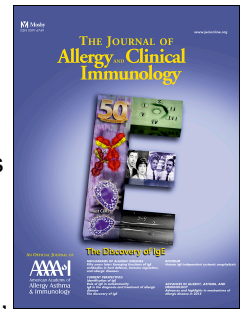


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Histamine and T helper cytokine driven epithelial barrier dysfunction in allergic rhinitis

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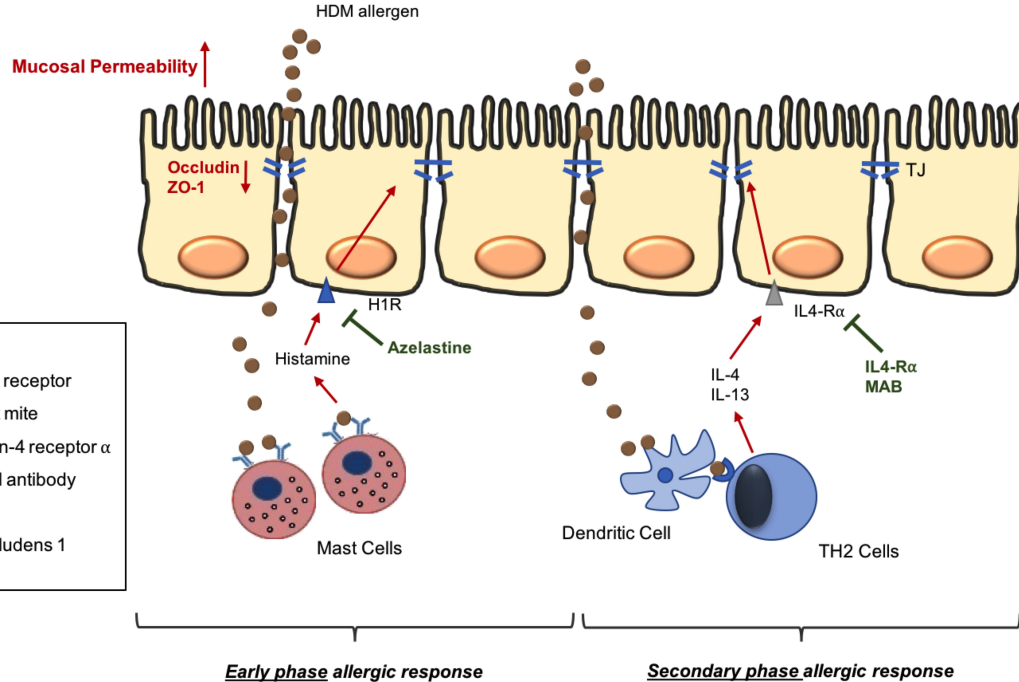
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## Histamine and T helper cytokine driven epithelial barrier dysfunction in allergic rhinitis



# **Title: Histamine and T helper cytokine driven epithelial barrier dysfunction in allergic rhinitis**

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**Abbreviations:** ALI: air-liquid interface, AR: allergic rhinitis; FITC: fluorescein isothiocyanate; HDM: house dust mite; IR: idiopathic rhinitis; pNEC: primary nasal epithelial cell; TER: trans-epithelial electrical resistance; TGN: trigeminal neuron; TJ: tight junction;

**Key words:** allergic rhinitis, idiopathic rhinitis, tight junctions, histamine, T<sub>H</sub>2 cells, primary nasal epithelial cells

48

49 **Word count:** 4170

ACCEPTED MANUSCRIPT

**Abstract**

**Background:** Allergic rhinitis (AR) is characterized by mucosal inflammation, driven by activated immune cells. Mast cells and T<sub>H</sub>2 cells might decrease epithelial barrier integrity in AR maintaining a leaky epithelial barrier. **Objective:** We sought to investigate the role of histamine and T<sub>H</sub>2 cells in driving epithelial barrier dysfunction in AR. **Methods:** Air-liquid interface (ALI) cultures of primary nasal epithelial cells (pNECs) were used to measure trans-epithelial electrical resistance, paracellular flux of FITC-dextran 4kDa and mRNA expression of tight junctions. Nasal secretions were collected from healthy controls, AR patients and idiopathic rhinitis (IR) patients and were tested *in vitro*. In addition, the effect of activated T<sub>H</sub>1 and T<sub>H</sub>2 cells, mast cells and neurons was tested *in vitro*. The effect of IL-4, IL-13, IFN- $\gamma$  and TNF $\alpha$  on mucosal permeability was tested *in vivo*. **Results:** Histamine as well as nasal secretions of AR but not IR patients rapidly decreased epithelial barrier integrity *in vitro*. Pretreatment with histamine receptor-1 antagonist, azelastine prevented the early effect of nasal secretions of AR patients on epithelial integrity. Supernatant of activated T<sub>H</sub>1 and T<sub>H</sub>2 cells impaired epithelial integrity, while treatment with anti-TNF $\alpha$  or anti-IL-4R $\alpha$  monoclonal antibodies restored the T<sub>H</sub>1 and T<sub>H</sub>2 induced epithelial barrier dysfunction respectively. IL-4, IFN- $\gamma$  and TNF $\alpha$  enhanced mucosal permeability in mice. Antagonizing IL-4 prevented mucosal barrier disruption and tight junction downregulation in a mouse model of house dust mite allergic airway inflammation. **Conclusion:** Our data indicate a key role for allergic inflammatory mediators in modulating nasal epithelial barrier integrity in the pathophysiology in AR.

**Word count abstract:** 250

74 **Key messages**

- 75       • Histamine initiates and T cell inflammation maintains defective epithelial barrier in  
76       allergic rhinitis
- 77       • Impaired barrier function is not found in idiopathic rhinitis

78

79 **Capsule summary:** A defective epithelial barrier is demonstrated in allergic rhinitis, and we here  
80 show that histamine, released during the early allergic immune reaction, together with T cell  
81 cytokines, released during chronic inflammation, contribute to this defect.

**[Main text]****Introduction**

The nasal epithelium is of vital importance in host defense since it not only provides a physical barrier between the environment and the submucosal region, but also contributes to the induction of an appropriate immune response towards pathogens, allergens and noxious stimuli that overcome the mucosal barrier<sup>1</sup>. The maintenance of an intact physical barrier depends on the coordinated expression and interaction of interepithelial protein complexes such as tight junctions (TJs) and adherence junctions<sup>2</sup>. TJs are the most apically located junctions and are important guards for maintaining selective permeability to ions and small molecules. Occludin, the family of claudins, tricellulin and junctional adhesion proteins make up the transmembrane proteins that seal off the paracellular space, whereas zonula occludens (ZO)-1, -2 and -3 are cytoplasmic linker proteins that connect the transmembrane proteins to the cytoskeleton<sup>1, 2</sup>.

Recently, studies have indicated that a defective epithelial barrier, due to TJ and/or adherence junction defects, is part of the underlying pathology in diseases such as atopic dermatitis<sup>3</sup>, asthma<sup>4</sup>, chronic rhinosinusitis<sup>5</sup> and allergic rhinitis (AR)<sup>6, 7</sup>. AR is characterized by symptoms such as nasal obstruction, rhinorrhea, itchy nose and/or sneezing upon contact with inhaled allergens in sensitized individuals<sup>8, 9</sup>. The inflammatory cascade in AR is initiated by an immunoglobulin E (IgE) dependent mast cell degranulation with release of histamine amongst other mediators, and is further orchestrated by T helper type 2 (T<sub>H</sub>2) cytokines interleukin-4 (IL-4), IL-5 and/or IL-13<sup>10-12</sup>. These cytokines maintain a continuous inflammation in the nasal mucosa by attracting inflammatory cells and by interfering with the establishment of an intact mucosal barrier. The dynamic nature of IL-4 and IL-13 is well demonstrated by their ability to modulate the expression and assembly of TJ proteins<sup>13, 14</sup>.

Noteworthy, idiopathic rhinitis (IR) is a subgroup of rhinitis characterized by an identical phenotype as AR though in the absence of an overt inflammation in the nasal mucosa<sup>15, 16</sup>. IR is assumed to be mediated by dysfunctional sensory C-fibers in the nasal mucosa, rather than by T<sub>H</sub>2 inflammation<sup>17-19</sup>. At present, the contribution of a defective epithelial barrier as underlying part in the pathology of AR and IR has not been investigated nor compared.

Therefore, we hypothesized that inflammation is a key node in initiating and sustaining defective epithelial barrier function in AR, which is unlikely to be the case in IR. For this purpose, nasal secretions from patient with AR and IR and from healthy controls were collected and tested on primary nasal epithelial cell (pNEC) cultures *in vitro*. In addition, we evaluated the effect of different inflammatory cells and their mediators on epithelial barrier function *in vitro* and in a mouse model of house dust mite (HDM) induced allergic airway inflammation. Patients with IR, in whom nasal inflammation is absent, showed an intact nasal epithelial barrier. Impaired epithelial barrier function in AR was induced by histamine during the early phase allergic immune response and is maintained in the late phase allergic immune response by T<sub>H</sub>2 cell inflammation. Antagonizing IL-4 and IL-13 *in vitro* and anti-IL-4 treatment in mice prevented epithelial barrier disruption and hence represents a possible strategic target for breaking persistent nasal inflammation in AR.

## Methods

### *Patients*

The study protocol was approved by the local ethics committee of UZ Leuven and was registered at clinicaltrial.gov (NCT02461797) (NCT02288156). Patient characteristics are

depicted in Table E1 in this article's Online Repository. Allergic status was determined via skin prick testing for the most frequent inhalant allergens in Belgium, i.e. birch pollen, grass pollen, cat dander, dog dander, *Dermatophagoides pteronyssinus* (Stallergenes, S.A., France), rabbit and spores of *Alternaria*, *Aspergillus* (HAL Allergy, Leiden, the Netherlands) as described previously<sup>20</sup>. All study participants were asked to score their rhinitis symptoms on a visual analog scale (VAS) (score 0-10). The major nasal symptom was selected based on the highest VAS score.

#### *Nasal secretions*

Nasal secretions were collected from each study participant. A nasal sponge (Ivalon Surgical products, San Diego, CA, USA) was weighed and inserted in both nostrils. After 10 minutes, the sponge was removed and weighed again. A volume of saline was added depending on the weight of the collected sponge (1/5 dilution). The sponge was then squeezed and centrifuged at 1500 g at 4°C for 5 minutes. Supernatant was stored at -20°C until analysis.

#### *Isolation of naïve CD4<sup>+</sup> T cells and T<sub>H</sub>1/T<sub>H</sub>2 polarization in vitro*

Details on the isolation and polarization of T<sub>H</sub>1/T<sub>H</sub>2 cells can be found in the Method section of this article's Online Repository.

#### *Isolation and culture of mast cells and eosinophils and murine trigeminal neurons (TGN)*

The method for isolation and culturing mast cells<sup>21</sup>, eosinophils<sup>22</sup> and murine TGN<sup>23</sup> has been described previously. Supernatant of the different activated cells was stored at -20°C until further analysis.

#### *Air-liquid interface (ALI) cultures of primary nasal epithelial cells (pNECs) and Calu-3 epithelial cells*

Inferior turbinates were used for isolation of pNECs from non-allergic, non-asthmatic healthy controls. A highly purified pNEC population was obtained as reported previously<sup>6</sup>. Isolated pNECs or Calu-3 epithelial cells were seeded on 0.4µm 0.33cm<sup>2</sup> polyester transwell inserts (Greiner Bio-One, Vilvoorde, Belgium) at a density of 100.000 cells/transwell. The culture medium used for pNECs was DMEM/F12, supplemented with antibiotics and ultrosor G (2%) (Pall Life Sciences, Belgium), Calu-3 culture medium was EMEM, supplemented with antibiotics, FCS (10%) and L-Glutamine (1%). Culture medium was refreshed every other day. Once the cells grew to complete confluence, the apical culture medium was removed to allow further cell differentiation at ALI. At day 21 in ALI, epithelial cell cultures were used for *in vitro* stimulation studies.

#### *Trans-epithelial electrical resistance (TER) measurement*

Epithelial integrity of ALI cultures was evaluated by TER measurements using an EVOM/Endohm (WPI Inc, Sarasota, USA) as described in the method section of this article's Online Repository. Wells not building up sufficiently (TER < 200 Ω x cm<sup>2</sup>) were not included in experiments.

#### *Stimulation experiments in vitro*

For long term experiments (up to 72 hours), ALI cultures of pNECs and Calu-3 cells were stimulated basolaterally for 3 days with IL-4, IL-13, TNF-α and IFN-γ (all 10 ng/ml, R&D Systems, Abingdon, United Kingdom), supernatant of activated T<sub>H</sub>1/T<sub>H</sub>2 cells, supernatant of IgE-activated mast cells, supernatant of C5a-activated eosinophils and supernatant of capsaicin-activated murine TGN. Two hours before addition of the above-mentioned stimuli, ALI cultures were pretreated with anti-IL-4Rα antibody, anti-IFN-γR antibody (both 2 µg/ml, Sigma-Aldrich, St Louis, Mo) or anti-TNF-α (Inflectra®, 40 µg/ml, Celltrion, South Korea).

For short term experiments (up to 4 hours), ALI cultures of Calu-3 epithelial cells were stimulated for 4 hours with nasal secretions of AR, IR and healthy control subjects, different doses of histamine (0.01–1 mM, Sigma-Aldrich, St Louis, Mo) or substance P (10–100 pM, R&D Systems, Abingdon, United Kingdom). To evaluate the effect of histamine in nasal secretions, Calu-3 epithelial cell cultures were pretreated for 2 hours with a selective histamine receptor-1 antagonist (Azelastine, Sigma-Aldrich, St Louis, Mo) before adding the nasal secretions. TER was measured in function of time and is expressed as relative change compared to baseline values.

#### *Ussing chamber experiments for the evaluation of mucosal explant integrity*

Nasal biopsy specimens were taken from the inferior turbinate after the application of a nasal spray with local anesthesia (cocaine 5%) by using a Fokkens forceps. Connective tissue was removed and biopsies were mounted in Ussing chambers (Mussler Scientific Instruments, Aachen, Germany) to evaluate mucosal integrity by measuring trans-tissue resistance (TTR) *ex vivo* as described previously<sup>6</sup>.

#### *RT-qPCR for tight junction genes*

The methods for mRNA isolation and RT-qPCR have been reported previously<sup>18</sup>. Detailed information and primer sequences can be found in the method section and Table E2 in this article's Online Repository.

#### *Mice*

Male Balb/c and C57Bl/6 mice (6-8 weeks) were obtained from Harlan (Horst, the Netherlands) and were kept under conventional conditions. Experimental procedures were approved by the Ethical Committee for Animal Research at the KU Leuven (P103/2013). Balb/c mice were 3 times endonasally instilled with IL-4, IL-13, IFN- $\gamma$ , TNF $\alpha$  (all 250 ng) or saline with 1 hour interval. One hour after the last endonasal instillation, 20  $\mu$ l fluorescein isothiocyanate–

dextran 4kDa (FD4) (50 mg/ml) was applied endonasally allowing evaluation of nasal mucosal permeability. One hour later, serum and nasal mucosa were collected for further analysis.

C57Bl/6 mice were endonasally sensitized with 50 µl house dust mite (HDM) extract (1 µg) (Greer Laboratories, Lenoir, North Carolina, USA) or 50 µl saline at day 1. From day 8 to 12, mice were endonasally challenged with 50 µl HDM extract (10 µg) or saline. One hour prior to each challenge, mice were injected intraperitoneally with either anti-IL-4 (250 µg) or anti-TNFα (100 µg) or sham (kind gift from L. Boon). One hour after FD4 (50 mg/ml) application, mice were sacrificed with intraperitoneal injection of Nembutal (Ceva, Brussels, Belgium). Levels of FD4 were determined in the serum by a fluorescence reader (FLUOstar Omega). Albumin leakage from the lungs to bronchoalveolar lavage (BAL) fluid, as a surrogate marker for lung permeability, was measured with ELISA according to the manufacturers protocol (Abcam, Cambridge, UK). IL-4 and IL-13 concentrations in BAL fluid were determined with ELISA.

Immunofluorescence staining of occludin and ZO-1

See the Methods section in this article's Online Repository for a detailed description of immunofluorescence staining of nasal mucosal biopsies.

### *Statistical analysis*

Data were analyzed using Graphpad Prism 7 (La Jolla, CA 92037 USA). Differences between two groups were analyzed using two-tailed unpaired t-test or Mann–Whitney U test, depending on normality. Data are presented as mean ± SD or median (IQR). One-way ANOVA or Kruskal–Wallis test with post-hoc analysis was used to compare multiple groups. Values were considered significantly different when  $P < .05$ .

## Results

*Decreased trans-tissue resistance and mRNA expression of occludin and ZO-1 in nasal biopsies from AR but not IR patients*

To address the potential role of inflammation in mucosal barrier dysfunction, trans-tissue resistance and FD4 permeability were measured on nasal mucosal biopsies from healthy subjects, IR and AR patients. A reduced trans-tissue resistance, accompanied with increased FD4 permeability was found in mucosal explants of AR patients (**Fig. 1a, b**). No difference in mucosal integrity or FD4 permeability was found in mucosal explants from IR patients compared to healthy subjects (**Fig. 1a, b**). VAS for major symptom was similar in IR and AR patients and significantly higher compared to healthy subjects (**Fig. 1c**). Histologically, AR patients showed a clear influx of inflammatory cells in the nasal mucosa, which was absent in the nasal mucosa of IR patients and healthy subjects (**Fig. 1d**).

Consistent with the absence of an impaired mucosal barrier in IR patients, no decreased mRNA expression for occludin and ZO-1 was observed compared to healthy subjects (**Fig. 2a**). Occludin and ZO-1 mRNA expression was significantly lower in AR patients compared to healthy subjects and IR patients. Interestingly, claudin-1 expression was significantly increased in IR patients compared to AR patients (**Fig. 2a**). Claudin-4 showed a similar, though nonsignificant increased expression in IR patients. Immunofluorescence of the TJ proteins occludin and ZO-1 of paraffin embedded mucosal biopsy specimens revealed an intact TJ layer in healthy mucosa and mucosa of IR patients. This layer was disrupted more severely in AR patients along with an irregular TJ protein expression (**Fig. 2b**).

*Nasal secretions of AR but not IR patients induce a rapid decrease in trans-epithelial resistance during the early allergic immune response which is prevented by azelastine*

To investigate the factors leading to decreased nasal epithelial integrity in AR patients, we first studied the effect of nasal secretions on Calu-3 epithelial cell cultures at ALI. Calu-3 epithelial cells were cultured for 4 hours with nasal secretions collected from healthy subjects, IR and AR patients and TER was measured. Nasal secretions from AR patients consistently decreased TER in a time dependent manner, whereas nasal secretions of IR patients and healthy subjects had no effect (**Fig. 3a**). Moreover, nasal secretions of AR patients only temporarily decreased TER of Calu-3 cell cultures at ALI as the effect was returned to baseline after 24 hours (see **Fig. E1 in the article's Online Repository**).

We next measured the presence of inflammatory mediators in the nasal secretions. Nasal secretions of both controls and IR patients contained low levels of histamine and type 2 cytokines IL-4 and IL-13, compared to nasal secretions of AR patients (**Fig. 3b-e**). The neuropeptide, substance P was significantly increased in nasal secretions of IR patients compared to controls and (**Fig. 3e**). Subsequently, we tested which inflammatory mediators in the nasal secretions of AR patients were responsible for the decreased TER during the early allergic immune response induced by nasal secretions of AR patients. Calu-3 epithelial cell cultures at ALI were stimulated for 4 hours with histamine, IL-4, IL-13 or substance P. Histamine rapidly decreased TER of Calu-3 epithelial cells in a dose-dependent manner (**Fig. 3f**). The supernatant of IgE-activated mast cells showed a similar rapid effect as histamine on TER, which had returned to baseline values after 24 hours (see **Fig. E2 in the article's Online Repository**). IL-4, IL-13 or substance P did not decrease of TER of Calu-3 epithelial cell cultures during the time window representing the early allergic immune response (**Fig. 3g, h**).

To verify that histamine in the AR nasal secretions is decreasing epithelial integrity *in vitro*, Calu-3 epithelial cell cultures at ALI were pretreated for 2 hours with different concentrations of azelastine, a selective histamine receptor-1 antagonist. Azelastine inhibited the effect of the nasal secretions of AR patients on TER in a dose-dependent way (**Fig. 3i**). Noteworthy, a 2-hour

pretreatment of Calu-3 epithelial cells with anti-IL-4R $\alpha$  (2 $\mu$ g/ml) to antagonize the effect of IL-4 and/or IL-13 did not inhibit the decrease in TER during the early allergic immune response induced by the nasal secretions of AR patients (see **Fig. E3 in the article's Online Repository**).

Together, these data indicate that histamine in nasal secretions of AR patients transiently decreases TER of Calu-3 epithelial cells in a time window representing the early allergic immune response. Azelastine prevented the AR nasal secretion mediated decrease in TER, indicating a possible role for histamine and the histamine receptor-1 signaling in mediating barrier dysfunction.

#### *T<sub>H</sub>1 and T<sub>H</sub>2 cytokine-mediated decrease in TER of epithelial cells in vitro during the delayed allergic immune response*

We next studied if other inflammatory cells can alter epithelial integrity though during a time window representing the delayed allergic immune response. To this end, T<sub>H</sub>1 and T<sub>H</sub>2 cells were polarized *in vitro* from naïve peripheral blood CD4<sup>+</sup> T cells and the supernatant of activated cells was collected to examine the effect on both Calu-3 and pNEC cultures at ALI for 3 days. Both supernatant of activated T<sub>H</sub>1 or T<sub>H</sub>2 cells decreased TER of Calu-3 epithelial cell and pNEC cultures (**Fig. 4a**). No decline in TER was found when Calu-3 and pNEC cultures were stimulated with supernatant of IgE-activated mast cells, C5a-activated eosinophils or capsaicin-activated murine TGN for 3 days (**Fig. 4b to d**).

#### *Anti-TNF $\alpha$ and anti-IL4-R $\alpha$ antibody prevent the decline in TER orchestrated by activated T<sub>H</sub>1 and T<sub>H</sub>2 cells*

Several cytokines were detected in the supernatant of activated T<sub>H</sub>1 and T<sub>H</sub>2 cells *in vitro* (see **Figure E4 in the article's Online Repository**). To study which cytokines released by T<sub>H</sub>1 and T<sub>H</sub>2 cells impair epithelial barrier integrity during the delayed allergic immune response, recombinant cytokines were used *in vitro*. Stimulation with TNF $\alpha$  (10 ng/ml) for 3 days decreased

TER of Calu-3 and pNEC cultures at ALI (**Fig. 5**), whereas IFN- $\gamma$  (10 ng/ml) did not alter TER (**Fig. 5**). In addition, T<sub>H</sub>2 derived IL-4 and IL-13 (10 ng/ml) also impaired TER (**Fig. 5b**). Pretreatment of Calu-3 and pNEC cultures at ALI for 2-hours with anti-IL-4R $\alpha$  (2  $\mu$ g/ml) or anti-TNF- $\alpha$  (40  $\mu$ g/ml) monoclonal antibodies prevented the cytokine-mediated barrier disruption. Likewise, pretreatment with anti-IFN- $\gamma$ R and anti-TNF- $\alpha$  monoclonal antibodies prevented the effect of supernatant from activated T<sub>H</sub>1 cells, while pretreatment with anti-IL-4R $\alpha$  monoclonal antibody prevented the effect of supernatant from activated T<sub>H</sub>2 cells (**Fig 5a**).

Collectively, these results indicate that both T<sub>H</sub>1- and T<sub>H</sub>2 cells can impair epithelial barrier function during the delayed allergic immune response, which is ascribed to either TNF $\alpha$  or to IL-4 and IL-13 respectively.

*IL-4, IL-13 and TNF $\alpha$  increase transmucosal FD4 permeability via decreasing expression of occludin and ZO-1 in vivo*

As IL-4, IL-13 and TNF $\alpha$  decreased epithelial barrier integrity *in vitro*, we next investigated if the cytokine-mediated barrier disruption could be confirmed *in vivo* and if it was associated with altered expression of occludin and ZO-1 *in vivo*. Wild type Balb/c mice were endonasally instilled with TNF $\alpha$ , IFN- $\gamma$ , IL-4 or IL-13 (all 250 ng/instillation) and mucosal permeability was evaluated by using FD4 (**Fig. 6a**). Instillation with IL-4, IFN- $\gamma$  and TNF $\alpha$  significantly increased FD4 permeability compared to saline instilled control mice (**Fig. 6b**). The increased FD4 mucosal permeability in IL-4, IFN- $\gamma$  and TNF $\alpha$  instilled mice was associated with decreased expression of occludin and ZO-1 compared to saline instilled mice (**Fig. 6c,d**). IL-13 had a modest, though non-significant effect on FD4 permeability and expression of occludin (**Fig. 6b,c**).

*Anti-IL-4 prevents induction of transmucosal FD4 permeability in a mouse model of house dust mite induced airway inflammation*

Given that our findings implicate a direct effect of IL-4 and TNF $\alpha$  on mucosal barrier integrity, we speculated that either treatment with anti-IL-4 or anti-TNF $\alpha$  monoclonal antibodies would prevent mucosal permeability disturbances in a mouse model of HDM-induced allergic airway inflammation (**Fig. 7a**). BAL fluid concentrations of IL-4 and to a lesser extent TNF $\alpha$  were significantly increased in HDM challenged mice compared to saline mice (**Fig. 7b**). Assessment of mucosal permeability by nasal application of FD4 showed elevated serum levels of FD4 in HDM challenged mice compared to saline challenged mice (**Fig. 7c**). Anti-IL-4 pretreatment of HDM challenged mice significantly decreased BAL IL-4 levels, without affecting BAL TNF $\alpha$  levels and completely normalized the HDM-induced increase in FD4 permeability (**Fig. 7b,c**). Moreover, albumin leakage to BAL fluid was significantly increased in HDM challenged mice, while pretreatment with anti-IL-4 prevented albumin leakage (**Fig. 7d**). Anti-IL-4 pretreatment of HDM challenged mice prevented loss-of-occludin and ZO-1 mRNA expression compared to HDM challenged mice receiving vehicle (**Fig. 7e, f**). Blocking TNF $\alpha$  in HDM challenged mice with anti-TNF $\alpha$  only partially prevented loss of nasal mucosal barrier integrity and albumin leakage to BAL fluid in the lungs (see **Fig. E5 in the article's Online Repository**). Anti-TNF $\alpha$  pretreatment did not restore ZO-1 expression in HDM challenged mice (see **Fig. E5 in the article's Online Repository**). Taken together, blocking IL-4 in HDM challenged mice protected mucosal barrier integrity by sustaining the expression of occludin and ZO-1.

## Discussion

A defective epithelial barrier has been associated with chronic inflammatory diseases such as asthma<sup>4</sup> and AR<sup>6</sup>, though evidence about the underlying mechanism is lacking. We here provide a link between inflammation and mucosal integrity. Mucosal permeability was increased

in mucosal explants from AR patients, which was associated with a decreased expression of occludin and ZO-1. In patients with IR, however, no defective epithelial barrier or decreased TJ expression was found (**Fig. 1** and **Fig. 2**). IR is a sub-classification of rhinitis characterized by symptoms like nasal obstruction, rhinorrhea and nasal hyperreactivity though in the absence of mucosal inflammation<sup>18, 24</sup>. Interestingly, claudin-1 and claudin-4 expression was elevated in IR patients compared to AR patients. Claudins can be divided into two broad categories: sealing and pore forming claudins<sup>25</sup>. Claudin-1 and claudin-4 are classified as sealing claudins<sup>26, 27</sup>, which might explain why patients with IR have a tight mucosal barrier. The fact that no disrupted barrier is present in IR emphasizes that other mechanisms are responsible for nasal symptoms compared to AR. Hence, local release of neuropeptides such as substance P by afferent nerves are assumed to induce symptoms in IR<sup>28</sup>.

Since AR patients show a defective epithelial barrier, we searched for the responsible mediators in nasal secretions from AR, IR and control subjects (**Fig. 3**). Only nasal secretions of AR patients rapidly decreased TER, which was ascribed to increased mast cell activity. Histamine levels in nasal secretions from AR patients were increased, suggesting a potential role for mast cell in modulating epithelial integrity during the early allergic immune response. Indeed, histamine transiently and rapidly decreases TER, which builds further on previous reports showing that histamine modulates epithelial permeability by interfering with expression of TJs<sup>29-31</sup> and/or adherence junctions<sup>32</sup>. Histamine exerts its effect via the histamine-1 receptor<sup>33</sup>, which is upregulated in AR<sup>34, 35</sup>. Blocking this receptor with azelastine prevented the decline in TER mediated by AR nasal secretions pointing towards a crucial role for histamine in rapidly decreasing epithelial barrier function during the early allergic reaction. Besides increased histamine levels, T<sub>H</sub>2 cytokines IL-4 and IL-13 were elevated in nasal secretions of AR patients but did not manifest a rapid effect on epithelial integrity. This is also supported by the finding that pretreatment of Calu-3 cells with anti-IL-4R $\alpha$  antagonist did not prevent the decrease in TER

mediated by nasal secretions of AR patients. Lastly, substance P was only elevated in nasal secretions of IR patients as shown by others<sup>18, 36</sup>. Interestingly, stimulation of Calu-3 cells with substance P modestly increased TER of Calu-3 cells, presumably by stimulating the expression of ZO-1 as shown by Ko and colleagues in corneal epithelial cells<sup>37</sup>. This finding might also support the assumption that IR patients have no defective epithelial barrier because of the barrier promoting effect of substance P.

Histamine, released during the early allergic immune response, is likely not the sole factor altering epithelial integrity. Other inflammatory cells may also interfere with epithelial barrier homeostasis, presumably during the delayed allergic immune response. The supernatant of activated T<sub>H</sub>1 and T<sub>H</sub>2 cells, mast cells, eosinophils and murine TGN were collected and tested on Calu-3 and pNEC cultures (**Fig. 4**). IgE-activated mast cells and capsaicin-activated murine TGN had no effect on TER during the delayed allergic immune response. Of note, of TGN increased TER of Calu-3 cells at 24 and 48 hours which was not found on pNECs. This disparity might be explained by morphological differences between cell lines and pNECs though needs further confirmation. Eosinophils are found in close proximity with epithelial cells in chronic rhinosinusitis<sup>38</sup> and are implicated in disease development and progression by increasing epithelial permeability<sup>39</sup>. We did not find any effect of eosinophils on epithelial integrity, which might be due to differences in activation methods of eosinophils and the subsequent released mediators. In agreement with previous publications, the supernatant of activated T<sub>H</sub>1 and T<sub>H</sub>2 cells significantly decreased TER in function of time of Calu-3 and pNEC cultures<sup>40, 41</sup>.

The secondary decline in TER during the delayed allergic immune response, mediated by activated T<sub>H</sub>1 cells is linked to TNF $\alpha$ . TNF $\alpha$  is an important cytokine in the pathology of inflammatory bowel disease<sup>42</sup> and rheumatoid arthritis<sup>43</sup>, though its role in AR is not yet extensively explored. TNF $\alpha$  is increased in the airways of corticosteroid-refractory asthmatic patients<sup>44-46</sup> and patients with chronic rhinosinusitis (unpublished data). TNF $\alpha$  decreases epithelial

integrity of Calu-3 and pNEC cultures as demonstrated previously on airway epithelial cells<sup>47</sup> and intestinal epithelial cells<sup>48-50</sup> (**Fig. 5**). Antagonizing TNF $\alpha$  prevented loss-of-epithelial barrier function *in vitro*. The observation that blocking TNF $\alpha$  protects epithelial barriers might help us understand why patients with Crohn's disease<sup>51</sup>, rheumatoid disease<sup>43</sup> or inflammatory bowel disease<sup>52</sup> treated with antagonizing TNF $\alpha$  have improved disease activity scores.

IL-4 and IL-13 are the main cytokines linked with decreased epithelial barrier integrity mediated by activated T<sub>H</sub>2 cells<sup>6, 40, 53, 54</sup>. Blocking IL-4 and IL-13 signaling with anti-IL-4R $\alpha$  antibodies restored epithelial barrier function during the delayed allergic immune response (**Fig. 5**). Interfering with IL-4/IL-13 is a novel biological treatment for asthma<sup>55</sup> and currently evaluated in phase III clinical trials for chronic rhinosinusitis with nasal polyps<sup>56</sup>. Anti-IL-4R $\alpha$  antibody treatment ameliorates symptom control in patients with asthma<sup>57</sup> and in patients with chronic rhinosinusitis<sup>56</sup>. Our data adds a new mode of action for anti-IL4R $\alpha$  treatment since this molecule not only inhibits type 2 inflammatory pathways but also prevents cytokine mediated barrier disruption. The barrier modulating capacity of T<sub>H</sub>1- and T<sub>H</sub>2-derived cytokines is also demonstrated *in vivo* (**Fig. 6**). Endonasal instillation of IL-4, IL-13, IFN- $\gamma$  or TNF $\alpha$  increased mucosal barrier permeability, which was associated with decreased expression of occludin and ZO-1. Importantly, we could confirm the crucial role of IL-4 on epithelial barrier homeostasis in a mouse model of HDM-induced allergic airway inflammation (**Fig. 7**). HDM challenged mice had an increased mucosal permeability as demonstrated with increased FD4 levels and BAL albumin levels, whereas pretreatment with anti-IL4 prevented mucosal barrier dysfunction. On the other hand, blocking TNF $\alpha$  only partially restored mucosal barrier integrity in HDM challenged mice since the expression of ZO-1 was not fully restored. We hypothesize that IL-4 is the major driver of mucosal barrier dysfunction and therefore overcomes the antagonistic effect on TNF $\alpha$ . Indeed, HDM challenged mice pretreated with anti-TNF $\alpha$  still showed elevated levels of IL-4 in BAL fluid, which might explain the increased mucosal permeability.

412 In summary, we have identified a pathogenic role for histamine and T<sub>H</sub>2 cells in decreasing  
413 mucosal barrier function in AR. Our findings suggest that histamine initiates loss-of-epithelial  
414 barrier function during the early allergic immune response which is then during the delayed  
415 allergic immune response is maintained by T cell inflammation. Targeting histamine via  
416 antagonizing H1-receptors or antagonizing T<sub>H</sub>1 cell-derived TNF $\alpha$  and/or T<sub>H</sub>2 cell-derived IL-4  
417 and IL-13 prevented the defective mucosal barrier dysfunction *in vitro* and *in vivo*.

**List of supplementary materials**

Fig. E1: Effect of nasal secretions of allergic rhinitis patients on TER of Calu-3 epithelial cells after 24 hours.

Fig. E2: Effect of histamine on TER of Calu-3 epithelial cells *in vitro*.

Fig. E3: Short term effect of allergic rhinitis nasal secretions on Calu-3 epithelial integrity in the presence or absence of anti-IL-4R $\alpha$  monoclonal antibody.

Fig. E4: Cytokines secretion by Th1 and Th2 primed cultures.

Fig. E5: Effect of anti-TNF $\alpha$  treatment in a mouse model of HDM induced allergic airway inflammation.

Table E1: Patient characteristics

Table E2: Primer and probe sequences used for RT-qPCR

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## Figures

**Fig. 1. Epithelial barrier function in patients with AR and IR. (A)** Trans-tissue resistance measured with Ussing chambers on nasal explants from AR, IR and controls. **(B)** FD4 permeability. **(C)** VAS scores for major nasal symptom. **(D)** H&E staining of nasal mucosal biopsies from healthy controls and patients with AR and IR. **(E to H)** mRNA expression of occludin, ZO-1, claudin-1 and -4 in patients with AR, IR and control subjects. Relative expression versus the housekeeping genes encoding  $\beta$ -actin and beta2-microglobulin is shown. \*  $P < .05$ , \*\*  $P < .01$ , \*\*\*  $P < .001$ .

**Fig. 2. Tight junction expression in mucosal biopsies from control subjects, AR and IR patients. A.** mRNA expression of occludin, ZO-1, claudin-1 and -4 in patients with AR, IR and control subjects. Relative mRNA expression versus the housekeeping genes encoding  $\beta$ -actin and beta2-microglobulin is shown. **B.** Representative images of protein expression of occludin and ZO-1. Data presented as median and interquartile range, \*  $P < .05$ , \*\*  $P < .01$ .

**Fig. 3. Effect of nasal fluid of AR patients on epithelial barrier function of Calu-3 cells. (A)** Effect of nasal fluid of healthy controls, AR patients and IR patients ( $n = 5/\text{group}$ ). **(B)** Histamine content measured in nasal fluid of healthy controls and patients with AR and IR. **(C)** IL-4 and IL-13 concentration in nasal fluid. **(D)** SP concentration in nasal fluid. **(E to G)** Effect of histamine, IL-4 (10 ng/ml), IL-13 (10 ng/ml) and SP on Calu-3 epithelial cell integrity after 4-hour stimulation. **(I)** Effect of 2-hour pretreatment with azelastine on the decrease of Calu-3 epithelial cell integrity induced by AR nasal secretions. \*  $P < .05$ , \*\*  $P < .01$ .

**Fig. 4. Effect of supernatant from activated immune cells on epithelial barrier function of Calu-3 cells and primary nasal epithelial cells. (A-B)** Effect of supernatant (SN) from activated

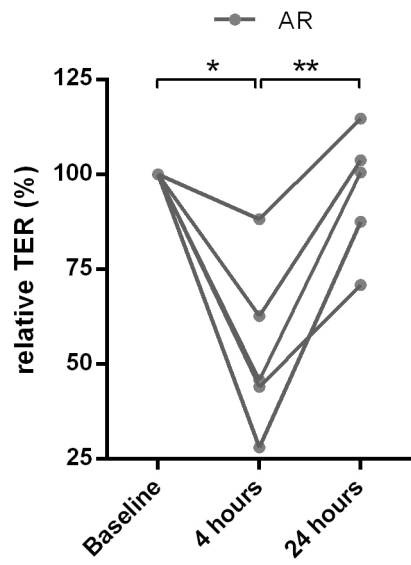
T<sub>H</sub>1 and T<sub>H</sub>2 cells. **(C-D)** Effect of SN from IgE-activated mast cells. **(E-F)** Effect of SN from capsaicin activated murine trigeminal neurons. **(G-H)** Effect of SN from C5a activated eosinophils. N = 5 for all experiments. Data presented as median and interquartile range. \*\*  $P < .01$ , \*\*\*  $P < .001$ .

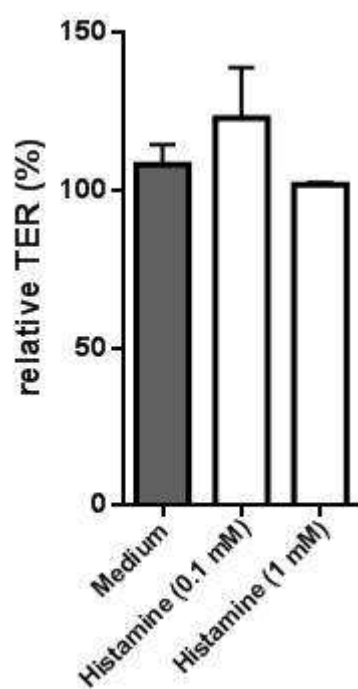
**Fig. 5. Effect of T<sub>H</sub>1 and T<sub>H</sub>2 cytokines on epithelial barrier function of Calu-3 cells and primary nasal epithelial cells. (A-B)** Calu-3 cells and pNECs were stimulated with SN of activated T<sub>H</sub>1 cells, T<sub>H</sub>1 cytokines and their receptor antagonists for 72 hours. **(C-D)** Calu-3 cells and pNECs were stimulated with SN of activated T<sub>H</sub>2 cells, T<sub>H</sub>2 cytokines and their receptor antagonists for 72 hours. n = 5 for Calu-3 cells and n = 4 for pNECs. Data presented as mean ± SD. \*  $P < .05$ , \*\*  $P < .01$ .

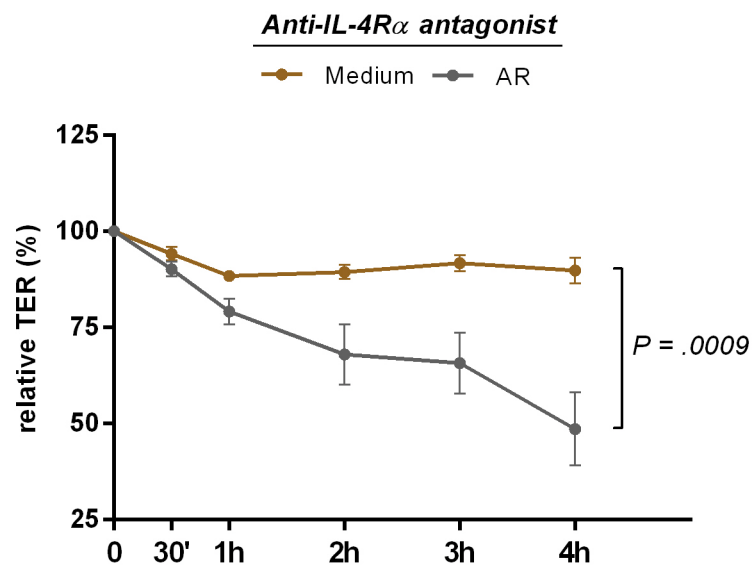
**Fig. 6. Effect of IL-4, IFN- $\gamma$  and TNF $\alpha$  on the mucosal barrier in naïve Balb/c mice. (A)** Study protocol. Mice received on day 1, 3 nasal instillations with 1 hour interval with IL-4, IL-13, IFN- $\gamma$ , TNF $\alpha$  (all 250 ng) or sham. One day after the last instillation, 20  $\mu$ l FD4 (50 mg/ml) was applied endonasally for evaluation of mucosal permeability. One hour later, mice were sacrificed for further analysis with an intraperitoneal injection of Nembutal. **(B)** Mucosal permeability for FD4. **(C)** mRNA expression for occludin. **(D)** mRNA expression for ZO-1. mRNA expression is relative to the housekeeping genes  $\beta$ -actin and b2-microglobulin. N = 5/group. \*  $P < .05$ , \*\*  $P < .01$ , \*\*\*  $P < .001$ .

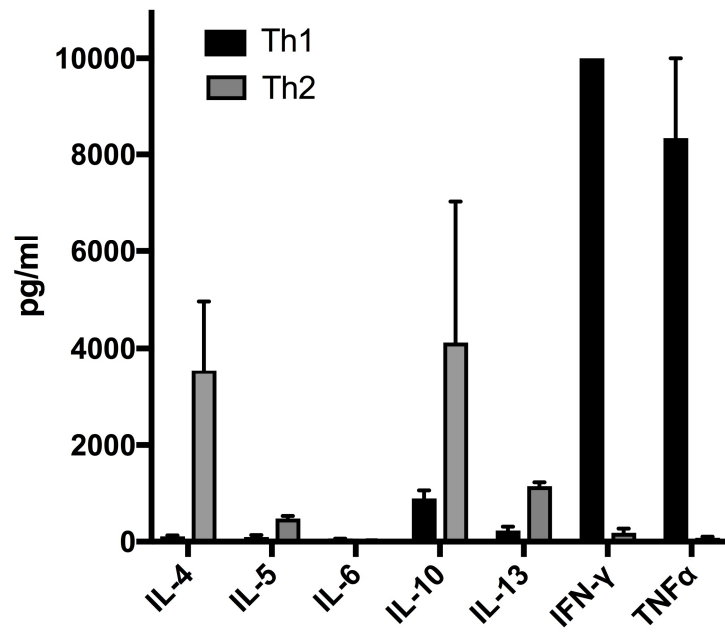
**Fig. 7. Effect of anti-IL-4 in mouse model of HDM-induced allergic airway inflammation. (A)** Mouse model. Mice were sensitized with 1  $\mu$ g HDM extract or saline control at day 1. One

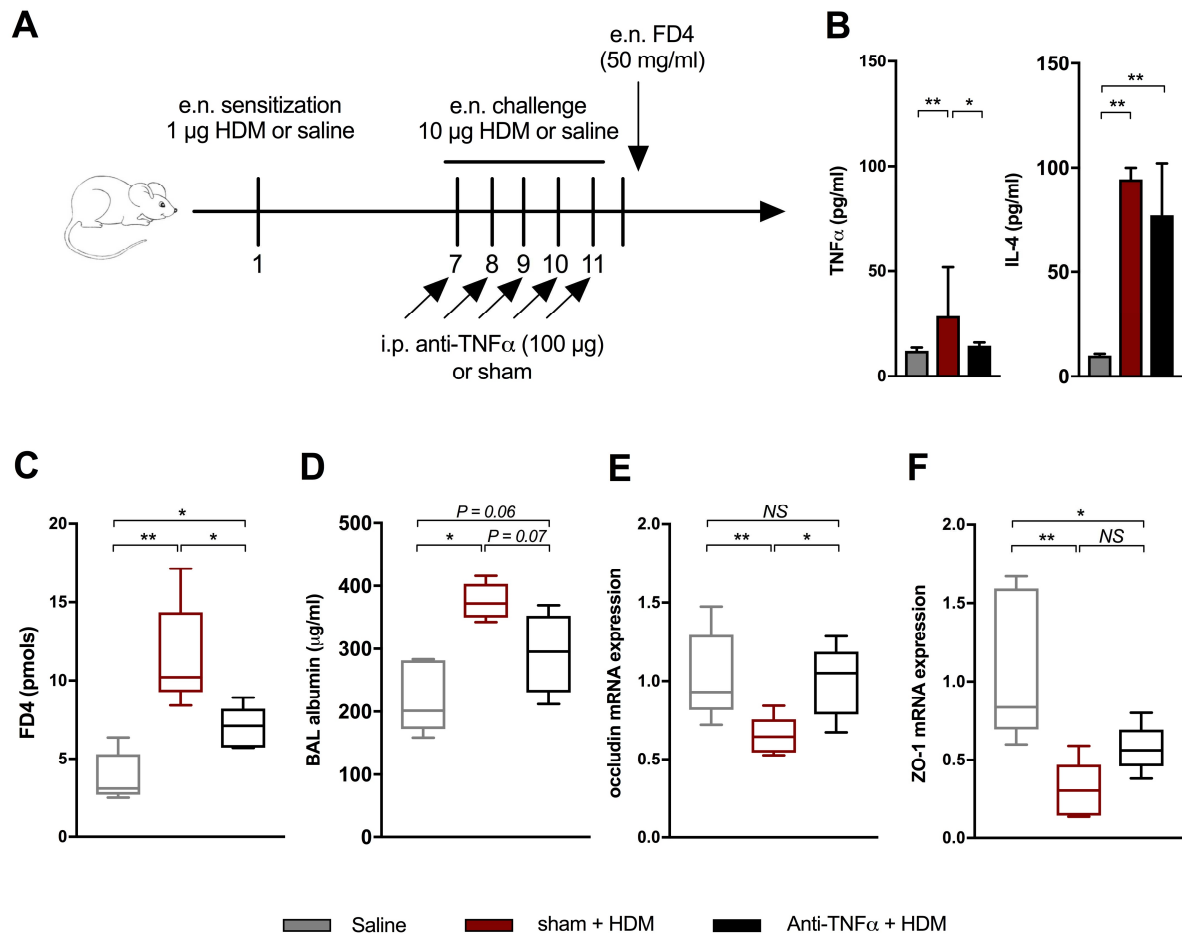
644 week later (day 7-11), mice were challenged endonasally with 10  $\mu$ g HDM extract or saline. One  
645 hour before each challenge, anti-IL-4 monoclonal antibody or vehicle was given intraperitoneally.  
646 One hour after the last challenge, 20  $\mu$ l FD4 (50 mg/ml) was applied followed one hour later with  
647 the sacrifice of the mice. **(B)** IL-4 levels in BAL fluid **(C-D)** Effect of anti-IL-4 treatment on FD4  
648 permeability and albumin leakage in the lungs. **(E-F)** mRNA expression of occludin and ZO-1.  
649 Relative expression versus the housekeeping B-actin and beta-2-microglobulin. N = 5/group. \*  $P$   
650  $< .05$ , \*\*  $P < .01$ .

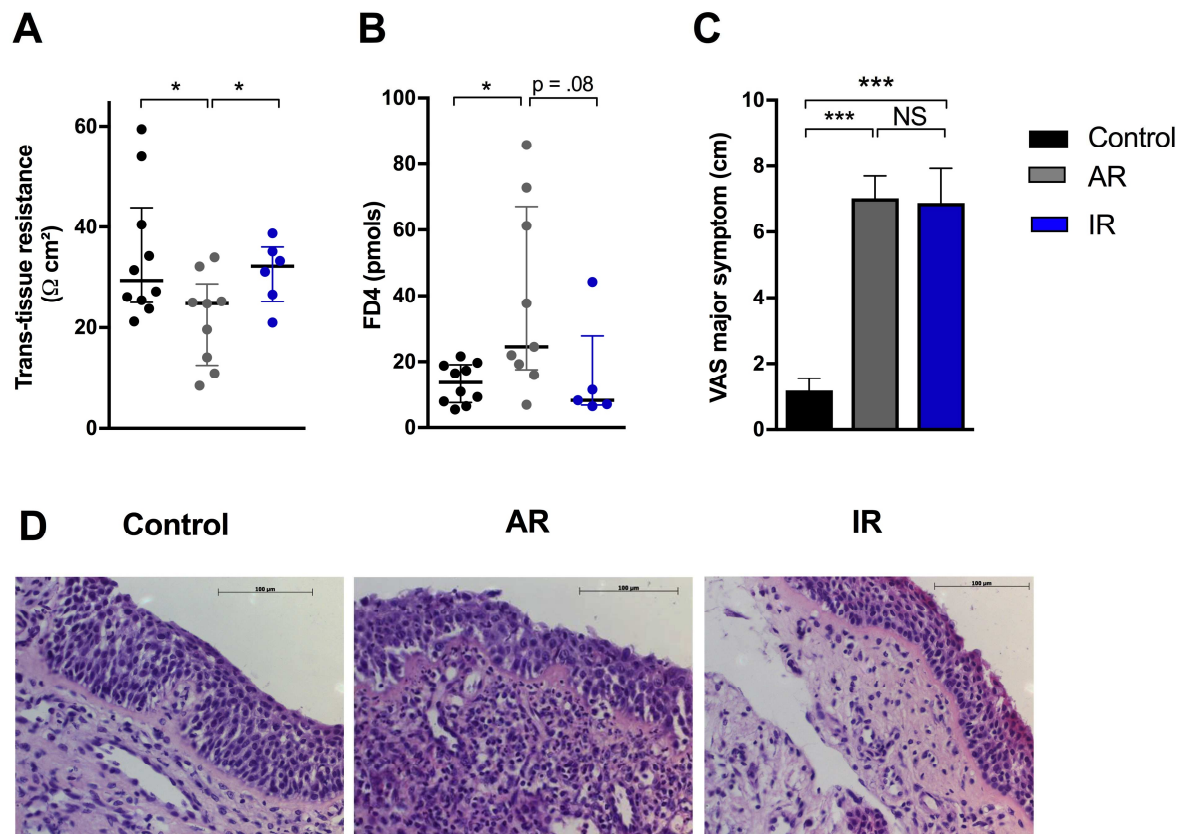




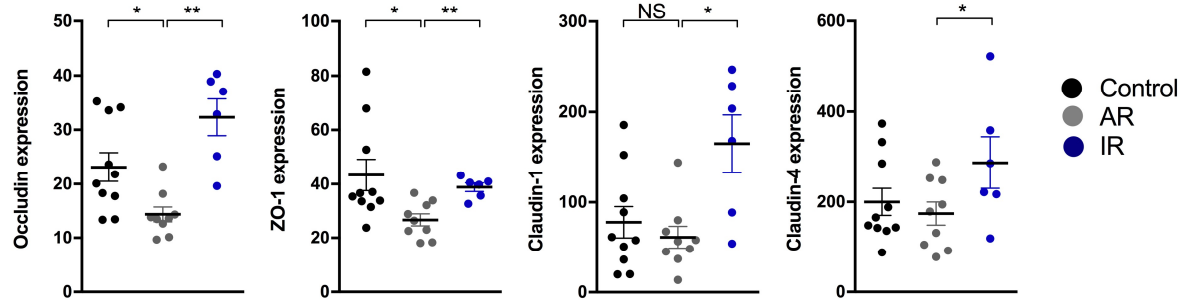




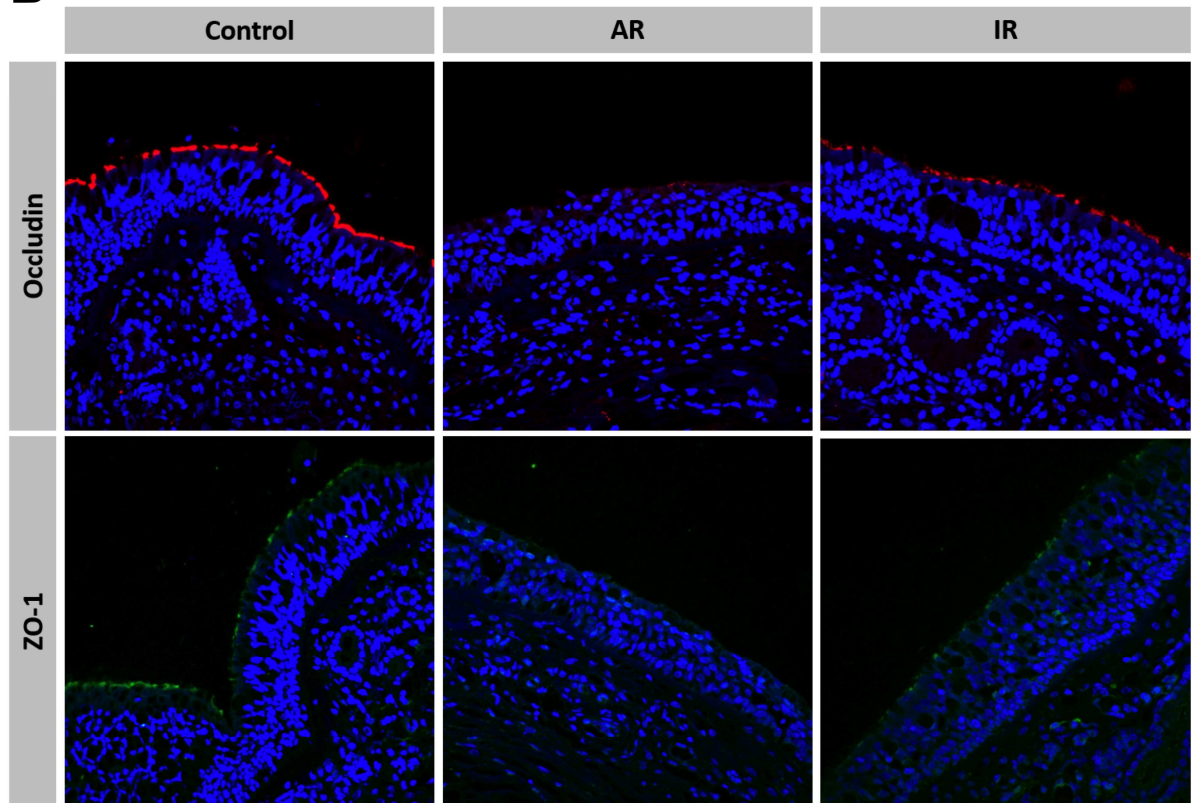


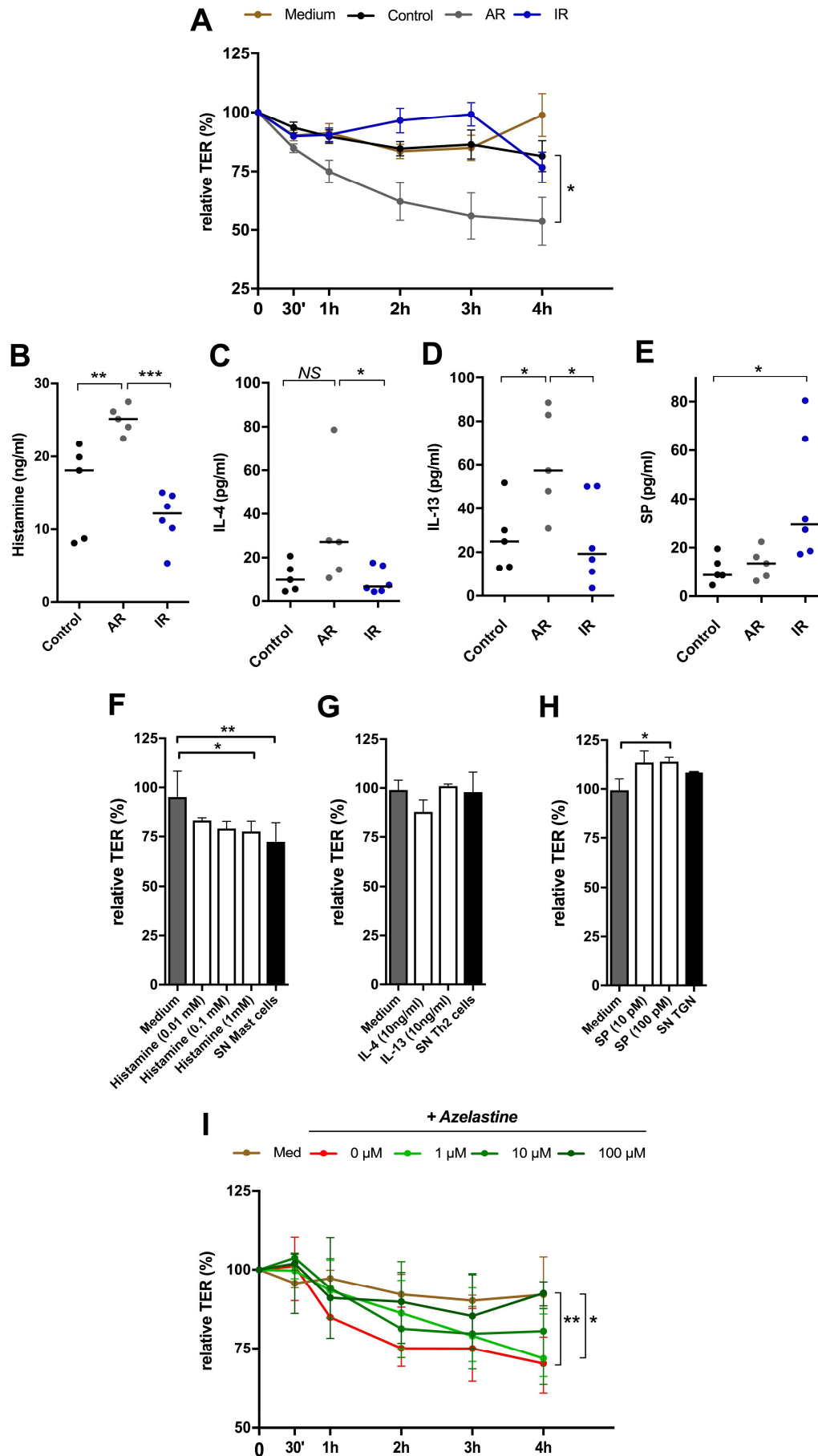


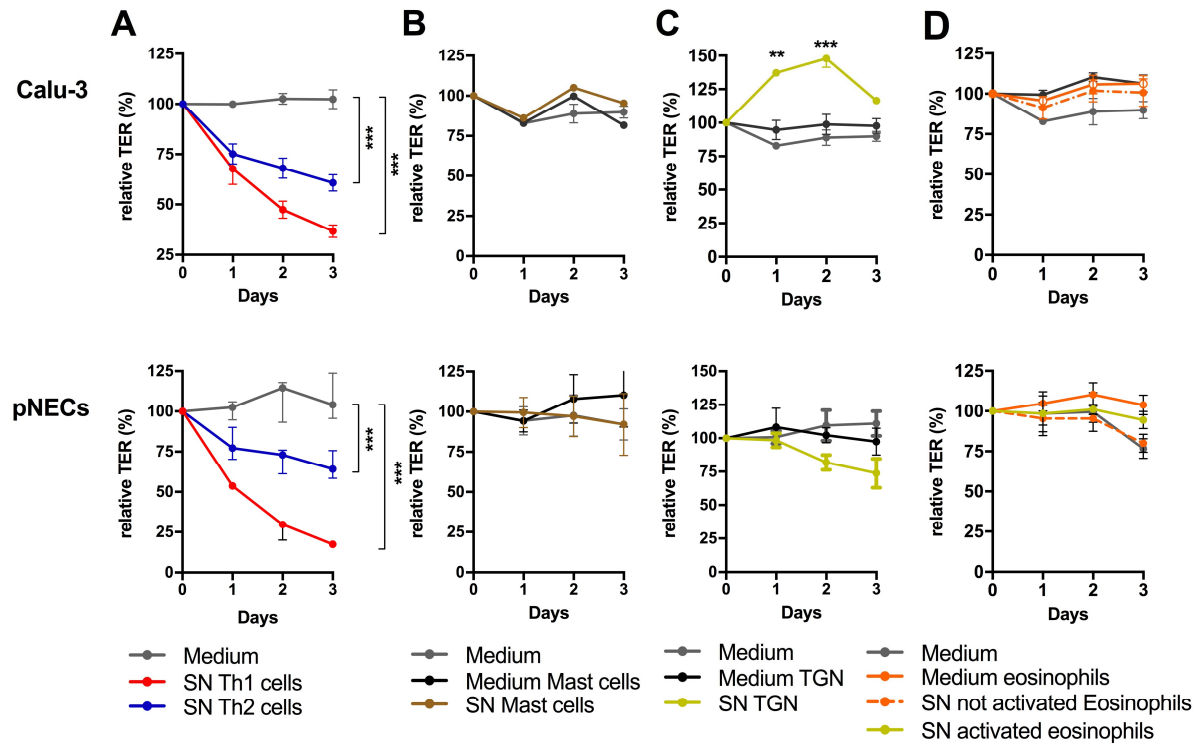
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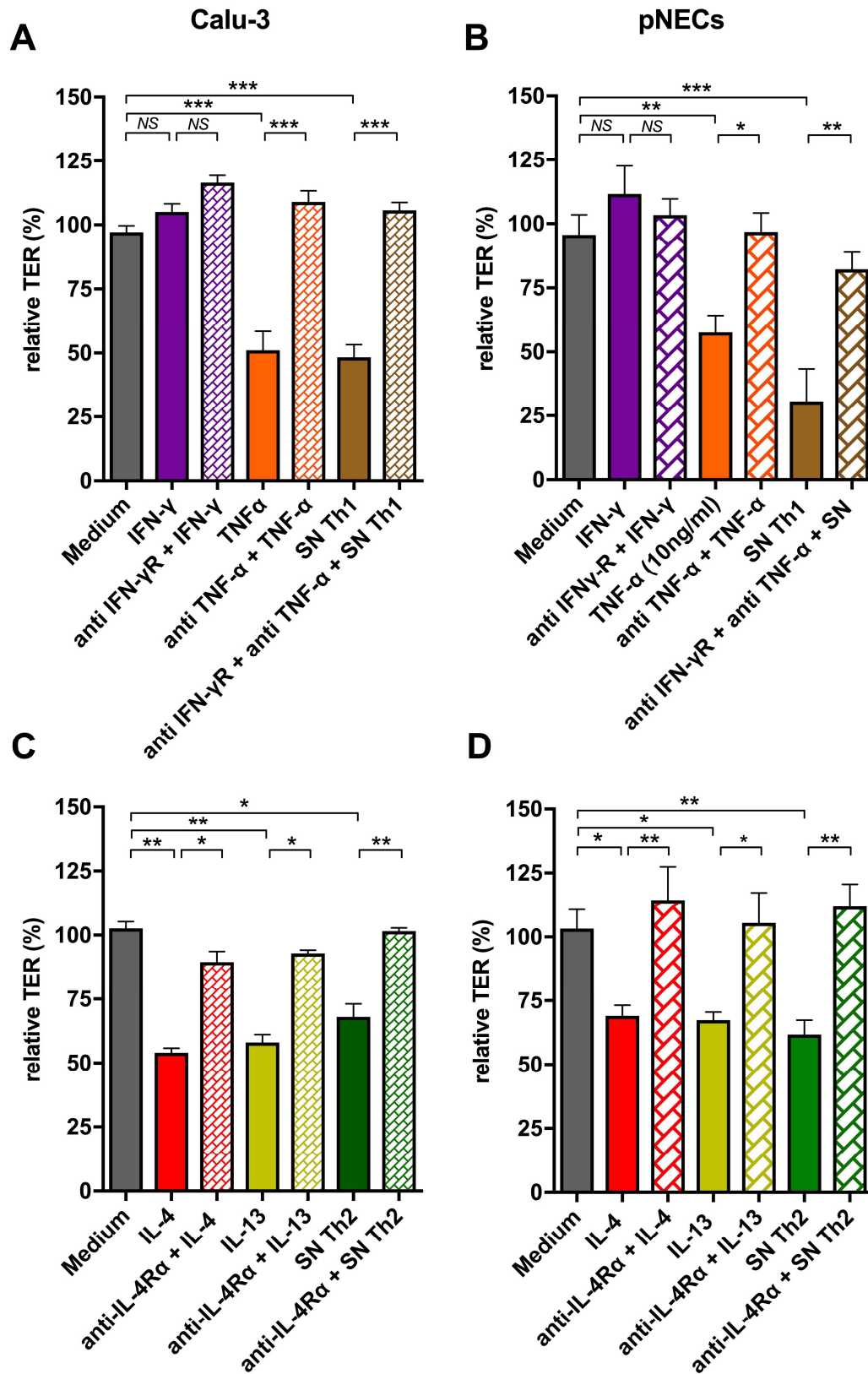


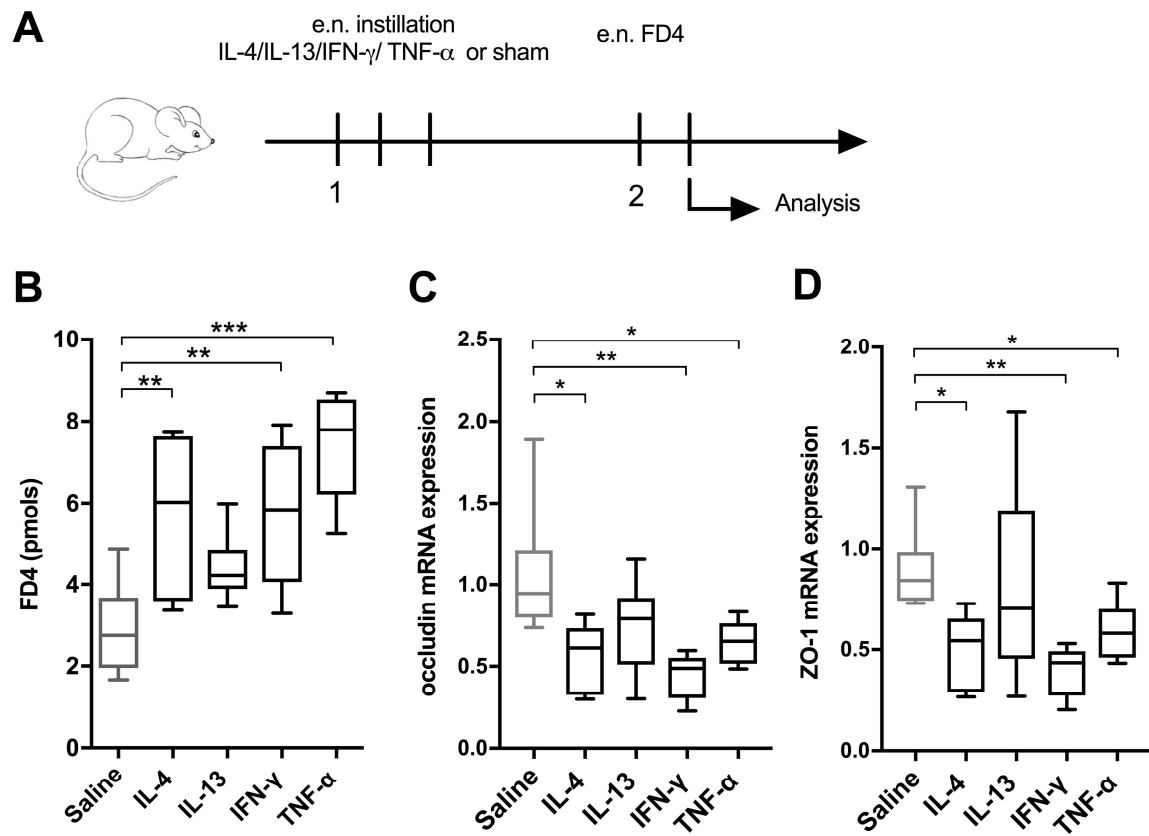
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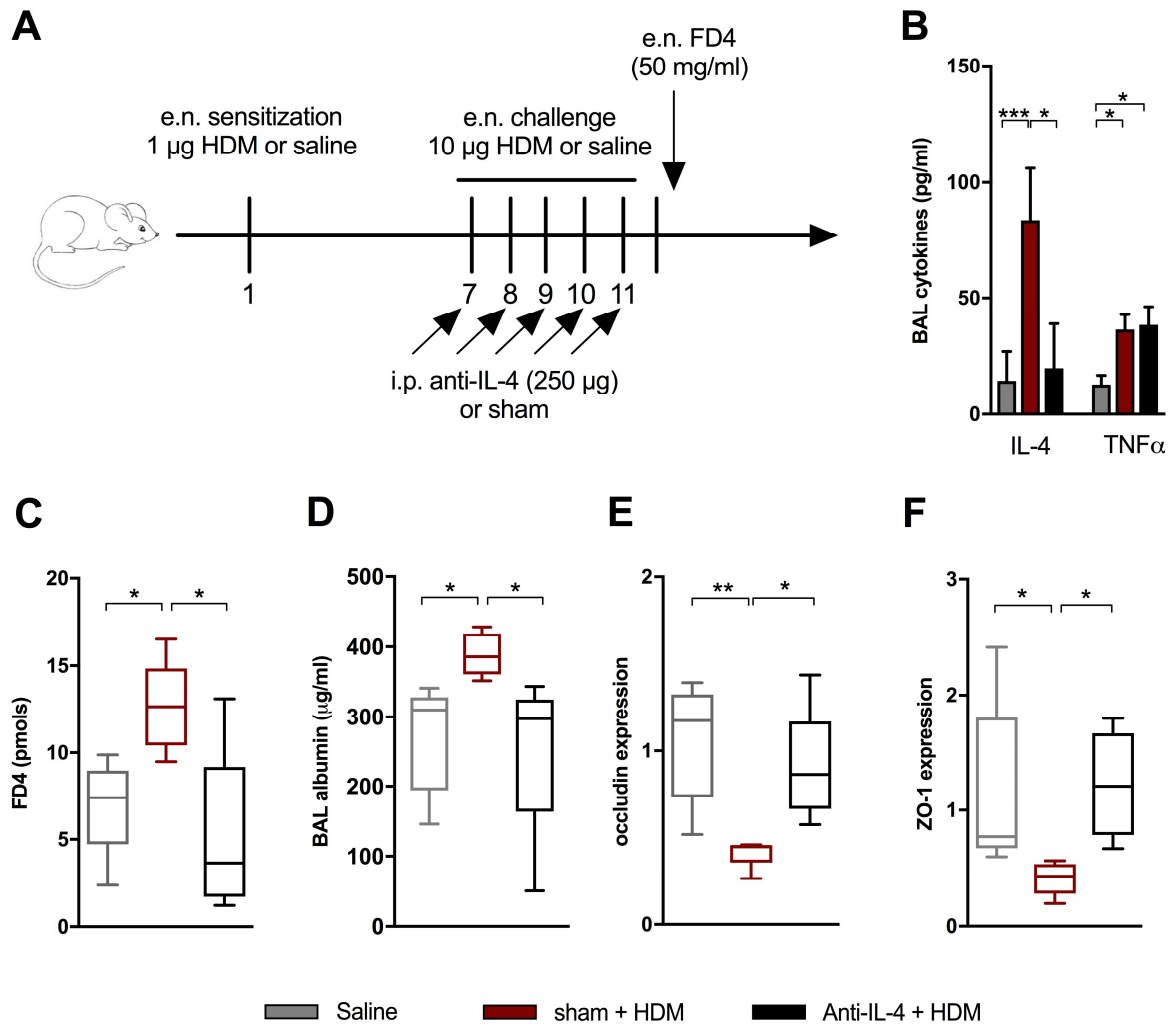












## Online Repository

### Title: Histamine and T helper cytokine driven epithelial barrier dysfunction in allergic rhinitis

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## Methods

### Isolation of naïve CD4<sup>+</sup> T cells and T<sub>H</sub>1/T<sub>H</sub>2 polarization *in vitro*

PBMCs were purified using Lymphoprep density centrifugation. The cells were washed twice with PBS. Human naïve CD4<sup>+</sup> T cells (“CD4<sup>+</sup>”) were isolated using the MagniSort Human CD4<sup>+</sup> naïve T cell enrichment kit (eBioscience) according to the manufacturer's instructions. Purity of CD4<sup>+</sup>CD45RO<sup>-</sup>CD45RA<sup>+</sup> T cells was > 97%. The purified CD4<sup>+</sup> T cells, were cultured at a density of 500.000 cells/ml at 37°C in pre-coated anti-CD3 (1 µg/ml, UCTH1) 48-well plates in RPMI 1640, supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, 1 mM l-glutamine and 10% FCS. For Th1 polarization *in vitro*, CD4<sup>+</sup> T cells were cultured in the presence of IL-12 (10 ng/ml), IL-2 (50 U/ml), anti-IL-4 (5 µg/ml) and anti-CD28 (1 µg/ml, CD28.2). For Th2 polarization *in vitro*, IL-4 (25 ng/ml), IL-2 (50 U/ml), anti-IFN-γ (5 µg/ml) anti-IL-12 (5 µg/ml) and anti-CD28 (1 µg/ml, CD28.2) were added. Th1/Th2 differentiation was evaluated by measuring the cytokine profile with ELISA. T cell cultures were stimulated with PMA (25 ng/ml) and ionomycin (1 µg/ml) for 6 hours. Afterwards, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and TNFα release by Th1 and Th2 cells was measured with ELISA (Figure E4). In parallel, for epithelial stimulation experiments, the supernatant (SN) of Th1 and Th2 cell cultures were collected and stored at – 80 °C for further analysis.

### Trans-epithelial electrical resistance measurements

Trans-epithelial electrical resistance (TER) of epithelial cell monolayers was measured using an EVOM/EndOhm (WPI Inc, Sarasota, USA). To eliminate the influence of temperature changes, TER measurements were performed within 5 minutes after taking the culture plates out of the incubator. Within this time frame, a relative stable TER were present. Before each measurement, electrodes were equilibrated and sterilized according to the manufacturer's recommendations. Two

hundred microliters of culture medium was added in the upper compartment of the transwell insert. The electrical resistance of a blank (transwell insert without cells) was measured in parallel. To obtain the sample resistance, the blank value was subtracted from the total resistance of the sample. The final unit area resistance ( $\Omega \times \text{cm}^2$ ) was calculated by multiplying the sample resistance by the effective area of the membrane ( $0.33 \text{ cm}^2$  for 24-well transwell inserts).

## ELISA

Cytokines IL-4 and IL-13 were measured in nasal secretions of AR, IR and healthy controls by sandwich ELISA. Capture monoclonal antibodies used were rat anti-human IL-4 (554515), rat anti-human IL-13 (554570). Biotinylated detection antibodies were anti-IL-4 (554483) and anti-IL-13 (555054). rhIL-4 and rhIL-13 were used as standard. All products were purchased from BD Pharmingen TM, BD Bioscience. Histamine and Substance P were measured in the nasal fluids according to the manufacture's protocols (LSBio, Seattle, WA, USA and Cayman chemicals, Michigan, USA respectively). The supernatants of activated Th1 and Th2 cells were assessed for IL-4, IL-5, IL-6, IL-10, IL-13, IFN- $\gamma$  and TNF $\alpha$ .

In murine experiments, IL-4 and TNF $\alpha$  were determined in BAL fluid with sandwich ELISA. Levels of BAL albumin were determined according to the manufacturer's protocol (Abcam, ab108792).

## RT-qPCR for the different genes

The primer and probe sequences for the specific genes were developed in the laboratory of Clinical Immunology using Primer Express (Applied Biosystems part of Thermo-Fisher Scientific). RT-qPCR was performed in an CFX Connect (Bio-Rad Laboratories Inc, California, USA) for all genes with specific Taqman probes and primers, and using Platinum® Quantitative PCR SuperMix-UDG w/ROX (Invitrogen part of Thermo Fisher Scientific, Merelbeke, Belgium).

Moreover, all probes are 5'FAM3'TAMRA labeled. Sequences for the probes and primers can be found in table E2.

### **Immunofluorescence staining of TJs**

Nasal biopsy specimens of healthy controls, AR and IR patients were stored in 4% paraformaldehyde (Fluka). Paraffin embedded tissue slides (5 µm) were subjected to antigen retrieval in citrate buffer, pH 6 (Fluka). Antibodies used for immunofluorescence: anti-occludin (rabbit, polyclonal, 1/100, abcam, 31721), anti-ZO-1 (rabbit, polyclonal, 1/100, Invitrogen, 61-7300), secondary antibody goat anti-rabbit AF488 (1/2000, Invitrogen). After staining, tissues were mounted with DAPI containing mounting media. Stained slides were stored at -20°C in the dark. Confocal images were taken using a Leica TCS SPE confocal microscope (Leica Microsystems, Heerbrugg, Switzerland).

### **Reuse of previously published material**

Parts of figure 1 have already been published previously<sup>1</sup>. The data obtained in controls and AR patients in figure 1 are reused to demonstrate the role of the epithelial barrier in idiopathic rhinitis patients. All the other data in this manuscript are uniquely for this study and are not presented somewhere else.

**References**

E1. Steelant B, Farre R, Wawrzyniak P, Belmans J, Dekimpe E, Vanheel H, et al. Impaired barrier function in patients with house dust mite-induced allergic rhinitis is accompanied by decreased occludin and zonula occludens-1 expression. *J Allergy Clin Immunol.* 2016;137(4):1043-53 e1-5.

**Figure Legends**

**Fig. E1: Effect of allergic rhinitis nasal secretions on TER of Calu-3 epithelial cell cultures at ALI.** Trans-epithelial electrical resistance (TER) was measured of Calu-3 epithelial cell cultures after stimulation with nasal secretions from allergic rhinitis (AR) patients. N = 5. Paired t-test, \*  $P < .05$ ; \*\*  $P < .01$ .

**Fig. E2. Histamine did not decrease TER after 24 hour stimulation of Calu-3 epithelial cell cultures at ALI.** Data presented as mean  $\pm$  SD.

**Fig. E3. Effect of pretreatment with anti-IL4R $\alpha$  monoclonal antibody on the decrease of Calu-3 epithelial cell integrity induced by AR nasal secretions.** Calu-3 epithelial cell cultures were pretreated for 2 hours with 2  $\mu$ g/ml anti-IL-4R $\alpha$  monoclonal antibody before stimulation with nasal secretions of allergic rhinitis (AR) patients. N = 5/group. Data presented as mean  $\pm$  SD. Two-way ANOVA with post-hoc analysis, \*\*\*  $P < .001$ .

**Fig. E4. Cytokines secretion by polarized Th1 and Th2 cell cultures *in vitro*.** Naïve CD4<sup>+</sup> T cells were polarized *in vitro* for 3 weeks into Th1 or Th2 cells. T cell cultures were stimulated for 6 hours with PMA (25 ng/ml) and ionomycin (1  $\mu$ g/ml). Supernatant was collected and cytokine profile was measured with ELISA. Data are presented from 3 independent experiments, using cells from the same donor. Data is shown as mean  $\pm$  SEM.

**Fig. E5. Effect of anti-TNF $\alpha$  treatment in a mouse model of HDM induced allergic airway inflammation.** **(A)** Mouse model. Mice were sensitized with 1  $\mu$ g HDM extract or saline at day 1. One week later (day 7-11), mice were challenged endonasally with 10  $\mu$ g HDM extract or saline. One hour before each challenge, anti-TNF $\alpha$  monoclonal antibody or vehicle was given intraperitoneally. One hour after the last challenge, 20  $\mu$ l FD4 (50 mg/ml) was applied followed one hour later with the sacrifice of the mice. **(B)** TNF $\alpha$  and IL-4 levels in BAL fluid. **(C-D)** Effect of anti-TNF $\alpha$  treatment on FD4 permeability and albumin leakage to BAL fluid in the lungs. **(E-F)** mRNA expression of occludin and ZO-1 in nasal mucosa. Relative expression versus the housekeeping B-actin and beta-2-microglobulin. N = 5 mice/group. Data presented as median and interquartile range. One-Way ANOVA with post-hoc analysis; \* P < .05, \*\* P < .01.

## Tables

**Table E1:** Patient characteristics

|                           | Controls   | AR         | IR         |
|---------------------------|------------|------------|------------|
| <b>n</b>                  | 10         | 9          | 6          |
| <b>Age (years)</b>        | 32 (28-36) | 25 (24-30) | 43 (35-48) |
| <b>Male (n)</b>           | 3          | 2          | 3          |
| <b>Smoking (n)</b>        | 0          | 0          | 0          |
| <b>INS usage (n)</b>      | 0          | 0          | 0          |
| <b>Asthma (n)</b>         | 0          | 0          | 0          |
| <b>HDM allergic (n)</b>   | 0          | 9          | 0          |
| <b>Monosensitized (n)</b> |            | 3          |            |
| <b>Polysensitized (n)</b> |            | 6          |            |

144 **Table E2:** Primer and probe sequences used for RT-qPCR

|                                 | Forward primer                    | Reverse primer                  | Probe                                         |
|---------------------------------|-----------------------------------|---------------------------------|-----------------------------------------------|
| <b><u>Human</u></b>             |                                   |                                 |                                               |
| <b>B-Actin</b>                  | gga cat ccg caa aga cct gt        | ctc agg agg agc aat gat ctt gat | ctg gcg gca cca cca tgt acc ct                |
| <b>Beta-2<br/>microglobulin</b> | ctg aag ctg aca gca ttc gg        | ctt tgg agt acg ctg gat agc c   | aga tgt ctc gct ccg tgg cct tag c             |
| <b>Claudin-1</b>                | cca gtc aat gcc agg tac gaa t     | ata ggg cct tgg tgt tgg gt      | tca ggc tct ctt cac tgg ctg ggc               |
| <b>Claudin-4</b>                | ggg ctg ctc aca ctt gct gg        | gac gga ctt aac gtt cgc aga g   | tgg ctt tat ctc ctg act cac ggt gca           |
| <b>Occludin</b>                 | cca atg tcg agg agt ggg tta a     | ttg cca ttg gaa gag tat gcc     | ctg cag gca cac agg acg tgc c                 |
| <b>ZO-1</b>                     | gtg cct aaa gct att cct gtg agt c | cta tgg aac tca gca cgc cc      | tgg cca cag ccc gag gca tat t                 |
| <b><u>Murine</u></b>            |                                   |                                 |                                               |
| <b>B-Actin</b>                  | aga ggg aaa tcg tgc gtg ac        | caa tag tga cct gcg cgt         | cac tgc cgc atc ctc ttc ctc cc                |
| <b>Beta-2<br/>microglobulin</b> | cca ctg aga ctg ata cat acg cct   | gat cac atg tct cga tcc cag tag | taa gca tgc cag tat ggc cga gcc               |
| <b>PPIA</b>                     | gcc gcg tct cct tcg ag            | gta aag tca cca ccc tgg cac     | atg cag aca aag ttc caa aga cag<br>cag aaa    |
| <b>GAPDH</b>                    | tca cca cca tgg aga agg c         | gct aag cag ttg gtg gtg ca      | atg ccc cca tgt ttg tga tgg gtg t             |
| <b>Occludin</b>                 | aca aga gaa att ttg atg cag gtc t | cat cag cag cag cca tgt act c   | aag agc tta cag gca gaa cta gac<br>gac gtc aa |
| <b>ZO-1</b>                     | ttc gag aag ctg gat tcc taa gac   | cag tcc cag cat ctc gtg g       | cat ctt tgg acc aat agc tga tgt tgt<br>tgc ca |

145 All probes are labeled with 5'FAM3'TAMRA. ZO-1: zonula occludens-1

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