

levels in PBMCs of patients with asthma. This result is supported by previous *in vivo* studies demonstrating that *NOS2* promoter activities were the highest in cells transfected with 12 or 14 CCTTT repeats, as indicated by bell-shaped activity profiles.^{7,8} Although it is quite conceivable that CCTTT repeat polymorphism influences asthma exacerbation through changing the level of gene expression, the pathophysiological role of this polymorphism in the inflammatory processes should be uncovered in further studies, which evaluate the *NOS2* mRNA expression level either in airway cells or in a longitudinal investigation.

In this study, there were no significant differences between the 2 genotype groups in terms of FENO levels or plasma NO_x concentrations. A recent investigation demonstrated that CCTTT repeat polymorphism in *NOS2* contributes to FENO levels in inhaled corticosteroid (ICS)-naïve patients with asthma⁴; however, another study reported no significant association between polymorphism and FENO levels in pediatric patients with asthma, approximately half of whom received ICS treatment.⁹ Although these studies differ in population and the means of division into genotype groups, our results may be attributed to the influence of ICS treatment on nitric oxide synthesis in bronchial cells, regardless of polymorphisms of *NOS2*.

In conclusion, the present study demonstrates the association between pentanucleotide repeat polymorphism in the *NOS2* promoter region and changing gene expression levels. Consequently, it is suggested that having alleles shorter than or equal to 11 repeats contributes to the worsening of clinical outcomes following asthma treatment in patients with moderate to severe asthma.

Keita Hirai, PhD^{a,b}

Toshihiro Shirai, MD, PhD^c

Masayuki Suzuki, BSc^a

Tatsuki Shimomura, BSc^a

Kunihiko Itoh, PhD^{a,b}

From ^athe Department of Clinical Pharmacology & Genetics, School of Pharmaceutical Sciences, University of Shizuoka, ^bthe Laboratory of Clinical Pharmacogenomics, Shizuoka General Hospital, and ^cthe Department of Respiratory Medicine, Shizuoka General Hospital, Shizuoka, Japan. E-mail: itohk@u-shizuoka-ken.ac.jp.

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Staphylococcus aureus enhances the tight junction barrier integrity in healthy nasal tissue, but not in nasal polyps



To the Editor:

Chronic rhinosinusitis (CRS) is characterized by mucosal inflammation of nasal and sinus cavities. Clinically relevant CRS phenotypes are defined by the presence (CRSwNP) or absence (CRSSNP) of nasal polyps (NPs). CRSwNP is mostly defined by a T_H2-type microenvironment and eosinophilia, whereas CRSSNP rather involves T_H1 responses and neutrophilia.¹ Previous studies have associated *Staphylococcus aureus* as one of the factors driving inflammation in NPs, but the relationship between the mucosal barrier and *S aureus* remains to be investigated.²

Tight junctions (TJs) represent transmembrane and adaptor proteins sealing the most apical part of intercellular junctions between adjacent epithelial cells. They regulate epithelial impermeability against pathogens and foreign particles, such as allergens. Members of the TJ proteins include the occludin, tricellulin, and the claudin families that form homodimers and heterodimers between neighboring cells, forming the intercellular barrier. Junctional adhesion molecules (JAMs) and the zonula occludens (ZO) proteins form the connection of transmembrane TJ molecules to the actin cytoskeleton.³

Defective expression of TJs and barrier leakiness have been reported in allergic disorders, such as asthma,³ atopic dermatitis,⁴ and CRS.⁵ TJs can be regulated by an inflammatory microenvironment in tissues; in CRS, by cytokines such as IFN-γ and IL-4.⁵ Recently, TJs have also been shown to be regulated by TLR-9 ligands, such as CpG-DNA, leading to an increase in TJ expression and epithelial barrier integrity.⁶ TJ genes respond to tissue factors and foreign factors to maintain epithelial integrity; however, the response of epithelial barrier molecules to common airway pathogens remains to be investigated. In this letter, we report that *S aureus* enhances barrier function of healthy nasal epithelial cells by increasing the expression of TJ molecules.

Nasal mucosa tissue explants of human inferior turbinate (IT) from healthy donors and NP tissue from patients with CRSwNP (see Table E1 in this article's Online Repository at www.jacionline.org) were infected with *S aureus* or *Staphylococcus epidermidis* with a dose of 2 × 10⁷ CFU/mL and observed for IL-1β and IL-6 response (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org). *S epidermidis*- and *S aureus*-stimulated samples did not differ in terms of IL-1β and IL-6 expression (Fig E1, C). Samples in

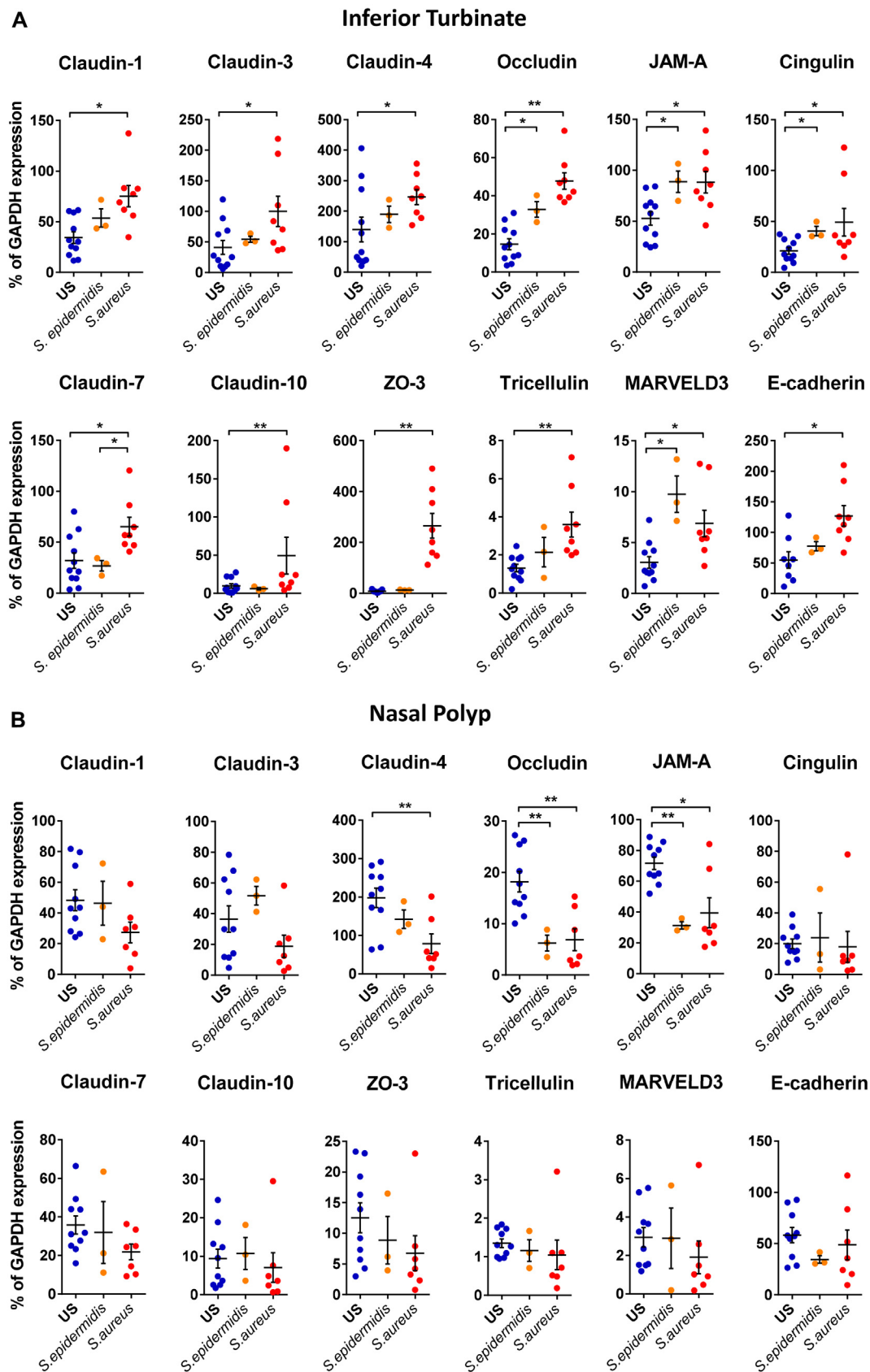


FIG 1. *S aureus* upregulates TJs and improves barrier integrity in ITs, but not in NPs. **A**, mRNA expression of TJ and junction-associated molecules in IT ($n = 11$) tissue infected with *S aureus* (2×10^7 CFU/mL) ($n = 8$) or *S epidermidis* (2×10^7 CFU/mL) ($n = 3$). **B**, mRNA expression of TJ and junction-associated molecules in NP ($n = 10$) tissue infected with *S aureus* (2×10^7 CFU/mL) ($n = 7$) or *S epidermidis* (2×10^7 CFU/mL) ($n = 3$). TJ gene expression measured by quantitative RT-PCR. Expression values calculated as arbitrary units ($2^{(-\Delta Ct)} \times 1000$), percentage of glyceraldehyde 3-phosphate dehydrogenase expression. * $P < .05$. ** $P < .01$.

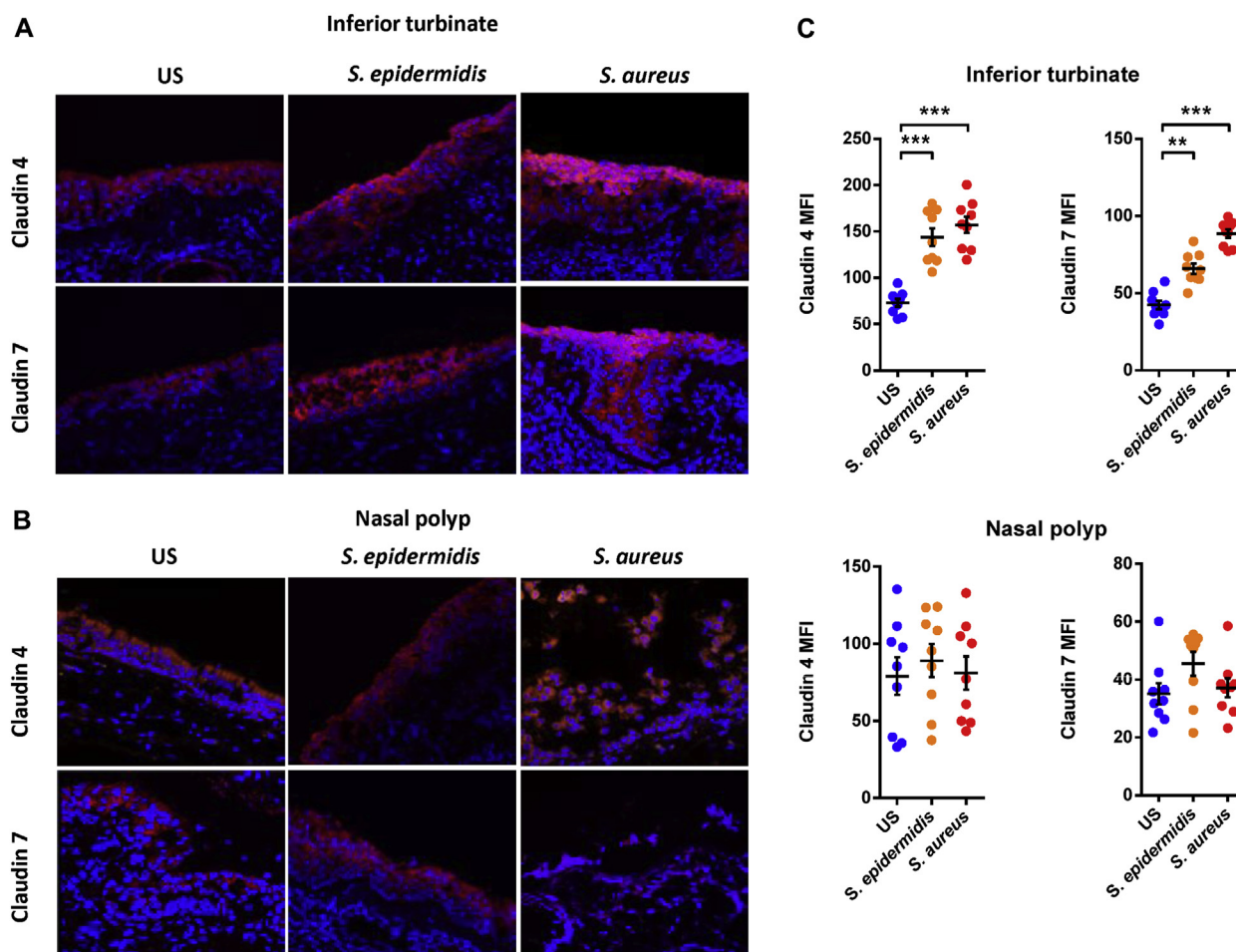


FIG 2. A, Immunofluorescence stainings of human claudin-4 and -7 molecules in IT (n = 3) tissue infected with *S. aureus* (2 × 10⁷ CFU/mL) or *S. epidermidis* (2 × 10⁷ CFU/mL) (n = 3). B, Immunofluorescence stainings of human claudin-4 and -7 molecules nasal tissue with NP (n = 3) infected with *S. aureus* (2 × 10⁷ CFU/mL) or *S. epidermidis* (2 × 10⁷ CFU/mL) (n = 3). C, Mean fluorescence intensity values of claudin-4 and -7 in ITs and NPs (n = 3). Measurements were taken from 3 separate epithelial regions of equal area from each donor. *P < .05. **P < .01. ***P < .001.

which *S. aureus* was absent from initial explant tissue in both IT and NP donors was selected (see Fig E2 in this article's Online Repository at www.jacionline.org). Expression levels of 21 TJ and related molecules known to be constitutively expressed in nasal mucosa and airways were assessed with quantitative PCR (Table E2 in this article's Online Repository at www.jacionline.org). We observed that upon *S. aureus* infection, there is an upregulation of the expression of several TJ molecules, namely, claudins-1, -3, -4, -7, -10, ZO-3, occludin, tricellulin, cingulin, JAM-A, MARVELD3, and E-cadherin in IT samples within 24 hours, suggesting that the healthy nasal mucosal tissue responds adequately to protect itself against bacterial invasion. Claudin-10, ZO-3, and occludin are upregulated 4.06, 2.06, and 2.26-fold in *S. aureus*-stimulated IT samples, respectively (see Table E3 in this article's Online Repository at www.jacionline.org). We also observed upregulation of occludin, JAM-A, and cingulin to a lesser extent with *S. epidermidis*. Furthermore, MARVELD3 showed an upregulation of 2.19-fold, greater than that of *S. aureus* (1.25 fold)-stimulated samples, in response to *S. epidermidis*. Although it is not a strong

pathogen as *S. aureus*, *S. epidermidis* also contains innate immune response-stimulating substances that may also affect barrier. In contrast, NP tissue shows a significant decrease in claudin-4, claudin-5, occluding, and JAM-A expression after *S. aureus* infection; expressional changes were not observed for other TJ molecules. Likewise, decrease in expression of claudin-4, claudin-5, occludin, and JAM-A was observed after stimulation with *S. epidermidis*. Overall, stimulation of IT tissue with *S. aureus* or with *S. epidermidis* leads to upregulation of various TJ molecules, whereas the NP tissue shows no change or down-regulation of barrier molecules in response to *Staphylococcus* species (Fig 1; see Fig E3 in this article's Online Repository at www.jacionline.org). Claudins-11, -17, -20, CLDN1, JAM-3, MAGI-1, -3, and ZO-1 were statistically not different in *S. aureus*- and *S. epidermidis*-infected tissue explants in both IT and NP tissue (Fig E3). Although it was not possible to perform Ussing chamber or macromolecule flux experiments in these tissue explants for a direct evidence of barrier integrity change, increased expression of TJ molecules may suggest an increased epithelial barrier. Next, we validated the observations seen on

mRNA expression by staining claudin-4 and claudin-7 proteins with immunohistochemistry. Cryosections taken from *ex vivo* human nasal tissue infected with *S aureus* were stained for the TJ molecules of the epithelial layer. Similar to the observations made for mRNA expressions, both claudin-4 and claudin-7 showed an increase in staining intensity in IT samples infected with *S aureus* for 24 hours (Fig 2, A) and to a lesser extent, samples infected with *S epidermidis*. In contrast, NP tissue showed a different expression profile of the TJs claudin-4 and claudin-7, at baseline and after infection. The epithelial layer was relatively intact and showed a low but uniform expression of both claudin-4 and claudin-7 in resting NP samples. However, we observed extensive epithelial shedding in NP samples infected with *S aureus* and only basal and subepithelial cells remained intact. Claudin-4 and claudin-7 expression cannot be observed in the epithelial layer, implicating a role for *S aureus* in the disrupted epithelial barrier of NP tissue (Fig 2, B). Such extensive epithelial layer shedding was not observed in NP tissue stimulated with *S epidermidis*. Mean fluorescence intensities of 3 donors confirmed the changes in protein expression in both IT and NP samples (Fig 2, C).

To assess the general state of tissue viability in tissues inoculated with *S aureus*, we stained cryosections with TUNEL staining, using terminal deoxynucleotidyl transferase to label cleaved DNA molecules. In IT unstimulated samples and in samples infected with *S epidermidis*, fluorescence intensity of cleaved DNA was very low and comparable between unstimulated tissue and tissue infected with *S aureus*. This indicates that healthy nasal tissue did not undergo tissue damage in the presence of *S aureus* and that the increased expression in TJ molecules and increased barrier protecting the tissue are unrelated to tissue damage. In contrast, we observed severe tissue damage and shedding, with apoptosis taking place in NPs (see Fig E4 in this article's Online Repository at www.jacionline.org).

S aureus colonization of the nasal mucosa is linked to the success of surgical outcome in patients with CRS.⁷ The amount of *S aureus* colonization and intramucosal immune reaction is also shown to be directly contributing to disease severity in NPs.⁸ Staphylococcal protein A was shown to induce IL-10 in CRSwNPs, possibly resulting in improved bacterial survival to further drive CRS pathogenesis.⁹ These findings, combined with our observations, suggest that CRSwNPs are prime sites for *S aureus* entry into the tissue, contributing to the pathogenesis of upper airway allergic diseases. Inherent dysbiosis in the nasal polyp epithelial barrier might contribute to the ability of *S aureus* to penetrate the nasal tissues, and contact of *S aureus* with immune cells may increase inflammatory cytokine production and contribute to persistence of inflammation in CRSwNP. Therapeutic mechanisms that specifically block bacterial proteolytic enzymes may contribute to the management of CRSwNP, through preservation of the tenuous epithelial barrier. The present study provides evidence for a strengthened barrier function in nasal epithelium in response to *S aureus* in healthy nasal tissue. This effect can also be observed with a commensal bacterium of the nasal mucosa, *S epidermidis*, which also contains innate immune response-stimulating substances that may also affect epithelial barrier to a lesser extent. In contrast, we demonstrated in an *in vitro* setting that the epithelial barrier has been disrupted in NPs of patients with CRSwNP, and might be unable to correctly

remodel in patients with CRS because of extensive epithelial damage and shedding induced by *S aureus*.

Can Altunbulakli, MSc^{a,b,*}

Rita Costa, MSc^{a,*}

Feng Lan, MD, PhD^{c,d,*}

Nan Zhang, MD, PhD^c

Mubeccel Akdis, MD, PhD^{a,b}

Claus Bachert, MD, PhD^{c,e}

Cezmi A. Akdis, MD^{a,b}

From ^athe Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich and ^bthe Christine Kühne-Center for Allergy Research and Education, Davos, Switzerland; ^cthe Department of Oto-Rhino-Laryngology, University of Ghent, Ghent, Belgium; ^dthe Department of Otolaryngology Head and Neck Surgery, Beijing Institute of Otolaryngology, Beijing TongRen Hospital, Capital Medical University, Beijing, China; and ^ethe Division of ENT diseases, CLINTEC, Karolinska Institute, Stockholm, Sweden. E-mail: akdisac@siaf.uzh.ch.

*These authors contributed equally to this work.

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METHODS

Tissue culture and infection experiments

Patients. Nasal tissue was obtained from 7 patients with CRSwNP and 8 control patients at the Department of Oto-Rhino-Laryngology, Ghent University Hospital. None of the patients had taken oral corticosteroids or antibiotics 4 weeks and topical corticosteroids 2 weeks before nasal tissue was biopsied. The diagnosis of CRSwNP was made according to the European Position Paper on Rhinosinusitis and Nasal polyps 2012 guideline. The IT tissues of patients with septal deviation were used as controls. The clinical data of all the patients are presented in Table E1. Informed consent was obtained from all patients before sample collection. The ethics committees of the Ghent University Hospital approved the study.

Preparation of *S aureus* stocks. *S aureus* RN6390, containing the plasmid pALC1743, a pSK236-derived shuttle plasmid carrying an active *S aureus* promoter (RNAIII promoter), was chosen as the infection strain in this study. In particular, this strain does not produce *S aureus* enterotoxins such as enterotoxin A, B, C, and E, and toxic shock syndrome toxin 1. The concentration of *S aureus* (2×10^7 CFU/well) was chosen according to the former *ex vivo* experiments in which we used bacterial concentrations of 10^7 to 10^8 CFU/mL and prepared pilot experiments to finally choose a concentration of 2×10^7 CFU/mL as suboptimal effective dose, which did not overgrow the mucosa.

***Ex vivo* human nasal mucosa infection model.** CRSwNP and control tissues were washed 3 times by tissue culture medium (TCM) (RPMI1640:Dulbecco modified Eagle medium = 1:1) with antibiotics (50 IU/mL penicillin [Invitrogen, Leideberg, Belgium] and 50 μ g/mL streptomycin [Invitrogen]). The washed tissue explant was cut into 24 small cubes approximately 25 mm² in size for further investigation. Each cube was placed on a mesh in a 6-well plate with 2.5 mL TCM supplemented with antibiotics in each well to create an air-liquid interface. All tissue cubes were incubated at 37°C in humidified air with 5% CO₂ overnight, and then the tissue cubes were transferred to a 24-well tissue culture plate (Falcon, BD Biosciences, Erembodegem, Belgium), infected separately with 2 mL inoculum containing 2×10^7 CFU *S aureus* and 2 mL TCM.

mRNA isolation and reverse transcription. RNA from nasal tissue samples was isolated using Rneasy Mini Kit and Rnase-free Dnase (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was obtained from RNA samples via reverse transcription using the RevertAid RT kit and random hexamers (Thermo Fisher Scientific, Waltham, Mass) as described by the manufacturer.

mRNA analysis by Taqman array microfluidic cards. Real-time quantitative PCR was performed using the Taq-man array microfluidic cards (Applied Biosystems, Foster City, Calif) using 25 predesigned probes including junctional molecules expressed in epithelial cells, chosen from the Applied Biosystems database (Table E2). The probes were selected as spanning exon-exon junctions of each gene and having an amplicon length of 200 bp maximum. A total cDNA amount of 400 ng per sample was mixed with the TaqMan Gene Expression Master Mix (Applied Biosystems) added

to the microfluidic card. The cards were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Arbitrary units for each gene were calculated according to the $2^{(-\Delta\Delta Ct)} \times 1000$ formula. Out of 25 genes, 21 that were found to be expressed in nasal mucosa tissue were quantified for further analysis.

Immunofluorescence staining of TJs

Cryosections taken from nasal tissue samples of *ex vivo* human nasal mucosa infection model were fixed with 4% paraformaldehyde (Fluka, Sigma-Aldrich, St Louis, Mo), permeabilized, and blocked with PBS with 0.1% Triton-X (Acros Organics, Gell, Belgium) and 10% goat serum (Dako, Glostrup, Denmark). After washing samples with PBS containing 0.1% Triton-X, they were incubated with rabbit anti-claudin-7 (Thermo Fisher Scientific) at room temperature for 60 minutes and goat anti-rabbit antibody labeled with alexa fluor 546 dye (Thermo Fisher Scientific) at room temperature for 45 minutes. Alternatively, the cryosections were incubated with mouse anti-claudin-4 (Thermo Fisher Scientific) at room temperature for 60 minutes and rabbit anti-mouse antibody labeled with alexa fluor 546 dye (Thermo Fisher Scientific) under the same conditions for 45 minutes. The nuclei were stained with diluted hoechst 33342 (Thermo Fisher Scientific) dye at room temperature for 15 minutes. The slides were then mounted with "Prolong Gold Antifade" mounting medium without 4'-6-diamidino-2-phenylindole, dihydrochloride (Thermo Fisher Scientific). Sections were examined with a confocal microscope (Leica TCS SPE, Leica Microsystems, Heerbrugg, Switzerland). Data were analyzed and fluorescence intensity was quantified using Image J software, by selecting 3 regions of equal area from each donor.^{E1}

TUNEL staining

TUNEL staining of nasal tissue samples was performed with the click-it TUNEL alexa fluor 647 imaging assay kit (Life Technologies) as described by the manufacturer. The nuclei were stained with diluted hoechst 33342 dye (Life Technologies) at room temperature for 15 minutes. Sections were examined with a confocal microscope (Leica TCS SPE, Leica Microsystems).

Statistics

All data analysis was performed using GraphPad Prism version 6.01 (GraphPad Software, San Diego, Calif). For comparison of experiments run in parallel, a Wilcoxon matched-pairs test was run. All data are presented as mean $\pm$ SD. *P* values (* $<.05$, ** $<.01$, and *** $<.001$) are indicated inside the graphs.

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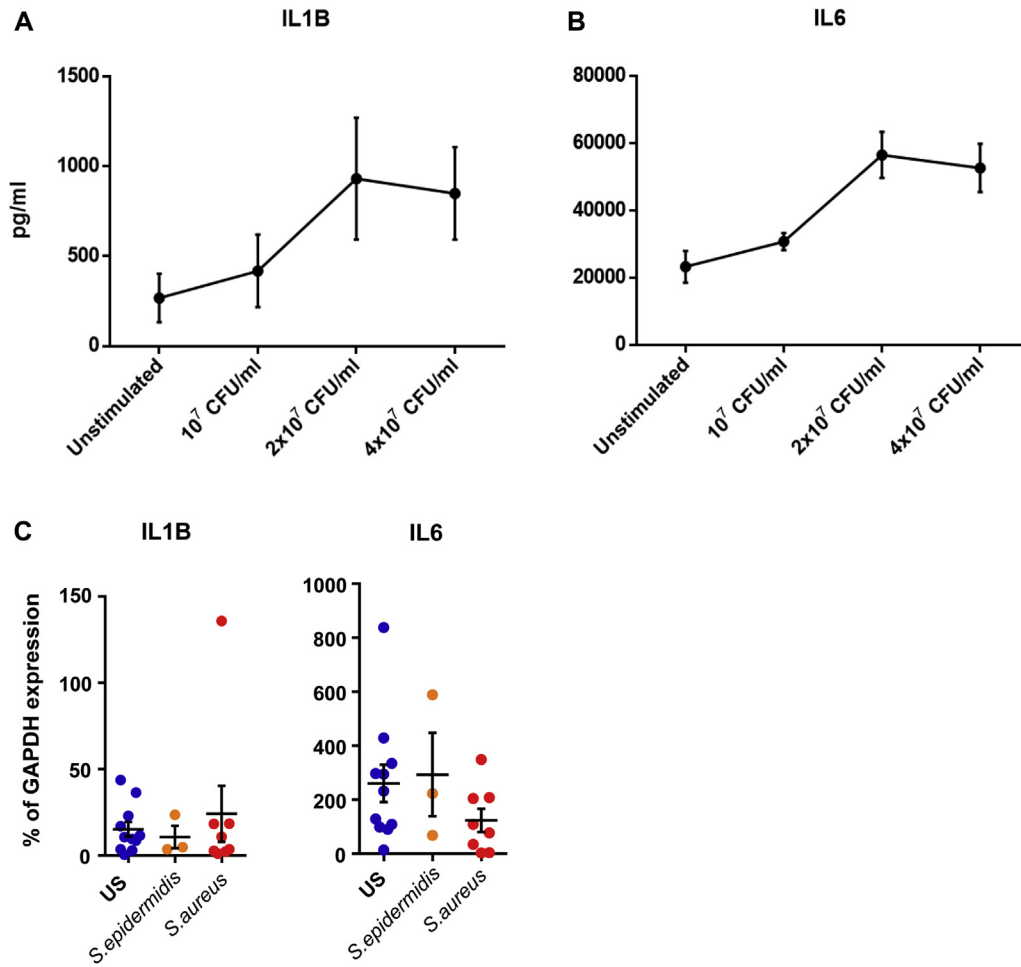


FIG E1. Protein measurement of (A) IL-1 β and (B) IL-6 on IT nasal tissue ($n = 3$) supernatants stimulated with increasing doses of *S aureus*. C, mRNA expression of IL-1 β and IL-6 in IT ($n = 11$) tissue infected with *S aureus* (2×10^7 CFU/mL) or *S epidermidis* (2×10^7 CFU/mL). Gene expression measured by quantitative RT-PCR. Expression values calculated as arbitrary units ($2^{(-\Delta Ct)} \times 1000$), percentage of glyceraldehyde 3-phosphate dehydrogenase expression.

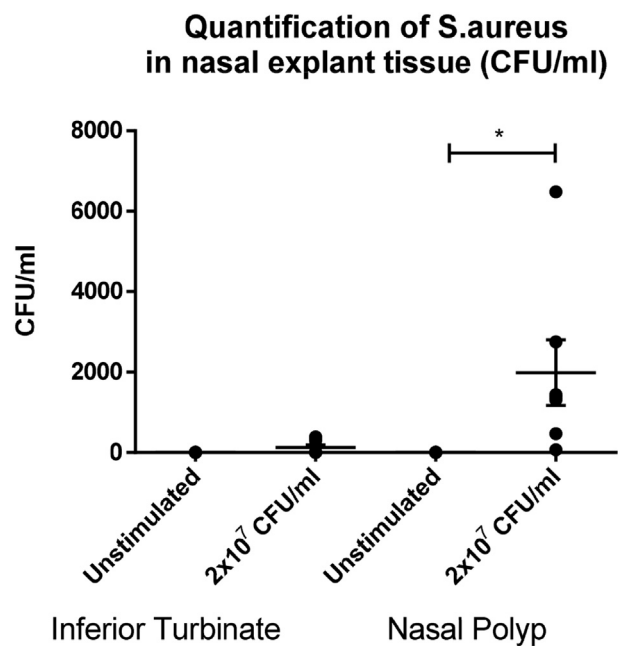


FIG E2. Quantification of *S aureus* abundance on IT and NP tissue after initial stimulation dose of 2×10^7 CFU/mL. * $P < .05$.

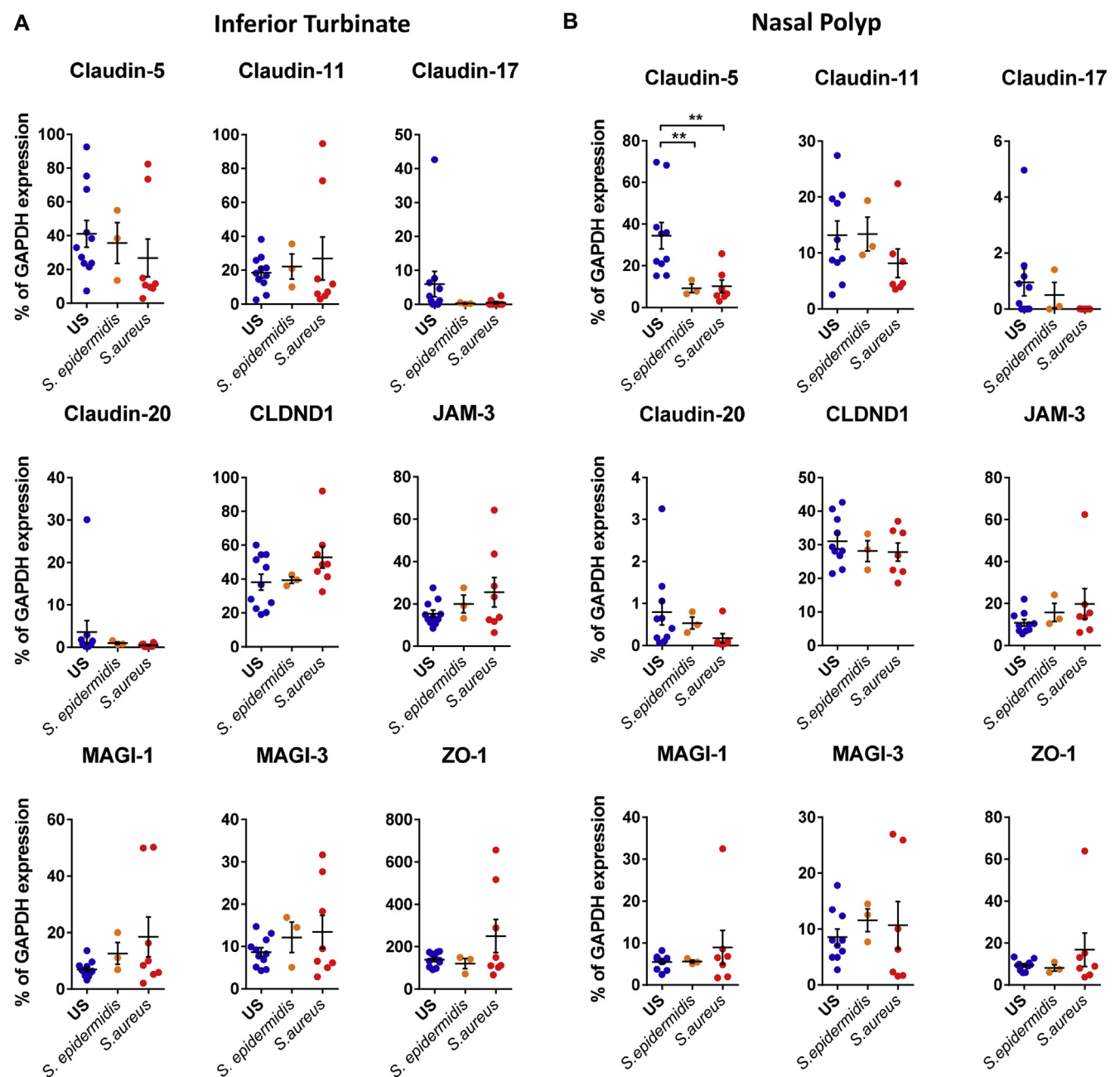


FIG E3. **A** and **B**, mRNA expression of other TJ and related molecules in IT (n = 11) and nasal NP (n = 11) tissue infected with *S aureus* (2×10^7 CFU/mL) or *S epidermidis* (2×10^7 CFU/mL). Tight junction gene expression measured by quantitative RT-PCR. Expression values calculated as arbitrary units ($2^{(-\Delta Ct)} \times 1000$), percentage of glyceraldehyde 3-phosphate dehydrogenase expression. * $P < .05$. ** $P < .01$.

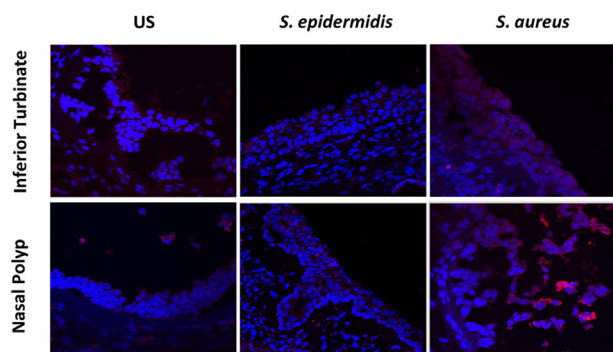


FIG E4. Immunofluorescence staining of TUNEL in IT tissue and NP tissue infected with *S aureus* (2×10^7 CFU/mL) or *S epidermidis* (2×10^7 CFU/mL).

TABLE E1. Clinical data of patients included in the study

Characteristic	Controls	CRSwNP
Number of patients	8	7
Age (y), median (range)	45.1	53.8
Sex: female/male	3/5	3/4
Atopy	0/8	4/7
Asthma	0/8	4/7
AERD	0/8	1/7

AERD, Aspirin-exacerbated respiratory disease.

TABLE E2. Screened predesigned probes chosen from the Applied Biosystems database

Gene symbol	Gene name	Assay ID
CDH1	E-cadherin	Hs01023894_m1
CGN	Cingulin	Hs00430426_m1
CLDN1	Claudin-1	Hs00221623_m1
CLDN3	Claudin-3	Hs00265816_m1
CLDN4	Claudin-4	Hs00976831_m1
CLDN5	Claudin-5	Hs00533949_m1
CLDN7	Claudin-7	Hs00600772_m1
CLDN10	Claudin-10	Hs00734479_m1
CLDN11	Claudin-11	Hs00194440_m1
CLDN17	Claudin-17	Hs01043467_s1
CLDN20	Claudin-20	Hs01851239_s1
CLDND1	Claudin-25	Hs00219886_m1
EGF	Epidermal growth factor	Hs01099999_m1
F11R, JAM-A	Junctional adhesion molecule 1	Hs00170991_m1
INADL	InaD-like	Hs00195106_m1
JAM3	Junctional adhesion molecule 3	Hs00230289_m1
MAGI1	Membrane associated guanylate kinase, WW and PDZ domain containing 1	Hs00191026_m1
MAGI3	Membrane associated guanylate kinase, WW and PDZ domain containing 1	Hs00326365_m1
MARVELD2	MARVEL domain containing 2, Tricellulin	Hs00376394_m1
MARVELD3	MARVEL domain containing 3	Hs00369354_m1
OCLN	Occludin	Hs00170162_m1
PGK1	Phosphoglycerate kinase 1	Hs00943178_g1
TJP1	Tight junction protein 1, ZO-1	Hs01551861_m1
TJP3	Tight junction protein 3, ZO-3	Hs00274276_m1
TMEM234	Transmembrane protein 1	Hs00963921_m1

TABLE E3. Fold change of TJ gene expression in IT and NP tissue

Gene name	IT tissue				NP tissue			
	<i>S aureus</i> vs US <i>P</i> value	<i>S aureus</i> vs US fold change	<i>S epidermidis</i> vs US <i>P</i> value	<i>S epidermidis</i> vs US fold change	<i>S aureus</i> vs US <i>P</i> value	<i>S aureus</i> vs US fold change	<i>S epidermidis</i> vs US <i>P</i> value	<i>S epidermidis</i> vs US fold change
Claudin 1	.0391	1.19	NS	0.56	NS	−0.43	NS	−0.03
Claudin 3	.0391	1.43	NS	0.32	NS	−0.48	NS	0.41
Claudin 4	.0391	0.75	NS	0.35	.0031	−0.60	NS	−0.28
Occludin	.0078	2.26	.022	1.24	.0046	−0.62	.007	−0.65
JAM-A	.0234	0.67	.0391	0.68	.0328	−0.44	.007	−0.56
Cingulin	.0409	1.32	.0386	0.91	NS	−0.10	NS	0.20
Claudin 7	.0157	1.04	NS	−0.15	NS	−0.39	NS	−0.10
Claudin 10	.0078	4.06	NS	−0.37	NS	−0.24	NS	0.14
ZO-3	.0078	2.06	NS	0.53	NS	−0.46	NS	−0.29
Tricellulin	.0078	1.74	NS	0.63	NS	−0.22	NS	−0.14
MARVELD3	.0156	1.25	.011	2.19	NS	−0.35	NS	−0.01
E-cadherin	.0234	1.31	NS	0.41	NS	−0.16	NS	−0.40
Claudin 5	NS	−0.34	NS	−0.13	.0046	−0.7	.007	−0.73