BAL.

Quantification of ovalbumin, IgG1, total IgE and β -hexosaminidase

Concentrations of OVA (0.625-20.0 ng/mL) were determined using ELISA. The protocol for OVA-specific IgG1 and for total IgE in serum can be found in the *Supplementary Materials*. Levels of β -hexosaminidase were quantified by hydrolysis of 1 mM of *p*-nitrophenyl *N*-acetyl- β -D glucosamide (Sigma Aldrich) in 0.1 M sodium citrate at 37°C for 60 minutes. The optical density (OD) was assessed at 405 nm and determined by subtracting the blank.

Immunofluorescence staining for occludin in murine nasal mucosa

Occludin staining in murine nasal mucosa was performed by deparaffinization of 5 μ m paraffin sections. After antigen retrieval, mucosal sections were incubated overnight at 4 °C with rabbit, anti-occludin unlabeled antibody (Thermo Fisher). The next day, mucosal sections were washed and incubated with anti-rabbit antibody Alexa Fluor 555 for 1h at room temperature in the dark. Lastly, nuclei were counterstained with 4',6-diamidino-2-phenylindole for 10 minutes and were mounted in ProLong Gold antifade. Samples were analyzed at x50 magnification with a Leica TCS SPE confocal microscope (Leica Microsystems, Heerbrugg, Switzerland). The microscope software was used for 3-dimensional visualization of TJs.

Statistical analysis

Data were analyzed using Graphpad Prism 7 (La Jolla, CA, USA). Kolmogorov–Smirnov test was used to test for normality of the data distribution. One-way Anova Sidak's multiple comparison was used for parametric data, and the Kruskal-Wallis test with Dunns test was used for non-parametric data. For analysis of *in vitro* data, a two-way Anova multiple comparison test was used. All data are presented with medians and interquartile ranges (IQR). Values were considered significant when $P < 0.05$.

Results

Chitosan impairs the epithelial barrier and facilitates transepithelial allergen passage *in vitro*

First, the effect of chitosan on trans-epithelial allergen passage was investigated. ALI cultures of pNECs were incubated in the presence or absence of chitosan and/or OVA (**Fig. 1, A**). Chitosan rapidly decreased TEER ($P<0.0001$) which recovered after 24h in presence of chitosan (**Fig. 1, B**). OVA had no effect on TEER. We measured OVA concentration in the culture medium to analyse transepithelial OVA passage. Transepithelial OVA passage was strongly increased when pNEC cultures were pretreated with chitosan compared to sham controls (**Fig. 1, C**).

Next, we evaluated changes in TEER and transepithelial passage when HDM allergens *rDer p1*, *rDer p2* or HDM-extract were added to pNEC cultures in the presence or absence of chitosan (**Fig. 1, D and S2, A**). Cell culture samples were collected. To analyse allergen passage, we used a bioassay in which the samples were incubated with primary human MCs coated with IgE to *rDer p2* or to *rDer p1*. MC activation was evaluated. **Fig. S1** shows that the level of CD63 expression and β -hexosaminidase release corresponded to the respective allergen concentrations. *rDer p2* had no effect on TEER (**Fig. 1, E**) while both *rDer p1* and HDM-extract decreased TEER in pNEC cultures ($P<0.0001$, **Fig. S2, B**, although to a lesser extent than chitosan ($P<0.0001$, **Fig. 1, E**). *rDer p2* passage across pNEC was observed only when pNECs were permeabilized with chitosan ($P<0.0001$, **Fig. 1, F**). In contrast, there was a high *rDer p1* passage to the basolateral medium even in absence of chitosan ($P<0.0001$, **Fig. S2, C**). After heat-inactivation, *rDer p1* alone could not pass the pNEC barrier (**Fig. S2, C**). Chitosan by itself did not interfere with the bioassay using MC. Indeed, conditioned medium taken after incubation of pNEC cultures with chitosan alone did not increase the percentage of CD63⁺ cells nor the levels of β -hexosaminidase when added to human MCs (data not shown).

Chitosan facilitates transepithelial allergen passage *in vivo*

We further explored the effect of chitosan on mucosal barrier integrity and on OVA passage *in vivo*. Naïve mice were exposed to a single intranasal application of OVA, 30 minutes after the nasal application of chitosan or sham (**Fig. 2, A**). OVA concentration in serum was elevated in chitosan-OVA mice compared to sham-OVA mice ($P<0.0001$, **Fig. 2, B**). After intranasal pretreatment with FP, OVA concentrations in serum of chitosan-OVA mice were lower than those from sham pretreated chitosan-OVA mice ($P<0.0001$, **Fig. 2, B**). These data demonstrate that chitosan increases mucosal permeability for OVA *in vivo*, and that pretreatment with FP prevents this. To elucidate the underlying mechanism by which chitosan affects the barrier, the expression of TJs was investigated with immunofluorescence. A strong expression of occludin

was found in nasal mucosal biopsies of sham-OVA mice. Intranasal application of chitosan resulted in a patchy occludin expression. Intranasal treatment with FP restored occludin expression (**Fig 2, C**).

Chitosan facilitates allergic sensitization *in vivo*

Next, we evaluated the consequences of an impaired epithelial barrier on allergic sensitization. It was previously reported by our research group that nasal application of OVA alone without adjuvant fails to induce allergic sensitization.⁶ Mice were intranasally exposed every second day over a 12-day period to OVA with or without chitosan (**Fig. 3, A**). On day 14, chitosan-OVA mice had increased OVA-specific IgG1 levels and elevated percentages of eosinophils in the peripheral blood compared to sham-OVA mice (**Fig. 3, B**).

In a repeat experiment (**Fig. 3, C**), we confirmed this effect on OVA specific IgG1 (**Fig. 3, D**) as well as on the increased percentages of eosinophils in blood (18 % vs. 3 %; $P < 0.001$). Moreover, we also observed an increase in total IgE, and increased eosinophils and IL-5 levels on day 14 in BAL of in chitosan-OVA mice compared to sham-OVA mice (**Fig 3, D-E**). Our next question addressed the effect of FP treatment on allergic sensitization. When treated with FP (**Fig. 3, D**), OVA-specific IgG1 levels and total IgE were lower, and the percentages of blood eosinophils were similar to those of mice exposed to OVA alone (3 % vs. 1.5 %; $P < 0.05$). Levels of eosinophils and IL-5 in BAL were reduced to control levels after treatment with FP (**Fig. 3, E**). Histologically, increased influx of eosinophils in the submucosa of chitosan-OVA exposed mice compared to sham-OVA mice was observed (**Fig. 3, F**). Treatment with FP reduced the number of infiltrating eosinophils. Epithelial barrier dysfunction facilitates allergic sensitization to a non-protease allergen, which is prevented with FP treatment.

***In vivo* mast cell degranulation is facilitated by an impaired epithelial barrier**

Acute nasal symptoms after allergen exposure in IgE sensitized subjects are mainly the result of MC degranulation. We evaluated whether epithelial barrier disturbance could also have a direct effect on MCs. Since an ongoing allergic inflammation directly contributes to an impaired epithelial barrier,^{11,18,19} an *in vivo* model of passive sensitization was developed to study the effects of barrier dysfunction on MC degranulation irrespective of inflammation. Mice were passively sensitized with rDer p2-specific murine IgE and subsequently exposed intranasally to chitosan (or sham) followed by rDer p2 (**Fig. 4, A**). Serum β -hexosaminidase levels were higher in chitosan-rDer p2 challenged mice compared to sham-rDer p2 challenged mice (**Fig. 4, B**). A similar experiment was performed to study the effect of FP treatment (**Fig. 4, C**). Sensitized mice exposed to chitosan and rDer p2 showed increased levels of BAL ($P = 0.001$) and serum ($P = 0.005$) β -hexosaminidase compared to sensitized mice treated with sham and rDer p2 (**Fig. 4,**

D). FP treatment prevented the increase of β -hexosaminidase in BAL ($P = 0.005$) and serum ($P = 0.003$) in passively sensitized mice exposed to chitosan and *rDer p2* (**Fig. 4, D**). Chitosan alone had no effect on serum β -hexosaminidase. Analysis of the BAL fluid and histology of the nasal mucosa showed no influx of eosinophils, neutrophils or lymphocytes in either of the groups (data not shown), which confirms that a local allergic inflammation was absent in this model. To conclude, an impaired epithelial barrier facilitates MC degranulation even in the absence of ongoing allergic inflammation.

Discussion

Defects in epithelial barriers are strongly associated with atopic diseases. Recent studies have shown that allergic inflammation can contribute to an impaired barrier function via pro-inflammatory mediators. Our group previously demonstrated that histamine and IL-4 drive epithelial barrier dysfunction.¹¹ In a study using primary human bronchial epithelial cells, IL-1 β impaired the epithelial barrier.¹⁸ Another study using bronchial epithelial cells found that the MC mediator chymase plays an important role in impairing epithelial monolayers via the disruption of cell junctions.¹⁹ However, the direct contribution of a defective epithelial barrier to allergic sensitization and allergic inflammation remained unclear. The present study elucidates the direct role of a defective epithelial barrier on MC degranulation and to allergic sensitization without the presence of inflammatory mediators. Pretreatment with FP neutralizes the effect of chitosan and prevents MC degranulation, most likely due to decreased allergen passage.

It has been demonstrated that chitosan causes a reversible, time- and dose-dependent decrease of epithelial integrity in both intestinal^{12,13} and bronchial epithelial¹² cell lines. Chitosan decreases epithelial integrity by inducing reversible redistribution of occludin and/or zonula occludens-1.²⁶ In line, chitosan rapidly decreased epithelial integrity of pNEC cultures. Recovery was seen after 24h, highlighting that this effect was reversible.^{26–28} Some studies suggest that chitins/chitosan are able to modulate immune responses,^{29,30} whereas other studies describe a rather inhibitory effect of chitosan on immune cells.^{31–33} We did not find any effect of chitosan alone on allergic inflammatory markers, although, more research is needed to fully exclude the effect on immune responses.

In ALI cultures of human pNEC, chitosan strongly increased the transepithelial passage of OVA or rDer p2, which, is devoid of enzymatic activity. The HDM allergen rDer p1, similar to papain, can act enzymatically on TJs to gain access to the host.⁴ In a comparable experimental setting (**Fig. S2**), rDer p1 and HDM-extract decreased epithelial integrity, although to a lower extent than chitosan. The ability to cross an epithelial barrier relies on proteolytic activity as heat-inactivated rDer p1 had no effect on epithelial integrity and was not able to pass the barrier (**Fig. S2**).

Chitosan also disrupted the epithelial barrier *in vivo* and facilitated the passage of OVA to the blood circulation. Repetitive nasal applications of chitosan and OVA in naïve mice resulted in allergic sensitization to OVA. Nasal application of OVA alone did not induce sensitization. These data show that easy access to antigen presenting cells in the context of a defective epithelial barrier is required as the initial step of the sensitization process, emphasizing a pathophysiologic role of the nasal epithelium in allergy.

To demonstrate that a dysfunctional nasal epithelial barrier facilitates MC degranulation upon exposure to allergens in absence of allergic inflammation, we designed a novel murine model of passive IgE sensitization to *rDer p2*. Mice that were passively sensitized with IgE antibodies to *rDer p2* were exposed to chitosan and challenged with *rDer p2* allergens. In these mice, increased MC degranulation was found compared to those challenged with sham + *rDer p2*. The increase in MC degranulation occurred as a result of the dysfunctional barrier and increased allergen passage and not secondary to allergic inflammation, which sheds a new light on the pathophysiology of AR. Other groups demonstrated that IL-33 has a priming effect on MCs which enhances IgE-dependent responses.^{34–36} We cannot rule out whether cytokines released by activated epithelial cells, such as IL-33, contributed to the effect of chitosan on the MCs. Whether MC degranulation (in the absence of Th2 cell priming) results in allergic inflammation at a later time point, was not investigated in this study.

Specific markers for indirect determination of MC degranulation are histamine and tryptase. It has been discussed that β -hexosaminidase may not be involved in allergic reactions.³⁷ However, β -hexosaminidase is released in parallel with other mediators, i.e. histamine and tryptase, upon degranulation of MCs or basophils. In comparison with mediators such as histamine and prostaglandin D2, β -hexosaminidase is a stable mediator, which makes it a reliable marker for MC degranulation. In addition, *in vitro* MC degranulation was confirmed by the measurement of CD63 expression using flow cytometry.³⁸

For *in vitro* differentiation of primary human MCs, blood from different donors was used, as the number of MCs is limited per culture. A previous study demonstrated that *in vitro* differentiated MC from patients with asthma and controls respond with similar sensitivity to *rDer p2*-induced, IgE-mediated activation.²² Therefore, the sensitivity of the MCs may depend on the actual microenvironment rather than the genetic background.

To prevent allergic sensitization and MC degranulation, maintenance of the barrier function should be aimed at. Intranasal steroids are used as first-line therapy in patients with (seasonal) AR. Recently, we showed that treatment with FP reconstituted a defective epithelial barrier in patients with AR.^{8,16} In a clinical trial we demonstrated that treatment with a nasal spray, containing both azelastine hydrochloride and FP, reduced local inflammatory mediators and nasal hyperreactivity in patients with HDM-induced AR.¹⁶ During ongoing allergic inflammation, FP may prevent barrier disruption and increase the expression of the TJ proteins zonula occludens-1 and occludin.^{8,16} In the present study, pretreatment with FP preserved the barrier function and thereby reduced transepithelial allergen passage in a setting without allergic inflammation. This contributes to reduction of sensitization and MC degranulation upon allergen

contact. Previous studies have shown suppressive effects of dexamethasone as therapeutic treatment on total IgE, OVA-specific IgE and IgG2a in an OVA-induced murine model of allergic asthma³⁹ and on OVA-specific IgE, IgG1, IgG2a production in a murine model of OVA-induced AR.^{40,41} Dexamethasone also reduced the numbers of eosinophils and Th2-cytokines (IL-4, IL-5 and IL-13).^{39–41} The inhibitory effect of FP on allergic sensitization might be related to its effect on dendritic cell function, but our results indicate that effects on the epithelial barrier also contribute to the protective effect of steroids against sensitization and MC degranulation. In our model of passive sensitization, β -hexosaminidase secretion was reduced in mice pretreated with FP. This is most likely due to preservation of barrier function as MC degranulation is not directly affected by steroids as described previously.¹⁶

To conclude, our data demonstrate that epithelial barrier dysfunction results in an increased allergen passage which in turn facilitates allergic sensitization and increases MC degranulation even in the absence of allergic inflammation. Maintenance of the barrier function is essential to prevent sensitization and to reduce MC degranulation after sensitization has occurred. Our data shed new light on the consequences of impaired epithelial barrier integrity in the airways, which may also be relevant for allergies related to other barriers, such as the gut and the skin. Future experiments should be performed to focus on additional barrier restoring molecules and to explore the preventive effects of such molecules on allergic sensitization and chronicity of inflammation in allergic diseases.

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Figure legends

Figure 1: Chitosan-induced nasal epithelial barrier dysfunction facilitates allergen passage *in vitro*

A. Flow chart of the cell culture model using ovalbumin (OVA) as allergen. Primary nasal epithelial cell (pNEC) monolayers in transwell inserts were incubated with chitosan and OVA. Trans-epithelial electrical resistance (TEER) was measured every hour for 5h, and at 24h. At timepoints 0, 3 and 5h culture medium samples were taken from the basolateral compartment for analysis of the OVA passage. **B.** Effect of chitosan and/or OVA on TEER (expressed as percentage from baseline) of pNEC air-liquid interface (ALI) cultures (n = 3). **C.** Concentration of OVA in cell culture medium by ELISA. ALI cultures of pNEC were incubated with chitosan + OVA or sham + OVA. The concentration of OVA was measured in the basolateral medium at baseline and at 3 and 5h of incubation to determine the transepithelial passage of OVA in the presence or absence of chitosan. **D.** Flow chart of the cell culture model using recombinant *Dermatophagoides pteronyssinus* 2 (rDer p2). pNEC monolayers in transwell inserts were incubated with chitosan and rDer p2. TEER was measured and cell culture samples were collected at different time points. Primary human mast cells (MCs) were pre-sensitized with IgE against rDer p2 and incubated with the cell culture samples to analyse MC degranulation as a bioassay for trans-epithelial allergen passage. **E.** Change in TEER (expressed as percentage from baseline) of pNECs treated with chitosan + rDer p2 or sham + rDer p2 at baseline and at 1, 2, 3, 6 and 24h of incubation. **F.** Transepithelial passage of rDer p2. pNEC were incubated with chitosan + rDer p2 or sham + rDer p2. Samples of basolateral medium were collected at baseline and at 3, 6 and 24h of incubation, and incubated with MCs pre-sensitized with IgE against rDer p2. Results are expressed as percentage of CD63⁺ cells and as β -hexosaminidase levels (optical density

(OD)) after 30 minutes of incubation. Data show the median and IQR of 6 individual measurements, two-way Anova multiple comparisons was used. *: $P < 0.05$, ****: $P < 0.0001$.

Figure 2: Intranasal chitosan increases permeability to ovalbumin and degrades occludin in mice, which is counteracted by pretreatment with fluticasone propionate

A. Mouse model. Male naive BALB/c mice were exposed intranasally (i.n.) to chitosan (1%) or sham (0.01M acetic acid) followed by i.n. OVA. Mice were pretreated on day -2, -1 and 0 with either i.n. FP or saline/DMSO. **B.** Concentrations of OVA (ng/mL) in serum in the experimental conditions explained in A. Data shown as median and interquartile range (IQR). Kruskal-Wallis test with posthoc analysis. ****: $P < 0.0001$. **C.** Representative immunofluorescence stainings for occludin in murine nasal mucosa. Red = occludin, Blue = DAPI. Magnification is 50x.

Figure 3: Epithelial barrier dysfunction facilitates allergic sensitization and inflammation

A. Murine model of allergic sensitization with intranasal (i.n.) application of ovalbumin (OVA) with or without chitosan every second day over a 12 day period. Blood was taken on day 14. **B.** OVA-specific IgG1 measured by ELISA and expressed as optical density (OD), and percentage of eosinophils in blood, both on day 14 as explained in A. **C.** Murine model of allergic sensitization with i.n. OVA with or without chitosan for 12 days and treatment with i.n. fluticasone propionate (FP) or sham (saline + DMSO 0.6%). Bronchoalveolar lavage (BAL) was performed and mice were sacrificed on day 14. **D.** OVA-specific IgG1 (OD), and concentration of total IgE (ng/mL) in serum. **E.** Percentage of eosinophils, and concentration of IL-5 (pg/mL) in BAL fluid. **F.** Giemsa staining of 5 μ m sections of paraffine-embedded nasal mucosa. Arrows indicate infiltration of eosinophils. Data show median and IQR. Student t-test (B) and one-way Anova (D, E) were used. Magnification = 10 x 40, bar = 100 μ m. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$.

Figure 4: Nasal epithelial barrier dysfunction facilitates mast cell degranulation in mice passively sensitized with IgE against recombinant *Der p2*

A. Male wild-type BALB/c mice were passively sensitized on day -1 by intravenous (i.v.) injection of 10 μ g of monoclonal mouse-human chimeric IgE against 3 different epitopes of recombinant *Dermatophagoides pteronyssinus* (rDer p2, clones H10, H12, and 4PE, ratio 1:1:1) or PBS. At day 0, mice were exposed to intranasal (i.n.) chitosan (1%) or sham (0.01M acetic acid), followed by i.n. rDer p2 (5 μ g). **B.** Serum β -hexosaminidase at 30 minutes after rDer p2 challenge as explained in A (results expressed as optical density (OD)). **C.** Murine model as explained in (A) but also including pre-treatment with fluticasone propionate (FP, 0.3 mg/kg) or sham (saline/DMSO 0.6%). **D.** serum β -hexosaminidase at baseline and at 30 minutes after challenge with rDer p2 (or sham), as explained in C. Data show median and IQR. One-way Anova was used. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$.

Supplementary materials

Methods

Culture of primary nasal epithelial cells

Inferior turbinates from healthy individuals were incubated overnight at 4 °C with 0.1 % protease (Protease XIV, Sigma-Aldrich) in culture medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM), Gibco, Thermo Fisher Scientific, Waltham, USA) supplemented with Ultrosor G (Pall Life Sciences, Zaventem, Belgium) and penicillin-streptomycin (200 µg/mL). Protease activity was inactivated by addition of FCS (10 %). Cells were incubated in a culture flask at 37 °C for 1 hour to remove fibroblasts. CD45 and CD15 magnetic beads (Dynabeads; Invitrogen, Merelbeke, Belgium) were used to remove leucocytes and monocytes. The highly purified pNEC population was seeded on transwell inserts with a pore size of 1.0 µm at a density of 10⁵ cells/well and cultured in culture medium (DMEM:F12 (Gibco, Thermo Fisher Scientific) supplemented with 2% Ultrosor-G (Pall Life Sciences, Belgium), 100 U/mL penicillin and 100 U/mL streptomycin) in a humidified incubator with 5 % CO₂ at 37 °C until a monolayer had been formed. Further cell differentiation was obtained by culture in air-liquid interface (ALI) for 2 weeks. The monolayers were used for experiments when sufficient integrity was obtained (trans-epithelial electrical resistance (TEER) >200 Ω x cm², EVOM/Endohm, World precision instruments, Sarasota, Fla).

Culture of primary human mast cells

Mast cells were generated in eight weeks by positive selection of CD133⁺ cells from buffy coats (CD133 Micro Bead kit, Miltenyi Biotec, Bergisch Gladbach, Germany) and differentiated using StemSpan medium (Stemcell Technologies, Vancouver, Canada) supplemented with recombinant human (rh) stem cell factor (100 ng/mL, Miltenyi Biotec), rhIL-6 (50 ng/mL, Gibco, Thermo Fisher Scientific), penicillin/streptomycin (200 µg/mL) in a humidified incubator with 5% CO₂ at 37°C. The first 3 weeks, the culture medium was supplemented with rhIL-3 (2 ng/mL, R&D systems Inc, Minneapolis, MN USA), and during the last 2 weeks of culture with 10% fetal bovine serum (Invitrogen, Thermo Fisher Scientific). Mature MCs were sensitized with 1, 10 or 20 kU/L IgE consisting of a mixture of chimeric murine-human IgE against recombinant *Dermatophagoides pteronyssinus* 1 (rDer p1, clones NH3 (10%) and NH5 (10%) or against rDer p2 (clones H10 (10%) and P4E (10%) and human myeloma IgE (80%) (Supplementary Figure 1 and 2).

ELISA

ELISA plates were coated overnight at 4 °C with 5 µg/mL OVA (for measurement of OVA specific IgG1) or 1 µg/mL anti-mouse IgE mAb (BD, Franklin Lakes USA) (for quantification of total IgE) or 1 µg/mL anti-human IL-5 mAb (BD) (for BAL IL-5). After blocking with PBS/2% bovine serum albumin for 1 hour at room temperature (rT), the plates were incubated with the 1:10 diluted serum samples and the standards (0.98 – 500 ng/mL, total IgE) for 2 hours at rT. After washing, biotin anti-mouse IgG1 (0.5 µg/mL, BD) or biotin anti-mouse IgE (0.5 µg/mL, BD) or biotin rat anti-human IL-5 (1 µg/mL, BD) was added to each well and incubated for 1 hour at rT. After washing, streptavidin-HRP was added and incubated for 1 hour at rT. TMB substrate was added for max 30 minutes and the color development was stopped by application of H₂SO₄. The optical density (OD) for IgG1 was determined by subtracting the blank (PBS/2% BSA/0.05% Tween 20). The concentration of total IgE was calculated using a standard curve and by multiplying with the dilution factor.

Giemsa staining

Giemsa staining was used to identify eosinophils in paraffin-embedded nasal mucosa tissue slides (5 µm). Sections were deparaffinized, stained with Giemsa solution in phosphate buffer at pH 6.5 and differentiated until showing red granules. Images were taken with a light microscope (Axiovert 200M, Zeiss, Oberkochen, Germany) with a AxioCam MRm camera at 40x magnification.

Supplementary Figures

Figure S1: Dose response of activation of primary human mast cells sensitized with specific IgE upon stimulation with recombinant *Der p1* or *Der p2* allergen

Primary human mast cells (MC) were sensitized with chimeric murine-human IgE against recombinant *Dermatophagoides pteronyssinus* (*rDer p1*, clones NH3 and NH5) or *rDer p2* (clones H10 and P4E). The specific IgE clones were used at a ratio 1:1 and mixed with myeloma IgE at a 1:5 ratio. This was followed by incubation with *rDer p1* or *rDer p2* at 10-log dilutions. As a positive control anti-IgE antibody was used to induce maximal mast cell degranulation. The mast cell response was normalized to 100% compared to the positive control. **A.** Percentage of CD63+ MCs after incubation with *rDer p1* or with *rDer p2*. **B.** Optical

density (OD) measurement of β -hexosaminidase after incubation with *rDer p1* or with *rDer p2*. Data show the median and IQR of 2 individual experiments.

Figure S2: Effect of *rDer p1* or house dust mite-mix on primary human nasal epithelial cell monolayers

A. Air liquid-interface cultures of primary nasal epithelial cells (pNEC) from inferior turbinates of 3 non-atopic, non-smoking healthy subjects were grown in transwell inserts. The pNEC monolayers were incubated in culture medium with recombinant *Dermatophagoides pteronyssinus* (*rDer p1*, 10 μ g/mL) or house dust mite (HDM)-mix (10 μ g/mL) or heat-inactivated *rDer p1* (10 μ g/mL) for 24h. The barrier integrity was evaluated by measurements of the trans-epithelial electrical resistance (TEER) at baseline and at 3, 6 and 24h of incubation. Samples of the cell culture medium were taken at baseline and at 3, 6 and 24h of incubation. **B.** Change in TEER of pNEC monolayers incubated with *rDer p1*, HDM-mix or heat inactivated *rDer p1*. Data show the median and IQR of 6 individual measurements. **C.** Primary human mast cells (MCs) pre-sensitized with IgE against *rDer p1* were incubated with the cell culture samples (as explained in A) to evaluate MC degranulation as a bioassay for the transepithelial allergen passage. Results are expressed as percentage of CD63⁺ cells and as β -hexosaminidase levels (optical density (OD)) after 30 minutes of incubation. Experiments were performed in duplicates. Data show the median and IQR a two-way Anova multiple comparisons was used **: P < 0.01, ***: P < 0.001, ****: P < 0.0001.

A

B

C

OVA + sham

OVA + chitosan

OVA + FP

OVA + chitosan + FP

A

C

D

B