# *Staphylococcus aureus* biofilm exoproteins are cytotoxic to human nasal epithelial barrier in chronic rhinosinusitis

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***Background:*** Chronic rhinosinusitis patients (CRS) suffer from chronic inflammation of the sinus mucosa associated with chronic relapsing infections. Mucosal biofilms, associated with *Staphylococcus aureus*, have been implicated as a cause. We compared the effect of exoproteins secreted from clinical isolates of *S aureus* from CRS patients in planktonic and biofilm form on the nasal epithelial barrier.

***Methods:*** Clinical *S aureus* isolates from 39 CRS patients were grown in planktonic and biofilm forms and their exoproteins concentrated. These were applied to primary human nasal epithelial cells grown at the air-liquid interface. Transepithelial electrical resistance, permeability of flourescein isothiocyanate-dextrans, and cytotoxicity were measured. Structure and expression of tight junctions zona occludens-1, and claudin-1 proteins were assessed by electron microscopy and immunofluorescence. The Wilcoxon signed rank test was used for statistical analyses.

***Results:*** *S aureus* biofilm exoproteins showed dose- and time-dependent reduction of transepithelial electrical resistance, increased cell toxicity, and increased permeability ($p < 0.001$) compared with equal concentrations of planktonic cultures. Discontinuity in zona occludens-1 and claudin-1 immunofluorescence was confirmed as disrupted tight junctions on electron microscopy.

***Conclusion:*** *S aureus* biofilm exoproteins disrupt the mucosal barrier structure in a time- and dose-dependent manner and are toxic. Damage to the mucosal barrier by *S aureus* biofilm exoproteins may play a major role in CRS etiopathogenesis. 

*Key Words:*
biofilm; chronic rhinosinusitis; claudin-1; mucosal barrier; *Staphylococcus aureus* exoproteins; tight junction; transepithelial electrical resistance; zona occludens-1

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Chronic rhinosinusitis (CRS) is defined as an inflammation of the mucosal lining of the nose and paranasal sinuses for >12 weeks.[1] The financial burden due to CRS, both from direct health expenditure and loss of productivity, is significant, as is its negative impact on quality of life.[2] *Staphylococcus aureus* is the most commonly isolated pathogenic bacteria in CRS patients[3–5]; however, *S aureus* sinonasal colonization can also occur in patients without CRS.[6,7] Phenotype switching in the small-colony variant form and the ability to form *S aureus*–dominant biofilms have nevertheless been linked to CRS disease recalcitrance.[8–10] It is thought that strain-specific differences in toxin and enzyme production could account for the variation in *S aureus* pathogenicity and type of inflammation.[11] Such virulence factors can influence the inflammatory process in different ways. These include allowing adherence and invasion into host cells enabling colonization and infection,[12,13] directly influencing and driving the inflammatory process,[11] or affecting the mucosal barrier structure and function.[14–16]

Biofilms enable the bacteria to survive within a self-produced matrix consisting of extracellular polymeric substances (EPS), consisting of proteins, polysaccharides, and extracellular DNA. These viscoelastic biopolymers

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facilitate attachment to inert or mucosal surfaces, enable intercellular communication and exchange of genetic information, and shield the bacteria from a hostile environment.[17] This makes the bacteria within biofilms less susceptible to antibiotics than their planktonic forms. Bacteria within the biofilm differ from their planktonic counterparts phenotypically and genotypically,[18,19] with enrichment of many proteins involved in the pathogenesis of inflammation in biofilm EPS.[20]

Bacterial products from different pathogens including *S aureus* have been shown to negatively affect mucosal barrier structure and function.[14–16,21] Tight junctions (TJs), cell adhesion complexes between epithelial cells composed of occludin, junction adhesion molecules, and zonula occludens (ZO) proteins, are necessary to maintain normal epithelial barrier function.[22] Hyperpermeability of the epithelial barrier is believed to allow submucosal penetration of antigens, leading to an exacerbated immune response.[23,24] This is the premise of the immune barrier hypothesis as a cause of CRS, and is also seen in other inflammatory conditions such as asthma, atopic dermatitis, inflammatory bowel disease, and allergic rhinitis.[25]

Despite evidence that *S aureus* is the most common organism in CRS,[26,27] that *S aureus* biofilms are associated with CRS disease recalcitrance,[9,10,28] and that *S aureus*–secreted products negatively affect the mucosal barrier,[14–16] the effect of biofilm exoproteins on the barrier structure and function and how that compares with exoproteins from planktonic forms is unclear and yet to be explored.

To address these issues, our study aimed to determine the effects of *S aureus* exoproteins from matched biofilm and planktonic clinical isolates from CRS patients on the nasal epithelial barrier.

## Materials and methods

### *S aureus* clinical isolate collection and clinical parameters

Our study was performed at The Queen Elizabeth Hospital in Adelaide, SA, with ethics approval and written consent obtained before the study for the use of *S aureus* clinical isolates (HREC/18/CALHN/69) and for the collection of primary human nasal epithelial cells (HNECs). *S aureus* clinical isolates were obtained from patients with CRS at the time of endoscopic sinus surgery and isolated by an independent pathology laboratory (Adelaide Pathology Partners, Adelaide, SA) and then stored at -80°C. CRS patients fulfilled the diagnostic criteria for CRS according to the recent position papers by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement (EPOS)[29] on CRS. Patients with CRS were further subclassified according the absence (CRSsNP) or presence (CRSwNP) of nasal polyps as, defined by the EPOS guidelines.[1] Clinical data from the patients were collected prospectively including age, gender, and history of asthma. Disease severity was measured based on completion of the preoperative patient-reported 22-item Sino-Nasal Outcome Test (SNOT-22) questionnaire,[30] the disease-specific 5-question-based Adelaide Severity score (ADS),[31] and the objective computed tomography (CT)-scan measure of Lund-Mackay score (LMS).[32]

### *S aureus* culture and collection of exoproteins

Clinical isolates of *S aureus* were streaked onto nutrient agar (Oxoid, UK) from glycerol stocks, cultured in nutrient broth (NB), and incubated on a shaking platform (180 revolutions per minute [rpm]) at 37°C for 14 to 16 hours. The next day, fresh NB was inoculated to a starting optical density of 0.01 at 600 nm. Growth was followed by measuring optical density on an hourly basis. At 6 hours[33,34] (late exponential phase of growth) liquid cultures were centrifuged at 4°C for 10 minutes at 1500*g*. Supernatants were removed and passed through a 0.22-$\mu$m syringe filter followed by passage through a 3-kDa filter to collect exoproteins (Pierce Protein Concentrator, Thermo Fisher Scientific, Rockford, IL), as described elsewhere.[35] Exoproteins were stored at -80°C for further analysis. Biofilm cultures for the same clinical isolates were set up with NB in 6-well plates after adjusting opacity to $1.0 \pm 0.1$ MacFarland unit and incubating at 37°C for 48 hours[36,37] on a rotating platform (3D Gyratory Mixer; Ratek Instruments, Boronia, Australia) at 70 rpm. Supernatants collected from the biofilm forms were centrifuged according to the same protocol used for the planktonic forms.

### Nano Orange protein assay

A protein quantitation kit (Nano Orange Protein Quantitation Kit, Molecular Probes, Eugene, OR) was used for the protein quantification for both planktonic and biofilm forms in black-walled 96-well plates, as per the manufacturer's instructions. Fluorescence was measured with excitation at 485 nm and emission at 590 nm using the plate reader (FLUOstar Optima; BMG Lab Tech, Ortenberg, Germany).

### Human nasal epithelial cell harvesting and culture

Nasal brushings were collected during surgery from patients with CRS from the inferior turbinate using cytology brushes (McFarlane Medical Equipment, Surrey Hills, VIC, Australia) as described elsewhere.[38] The nasal brushings were transported using basal medium (PneumaCult Ex Plus; STEMCELL Technologies, Cambridge, UK). Processing of the cells from the brushes was performed after centrifugation of the brushes at 300*g* for 5 minutes at 4°C and suspending in 2 mL of basal medium (PneumaCult™ Ex Plus). The pellet was resuspended using a 10-mL syringe to create a single-cell suspension in a Petri dish containing anti-CD68 antibody (Dako, Carpinteria, CA) to deplete the cell suspension of monocytes. HNECs were expanded on type 1 collagen–coated T25 flasks (Thermo Fisher Scientific, Waltham, MA) and incubated at 37°C with 5% $CO_2$.[39]

**TABLE 1.** Demographics of *Staphyloccocus aureus* clinical isolates

| ID | Age(years) | Sex | Polyp status | Asthma | SNOT-22 | Lund-Mackay score | Adelaide Disease Severity Score |
|---|---|---|---|---|---|---|---|
| CL1 | 52 | M | CRSsNP | Positive | 38 | 21 | NA |
| CL2 | 29 | F | CRSsNP | Negative | NA | 4 | NA |
| CL3 | 73 | F | CRSwNP | Negative | 25 | NA | 13 |
| CL4 | 59 | M | CRSwNP | Positive | 34 | 19 | 12 |
| CL5 | 50 | F | CRSwNP | Negative | 63 | 14 | 16 |
| CL6 | 50 | M | CRSwNP | Positive | 36 | 18 | 14 |
| CL7 | 58 | M | CRSwNP | Positive | 78 | NA | 18 |
| CL8 | 64 | F | CRSwNP | Positive | 34 | 19 | 25 |
| CL9 | 48 | F | CRSsNP | Negative | 76 | 10 | NA |
| CL10 | 58 | M | CRSwNP | Positive | NA | NA | NA |
| CL11 | 37 | F | CRSwNP | Positive | NA | 20 | NA |
| CL12 | 56 | M | CRSwNP | Negative | NA | 12 | NA |
| CL13 | 75 | F | CRSwNP | Positive | 49 | 18 | 19 |
| CL14 | 54 | M | CRSsNP | Negative | 46 | 3 | 18 |
| CL15 | 60 | M | CRSwNP | Positive | NA | 19 | NA |
| CL16 | 76 | M | CRSsNP | Negative | NA | 7 | NA |
| CL17 | 74 | M | CRSsNP | Negative | 67 | NA | 19 |
| CL18 | 40 | F | CRSwNP | Positive | 44 | 23 | 19 |
| CL19 | 47 | M | CRSwNP | Positive | 36 | NA | 7 |
| CL20 | 77 | F | CRSsNP | Positive | NA | NA | NA |
| CL21 | 55 | F | CRSwNP | Positive | 79 | 18 | 19 |
| CL22 | 76 | M | CRSwNP | Negative | 15 | 19 | 13 |
| CL23 | 77 | M | CRSsNP | Negative | NA | 5 | NA |
| CL24 | 29 | F | CRSwNP | Positive | NA | 11 | NA |
| CL25 | 54 | M | CRSsNP | Negative | 65 | 14 | 17 |
| CL26 | 49 | M | CRSsNP | Positive | 46 | 13 | 12 |
| CL27 | 45 | F | CRSsNP | Negative | NA | 15 | NA |
| CL28 | 70 | M | CRSwNP | Positive | NA | 14 | NA |
| CL29 | 35 | F | CRSwNP | Negative | 33 | 17 | 8 |
| CL30 | 73 | M | CRSsNP | Negative | NA | 13 | NA |
| CL31 | 39 | M | CRSsNP | Negative | 32 | NA | 8 |
| CL32 | 79 | M | CRSsNP | Negative | NA | 13 | NA |
| CL33 | 61 | F | CRSwNP | Negative | NA | 18 | NA |
| CL34 | 57 | M | CRSwNP | Negative | 36 | NA | 8 |
| CL35 | 89 | F | CRSwNP | Negative | 38 | 15 | 18 |
| CL36 | 77 | M | CRSwNP | Positive | 6 | NA | 4 |

(*Continued*)

**TABLE 1.** Continued

| ID | Age(years) | Sex | Polyp status | Asthma | SNOT-22 | Lund-Mackay score | Adelaide Disease Severity Score |
|---|---|---|---|---|---|---|---|
| CL37 | 77 | M | CRSwNP | Positive | 43 | 22 | 17 |
| CL38 | 74 | M | CRSsNP | Negative | NA | 1 | NA |
| CL39 | 37 | F | CRSwNP | Negative | 46 | NA | 14 |

CL = clinical isolate; CRSsNP = chronic rhinosinusitis without nasal polyps; CRSwNP = chronic rhinosinusitis with nasal polyps; NA = not available.

## Air-liquid interface culture

HNECs reaching 80% to 90% confluence were maintained at air-liquid interface (ALI) using an ALI culture method (PneumaCult™ Ex Plus) as described elsewhere.[40,41] Transwells, 6.5 mm and permeable (Costar), were seeded with 70,000 cells in 100 $\mu$L of PneumaCult-EX medium (in the apical chamber) and 500 $\mu$L of Pneuma Cult-EX growth medium were added into the basal chambers. The Transwells were incubated at 37°C with 5% $CO_2$. The medium in the apical chambers was removed after 72 hours

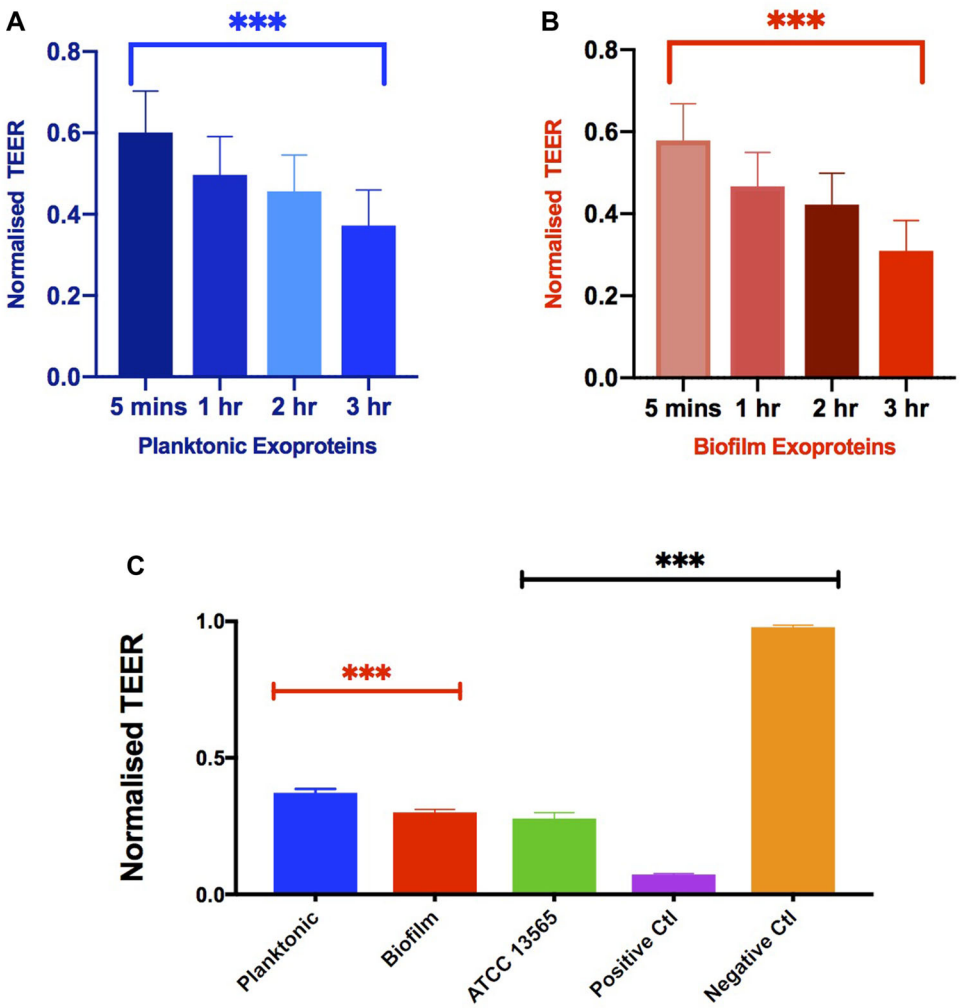


**FIGURE 1.** Time-dependent decrease in TEER on addition of *S aureus* exoproteins over 3 hours.
Exoproteins from 39 clinical isolates planktonic forms (A) and biofilm forms (B) were added to HNEC-ALI cultures at a concentration of 20 $\mu$g/mL. TEER was measured at baseline and 5 minutes and 1, 2, and 3 hours. TEER values were normalized to values at baseline with significant reductions at 5 minutes and a further reduction at 3 hours for both planktonic and biofilm exoproteins. Mean normalized TEER measurements at 3 hours were lower for biofilm exoproteins than for planktonic exoproteins (C). Planktonic ATCC 13565 exoproteins and positive control (2% Triton-X 100) reduced TEER compared with negative control (undiluted nutrient broth). TEER measurements are expressed as mean ± SEM. *** in (A) and (B) and *** in (C) $p < 0.001$, Wilcoxon signed rank test; ***$p < 0.001$, Mann-Whitney *U* test in (C). HNEC-ALI = human nasal epithelial cells grown at air-liquid interface; SEM = standard error of the mean; TEER = transepithelial electrical resistance.

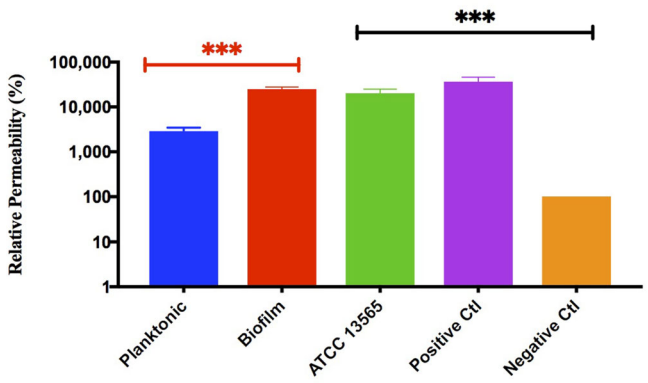


**FIGURE 2.** Paracellular permeability of HNEC-ALI cultures exposed to *S aureus* exoproteins. Equal concentrations of planktonic and biofilm exoproteins from *S aureus* CIs (n = 39) and planktonic ATCC 13565 as well as positive control (2% Triton-X 100) caused a significant increase in permeability of FITC-dextrans relative to the negative control (undiluted nutrient broth). Alterations in paracellular permeability to FITC-dextrans reported as mean ± SEM. $^{***}p < 0.001$, Wilcoxon signed rank test; $^{***}p < 0.001$, Mann-Whitney *U* test. CI, clinical isolate; FITC = fluorescein isothiocyanate; SEM = standard error of the mean.

and 500 $\mu$L of PneumaCult-ALI differentiation medium (STEMCELL Technologies) was added to the basal chamber. PneumaCult-ALI differentiation medium was changed from the basal chamber every second day. HNECs at the ALI (HNEC-ALI) were maintained for 4 to 6 weeks for development of TJs.

### Transepithelial electrical resistance

Transepithelial electrical resistance (TEER) was measured from the apical chambers using the EVOM2 epithelial volt-ohmmeter (World Precision Instruments, Sarasota, FL). PneumaCult-ALI medium (100 $\mu$L) was added into the apical chamber to produce an electrical circuit transmitted from the apical cells to the basal chambers. Those cells that were mature and with resistance measuring >1000 $\Omega/cm^2$ were considered appropriate for the experiment. ALI cultures were rinsed with phosphate-buffered saline (PBS) to remove all accumulated soluble mucus, and baseline TEER (timepoint = 0) was recorded, after which the exoproteins were added and the TEER noted at different time intervals. First, optimization experiments were performed in triplicates using *S aureus* ATCC 13565 (American Type Culture Collection, Manassas, VA), known to disrupt the mucosal barrier. Filtered exoproteins at various concentrations (200, 100, 50, 20, 10, and 5 $\mu$g/mL) were applied to the HNEC-ALI cultures to determine the optimal concentration that produced a consistent decrease in TEER. For the remainder of the experiments, exoproteins were applied to the HNEC-ALI cultures at a concentration of 20 $\mu$g/mL of planktonic or matched biofilm protein, followed by measurement of TEER, paracellular permeability, and cell viability (lactate dehydrogenase assay [LDH]). A positive control of 2% Triton-X 100 and a negative control of undiluted NB were used. TEER was normalized against the negative control and expressed as ohms per square centimeter ($\Omega/cm^2$). TEER measurements were taken after 5 minutes and 1, 2, and 3 hours, each time placing the HNEC-ALI plate on a heating platform maintained at 37°C.

### Proteinase K and heat treatment of exoproteins

To confirm that the effects of the exoproteins on ALI cultures were indeed due to proteins, inactivation experiments were performed. Exoproteins of clinical isolates, both planktonic and biofilm forms, were subjected to heat treatment at 100°C for 30 minutes, after which they were allowed to cool to room temperature for 15 minutes and added to the ALI culture to document the effect on the TEER. To a separate aliquot of exoproteins, 1 mg/mL of proteinase K (Sigma-Aldrich, St Louis, MO) was added and incubated overnight at 37°C, followed by addition of 5 mmol/L phenylmethylsulfonylfluoride (Sigma-Aldrich) to inactivate the activity of the enzyme before addition to ALI cultures. A separate control, replacing proteinase K with PBS, was included along with a positive control (2% Triton-X 100) and a negative control (undiluted NB).

### Permeability assay

The paracellular permeability across the HNEC-ALI cultures was measured by adding a 4-kDa fluorescein isothiocyanate (FITC)-labeled dextran (Sigma-Aldrich). After exoprotein treatment of the cells for 3 hours, the upper chambers were filled with 0.3 mg/mL FITC-dextran in 100 $\mu$L of PneumaCult-ALI medium and incubated for 2 hours at 37°C. The amount of dextran present in the basolateral compartment was measured using a 96-well fluorescence microplate reader (FLUOstar Optima; BMG Labtech, Ortenberg, Germany) with excitation and emission wavelengths of 485 and 520 nm, respectively.

### Lactate dehydrogenase assay

Cell viability of the HNEC-ALI cells was determined using a cytotoxicity detection kit (Promega CytoTox 96; Promega, Fitchburg, WI) after 20 $\mu$g/mL of exoprotein treatment of the cells for 3 hours. LDH (50 $\mu$L) reagent was added to 50 $\mu$L of the ALI supernatants in a 96-well clear bottom plate and incubated for 30 minutes at 37°C after adding 50 $\mu$L of stop solution to each well. The absorbance was read using a plate reader (FLUOstar Optima) at 490 nm. The results were calculated as a percentage viability relative to the negative control.

### Immunofluorescence of claudin-1 and ZO-1

After 20-$\mu$g/mL exoprotein treatment of the cells for 3 hours HNEC-ALI cultures were fixed using 2.5% formalin in phosphate-buffered saline (PBS) for 15 minutes, permeated using 0.1% Triton X-100 in PBS, and blocked using serum-free blocker (SFB; Dako, Glostrup, Denmark) at room temperature (RT) for 60 minutes. Culture support membranes were incubated overnight with 10 $\mu$g/mL claudin-1 rabbit polyclonal antibody and 5 $\mu$g/mL ZO-1 mouse monoclonal antibodies (both Invitrogen, Carlsbad,

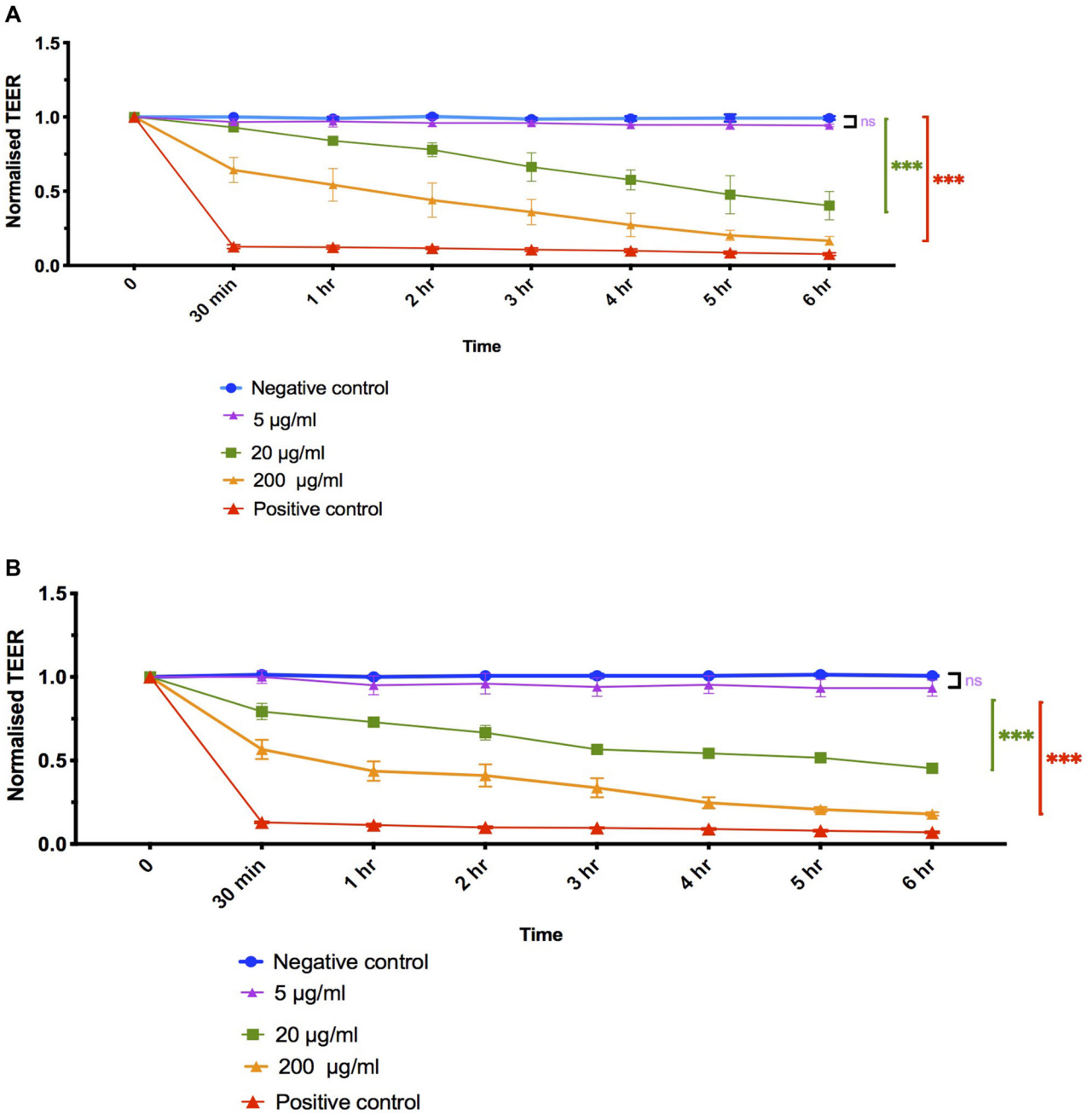


**FIGURE 3.** Dose-dependent reduction in TEER on addition of *S aureus* exoproteins. The positive control was 2% Triton-X 100 and negative control was undiluted nutrient broth. TEER measurements expressed as mean ± SEM for 3 biologically independent clinical isolates in 3 replicates each (A) (planktonic) and (B) (biofilm). $^{***}p < 0.001$, Mann–Whitney *U* test. ns = not significant; TEER = transepithelial electrical resistance.

CA) diluted in Tris-buffered saline with 0.5% Tween (TBST) and 10% SFB (both Invitrogen). Cells were washed 2 × 10 minutes with TBST followed by addition of 2 $\mu$g/mL anti-mouse Cy3 and anti-rabbit Alexa 488–conjugated secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) for 60 minutes at RT. After the third wash 200 ng/mL of 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) was added and incubated for 10 minutes at RT in a humid chamber, protected from light to visualize the nucleus. Membranes were mounted onto a glass slide using Anti-Fade medium (Dako), and imaging was performed using a confocal laser scanning microscope (LSM700; Carl Zeiss, Jena, Germany).

## Transmission electron microscopy

### *Fixation and embedding of HNEC-ALI cells*

HNEC-ALI cells on culture support membranes were fixed by adding 1.25% glutaraldehyde and 4% paraformaldehyde in PBS containing 4% sucrose to both apical (0.5 mL) and basal (1.5 mL) chambers and incubated overnight at 4°C. Cells were washed in PBS containing 4% sucrose

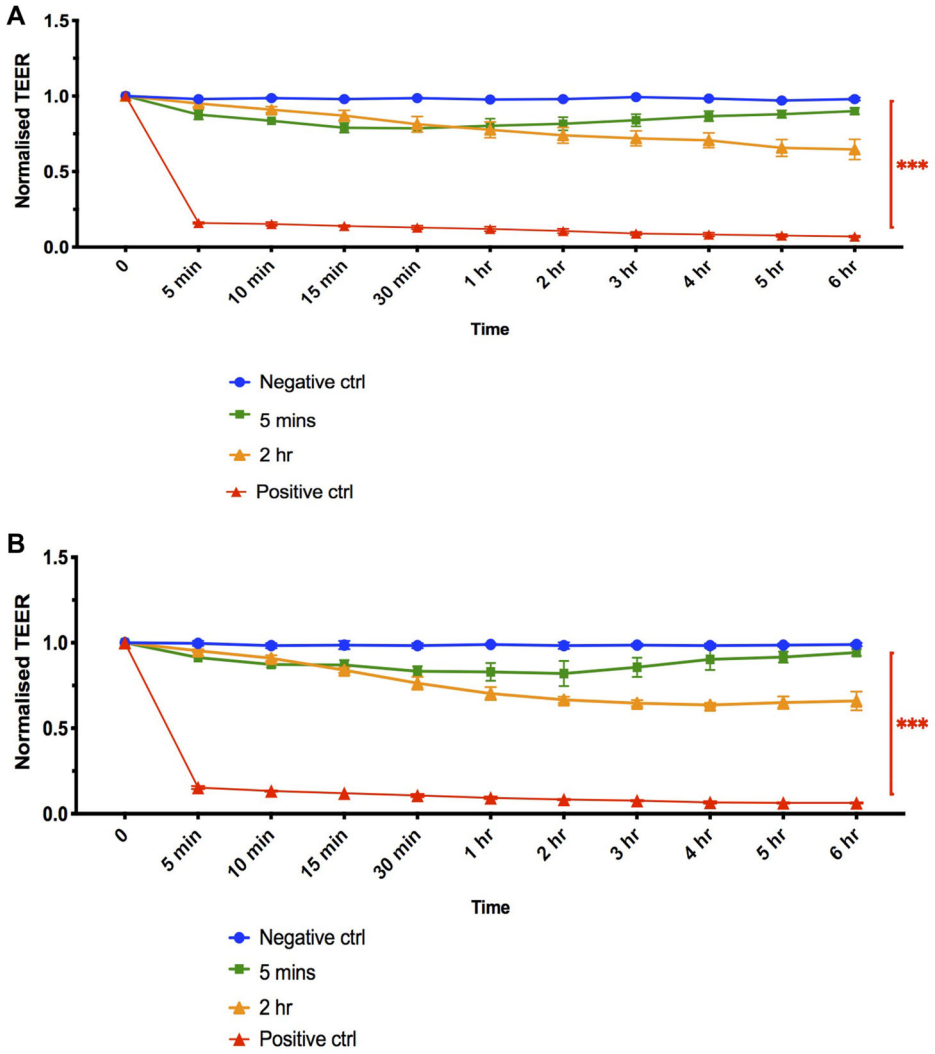


**FIGURE 4.** Recovery in TEER after short but not prolonged exposure to *S aureus* exoproteins. *S aureus* exoproteins (20 $\mu$g/mL) from planktonic (A) and biofilm (B) forms were added at time = 0 and removed after 5 minutes (green line) or 2 hours (red line), followed by measuring TEER to 6 hours; positive control is 2% Triton-X 100 and negative control is undiluted nutrient broth. TEER expressed as mean ± SEM for 3 biologically independent clinical isolates in 3 replicates, normalized to time = 0. Significance denoted as $^{***}p < 0.001$ (Mann-Whitney *U* test). SEM = standard error of the mean; TEER = transepithelial electrical resistance.

and postfixed in 2% osmium tetroxide ($OsO_4$) for 1 hour (ProSciTech, Kirwan, QLD, Australia). The wash step was repeated, and cells were dehydrated through a graded ethanol series (70%, 90%, 100%, 3 × 10 minutes each, and final 20 minutes in 100% ethanol). After dehydration, the cell monolayers were infiltrated with 1:1 ethanol (100%):resin (100%, Epon-Araldite) overnight and 100% resin (3 × 3 hours). The transmembrane was embedded in fresh resin and polymerized at 70°C for 24 hours.

### *Sectioning and imaging*

Areas of interest were noted, and 1-$\mu$m sections were made followed by ultrathin (70-nm) sections. These were placed on 200-mesh copper grids (ProSciTech). Sections were stained with uranyl acetate (4%) followed by Reynolds lead citrate (ProSciTech), each for 10 minutes. Transmission electron microscopy (FEI Tecnai G2 Spirit, Hillsboro, OR) was used to image TJs between cells in control and experimental (planktonic and biofilm) treatments.

## Statistical analysis

Mean results of TEER, FITC-dextran permeability assays, and LDH measurements were compared between the planktonic and biofilm *S aureus* treatments using a paired Wilcoxon signed rank test. Comparison of (nonpaired) means of standard negative control, positive control, and standard ATCC planktonic samples were compared against the remainder of the groups using a pairwise nonparametric Mann-Whitney *U* test with the Benjamini-Hochberg false discovery rate (FDR) correction. All statistics were performed using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, CA). Statistical significance was taken at the traditional $\alpha = 0.05$ level.

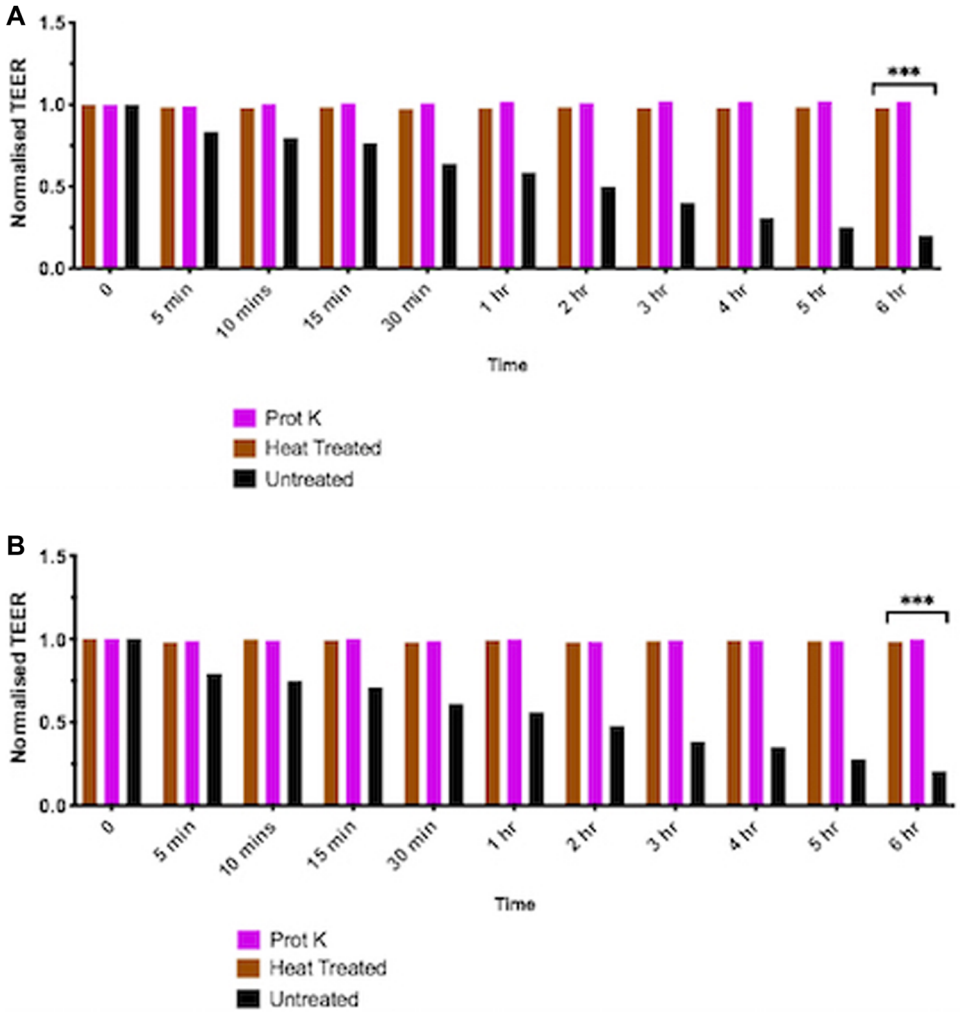


**FIGURE 5.** Effect of proteinase K and heat inactivation of *S aureus* planktonic and biofilm exoproteins on their barrier disrupting ability. HNEC-ALI cultures were treated with exoproteins (20 $\mu$g/mL) from planktonic (A) and biofilm (B) forms of *S aureus* left untreated or exposed to 100°C for 30 minutes, to proteinase K, positive control was 2% Triton-X 100 and negative control was undiluted nutrient broth. The TEER was measured at 5, 10, 15, and 30 minutes and 1, 2, 3, 4, 5, and 6 hours. Values are shown as mean ± SEM for 3 biologically independent clinical isolates. Significance denoted as $^{***}p < 0.001$ (Mann–Whitney *U* test). HNEC-ALI = human nasal epithelial cells air-liquid interface; SEM = standard error of the mean; TEER = transepithelial electrical resistance.

# Results

## Clinical characteristics

A total of 39 *S aureus* clinical isolates (CIs) from CRS patients were used in the study, including 16 women and 23 men and with a mean age of 58 (range, 29-89) years. The patient cohort consisted of 23 patients with CRSwNP and 16 with CRSsNP. The symptoms scores (SNOT-22 and ADS) and the LMS for each group are summarized in Table 1

## *Staphylococcus aureus* exoproteins reduce the transepithelial electrical resistance of HNEC-ALI cultures

For uniformity, the HNEC-ALI cells used in the experiments were obtained from CRSsNP patients (n = 7; age range, 35-75 years) without a history of smoking, asthma, or allergy. The TEER was measured at time = 0 and at 5 minutes and 1, 2, and 3 hours after adding 20 $\mu$g/mL of *S aureus* exoproteins of matched planktonic and biofilm forms to HNEC-ALI cultures. Both biofilm- and planktonic-derived *S aureus* exoproteins showed an immediate reduction in TEER values within 5 minutes of application ($p < 0.001$, one-sample Wilcoxon signed rank test). There was a further time-dependent decrease in TEER over 3 hours (Friedman with post hoc Wilcoxon test, multiple comparisons $p < 0.001$, original FDR method of Benjamini and Hochberg) (Fig. 1A and B).

Compared with negative control, both the planktonic and biofilm exoproteins caused a significant decrease in normalized (to time = 0) TEER values after 3 hours from 0.97 $\Omega/cm^2$ in the negative control to 0.37 and 0.29 $\Omega/cm^2$ in planktonic and biofilm forms, respectively ($p < 0.001$, Mann-Whitney *U* test). Two percent Triton-X 100 along with 20 $\mu$g/mL planktonic ATCC 13565 exoproteins, known to disrupt the mucosal barrier, were used as positive control and significantly reduced TEER levels compared with negative control, as expected.[35,42] Compared with

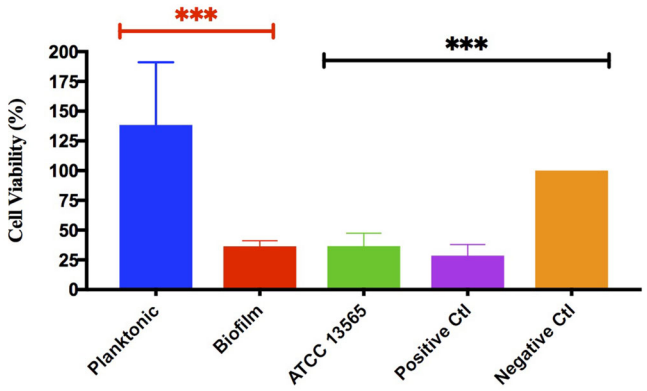


**FIGURE 6.** Cytotoxic effects of *S aureus* exoproteins on HNEC-ALI cultures. Exoproteins from *S aureus* planktonic and biofilm forms (n = 39), ATCC 13565 planktonic exoproteins, positive control (2% Triton-X 100), and negative control (undiluted nutrient broth) were applied for 3 hours to HNEC-ALI cultures, followed by measuring cell viability using an LDH assay. Cell viability is expressed as relative to untreated cells (negative control set at 100%). ATCC = American Type Culture Collection; HNEC-ALI = human nasal epithelial cells air-liquid interface; LDH = lactate dehydrogenase. $^{***}p < 0.001$, Wilcoxon signed rank test; $^{***}p < 0.001$, Mann-Whitney *U* test.

exoproteins from planktonic cultures, equal concentrations of exoproteins from matched biofilm cultures significantly reduced TEER readings ($p < 0.001$) (Fig. 1C).

### *S aureus* exoproteins increased paracellular permeability of FITC-dextrans

HNEC-ALI cultures were then treated with *S aureus* exoproteins of planktonic and biofilm forms for 3 hours, followed by measuring the paracellular permeability of FITC-dextrans. Compared with the negative control, results showed a significant increase in permeability induced by exoproteins from planktonic (29-fold) and biofilm (250-fold) forms ($p < 0.001$, Wilcoxon rank sum) as well as positive control (360-fold) and planktonic ATCC 13565 (200-fold) forms ($p < 0.001$; Mann-Whitney *U* test). Biofilm exoproteins significantly increased the relative permeability of FITC-dextrans compared with their planktonic counterparts ($p < 0.001$) (Fig. 2).

### Dose-dependent reduction in TEER

To assess the dose-dependent effect of the exoproteins on TEER, exoproteins from 3 *S aureus* CIs in planktonic and biofilm form were applied at 5, 20, and 200 $\mu$g/mL for up to 6 hours. The results show that the lowest concentration of 5 $\mu$g/mL did not cause a reduction in TEER. Concentrations of 20 and 200 $\mu$g/mL, however, caused a significant dose- and time-dependent reduction in TEER values (Fig. 3A and B for planktonic and biofilm forms, respectively).

### Prolonged exposure leads to increased loss in TEER of HNEC-ALI cultures

To assess the reversibility of the effect of exoproteins on TEER measurements, exoproteins from 3 CIs in planktonic and biofilm forms were added to HNEC-ALI cultures at a concentration of 20 $\mu$g/mL for different exposure times (5 minutes and 2 hours) followed by measuring TEER for up to 6 hours. TEER measurements at the 6-hour timepoint normalized to t = 0 showed a significant reduction ($p < 0.001$, Mann-Whitney *U* test) in HNEC-ALI cultures exposed to exoproteins for 2 hours compared with those exposed for 5 minutes (Fig. 4A and B for planktonic and biofilm forms). After a short 5-minute exposure time to exoproteins from planktonic cells and biofilms, TEER values decreased further with a maximal reduction after 15 minutes and 2 hours for the planktonic and biofilm exoproteins, respectively. TEER values reversed to back to normal at the 6-hour timepoint for both planktonic and biofilm exoproteins, with no significant differences between TEER values of short 5-minute *S aureus* exoprotein–challenged cells and control cells. In contrast, there was no evidence of recovery of TEER values at the 6-hour timepoint after prolonged exposure times of 2 hours.

### Proteinase K and heat treatment abort the TEER-reducing effects

To confirm that the detrimental effect of the exoproteins on the mucosal barrier was in fact due to proteinaceous particles, exoproteins were inactivated by heat or proteinase K treatment. The TEER-reducing effect on the ALI cultures was lost when exoproteins from planktonic forms and biofilms were heat treated to 100°C for 30 minutes or incubated overnight at 37°C with proteinase K (Fig. 5A and B). Exoproteins incubated overnight in PBS retained their ability to reduce the TEER on the HNEC-ALI culture, similar to the effect seen with the untreated exoproteins.

### *S aureus* biofilm exoproteins decrease cell viability

The cytotoxic effect of *S aureus* exoproteins (n = 39) on HNECs was assessed by measuring the concentration of LDH in the medium 3 hours after application of the exoproteins. Planktonic exoproteins did not significantly affect the cell viability of HNEC-ALI cultures compared with negative control. In contrast, biofilm exoproteins significantly reduced the viability of HNEC-ALI cultures compared with their planktonic counterparts and compared with the negative control (Fig. 6).

### Exoproteins from *S aureus* cause disruption of TJ proteins ZO-1 and claudin-1

IF staining and confocal laser scanning microscopy were used to assess the localization of zona occludens-1 (ZO-1) and claudin-1 after exposing the HNEC-ALI cultures to the exoproteins from biofilm and planktonic forms for 3 hours. In control cells, ZO-1 and claudin-1 showed clear colocalization, but application of planktonic exoproteins reduced the colocalization of both proteins. Application of biofilm exoproteins induced a complete degradation of these TJ proteins compared with negative control NB, as shown in Figure 7.

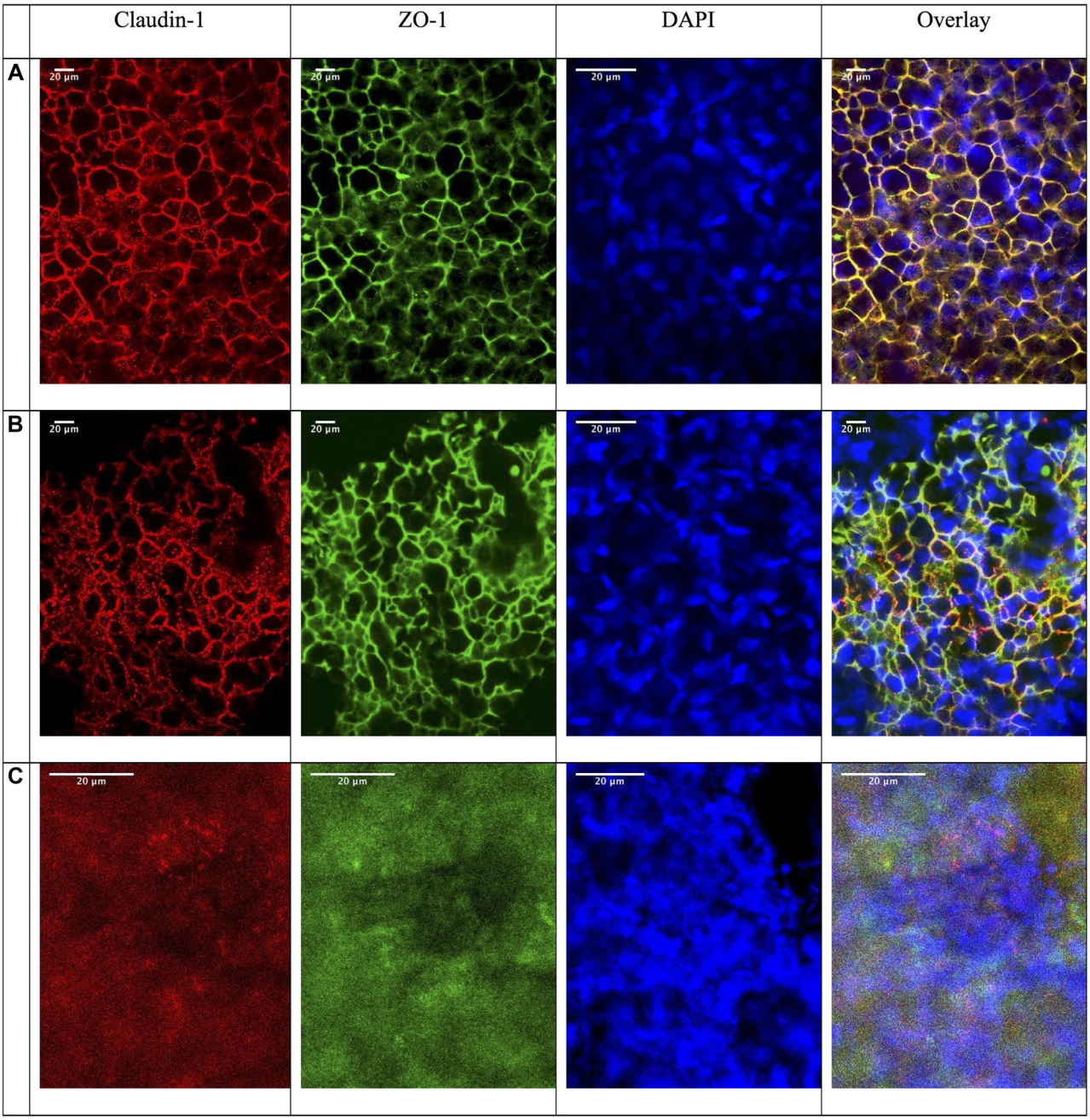


**FIGURE 7.** Representative confocal scanning laser microscopy images. Control cells (A) showed a consistent colocalization of claudin-1 (red) and ZO-1 (green) proteins evidenced by orange color in the overlay. In contrast, cells exposed to *S aureus* planktonic exoproteins (B) showed reduced colocalization of both proteins (mixed green, orange, and red colors in overlay). Cells exposed to *S aureus* biofilm exoproteins (C) showed a complete degradation of ZO-1 and claudin-1 with absence of distinct immunostaining patterns. DAPI stains nuclei blue. All images were viewed with 20× objective power (scale bar = 20 $\mu$m). DAPI = 4′,6-diamidino-2-phenylindole.

### Transmission electron microscopy shows disruption of TJs

The apical junctional complexes of the negative controls were seen as "kissing" cells, illustrating the close contact of the apical region of adjacent cells with normal and intact TJs (Fig. 8A). In contrast, HNECs exposed to planktonic *S aureus* exoproteins exhibited a gap between adjacent cells, characteristic of TJ disruption (Fig. 8B). HNECs exposed to the biofilm exoproteins exhibited severe destruction of the cell membrane with no trace of TJ or scattered cilia and loss of normal cell structures, with presence of cell death seen as reduced nuclear chromatin density (Fig. 8C).

## Discussion

This study is the first to use *S aureus* clinical isolates from CRS patients to demonstrate that *S aureus* exoproteins have dose- and time-dependent detrimental effects on

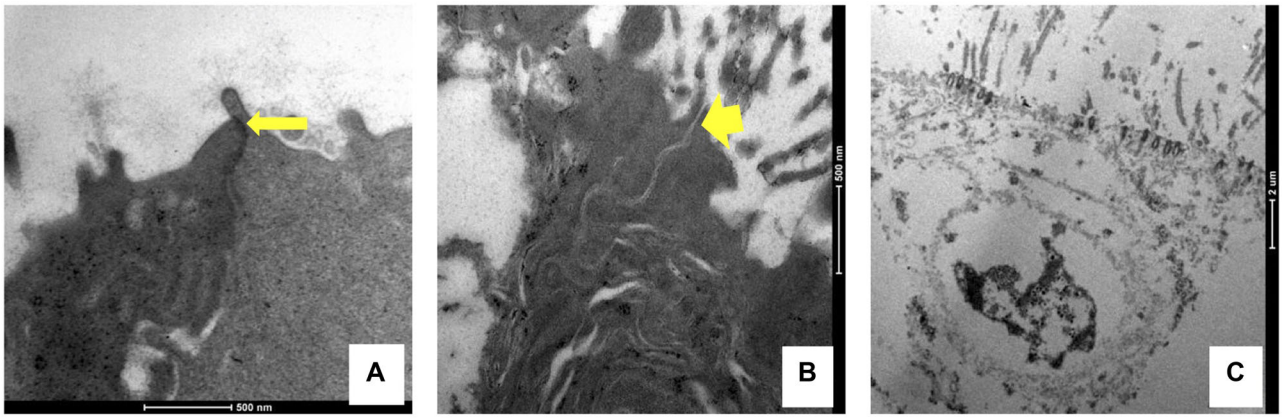


**FIGURE 8.** Representative transmission electron microscopic images of HNEC-ALI cultures exposed to control solution (A) planktonic exoproteins (B) and biofilm exoproteins (C). Tight junctions in cells exposed to control solution (kissing cells) [yellow arrow in (A), scale bar = 500 nm]. In cells exposed to planktonic exoproteins, disrupted tight junctions are seen as increased space between cells, noted as a white line extending between adjacent cells [yellow arrow in (B), scale bar = 500 nm]. HNEC-ALI–cultured cells treated with biofilm exoproteins show destruction of cell morphology along with nuclear chromatin condensation [in (C), scale bar = 20 $\mu$m]. HNEC-ALI = human nasal epithelial cells air-liquid interface.

the mucosal barrier structure of HNEC-ALI cultures in vitro. Our results indicate that, compared with *S aureus* planktonic exoproteins, equal concentrations of exoproteins derived from their biofilm counterparts have more profound detrimental effects on the mucosal barrier structure and function, and are cytotoxic.

Chronic relapsing bacterial infections are a hallmark of CRS. Oral antibiotics, often combined with topical rinses, form the cornerstone of CRS management, and in fact adult rhinosinusitis is the main diagnosis for which antibiotics are prescribed.[43] *S aureus* remains the most common pathogen isolated in CRS patients, particularly those with nasal polyps.[26,27] Recently, CRSwNP patients have been shown to have a defective mucosal barrier, with reduced expression of TJ proteins.[44,45] Interestingly, an association has also been demonstrated between *S aureus* infection in CRSwNP patients and decreased levels of tight junction proteins.[44] The respiratory epithelium protects the airway against allergens[46] and pathogens by creating a barrier held together by intercellular TJs. Destruction of these TJ proteins allow direct exposure of the submucosal tissue to antigens, thereby contributing to the development of inflammation. Hence, TJ dysfunction and loss of barrier integrity is thought to play a crucial role in the pathogenesis of CRS by forming a leaky junction between opposing cells.[47]

In line with our current findings, previous studies have shown exoproteins from *S aureus* planktonic cells and specific proteases disrupt the mucosal barrier of HNEC-ALI cultures.[14–16] However, those studies used laboratory strains and the relevance of those findings in strains that are isolated from the sinonasal cavities is unclear. Interestingly, although 5-$\mu$g/mL *S aureus* exoproteins did not affect the mucosal barrier, 20-$\mu$g/mL exoproteins harvested from all *S aureus* clinical isolates tested had acute detrimental effects on the mucosal barrier, regardless of whether those exoproteins were harvested from planktonic cells or biofilms. These effects were long-lasting and dose-dependent, and were abrogated with heat or proteinase K inactivation. These results indicate that *S aureus* clinical isolates from the sinonasal cavities of CRS patients universally secrete proteins that have strong immediate barrier disruptive effects. The identity of those proteins and their exact mechanism of action are not known and warrant further investigation.

Exoproteins express staphylococcal virulence[48] and induction of inflammation; however, this is not restricted to the effect of a single toxin. Rather, a repertoire of proteins, such as enterotoxins, hemolysins, and proteases,[49] are thought to contribute to the inflammation observed, but only some of these are likely contributing to the actual barrier disruption. This was also shown in our previous studies, where, from a number of toxins and virulence factors tested, only the V8 protease was shown to disrupt the integrity of HNEC-ALI cultures.[16] Interestingly, our results indicate planktonic exoproteins, at a concentration of 20 $\mu$g/mL, could disrupt the barrier without causing significant cytotoxicity. Those exoproteins induced a 29-fold increase in paracellular permeability of 4-kDa dextrans, indicating that antigens and proteins of similar size may traverse the mucosal layer in the presence of similar planktonic cell exoprotein concentrations. Exoproteins such as lipoteichoic acid (LTA) are released in substantial amounts during *S aureus* growth and infection with levels as high as 10 $\mu$g/mL present in wash-fluid samples of atopic dermatitis (AD) patients with levels correlating with *S aureus* colony-forming unit (CFU) counts and AD disease severity.[50] Hence, taking into consideration that LTA would be expected to be only one of many proteins present in *S aureus* exoproteins, the exoprotein concentrations found to affect the barrier in this study may be relevant also in vivo. This could occur, for example, during the course of acute *S aureus* infections, where relatively large quantities of exoproteins can be produced.[50]

*S aureus*, however, often resides within mucosal biofilms, protecting the bacteria from a hostile environment and

rendering them less susceptible to antibiotics. *S aureus* biofilms have therefore been associated with recurrence of disease and poor postoperative outcomes.[51,52] Although many studies have demonstrated an association between mucosal biofilms and CRS disease recalcitrance,[53] a causative link, their toxicity and how they interact with the host mucosa is largely unknown.

Our study indicates *S aureus* biofilm exoproteins had more profound effects on the mucosal barrier and were more toxic than equal concentrations of matched planktonic exoproteins, increasing the paracellular permeability >250-fold.

The increased permeability could at least in part be due to the cell toxicity induced by biofilm exoproteins. Together, these results indicate that qualitative differences exist between exoproteins secreted by planktonic cells and biofilms. Indeed, studies have shown that bacteria living in a biofilm form express a different set of genes and more virulence factors compared with their planktonic counterparts.[20,54] Further research is needed to determine the specific *S aureus* biofilm proteins that cause toxicity and disrupt the mucosal barrier and to evaluate how that relates to the extent and type of inflammation in the context of CRS. Antitoxin treatment and targeted barrier protection could be avenues of novel therapies in the treatment of *S aureus*–associated CRS.

## Conclusion

*S aureus* exoproteins produce a dose- and time-dependent detrimental effect on HNEC-ALI barrier structure and function. In comparison to planktonic exoproteins, biofilm exoproteins produced irreversible damage to the HNEC-ALI cells with increased paracellular permeability and decreased cell viability. Unraveling the identity and role of exoproteins produced by *S aureus* biofilms in the inflammatory process is crucial toward understanding the pathophysiology of CRS.

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