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Staphylococcus aureus controls interleukin-5 release in upper airway inflammation

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ABSTRACT

Staphylococcus aureus is a frequent colonizer of the upper airways in chronic rhinosinusitis with nasal polyps, but also resides intramucosally; it has been shown that secreted staphylococcal proteins such as enterotoxins and serine proteases induce the release of cytokines such as IL-5. We have analyzed nasal polyp tissue freshly obtained during routine surgery, which did or did not contain cultivatable *S. aureus*, to study spontaneous IL-5 production by nasal polyp tissue over 24 and 72 h in tissue culture. In *S. aureus*-positive samples we interfered by killing the bacteria using antibiotics or *S. aureus* specific intravenous staphylococcal phages (ISP), active or heat-inactivated. Phage-neutralizing antibodies were used to demonstrate the specificity of the phage-mediated effects. We monitored *S. aureus* colony forming units, and identified *S. aureus* proteins by mass spectrometry. We demonstrate that cultivatable *S. aureus* may be found in type-2 inflamed nasal polyps; the pathogen is replicating within 24 h and secretes proteins, including enterotoxins and serine proteases. The presence of *S. aureus* was associated with a significantly higher release of IL-5. Killing of *S. aureus* by antibiotics or specific ISP significantly reduced the IL-5 release. The suppressive activity of the bacteriophage on IL-5 be abolished by heat inactivation or anti-phage antibodies.

Biological significance: In this study, we used high resolution mass spectrometry to identify *S. aureus* proteins directly in infected nasal polyp tissue and nasal polyp tissue incubated over 24 and 72 h in culture. We discovered bacterial proteins including enterotoxins and serine proteases like proteins. These experiments indicate a direct role of *S. aureus* in the regulation of IL-5 production in nasal polyps and may suggest the involvement of bacterial proteins detected in the tissues.

1. Introduction

Staphylococcus aureus has been associated with chronic inflammatory airway diseases, as the bacteria may colonize the airways and release proteins such as staphylococcal enterotoxins (SEs) and related molecules which might severely impact the mouse and human immune system [1,2]. SE-IgE, specific IgE antibodies to staphylococcal enterotoxins (SEs), as a marker of immune contact with *S. aureus*, have been associated with asthma throughout Europe [3], and specifically severe asthma [4,5], as well as chronic rhinosinusitis with nasal polyps (CRSwNP; [2,6]). The abundancy of SE-IgE was related to more severe disease, as manifested by the presence of comorbid asthma and recurrence of disease after surgery [6,7]. Recently, Chen et al. [7a] demonstrated that SEs could function as an allergen and as a superallergen in nasal polyp tissue. The release of high concentrations of cytokines, including type 2 cytokines such as IL-5, has been observed upon exposure of mucosal tissue to SEs [8] and serine proteases (spl's) [18].

Abbreviations: IL, interleukin; *S. aureus*, *Staphylococcus aureus*; CRSwNP, chronic rhinosinusitis with nasal polyps (nasal polyps); MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; ISP, Intravenous Staphylococcal Phage; ECP, eosinophil-cationic protein; APS, anti-ISP phage serum; CFU, colony-forming units; Spl, serine protease like protein; SE, staphylococcal enterotoxin; TSST-1, toxic-shock-syndrome toxin-1; PNA-FISH, peptide nucleic acid–fluorescence in situ hybridization

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S. aureus has been shown to permanently colonize the nose in about a third of European adults, but can be found in up to 85% of patients with chronic rhinosinusitis with nasal polyps [9]. However, no difference in the ability of production of classical superantigens has been found between colonizing *S. aureus* strains obtained from CRSwNP patients vs. healthy controls [10]. In contrast to healthy mucosal tissue, however, *S. aureus* is frequently found intramucosally and intracellularly in CRSwNP tissues [11]; furthermore, macrophages in severely inflamed polyp tissues are at least in part alternatively activated and unable to effectively phagocytize and kill *S. aureus*, as shown by Krysko et al. [12], and may allow the pathogen to survive and to secrete proteins intramucosally. Although we have shown recently that secreted proteins from *S. aureus* can be detected in nasal polyp tissue [12a], and that both SEs and spl's have the potential to induce the release of IL-5 upon exposure to human nasal mucosal tissue [8,18], it has not been demonstrated yet that *S. aureus* can be cultured from nasal polyp tissue and is spontaneously producing and releasing proteins "ex-vivo" into the mucosal tissue of CRSwNP patients, associated with the release of type-2 inflammatory cytokines.

We therefore aimed to use ex vivo human tissue, freshly obtained from CRSwNP patients during surgery for their disease, to study the presence of *S. aureus*, its secretome, and effects of the released proteins on the mucosal inflammation, measured here as spontaneous release of Interleukin-5 (IL-5). To demonstrate the direct link between the activity of the germ and cytokine release, we intended to eradicate tissue *S. aureus* using both antibiotics and germ-specific bacteriophages; and to evaluate the release of secreted staphylococcal proteins into the mucosal tissues by proteomics.

2. Material and methods

2.1. Patients and sample collection

Study subjects were selected on the basis of a documented medical history of chronic rhinosinusitis with nasal polyps (CRSwNP), a pathological nasal endoscopy confirming bilateral nasal polyps and a pathological CT-Scan of the sinuses, according to the current European position paper on sinusitis and nasal polyps [13]. Tissue samples from the ethmoidal sinuses were collected from patients during endoscopic sinus surgery procedures, which were indicated for clinical reasons, independent of this study. Surgery was performed at the Department of Otorhinolaryngology at Ghent University Hospital, Belgium. The local Ethics Committee approved the study (B670201112019), and written informed consent was obtained from all patients prior to surgery. Clinical data of the patients are summarized in Table 1.

2.2. Tissue culture for identification of bacteria

Several nasal polyps from each patient with a diameter of at least 5 mm were washed vigorously in 0.9% NaCl, cut open with a sterile scalpel to obtain small tissue pieces from the center of the polyp, which were cultured overnight at 37 °C in Tryptic Soy Broth (TSB). This was followed by plating 10 µL of the TSB-overnight culture on Columbia agar with 5% sheep blood as well as on Mannitol Salt Agar (MSA), a selective growth medium for *S. aureus* (all media from Becton Dickinson, Erembodegem, Belgium). After further incubation for 24 h at 37 °C, bacterial colonies were identified by MALDI-MS (Bruker Daltonics, Germany).

For quantifying *S. aureus* in the tissue culture experiments, decinormal serial dilutions from the ex vivo nasal polyp supernatant samples were prepared and 100 µL of each dilution was inoculated onto MSA plates. Colonies were counted after incubation for 24 h at 37 °C. All culture dilutions were done in triplicate. The detection threshold for *S. aureus* and *Staphylococcus epidermidis* in the overnight cultures was 100 CFU/mL.

After overnight culture several colonies were picked, using a 1 µL disposable loop, and spotted evenly over the wells of the MALDI target plate. The preparations were covered with 1 µL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and dried for 2 min at RT. A bacterial test standard (BTS 255343, Bruker Daltonics, Germany) was used as positive control for MALDI spectra, and an empty well covered with matrix served as a negative control. Mass spectra were generated and analyzed with a Microflex Biotyper™ spectrometer (Bruker Daltonics) and Bruker Daltonics' flexControl version 3.0 software, using the manufacturer's standard settings [13a].

2.3. Bacteriophages

The LPS-free *S. aureus* bacteriophage ISP was received from Eliava IBMV (Tbilisi, Georgia) in 2002 and is currently maintained in the phage collection of the Laboratory Bacteriology Research Ghent University, Belgium. For the phage propagation we used the clinical *S. aureus* strain '13 S44 S9' isolated from a burn wound at the Burn Wound Centre (Queen Astrid Military Hospital, Brussels, Belgium) in 2006. The bacterial strain and the phage were cultured on Select Alternative Protein Source Luria Bertani media (Becton Dickinson, Erembodegem, Belgium). The agar overlay method with modifications as described earlier [14] was used to obtain a high titer (11 log plaque-forming units (PFU)/mL) phage lysate. Briefly, 1 mL of phage suspension containing 4 log PFU of ISP was mixed with 3.0 mL of molten (45 °C) Alternative Protein Source Luria Bertani top agar (0.7%) and 0.1 mL of a host bacterial suspension (end concentration of 8 log CFU/mL). This mixture was plated onto Petri dishes, filled with a bottom layer of 1.5% Alternative Protein Source Luria Bertani agar and incubated at 37 °C for 16–18 h. The top agar layer was scraped off and centrifuged for 20 min at 6000 × *g*. The supernatant was filtered through a 0.45 µm membrane filter (Sartorius Stedim Biotech, Göttingen, Germany). Phage lysate was subsequently purified from endotoxins using a commercially available kit (Endotrap Blue, Hyglos, Germany) according to the instructions of manufacturer.

The obtained phage lysate was ultracentrifuged at 25,000 × *g* for 1 h at 4 °C and the pellet was resuspended in ten times less volume of a 0.9% NaCl solution. Phage particles were enumerated by the agar overlay method [14]. Briefly, decinormal serial dilutions (from log(0) to log(−10)) of the bacteriophage suspension were prepared. One milliliter of each dilution was mixed with 3.0 mL of molten (45 °C) 0.7% Alternative Protein Source Luria Bertani LB top agar and 0.1 mL of a host bacterial suspension (end concentration of 8 log CFU/mL) and plated in triplicate onto 90 mm diameter Petri dishes (Plastiques Gosselin, Menen, Belgium), filled with a bottom layer of 1.5% Alternative Protein Source Luria Bertani agar and incubated for 18–24 h at 37 °C. To estimate the original bacteriophage concentration, plates with 100–1000 plaques were counted. Each titration was performed in triplicate and the means were calculated.

Heat inactivation of ISP was performed at 90 °C for 15 min. Anti-ISP

Table 1
Patient's clinical data.

Patient clinical data	*S. aureus* negative CRSwNP	*S. aureus* positive CRSwNP
Number of subjects	8	9
Age (y), median (range)	47 (31–77)	47 (28–81)
Gender (male/female)	4/4	5/4
Atopy	6	6
Asthma	6	5
IgE (kU/l)	350.9 (IQR: 251.4–432.1)	428.5 (IQR: 171.4–571.9)
SE-IgE positive	3	3
IL-5 (pg/mL)	270.9 (IQR: 216.2–956.6)	151.6 (IQR: 61.3–326.0)
ECP (µg/l)	13,365 (IQR: 5918–20,075)	10,846 (IQR: 3461–32,230)

phage serum (APS) was produced by Eurogentec (Seraing, Belgium). The optimal volume of APS to inactivate phage lysates in the range of 7–9 log PFU/mL was defined by the neutralization method [15].

2.4. Preparation and treatment of human sinonasal tissue fragments

Tissue fragments were prepared by cutting sinonasal tissue into fragments of ± 0.9 mm^3. These tissue fragments were suspended at concentrations of 0.04 g per mL in tissue culture medium RPMI-1640 (Life Technologies, Ghent, Belgium), which contained 2 mM L-glutamine (Life Technologies) and 0.1% BSA (Sigma-Aldrich, Bornem, Belgium) and were seeded into a 48-well plate at a volume of 1 mL/well.

Tissue fragments were incubated for 24 or 72 h in tissue culture medium in a final sample volume of 50 µL. To interfere with bacterial growth, antibiotics (50 IU/mL penicillin, 50 µg/mL streptomycin (P/S)) or active resp. heat inactivated Phage ISP were added to the wells at concentrations of 9 log PFU/mL, 8 log PFU/mL or 7 log PFU/mL as indicated. In some cases the phage suspensions were preincubated for 30 min with 5 µL of APS, either non-diluted (APS1) or diluted 1:10 in tissue culture medium (APS2). Following incubation, tissue fragments and culture supernatants were collected by centrifugation, immediately snap frozen and stored at − 20 °C/− 80 °C for proteomics analysis.

2.5. Tissue homogenates and IgE, SE-IgE, ECP and IL-5 assessment

Snap-frozen tissue specimens were weighed, pulverized by means of a mechanical Tissue Lyser LT (Qiagen, Hilden, Germany) at 50 oscillations per second for 2 min in prechilled eppendorfs and then suspended in a 10 times volume of 0.9% NaCl solution with Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) as described before [13b].

Tissue homogenate concentrations of total IgE, specific IgE to a mixture of *S. aureus* enterotoxins (staphylococcal enterotoxins A and C and toxic shock syndrome toxin 1) and ECP were assayed by using the UniCAP system (Phadia, Uppsala, Sweden). Concentrations of IL-5 were assayed with commercially available Luminex Performance Assay Kits and measured on a Bio-Plex 200 Platform (Bio-Rad Laboratories S.A.-N.V., Nazareth Eke, Belgium).

2.6. Tissue sample preparation for gel-based and gel-free-MS analysis

Following ex vivo cultivation under different conditions as described above, three snap-frozen nasal polyp tissue fragments were transferred to teflon vessels – pre-cooled in liquid nitrogen and containing a tungsten carbide bead – and disrupted in a bead mill (Mikrodismembrator S, B. Braun Biotech International GmbH, Melsungen, Germany; part of Sartorius AG, Göttingen, Germany) at 2600 rpm for 2 min. Tissue powder was resuspended in phosphate-buffered saline (PBS) and subjected to nine cycles of ultrasonication at 50% power (Sonopuls, BANDELIN electronic GmbH & Co. KG, Berlin, Germany). After pelleting insoluble material by centrifugation, the protein concentration of the supernatant was determined using a Bradford assay (Bio-Rad, Hercules, CA, USA).

2.7. Tryptic digestion and sample preparation for MS analysis (in-gel digestion)

For gel-based analyses, proteins were separated by SDS-polyacrylamide gel electrophoresis using NuPAGE precast gels (Invitrogen/Life Technologies/Thermo Fisher Scientific Inc., Waltham, MA, USA). After staining the NuPAGE precast gels with Coomassie brilliant blue G-250, the complete protein-containing region of the gel was cut and divided vertically into 10 pieces. Subsequently, gel pieces were destained and subjected to an in-gel tryptic digestion using a trypsin:-protein mass ratio of 1:15. Peptides were extracted from the gel pieces by two cycles of 30 min of ultrasonication, first using 0.1% (v/v) acetic acid in water, second using 50% (v/v) acetonitrile/0.05% (v/v) acetic acid in water. Peptide eluates were afterwards lyophilized, and re-suspended in Buffer A1 (0.1% (v/v) acetic acid, 2% (v/v) acetonitrile in water). Finally, tryptic peptides were purified using ZipTip C18 (Millipore Corp., Billerica, MA, USA).

2.8. Tryptic digestion and sample preparation for MS analysis (gel-free digestion)

Four µg of protein extract buffered by 20 mM ammonium bicarbonate were reduced in the presence of 2.5 mM dithiothreitol (DTT) and then alkylated using 10 mM 2-iodoacetamide (IAA). Finally, proteins were digested into peptides by 160 ng sequencing-grade modified trypsin (Promega GmbH, Mannheim, Germany) per sample overnight. The digestion was stopped with acetic acid at a final concentration of 1%. Tryptic peptides were purified using ZipTipµ-C18 pipette tips (Merck Millipore, Billerica, MA, USA).

2.9. Mass spectrometry

LC-MS/MS analyses of gel-free digests were performed by a reverse phase peptide separation on a Dionex UltiMate 3000 nano-LC system (Dionex/Thermo Fisher Scientific, Idstein, Germany). For MS data generation a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a TriVersa NanoMate (Advion, Ltd., Harlow, UK) was used as described before [16]. Briefly, peptides were separated on a 25 cm Acclaim PepMap RSLC analytical column (2 µm C18 particles, Thermo Scientific, Waltham, MA, USA) with a linear gradient ranging from 2 to 25% buffer (0.1% (v/v) acetic acid in acetonitrile) in 120 min at a flow rate of 300 nL/min.

For samples from in-gel digestion a reverse phase separation of peptides was carry out using a nano UPLC (Acquity UPLC system, Waters, Milford, MA, USA) system coupled with an Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA) as described in detail by Jehmlich et al. [17].

2.10. Identification of proteins

In order to improve the sensitivity of the detection and identification of bacterial proteins in the presence of a vast excess of human proteins, an iterative search profile was applied, focusing initially on the human and then on the bacterial proteins. First, a search was performed with the UniProt human database, containing 40,460 sequences and 22,585,166 residues, using a Mascot in-house version 2.3.02. Trypsin was selected as enzyme without any missed cleavage site and a mass tolerance of 10 ppm for the precursor ion (including 13C shift) was applied. For fragment ion search, 0.05 Da for the HCD-MS/MS spectra and 0.5 for CID-MS/MS spectra were applied and carbamidomethyl (C) conversion was allowed as fixed modification. In the second search *S. aureus* specific peptides were identified by focusing on tandem-MS spectra which did not pass the significance threshold filter ($p < 0.05$) in the first iteration. These spectra were researched against the MSMSpdbb1.1 *Staphylococcus aureus* database version 2.0 [MSMSpdbb: providing databases of closely related organisms to improve proteomic characterization of prokaryotic microbes. Bioinformatics 26 [5], 698–9, 2010]. This database contains 14 *S. aureus* strains and 13 plasmids namely Mu3 AP009324, *S. aureus* Mu50BA000017, AP003367, *S. aureus* NCTC 8325CP000253, *S. aureus* Newman AP009351, *S. aureus* RF122 AJ938182, *S. aureus* USA300_FRP3757, CP000255, CP000256, CP000257, CP000258, *S. aureus* USA300_TCH1516, CP000730, CP000731, CP001544, *S. aureus aureus* MRSA252 BX571856, *S. aureus aureus* MSSA476 BX571857, BX571858, *S. aureus* COL CP000046, CP000045, *S. aureus* JH1 CP000736, CP000737, *S. aureus* JH9 CP000703, CP000704, *S. aureus* ED98 CP001781, CP001782, CP001783, CP001784, *S. aureus* N315,

BA000018, AP003139. Identical sequences are merged and strain specific ones were added by a specific enzymatic cleavage rule (cKcRcJcOnJnO). The resulting *S. aureus* database contained 4374 sequences and 1,923,939 residues. It was searched with the Mascot in-house version 2.3.02 using the following filter settings: a minimum protein length of 50 aa, a minimum for J-peptide length of 7 aa, a maximum for J-peptide length of 35 aa, a minimum for O-peptide length of 7 aa and a maximum for O-peptide length of 35 aa was applied. The non-significant option from the *Re-search tool* was used in order to resubmit unmatched spectra from the dataset obtained from the first search against human sequences. TrypsinJO/P was selected as enzyme with 2 missed cleavage sites and a mass tolerance of 10 ppm for the precursor ion (including 13C shift) was allowed. For fragment search, 0.05 Da for the HCD-MS/MS fragments and 0.5 for CID-MS/MS fragments were applied. Carbamidomethyl (C) conversion was allowed as fixed modification. A protein hit was accepted if the significance threshold was less then $p < 0.05$ which is roughly equal to an ion score > 14–20. Proteins were further gene ontology-based (GO) categorized using the program ProteinCenter, version 3.13.10011 (Thermo Scientific, Waltham, MA, USA).

2.11. Statistical analysis

The data obtained from the two patients groups (*S. aureus* negative vs. *S. aureus* positive) were compared by a non-parametric Mann-Whitney test. Data derived from human sinonasal ex vivo tissue fragment assays were compared by means of a Wilcoxon signed-rank test after achieving significance in a Friedman test (paired test for > 2 groups). A cutoff of $p < 0.05$ was used to establish statistical significance. All statistics were performed using SPSS Statistics 22 software (IBM, Germany).

3. Results

3.1. Patients and presence of S. aureus

Seventeen consecutive patients undergoing surgery for CRSwNP provided material for research. From the polyp samples of 9 of the patients, we could isolate and culture *S. aureus* from the nasal polyp tissue. There was no statistically significant difference between the clinical data of the two groups in terms of age, atopy or comorbid asthma. Furthermore, the groups did not differ significantly in total serum IgE concentrations, specific IgE-antibodies to staphylococcal enterotoxins nor in serum concentrations of eosinophil cationic protein (ECP) or IL-5 (Table 1). Among the cultivatable germs, *S. aureus* was the most frequent, followed by *S. epidermidis* (Table 2).

3.2. IL-5 release in nasal tissue culture

When tissue fragments were taken into culture, we observed spontaneous release of IL-5 in all nasal polyp samples, which was > 3-fold higher in those nasal polyps containing, *S. aureus* compared to *S. aureus*-negative tissues (Fig. 1).

In *S. aureus*-positive nasal polyp tissue the spontaneous release of IL-5 was significantly reduced by antibiotics as well as by the bacteriophage ISP (Fig. 2). 8 log PFU/mL phage ISP was the concentration as determined before based on its anti-staphylococcal effect. The antibiotic treatment reduced spontaneous IL-5 concentrations after 24 and 72 h to the levels seen without *S. aureus*, whereas the effect of the ISP phage was less pronounced, but significant at 72 h. Heat-inactivation of the phage or co-incubation with Anti-ISP phage serum (APS) abolished the effect of the phage. By contrast, antibiotics or ISP phage treatment did not change IL-5 secretion in *S. aureus*-negative tissue cultures.

Table 2
Bacterial strains present in nasal polyps (culture).

	S. aureus negative CRSwNP	*S. aureus* positive CRSwNP
S. aureus	0/8	9/9
S. epidermidis	3/8	1/9
Escherichia coli	3/8	0/9
Pseudomonas aeruginosa	1/8	0/9

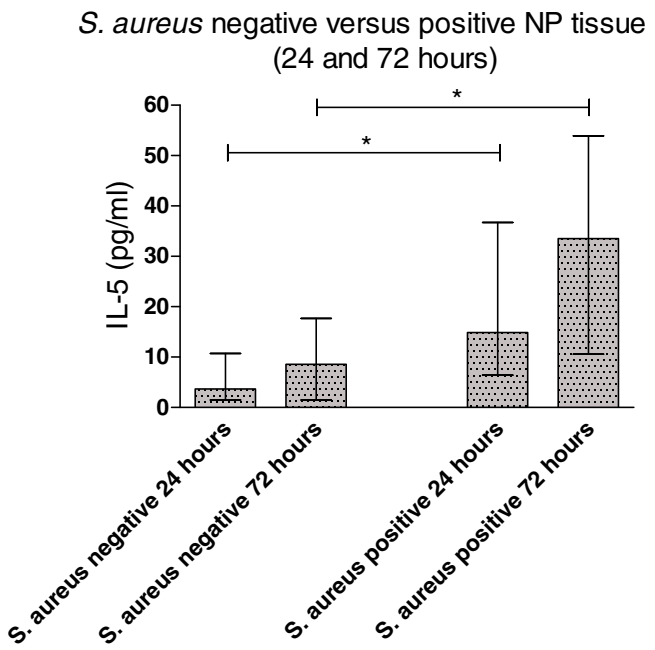

Fig. 1. Spontaneous IL-5 protein release into the supernatants of human nasal polyp tissue ex-vivo. Data are presented as bar charts showing the median and the interquartile range. *$p < 0.05$ (Mann-Whitney test).

3.3. Quantification of S. aureus after ex vivo nasal tissue culture

At the start of experiments (time point zero) no bacteria could be detected in any samples at/above the detection threshold level defined as 2 log CFU/mL (Fig. 3). In cultures of *S. aureus*-positive tissue fragments, massive growth of *S. aureus* was noticed over 24 to 72 h (medians 6 and 8 log CFU/mL, respectively). Bacterial growth was significantly reduced by antibiotics as well as active phage treatment, but not when phages had been heat-inactivated or neutralized by pre-incubation with APS. ISP at the concentrations of 9 and 8 log/mL could completely prevent the growth of *S. aureus* strains for up to 48 h. After 72 h, average bacterial load was in the range 3.4–4.2 log CFU/mL. With lower concentrations of phage ISP, 7 log PFU/mL, bacterial growth was already detectable after 24 h; after 72 h it reached an average of 5.7 log CFU/mL, which was still significantly less than the average 8 log CFU/mL observed in the absence of phages.

3.4. Identification and classification of cytoplasmic and secreted S. aureus proteins in ex vivo cultured nasal tissue

Three independent nasal tissue fragments (BOF1129, BOF1132 and BOF1209), obtained freshly or after cultivation as described above, were homogenized, and protein extracts were subjected to MS analysis to search for the presence of *S. aureus* proteins. In total, 1.458 *S. aureus*-specific proteins were identified despite the presence of a large excess of human proteins (Supplementary Tables 1–3). This high sensitivity was achieved by extensive pre-fractionation of the samples (175 gel bands and 18 gel-free samples) in combination with the specific iterative

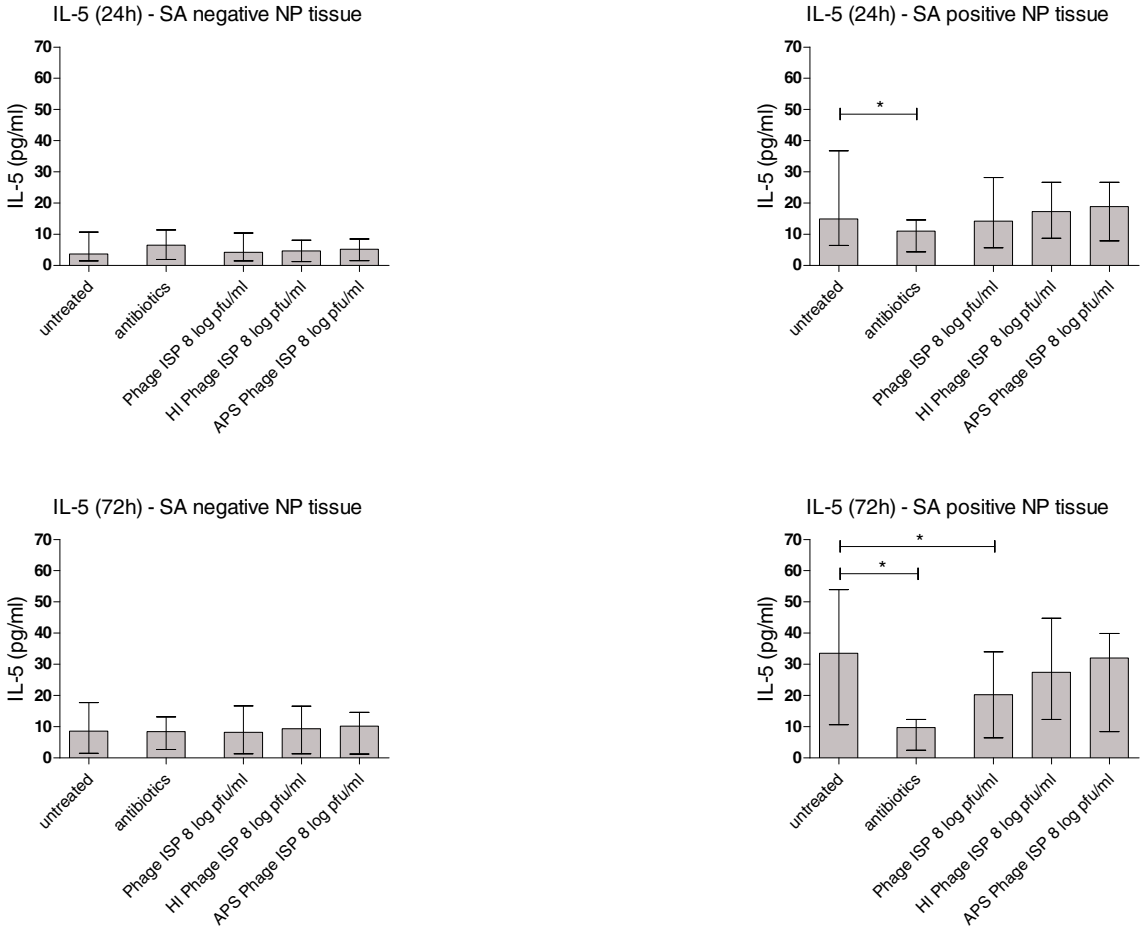

Fig. 2. Effects of antibiotics, Phage ISP, heat-inactivated Phage ISP and APS Phage ISP (all 8 log PFU/mL) on the release of IL-5 protein from human nasal polyp tissue ex-vivo. Data are presented as bar charts showing the median and interquartile range. *$p < 0.05$ (Wilcoxon test).

search strategy (see Materials and Methods), which first identified and excluded the human proteins from the dataset and then searched the remaining profiles against the MSMSpdbb database which contains *S. aureus* proteins selectively. Since we also found additional bacteria in the PCR analyzes (Table 2), a proteome-specific analysis was also performed. Taking all data together, we found additional proteins belonging to *S. epidermidis* and *E. coli*. However, most of the identifications were characterized by single peptide identification and Mowse scores slightly above the threshold. When comparing the median Mowse scores it is obvious that human protein identifications (64) and *S. aureus* protein identifications (68) were clearly higher in median than *E. coli* [21] or *S. epidermidis* [19] (Supplementary Fig. 1a). In terms of these results we believe that the majority of the reliable and significant identifications belonging to human and *S. aureus* but not to *E. coli* and *S. epidermidis*.

With further applied different types of classification analyses. In order to perform such analyses in a reliable fashion, we first calculated how many proteins fitting to which subspecies in the *S. aureus* specific MSMSpdbb database. This was done for each nasal polyp sample (Supplementary Fig. 1b). As a result, roughly 86% of the identified proteins from the three *S. aureus* isolates showed homology to the subspecies *S. aureus* JH90 and based on this, we performed a percentagewise comparison on this strain only. In summary, the majority of the proteins were unannotated (50%) in the category cellular components from GO. The annotated proteins showed a nearly identical distribution in all isolates. Most of the proteins were assigned to cytoplasm (15%) followed by membrane and intracellular ones (5%). A similar percentage of about 4% was detected for integral component of membrane, ribosome or ribonucleoprotein complex. Roughly 1–2% of the proteins were belong the plasma membrane, extracellular region and cell wall complex (Supplementary Fig. 1c). If one compares the biological processes from GO, a similar distribution between the isolates can be seen. Again, the majority of proteins cannot be assigned to any category. The subsequent dominant group was metabolic processes followed by oxidation and translation. With about 2% or less followed the other groups, such regulation, transport or transcription (Supplementary Fig. 1d).

Due to the particular interest in proteins which can be secreted into the intramucosal or intracellular space of CRSwNP tissues, we focused now on proteins containing a signal peptide; 177 such proteins were detected. We focused our attention on superantigens and Spls A–F because of their known IL-5-inducing potential in nasal tissue culture [6,8,18]. Considering the minute numbers of *S. aureus* in fresh nasal polyps, only a small number of *S. aureus* secreted proteins was detectable, among them SplF. 72 h post-cultivation without anti-*S. aureus* treatment, three superantigens were detected, SEA(P), SEI(Q) and TSST-1. After treatment with antibiotics, no bacterial toxins or proteases were detectable. In comparison the ISP phage treatment two superantigen-like proteins could be detected (staphylococcal exotoxin 3 and 7). Neutralization of phages by Anti-ISP Phage Serum (APS) or heat-inactivation did not inhibit the secretion of toxins or serine proteases. The total number of secreted *S. aureus* proteins detected in the tissues was highest following tissue culture without inhibition and significantly reduced by antibiotic and ISP treatment. Inactivation of the phages restored the number of bacterial proteins that could be identified (Fig. 4, Table 3).

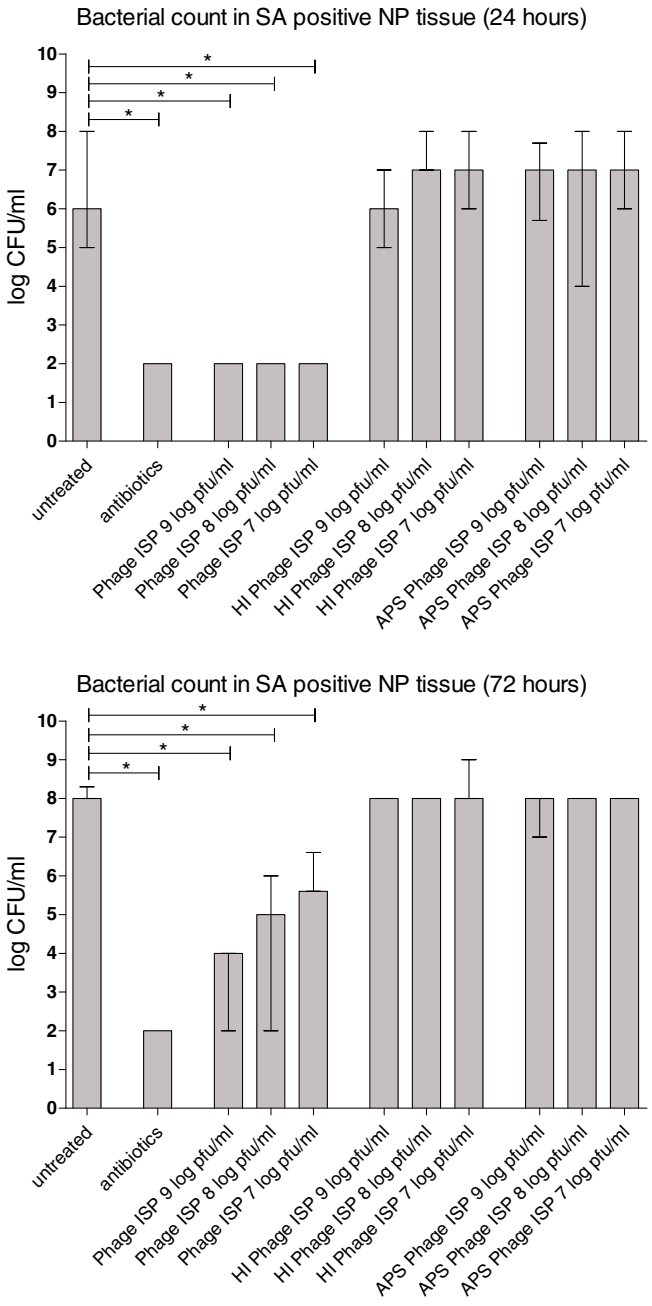

Fig. 3. Quantification of *S. aureus* strains by culturing method in ex-vivo nasal tissue experiments. ISP Phage, heat inactivated ISP; APS ISP; Antibiotics-50 IU/mL penicillin, 50 µg/mL streptomycin and untreated. The sample volume was 1 mL, the bacterial detection threshold was 2 log CFU/mL. The chart is a summary of results of nasal tissue samples obtained from six patients infected with *S. aureus*. Data are presented as bar charts showing the median and interquartile range. $*p < 0.05$ (Wilcoxon test). *p*-Values vs. baseline for 24 h: antibiotics 0.018, phage ISP 9 log 0.018, phage ISP 8 log 0.018, phage ISP 7 log 0.018. *p*-Values vs. baseline for 72 h: antibiotics 0.017, phage ISP 9 log 0.017, phage ISP 8 log 0.027, phage ISP 7 log 0.044.

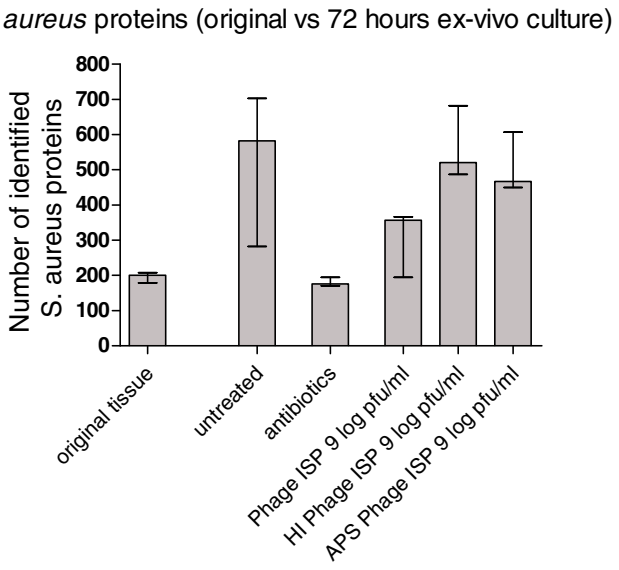

Fig. 4. Numbers of secreted *S. aureus* proteins identified from three polyp samples (BOF1129, BOF1132 and BOF1209). Fresh tissue fragments were analyzed as well as tissue fragments that had been cultured for 72 h with the following additives: (i) none (untreated), (ii) penicillin/streptomycin (antibiotics), (iii) ISP phages; (iv) heat inactivated (HI) phages and (v) ISP phages and anti-phage serum (APS). The nLC-MS/MS analysis was performed with the Orbitrap Velos or Q Exactive using gel-based and gel-free approaches.

4. Discussion

We here for the first time demonstrate that cultivatable *Staphylococcus aureus* can be found in type-2 inflamed upper airway mucosal tissue, freshly harvested nasal polyps. In tissue culture those bacteria are able to replicate within 24 h, they are metabolically active and are able to secrete proteins, comprising enterotoxins, exotoxins, serine proteases as well as other proteins, which were detected and identified by a sophisticated mass spectrometry approach. The presence of *S. aureus* was associated with a significantly higher spontaneous release of interleukin-5, a key cytokine for activation and survival of eosinophils, by polyp tissue. Killing of *S. aureus* by antibiotics as well as by the *S. aureus* specific ISP bacteriophage significantly reduced or abolished the spontaneous IL-5 release. The suppressive activity of the bacteriophage could be reversed by heat inactivation or co-administration of phage-neutralizing antibodies, indicating the specificity of the suppression. These experiments unequivocally demonstrate the ability of *S. aureus* to induce IL-5 production in nasal polyps. It is very likely that some of the proteins produced by growing bacteria and detected here contribute to this up-regulation of type 2 inflammation, as in the past we have shown that enterotoxins [8] and serine protease-like proteins [18], both detected in the tissues after spontaneous bacterial growth, are capable of inducing IL-5 release from nasal polyp tissues.

S. aureus colonization has been demonstrated to be frequent and persisting in eosinophilic nasal polyp disease [9,10,19] and was also associated with recurrence of disease after surgical interventions, possibly also related to biofilm formation [20]. We and others have shown before that *S. aureus* can be found intra-mucosally and intra-cellularly in nasal polyp tissues [11,21,22]; *S. aureus* may reside within the epithelial cells, macrophages and mast cells, as demonstrated by PNA-FISH [11], and may replicate intra-epithelially, as shown by electron microscopy [21]. Type-2 biased nasal polyp tissues have been suggested to have a marked deficit in defense against this bacterial species, as increased numbers of alternatively activated macrophages have been found in this environment, being incompetent to phagocytose and kill intracellular *S. aureus* [12]. The same phenomenon also has been documented in severe asthma [23].

In a mouse asthma model, we studied the role of bacterial enterotoxins such as SEB on nasal and bronchial allergic inflammation [24]. Both nasal and bronchial application of SEB enhanced allergen-induced eosinophilic inflammation, correlating with higher transcription of mRNA for IL-5, IL-4, and other cytokines, and also increased titers of OVA-specific and total IgE in serum. Classical enterotoxins, especially staphylococcal enterotoxin B (SEB), have been shown to be capable of inducing Th1, Th2 and Th17-related cytokine release in nasal mucosal and polyp tissue fragments (8). SEB stimulation over a

Table 3
Number of independently identified *S. aureus* proteins in three fresh and treated nasal polyp samples by gel-based Orbitrap VELOS MS (OV) and gel-free Q Exactive MS (QE).

	fresh nasal polyp		TP27 - 72h no treatment		TP28 - 72 h penicillin/streptomycin		TP31 - 72 h phages		TP34 - 72 h ISP phages		TP37 - 72 h heat-inactivated phages	
	OV	QE	OV	QE	OV	QE	OV	QE	OV	QE	OV	QE
BOF 1209	52	41	396	544	41	43	56	174	65	319	132	335
BOF1129	44	50	57	193	19	41	161	217	80	307	276	430
BOF 1132	32	47	704	819	35	45	27	39	326	488	524	691
Average	43	46	386	519	32	43	81	143	157	371	311	485

period of 24 h induced significant increases of IL-4, IL-5, and IL-13, among other cytokines. We may, therefore, assume that the enterotoxins found in of ex-vivo cultures of *S. aureus*-positive nasal polyp tissue also contributed to the release of IL-5 in the current experiment. Recently, screening for bacterial allergens, we identified staphylococcal serine protease-like proteins (Spls) eliciting IgE antibody responses in asthmatic patients [18]. In mice, inhalation of SplD induced lung inflammation characterized by Th2 cytokines and eosinophil infiltration. Following stimulation with Spls, peripheral blood T cells elaborated Th2 cytokines as is typical for allergens. Also, making use of an ex vivo tissue culture model, the local cytokine response was examined in the nasal polyp tissue model. As expected, SEB induced significant amounts of IL-5, IL-17 and IFN-γ at a low concentration (0.1 μg/mL); SplD increased IL-5 production to the same extent as SEB at higher concentrations, but had only marginal effect on IL-17 and IFN-γ. The cytokine response elicited by SplD in the human airway tissue model is shifted toward Th2 in comparison to SEB. Hence, Spls, which were also present in the *S. aureus*-positive tissue cultures, may also contribute to the observed up-regulation of IL-5.

The current experiments made use of freshly collected human diseased polyp tissue and the spontaneous cytokine release from the tissues was measured over 72 h, without the addition of any stimuli or bacteria; this way we were able to approach the real-life situation as closely as possible. These findings confirm unequivocally that *S. aureus* is present within or at the epithelial surfaces of nasal polyp tissue, as has been shown earlier, but adds knowledge in that this germ is culturable and replicates fast. Furthermore, we could demonstrate that the germ secrets proteins ("the secretome") into tissues, in some of which we and others have demonstrated their activity on human immune tissue before. Interestingly, although the spontaneous release of IL-5 was significantly higher in *S. aureus* positive samples after 24 and 72 h in comparison to *S. aureus* negative samples, there was no difference in baseline mucosal cytokine secretion, of SE-IgE antibodies and total IgE concentrations in serum nor in the clinical presence of comorbid asthma (see Table 1). This suggests previous exposure and sensitization to *S. aureus* also in patients with *S. aureus*-negative polyps. The presence of culturable *S. aureus* may vary over time, and the same patient's tissues may be positive at another time point, depending on the number of germs present at that specific moment. Bacterial counts at baseline were below 2 log CFU/mL and increased to 6 log and 8 log CFU/mL within 24 h and 72 h in tissue culture, respectively, when untreated. The growth properties of *S. aureus* in nasal polyp tissue may also be influenced by competition with other bacterial species, as Table 2 indicates, since 7 out of 8 *S. aureus*-negative tissue samples grew *S. epidermidis, E. coli* or *P. aeruginosa* in culture at the moment of sampling, whereas only 1 out of the 9 *S. aureus*-positive samples was positive for another species (i.e. *S. epidermidis*).

Whereas bacterial growth and IL-5 production remained low over time in *S. aureus* negative samples, increased bacterial growth and increased IL-5 production was observed in *S. aureus* positive tissue samples, which could be reduced to baseline values by antibiotic treatment. As antibiotics may target many bacteria and possess anti-inflammatory activities, we included a highly *S. aureus*-specific intervention in addition to antibiotics, and made use of ISP bacteriophages [14]. Increased bacterial growth and *S. aureus* induced IL-5 production were dose-dependently reduced by the specific bacteriophage treatment over 24 and 72 h, reaching significance at 72 h. The inactivation of the suppressive phages by heat-inactivation or by antibodies directed against the phages abolished this effect, underlining the specificity of the model. In parallel, the number of bacterial proteins that could be identified in *S. aureus* positive samples increased from 10 at baseline to over 50 proteins at 24 h without treatment, but was reduced by antibiotic and phage treatment ($n = 3$), indicating the specific involvement of *S. aureus*. Among those proteins were SEA(P), SEI(Q) and TSST1 as well as the serine protease SplF, members of protein families that have been demonstrated to induce IL5 in nasal tissues [8,18]. In untreated fresh nasal polyp tissue samples from our hospital, but unrelated to the current experiments, the following enterotoxins were detected at least once using high resolution mass spectrometry: SEB, SEG, SEH, SEO, SEL, and after cultivation the serine protease-like proteins SplA and SplF in addition. This underlines the secretory activity of *S. aureus* in nasal polyp tissue and underlines its possible role in driving the inflammatory process.

Although the evidence for a direct role of *S. aureus* in the regulation of IL-5 in patients with upper airway disease is striking, we can only provide indirect evidence for the involvement of secreted proteins. The only feasible way to inhibit the interaction between the germ and the mucosa seems to be the killing of the producing bacteria, the effect of which we clearly demonstrated here using antibiotics and ISP bacteriophages. Specific inhibition, e.g., by neutralizing antibodies, could be a way of dissecting the individual roles of various secreted proteins in the process, which, however, are likely to be highly redundant. Probably, both the bacteria themselves and their secretion products contribute to the chronic inflammatory process in nasal polyposis.

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