

Defective epithelial barrier in chronic rhinosinusitis: The regulation of tight junctions by IFN- γ and IL-4

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Background: Chronic rhinosinusitis (CRS) is a common disease with still unclear pathophysiologic mechanisms. Epithelial tight junctions (TJs) have been shown to be involved in different chronic disorders, including bronchial asthma, inflammatory bowel diseases, and skin disorders. The regulation of epithelial barrier function and TJ expression has not been extensively studied in patients with CRS and in the paranasal sinus epithelium thus far.

Objective: We sought to elucidate the TJ expression pattern in the epithelium of the sinonasal mucosa and its regulation in patients with CRS.

Methods: Trans-tissue resistance was measured in biopsy specimens from healthy control subjects and patients with CRS with and without nasal polyps. TJ protein expression was determined by using immunofluorescence, Western blotting, and real-time PCR. Primary epithelial cell cultures from patients with CRS and control subjects were used in air-liquid interface (ALI) cultures for the measurement of transepithelial resistance (TER) and TJ expression. The effect of IFN- γ , IL-4, and IL-17 on ALI cultures was assessed.

Results: A decreased trans-tissue resistance was found in biopsy specimens from patients with CRS with nasal polyps along with an irregular, patchy, and decreased expression of the TJ molecules occludin and zonula occludens 1. TER was reduced in ALI cultures from patients with CRS with nasal polyps. The cytokines IFN- γ and IL-4 decreased TER, whereas IL-17 did not have any influence on epithelial integrity.

Conclusion: A defective epithelial barrier was found in patients with CRS with nasal polyps along with a decreased expression of TJ proteins. The disruption of epithelial integrity by IFN- γ and IL-4 *in vitro* indicates a possible role for these proinflammatory cytokines in the pathogenesis of patients with CRS. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Chronic rhinosinusitis, chronic sinusitis, tight junctions, occludin, claudin, zonula occludens, regulation, cytokines, leaky epithelium

Chronic rhinosinusitis (CRS) is characterized by mucosal inflammation involving both the nasal cavity and paranasal sinuses, with potentially diverse causes.¹ It affects approximately 15% of the general population, leading to an immense effect on the quality of life of patients, as well as creating a large financial burden on health care systems worldwide.^{2,3} According to the presence or absence of polyps in the sinonasal cavities, 2 clinical entities are distinguished. These subgroups not only reflect different clinical features but also show distinct immunologic patterns. In the Western population patients with chronic rhinosinusitis with nasal polyps (CRSwNP) have a T_H2-predominant type of inflammation,⁴ whereas patients with chronic rhinosinusitis without nasal polyps (CRSsNP) display a T_H1 type.⁵ Different disease-related processes have been identified in patients with CRS, yet its exact pathogenesis still remains unknown.

Tight junctions (TJs) consist of different transmembrane and scaffold adaptor proteins. They form the most apical intercellular junction between epithelial cells, providing an apicobasolateral differentiation pattern. On the one hand, they are responsible for the regulation of paracellular flux and epithelial impermeability. In addition, they also prevent foreign particles, such as allergens, from entering the subepithelial layers. On the other hand, an opening of TJs can lead to drainage of inflammatory cells toward the lumen, supporting the resolution of phlogistic processes. Consequently, they can be considered gatekeepers that could contribute to both aggravation of inflammation-related tissue damage or resolution of inflammation through drainage. Different members of the TJ proteins have been identified, including occludin, tricellulin, the family of claudins, and junctional adhesion molecules.⁶ They form intercellular homodimers/heterodimers between neighboring cells. On the cytoplasmic side, they bind to the actin cytoskeleton through associated proteins, such as the zonula occludens (ZO) family and cingulin.

Disturbed TJs can lead to the entrance of pathogens and environmental antigens, including allergens, into the organism. Multiple disorders have been linked to defective or altered TJs, such as seen in patients with inflammatory intestinal diseases,⁷ including Crohn disease; skin diseases, such as atopic dermatitis⁸ and psoriasis⁹; and bronchial asthma.¹⁰ Very recently, a disrupted

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The laboratory of C.A.A. is supported by the European Allergy and Asthma Center Davos (EACD), Swiss National Science Foundation grants 32-132899 and 32-112306, the Christine Kühne Center for Allergy Research and Education (CK-CARE), the Müller-Gierok-Foundation, and University Hospital Zurich.

Disclosure of potential conflict of interest: M. B. Soyka has received research support from the Müller-Gierok Foundation, the European Allergy and Asthma Center Davos, the Swiss National Foundation, University Hospital Zurich, and CK-CARE and is employed by University Hospital Zurich. C. A. Akdis has received research support from Novartis, PREDICTA, the Swiss National Science Foundation, MeDALL, the Global Allergy and Asthma European Network, and CK-CARE; has provided legal consultation/expert witness testimony on the topics of Acellion T_H2-specific receptors, Aventis T-cell Bell regulation, and allergen-specific immunotherapy (for Stallergenes and Allergopharma); is president of the European Academy of Allergy and Clinical Immunology, a GA²LEN ex-committee member, director of CK-CARE, and a fellow and interest group member for the American Academy of Allergy, Asthma & Immunology. The rest of the authors declare that they have no relevant conflicts of interest. Received for publication October 30, 2011; revised May 25, 2012; accepted for publication May 30, 2012.

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0091-6749/\$36.00

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http://dx.doi.org/10.1016/j.jaci.2012.05.052

Abbreviations used

ALI:	Air-liquid interface
CRS:	Chronic rhinosinusitis
CRSsNP:	Chronic rhinosinusitis without nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
ECP:	Eosinophil cationic protein
FITC:	Fluorescein isothiocyanate
HSEC:	Human primary sinonasal epithelial cell
MMP:	Matrix metalloproteinase
TER:	Transepithelial resistance
TJ:	Tight junction
ZO:	Zonula occludens

TJ layer in biopsy specimens and increased permeability *in vitro* were found in asthmatic patients.¹¹

However, changes in TJ arrangement in the nasal cavity, a region heavily exposed to environmental antigens, are not understood in the context of chronic inflammation. A defective barrier function has been suggested in patients with CRS.¹² TJs have not been studied extensively in the context of the nose and paranasal sinuses thus far. It is known that in patients with acute rhinitis involving rhinovirus, transepithelial resistance (TER) is decreased and ZO-1 is disrupted.¹³ ZO-1 was also shown to be downregulated in nasal polyposis along with epithelial dedifferentiation.¹⁴ Weakened desmosomal junctions were present in patients with CRSwNP.¹⁵ However, a clear comparison of the 2 disease entities with regard to TJs has been lacking.

This study aims to investigate TJ function, expression, and regulation in patients with CRSwNP and those with CRSsNP. We demonstrate that leaky epithelium is present *in vivo* and *in vitro* in patients with CRSwNP along with a downregulation of claudin-4 and occludin mRNA in biopsy specimens from patients with CRSwNP. In view of the inflammatory processes in patients with CRS, the regulation by cytokines was assessed.¹⁶ TER of air-liquid interface (ALI) cultures was decreased by the T_H1 cytokine IFN- γ and the T_H2 cytokine IL-4, whereas the T_H17 cytokine IL-17A had no effect.

METHODS

Patients

Patients undergoing paranasal sinus surgery because of CRS with and without nasal polyposis were enrolled as study patients. Patients undergoing paranasal sinus surgery for noninflammatory reasons (ie, cerebrospinal fluid leak, bullous middle turbinate, and those undergoing septal surgery) were used as healthy control subjects. Nasal or systemic corticosteroid administration up to 4 weeks before surgery was considered an exclusion criterion. Patients with CRS caused by underlying systemic disorders were not included. Total serum IgE and specific IgE levels to common aeroallergens were obtained, when feasible. Patients were considered allergic if total IgE levels exceeded 100 kU/L or the allergen-specific IgE level was greater than 3.51 kU/L in addition to the patient's history being suggestive for allergies (see Table E1 in this article's Online Repository at www.jacionline.org). The study protocol was approved by the ethics committee and review board of the canton Zurich and was conducted according to the latest version of the Declaration of Helsinki.

Biopsy specimens were taken during paranasal sinus or skull base surgeries and septo-/septorhinoplasties after achievement of general anesthesia. In patients with CRSwNP, polypoid tissue was used for all analyses, whereas in patients with CRSsNP, biopsy specimens were obtained from the affected maxillary/ethmoidal or sphenoidal mucosae. Biopsy specimens in control subjects were

obtained from different locations, including the inferior/middle turbinates, uncinate process, and paranasal sinuses, to minimize the effects of a potential bias caused by the site of tissue origin. In a subgroup of patients, we decided to only collect the surface layers of the tissue by means of curettage and scraping.

Ussing chamber and trans-tissue resistance

Tissue samples with a diameter of approximately 7 mm were transported in 0.9% NaCl on ice. They were placed to cover the 4-mm-wide opening of a CHM8 Ussing chamber (World Precision Instruments, Berlin, Germany). Two percent Agarose (Gibco-BRL, Invitrogen, Basel, Switzerland) in 150 mmol/L KCl (Fluka, Sigma-Aldrich, St Louis, Mo) was used to fill the electrodes, and PBS (Gibco-BRL, Invitrogen) was used in the chamber bath. Measurements were obtained in $\Omega \times \text{cm}^2$ by using a Millicell-ERS volt ohm meter (Millipore, Temecula, Calif).

Human primary sinonasal epithelial cell lines

Tissue samples were cut into pieces of approximately 1 to 2 mm and trypsinized for 3 hours at 37°C (5% CO₂; Trypsin EDTA 0.05%, Invitrogen). Trypsin was neutralized with TNS (Lonza, Basel, Switzerland), and the tissue was passed through a 70- μm nylon mesh. The obtained cells were seeded in 75-cm² plastic culture flasks and cultured in bronchial epithelial growth medium including the Single Quot Bullet Kit (Lonza). Medium was changed after 24 hours and every second day from then on. Cells were harvested at a confluence of 90% by using trypsinization.

Cell purity

Cell purity was determined by using vimentin/cytokeratin staining for all human primary sinonasal epithelial cells (HSECs) that were isolated in this study (see Fig E1 in this article's Online Repository at www.jacionline.org). The full methodology is provided in the Methods section in this article's Online Repository at www.jacionline.org.

ALI cultures, cytokine stimulations, and TER

HSECs obtained from healthy subjects and patients with CRS in passages 2 or 3 were seeded onto 6.5-mm-diameter polyester membranes with a pore size of 0.4 μm (Costar; Corning, Corning, NY) at a density of 110,000 cells per well. Dulbecco modified Eagle medium (Gibco-BRL, Invitrogen) with fresh retinoic acid was mixed 1:1 with bronchial epithelium basal medium (BEBM, Lonza) supplemented with the Single Quot kit except for triiodothyronine and retinoic acid (Lonza) and used as the medium in ALI cultures. Experiments were conducted with cells from different donors. Each donor culture was measured in a minimum of duplicates in multiple well systems to minimize variation within 1 experiment, and average numbers were used for analyses. Cell cultures were performed with primary cell lines from comparable passage numbers. Passage 2 was used in 16 experiments, and third-passage HSECs were used in 4 different cultures (2 control subjects and 2 patients with CRSwNP, respectively). Medium was changed every second day. Once the cells grew to complete confluence, the apical compartment was freed of any medium to allow further cell differentiation at the ALI to take place. TER was measured daily in $\Omega \times \text{cm}^2$ by using a Millicell ERS Volt-Ohm Meter (Millipore). The highest measurement in the time course was used for comparison between different cell lines. Wells not building up sufficient TER (<200 $\Omega \times \text{cm}^2$) were not used in experiments. Hematoxylin and eosin staining of ALI culture cross-sections can be seen in Fig E2 in this article's Online Repository at www.jacionline.org.

Stimulation experiments were initiated 2 days after reaching maximal TER. All ALI cultures from control subjects, patients with CRSwNP, and patients with CRSsNP were used for experiments. The abovementioned medium without the addition of corticosteroids was used from then on. ALI cultures were stimulated by adding different cytokines to the basolateral compartment: IFN- γ , 10 ng/mL (R&D Systems, Abingdon, United Kingdom); IL-4, 10 ng/mL (R&D Systems); and IL-17A, 10 ng/mL (eBioscience, San Diego, Calif). Measurements were done at 0, 12, 24, 36, and 48 hours after

stimulation. The choice of concentrations for the different experiments was based on preceding dose titration experiments in ALI cultures and monolayers from bronchial and sinonasal epithelial cell cultures. For the use of ALI cultures in immunofluorescence, cells were fixed with paraformaldehyde 4% (Fluka). For mRNA isolation, cells were stored in RNeasy lysis buffer (RLT; Qiagen, Basel, Switzerland) plus β -mercaptoethanol (Sigma-Aldrich).

Paracellular flux measurements

Epithelial permeability as a surrogate marker of layer integrity was measured by using fluorescein isothiocyanate (FITC)–dextran (Sigma-Aldrich) flux measurements. FITC-dextran (2 mg/mL) was added apically at given time points. Twelve or 24 hours after addition, the FITC intensity of basolateral fluids was assessed with an ELISA reader (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany) at 480 nm.

Immunofluorescence staining of TJs

Biopsy specimens were directly frozen in OCT compound Tissue Tek (Sakura, Finetek, The Netherlands). Sections were cut to a thickness of 8 μ m on a cryomicrotome (Microm; Carl Zeiss, Feldbach, Switzerland) at -25°C and fixed with 4% paraformaldehyde (Fluka). The staining procedure was the same for cryosections and ALI cultures. Permeabilization and blocking were accomplished by means of incubation with a mixture of 10% goat serum (DakoCytomation, Glostrup, Denmark), PBS containing 0.2% Triton X (Acros Organics, Geel, Belgium), and 1% BSA (Sigma-Aldrich) in PBS. Specimens were then incubated with Alexa Fluor 488–labeled anti-occludin mAb (Invitrogen) 1:100 in 0.2% Triton X and 1% BSA in PBS, followed by incubation with polyclonal rabbit anti ZO-1 (Invitrogen) and Alexa Fluor 546–labeled goat anti-rabbit antibody. 4',6-Diamidino-2-phenylindole dihydrochloride was premixed in the mounting medium. Slides were mounted with ProLong-Gold containing 4',6-diamidino-2-phenylindol (Prolong-Gold DAPI, Invitrogen) and analyzed with a Leica TCS SPE confocal microscope (Leica Microsystems, Heerbrugg, Switzerland).

Western blotting

Samples were stored in C/D Buffer containing 140 mmol/L NaCl (Fluka), 1.5 mmol/L MgCl_2 (Fluka), 1 mmol/L dithiothreitol (Fermentas, Glen Burnie, Md), 20 mmol/L Hepes (Fluka), 0.2 mmol/L EDTA (Sigma-Aldrich), 0.1% NP-40 (Biochemika, Sigma-Aldrich), and proteinase inhibitor (Roche, Basel, Switzerland). Samples were sonicated on ice for protein extraction. SDS-PAGE was performed on 10% gels by using a Mini-Protean Electrophoresis system (Bio-Rad Laboratories, Hercules, Calif) and blotted onto a Hybond-P polyvinylidene difluoride membrane (GE Healthcare, Fairfield, Conn) in the same device. Membranes were blocked with 10% FCS (Sigma-Aldrich) in TBS-T (50 mmol/L Tris [pH 7.6; Calbiochem, San Diego, Calif], 150 mmol/L NaCl, and 0.1% Tween-20 [Sigma-Aldrich]) and incubated overnight at 4°C with mouse anti-occludin mAb (Invitrogen) or glyceraldehyde-3-phosphate dehydrogenase antibodies (Ambion, Life Technologies, Carlsbad, Calif) in 10% FCS in TBS-T. Membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (anti-mouse; Cell Signalling, Danvers, Mass) for 1 hour at room temperature. Proteins were visualized with a chemiluminescent reagent (ECL-Plus agent, GE Healthcare) with Image Reader LAS-1000 Pro version 2.5 software.

mRNA isolation and RT-PCR

The methods for mRNA isolation and RT-PCR have been reported previously.¹⁷ Details and primer sequences can be found in the **Methods** section and **Table E2** in this article's Online Repository.

Statistics

Data analysis was performed with Prism Version 5 software (GraphPad Software, La Jolla, Calif). Differences between independent variables were computed by using the Mann-Whitney *U* test. In paired values the Wilcoxon

matched pairs test was applied. For correlations, the Spearman coefficient was used. The significance level α was set to .05.

RESULTS

Disrupted epithelial integrity and TJs in patients with CRS

We first investigated whether there is any difference in tissue resistance in patients with CRS. Large and intact biopsy specimens were used for resistance assessments in an Ussing chamber to quantify the epithelial integrity directly in affected tissues. The measurement revealed significantly ($P = .03$) higher trans-tissue resistances in control subjects ($105.8 \pm 6.4 \Omega \times \text{cm}^2$) compared with that seen in patients with CRSwNP ($48.8 \pm 9.6 \Omega \times \text{cm}^2$). Samples from patients with CRSsNP ($81.5 \pm 7.9 \Omega \times \text{cm}^2$) also showed a trend toward a lower trans-tissue resistance compared with that seen in control subjects ($P = .1$; **Fig 1, A**). Supporting these findings, the TER of ALI cultures from patients with CRSwNP ($958 \pm 384 \Omega \times \text{cm}^2$) was significantly lower compared with that of control subjects ($2547 \pm 580 \Omega \times \text{cm}^2$, $P = .05$). Patients with CRSsNP ($2143 \pm 496 \Omega \times \text{cm}^2$) exerted higher resistances than patients with CRSwNP but lower than that seen in control subjects without reaching significance (**Fig 1, B**).

Immunofluorescence of the TJ proteins occludin and ZO-1 of frozen biopsy specimens revealed an intact TJ layer in healthy mucosa. This layer was disrupted more severely in patients with CRSwNP than in patients with CRSsNP along with an irregular TJ protein expression (**Fig 1, C**, and see **Fig E3** in this article's Online Repository at www.jacionline.org). In accordance with these findings, ALI cultures from control cell lines showed regular and strong expression of occludin and ZO-1. A similar but less clear picture was observed in patients with CRSsNP. However, patients with CRSwNP had a patchy, disturbed, and less dense arrangement of TJs with a reduced expression of occludin and ZO-1 (**Fig 1, D**). Taken together, a disrupted epithelial barrier was found directly *in vivo* and *in vitro* in ALI cultures of epithelia from patients with CRS with an altered expression pattern in TJs. This observation was more prominent in patients with CRSwNP.

Decreased claudin-4 and occludin TJ mRNA and protein expression in patients with CRS

The next step was to investigate the whole junctional network of sinus epithelia. A screening for 62 different junctional proteins was performed with a TaqMan Micro Fluidic Card (Applied Biosystems, Foster City, Calif) system, a customized PCR-based mRNA expression profiling system. Biopsy specimens from patients and control subjects were obtained, and mRNA expression was assessed (see **Fig E4** in this article's Online Repository at www.jacionline.org) as described in the **Methods** section in this article's Online Repository. Only those mRNAs with intermediate and high expression levels were considered. According to previous reports,^{13,18-20} we decided to choose the TJ proteins claudin-1, claudin-4, and occludin, as well as the associated proteins ZO-1 and ZO-2, for further analyses. Tissues from patients with CRSwNP had 2.2-fold lower claudin-4 and 1.6-fold lower occludin mRNA expression levels compared with control tissues ($P = .04$ and $P = .02$, respectively). Claudin-4 mRNA expression was also 2.1 times lower in patients with CRSsNP than in control subjects ($P = .04$;

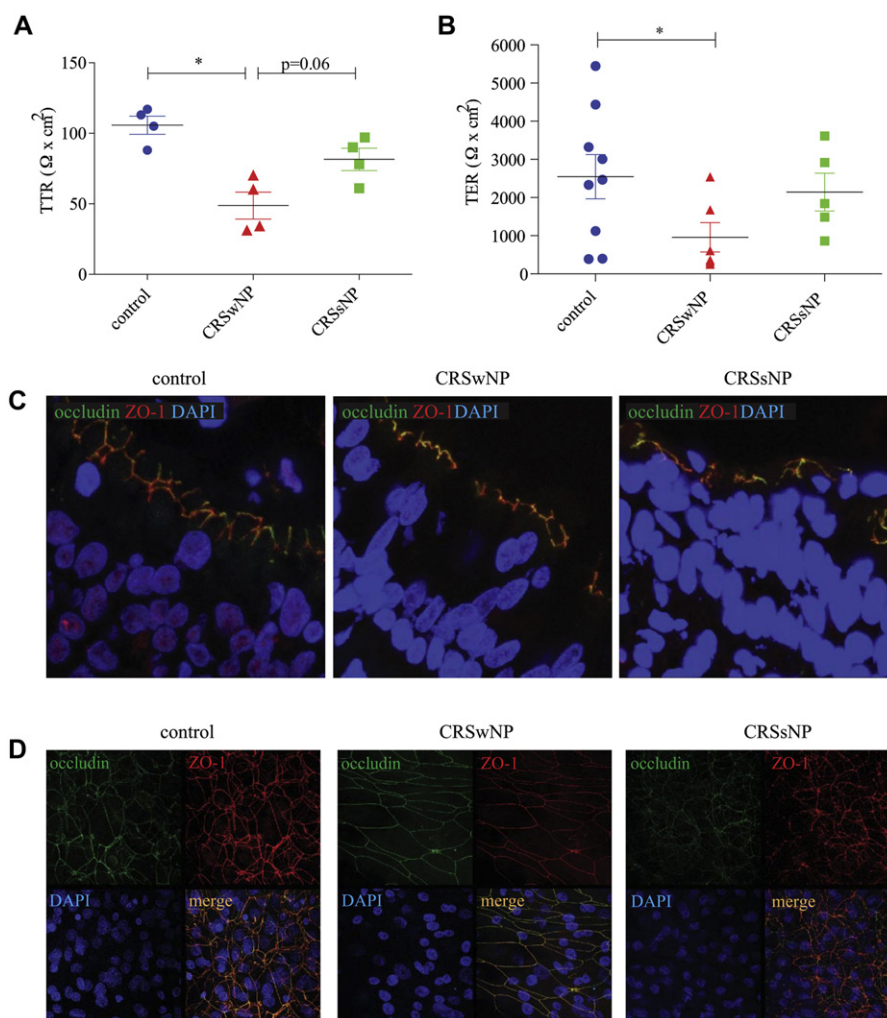


FIG 1. TJ integrity and barrier function is disturbed in patients with CRSwNP: **A** and **B**, Trans-tissue resistance (*TTR*) measured with an Ussing chamber in biopsy specimens (Fig 1, **A**) and TER measured in ALI cultures from control subjects and patients with CRS (Fig 1, **B**; mean $\pm$ SEM). **C** and **D**, Immunohistochemistry for occludin and ZO-1 in biopsy specimens (Fig 1, **C**) and ALI cultures (Fig 1, **D**) from control subjects and patients with CRS. Fig 1, **A** and **C**, Same effects for at least 4 biopsy specimens in each group. Fig 1, **B** and **D**, control subjects, $n = 9$; patients with CRSwNP, $n = 6$; and patients with CRSsNP, $n = 5$ cultures. DAPI, 4',6-Diamidino-2-phenylindole dihydrochloride. * $P \leq .05$.

Fig 2, **A**). A less pronounced trend toward the same direction was observed in claudin-1, ZO-1, and ZO-2 expression. An intraoperative surface scraping/curettage was taken in a subgroup of patients and analyzed the same way to exclude potentially misleading results by TJ protein-bearing cells from the subepithelial layer. In this way the experiments were focused only on the epithelium. Again, a trend toward a lower expression of TJ mRNA of claudin-1, claudin-4, occludin, and ZO-2 was found in patients with CRS (see Fig E5 in this article's Online Repository at www.jacionline.org), confirming that changes in TJ mRNA expression directly involve changes in the epithelium but not cells from subepithelial tissue. For a better description of the effects by the different disease types on TJ protein expression, Western blot analysis for occludin was performed in lysates of biopsy specimens. Very low levels or no expression of occludin were observed in tissues from patients with CRS compared with control subjects (Fig 2, **B**). In conclusion, a significant downregulation of TJ proteins and their mRNA levels was observed in patients with CRS.

TJ mRNA expression negatively correlates with eosinophil cationic protein and IFN- γ levels in sinonasal biopsy specimens

Eosinophil cationic protein (ECP) represents the level of eosinophilic inflammation and correlates with severity in patients with CRSwNP.²¹ TJ mRNA showed significant negative correlation with ECP mRNA determined in biopsy specimens, suggesting that eosinophilic inflammation has a negative regulatory effect on epithelial TJ expression. Similarly, a trend toward a negative correlation was also seen between IFN- γ and claudin-1 mRNA (see Fig E6 in this article's Online Repository at www.jacionline.org). There was no correlation of IFN- γ mRNA and occludin/ZO-2 mRNA.

TJ mRNA upregulation in ALI cultures from patients with CRS

An interesting finding was observed when primary epithelial cells were put into ALI cultures. In contrast to the findings in

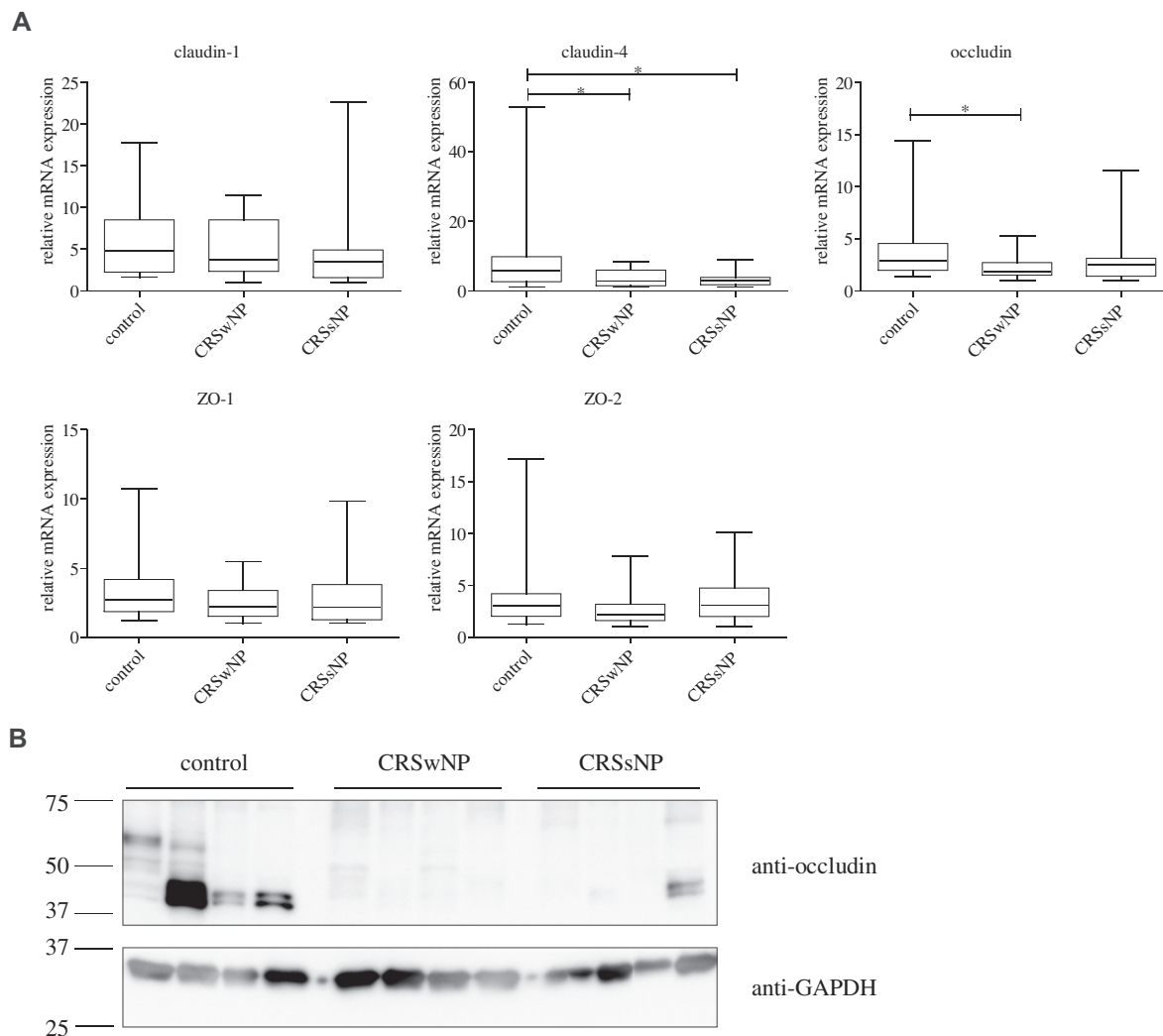


FIG 2. Low expression of TJ mRNA and proteins in patients with CRSwNP: **A**, mRNA expression for claudin-1, claudin-4, occludin, ZO-1, and ZO-2 in biopsy specimens reveals a significantly higher expression of claudin-4 and occludin in control subjects than in patients with CRS (control subjects, $n = 17$; patients with CRSwNP, $n = 14$; patients with CRSsNP, $n = 15$). **B**, Western blot showing a specific occludin band or cleaved occludin in all control biopsy specimens and in 1 patient with CRSsNP, whereas it is not detectable in patients with CRSwNP ($n = 4$ per group). *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase. * $P \leq .05$.

biopsy specimens, cells taken out of their inflammatory conditions showed relatively increased TJ expression (Fig 3, A). Claudin-4 mRNA expression in patients with CRSwNP was 2.3-fold higher in ALI cultures from patients with CRSwNP compared with that seen in control subjects ($P = .01$). On the other hand, claudin-1 expression was decreased in patients with CRSsNP by a factor of 1.7 compared with that seen in control subjects ($P = .03$). Cell proliferation measured in a subgroup of 9 ALI cultures based on Ki67 mRNA expression did not show any statistically significant difference (Fig 3, B).

Downregulation of epithelial integrity and TJs by IFN- γ and IL-4 in ALI cultures

We hypothesized that the decreased TJ expression in patients with CRS is due to cytokines released from inflammatory cells. Although many different cytokines can be related to epithelial

cell TJs, we focused on prototype T-cell cytokines and chose IFN- γ as a T_H1 , IL-4 as a T_H2 , and IL-17A as a T_H17 cytokine to stimulate ALI cultures that have been developed by using cells from control subjects and patients with CRS. Significantly decreased TER was measured during 48 hours in IFN- γ - and IL-4-stimulated cultures, whereas IL-17 showed no influence on TER (Fig 4, A). When comparing the different effects of the cytokines on ALI cultures from patients with CRS and control subjects, similar responses are observed independently of HSEC origin. Therefore the obtained results are not disease specific and can be induced in any sinonasal epithelial cell culture (see Fig E7 in this article's Online Repository at www.jacionline.org).

The effects of the cytokines were also demonstrated on the protein expression level of junctional molecules by using confocal microscopy (Fig 4, B). The IFN- γ -stimulated ALI cultures showed an opening of the TJs between neighboring cells by stratification compared with control ALI cultures. Similarly, a

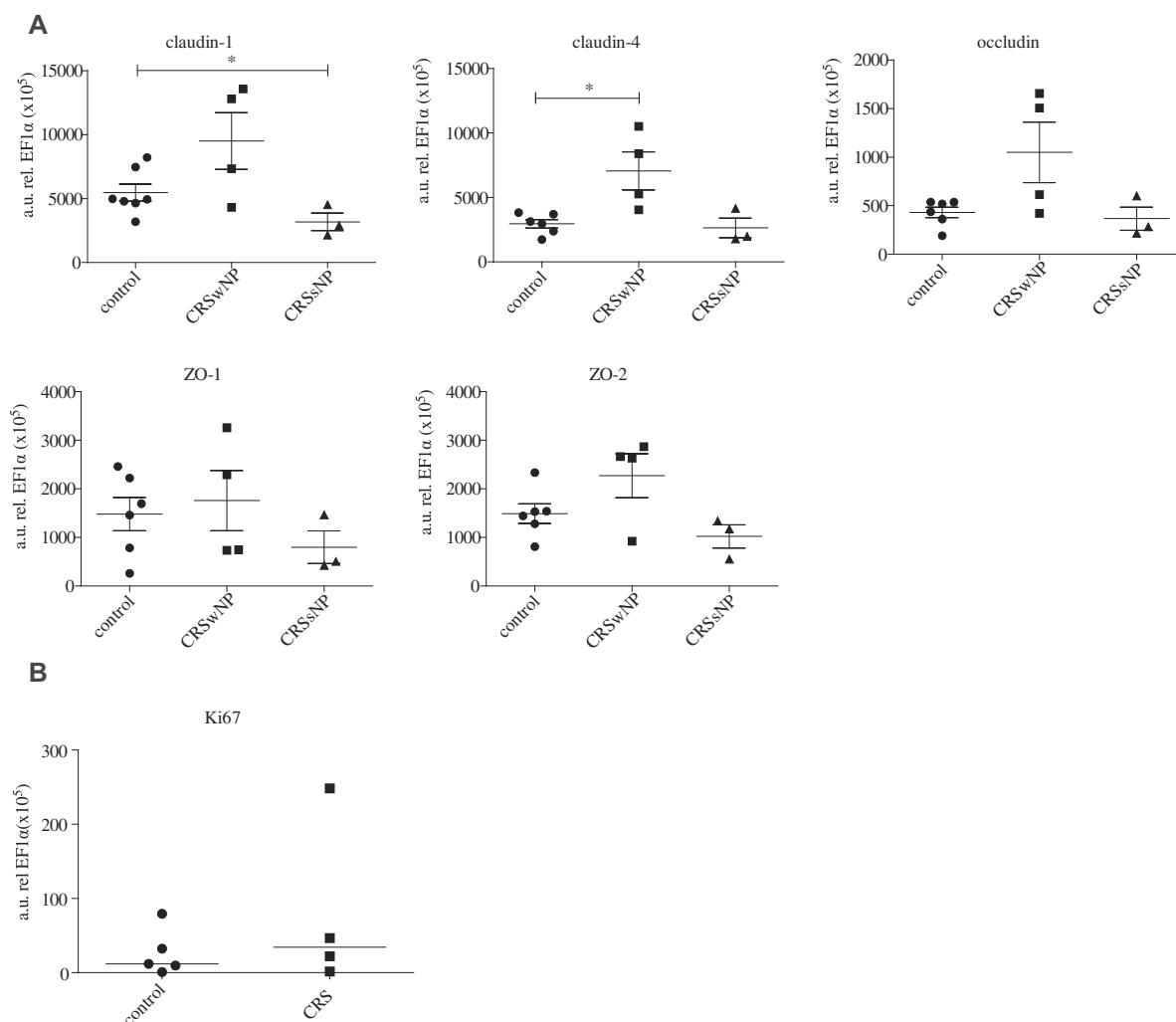


FIG 3. TJ mRNA expression is increased in ALI cultures of patients with CRSwNP. **A**, Relative mRNA expression for claudin-1, claudin-4, occludin, ZO-1, and ZO-2 in ALI cultures shows higher levels in patients with CRSwNP compared with that seen in control subjects, with a significant difference for claudin-4 (control subjects, $n = 6$; patients with CRSwNP, $n = 4$; and patients with CRSsNP, $n = 3$). **B**, Cell proliferation was measured based on Ki67 mRNA expression in a subgroup of ALI cultures (control subjects, $n = 5$; patients with CRS, $n = 4$). *EF1α*, Elongation factor 1α. * $P \leq .05$.

disruption of the TJs and an irregular expression pattern was observed in the IL-4-stimulated ALI cultures.

Paracellular flux was measured based on FITC-dextran passage across the epithelial layer to confirm the negative correlation between permeability and TER. Measurements were performed in a group of 5 ALI cultures (2 from healthy subjects, 2 from patients with CRSsNP, and 1 from a patient with CRSwNP). In general, TER negatively correlated with FITC-dextran permeability levels, as shown in Fig 4, C. IL-4 (+9.6%, $P = .02$) and IFN- γ (+5.0%, $P = .4$) stimulations increased flux, whereas IL-17 (0% change, $P = .7$) had no effect (data not shown in figures).

Increased claudin-4 and ZO-2 mRNA expression was induced on stimulation with IFN- γ in ALI cultures (Fig 5, A). IL-4 and IL-17 showed no effect. Individual analysis of the different patient groups revealed that TJ mRNA expression in ALI cultures from control subjects was not affected, whereas the CRS cultures increased TJ mRNA expression by IFN- γ stimulation (Fig 5, B). These results show that proinflammatory cytokines regulate the barrier function in epithelial cells from patients with CRS.

DISCUSSION

The present study analyzes the function and expression of TJs in patients with CRS. We provide direct *in vivo* evidence for a defective barrier function in patients with CRSwNP in conjunction with a decreased expression of TJ proteins and mRNA levels compared with that seen in control subjects. Thus far, most of the research has focused on the inflammatory pathomechanisms and identification of proinflammatory mediators in patients with CRS rather than on the effects on the nasal/paranasal sinus epithelial integrity.²² Therefore the goal of our study was to investigate an essential component of epithelial barrier function, namely TJ expression in the nasal and paranasal sinus mucosa. Epithelial TJ integrity is decisive for the barrier function of the epithelium.²³ TJs are able to form homodimer bands that are too tight for cells and proteins to pass through while selectively regulating the paracellular water permeability. The pathogenesis of cholera-induced diarrhea and TJ regulation by *Vibrio cholerae* toxins emphasizes the importance of a correct molecular arrangement of TJs in the maintenance of a tight barrier function.²⁴ An intact mucosal

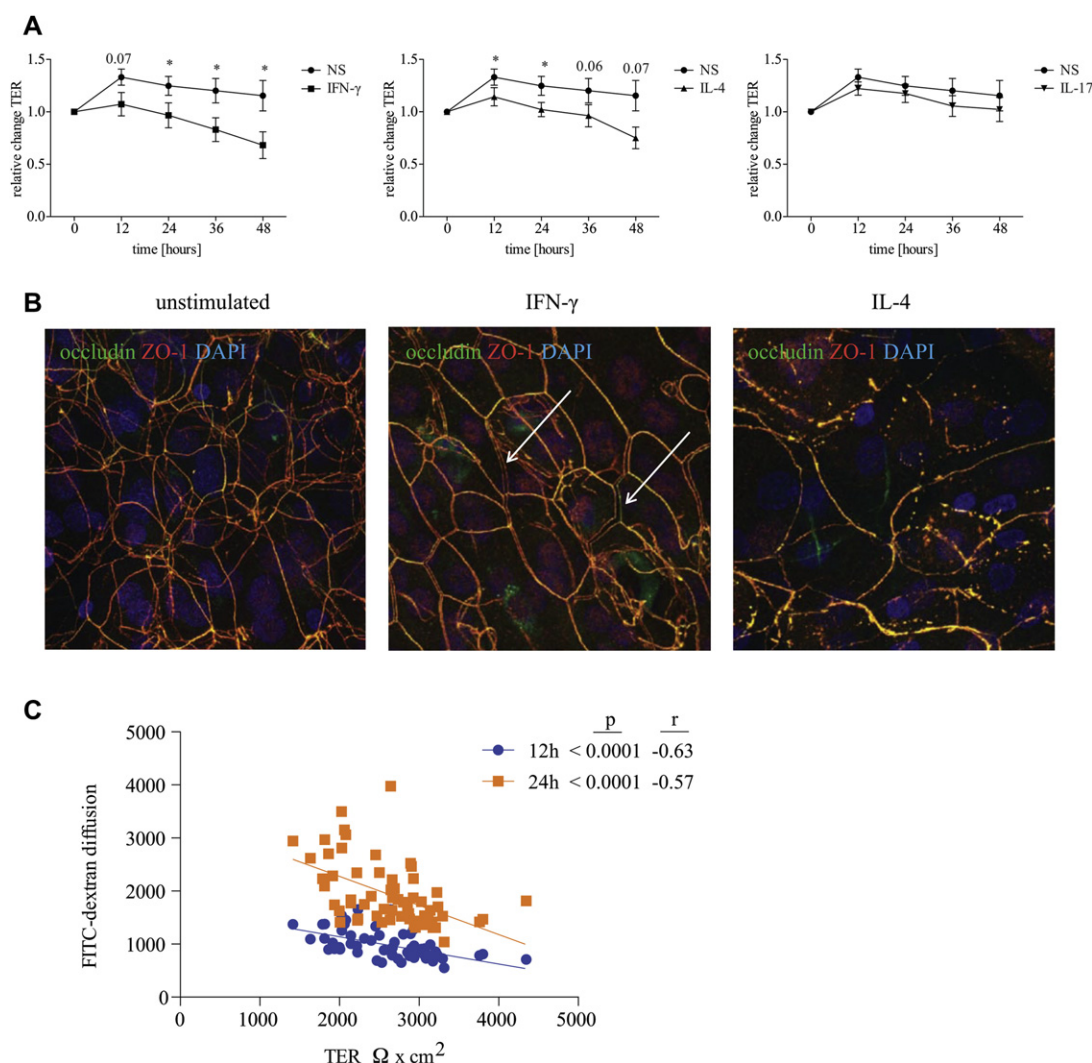


FIG 4. Proinflammatory cytokines regulate TER in ALI cultures: **A**, Relative TER in ALI cultures during 48 hours after stimulation compared with the starting point. Significantly decreased TER by IFN- γ and IL-4 but not by IL-17 was shown. **B**, Immunofluorescence staining for occludin and ZO-1 in stimulated ALI cultures. IFN- γ leads to epithelial stratification (white arrows). The IL-4-stimulated culture shows disruption of the TJ stands. DAPI, 4'-6-Diamidino-2-phenylindole dihydrochloride. **C**, TER negatively correlates with FITC-dextran permeability in ALI cultures (12 and 24 hours' incubation of FITC-dextran). * $P \leq .05$.

barrier function is required as the first-line defense mechanisms against pathogens and antigens.²⁵

It appears that inflammation downregulates TJ integrity. Expression of TJ mRNA, namely claudin-1, occludin, ZO-1, and ZO-2, negatively correlates with expression of ECP, one of the markers of eosinophilic inflammation and disease severity in patients with CRSwNP. A decreased epithelial barrier could cause a higher absorbance of *Staphylococcus aureus* endotoxins, which might play a central role in the pathogenesis of patients with CRS, as discussed below. In addition, a deliberate opening of TJs might help to resolve ongoing subepithelial inflammatory processes and drainage toward the mucosal lumen, as has been observed in neutrophils, representing a second very important hypothesis in the function of TJs.²⁶ A better understanding of this pathophysiologic aspect in patients with CRS is required to improve therapeutic effects.

A defective barrier function could be shown in patients with CRS, and all of the observed effects on TJ and barrier function

were more pronounced in patients with CRSwNP than in patients with CRSsNP. The fact that patients with CRSsNP are usually operated on in an oligosymptomatic interval might play a role in the appearance of less pronounced differences in this subgroup.

TJs play a role in different mucosal disorders. Recently, several studies highlighted defective TJs in asthmatic patients.^{10,11,19,27} Reduced TER measurements in ALI cultures from asthmatic patients corresponded with a decreased expression in biopsy specimens.¹¹ A common pathophysiologic pathway between the lower and upper respiratory tract diseases has been suggested. Patients with CRSwNP are most notably associated with nonatopic asthma.²⁸ In 1997, Bernstein et al²⁹ made the first description of TER measurements in patients with CRSwNP. In a later study in which TER was measured in ALI cultures, no difference was observed between cell cultures from healthy subjects and those derived from patients with CRS. However, no distinction between patients with CRSsNP and patients with CRSwNP was

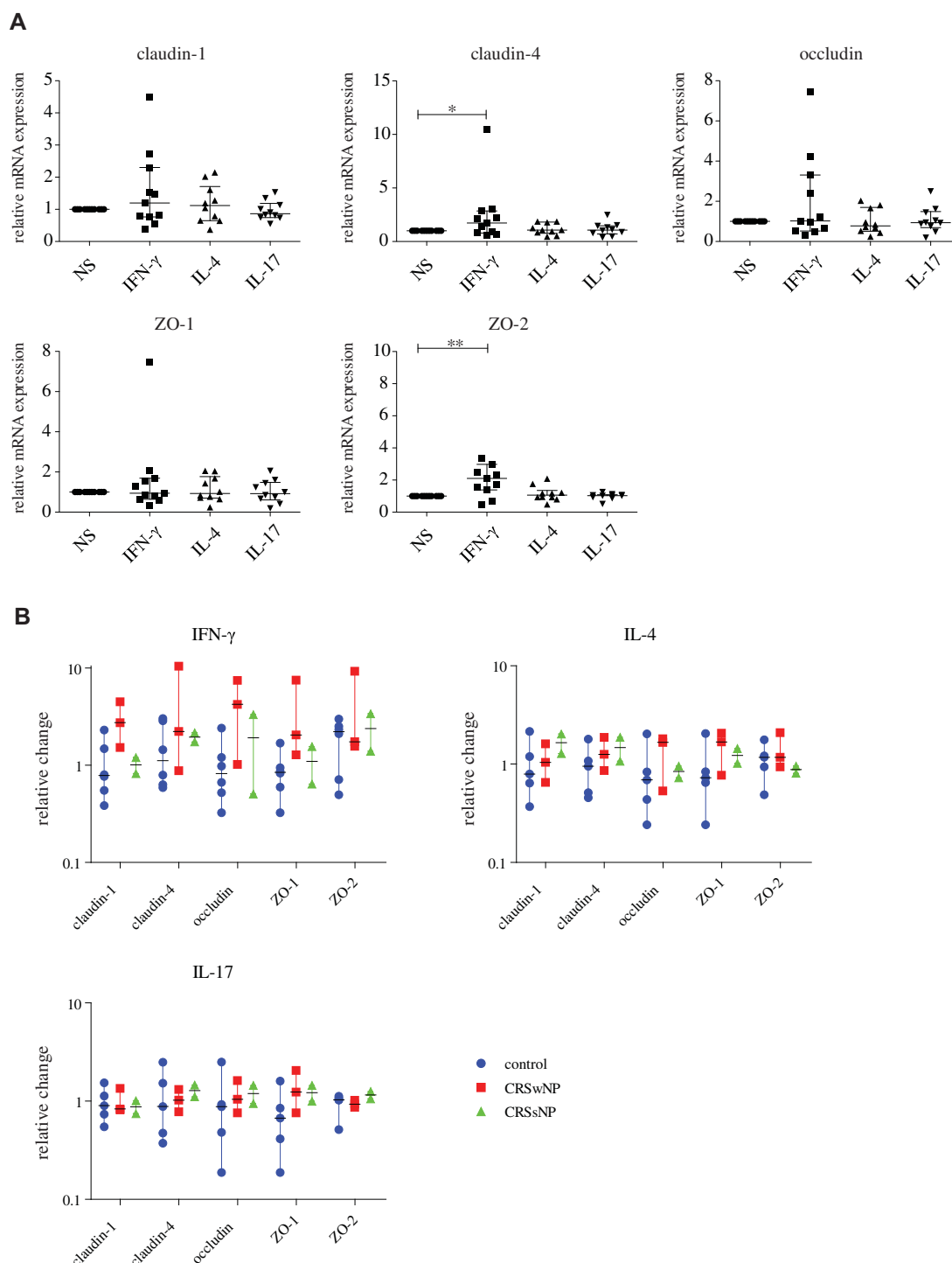


FIG 5. IFN- γ upregulates claudin-4 and ZO-2 mRNA expression in ALI cultures: **A**, Relative mRNA expression of claudin-1, claudin-4, occludin, ZO-1, and ZO-2 compared with that seen in nonstimulated (NS) ALI cultures. IFN- γ leading to a significant upregulation of claudin-4 and ZO-2 mRNAs is shown ($n = 11$). **B**, Divided according to the disease, no changes in TJ mRNA expression for IL-4- and IL-17-stimulated ALI cultures were found. IFN- γ -stimulated ALI cultures from patients with CRSwNP seem to have higher TJ mRNA expression than those of control subjects. * $P \leq .05$, ** $P \leq .01$.

performed.³⁰ Desmosomal proteins were found to be reduced in patients with CRSwNP.¹⁵ This study did not show any difference in claudin-1 or ZO-1 protein expression between control subjects and systemically steroid-treated patients with CRSwNP.¹⁵ Dexamethasone suppresses an IL-1 β - and TNF- α -dependent

claudin-1 upregulation in human smooth muscle cells.³¹ In line with our findings, the TJ-associated protein ZO-1 has been described to be downregulated in parallel to the worsening of epithelial dedifferentiation in patients with CRSwNP by means of immunohistochemistry.¹⁴

A broad screening analysis with the use of a microfluidic card PCR of 62 TJs and associated proteins was performed in biopsy specimens to find the TJ genes and proteins of interest that are involved in barrier function of the sinonasal epithelium. This approach not only helped to identify the 5 junctional proteins analyzed in this study but also showed mRNA expression levels specific for this type of tissue. Knowledge about the different expression levels of junctional molecules in nasal and paranasal sinus epithelium will support the choice of investigational targets in further studies.

The causative agent or condition that drives the epithelium to become leaky remains unknown. Differences in proliferation and cell density of the different cultures depending on their disease origin could be partially responsible. The repeatedly observed lower numbers of nuclei found in staining of ALI cultures from patients with CRSwNP compared with the other cultures in Fig 1 might represent differences in the epithelial turnover but could also be due to different planes of the section, in which all nuclei could not be captured. Furthermore, a moderate interindividual variation of donors could be the cause of the different cell numbers, despite the use of a unified study protocol. Genetic causes or epigenetic changes could be responsible because epithelial cells from patients with CRS taken out of their inflammatory condition and surrounding tissue did not change the profile of their ill-functioning barrier to a certain extent. Apart from the proinflammatory milieu discussed in this part, viral infections have been shown to be present in sinonasal tissues³² and are also known to directly disrupt epithelial TJs in patients with rhinitis.¹³ Therefore infective agents could also be the cause of barrier dysfunction in patients with CRS.

Different cytokines and matrix metalloproteinases (MMPs) are dysregulated in patients with CRS.³³ MMP-9 levels are increased in patients with both types of CRS, and MMP-7 levels are increased in patients with CRSwNP, whereas levels of the tissue inhibitor of MMP-1 protein is decreased in patients with CRS compared with control subjects.³⁴ MMPs are involved in the regulation of TJs and can promote leakiness in the bronchial epithelium.³⁵ Occludin is cleaved by MMP-2 in cerebral endothelium under pathologic conditions.³⁶ Macrolides are known to suppress MMPs.³⁷ Azithromycin, an antibiotic of the group of macrolides that is frequently used in the long-term therapy of CRS, has been shown to increase epithelial resistance and influence TJ regulation in airway epithelial cells.¹⁸

Cytokines are known to influence the integrity and expression of TJs in different tissues.³⁸ ECP, the marker of eosinophilic inflammation, and, to lesser extent, IFN- γ , representing a T_H1 environment, show negative correlation with TJ mRNA in our human sinonasal biopsy specimens. This provides evidence for lower mRNA expression of TJs in both proinflammatory conditions. According to the predominant inflammatory cell types in the CRS subgroups, we chose IFN- γ , IL-4, and IL-17 as typical T_H1, T_H2, and T_H17 cytokines to stimulate ALI cultures. Although a consistent decrease in TER by IFN- γ and IL-4 along with changes in the expression of TJ protein expression in immunofluorescent staining was observed, the differences in mRNA expression were inconsistent. The results underline the importance of other non-mRNA-related factors in the regulation of the epithelial barrier in patients with CRS. A trend toward higher expression of TJ mRNA was observed in cultures from patients with CRS that can also confound mRNA expression levels in stimulated ALI cultures. This finding was in

accordance with the overall higher expression of TJ mRNA in unstimulated ALI cultures from patients with CRSwNP. From previous studies, we know that activated T cells lead to the activation and induction of proinflammatory functions of epithelial cells and their apoptotic death.³⁹⁻⁴¹ It appears that IFN- γ shows a 3-dimensional effect on the epithelium, which sequentially induces activation and increased cell turnover together with cytokine and chemokine production. This is followed by an opening of TJs, which extends to epithelial apoptosis and severe tissue damage.

S aureus and its enterotoxins have been suggested as one possible cause of CRSwNP.⁴² Leaky epithelium could contribute to the invasion of the pathogen into deeper tissue layers in patients with CRS. A similar mechanism could be implicated in pollens that were shown to be capable of disrupting TJs.⁴³ It is not known whether genetic differences exist in TJs between patients with CRS and healthy subjects. However, we observed a dysregulation of TJs not only in biopsy specimens but also *in vitro* when the epithelium was cultured in the absence of any inflammatory stimulus for several weeks. This either suggests an intrinsic defect in the production or degradation of TJs in paranasal sinus epithelium or a certain “memory” effect of the previously inflamed epithelium. The fact that ALI cultures from patients with CRSwNP had low TERs despite high TJ mRNA levels supports the idea of an ineffective TJ production, yet it remains unclear whether dysfunctional TJs are the cause or a result of underlying inflammation in patients with CRS.

Key messages

- A defective epithelial barrier was found in patients with CRSwNP.
- Knowledge about the dysregulation of TJs in patients with CRS will help us better understand its pathophysiology.

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METHODS

Cell purity

HSECs were grown to 85% to 90% confluence on glass slides. Human smooth muscle cells grown on cover slips were used as a positive control for mesenchymal cells. Cells were fixed with 4% paraformaldehyde in PBS (Fluka) and stained with anti-cytokeratin mAbs (DakoCytomation) and anti-vimentin mAb (DakoCytomation) according to the immunofluorescent staining procedure provided later in the Methods section. Alexa Fluor 488 and 546 goat anti-mouse antibodies (Invitrogen) were used as secondary antibodies. Appropriate isotype controls were applied (DakoCytomation). A purity of greater than 95% could be proven in all samples based on cytokeratin positivity and the absence of vimentin (Fig E1).

Furthermore, biopsy specimens were screened for the presence of rhinovirus, respiratory syncytial virus, and influenza types A and B according to the method of Zhang et al^{E1} using real-time PCR. However, despite their presence in a positive control subject, we have not found these viruses in any of our samples.

mRNA isolation and RT-PCR

Tissue samples were immediately put into RNeasy lysis buffer (Qiagen) and stored for later use. Biopsy specimens were then shredded with ceramic beads (Precellys; LabForce, Nunningen, Switzerland), and mRNA was subsequently extracted with the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. Reverse transcription was accomplished with the use of reverse transcription reagents containing random hexamers (Fermentas).

TaqMan low-density array Micro Fluidic Card PCR

A TaqMan PCR-based screening was performed to identify the expressed genes in sinonasal tissue. Sixty-two genes (Fig E4) were chosen for analysis from the manufacturer's database, and ready-to-use plates were provided by the company. From the 23 measured claudins, there was a high expression level in claudins 1, 3, 4, 5, 7, 10, 11, 12, 25, and 27 in paranasal sinus tissues. Desmosomal, adherens, and gap junctional mRNAs were highest for E-cadherin, nectin-2, and connexin 43. In associated proteins all ZO proteins, β -catenin, cingulin, plakoglobin, and desmoplakin, have highly expressed mRNAs.

From these genes, we decided to analyze the collected samples with the following different primer pairs that were designed to flank or cross intron-exon borders. The used sequences can be found in Table E2.

In fluidic card PCR glyceraldehyde-3-phosphate dehydrogenase was used as the reference housekeeping gene, and in all others elongation factor 1 α was used as the reference housekeeping gene. Relative quantification was done on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with the $2^{-\Delta\Delta CT}$ formula. Because only 4 control biopsy specimens and 3 from each disease group were used in this analysis, the differences between groups are not statistically comparable and should only represent general expression levels. Comparison between groups was done by using conventional real-time PCR, as shown in Fig 2.

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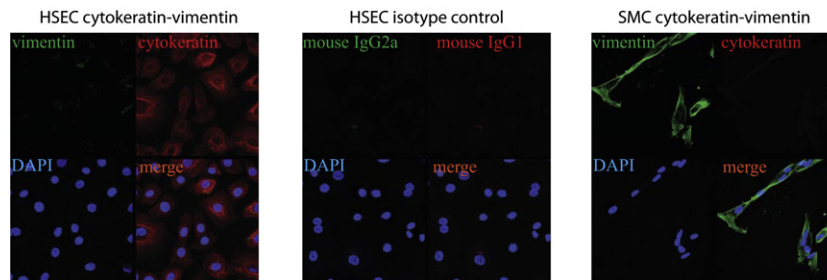


FIG E1. Cell purity of HSECs was confirmed by using cytokeratin and vimentin costaining: HSEC monolayer staining for vimentin and cytokeratin was performed for all isolated epithelial cell lines. Purity was found to be greater than 95% in all HSEC cell lines based on cytokeratin positivity and the absence of vimentin. Isotype and positive controls for vimentin with human bronchial smooth muscle cells are provided. *DAPI*, 4'-6-Diamidino-2-phenylindole dihydrochloride.

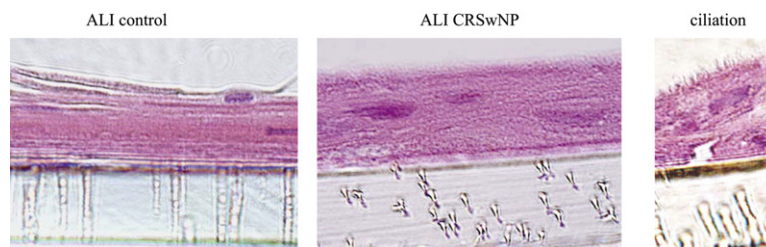


FIG E2. ALI cultures from healthy control subjects and patients with CRSwNP show the same thickness and are able to develop cilia: ALI cultures were embedded in paraffin and cut perpendicularly into 10- μ m sections on a microtome. Hematoxylin and eosin staining of ALI culture cross-sections shows the same height for cultures from patients with CRSwNP and healthy control subjects, confirming the similar assembly of cells in both cultures. Ciliation is detectable on some parts of the ALI cultures.

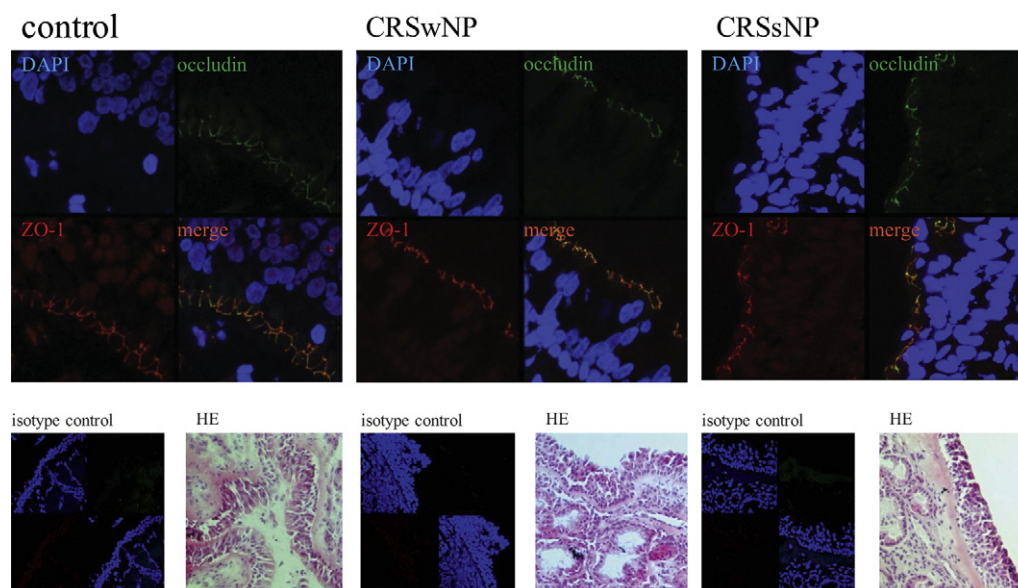


FIG E3. TJ expression is altered in biopsy specimens from patients with CRS: Immunohistochemistry for the TJ protein occludin and the associated protein ZO-1 in biopsy specimens from healthy control subjects and patients with CRS from Fig 1, B, is shown. Single-color staining for occludin and ZO-1, including isotype controls and hematoxylin and eosin (HE) staining, is provided. In the control biopsy specimen both occludin and ZO-1 are regularly expressed and show a tight pattern. In both CRS samples, the expression is disrupted and less intense for occludin, especially in patients with CRSwNP. The results represent the faulty TJ arrangement in patients with CRS, indicating leaky epithelium. Hematoxylin and eosin staining shows a preserved epithelium in all samples. DAPI, 4'-6-Diamidino-2-phenylindole dihydrochloride.

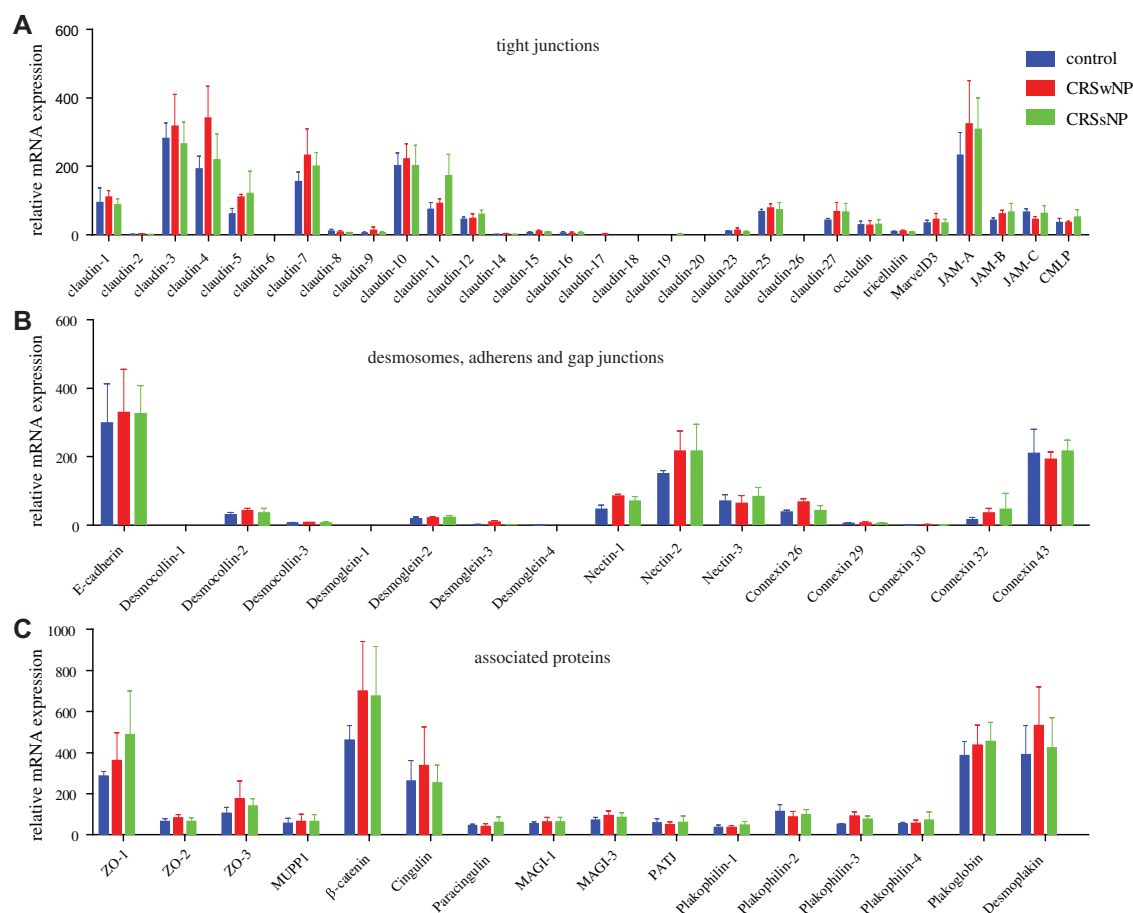


FIG E4. Specific mRNA expression pattern in biopsy specimens from patients with CRS of TJs, desmosomes, adherens, and gap junctions, as well as associated proteins: Microfluidic card PCR was performed in biopsy specimens from patients with CRS for a total of 62 TJs and related genes. **A**, Different expression patterns for TJ mRNA. **B**, Desmosomal, adherens, and gap junctional mRNA expression. **C**, Associated protein gene expression. The differences between disease subtypes were quantified by using RT-PCR for a selection of genes and are shown in the main text. Control subjects, $n = 4$; patients with CRSwNP, $n = 3$; and patients with CRSsNP, $n = 3$.

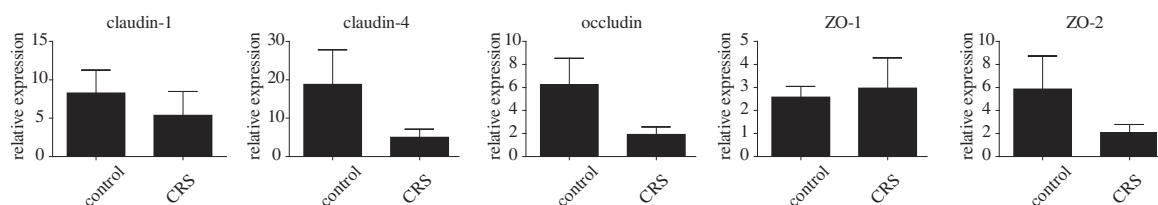


FIG E5. TJ mRNA expression tends to be decreased in patients with CRS compared with that seen in healthy control subjects in epithelial scrapings/curettage: mRNA analysis by using real-time PCR of epithelial scrapings/curettage of patients with CRS and control subjects shows a trend toward lower expression levels of claudin-1, claudin-4, occludin, and ZO-2 in patients with CRS compared with those seen in control subjects. Because of the low number of samples of epithelium only, statistical significance could not be reached. In analogy to the results from Fig 2, A, this indicates that TJ mRNA expression shows the same results in full-thickness biopsy specimens and samples of epithelium only. Therefore it is not the subepithelial TJ-carrying cells that influence our measurements. Healthy control subjects, n = 5; patients with CRS, n = 3.

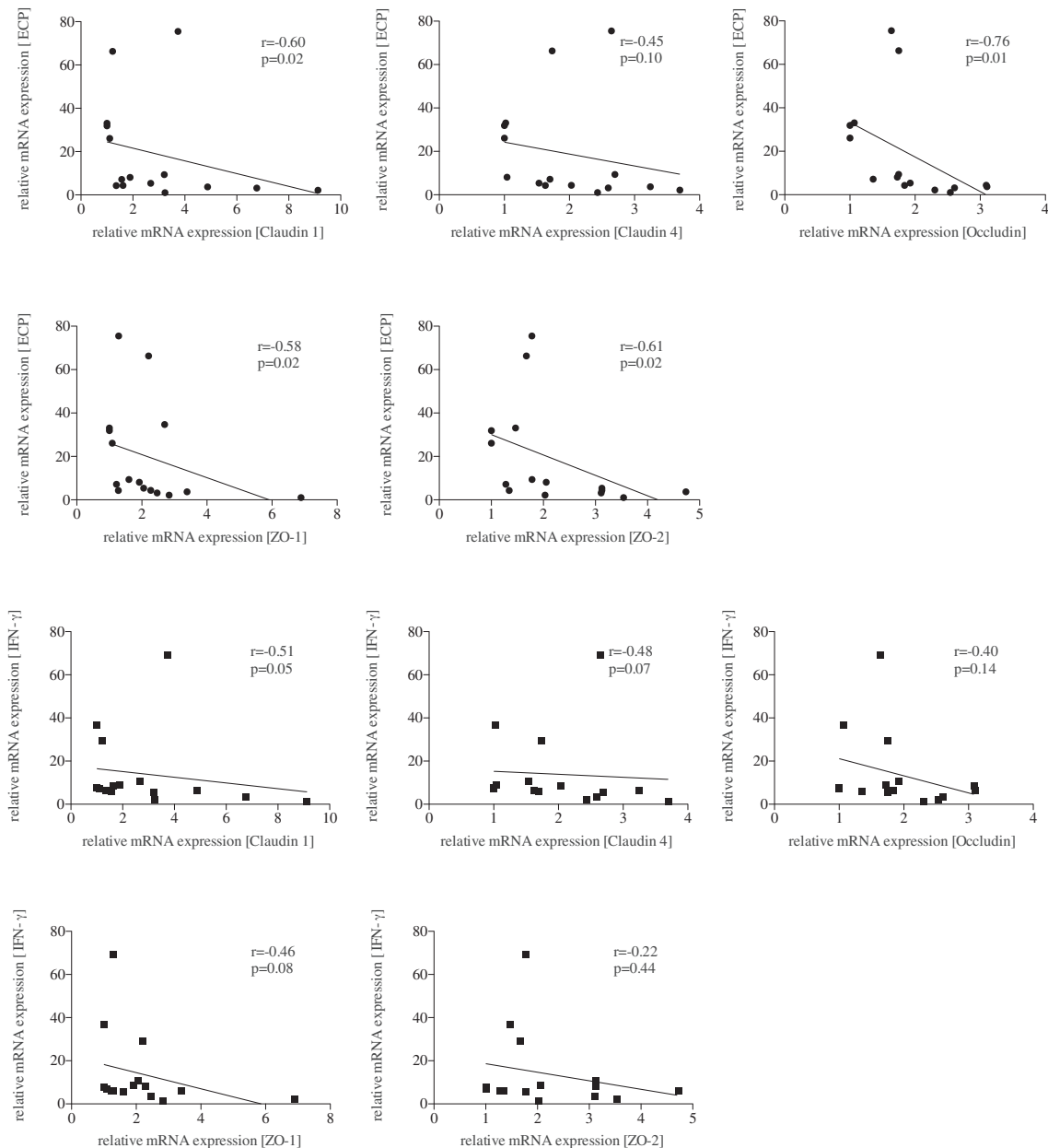


FIG E6. ECP and IFN- γ mRNAs negatively correlate with TJ mRNA in biopsy specimens: ECP shows a significant negative correlation with expression of measured TJ mRNAs, and IFN- γ demonstrates a trend-wise negative connection with TJ mRNA expression in whole-tissue biopsy specimens. Therefore the level of inflammation negatively correlates with TJ expression on mRNA level. N = 15; control subjects, n = 6; patients with CRSwNP, n = 5; and patients with CRSsNP, n = 4.

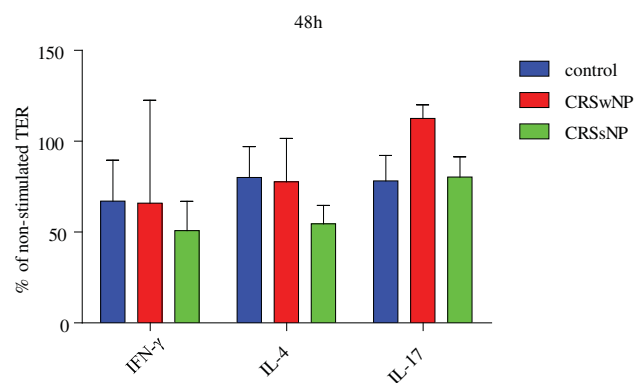


FIG E7. Proinflammatory cytokines influence the tightness of ALI cultures: The TER data from Fig 4, A, were analyzed according to the disease type of the originating cell line used in the ALI cultures after 48 hours of stimulation with either IFN- γ , IL-4, or IL-17. TER is indicated as a percentage of the non-stimulated ALI cultures. Again, decrease in TER by IFN- γ and IL-4 is seen without any relevant differences among the 3 groups. In IL-17 the cultures from patients with CRSwNP exerted the highest TER. Therefore the obtained results are not disease specific and can be induced in any sinonasal epithelial cell culture. Control subjects, n = 4; patients with CRSwNP, n = 2; and patients with CRSsNP, n = 2.

TABLE E1. Demographics of patients and use of biopsy specimens in different experiments

Patient group	Mean age (y)	Allergy	Asthma	Aspirin intolerance	PCR	ALI	Ussing chamber	WB
Control subjects (n = 29)	41	11 (38%)	1 (3%)	1 (3%)	17	9	4	6
Patients with CRSwNP (n = 23)	44	8 (35%)	7 (30%)	2 (10%)	14	5	4	8
Patients with CRSsNP (n = 20)	45	6 (30%)	1 (5%)	0 (0%)	15	5	4	6
Total (n = 72)	43	25 (35%)	9 (13%)	3 (4%)	46	19	12	20

Mean ages and numbers of patients affected by allergies, asthma, or aspirin hypersensitivity are provided in the first 4 columns, including percentages. Samples were used for different analyses indicated in the last 4 columns (in number of patients).

WB, Western blot.

TABLE E2. Primer sequences used for PCR (indicated as 5'-3')

Gene	Forward primer	Reverse primer
EF1 α	CTG AAC CAT CCA GGC CAA AT	GCC GTG TGG CAA TCC AAT
Claudin-1	CAG TCA ATG CCA GGT ACG AAT TT	AAG TAG GGC ACC TCC CAG AAG
Claudin-4	TGT ACC AAC TGC CTG GAG GAT	GAC ACC GGC ACT ATC ACC ATA A
Occludin	GAT GAG CAG CCC CCC AAT	GGT GAA GGC ACG TCC TGT GT
ZO-1	ACA GTG CCT AAA GCT ATT CCT GTG A	TCG GGA ATG GCT CCT TGA G
ZO-2	CGG TTA AAT ACC GTG AGG CAA A	GGG AAC CAC TGG GTG TAA TTC A
IFN- γ	TCT CGG AAA CGA TGAA ATA TAC AAG TTA T	GTA ACA GCC AAG AGA ACC CAA AA
ECP	AGT AGA TTC CGG GTG CCT TT	AGG TGA ACT GGA ACC ACA GG
Picornavirus	CGG ACA CCC AAA GTA G	GCA CTT CTG TTT CCC C
Influenza A	AAG GGC TTT CAC CGA AGA GG	CCC ATT CTC ATT ACT GCT TC
Influenza B	ATG GCC ATC GGA TCC TCA AC	TGT CAG CTA TTA TGG AGC TG
RSV	GCG ATG TCT AGG TTA GGA AGA A	GCT ATG TCC TTG GGT AGT AAG CCT

EF1 α , Elongation factor 1; *RSV*, respiratory syncytial virus.