Title: *Staphylococcus aureus* enterotoxin B disrupts nasal epithelial barrier integrity

Running title: SEB impairs epithelial barrier integrity

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Abbreviations: ALI: air-liquid interface; CRSwNP: chronic rhinosinusitis with nasal polyps; SEB: Staphylococcus aureus enterotoxin B; TEER: trans-epithelial electrical resistance; TLR2: toll-like receptor 2; ZO-1: zonula occludens-1

Key words: Chronic rhinosinusitis; epithelial permeability; Staphylococcus aureus enterotoxin B; tight junction; toll-like receptor 2
Abstract

Background: *Staphylococcus aureus* colonization and release of enterotoxin B (SEB) has been associated with severe chronic rhinosinusitis with nasal polyps (CRSwNP). The pathogenic mechanism of SEB on epithelial barriers, however, is largely unexplored. **Objective:** We investigated the effect of SEB on nasal epithelial barrier function. **Methods:** SEB was apically administered to air-liquid interface (ALI) cultures of primary polyp and nasal epithelial cells of CRSwNP patients and healthy controls, respectively. Epithelial cell integrity and tight junction expression were evaluated. The involvement of toll-like receptor 2 (TLR2) activation was studied *in vitro* with TLR2 monoclonal antibodies and *in vivo* in *tlr2*−/− knock-out mice. **Results:** SEB applied to ALI cultures of polyp epithelial cells decreased epithelial cell integrity by diminishing occludin and zonula occludens (ZO)-1 protein expression. Antagonizing TLR2 prevented SEB-induced barrier disruption. SEB applied in the nose of control mice increased mucosal permeability and decreased mRNA expression of occludin and ZO-1, whereas mucosal integrity and tight junction expression remained unaltered in *tlr2*−/− mice. Furthermore, *in vitro* SEB stimulation resulted in epithelial production of IL-6 and IL-8, which was prevented by TLR2 antagonization. **Conclusion & Clinical relevance:** SEB damages nasal polyp epithelial cell integrity by triggering TLR2 in CRSwNP. Our results suggest that SEB might represent a driving factor of disease exacerbation, rather than a causal factor for epithelial defects in CRSwNP. Interfering with TLR2 triggering might provide a way to avoid the pathophysiological consequences of *S. aureus* on inflammation in CRSwNP.
Introduction

It is increasingly recognized that epithelial barrier function and innate immunity are fundamentally important in the pathogenesis of chronic rhinosinusitis (CRS). CRS affects approximately 15% of the general population, impairs quality of life and has a high socio-economic impact. Clinically, two phenotypes can be distinguished based on the presence (CRSwNP) or the absence (CRSsNP) of nasal polyps (NP). The airway epithelium protects the host from pathogens and other potential harmful molecules by creating a physical barrier, by secreting antimicrobial peptides and by mucociliary clearance of foreign molecules. Neighboring airway epithelial cells are connected to one another via apical junctional complexes, such as tight junctions (TJs) that restrict the paracellular transport of certain macromolecules and ions. TJs are composed of transmembrane proteins including the claudin family and occludin, and adaptor proteins such as zonula occludens (ZO) proteins, that seal the intercellular space of adjacent cells. To gain access to the subepithelial regions, pathogens need to breach the epithelial barrier by cleaving TJ proteins directly or indirectly. Loss of TJ function and/or expression has been observed in asthma, CRS and allergic rhinitis. Maintaining an intact epithelial barrier is, therefore, of fundamental importance to prevent the development and progression of disease.

Staphylococcus aureus is a common human microorganism often found in the normal nasal microbiota of healthy individuals and more frequently in patients with CRS. Under certain circumstances, S. aureus colonization of the nasal mucosa can facilitate its invasion into the subepithelial regions where S. aureus secretes proteins that act as superantigens to activate T and B cells. Superantigens like S. aureus enterotoxin B (SEB) promote the release of type 2 cytokines IL-4 and IL-13 by acting on Th 2 cells and promote eosinophilic infiltration. Several diseases such as asthma, atopic dermatitis and allergic rhinitis are associated with increased presence of S. aureus enterotoxins on skin or respiratory mucosa, respectively. In this regard, it is important to understand the interaction between S. aureus and the epithelial barrier. There is some evidence that S. aureus enterotoxins impact ciliary structure and function. Furthermore, it has been shown that SEB can activate toll-like receptor 2 (TLR2) and TLRs are innate receptors, essential in recognizing bacterial components to induce an appropriate immune response against the encountered microorganism. Activation of TLR2 triggers the production of pro-inflammatory cytokines such as IL-6 and IL-8, leading to mucosal inflammation and barrier...
dysfunction, illustrating a crucial role for TLR2 signaling in maintaining epithelial barrier homeostasis.

Nevertheless, the interaction between SEB and the epithelial barrier is not completely understood. In this paper, therefore, we examined the effect of SEB on epithelial barrier function in CRSwNP and whether TLR2 is involved in this process.
Materials and Methods

Study approval
All experiments were approved by the Medical Ethical Committee of the University Hospitals Leuven (S59865), and the Ethical Committee for Animal Research at the KU Leuven (P103/2013 and P150/2017).

Isolation of primary epithelial cells from nasal polyps and inferior turbinate
Nasal polyp tissue of patients with CRSwNP undergoing functional endoscopic sinus surgery or inferior turbinate of non-allergic of patients undergoing functional surgery was used for isolation of primary epithelial cells. Diagnosis of CRSwNP was made according to the European Position Paper on Rhinosinusitis and Nasal polyps24. Patient demographics are depicted in Supplementary table 1. A highly purified epithelial cell population was obtained as reported previously7. Briefly, inferior turbinates or nasal polyp tissues were enzymatically digested in 0.1% pronase (Protease XIV, Sigma, Belgium) solution in DMEM-F12 culture medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% Ultroser G (Pall Life Sciences, Belgium). After overnight incubation at 4°C while shaking, the protease reaction was stopped by the addition of 10% fetal bovine serum (FBS). Cells were washed in culture medium and pelleted by centrifugation for 5 min at 100 g. Cells were then resuspended in 10 ml culture medium and incubated in a plastic culture flask for one hour at 37°C to remove fibroblasts. The cell suspension was mixed with 2 x 10^7 prewashed CD45- and CD15-magnetic beads (Dynabeads®, Invitrogen, Merelbeke, Belgium) and epithelial cells were purified by negative selection following the manufacturer’s instructions.

Calu-3 epithelial cell line
Calu-3 epithelial cells were used for Western blot experiments. The cells were cultured in EMEM medium (ATCC, Molsheim Cedex, France), supplemented with 10% FCS, 1% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Calu-3 cells were plated in T75 culture flasks and were split 1/3 before reaching confluency using a 0.25% trypsin solution in EDTA (Sigma-Aldrich, St Louis, Missouri, USA). The culture medium was changed every other day. Calu-3 cells between passages 10-20 were used for experiments.
Air-liquid interface cultures

Primary epithelial cells from healthy controls and CRSwNP patients were grown in bronchial epithelial basal medium (Lonza, Basel, Switzerland) supplemented with the SingleQuot bullit kit, placed in a T75 culture flask at 37°C. Calu-3 epithelial cells were grown in EMEM medium (Lonza), supplemented with 10% FBS, 1% L-glutamine and 100U/ml penicillin, 100μg/ml streptomycin in a T75 culture flasks. Calu-3 cells between passages 10-20 were used during the different experiments. Once cells reached 75-80% confluency, cells were detached and were seeded on 0.4 μm polyester transwell inserts (Greiner Bio-One, Austria) at a density of 110,000 cells/well. Cells were grown submerged for 5-7 days until a confluent monolayer was formed. Afterwards, cells were placed in air-liquid interface (ALI) for 21 days for further differentiation. Nasal epithelial cells were cultured in DMEM-F12 culture medium supplemented with 100U/ml penicillin, 100μg/ml streptomycin, and 2% Ultroser G (Pall Life Sciences, Belgium). Medium of Calu-3 and nasal epithelial cells was changed every other day.

Trans-epithelial electrical resistance and paracellular flux measurements

At day 21 in ALI, epithelial integrity was evaluated by TEER measurements using an EVOM/Endohm (WPI Inc, Sarasota, USA). Fluorescein isothiocyanate dextran 4 kDa (FD4) (Sigma-Aldrich, St Louis, Missouri, USA) was used to measure epithelial permeability. FD4 (2 mg/mL) was added apically to the ALI cultures and the fluorescein intensity of basolateral fluid was measured using a fluorescence reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany). FD4 concentration was calculated and is expressed in pmol.

Stimulation experiments of primary nasal and Calu-3 epithelial cell cultures

ALI cultures of primary nasal epithelial cells or Calu-3 epithelial cells were stimulated at day 21 with 1 or 10 μg SEB (Sigma-Aldrich, St Louis, Missouri, USA) at the apical site for four hours. TEER and FD4 were measured every hour. After four hours, SEB was removed, fresh culture medium was added, and TEER was measured 24 hours later. The neutralizing TLR2 antibody (10 μg/ml, R&D Systems, Abingdon, United Kingdom) was added apically two hours prior to stimulation with 10 μg SEB. TEER and FD4 permeability were determined every hour. For each stimulus, TEER is represented as a percentage change to timepoint 0.

mRNA isolation and RT-qPCR

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Epithelial cells from ALI cultures or nasal mucosae from mice were homogenized in lysis buffer from the Qiagen Mini RNeasy kit (Maryland, USA). RNA was isolated with the Qiagen Mini RNeasy kit (Maryland, USA) and first-strand cDNA was reverse transcribed by SuperScriptII RT (Invitrogen, Carlsbad, CA, USA) using 1000 ng of total RNA for epithelial cells and 500 ng of total RNA from nasal mucosae according to manufacturer’s protocol. cDNA plasmid standards, consisting of purified plasmid DNA specific for each individual target, were used to quantify the target gene in the unknown samples, as described before\(^1\). The housekeeping genes to evaluate TJ expression in ALI cultures were β-Actin and β-2-microglobulin. The housekeeping genes to evaluate the TJ expression in nasal mucosae of mice were β-actin and ppia. The target genes for both ALI cultures and the nasal mucosae were occludin and ZO-1. The primer and probe sequences for the specific genes were determined in our laboratory using Primer Express (Applied Biosystems part of Thermo Fisher Scientific). RT-qPCR was performed in an CFX Connect (Bio-Rad Laboratories Inc, California, USA) for all genes with specific Taqman probes and primers and using Platinum® Quantitative PCR SuperMix-UDG w/ROX (Invitrogen part of Thermo Fisher Scientific, Merelbeke, Belgium). All probes were 5’FAM3’TAMRA labeled. Sequences for the probes and primers can be found in Supplementary table 2.

Western Blot

Stimulated Calu-3 epithelial cells were harvested four hours post-stimulation and proteins were isolated. Equal amounts of protein were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Membranes were incubated overnight with primary antibodies: rabbit polyclonal anti-claudin-1 (1:250; Abcam, Cambridge, UK) and rabbit polyclonal anti-occludin (1:200; Invitrogen). As protein-loading control, blots were stained with rabbit anti-β-Actin (1:5000; Abcam). Secondary antibody was peroxidase-conjugated goat anti-rabbit IgG (1:5000; Dako). Visualization was performed using chemiluminescence (Western Lightening, Perkin Elmer). Protein bands were quantified by densitometry using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/). The amount of occludin, phosphor-occludin and claudin-1 proteins is expressed relatively to β-Actin for the different stimuli.

Immunofluorescence staining of tight junctions

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ALI cultures were fixed with 4% paraformaldehyde (Sigma-Aldrich, St Louis, Missouri, USA) after four hours of stimulation. Epithelial cell cultures were blocked with goat serum in 1% BSA/PBS and stained for occludin (mouse anti-occludin antibody, Alexa Fluor 488, 100 mg/mL, Invitrogen) and ZO-1 (rabbit, anti–ZO-1 unlabeled antibody, Invitrogen), detected by using goat anti-rabbit antibody Alexa Fluor 555 (Invitrogen). Samples were mounted and stored at -20°C.

For immunofluorescence staining of the nasal mucosa of mice, paraffin-embedded tissue slides (5 µm) were subjected to antigen retrieval in citrate buffer. Antibodies used for immunofluorescence were anti-occludin (rabbit, polyclonal, 1/100, Invitrogen) and secondary antibody goat anti-rabbit Alexa Fluor 488 (1/2000, Invitrogen). After staining, tissues were mounted with 4’-6-diamidino-phenylindole dihydrochloride-containing mounting media and stored at -20°C. Images were recorded on a Zeiss LSM 780 – SP Mai Tai HP DS with Z-stacking (Cell and Tissue Imaging Cluster (CIC), Images were processed using ImageJ (Java).

**TLR2 signaling assay**

Human TLR2+TLR6/NF-κB/SEAP reporter HEK293 cells and human TLR2+TLR1/NF-κB/SEAP reporter HEK293 cells (InvivoGen, San Diego, California, USA) were grown in a T75 flask in Dulbecco’s modified Eagle's medium (Life Technologies, Invitrogen) with 10% FBS, supplemented with 4.5g/l glucose, 2mM L-glutamine, Pen-Strep (100U/ml-100µg/ml) and normocin™ (100µg/ml, InvivoGen), at 37°C. HEK293 cells were seeded in a 96 well plate when 70-80% confluency was reached. Afterwards, HEK293 cells were stimulated for 24 hours with SEB (10 µg), Pam2CSK4 (10 ng/ml) or Pam3CSK (1µg/ml). The induction of TLR2 signaling was assessed by measuring the pNPP in supernatant of stimulated HEK293 cells. Briefly, 50µl of the supernatant from stimulated cells was mixed with 50µl of pNPP solution and absorbance was measured after 20 minutes at 405 nm.

**Mouse model to evaluate effect of SEB on mucosal permeability**

Male C57Bl/6 mice (6-8 weeks) were obtained from Envigo (Horst, The Netherlands) and were kept under conventional conditions in filter top cages. Mice (n=5/group) received three times, with one-hour interval 50 µl of SEB (1 or 10 µg/ml, in saline), or 50µl saline endonasally. One hour after the last nasal application, 20 µl FD4 (50 mg/ml) was applied endonasally for evaluation of mucosal permeability. One hour after FD4 application, mice were sacrificed with an intraperitoneal injection of Dolethal (Vétoquinol, S.A., Lure, France). Blood and nasal mucosa...
were collected for further analysis. Blood was centrifuged and serum was collected to determine the levels of FD4 with a fluorescence reader.

Pathogen-free male \( tlr2^-/- \) mice (B6.B10ScN-Tlr2lps-del/JthJ, The Jackson Laboratory) and the wild type littermates were used to investigate the involvement of TLR2 in SEB effects on epithelial barrier permeability.

**Evaluation of IL-6 and IL-8 production by primary nasal epithelial cells**

After four hours of incubation of ALI cultures with SEB, basolateral supernatants were harvested and stored at -20°C until subsequent analysis. IL-10, IL-6 and IL-8 were measured in the supernatants of stimulated primary nasal epithelial cells by sandwich ELISA. Capture monoclonal antibodies used were rat anti-human/viral IL-10 (554497), rat anti-human IL-6 (554543) and mouse anti-human IL-8 (554716). Biotinylated detection antibodies were anti-IL-10 (554499), anti-IL-6 (554546) and anti-IL-8 (554718). rhIL-10, rhIL-6 and rhIL-8 were used as standard. All products were purchased from BD Pharmingen TM, BD Bioscience.

**MTT assay**

Metabolic activity as surrogate for cell viability was evaluated as described previously\(^2\)\(^5\).

**Statistics**

Data were analyzed using Graphpad Prism 8 (La Jolla, CA 92037 USA). Normality was determined with the Shapiro-Wilk test. Differences between groups were analyzed using two-tailed Student’s t-test (normally distributed) or Mann-Whitney U test. One-way ANOVA or Kruskal-Wallis test with post-hoc analysis was used to compare multiple groups. Values were considered significantly different when \(p < 0.05\).
Results

*Staphylococcus aureus enterotoxin B decreases integrity of polyp epithelial cells*

To investigate the effect of SEB on epithelial cell integrity, primary polyp epithelial cells from CRSwNP patients and nasal epithelial cells from inferior turbinates of controls were cultured in ALI for 21 days[^6], and the effect of SEB on epithelial integrity was evaluated by measuring TEER[^7]. TEER of ALI cultures of patients was significantly lower compared to ALI cultures of healthy controls (p < 0.01) ([Fig. 1A](#)). The lower TEER was associated with decreased mRNA expression of occludin and ZO-1 (both p < 0.05) ([Fig. 1B](#)). On immunofluorescence stainings of ALI cultures, we observed an intact epithelial architecture for ZO-1 and occludin at cell membranes of controls compared to CRSwNP patients ([Fig. 1C](#)).

Stimulation of ALI cultures of CRSwNP patients with increasing SEB concentrations for four hours resulted in a further dose-dependent decrease in TEER, while there was no effect on ALI cultures of controls ([Fig. 2A](#)). Diffusion of FD4 across the epithelial layer was significantly increased with the highest SEB dose (10µg) in ALI cultures from CRSwNP patients compared to medium control (p < 0.01) ([Fig. 2B](#)). The SEB effect on epithelial integrity was transient as TEER was restored to baseline levels after 24 hours of stimulation (Supplemental Figure 1). Additionally, SEB did not affect cell viability as measured with MTT assay[^25] (Supplemental Figure 2).

To investigate putative causes for decreased epithelial cell integrity after exposure to SEB, we performed Western Blot analyses and immunostainings of TJs. We observed decreased expression of claudin-1 after SEB stimulation. Additionally, the phosphorylated form of occludin was less present compared to the unphosphorylated occludin after SEB stimulation ([Fig. 2C-D](#)). Assembly and disassembly of TJs are associated with reversible phosphorylation of occludin on serine and threonine residues[^28]. Particularly, removal of phosphoryl groups from occludin favors protein internalization, whereas phosphorylation of occludin results in membrane accumulation. Lastly, ALI cultures stimulated with SEB showed an altered occludin and ZO-1 architecture in patients compared to controls ([Fig. 2E](#)). To conclude, SEB alters epithelial barrier integrity in CRSwNP and not in healthy controls, by dephosphorylation of occludin.

*SEB induces barrier defects through TLR2 signaling*

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We next investigated how SEB interacts with epithelial cells. *S. aureus* infection at mucosal sites has been associated with activation of TLR family members. To assess the possible involvement of TLR2 signaling in SEB-induced barrier disruption, we first studied TLR2 and TLR4 mRNA expression in ALI cultures of controls and patients. Elevated mRNA levels of TLR2 and TLR4 were found in ALI cultures of patients compared to controls (p < 0.05) (Fig. 3A). Stimulation of ALI cultures of patients with SEB dose-dependently increased TLR2 mRNA expression (p < 0.05) while TLR4 expression was not altered. SEB did not affect mRNA expression of TLR2 and TLR4 in ALI cultures of controls (Fig. 3C). To confirm that SEB induced TLR2 signaling, TLR2+TLR6 and TLR2+TLR1 transfected HEK293 cells were stimulated with SEB (10 µg) and the absorbance was measured. TLR2/6 agonist Pam2CSK4 and TLR2/1 agonist Pam3CSK4 were used as a positive control whereas medium was used as a negative control. SEB activated TLR2/TLR1 signaling pathway, while TLR2/6 was not activated (Fig. 3B).

To confirm the involvement of TLR2 triggering in SEB-induced barrier dysfunction, ALI cultures of controls and patients were pre-incubated for two hours with a TLR2 blocking monoclonal antibody before stimulation with 10 µg SEB for four hours. Pre-treatment with anti-TLR2 antibodies prevented the SEB-induced decrease in TEER in ALI cultures of polyp epithelial cells (Fig. 3D). mRNA expression of occludin and ZO-1 was not altered when ALI cultures were pre-treated with anti-TLR2 antibodies (Fig. 3E). On immunofluorescence, a clear opening of the TJ layer was observed in ALI cultures of polyp epithelial cells stimulated with SEB, which is indicated by the white arrows. Pre-treatment with anti-TLR2 antibodies prevented SEB-induced opening of TJs (Fig. 3F). SEB treatment nor pre-treatment with anti-TLR2 altered epithelial integrity of primary nasal epithelial cells of healthy controls (data not shown). In summary, SEB alters the expression of TJs via the activation of TLR2 signaling.

**SEB increases FD4 mucosal permeability and alters occludin expression in vivo**

To further explore the effect of SEB on mucosal integrity, we examined the mucosal permeability for FD4 together with the nasal mRNA expression of TJs in mice following the nasal administration of different SEB doses or saline as a negative control (Fig. 4A). SEB significantly increased mucosal FD4 permeability (p < 0.01) and was associated with a significant decrease in mRNA expression of occludin and ZO-1 (both p < 0.05) (Fig. 4B-C). In *tlr2−/−* mice, no effect of SEB on mucosal permeability nor on occludin and ZO-1 expression was observed (Fig. 4D-E). Immunofluorescence staining for occludin and ZO-1 showed an intact nasal mucosa of saline
instilled wild type littermates, while the mucosa was severely disrupted after SEB application. *Thr2*−/− mice did not show a disrupted nasal mucosa after SEB administration (Fig. 4F). Taken together, SEB disrupts epithelial barrier integrity *in vivo*, which is TLR2-dependent.

**SEB promotes the secretion of IL-6 and IL-8 which decreases epithelial integrity *in vitro***

Lastly, we investigated if activation of TLR2 by SEB resulted in the release of pro-inflammatory cytokines from the epithelium. ALI cultures of polyp epithelial cells stimulated with 10 μg SEB showed significantly elevated levels of IL-6 and IL-8 compared to medium control (Fig. 5A). Blocking TLR2 with a monoclonal antibody significantly decreased the SEB-induced secretion of IL-6 and IL-8 (Fig. 5B). Lastly, we verified if IL-6 (p < 0.05), IL-8 (p < 0.001) or the combination of both cytokines were responsible for the decreased TEER. Recombinant IL-6 or IL-8 alone did not decrease TEER nor increased FD4 permeability, while the combination of IL-6 and IL-8 significantly decreased TEER (p < 0.05) and increased FD4 permeability (P < 0.05) (Fig. 5C). To conclude, SEB promotes the release of pro-inflammatory cytokines IL-6 and IL-8, which is TLR2 dependent. IL-6 and IL-8 affect TEER and FD4 passage.
Discussion

Bacterial infections with \textit{S. aureus} are serious problems in airways with compromised barrier function such as in patients with CRSwNP. \textit{S. aureus} is able to manipulate host immune responses by producing superantigens that facilitates its invasion and colonization\textsuperscript{30}. An increased colonization rate of \textit{S. aureus} has been reported in nasal polyp tissue\textsuperscript{9}, whereby \textit{S. aureus} superantigens shifts the cytokine pattern in nasal polyps tissue to type 2 cytokines (increased production of IL-4 and IL-5, among others)\textsuperscript{31}. Previously, it was shown that TJ proteins in the skin are influenced by staphylococcal infections\textsuperscript{32}, an event that has not been extensively studied in airway epithelial cells.

We here show the effect of SEB inoculation on epithelial barrier function and TJs in primary polyp and nasal epithelial cells of patients with CRSwNP and healthy controls respectively, and \textit{in vivo} in a mouse model.

In primary polyp epithelial cells from patients with CRSwNP, we observed a decreased TEER together with an increased FD4 permeability compared to primary nasal epithelial cells of healthy controls. The decrease in TEER in CRSwNP was accompanied by decreased expression of occludin and confirms previous findings\textsuperscript{6}.

Stimulation of primary polyp epithelial cells from CRSwNP patients with SEB decreased TEER and increased FD4 permeability in a dose-dependent manner. These results might impose SEB as an initial trigger to impair epithelial barrier integrity. However, this is likely not the case as TEER of primary nasal epithelial cells of controls was not changed by SEB. This is also supported by the finding that TEER normalizes again after 24 hours. We propose SEB as a driving factor of epithelial barrier defects, rather than a causal factor.

Comparing epithelial barrier function of actively \textit{S. aureus} colonized patients with patients without \textit{S. aureus} colonization could potentially confirm this notion, though was not evaluated in our study. The observation that SEB influences epithelial integrity and permeability fits with a previous report which showed increased epithelial permeability to ovalbumin in a SEB-stimulated T84 cells. However, Yan \textit{et al.} did not report changes in TEER\textsuperscript{33}. We ascribe this discrepancy to differences in epithelial cell methodology. We have previously observed differences in TEER and cytokine release between cell lines and primary nasal epithelial cells stimulated with the same triggers\textsuperscript{34}.

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The decrease in TEER of polyp epithelial cells after SEB stimulation was associated with changes in tight junction proteins. More specifically, ZO-1 and occludin localization at cell membranes was decreased after SEB stimulation. On Western Blot, we observed less claudin-1 and phosphorylated occludin. TJ assembly and disassembly are associated with reversible phosphorylation of occludin on serine and threonine residues\textsuperscript{35, 36}. This observation is in agreement with previous studies\textsuperscript{32}. Bäsler et al. demonstrated that inoculation of primary human keratinocytes with \textit{S. aureus} resulted in increased levels of phosphorylated occludin and increased membrane localization of occludin\textsuperscript{32}. Of note, occludin and ZO-1 mRNA expression remained unaltered after SEB stimulation. In mice, nasal challenge with SEB significantly increased mucosal permeability for FD4 and decreased occludin mRNA expression compared to saline controls. Collectively, these data suggest that SEB impairs epithelial integrity by altering the expression and function of TJs and especially occludin.

Concerning upstream mechanisms responsible for TJ defects, it was shown that enterotoxins released by \textit{S. aureus} activate TLR2 and/or TLR4\textsuperscript{37-41}. Mandron et al., demonstrated that SEB-activated dendritic cells could drive polarization of naïve T cells towards type 2 immune cells, which was TLR2 dependent\textsuperscript{16}. With the use of TLR2/TLR1 transfected HEK293 cells, we confirmed that SEB activates TLR2/TLR1. It is generally accepted that upon activation of TLR2, this receptor will dimerize with TLR1 to induce a pro-inflammatory response or with TLR6 to generate an anti-inflammatory response, depending on the bacterial ligand\textsuperscript{42}. Additionally, altered TLR expression is reported in the pathogenesis of CRS\textsuperscript{43}. We observed increased mRNA levels of TLR2 and TLR4 in primary polyp epithelial cells of CRSwNP patients compared to nasal epithelial cells of controls\textsuperscript{44}. The notion of increased TLR2 and the fact that SEB binds to TLR2 might explain why SEB only decreases TEER in polyp epithelial cells from patients and not in healthy controls. Furthermore, polymorphisms in TLR1, TLR2 and TLR6 are associated with altered susceptibility to \textit{S. aureus} infections, and might support our observations\textsuperscript{45}. Lastly, \textit{in vivo}, \textit{tlr}\textsuperscript{2-/-} mice instilled with SEB showed no increase in FD4 passage compared to wild type littermates, confirming a role for TLR2 in the effect of SEB on epithelial barrier function.

Another factor contributing to decreased epithelial integrity could be inflammation. Toxins released by \textit{S. aureus} also promote the production of pro-inflammatory cytokines such as IL-6 and IL-8, possibly by activation TLR2\textsuperscript{38, 40}. We show that SEB results in an induction of IL-6 and IL-8 by primary polyp epithelial cells of patients with CRSwNP. Additionally, we observed that stimulation of ALI cultures with IL-6 and IL-8 rapidly decreased epithelial integrity. This
indicates a crucial influence of these pro-inflammatory cytokines on TJ function. It was shown that IL-6 and IL-8 increased endothelial permeability by decreasing the expression of occludin, ZO-1 and claudin-5. Furthermore, we show that blocking TLR2 decreased the release of IL-6 and IL-8, implying a potential role for TLR2 activation in SEB-mediated inflammation.

In summary, we have shown that SEB rapidly decreases epithelial barrier integrity and TJs in primary polyp epithelial cells of patients with CRSwNP. The SEB-mediated disruption of TJs depends on activation of TLR2 signaling and the subsequent release of pro-inflammatory cytokines IL-6 and IL-8. Sensing of SEB by TLR2 provides new insights on how S. aureus might impair epithelial integrity in CRSwNP. Interfering with TLR2 triggering might provide a novel therapeutic avenue to avoid the pathophysiological consequences of S. aureus on inflammation in CRSwNP.
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References


Figures and figure legends

Figure 1. Epithelial barrier function in patients with chronic rhinosinusitis with nasal polyps. A. Trans-epithelial electrical resistance and FD4 permeability in air-liquid interface cultures of nasal epithelial cells from CRSwNP patients (n = 6) compared to healthy controls (n = 5) at day 21. B. mRNA expression of occludin and ZO-1 in ALI cultures of patients (n=5) and controls (n=5). Expression is normalized to housekeeping genes β-actin and β-2-microglobulin. C. Representative immunofluorescence staining for occludin and ZO-1 in ALI cultures of patients and controls. Data presented as median. Significance was calculated by the Mann-Whitney U-test. *, P < 0.05; **, P < 0.01.

Figure 2. Effect of S. aureus enterotoxin B on nasal epithelial barrier integrity in vitro. A. SEB was added to air-liquid interface (ALI) cultures of epithelial cells derived from patients (n = 6) and controls (n = 5). Trans-epithelial electrical resistance was measured in duplicate for the different concentrations for each donor. B. Effect of SEB on FD4 passage in ALI cultures of patients and controls. C. Representative Western Blot for claudin-1, phosphorylated-occludin and occludin on Calu-3 epithelial cells stimulated with SEB for 4 hours. D. Quantification of claudin-1, occludin and ZO-1 protein levels, expressed relatively to the housekeeping protein β-actin as quantified by Image J. E. Representative immunofluorescence staining for occludin and ZO-1 in ALI cultures of patients and controls with/without SEB (10 µg) stimulation. Error bars in figures A and B show mean ± SEM. Significance was calculated by the 2-Way ANOVA with post-hoc analysis for figure A. One-Way ANOVA with post-hoc analysis was used for figures B, C and E, while the paired variant was used for figure C. *, P < 0.05; **, P < 0.01; *** P < 0.001.

Figure 3. Role of TLR2 triggering in S. aureus enterotoxin B -induced epithelial barrier disruption in vitro. A. mRNA expression of TLR2 and TLR4 in nasal epithelial cells from controls (n = 5) and patients (n = 5). mRNA expression is relative vs. housekeeping genes β-actin and β-2 microglobulin. B. Effect of SEB on TLR2/1 and on TLR2/6 transfected reporter HEK cells. Pam3CSK4 and Pam2CSK4 were used as positive controls respectively. Pooled date from two experiments. Absorbance of pNPP was measured in the supernatant of HEK stimulated cells. C. Effect of SEB stimulation on the mRNA expression of TLR2 and TLR4 in ALI cultures of controls and patients. D. Effect of two-hour pre-treatment with antagonistic anti-TLR2 monoclonal
antibodies on SEB-induced changes of TER in ALI cultures of controls (n = 4) and patients (n = 5). E. mRNA expression of occludin and ZO-1 in relation to SEB stimulation with/without anti-TLR2 pre-treatment in controls (n = 4) and patients (n = 5). F. Representative Immunostainings for occludin and ZO-1 in ALI cultures from patients. Error bars show mean ± SEM. Significance was calculated using a student’s t test for figure A, One-way ANOVA for figures B, C and E., and 2-Way ANOVA with post-hoc analysis for figure D. * P < .05, ** P < .01.

Figure 4. Effect of *S. aureus* enterotoxin B on FD4 permeability and tight junction expression in vivo. A. Mice received 3 nasal applications with one-hour interval of 50 µl of SEB (1 or 10 µg/ml), or saline. One hour later, 20 µl of FD4 (50 mg/ml) was instilled in the nose. One hour after FD4 application, blood was collected to measure the FD4 passage. B. FD4 concentration measured in blood. C. mRNA expression of occludin and ZO-1 in the nasal mucosa, relative to housekeeping genes β-actin and ppia. D. FD4 concentration measured in blood of wild type compared *tlr2*⁻/⁻ mice. E. mRNA expression of occludin and ZO-1 in the nasal mucosa of wild type and *tlr2*⁻/⁻ mice, relatively to housekeeping genes β-actin and Ppia. F. Representative Immunostainings for occludin in nasal mucosa of wild type and *tlr2*⁻/⁻ mice. Error bars show mean ± SEM. One-way ANOVA with post-hoc analysis. N = 5 mice/group. * P < .05, ** P < .01, *** P < 0.001.

Figure 5. Effect of *S. aureus* enterotoxin B on the secretion of IL-6 and IL-8 in vitro. A. Secretion of IL-6 and IL-8 in ALI cultures of patients after stimulation with SEB or medium for four hours. B. Effect of two-hour pre-treatment with anti-TLR2 monoclonal antibody on SEB-induced release of IL-6 and IL-8. C. Effect of four-hour stimulation with recombinant IL-6, IL-8 or the combination on trans-epithelial electrical resistance and FD4 permeability of ALI cultures of CRSwNP patients. N = 3-5 donors. Error bars show mean ± SEM. Significance was calculated using Kruskal-Wallis test with post-hoc analysis. * P < .05, *** P < .001.