

Staphylococcus aureus* invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells *in vitro

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Abstract

Background: *Staphylococcus aureus* has been associated with chronic rhinosinusitis with nasal polyps (CRSwNP) pathogenesis but its role is still controversially discussed. Here, we demonstrate *S. aureus* detection in the mucosa of CRSwNP. In addition, intracellular residency of *S. aureus* in nasal polyp epithelial cells (NPECs) and its capability to induce TH-2 cytokines were analyzed *in vitro*.

Methods: *Staphylococcus aureus* detection in CRSwNP ($n = 25$), CRS without polyps (CRSsNP, $n = 5$), and turbinate mucosa (TM, $n = 10$) was performed by peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) and microbial cultivation from tissue biopsies. Intracellular residency was examined by intracellular persistence assay and electron microscopy. IL-6 and IL-13 responses to *S. aureus* infection and supernatants were quantified by ELISA.

Results: Peptide nucleic acid-fluorescence *in situ* hybridization positive bacterial cells were significantly increased in the epithelium of CRSwNP (17/25) compared to CRSsNP (0/5) and TM (1/10). Good concordance of PNA-FISH results and *S. aureus* cultivation was found applying Cohen's κ for CRSwNP ($\kappa = 0.841$) and TM ($\kappa = 1.0$). Intracellular persistence assay with *S. aureus* strain Newman and its corresponding small-colony variant mutant strain III33 demonstrated intracellular survival and replication of *S. aureus* within NPECs. Both *S. aureus* strains significantly induced IL-6 but not IL-13 in infected NPECs and in NPECs challenged with corresponding staphylococcal supernatants.

Conclusion: Invasion of the epithelium by *S. aureus* was a phenomenon seen predominantly in CRSwNP. Regardless of an intra- or extracellular localization in the epithelium, *S. aureus* is capable to induce IL-6 synthesis *in vitro* and thus may contribute to the TH-2 cytokine pattern in CRSwNP.

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nasal and paranasal mucosa of yet unknown etiology. Although much attention has been paid to the presence of bacteria and fungi in CRS, a microbial etiology has not been demonstrated so far. One pathogen that has been intensively focused on in the past years is *Staphylococcus aureus*, a gram-positive bacterium that can be frequently cultivated from the nasal vestibulum and that can potentially cause systemic *S. aureus* bacteraemia (1).

A pathogenic role for *S. aureus* in CRS appears possible as several studies demonstrated increased incidence of *S. aureus* in patients with CRS (2–5). Pathogenic impact of *S. aureus* in CRS has been mainly attributed to virulence factors

secreted by *S. aureus* such as staphylococcal enterotoxins (SEs). In CRS with nasal polyps (CRSwNP), IgE antibodies against SEs have been associated with local superantigen activation of T cells, tissue eosinophilia, and a TH-2-dominated cytokine pattern (6, 7). In this context, it is worth mentioning that the concept of a localized nasal inflammation has been also demonstrated in nonatopic rhinitis (8). Most recently, *S. aureus* was detected in the submucosal space by peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) in a subgroup of CRSwNP (9). By contrast, Niederfuhr et al. (10, 11) detected *S. aureus* using PNA-FISH in one case only. Hence, they questioned the immunomodulatory potential of *S. aureus* to contribute to the TH-2 cytokine pattern in

CRSwNP. Overall, presence and impact of *S. aureus* in CRS are still controversially discussed.

In this study, we demonstrated *S. aureus* invasion of epithelial layer in CRSwNP by PNA-FISH. In addition, we examined the possibility of intraepithelial residency of *S. aureus in vitro* by performing intracellular persistence assays. For that purpose, nasal polyp epithelial cells (NPECs) were co-cultivated with the well-characterized laboratory strain *S. aureus* Newman and its site-directed *hemB* mutant displaying the typical properties of small-colony variants (SCVs). These variants represent a subpopulation of *S. aureus* characterized by formation of tiny colonies on solid agar media, low proliferation rate, reduced alpha-toxin production, and decreased susceptibility to aminoglycosides and other antibiotics. Small-colony variants have been reported to play an important role in chronic, recurrent, and antibiotic-refractory infections such as chronic osteomyelitis, persistent wound infection, and recurrent endocarditis (12). Infected NPECs were analyzed for their immunomodulatory capacity to synthesize TH-2 cytokines IL-6 and IL-13. IL-6 is known as a key cytokine mediating the transition from innate to acquired immunity (13). In addition, it has been demonstrated that *S. aureus* enterotoxin B was able to induce IL-6 and may be relevant for insufficiency of regulatory T cells (Treg) in nasal polyps (14, 15). IL-13 has recently been shown to

down-regulate short palate, lung and nasal epithelial clone 1 (SPLUNC1) protein, an airway epithelial cell-derived protein with host-defense effects against pathogens (16).

Methods

Patients

Forty consecutive patients with CRS or undergoing either septoplasty or septorhinoplasty were included in this study (Table 1). Chronic rhinosinusitis was diagnosed according to the criteria defined by the European position paper on rhinosinusitis and nasal polyps guidelines (17).

All tissue samples were obtained during routine surgery at the clinic of Otorhinolaryngology of the University Hospital of Münster, Germany. To avoid bacterial contamination during tissue removal via the nasal vestibule, the vestibular skin and the mucocutaneous junction area were disinfected using swabs of Octenisept solution (Schülke & Mayr, Norderstedt, Germany). A sterile self-fixing nasal speculum was used to create enough space for a contact-free removal of nasal tissue using sterile instruments. Tissue samples from CRSwNP and CRS without polyps (CRSsNP) were harvested from the middle nasal meatus/anterior ethmoid. Inferior turbinate mucosa (TM), which served as control tissue, was obtained from

Table 1 Characterization of the study population. The mean polyp score was calculated based on the results of nasal endoscopy

Patient group	A CRSwNP	B CRSwNP/ASI	C CRSsNP	D TM	<i>P</i> < 0.05
<i>n</i>	25	6	5	10	
Mean age (± SD)	48.9 (13.2)	55.6 (16.9)	56 (17.4)	38.1 (10.6)	A vs D; C vs D
Gender					
Males	14 (56%)	3 (50%)	5 (100%)	5 (50%)	
Females	11 (44%)	3 (50%)	0	5 (50%)	
Asthma	7 (28%)	4 (67%)	1 (20%)	0	A vs D; B vs D
ASI	6 (24%)	6 (100%)	0	0	B vs C; B vs D
Atopy	13 (52%)	2 (33%)	1 (20%)	3 (30%)	
Sa+	7/13 (54%)	1/2 (50%)	1/1 (100%)	1/3 (33%)	
Mean Polyp score (± SD)	4.0 (2.3)	5.2 (0.8)	0	0	A vs C/D; B vs C/D
Mean CT score (± SD)	17.9 (3.5)	17.6 (2.1)	2.6 (3.1)	–	A vs C; B vs C
Sa+ PNA-FISH					
Epithelial layer	17 (68%)	5 (83%)	0 (0%)	1 (10%)	A vs C/D; B vs C/D
Submucosa	12 (48%)	2 (33%)	0 (0%)	1 (10%)	
Sa+ cultivation	11 (44%)	1 (16%)	1 (20%)	1 (10%)	
Sa+ cult./PNA-FISH+	11	1	0	1	
Sa– cult./PNA-FISH+	2	1	0	0	
Sa+ cult./PNA-FISH–	0	0	1	0	
Sa– cult./PNA-FISH–	12	4	4	9	
CI (95%)	0.63–1.05	–0.20 to 1.34	–1.75 to 1.75	1.00–1.00	
Cohen's κ	0.841	0.571	0.00	1.0	

The mean CT score was calculated according to the Lund–McKay scoring system. Categorical data were analyzed by Fisher-exact test. Ordinal data were analyzed by Mann–Whitney test. Cohen's κ was applied as a measure of concordance. Only significant differences between patient's groups (*P* < 0.05) are indicated.

ASI, aspirin salicylate intolerance; PNA-FISH, peptide nucleic acid-fluorescence *in situ* hybridization; Sa+, *Staphylococcus aureus* detection by PNA-FISH or cultivation; SD, standard deviation; CRSwNP, chronic rhinosinusitis with nasal polyps; CRSsNP, chronic rhinosinusitis without polyps; TM, turbinate mucosa.

patients undergoing septoplasty or septorhinoplasty without any findings of nasal inflammation.

Allergy was evaluated by allergic history and skin-prick test. If results were ambiguous, specific IgE detection and/or nasal provocation test were additionally performed. Asthma was diagnosed by a pulmonary specialist. Aspirin sensitivity was evaluated by typical medical history and nasal provocation test. None of the patients underwent prior sinus surgery or turbinate surgery. In addition, none of the patients received medical treatment 4 weeks prior to surgery. All patients were nonsmokers.

Informed consent was obtained from all patients, and the study was approved by the ethics committee of the University of Münster.

S. aureus peptide nucleic acid-fluorescence *in situ* hybridization

Unless declared otherwise, all reagents were purchased from Sigma (Sigma, Deisenhofen, Germany). Nasal tissue was fixed in 4% buffered formalin and embedded in paraffin. Subsequently, 8- μ m-thick sections were prepared and mounted on glass slides (Superfrost, Langenbrinck, Germany). Sections were then deparaffinized and hydrated using xylene and graded concentrations of ethanol.

Following air-drying, sections were hybridized at 55°C for 90 min in a humidified chamber with 30 μ l of specific *S. aureus* PNA-FISH probe (*S. aureus* PNA-FISH, AdvanDX, Woburn, MA, USA) according to the instructions of the manufacturer. Counterstaining of nuclei was achieved by 4,6 diamidino-2-phenylindole dihydrochloride (DAPI). Slides were immediately examined using a Zeiss microscope (Zeiss Imager M1, Carl Zeiss, Jena, Germany) equipped with a Hamamatsu camera (ORCA-ER, Hamamatsu Photonics KK, Hamamatsu City, Japan) connected with an iMac workstation (Apple Inc., Cupertino, CA, USA). For picture analysis, Openlab 5.5 (Perkin Elmer, Waltham, MA, USA) was used.

In negative controls, the PNA-FISH probe was omitted. A positive control was generated by incubation of nasal polyp tissue with *S. aureus* strain Newman (ATCC25904) for 24 h.

Enumeration of PNA-positive bacterial cells in the epithelial layer and submucosa was performed by two independent observers at 630 $\times$ magnification. Only those PNA-FISH signals were considered as *S. aureus* cells that displayed the typical morphology and staining intensity as demonstrated in the positive control (Fig. 1D) and that were concordantly identified by both observers.

Because of the rare incidence of *S. aureus* at all, statistical analysis was confined to *S. aureus* presence/absence. Finally, group-specific total numbers of *S. aureus*-positive and *S. aureus*-negative cases were determined and statistically analyzed.

S. aureus cultivation

Specimens were cultured aerobically using a set consisting of Columbia sheep blood agar (Becton Dickinson, Heidelberg, Germany), Endo agar (Merck, Darmstadt, Germany) and chocolate agar (Mast, Rheinfeld, Germany). In addition, cul-

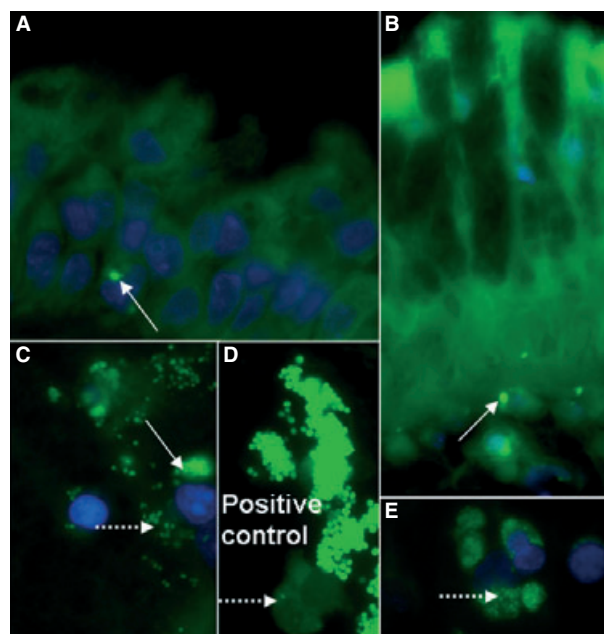


Figure 1 Peptide nucleic acid-fluorescence *in situ* hybridization/4,6 diamidino-2-phenylindole dihydrochloride stain. (A) Epithelial layer in chronic rhinosinusitis with nasal polyps (CRSwNP) showing a single PNA-positive bacterial cell (arrow). (B) Subepithelial border in CRSwNP showing a single PNA-positive bacterial cell (arrow). (C) Submucosal polyp stoma with a cluster of smaller bacterial cells (arrow). (D) CRSwNP infected by *Staphylococcus aureus* Newman (positive control). (E) Inflammatory cells in the submucosal polyp stroma in CRSwNP. Extracellular and cytoplasmic granules (C, D; E, dotted arrow) display weaker staining compared to specific bacterial staining (D). Screenshots represent representative staining results and were taken at 630 $\times$ magnification.

tivation was performed on brain heart infusion agar (Becton Dickinson) anaerobically and with 5% CO₂. All plates were incubated at 35°C for up to 2–3 days. Furthermore, dextrose broth was inoculated and incubated at 35°C for 2 days. Isolated colonies were identified biochemically according to standard procedures (18). If the identification of bacterial isolates by the use of biochemical procedures was ambiguous or rated unacceptable, 16S rDNA sequencing was performed as previously described (19, 20).

Nasal polyp epithelial cell culture

Chronic rhinosinusitis with nasal polyps specimens were dissected for individual experiments under sterile conditions. Subsequently, tissue was washed with phosphate-buffered saline (PBS) and incubated with trypsin (0.5%) overnight at 4°C. The epithelial layer was resuspended in PBS (pH 7.4). Following centrifugation at 150 g for 10 min, cells were washed again with PBS, pelleted, and resuspended in a serum-free airway epithelial cell growth (AECG) medium (AECG Medium, Promocell, Heidelberg, Germany), supplemented with a ready-to-use supplement mix according to the

manufacturer's recommendation. Penicillin was applied at a dilution of 200/ml medium and streptomycin applied to a dilution of 0.2 µg/ml medium (Biochem, Berlin, Germany). Nasal polyp epithelial cells were grown to 80% confluence and passaged two more times. The epithelial phenotype of cells was confirmed by staining of epithelial cells with a monoclonal anti-pan cytokeratin antibody as previously shown (21). Viability of NPECs as assessed by trypan blue dye exclusion was greater than 90% in all experiments.

***In vitro* intracellular persistence assay**

The assay was performed according to the method described by Balwit and coworkers with minor modifications (22). Briefly, NPECs were grown in AECG medium in 24-well tissue culture plates to confluence (10^5 cells per well). The numbers of washed bacteria were adjusted to be nearly equal for strain *S. aureus* Newman (normal phenotype) and its site-directed *hemB* mutant III33 displaying the stable SCV phenotype (23). Bacteria were then added to the washed monolayers. The infected monolayers were incubated for 3.5 h at 37°C in 5% CO₂ to allow adhesion and phagocytosis of the bacteria. Subsequently, monolayers were washed three times with AECG medium to remove nonattached organisms. Then, 1 ml of medium containing 10 µg of lysostaphin (AMBI, Inc., Lawrence, NY, USA) was added, which effectively eliminated extracellular staphylococci. Incubation in the presence of lysostaphin was continued for 30 min, 24, 48, and 72 h. At these time points, the monolayers were washed 3 times with AECG medium to remove lysostaphin. Viability of cells was evaluated by trypan blue exclusion test. Finally, 1 ml of sterile water was added to disrupt epithelial cells and to release intracellular organisms. Serial dilutions were made in sterile water.

The number of intracellular cfu was determined by plating 100-µl aliquots on trypticase soy agar in duplicate. The detection limit was 10 CFU. The number of intracellular cfu at each time point was determined in triplicate, and the mean $\pm$ SD from three experiments was calculated.

Electron microscopy

Following incubation of the infected NPECs in the presence of lysostaphin for 30 min and 48 h, cells were washed twice in PBS, dehydrated in ethanol, fixed in glutaraldehyde and osmium tetroxide, and embedded in Epon. Ultrathin sections were counterstained with lead citrate and uranyl acetate and examined with the use of a Philips EM10 electron microscope.

IL-6 and IL-13 synthesis by infected NPECs

For the determination of cytokine synthesis by infected NPECs, culture supernatants of invasion experiments using 1.5×10^6 NPECs were collected at 12 h and analyzed for IL-6 protein (IL-6, R&D, Wiesbaden, Germany) or IL-13 protein (IL-13, R&D). For the negative control, not-infected NPECs were cultured for 12 h and analyzed as aforementioned.

IL-6 and IL-13 synthesis by NPECs in response to staphylococcal supernatants

Nasal polyp epithelial cells (5×10^6 cells) were stimulated for 12 h with staphylococcal supernatants derived from *S. aureus* strain Newman and the *hemB* mutant *S. aureus* III33 (23). Culture supernatants were analyzed for IL-6 or IL-13 protein after 12 h. For the negative control, NPECs were cultured for 12 h without addition of staphylococcal supernatants.

Statistical analysis

Categorical and ordinal data were collected and summarized in Table 1. Frequencies of categorical data were analyzed using Fisher-exact test. As a method to evaluate concordance of *S. aureus* cultivation and PNA-FISH results, Cohen's κ test was applied. Out of ordinal data, means with standard deviation were calculated and analyzed by Mann-Whitney test. IL-6 and IL-13 concentrations were analyzed by ANOVA followed by Dunnett's post-test. Significance was always taken where $P < 0.05$.

Results

PNA-FISH/DAPI stain of nasal tissues

Peptide nucleic acid-fluorescence *in situ* hybridization positive signals were detected in the epithelial layer or subepithelial border of 17/25 (68%) patients with CRSwNP and 5/6 (83%) cases of CRSwNP/aspirin salicylate intolerance (ASI), whereas no PNA-FISH signals were observed in CRSsNP. In TM, the PNA-FISH signal was observed in one case (1/10, 10%) only. Epithelial PNA-FISH signals were significantly increased in CRSwNP and CRSwNP/ASI compared to CRSsNP or TM (Table 1). In the submucosa, the highest rate of PNA-FISH detection was found in CRSwNP (12/25, 48%), but significance was not reached when results were compared to CRSsNP or TM. A similar finding was observed for the *S. aureus* cultivation rate. Positive *S. aureus* detection by cultivation was found in 11/25 (44%) cases of CRSwNP, whereas in CRSwNP/ASI, CRSsNP, and TM, cultivation rates were 16%, 20%, and 10%, respectively. However, analysis of PNA-FISH results and *S. aureus* cultivation rates applying Cohen's κ revealed good concordance for the CRSwNP ($\kappa = 0.841$) and the TM ($\kappa = 1.0$) groups, whereas moderate or poor concordance was observed for CRSwNP/ASI ($\kappa = 0.571$) and the CRSsNP ($\kappa = 0.0$) groups, respectively (Table 1).

No difference was found between the patient's groups concerning gender. Mean ages of patients with CRSwNP or CRSsNP significantly differed from TM. An analysis of the patient's co-morbidities revealed the well-known finding that patients with asthma were more frequently observed in the group of CRSwNP (7/25, 28%) and CRSwNP/ASI (4/6, 67%) compared to controls (0%). Aspirin salicylate intolerance was never observed in CRSsNP or controls. Atopy was not related to CRSwNP, CRSwNP/ASI, or CRSsNP. In addition, *S. aureus*-positive atopics were not increased in the CRSwNP or CRSwNP/ASI group. As expected, endoscopic

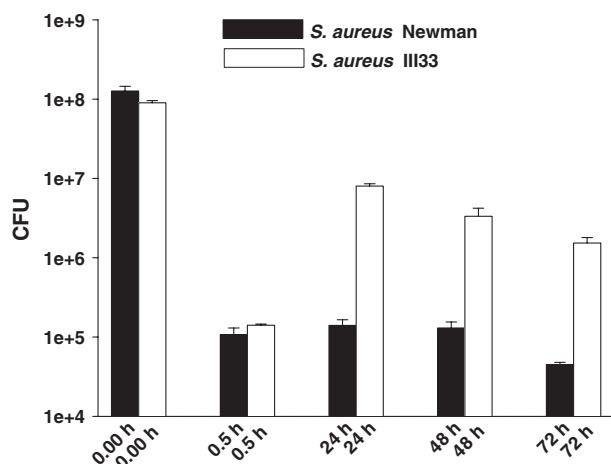


Figure 2 Nasal polyp epithelial cells (NPECs) intracellular persistence assay. Bars represent CFU means $\pm$ SEM of three independent experiments with *Staphylococcus aureus* strain Newman (black bars) or with its *hemB* mutant III33 (white bars). At time zero, 10⁸ CFUs were added to 10⁵ NPECs for a single experiment.

polyp scores as well as CT scores were significantly increased in CRSwNP and CRSwNP/ASI (Table 1).

S. aureus replicates in NPECs in vitro

Co-cultivation of NPECs with 10⁸ CFUs of strain *S. aureus* Newman or of the *hemB* mutant III33 resulted in infection of NPECs. Intracellular replication of cells of the *hemB* mutant III33 was detectable after 24 h (Fig. 2). Invasion of NPECs with strain Newman was associated with weaker intracellular replication compared to the *hemB* mutant. Noteworthy, viability of NPECs as determined by trypan blue exclusion test was over 90% in all experiments even after 72 h (results not shown).

Ultrastructural examination of the NPECs cells confirmed persistence of *S. aureus* III33 within the epithelial cells. After 30 min and 48 h, *hemB* mutant cells appeared to be within the cytoplasm (Fig. 3). Nasal polyp epithelial cells appeared viable and showed no signs of degeneration. Likewise, the corresponding parental strain *S. aureus* Newman displaying the normal phenotype was incorporated after 30 min by NPECs.

IL-6 but not IL-13 is synthesized by infected NPECs and in response to *S. aureus* supernatants

Cell culture supernatants collected after 12 h from intracellular persistence assay were analyzed for IL-6 and IL-13 synthesis. A significant increase of IL-6 synthesis was detected after 12 h compared to unstimulated controls. Correspondingly, IL-6 was significantly induced in response to staphylococcal supernatants of strain *S. aureus* Newman or the corresponding *hemB* mutant III33 (Fig. 4). IL-13 was not produced by infected NPECs nor in response to staphylococcal supernatants.

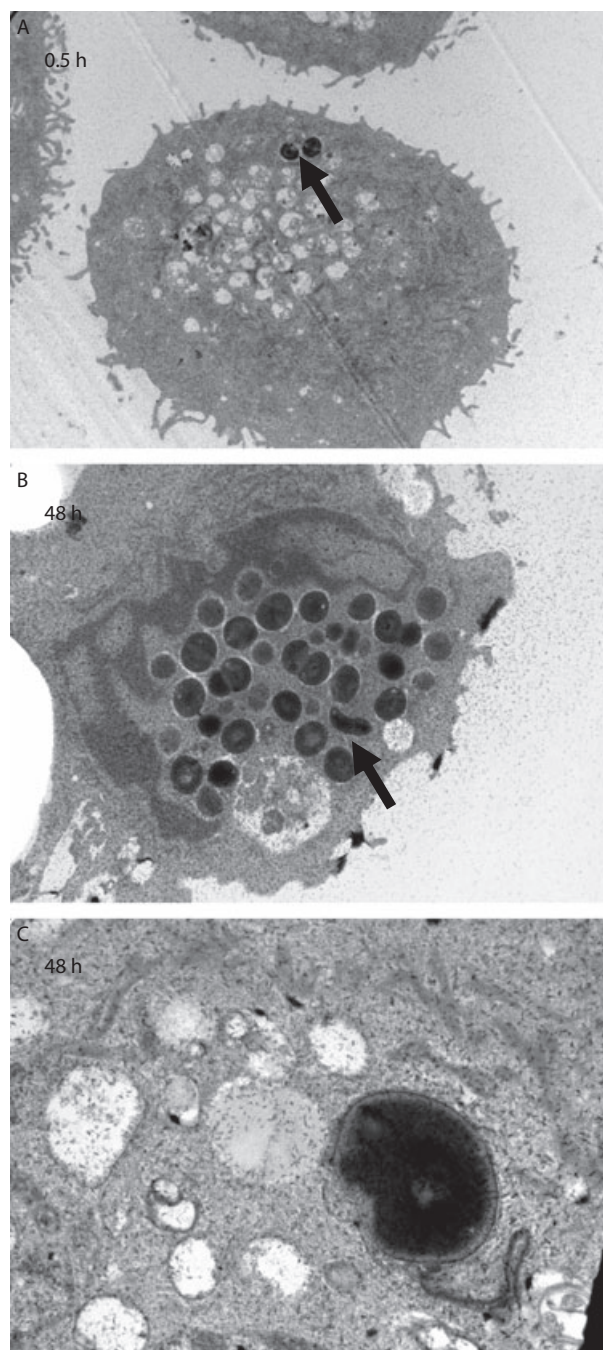


Figure 3 Electron microscopy. Nasal polyp epithelial cells were infected with the *Staphylococcus aureus* *hemB* mutant III33 (A and B) or *S. aureus* Newman (C). Screenshots were taken after 0.5 and 48 h. The black arrow indicates bacterial cells.

Discussion

In this study, presence of *S. aureus* in CRS using *S. aureus*-specific PNA-FISH and microbial cultivation was studied. To analyze intracellular persistence of *S. aureus* in the nasal

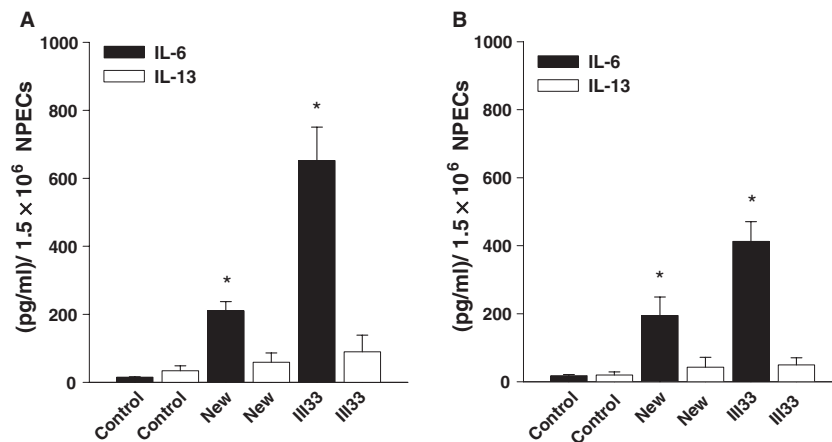


Figure 4 IL-6 and IL-13 synthesis [pg/ml/1.5 × 10⁶ Nasal polyp epithelial cells (NPECs) (ELISA)]. (A) NPECs were infected by *Staphylococcus aureus* Newman (New) or by its *hemB* mutant III33 (III33). For the negative control, not-infected NPECs were cultured for 12 h. (B) Induction of IL-6 and IL-13 synthesis by *S. aureus*

Newman (New) or by *S. aureus* III33 (III33) supernatants. For the negative control, NPECs were cultured for 12 h without addition of staphylococcal supernatants. Bars represent means ± SEM of three independent experiments. (*) indicates significant observations ($P < 0.05$ as determined by ANOVA).

epithelium, NPECs were cultivated and intracellular persistence assays were performed. Immunomodulatory effects of *S. aureus* were characterized by its potential to induce TH-2 cytokines IL-6 and IL-13.

DNA-FISH probes have been used for the identification of bacteria in a variety of tissues. In this study, a PNA-probe specific for *S. aureus* was used. PNA-probes differ from DNA-probes in their molecular structure as the phosphodiester backbone of the DNA is replaced by 2-aminoethylglycine linkage. This modification allows for better penetration of the bacterial cell wall and contributes to the high sensitivity and specificity observed for this probe (24–26). Similar to respective DNA-probes, the *S. aureus* PNA-probe used here targets a species-specific region of the 16sRNA gene. This technique is sensitive enough to detect single bacterial cells of a single bacterial species. In addition, detection of the 16sRNA gene is independent from bacterial metabolism and growth rates, thereby allowing detection of dormant and inactive bacteria. Furthermore, it has been shown that intracellular-located *S. aureus* SCVs could also be detected using *S. aureus*-specific DNA-probes (27, 28).

So far, two studies have aimed at detecting *S. aureus* in CRSwNP using a PNA-FISH probe. While Niederfuhr had difficulties in detecting *S. aureus* with PNA-FISH and reported *S. aureus* detection in just one of 51 cases of CRSwNP, Corriveau reported submucosal presence of *S. aureus* in the aspirin-sensitive subgroup of CRSwNP. In addition, the authors reported that TH-2 markers such as eosinophil cationic protein and total IgE were increased related to the status of specific IgE antibodies against *S. aureus* enterotoxins but not related to the presence of *S. aureus* in the tissue (9). Niederfuhr et al. (10) concluded that *S. aureus* positivity did not influence biomarker concentrations in nasal lavages and that *S. aureus* did not intensify the TH2 shift in patients with CRSwNP.

According to our results, *S. aureus* invasion of the epithelial layer in CRSwNP did occur, even if only single bacterial cells could be detected. By contrast, *S. aureus* was almost not detectable in the epithelium of CRSwNP or TM suggesting that invasion of the epithelial layer is a phenomenon predominantly seen in CRSwNP. Similar as reported by Corriveau, we observed submucosal presence of bacterial clusters in CRSwNP (9). Of particular importance, PNA-FISH results and those generated by microbial cultivation were in good concordance as demonstrated by Cohen's κ test.

Principally, invasion of the nasal epithelium by microbes depends on microbial factors of virulence and the epithelial barrier function. Recently, it has been reported that desmosomal proteins DSG2 and DSG3 were significantly decreased in nasal polyps vs controls. *In vitro* studies showed that DSG2 was down-regulated by IL-13 in human bronchial epithelial cells. Thus, weakened desmosomal junctions in the nasal mucosa may contribute to the formation of nasal polyps (29). Moreover, IL-13 has been shown to down-regulate SPLUNC1 protein, an airway epithelial cell-derived protein with host-defense effects against pathogens (16). In addition, it was found that the S100 family of genes associated with epithelial defense and repair was decreased in CRS (30). Moreover, SPINK5, a secreted antiprotease that may protect gap junctions from the attack of proteases derived from host sources as well as microbes and allergens, may be diminished in CRSwNP (30, 31). These events may result in a loss of epithelial integrity through gap junction degradation (32). In this context and in association with the results presented in this study, we hypothesize that *S. aureus* not only colonizes the nasal mucosa in CRSwNP but is also capable of invading the epithelial layer and of migrating to the submucosal space.

In the past decade, several studies have shown that *S. aureus* is not only an extracellular pathogen but also an intra-

cellular pathogen capable of invading and surviving within a broad range of nonphagocytic cells (33–36). Similar as reported by Corriveau and coworkers, we could not prove intracellular localization of *S. aureus* in CRSwNP as we used epifluorescence microscopy with PNA-FISH.

However, establishing an *in vitro* cell culture model using NPECs and performing an intracellular persistence assay, we found that *S. aureus* Newman and the corresponding *hemB* mutant-infected NPECs survived intracellularly for more than 48 h. Intracellular replication was mainly observed in NPECs infected with *S. aureus* III33 displaying the SCV phenotype. Surprisingly, vitality of NPECs was around 90% in all of these experiments. These *in vitro* results, at least theoretically, may argue for the possibility of intraepithelial residency of *S. aureus* in CRSwNP.

In addition, we found increased synthesis of the TH-2 cytokine IL-6 but not of IL-13 in cell culture supernatants of infected NPECs. These IL-6 concentrations were as high as induced by extracellular stimulation with staphylococcal supernatants which did not contain any bacterial cells. Thus, regardless of intra- or extracellular presence, *S. aureus* exerted immunomodulatory effects as demonstrated by IL-6 synthesis *in vitro*. As *S. aureus* was detected in CRSwNP by PNA-FISH, immunomodulatory effects of *S. aureus* may potentially contribute to the TH-2 cytokine pattern in CRSwNP via IL-6 induction. Even if there exist any studies analyzing pro-inflammatory effects caused by single bacterial cells, it has been demonstrated that very small compounds of the bacterial cell wall such as lipoteichoic acid and peptidoglycan derived from *S. aureus* could induce IL-6 synthesis (37, 38). Furthermore, previous studies have demonstrated significantly increased levels of IL-6 protein and the soluble IL-6 receptor protein in CRSwNP when compared with CRSNP and controls (39). Similar as reported by Damm et al., we confirmed that nasal epithelial cells are a source of IL-6 following stimulation with *S. aureus* and/or SEs (40).

Most important, IL-6 frees helper and effector T cells from the suppressive effects of IL-10 secreted by regulatory T cells (Tregs) (15, 41). Most recently, Xu and coworkers demonstrated that *S. aureus* enterotoxin B was able to induce IL-6 and thereby suppress the activity of Tregs in cultured nasal polyps, which were rescued by blocking IL-6 activity. The authors concluded that IL-6 is essential for *S. aureus* enterotoxin B-induced Treg insufficiency in nasal polyps (14).

In summary, we demonstrated *S. aureus* invasion of the nasal epithelium and submucosa in CRSwNP by PNA-FISH. Regardless of intra- or extracellular localization, *S. aureus* exerted immunomodulatory effects *in vitro* as evaluated by IL-6 synthesis. Thus, *S. aureus* and IL-6 may contribute to the TH-2 cytokine pattern found in CRSwNP. According to the results of this study, we suggest a concept where single staphylococcal cells can invade the nasal mucosa in CRSwNP. However, it remains unclear whether the virulence of *S. aureus* contributes to impaired mucosal integrity or whether host factors play a main role. Infection processes of single staphylococcal cells could be contained by the immune system and would result in a localized mucosal inflammatory TH-2 response. In case of repetition of such events because of an impaired mucosal barrier, a 'priming effect' against the pathogen may represent a basis for chronic mucosal inflammation in CRSwNP.

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