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Immunomodulation of Nasal Epithelial Cells by Staphylococcus aureus-Derived Serine Proteases

Claudia Rudack,* Florian Sachse,* Nils Albert,* Karsten Becker,† and Christof von Eiff2,3†

The impact of Staphylococcus aureus in the pathogenesis of chronic rhinosinusitis is not well understood. Therefore, we investigated primary human nasal epithelial cell cultures for their ability to produce IL-8, growth-related oncogene-α, and IL-6 via stimulation with trypsin and culture supernatants of different S. aureus strains and phenotypes. Inhibition of cytokine synthesis was performed using a glucocorticoid, a serine protease inhibitor, and a cytokine protease inhibitor. Finally, signal transduction pathways were analyzed by quantifying phosphorylated forms of MAPKs (PI3K, ERK, and p38) and DNA-binding assays that quantified NF-κB and its inhibition using BAY11-7085. In vitro studies showed that the induction of IL-8, growth-related oncogene-α, and IL-6 by S. aureus culture supernatants was significantly inhibited by the serine protease inhibitor. In contrast, steroids and the cytokine protease inhibitor had little effect. Activation of NF-κB was observed after cell treatment with trypsin and bacterial supernatants, and was inhibited by BAY11-7085 and the serine protease inhibitor. S. aureus serine proteases were identified to modulate chemokine synthesis and activate NF-κB in nasal epithelial cells, and may therefore be relevant for the pathophysiology of chronic rhinosinusitis. The Journal of Immunology, 2009, 183: 7592–7601.

C hronic rhinosinusitis (CRS) is an inflammation of unknown etiology of one or more paranasal sinuses lasting for at least 12 wk that involves the mucosal lining of the nasal cavity. Recent studies revealed that the opportunistic pathogen Staphylococcus aureus may be regularly recovered from nasal lavages and mucosal biopsies of CRS patients (1,2). However, the role of this microorganism as causative agent for CRS has not been defined yet. In addition, the impact of small-colony variants (SCVs), a phenotypic population of S. aureus, known to be associated with recurrent and persistent infections (recently reviewed in Ref. 3), has not been adequately determined for this chronic disease (1,4).

Mechanisms that cause rhinosinusitis to become recurrent and ultimately chronic have been partially attributed to a release of microbial products (5). Members of the S. aureus pyrogenic toxin superantigen family such as staphylococcal enterotoxins have been identified to induce the release of a Th2-skewed pattern of cytokines in CRS tissue biopsies (6–8).

Besides a broad range of exotoxins, adhesins, and other virulence factors affecting the host, S. aureus produces 10 proteolytic enzymes comprising a metalloproteinase (aureolysin), two related cytosteine proteases ScpA and SspB, a serine glutamyl endopeptidase SspA, and six other serine proteases (splABCDEF) (9,10).

Generally, serine proteases such as trypsin and tryptase are considered to play a role in inflammation and immunity under physiological and pathophysiological conditions by cleavage of protease-activated receptors (PARs) and subsequent activation of various signal transduction cascades such as the mobilization of calcium from endoplasmatic reticula (11). Noteworthily, PAR2 expression is increased in nasal biopsies from patients with CRS (12). Consistent with this, stimulation of cultured nasal epithelial cells with either PAR2-activating peptide or trypsin induced G protein- and NF-κB-dependent expression of the neutrophil chemottractants IL-8/CXCL8 and growth-related oncogene (GRO)-α/CXCL1, but not the eosinophil and T cell chemottractants eotaxin/CCL11, CCL5, or CCL17 (12).

Therefore, this study was undertaken as follows: 1) to determine S. aureus supernatant modulation of cytokine generation and cytokine signaling in an in vitro cell culture system of primary nasal epithelial cells, and 2) to elucidate the role of S. aureus-derived serine proteases in initiating and maintaining inflammatory mechanisms in nasal epithelial cells. For these purposes, two well-characterized S. aureus strains with normal phenotype as well as corresponding site-directed hemB mutants displaying a stable SCV phenotype were used to assess modulation of cytokine synthesis by cysteine and serine protease inhibitors as well as by a glucocorticoid. Cytokine signaling pathways (p38, the ERK 1/2, and the PI3K) as well as the involvement of NF-κB during cytokine synthesis were examined in nasal epithelial cells by means of NF-κB-DNA-binding assays and quantifying phosphorylation of different kinases. In addition, kinases were blocked by specific inhibitors to study regulation mechanisms of cytokine synthesis.

Materials and Methods

Materials

Trypsin and all other reagents were purchased from Sigma-Aldrich, unless otherwise stated.
Primary nasal epithelial cell culture from turbinate mucosa (TM) of the nasal cavity

For respective experiments, human nasal epithelial cells were cultured from the inferior TM of a total of 23 subjects undergoing conchotomy without any signs of rhinosinusitis (mean age 49.8 ± 15.5 years) at the Department of Otorhinolaryngology in the University Hospital of Münster. Informed consents were obtained from all patients, and the ethics committee of the University of Münster approved the study (Reg. Nr. 5XII Rudack).

The tissue was washed with PBS and incubated with trypsin (0.5%) overnight at 4°C, as previously described, with minor modifications (13). Cells were grown to confluence (~2 wk) and passaged two further times before they were used for experiments. Epithelial cells were split and grown in six-well plates (each well containing ~1.8 × 10^5 cells) to confluence. Twenty-four hours before the studies were initiated, the medium was replaced by serum-free medium. Cytokertins were expressed until five passages of the cells.

Stimulation experiments

Primary nasal epithelial cells were exposed to the supernatants of two well-characterized strains of \textit{S. aureus} and their isogenic site-directed \textit{S. aureus hemB} mutants displaying the SCV phenotype (Table I). \textit{S. aureus} COL, a methicillin-resistant strain (mecA gene positive), was tested PCR positive for the enterotoxin B gene (seb), whereas the strain \textit{S. aureus} Newman (ATCC 25904) was tested PCR positive for the enterotoxin A gene (sea), as previously described (14).

To yield the staphylococcal supernatants, strains were grown in 150 ml of tryptic soy broth for 12 h and adjusted to OD of 1.0 with sterile tryptic soy broth. Then, an aliquot was removed for CFU determination (5 × 10^8–1 × 10^9). Bacterial cells were removed by centrifugation (4000 rpm for 20 min, 4°C). Except for \textit{S. aureus} strain Newman (dilution of 1/10), supernatants were added at a dilution ratio of 1/5.

In addition, cells were also exposed to trypsin at a concentration of 10^{-7} M during the stimulation experiments. Trypsin belongs to the group of serine proteases and serves a positive control stimulus. Inhibition investigations due to specific protease inhibition were performed with 1 mM serine protease inhibitor 4-[(2-aminoethyl)-benzenesulfonyl] fluoride (AEBSF; Roche Diagnostics), cysteine protease inhibitor \textit{trans}-epoxysuccinyl-leucylamido(4-guanidino)butane (E64; Sigma-Aldrich) at a concentration of 10 μM, and prednisolone (Merck Pharma) at a concentration of 1 × 10^{-5} M.

BAY 11-7085, a (2E)-3-[[4-(1,1-dimethylylethyl)phenyl][sulfonfonyl]-2-propenentitrile (Calbiochem), is an inhibitor of NF-κB that was used at a concentration of 10 mM to block NF-κB-dependent synthesis of IL-8 and IL-6. BAY 11-7085 is an irreversible inhibitor of TNF-α-activated IκBα phosphorylation. For further inhibitor experiments, primary nasal epithelial cells were incubated with cortisone (prednisolone; 10^{-8} M; Merck Pharma).

MAPKs were inhibited by pretreatment with either PD98059 (Calbiochem), at a concentration of 50 μM and wortmannin (Calbiochem) at a concentration of 100 nM or the selective p38 inibitor SB-239603 (trans-1-(4-hydroxycyclohexyl)-4-fluorophenyl)-5(2-methoxy-pyrimidin-4-yl) imidazole) at a concentration of 30 μM, 30 min before challenge with bacterial supernatants (BioSource International) (15).

All supernatants and inhibitors were pretested at different concentrations of relevance to cell vitality (trypan blue dye exclusion) and chemokine production in the control studies. All experiments were conducted four to eight times.

Determination of proteolytic activities in bacterial supernatants

Proteolytic activities from four different \textit{S. aureus} strains (for characterization, see Table I) were determined using the insoluble proteolytic substate azocoll (Calbiochem; n = 6 independent experiments for each strain), as previously described, but with some minor modifications (16). The substrate (4 mg/ml) was suspended in 100 mM phosphate buffer (pH 7.0). A total of 750 μl of an overnight culture supernatant was added to 500 μl of the substrate suspension. The mixtures were incubated for 2 h at 37°C with shaking. After incubation, the assay mixtures were centrifuged and the absorbances at 520 nm were determined. Proteases were distinguished through the use of different protease inhibitors, AEBSF, EDTA (Calbiochem), and E64. These inhibitors were added to the culture supernatant for 45 min before adding the proteolytic substrate azocoll. One unit of protease activity was arbitrarily defined as the proteolysis that resulted in the release of 0.001 absorbance unit of dye for 2 h. Protease-specific activities were expressed as units per milligram of protein.

ELISA measurements of cytokines

ELISA tests for cytokines IL-8 (minimum detectable concentration 3.5 pg/ml), GRO-α (minimum detectable concentration 10 pg/ml), and IL-6 (minimum detectable concentration 3.5 pg/ml) were performed according to the manufacturer’s recommendations (R&D Systems), respectively.

Activation/Inhibition of MAPK (p38a, ERK1/2) and PI3K in epithelial cells treated with bacterial supernatants

To determine kinase phosphorylation, SDS-PAGE and immunoblotting were first established to identify optimal time of stimulation before the quantitative measurement of phosphorylated kinases.

ELISA phospha-p38a (T180/Y182)

The Surveyor IC Immunoassay employs a two-site sandwich ELISA to quantify p38a phosphorylated at T180/Y182 in cell lysate. Before stimulation with bacterial supernatants or trypsin for 15 min, primary human epithelial cells were seeded overnight in 25-cm² culture flasks and incubated at 37°C (5% CO2). Twenty-four hours before the studies were started, the medium was replaced by serum-free medium. After stimulation for 15 min, 25-cm² culture flasks were placed on an ice bath and washed three times with ice-cold PBS. Lysates were collected by adding 100 μl of Nonidet P-40 lysis solution (1% Nonidet P-40, 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 10 μg/ml leupeptin and aprotonin protease inhibitors, 50 mM NaF, and 1 mM sodium vanadate; Roche Diagnostics) to each flask, scraping the cells from the flasks, and pipetting the lysate into 5.0-mL centrifuge tubes. The lysates were placed on ice for 20–30 min and vortexed briefly every 7 min. All lysates were then centrifuged at 4°C for 30 min, and the supernatants were transferred to a new 1.5-mL microcentrifuge tube and stored at −80°C. Protein concentration of the cellular extracts was determined using the Bio-Rad protein assay. The test was conducted according to the manufacturer’s recommendations (R&D Systems).

Face PI3K p85

Face PI3K p85 (Active Motif) was tested for sensitivity and specificity in detecting the phosphorylation of PI3K. Before stimulation with bacterial supernatants or trypsin, primary human epithelial cells were seeded overnight in 96-well plates and incubated at 37°C (5% CO2). A gain, 24 h before the studies, the medium was replaced by serum-free medium. After the cells were fixed, one plate was studied with the phospho-PI3K Ab, whereas the other plate was analyzed with the total PI3K Ab. The relative number of cells in each well was subsequently determined by the use of the Crystal Violet reagent. Once the phospho-PI3K and total PI3K signals were normalized for cell number, a comparison of the ratio of phosphorylated PI3K to total PI3K for each of the cell growth conditions was performed. The total PI3K p85 Ab can be used to detect the total level of PI3K p85 proteins regardless of phosphorylation state.
Table II. Extracellular protease activity determined in the culture supernatant

<table>
<thead>
<tr>
<th>Pro tease</th>
<th>Additive</th>
<th>Activity* (U/mg protein) of Strains:</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Parental Strain</td>
<td><em>hemB</em> Mutant III3</td>
<td>Parental Strain</td>
<td><em>hemB</em> Mutant IA48</td>
<td></td>
</tr>
<tr>
<td>Total protease</td>
<td>590</td>
<td>2769* p = 0.026</td>
<td>571</td>
<td>1410 NS</td>
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<tr>
<td>Serine protease</td>
<td>289</td>
<td>1847* p = 0.041</td>
<td>356</td>
<td>1000 NS</td>
<td></td>
</tr>
<tr>
<td>Metalloprotease</td>
<td>94</td>
<td>953* p = 0.009</td>
<td>214</td>
<td>20 NS</td>
<td></td>
</tr>
<tr>
<td>Thiol protease</td>
<td>248</td>
<td>615* p = 0.041</td>
<td>179</td>
<td>705 NS</td>
<td></td>
</tr>
<tr>
<td>DT, EDTA, E64  (10 μM)</td>
<td>82</td>
<td>230 NS</td>
<td>0</td>
<td>101 NS</td>
<td></td>
</tr>
</tbody>
</table>

* Units of serine protease and metalloprotease were determined by subtraction of units obtained with AEBSF (1 mM) and EDTA (1 mM), respectively, from the total units of proteolytic activity (n = 6). Thiol protease activities were measured in the presence of DT (1 mM), EDTA, and the cysteine inhibitor E64 (1 mM). *p Displays significance of means obtained from six measurements of proteolytic activities in supernatants of four different _S. aureus_ strains calculated by comparing activity from parental and mutant strain (Mann-Whitney U' sum rank test).

Statistical analysis

All results are presented as box plots using the SigmaPlot 11 software package (Systat software). The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles; outliers are displayed as dots.

Applying the nonparametrical Mann-Whitney rank sum test, our null hypothesis was that the two samples were not drawn from populations with different medians. Value of p < 0.05 was considered statistically significant.

**Results**

**Extracellular proteolytic activity of bacterial _S. aureus_ strains**

To specify extracellular proteolytic activity in bacterial supernatants (16), overnight cultures were assayed using the azocoll substrate. For six subsequent experiments, supernatants of both normal phenotype _S. aureus_ (strains COL and Newman) and their isogenic _S. aureus hemB_ mutants were used (Table I) (17, 18). These site-directed mutants have been constructed to generate a stable SCV phenotype because SCVs recovered from clinical specimens are genetically undefined and often spontaneously revert to the normal phenotype (3).

In fact, total extracellular proteolytic activity significantly differed in the _hemB_ mutant derived from _S. aureus_ Newman compared with its parent strain (Table II). Noteworthy, the same phenomenon was observed comparing _S. aureus_ COL proteolytic activity with its _hemB_ mutant IA48, but reached no statistical significance (Table II).

By using different protease inhibitors, we were able to quantify different types of proteases present in the culture supernatant. Serine proteases were inhibited by AEBSF, a hydroxyl-reactive organofluoride, whereas the metalloprotease required a chelator EDTA for its inhibition. Serine proteases and the metalloprotease were secreted by all _S. aureus_ strains and phenotypes; however, production of the serine proteases was significantly increased in supernatant of mutant III3 compared with the parental strain (Fig. 1; Table II). Metalloprotease activity was also mostly detected in III3. Because cysteine (=thiol) proteases contain a sulfhydryl group in the active site, they are only active in the presence of reducing agents such as DTT. Thus, thiol protease activities were determined in the presence of DTT (Table II) and the specific inhibitor E64 as control. Cysteine protease activity was mostly produced in _hemB_ mutant IA48, but reached no statistical significance.

**Stimulation of nasal epithelial cells by trypsin and _S. aureus_ supernatants**

Primary nasal epithelial cell cultures were established from TM of six subjects undergoing conchotomy without any signs of rhinosinusitis. To examine the capacity of nasal epithelial cells to...
synthesize CXC chemokines and IL-6 time dependently, cells were stimulated in a different manner. Besides the supernatants of *S. aureus* strains as indicated in Table II, trypsin was applied as a positive control to the stimulation experiments.

Our results revealed that nonstimulated nasal epithelial cells released IL-8, GRO-α, and IL-6 spontaneously over time. Compared with nonstimulated controls, significantly elevated levels of IL-8 and GRO-α protein were registered after stimulation periods with trypsin at concentrations of $10^{-7}$ M for 12 and 24 h (Fig. 2). IL-6 was not detected.

Supernatants of *S. aureus* strain Newman and its corresponding *hemB* mutant III33 induced a significant increase in IL-8, GRO-α, and IL-6 production compared with nonstimulated controls after 12 and 24 h (Fig. 2).

FIGURE 1. Units of total protease, serine protease, metalloprotease, and thiol protease activity in four different *S. aureus* strains and phenotypes (see Table I). Serine protease and metalloprotease activity were determined by adding AEBSF (1 mM) and EDTA (1 mM), respectively. Thiol protease activities were measured in the presence of DTT (1 mM) and EDTA (1 mM). The data are summarized from six different experiments (*n* = 6) and represented as box plots showing the median as straight line.

FIGURE 2. Production of IL-8, GRO-α, and IL-6 by primary nasal epithelial cells stimulated with trypsin or with bacterial supernatants after 12 and 24 h. Epithelial cell cultures were obtained from six different inferior turbinates taken from sinuses of subjects undergoing conchotomy (*n* = 6 for each series). The box plots represent IL-8, GRO-α, or IL-6 levels (pg/ml) obtained by stimulation with trypsin and the different *S. aureus* strains and phenotypes for 12 and 24 h (straight line = median). The box plot indicates the 25th and 75th percentiles within the box. The whiskers above and below the box indicate the 90th and 10th percentiles. Different colors, white, gray, and dark gray, were used to identify cytokine levels with their corresponding inductors according to the legend. Value of *p* < 0.05 (*) was considered statistically significant according to the Mann-Whitney rank sum test comparing nonstimulated controls (con) with cells stimulated with trypsin and bacterial supernatants (*n* = 6). Cytokine levels of IL-8 and GRO-α generated by Col and IA48 after 12 are significantly higher than those of New and III33 (*p* < 0.001). Abbreviations: con, control; New, *S. aureus* strain Newman; III33, *S. aureus hemB* mutant with strain Newman as parental strain; COL, *S. aureus* strain COL; and IA48, *S. aureus hemB* mutant with strain COL as parental strain.
Supernatants of the methicillin-resistant *S. aureus* strain COL and its *hemB* mutant IA48 induced significant IL-8 and GRO-α responses compared with nonstimulated controls after 12 and 24 h (Fig. 2). Interestingly, stimulation of IL-8 and GRO-α synthesis in *S. aureus* strain COL and its *hemB* mutant IA48 significantly differed from those in *S. aureus* strain Newman and its corresponding *hemB* mutant III33 (*p* < 0.001) after 12 h.

Inhibition of chemokines IL-8 and IL-6 by serine protease inhibitor AEBSF and cysteine inhibitor E64

The impact of using serine proteases of different origin to generate IL-8 and IL-6 from nasal epithelial cells was examined by different inhibitory investigations. Twelve-hour stimulation of nasal epithelial cells obtained from TM tissue of six subjects with trypsin and supernatants derived from the *S. aureus* mutants and their parental strains revealed a significant increase in IL-8 (Fig. 3). In contrast, applying AEBSF, IL-8 induction was significantly inhibited with IL-8 reduced by more than 90% (Fig. 3). The addition of the cysteine inhibitor E64 at a concentration of 10 μM led to a significant inhibition of IL-8 release upon stimulation with *S. aureus* Newman, but generally failed to inhibit IL-8 responses due to the other staphylococcal supernatants. Similar results were obtained for stimulation and inhibition of IL-6 and GRO-α responses (data not presented). Stimulation by trypsin did not result in IL-6 generation.

**Induction of phosphorylation of PI3K and p38 MAPK by *S. aureus* supernatants**

Activation of PI3K, ERK1/2, and p38 MAPK was examined using Abs raised against the phosphorylated forms of MAPKs in three independent experiments for each kinase. Although none of the three experimental series showed any statistical significance, some trends in induction of the phosphorylation became visible. Highest amounts of phospho-PI3K 2-fold were obtained after induction with trypsin as well as after challenge with the *hemB* mutants III33 and IA48, whereas phosphorylated ERK1/2 MAPKs did not differ after exposure to the different bacterial supernatants and trypsin compared with controls (Fig. 4). By contrast, phospho-p38 was increased after induction with the supernatants of all bacterial strains.

To determine the role of AEBSF in signal transduction, the cells were incubated with all stimulants again in three independent experiments for each kinase. Preparations included an additional 1 mM AEBSF. Both phospho-PI3K and phospho-p38 kinase were reduced to baseline levels, indicating that both pathways were dependent on AEBSF, as shown in Fig. 4. Remarkably, trypsin had...
no influence on the expression of phospho-p38. Again, no effect was observed on the expression of ERK1/2.

**Influence of kinase inhibitors on the de novo cytokine synthesis of IL-8 and IL-6**

To investigate the mechanism involved in staphylococcal supernatant-induced IL-8 and IL-6 release, nasal epithelial cells were pretreated with different types of protein kinase inhibitors, PD98059, wortmannin, and SB-239063, for 30 min before bacterial supernatant challenge. After 10 h of stimulation, de novo cytokine synthesis was measured. Wortmannin decreased IL-8 production in experiments using supernatants of mutants III33 (\(*, p = 0.008\)) and IA48 (\(*, p = 0.008\)) as well as trypsin as stimulants. No influence on IL-6 synthesis was detected. In addition, IL-6 was not expressed following stimulation with trypsin (Fig. 5).

As shown in five independent experiments, pretreatment with the MEK inhibitor PD98059 reduced the stimulatory effect of bacterial supernatants on IL-8 release by 50% (\(*, p = 0.004\); Fig. 5). A p38 MAPK inhibitor, SB-239063, had nearly the same effect as did a MEK inhibitor (\(*, p = 0.008\)). Notably, IL-6 de novo synthesis was completely abolished after 12 h.

**Activation of the transcription factor NF-κB**

To confirm the biological functionality of different bacterial supernatants in activation of the NF-κB complex, we investigated the protein composition of nuclear extracts using an EMSA and a quantitative DNA-binding assay.

The activity of the NF-κB transcription factor on *S. aureus* supernatants was investigated in triplicate with a DNA-binding assay on nuclear cell extracts. Comparison was made in six independent experiments between stimulated and nonstimulated nasal epithelial cells. TNF-α, trypsin, and *S. aureus* supernatants mediated the level of stimulation and resulted in activation of NF-κB heterodimers p50 and p65. The extent of p50 activation was more pronounced than that of p65. AEBSF at a concentration of 1 mM caused a reduction in NF-κB activation, although controls treated with AEBSF showed slight increase in NF-κB activation.

To determine whether prednisolone inhibits the activation of NF-κB in primary nasal epithelial cells, we also performed EMSAs with nuclear cell extracts from stimulated and nonstimulated primary nasal epithelial cells that were pretreated with prednisolone. Supernatants of the *S. aureus hemB* mutants (Fig. 6B) and...
their isogenic parental strains (Fig. 6B) showed a protein-DNA complex band at the same level as the positive control. Prednisolone (10^{-5} M) had no inhibitory effect on the NF-κB-DNA complex generated by stimulation with S. aureus Newman; however, a minimal inhibitory effect was detectable following stimulation with its hemB mutant III33.

Cytokine synthesis in primary nasal epithelial cells via transcription factor NF-κB

Because glucocorticoids are strongly recommended in the pharmacological treatment of chronic rhinosinusitis, inhibitory experiments with cultured nasal epithelial cells from six TM specimens were performed with prednisolone. Neither GRO-α nor IL-6 or IL-8 induction was significantly inhibited compared with corresponding stimulation experiments without addition of prednisolone (data for IL-8, see Fig. 7). The maximal inhibition of IL-8 and IL-6 responses by prednisolone was \sim 10–20%, as shown in eight independent experiments. These results imply that prednisolone is unable to inhibit IL-8 and IL-6 responses induced by bacterial supernatants and trypsin in primary nasal epithelial cells.

The NF-κB inhibitor BAY11-7085 reduced dramatically the production of both cytokines IL-8 and IL-6 during stimulation with bacterial supernatants. Again, trypsin had no capacity to induce IL-6. Cytokine synthesis was inhibited by 85–95% of the stimulation value (Fig. 7). Preincubation of cells with BAY11-7085 had no influence on the control values of IL-6 and IL-8.

Discussion

S. aureus is one of the most common causes of both endemic and epidemic infections acquired in hospitals and in the community, which result in substantial morbidity and mortality (19). Although the significance of this pathogen has been demonstrated in numerous infectious entities with the particular role of the SCV phenotype for chronic and relapsing infections, reliable data on the role of S. aureus in CRS are very limited. S. aureus has been recovered in patients with CRS; however, its role as causative agent for this disease has not been elucidated (1, 5).

To study responses of nasal epithelial cells due to soluble extracellular proteases derived from different S. aureus strains and phenotypes, we analyzed the expression of inflammatory cytokines IL-8, GRO-α, and IL-6 in a two-dimensional cell culture model of primary nasal epithelial cells.

For our purpose and with particular interest to the interaction between bacterial extracellular protease activity and cytokine generation in nasal epithelium during immune response, culture
supernatants of well-characterized *S. aureus* strains and mutants exhibiting the normal and the SCV phenotype were used. The site-directed hemB mutants represent a stable and genetically defined SCV phenotype (18, 20, 21), and mutant III33 has been shown to produce almost 20 times more protease than its parental strain (16). Of particular interest, in this study, is that both site-directed hemB mutants exhibited more metalloprotease, serine protease, and thiol protease activity than did their parent strains.

In a previous study, PAR-2 activation was shown to regulate IL-8 synthesis, but not IL-6 expression in nasal epithelium (12). To mimic and to compare immune responses and signaling processes in nasal epithelial cells due to serine proteases in bacterial supernatants, trypsin, a member of serine protease family, was applied to the experiments as a stimulator of cytokine synthesis. Two-dimensionally cultured primary nasal epithelial cells were able to respond to all applied *S. aureus*-derived supernatants. Noteworthy, the hemB mutant IA48 and its parental strain COL produced higher amounts of IL-8, GRO-α, and IL-6 than did strain Newman and its corresponding mutant, which may be partially referred to the dilution factor. Because initial investigations had revealed higher cytotoxicity, but similar response of IL-8, applying 1/5 dilutions of *S. aureus* Newman supernatant, a higher dilution factor for this strain (1/10 vs 1/5 for strain COL and both mutants) was used for the subsequent experiments.

The same experimental design was used to demonstrate the impact of serine proteases. Nearly complete suppression of cytokine production was achieved by applying the serine protease inhibitor AEBSF in all experiments. Inhibition of the cysteine protease activity resulted in significant cytokine reduction applying *S. aureus* Newman.

**FIGURE 6.** Activation of NF-κB induction in human nasal epithelial cells. A, Nuclear cell extracts (*n* = 6) from nasal epithelial cells stimulated with TNF-α, trypsin, and supernatants from different *S. aureus* strains, and isogenic hemB mutants were analyzed by NF-κB-binding assay. The goal was to determine activation of NF-κB heterodimer p50/p65 in a quantitative manner (each experiment featured six different nuclear extracts). p50 and p65 activation values are represented in box plots. Value of *p* < 0.05 (asterisk) was considered statistically significant according to the Mann-Whitney *U* rank sum test comparing nonstimulated epithelial cells with stimulated cells (*p* in black) and comparing nonstimulated epithelial cells pretreated with AEBSF with stimulated cells pretreated with AEBSF (*p* in dark gray). B, EMSA investigations were performed using nuclear cell extracts (*n* = 3). Cells were initially stimulated with the bacterial supernatants (positive control: TNF-α) and in combination with prednisolone (negative control: prednisolone alone). In all experiments, the negative control demonstrated weak concentrations of the NF-κB-DNA complex. The notation NS indicates bands of nonspecific DNA-binding complex. Abbreviations: New, *S. aureus* strain Newman; III33, *S. aureus* hemB mutant with strain Newman as parental strain; COL, *S. aureus* strain COL; and IA48, *S. aureus* hemB mutant with strain COL as parental strain.
For further understanding of underlying mechanisms of cytokine responses through bacterial proteases in nasal epithelial cells, host epithelial signaling pathways were analyzed in different cell compartments comprising the cytosol and the nucleus. Cell signaling due to bacteria is poorly understood, and most data in airway epithelial cells were raised from infection with *Haemophilus influenzae* and not from *S. aureus* (22). The p38 MAPK, PI3K, and ERK1/2 pathways represent cytosolic located key signaling molecules involved in mediating cellular stress responses during bacterial infections, which are directly induced within some minutes. In particular, trypsin and the serine protease-rich supernatant of *S. aureus hemB* mutant III33 seemed to activate the PI3K pathway. Phosphorylation of p38 was induced by bacterial supernatants and inhibited by AEBSF in all experiments, with exception of trypsin. Finally, ERK1/2 phosphorylation was not measured.

To confirm these observations and to elucidate the impact of cell signaling on cytokine synthesis in our model, cell signaling inhibition experiments have been performed using MAPK inhibitors PD98059, wortmannin, and SB-239063 (15). From these experiments, we learned that particularly PI3K plays a role in the transduction pathway triggered by *S. aureus hemB* mutants and trypsin for gene transcription of IL-8. The p38 pathway is involved in both IL-8 and IL-6 gene transcriptions triggered by all *S. aureus* strains independent of the phenotypes. Furthermore, the ERK1/2 pathway was activated to take part in cytokine synthesis of IL-8 and IL-6, although the phosphorylated form of ERK1/2 was initially not detected after 20 min of stimulation. We speculate that because cytokines were produced through activation of p38 and PI3K after 7–8 h, the classical ERK1/2 signaling pathway may act as an amplifier of cytokine generation, as indicated by Imasato et al. (23).

A major step in the inflammatory response cascade is activation of NF-κB. Based on our findings, we propose a NF-κB translocation-dependent pathway in inflammatory responses through serine proteases, because AEBSF dramatically represses activation of NF-κB under all conditions of stimulation with *S. aureus* supernatants. To exclude an additional blockage of the serine proteases responsible for the proteolysis of the inhibitory protein IκB-α (24) by AEBSF, stimulation of nasal epithelial cells with TNF-α facilitating NF-κB expression was investigated. Blocking with AEBSF resulted not in a significant reduction of NF-κB expression. One possible explanation for the AEBSF-induced NF-κB reduction not only acts on direct serine protease inhibition, but may secondarily have a protective function on changes in permeability of the epithelial barrier. AEBSF exhibits the capacity to prevent the disruption of tight junctions and, thereby, blocks secondary signaling processes in vitro; it not only acts on direct serine protease inhibition, but may secondarily have a protective function on changes in permeability of the epithelial barrier (25).

Glucocorticoids are strongly recommended for the treatment of chronic rhinosinusitis because they reduce the expression of proinflammatory cytokines and CC chemokines (26–28). Actually, glucocorticoids have been reported to inhibit NF-κB-driven gene expression, which may explain, at least in part, the anti-inflammatory action (29–32). In addition, to compare effects of prednisolone, the irreversible inhibitor of the NF-κB/ IκB-α signaling, BAY11-7085, was tested. This agent reduced swelling in a dose-dependent manner in both the rat carrageenan paw edema assay and a rat adjuvant arthritis model by reducing expression of adhesion molecules (33).

In this study, its potential to inhibit the release of IL-8 and IL-6 in stimulated nasal epithelial cells clearly indicates the regulatory role for the NF-κB pathway through bacterial supernatants. In contrast, prednisolone, even in high concentrations, was unable to abolish the cytokine response in vitro. One explanation might be that steroids differentially regulate neutrophil and eosinophil cytokines. In asthma, it has been reported that corticosteroids downregulate the number of eosinophils and the level of CC chemokines in bronchial lavage, whereas neither the number of neutrophils nor IL-8 levels were affected (26). The mechanisms involved are currently unknown.

In summary, the inflammatory impact of *S. aureus*-derived supernatants was clearly evident. *S. aureus* strains with normal phenotype as well as isogenic hemB mutants displaying the SCV phenotype were potent inducers of proinflammatory cytokines with strain-specific potency. The regulation of cytokines through serine proteases derived from supernatants was identified in a cell culture of primary nasal epithelial cells. Serine proteases have the capacity...
to regulate phosphorylation of PI3K, but additional effects by sec-
ondary signaling processes on p38 and on ERK pathway by means of secreted cytokines have to be taken into account when consid-
ering the mechanisms of signaling pathways. NF-κB seems to be the key transcription factor required for the release of both IL-8 and IL-6 through S. aureus-derived serine proteases. Neither hy-
drocortisone nor cysteine protease inhibitors weakened the cyto-
kine production.

Our findings suggest that proteases may have implications for the development of therapeutic strategies in CRS. Protease inhib-
itors and signaling inhibitors present new candidates for pharma-
cological approaches in CRS. However, further studies are war-
anted to establish whether S. aureus may persist intracellularly and may be responsible for the persistent character of the infection.

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