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

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Abstract: Background Allergic rhinitis (AR) is characterized by mucosal inflammation, driven by activated immune cells. Mast cells and TH2 cells might decrease epithelial barrier integrity in AR, maintaining a leaky epithelial barrier. Objective We sought to investigate the role of histamine and TH2 cells in driving epithelial barrier dysfunction in AR. Methods Air-liquid interface cultures of primary nasal epithelial cells were used to measure transepithelial electrical resistance, paracellular flux of fluorescein isothiocyanate-dextran 4 kDa, and mRNA expression of tight junctions. Nasal secretions were collected from healthy control subjects, AR patients, and idiopathic rhinitis patients and were tested in vitro. In addition, the effect of activated TH1 and TH2 cells, mast cells, and neurons was tested in vitro. The effect of IL-4, IL-13, IFN- , and TNF- on mucosal permeability was tested in vivo. Results Histamine as well as nasal secretions of AR but not idiopathic rhinitis 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 TH1 and TH2 cells impaired epithelial integrity, while treatment with anti-TNF- or anti-IL-4R monoclonal antibodies restored the TH1- and TH2-induced epithelial barrier dysfunction, respectively. IL-4, IFN- , and TNF- 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. Conclusions Our data indicate a key role for allergic inflammatory mediators in modulating nasal epithelial barrier integrity in the pathophysiology in AR.

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

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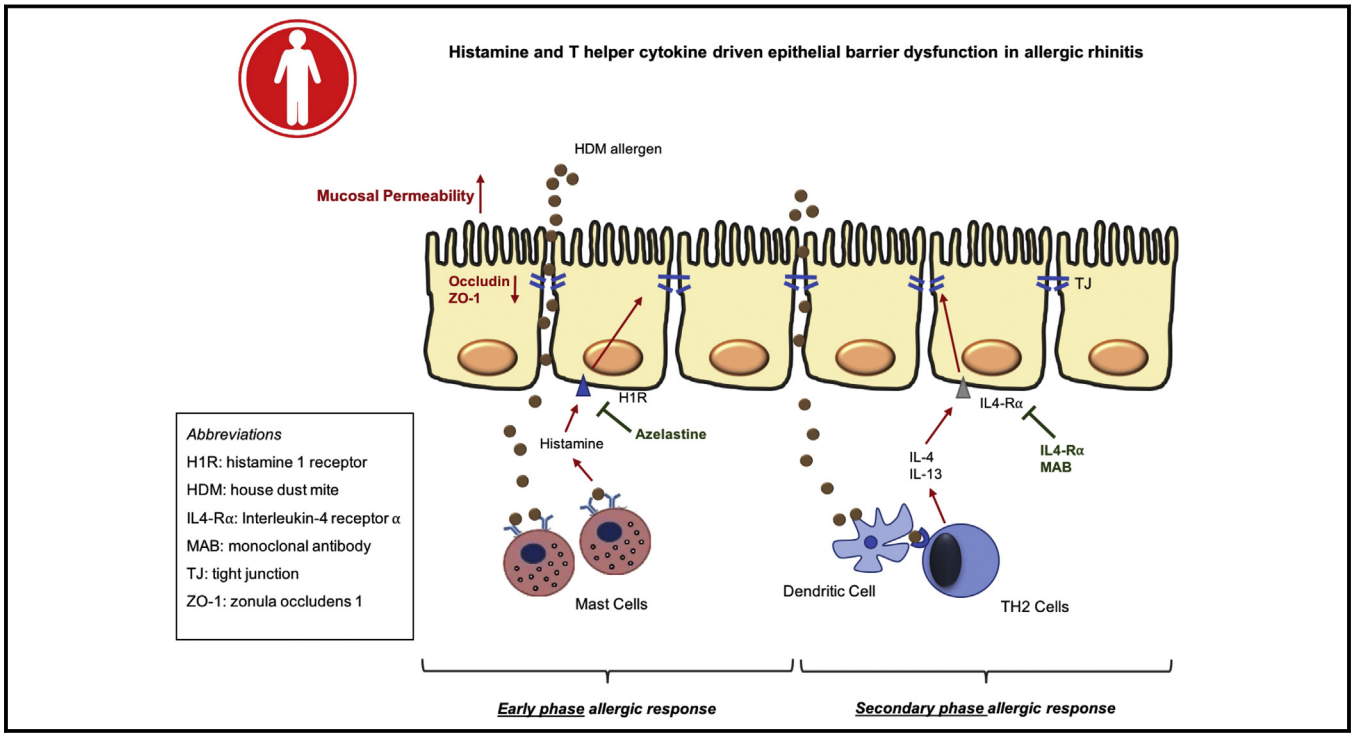
## GRAPHICAL ABSTRACT

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**Background: Allergic rhinitis (AR) is characterized by mucosal inflammation, driven by activated immune cells. Mast cells and $T_H2$ cells might decrease epithelial barrier integrity in AR, maintaining a leaky epithelial barrier.**

**Objective: We sought to investigate the role of histamine and $T_H2$ cells in driving epithelial barrier dysfunction in AR.**

**Methods: Air-liquid interface cultures of primary nasal epithelial cells were used to measure transepithelial electrical resistance, paracellular flux of fluorescein isothiocyanate-dextran 4 kDa, and mRNA expression of tight junctions. Nasal secretions were collected from healthy control subjects, AR patients, and idiopathic rhinitis patients and were tested *in vitro*. In addition, the effect of activated $T_H1$ and $T_H2$ cells, mast cells, and neurons was tested *in vitro*. The effect of IL-4, IL-13, IFN-γ, and TNF-α on mucosal permeability was tested *in vivo*.**

**Results: Histamine as well as nasal secretions of AR but not idiopathic rhinitis 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 $T_H1$ and $T_H2$ cells impaired epithelial integrity, while treatment with anti-TNF-α or anti-IL-4Rα monoclonal antibodies restored the $T_H1$- and $T_H2$-induced epithelial barrier dysfunction, respectively. IL-4, IFN-γ, and TNF-α 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.**

**Conclusions: Our data indicate a key role for allergic inflammatory mediators in modulating nasal epithelial barrier integrity in the pathophysiology in AR. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)**

***Key words:*** *Allergic rhinitis, idiopathic rhinitis, tight junctions, histamine, $T_H2$ cells, primary nasal epithelial cells*

*Abbreviations used*
ALI: Air-liquid interface
AR: Allergic rhinitis
BAL: Bronchoalveolar lavage
FD4: Fluorescein isothiocyanate–dextran 4 kDa
HDM: House dust mite
IR: Idiopathic rhinitis
KU: Katholieke Universiteit
pNEC: Primary nasal epithelial cell
TER: Transepithelial electrical resistance
TGN: Trigeminal neuron
TJ: Tight junction
ZO: Zonula occludens

The nasal epithelium is of vital importance in host defense because it not only provides a physical barrier between the environment and the submucosal region, but it also contributes to the induction of an appropriate immune response toward pathogens, allergens, and noxious stimuli that overcome the mucosal barrier.[1] 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.[2] 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.[1,2]

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,[3] asthma,[4] chronic rhinosinusitis,[5] and allergic rhinitis (AR).[6,7] AR is characterized by symptoms such as nasal obstruction, rhinorrhea, itchy nose, and/or sneezing on contact with inhaled allergens in sensitized individuals.[8,9] The inflammatory cascade in AR is initiated by an IgE-dependent mast cell degranulation with release of histamine among other mediators and is further orchestrated by $T_H2$ cytokines IL-4, IL-5, and/or IL-13.[10-12] 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.[13,14]

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.[15,16] IR is assumed to be mediated by dysfunctional sensory C-fibers in the nasal mucosa, rather than by $T_H2$ inflammation.[17-19] At present, the contribution of a defective epithelial barrier as an underlying part in the pathology of AR and IR has not been investigated or 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 patients with AR and IR and from healthy control subjects 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 $T_H2$ 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 University Hospitals Leuven and was registered at clinicaltrials.gov (NCT02461797, NCT02288156). Patient characteristics are depicted in Table E1 in this article's Online Repository at www.jacionline.org. Allergic status was determined via skin prick testing for the most frequent inhalant allergens in Belgium—birch pollen, grass pollen, cat dander, dog dander, *Dermatophagoides pteronyssinus* (Stallergenes, Antony, France), rabbit and spores of *Alternaria*, *Aspergillus* (HAL Allergy, Leiden, The Netherlands)—as described previously.[20] All study participants were

asked to score their rhinitis symptoms on a visual analog scale (score 0-10). The major nasal symptom was selected based on the highest visual analog scale score.

## Nasal secretions

Nasal secretions were collected from each study participant. A nasal sponge (Ivalon Surgical products, San Diego, Calif) 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 naive $CD4^+$ T cells and $T_H1/T_H2$ polarization *in vitro*

Details on the isolation and polarization of $T_H1/T_H2$ cells can be found in the Methods section of this article's Online Repository at www.jacionline.org.

## Isolation and culture of mast cells and eosinophils and murine TGN

The method for isolation and culturing mast cells,[21] eosinophils,[22] and murine trigeminal neurons (TGNs)[23] has been described previously. Supernatant of the different activated cells was stored at −20°C until further analysis.

## ALI cultures of pNECs and Calu-3 epithelial cells

Inferior turbinates were used for isolation of pNECs from nonallergic, nonasthmatic healthy control subjects. A highly purified pNEC population was obtained as reported previously.[6] Isolated pNECs or Calu-3 epithelial cells were seeded on 0.4-μm × 0.33-$cm^2$ polyester Transwell inserts (Greiner Bio-One, Vilvoorde, Belgium) at a density of 100,000 cells per Transwell. The culture medium used for pNECs was Dulbecco modified Eagle medium/F12, supplemented with antibiotics and Ultroser G (2%) (Pall Life Sciences, Tienen, Belgium), Calu-3 culture medium was Eagle minimal essential medium, 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 air-liquid interface (ALI). At day 21 in ALI, epithelial cell cultures were used for *in vitro* stimulation studies.

## TER measurement

Epithelial integrity of ALI cultures was evaluated by transepithelial electrical resistance (TER) measurements using an EVOM/Endohm (World Precision Instruments, Sarasota, Fla) as described in the Methods section of this article's Online Repository. Wells not building up sufficiently (TER < 200 Ω × $cm^2$) 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 $T_H1/T_H2$ 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 Healthcare, Incheon, 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 and IR patients and healthy control subjects using different doses of histamine (0.01-1 mmol/L; Sigma-Aldrich) or substance P (10-100 pmol/L; R&D Systems). 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) before adding the nasal secretions. TER was measured as a function of time and is expressed as relative change compared with 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 transtissue resistance *ex vivo* as described previously.[6]

## RT-quantitative PCR for TJ genes

The methods for mRNA isolation and RT-quantitative PCR have been reported previously.[18] Detailed information and primer sequences can be found in the Methods section and Table E2 in this article's Online Repository at www.jacionline.org.

## 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 Katholieke Universiteit (KU) Leuven (P103/2013). BALB/c mice were 3 times endonasally instilled with IL-4, IL-13, IFN-γ, TNF-α (all 250 ng), or saline at 1-hour intervals. One hour after the last endonasal instillation, 20 μL fluorescein isothiocyanate–dextran 4 kDa (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 HDM extract (1 μg) (Greer Laboratories, Lenoir, NC) or 50 μL saline at day 1. From days 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-TNF-α (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; BMG Labtech, Cary, NC). Albumin leakage from the lungs to bronchoalveolar lavage (BAL) fluid, as a surrogate marker for lung permeability, was measured with ELISA according to the manufacturer's protocol (Abcam, Cambridge, United Kingdom). 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, Calif). Differences between 2 groups were analyzed using 2-tailed unpaired *t*-test or Mann-Whitney *U* test, depending on normality. Data are presented as mean ± SD or median (interquartile range). 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 transtissue 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, transtissue resistance and FD4 permeability were measured on nasal mucosal biopsies from healthy subjects and IR

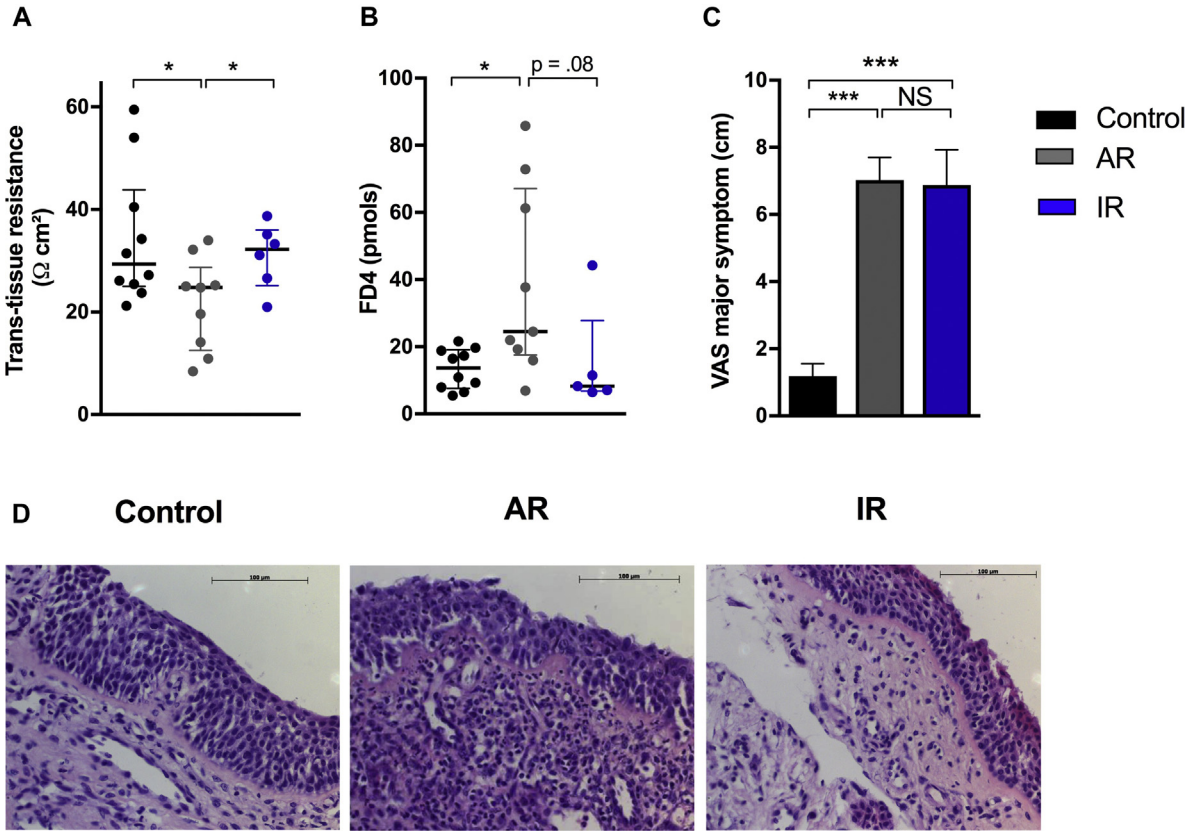


**FIG 1.** Epithelial barrier function in patients with AR and IR. **A,** Transtissue resistance measured with Ussing chambers on nasal explants from AR and IR patients and control subjects. **B,** FD4 permeability. **C,** Visual analog scale (VAS) scores for major nasal symptom. **D,** Hematoxylin and eosin staining of nasal mucosal biopsies from healthy control subjects and patients with AR and IR. Bars = 100 μm. **P* < .05 and ****P* < .001. *NS,* Not significant.

and AR patients. A reduced transtissue resistance, accompanied with increased FD4 permeability was found in mucosal explants of AR patients (Fig 1, *A* and *B*). No difference in mucosal integrity or FD4 permeability was found in mucosal explants from IR patients compared with those of healthy subjects (Fig 1, *A* and *B*). The visual analog scale scores for major symptoms were similar in IR and AR patients and significantly higher than for healthy subjects (Fig 1, *C*). 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 1, *D*).

Consistent with the absence of an impaired mucosal barrier in IR patients, no decreased mRNA expression for occludin and ZO-1 was observed compared with that of healthy subjects (Fig 2, *A*). Occludin and ZO-1 mRNA expression was significantly lower in AR patients than in healthy subjects and IR patients. Interestingly, claudin-1 expression was significantly increased in IR patients than in AR patients (Fig 2, *A*). 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 2, *B*).

## Nasal secretions of AR but not IR patients induce a rapid decrease in TER 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 and 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 3, *A*). 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 this article's Online Repository at www.jacionline.org).

We next measured the presence of inflammatory mediators in the nasal secretions. Nasal secretions of both control subjects and IR patients, compared with those of AR patients, contained low levels of histamine and type 2 cytokines IL-4 and IL-13 (Fig 3, *B-E*). The neuropeptide, substance P, was significantly increased in nasal secretions of IR patients compared with those of control subjects (Fig 3, *E*). Subsequently, we tested which inflammatory mediators in the nasal secretions of AR patients were responsible for the decreased TER during the early phase allergic immune

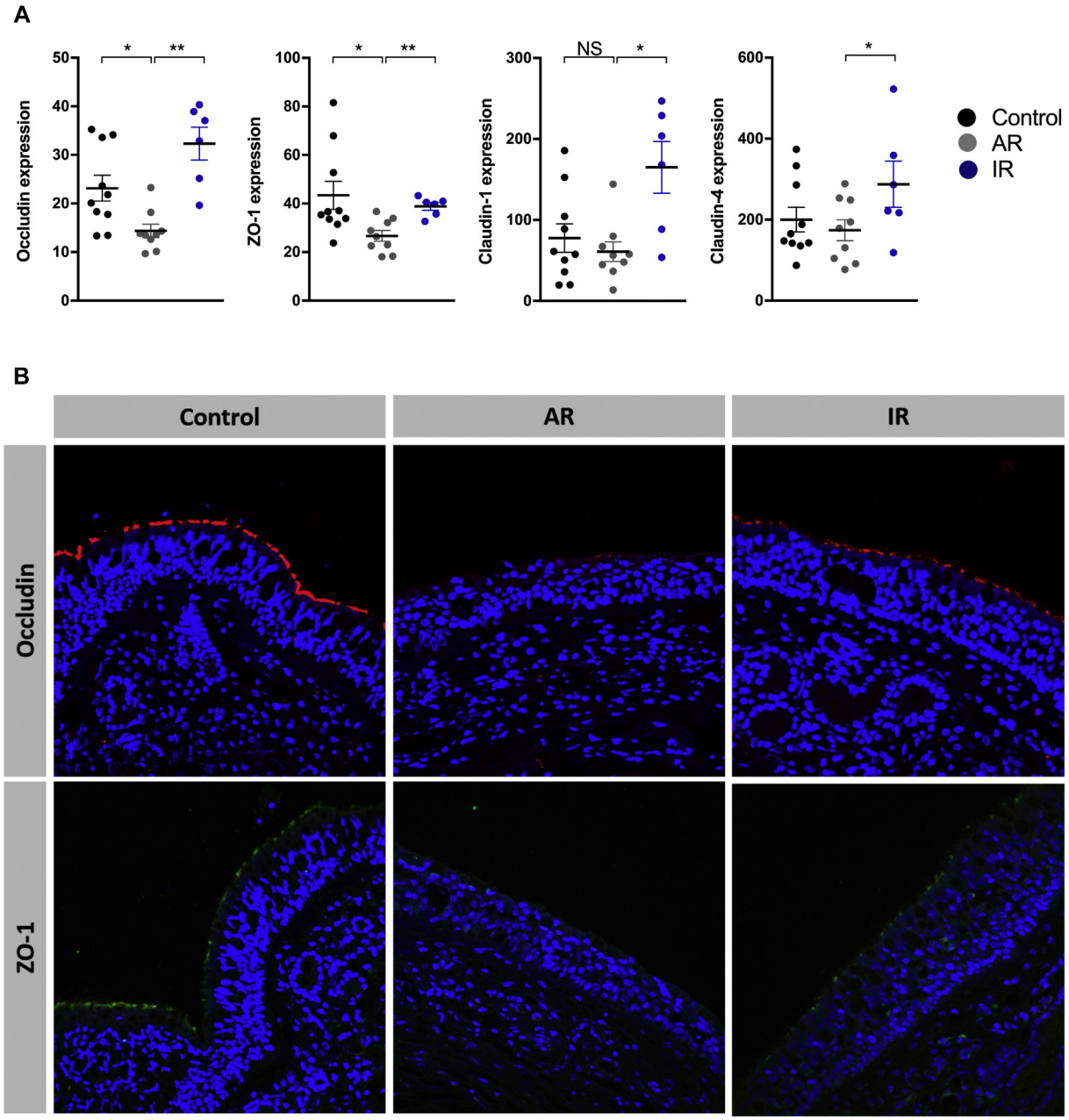


**FIG 2.** Tight junction expression in mucosal biopsies from control subjects and AR and IR patients. **A,** mRNA expression of occludin, ZO-1, and claudin-1 and -4 in patients with AR and IR and control subjects. Relative mRNA expression versus the housekeeping genes encoding β-actin and $\beta_2$ microglobulin is shown. **B,** Representative images of protein expression of occludin and ZO-1. Original magnification 40×. Data presented as medians and interquartile ranges. **P* < .05 and ***P* < .01.

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 3, *F*). The supernatant of IgE-activated mast cells showed a similarly rapid effect as that of histamine on TER, which had returned to baseline values after 24 hours (see Fig E2 in this article's Online Repository at www.jacionline.org). IL-4, IL-13, or substance P did not decrease TER of Calu-3 epithelial cell cultures during the time window representing the early phase allergic immune response (Fig 3, *G* and *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 3, *I*). Noteworthily, a 2-hour pretreatment of Calu-3 epithelial cells with anti-IL-4Rα (2 μg/mL) to antagonize the effect of IL-4 and/or IL-13 did not inhibit the decrease in TER during the early phase allergic immune response induced by the nasal secretions of AR patients (see Fig E3 in this article's Online Repository at www.jacionline.org).

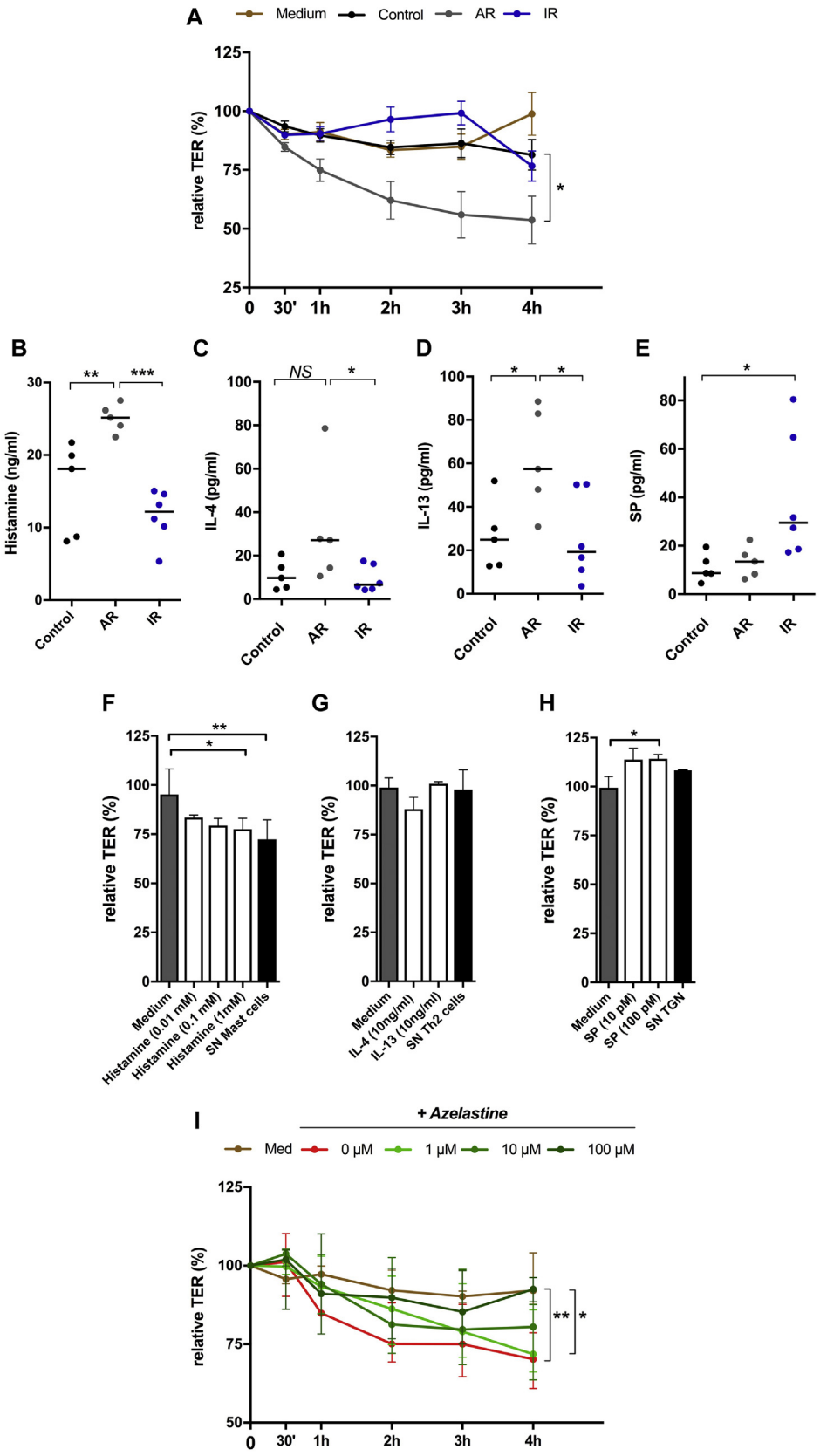


**FIG 3.** Effect of nasal fluid of AR patients on epithelial barrier function of Calu-3 cells. **A,** Effect of nasal fluid of healthy control subjects and AR and IR patients (n = 5/group). **B,** Histamine content measured in nasal fluid of healthy control subjects and patients with AR and IR. **C** and **D,** IL-4 and IL-13 concentration in nasal fluid. **E,** Substance P (SP) concentration in nasal fluid. **F-H,** 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, and ****P* < .001. *M*, Mol/L; *SN*, supernatant.

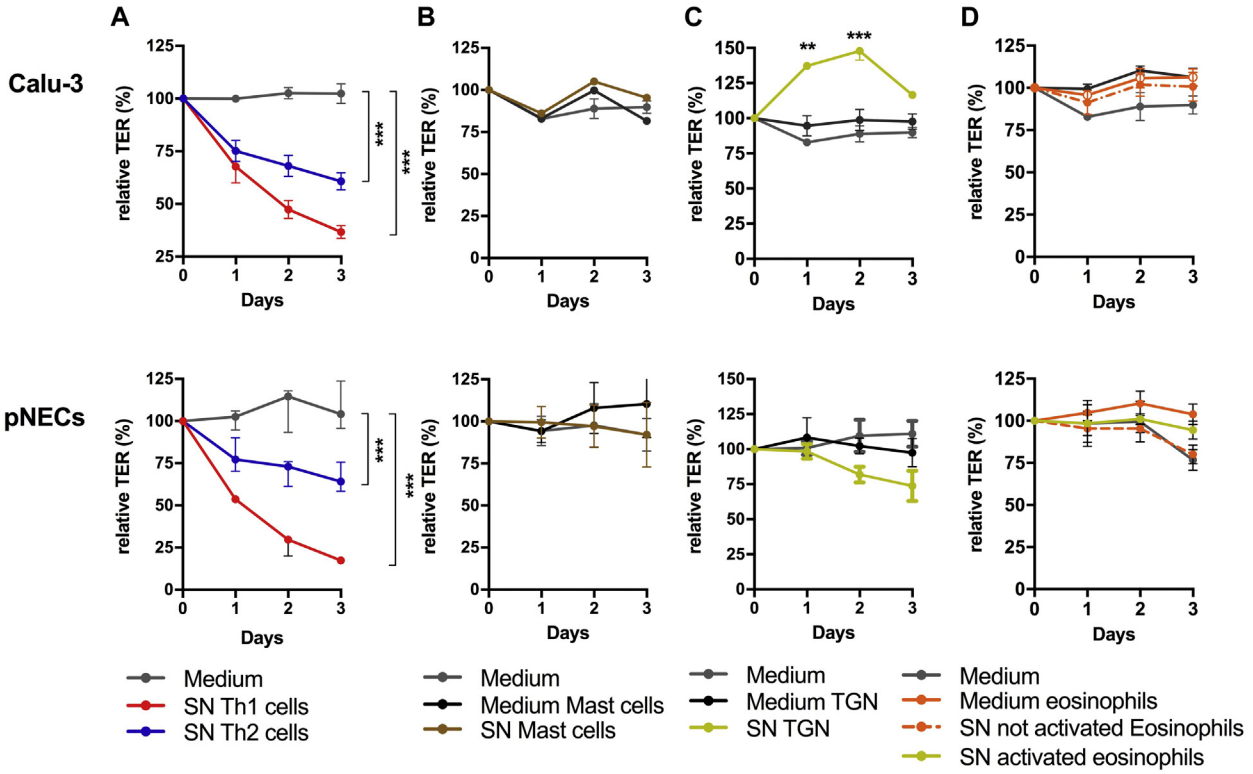


**FIG 4.** Effect of SN from activated immune cells on epithelial barrier function of Calu-3 cells and pNECs. **A**, Effect of SN from activated $T_H1$ and $T_H2$ cells. **B**, Effect of SN from IgE-activated mast cells. **C**, Effect of SN from capsaicin-activated murine TGNs. **D**, Effect of SN from C5a-activated eosinophils. n = 5 for all experiments. Data presented as medians and interquartile ranges. $^{**}P < .01$ and $^{***}P < .001$.

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 phase 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.

## $T_H1$ and $T_H2$ cytokine-mediated decrease in TER of epithelial cells *in vitro* during the delayed allergic immune response

We next studied whether other inflammatory cells can alter epithelial integrity during a time window representing the secondary phase allergic immune response. To this end, $T_H1$ and $T_H2$ cells were polarized *in vitro* from naive peripheral blood $CD4^+$ 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 supernatants of activated $T_H1$ or $T_H2$ cells decreased TER of Calu-3 epithelial cell and pNEC cultures (Fig 4, *A*). 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 4, *B-D*).

## Anti-TNF-α and anti-IL4-Rα antibody prevent the decline in TER orchestrated by activated $T_H1$ and $T_H2$ cells

Several cytokines were detected in the supernatant of activated $T_H1$ and $T_H2$ cells *in vitro* (see Fig E4 in this article's Online Repository at www.jacionline.org). To study which cytokines released by $T_H1$ and $T_H2$ cells impair epithelial barrier integrity during the secondary phase allergic immune response, recombinant cytokines were used *in vitro*. Stimulation with TNF-α (10 ng/mL) for 3 days decreased TER of Calu-3 and pNEC cultures at ALI (Fig 5), whereas IFN-γ (10 ng/mL) did not alter TER (Fig 5). In addition, $T_H2$-derived IL-4 and IL-13 (10 ng/mL) also impaired TER (Fig 5, *B*). Pretreatment of Calu-3 and pNEC cultures at ALI for 2 hours with anti-IL-4Rα (2 μg/mL) or anti-TNF-α (40 μg/mL) monoclonal antibodies prevented the cytokine-mediated barrier disruption. Likewise, pretreatment with anti-IFN-γR and anti-TNF-α monoclonal antibodies prevented the effect of supernatant from activated $T_H1$ cells, while pretreatment with anti-IL-4Rα monoclonal antibody prevented the effect of supernatant from activated $T_H2$ cells (Fig 5, *A*).

Collectively, these results indicate that both $T_H1$ and $T_H2$ cells can impair epithelial barrier function during the secondary phase allergic immune response, which is ascribed to either TNF-α or to IL-4 and IL-13, respectively.

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

As IL-4, IL-13, and TNF-α decreased epithelial barrier integrity *in vitro*, we next investigated whether the cytokine-mediated barrier disruption could be confirmed *in vivo* and whether it was associated with altered expression of occludin

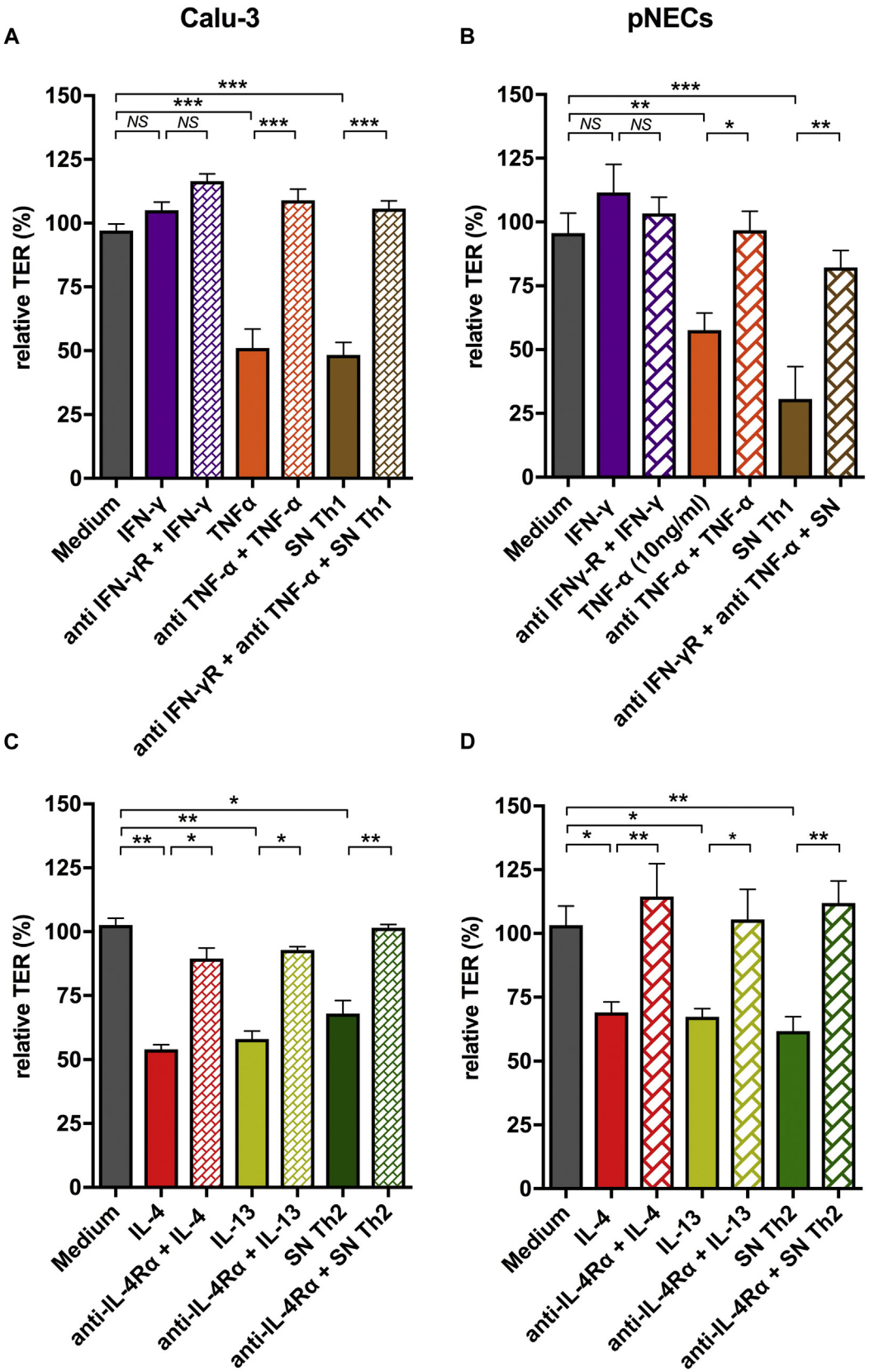


**FIG 5.** Effect of $T_H1$ and $T_H2$ cytokines on epithelial barrier function of Calu-3 cells and pNECs. **A** and **B,** Calu-3 cells and pNECs were stimulated with SN of activated $T_H1$ cells, $T_H1$ cytokines, and their receptor antagonists for 72 hours. **C** and **D,** Calu-3 cells and pNECs were stimulated with SN of activated $T_H2$ cells, $T_H2$ cytokines, and their receptor antagonists for 72 hours. n = 5 for Calu-3 cells and n = 4 for pNECs. Data presented as means ± SD. $*P < .05$, $**P < .01$, and $***P < .001$.

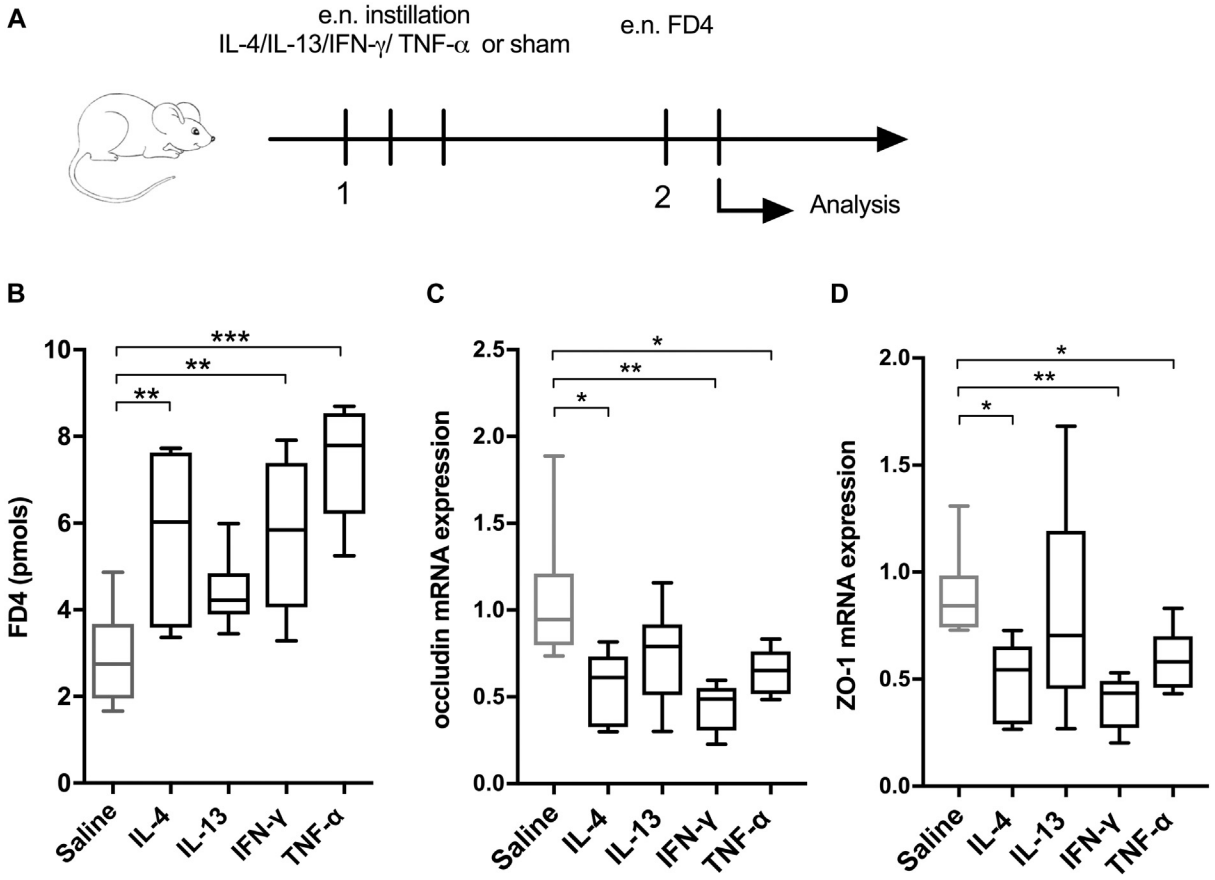


**FIG 6.** Effect of IL-4, IFN-γ, and TNF-α on the mucosal barrier in naive BALB/c mice. **A,** Study protocol. On day 1, mice received 3 nasal instillations at 1-hour intervals with IL-4, IL-13, IFN-γ, TNF-α (all 250 ng), or sham. One day after the last instillation, 20 μL FD4 (50 mg/mL) was applied endonasally (en) 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 β-actin and $\beta_2$ microglobulin. n = 5/group. *$P < .05$, **$P < .01$, and ***$P < .001$.

and ZO-1 *in vivo*. Wild-type BALB/c mice were endonasally instilled with TNF-α, IFN-γ, IL-4, or IL-13 (all 250 ng per instillation) and mucosal permeability was evaluated by using FD4 (Fig 6, *A*). Instillation with IL-4, IFN-γ, and TNF-α, compared with instillation of saline in control mice, significantly increased FD4 permeability (Fig 6, *B*). The increased FD4 mucosal permeability in IL-4-, IFN-γ-, and TNF-α-instilled mice, compared with saline instilled mice, was associated with decreased expression of occludin and ZO-1 (Fig 6, *C* and *D*). IL-13 had a modest, though nonsignificant effect on FD4 permeability and expression of occludin (Fig 6, *B* and *C*).

## Anti-IL-4 prevents induction of transmucosal FD4 permeability in a mouse model of HDM-induced airway inflammation

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

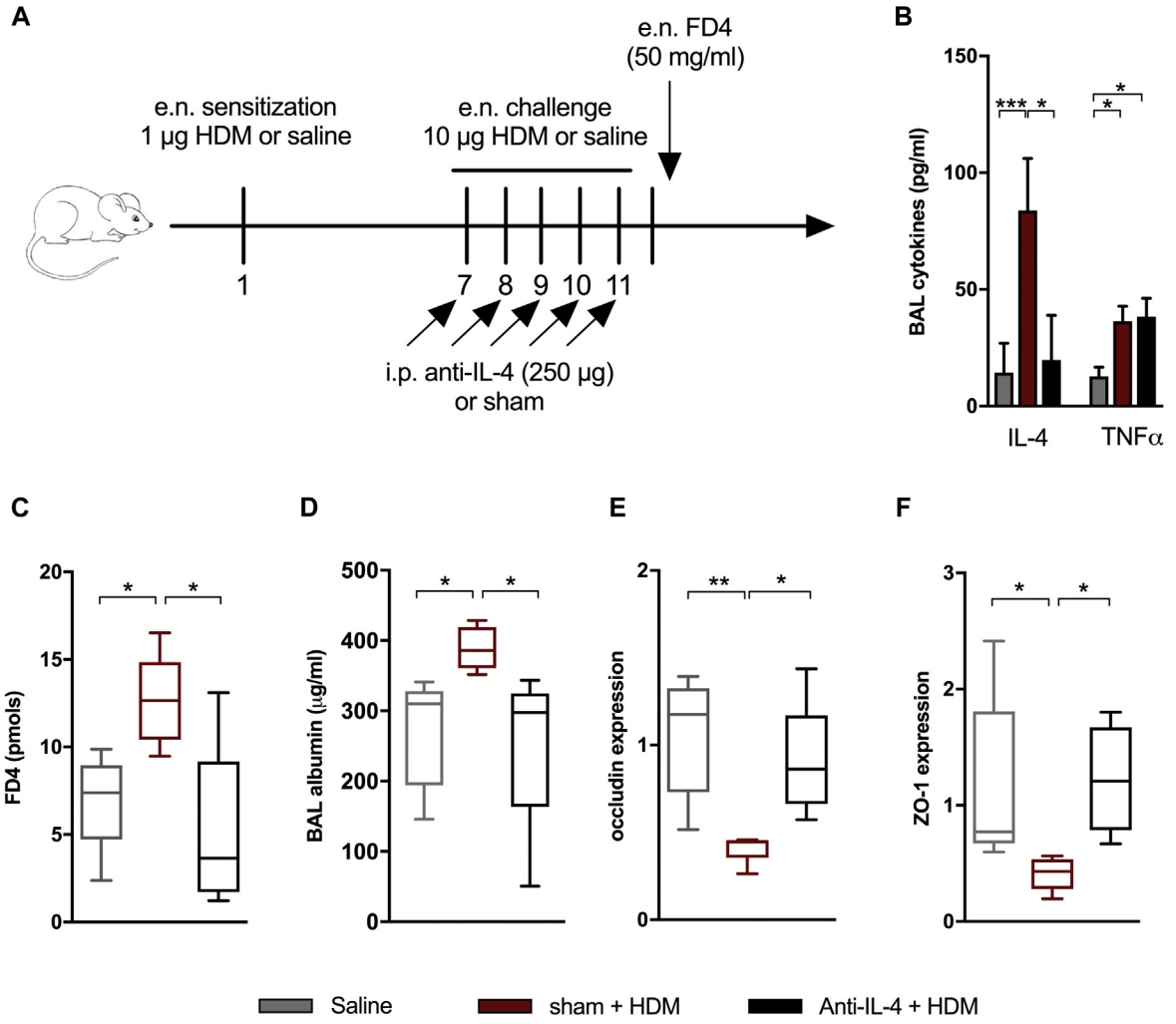


**FIG 7.** Effect of anti-IL-4 in mouse model of HDM-induced allergic airway inflammation. **A,** Mouse model. Mice were sensitized with 1 µg HDM extract or saline control at day 1. One week later (days 7-11), mice were challenged endonasally (en) with 10 µg HDM extract or saline. One hour before each challenge, anti-IL-4 monoclonal antibody or vehicle was given intraperitoneally (ip). One hour after the last challenge, 20 µL FD4 (50 mg/mL) was applied, followed 1 hour later with the sacrifice of the mice. **B,** IL-4 levels in BAL fluid. **C** and **D,** Effect of anti-IL-4 treatment on FD4 permeability and albumin leakage in the lungs. **E** and **F,** mRNA expression of occludin and ZO-1. Relative expression versus the housekeeping β-actin and $\beta_2$ microglobulin. n = 5/group. **P* < .05, ***P* < .01, and ****P* < .001.

## DISCUSSION

A defective epithelial barrier has been associated with chronic inflammatory diseases such as asthma[4] and AR,[6] 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 (Figs 1 and 2). IR is a subclassification of rhinitis characterized by symptoms such as nasal obstruction, rhinorrhea, and nasal hyperreactivity, though in the absence of mucosal inflammation.[18,24] Interestingly, claudin-1 and claudin-4 expression was elevated in IR patients compared with in AR patients. Claudins can be divided into 2 broad categories: sealing and pore-forming claudins.[25] Claudin-1 and claudin-4 are classified as sealing claudins,[26,27] 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. Hence, local release of neuropeptides such as substance P by afferent nerves are assumed to induce symptoms in IR.[28]

Because AR patients show a defective epithelial barrier, we searched for the responsible mediators in nasal secretions from AR and IR patients 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 phase 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[29-31] and/or adherence junctions.[32] Histamine exerts its effect via the histamine-1 receptor,[33] which is upregulated in AR.[34,35] Blocking this receptor with azelastine prevented the decline in TER mediated by AR nasal secretions pointing toward a crucial role for histamine in rapidly decreasing epithelial barrier function during the early phase allergic immune response. Besides increased histamine levels, $T_H2$ 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-4Rα 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.[18,36] 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 et al.[37] in corneal epithelial cells. 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 phase 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 secondary phase allergic immune response. The supernatants of activated $T_H1$ and $T_H2$ cells, mast cells, eosinophils, and murine TGNs 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, 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 this needs further confirmation. Eosinophils are found in close proximity with epithelial cells in chronic rhinosinusitis[38] and are implicated in disease development and progression by increasing epithelial permeability.[39] We did not find any effect of eosinophils on epithelial integrity, which might be due to differences in activation methods of eosinophils and the subsequently released mediators. In agreement with previous publications, the supernatants of activated $T_H1$ and $T_H2$ cells significantly decreased TER in function of time of Calu-3 and pNEC cultures.[40,41]

The secondary decline in TER during the secondary phase allergic immune response, mediated by activated $T_H1$ cells is linked to TNF-α. TNF-α is an important cytokine in the pathology of inflammatory bowel disease[42] and rheumatoid arthritis,[43] though its role in AR is not yet extensively explored. TNF-α is increased in the airways of corticosteroid-refractory asthmatic patients[44-46] and patients with chronic rhinosinusitis (unpublished data). TNF-α decreases epithelial integrity of Calu-3 and pNEC cultures as demonstrated previously on airway epithelial cells[47] and intestinal epithelial cells[48-50] (Fig 5). Antagonizing TNF-α prevented loss-of-epithelial barrier function *in vitro*. The observation that blocking TNF-α protects epithelial barriers might help us understand why patients with Crohn disease,[51] rheumatoid disease,[43] or inflammatory bowel disease[52] treated with antagonizing TNF-α have improved disease activity scores.

IL-4 and IL-13 are the main cytokines linked with decreased epithelial barrier integrity mediated by activated $T_H2$ cells.[6,40,53,54] Blocking IL-4 and IL-13 signaling with anti-IL-4Rα antibodies restored epithelial barrier function during the secondary phase allergic immune response (Fig 5). Interfering with IL-4/IL-13 is a novel biological treatment for asthma[55] and is currently being evaluated in phase III clinical trials for chronic rhinosinusitis with nasal polyps.[56] Anti-IL-4Rα antibody treatment ameliorates symptom control in patients with asthma[57] and in patients with chronic rhinosinusitis.[56] Our data add a new mode of action for anti-IL4Rα treatment because this molecule not only inhibits type 2 inflammatory pathways but also prevents cytokine-mediated barrier disruption. The barrier modulating capacity of $T_H1$- and $T_H2$-derived cytokines is also demonstrated *in vivo* (Fig 6). Endonasal instillation of IL-4, IL-13, IFN-γ, or TNF-α 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-α only partially restored mucosal barrier integrity in HDM-challenged mice because 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-α. Indeed, HDM-challenged mice pretreated with anti-TNF-α still showed elevated levels of IL-4 in BAL fluid, which might explain the increased mucosal permeability.

In summary, we have identified a pathogenic role for histamine and $T_H2$ cells in decreasing mucosal barrier function in AR. Our findings suggest that histamine initiates loss-of-epithelial barrier function during the early phase allergic immune response, which during the secondary phase allergic immune response is maintained by T-cell inflammation. Targeting histamine via antagonizing H1-receptors or antagonizing $T_H1$ cell-derived TNF-α and/or $T_H2$ cell-derived IL-4 and IL-13 prevented the defective mucosal barrier dysfunction *in vitro* and *in vivo*.

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**Key messages**

- **Histamine initiates and T-cell inflammation maintains defective epithelial barrier in AR**
- **Impaired barrier function is not found in IR**

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# METHODS

## Isolation of naive $CD4^+$ T cells and $T_H1/T_H2$ polarization *in vitro*

PBMCs were purified using Lymphoprep density centrifugation (STEMCELL Technologies, Cambridge, Mass). The cells were washed twice with PBS. Human naive $CD4^+$ T cells ($CD4^+$) were isolated using the MagniSort Human $CD4^+$ naive T-cell enrichment kit (eBioscience, Thermo Fisher Scientific, Waltham, Mass) according to the manufacturer's instructions. Purity of $CD4^+CD45RO^-CD45RA^+$ T cells was > 97%. The purified $CD4^+$ T cells, were cultured at a density of 500.000 cells/mL at 37°C in precoated anti-CD3 (1 μg/mL, UCTH1) 48-well plates in RPMI 1640, supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin, 1 mmol/L L-glutamine and 10% FCS. For $T_H1$ polarization *in vitro*, $CD4^+$ 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 $T_H2$ 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. $T_H1/T_H2$ differentiation was evaluated by measuring the cytokine profile with ELISA. T-cell cultures were stimulated with phorbol 12-myristate 13-acetate (25 ng/mL) and ionomycin (1 μg/mL) for 6 hours. Afterward, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, and TNF-α release by $T_H1$ and $T_H2$ cells was measured with ELISA (Fig E4). In parallel, for epithelial stimulation experiments, the SNs of $T_H1$ and $T_H2$ cell cultures were collected and stored at −80°C for further analysis.

## TER measurements

TER of epithelial cell monolayers was measured using an EVOM/EndOhm (World Precision Instruments). 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 relatively stable TER was 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 ($\Omega \times cm^2$) was calculated by multiplying the sample resistance by the effective area of the membrane (0.33 $cm^2$ for 24-well Transwell inserts).

## ELISA

Cytokines IL-4 and IL-13 were measured in nasal secretions of AR and IR patients and healthy control subjects 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 (BD Bioscience, San Diego, Calif). Histamine and substance P were measured in the nasal fluids according to the manufacturers' protocols (LSBio, Seattle, Wash; and Cayman Chemical, Ann Arbor, Mich, respectively). The SNs of activated $T_H1$ and $T_H2$ cells were assessed for IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, and TNF-α.

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

## RT-quantitative PCR 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, Thermo-Fisher Scientific). RT-quantitative PCR was performed in a CFX Connect (Bio-Rad Laboratories, Hercules, Calif) for all genes with specific TaqMan probes and primers and using Platinum Quantitative PCR SuperMix-UDG w/ROX (Invitrogen, Thermo Fisher Scientific). 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 control subjects and AR and IR patients were stored in 4% paraformaldehyde (Fluka, Sigma Aldrich, Buch, Switzerland). 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, 31721; Abcam), anti-ZO-1 (rabbit, polyclonal, 1/100, 31721; Invitrogen), secondary antibody goat antirabbit AF488 (1/2000; Invitrogen). After staining, tissues were mounted with 4′-6-diamidino-2-phenylindole, dihydrochloride 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 Fig 1 have already been published previously.[E1] The data obtained in control subjects and AR patients in Fig 1 are reused to demonstrate the role of the epithelial barrier in IR patients. All the other data in this manuscript are uniquely for this study and are not presented somewhere else.

### REFERENCE

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;137:1043-53, e1-5.

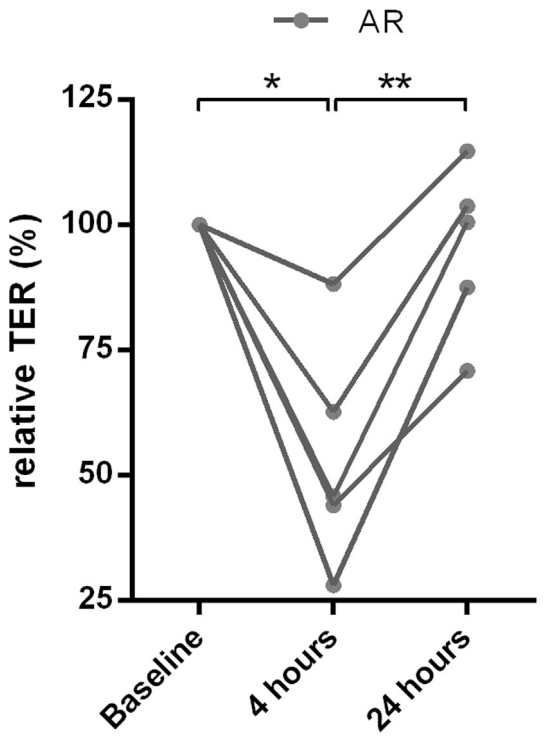


**FIG E1.** Effect of AR nasal secretions on TER of Calu-3 epithelial cell cultures at ALI. TER was measured of Calu-3 epithelial cell cultures after stimulation with nasal secretions from AR patients. n = 5. Paired *t*-test, $*P < .05$; $**P < .01$.

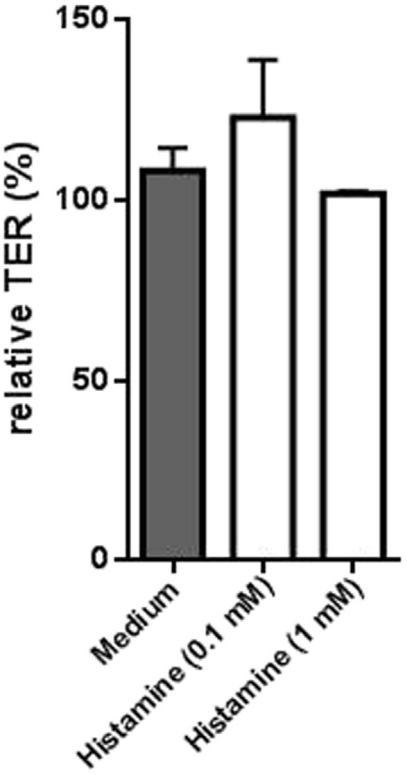


**FIG E2.** Histamine did not decrease TER after 24-hour stimulation of Calu-3 epithelial cell cultures at ALI. Data presented as means ± SDs. *M,* Mol/L.

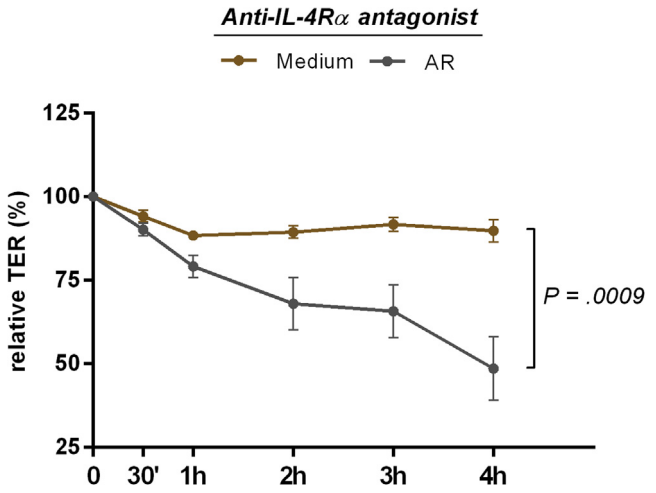


**FIG E3.** Effect of pretreatment with anti-IL-4Rα monoclonal antibody on the decrease of Calu-3 epithelial cell integrity induced by AR nasal secretions. Calu-3 epithelial cell cultures were pretreated for 2 hours with 2 μg/mL anti-IL-4Rα monoclonal antibody before stimulation with nasal secretions of AR patients. n = 5/group. Data presented as means ± SDs. Two-way ANOVA with *post-hoc* analysis, ***$P < .001$.

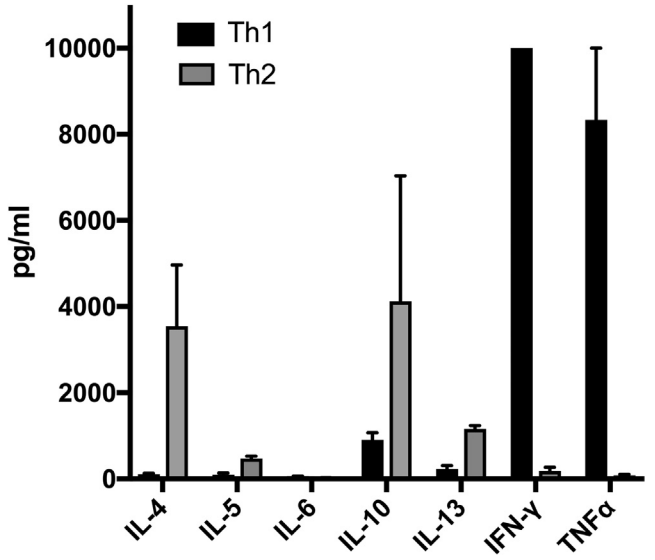


**FIG E4.** Cytokines secretion by polarized $T_H1$ and $T_H2$ cell cultures *in vitro*. Naive $CD4^+$ T cells were polarized *in vitro* for 3 weeks into $T_H1$ or $T_H2$ cells. T-cell cultures were stimulated for 6 hours with phorbol 12-myristate 13-acetate (25 ng/mL) and ionomycin (1 μg/mL). SN was collected and cytokine profile was measured with ELISA. Data are presented from 3 independent experiments, using cells from the same donor. Data are shown as means ± SEMs.

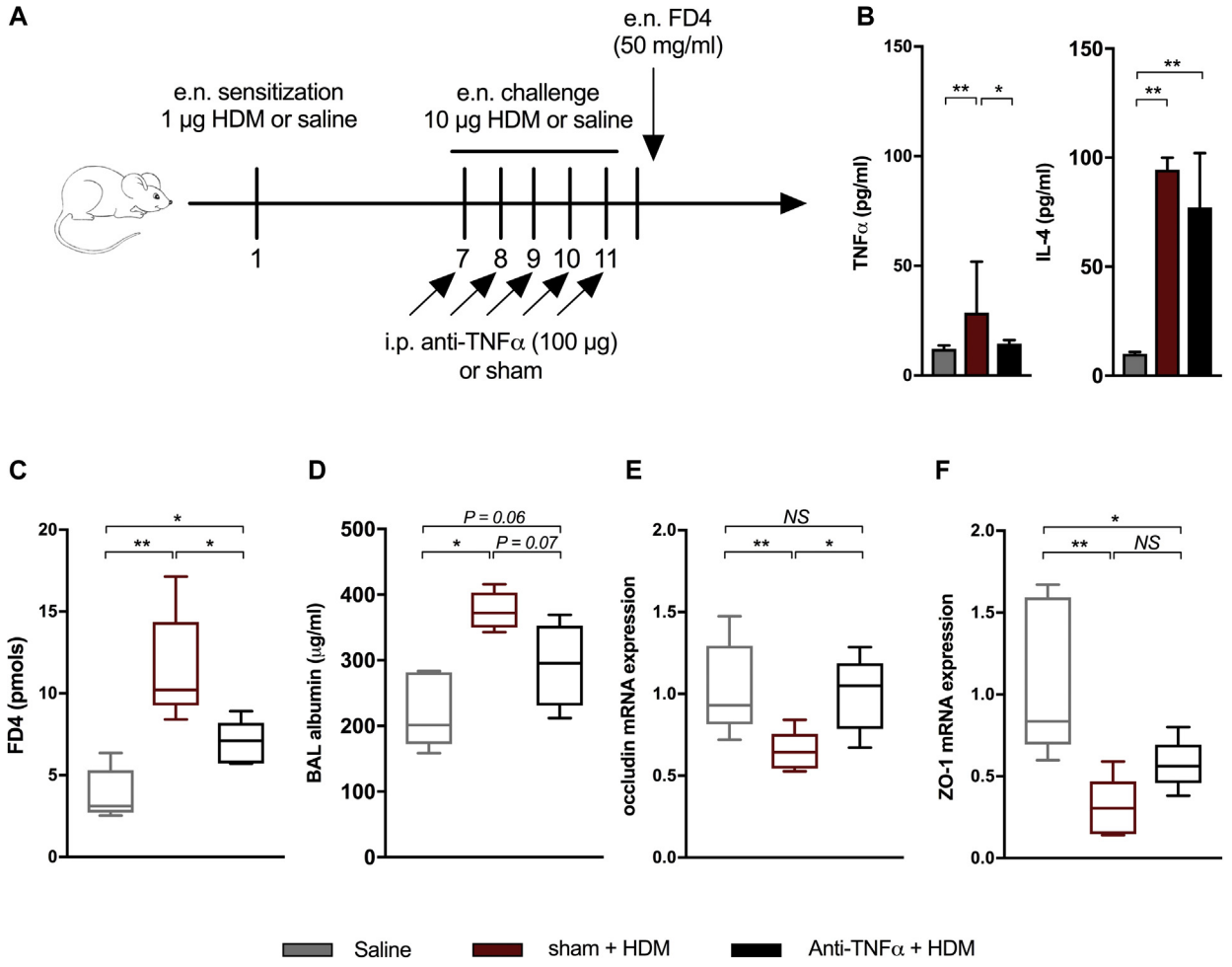


**FIG E5.** Effect of anti-TNF-α treatment in a mouse model of HDM-induced allergic airway inflammation. **A,** Mouse model. Mice were sensitized with 1 µg HDM extract or saline at day 1. One week later (days 7-11), mice were challenged endonasally with 10 µg HDM extract or saline. One hour before each challenge, anti-TNF-α monoclonal antibody or vehicle was given intraperitoneally. One hour after the last challenge, 20 µL FD4 (50 mg/mL) was applied followed 1 hour later with the sacrifice of the mice. **B,** TNF-α and IL-4 levels in BAL fluid. **C** and **D,** Effect of anti-TNF-α treatment on FD4 permeability and albumin leakage to BAL fluid in the lungs. **E** and **F,** mRNA expression of occludin and ZO-1 in nasal mucosa. Relative expression versus the housekeeping β-actin and $\beta_2$ microglobulin. n = 5 mice/group. Data presented as medians and interquartile ranges. One-Way ANOVA with *post-hoc* analysis; **P* < .05, ***P* < .01.

**TABLE E1.** Patient characteristics

| | Control | AR | IR |
|---|---|---|---|
| N | 10 | 9 | 6 |
| Age (y), median (IQR) | 32 (28-36) | 25 (24-30) | 43 (35-48) |
| Male, n | 3 | 2 | 3 |
| Smoking, n | 0 | 0 | 0 |
| INS usage, n | 0 | 0 | 0 |
| Asthma, n | 0 | 0 | 0 |
| HDM allergic, n | 0 | 9 | 0 |
| Monosensitized, n | | 3 | |
| Polysensitized, n | | 6 | |

*INS*, Intranasal steroids; *IQR*, interquartile range.

**TABLE E2.** Primer and probe sequences used for RT-quantitative PCR

| | Forward primer | Reverse primer | Probe |
|---|---|---|---|
| Human | | | |
| β-actin | GGA CAT CCG CAA AGA CCT GT | CTC AGG AGG AGC AAT GAT CTT GAT | CTG GCG GCA CCA CCA TGT ACC CT |
| $\beta_2$ microglobulin | CTG AAG CTG ACA GCA TTC GG | CTT TGG AGT ACG CTG GAT AGC C | AGA TGT CTC GCT CCG TGG CCT TAG C |
| Claudin-1 | CCA GTC AAT GCC AGG TAC GAA T | ATA GGG CCT TGG TGT TGG GT | TCA GGC TCT CTT CAC TGG CTG GGC |
| Claudin-4 | GGT CTG CTC ACA CTT GCT GG | GAC GGA CTT AAC GTT CGC AGA G | TGG CTT TAT CTC CTG ACT CAC GGT GCA |
| Occludin | CCA ATG TCG AGG AGT GGG TTA A | TTG CCA TTG GAA GAG TAT GCC | CTG CAG GCA CAC AGG ACG TGC C |
| ZO-1 | GTG CCT AAA GCT ATT CCT GTG AGT C | CTA TGG AAC TCA GCA CGC CC | TGG CCA CAG CCC GAG GCA TAT T |
| Murine | | | |
| β-actin | AGA GGG AAA TCG TGC GTG AC | CAA TAG TGA CCT GCG CGT | CAC TGC CGC ATC CTC TTC CTC CC |
| $\beta_2$ microglobulin | CCA CTG AGA CTG ATA CAT ACG CCT | GAT CAC ATG TCT CGA TCC CAG TAG | TAA GCA TGC CAG TAT GGC CGA GCC |
| PPIA | GCC GCG TCT CCT TCG AG | GTA AAG TCA CCA CCC TGG CAC | ATG CAG ACA AAG TTC CAA AGA CAG CAG AAA |
| GAPDH | TCA CCA CCA TGG AGA AGG C | GCT AAG CAG TTG GTG GTG CA | ATG CCC CCA TGT TTG TGA TGG GTG T |
| Occludin | 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 GAC GTC AA |
| ZO-1 | TTC GAG AAG CTG GAT TCC TAA GAC | CAG TCC CAG CAT CTC GTG G | CAT CTT TGG ACC AAT AGC TGA TGT TGT TGC CA |

All probes are labeled with 5′FAM3′TAMRA.
*GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *PPIA*, peptidyl prolyl isomerase A.