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Immunomodulation of Nasal Epithelial Cells by *Staphylococcus aureus*-Derived Serine Proteases¹

Claudia Rudack,* Florian Sachse,* Nils Albert,* Karsten Becker,[†] and Christof von Eiff^{2,3,†}

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 cysteine 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- κ B 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 cysteine protease inhibitor had little effect. Activation of NF- κ B 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- κ B in nasal epithelial cells, and may therefore be relevant for the pathophysiology of chronic rhinosinusitis. *The Journal of Immunology*, 2009, 183: 7592–7601.

Chronic rhinosinusitis (CRS)⁴ 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 subpopulation 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 cysteine 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 endoplasmic reticulum (11). Noteworthy, 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_i protein- and NF- κ B-dependent expression of the neutrophil chemoattractants IL-8/CXCL8 and growth-related oncogene (GRO)- α CXCL1, but not the eosinophil and T cell chemoattractants 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- κ B during cytokine synthesis were examined in nasal epithelial cells by means of NF- κ B-DNA-binding assays and quantifying phosphorylation of different kinases. In addition, kinases were blocked by specific inhibitors to study regulation mechanisms of cytokine synthesis.

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Materials and Methods

Materials

Trypsin and all other reagents were purchased from Sigma-Aldrich, unless otherwise stated.

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⁴ Abbreviations used in this paper: CRS, chronic rhinosinusitis; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; E64, *trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane; GRO, growth-related oncogene; PAR, protease-activated receptor; SCV, small-colony variant; TM, turbinate mucosa.

Table I. Bacterial strains used in this study

Strain	Characteristics	Source or Reference
<i>S. aureus</i> Newman	Parent strain of III33, <i>sea</i> positive	ATCC 25904
<i>S. aureus</i> III33	<i>hemB::ermB</i> , site-directed mutant displaying a stable SCV phenotype	16
<i>S. aureus</i> COL	Parent strain of IA48, methicillin resistant, <i>seb</i> positive	www.tigr.org
<i>S. aureus</i> IA48	<i>hemB::ermB</i> , site-directed mutant displaying a stable SCV phenotype	17

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 $\sim 1.8 \times 10^5$ cells) to confluence. Twenty-four hours before the studies were initiated, the medium was replaced by serum-free medium. Cytokeratins 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 *S. aureus* and their isogenic site-directed *S. aureus hemB* mutants displaying the SCV phenotype (Table I).

S. aureus COL, a methicillin-resistant strain (*mecA* gene positive), was tested PCR positive for the enterotoxin B gene (*seb*), whereas the strain *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 = 1.0 with sterile tryptic soy broth. Then, an aliquot was removed for CFU determination (5×10^3 – 1×10^9). Bacterial cells were removed by centrifugation (4000 rpm for 20 min, 4°C). Except for *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 *trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E64; Sigma-Aldrich) at a concentration of 10 μ M, and prednisolone (Merck Pharma) at a concentration of 1×10^{-5} M.

BAY 11-7085, a (2*E*)-3-[[4-(1,1-dimethylethyl)phenyl]sulfonyl]-2-propenenitrile (Calbiochem), is an inhibitor of NF- κ B that was used at a concentration of 10 mM to block NF- κ B-dependent synthesis of IL-8 and IL-6. Bay 11-7085 is an irreversible inhibitor of TNF- α -activated I κ B α phosphorylation. For further inhibitor experiments, primary nasal epithelial cells were incubated with cortisone (prednisolone; 10^{-5} M; Merck Pharma).

MAPKs were inhibited by pretreatment with either PD98059 (Calbiochem) at a concentration of 50 μ M and wortmannin (Calbiochem) at a concentration of 100 nM or the selective p38 inhibitor SB-239063 (*trans*-1-(4-hydroxycyclohexyl)-4-(fluorophenyl)-5-(2-methoxy-pyrimidin-4-yl)imidazole) at a concentration of 30 μ M, 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 *S. aureus* strains (for characterization, see Table I) were determined using the insoluble proteolytic sub-

strate 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 μ l of an overnight culture supernatant was added to 500 μ l 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 (p38 α , 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 phospho-p38 α (T180/Y182)

The Surveyor IC Immunoassay employs a two-site sandwich ELISA to quantify p38 α 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 μ l 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 μ g/ml leupeptin and aprotinin 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). Again, 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

Protease	Additive	Activity ^a (U/mg protein) of Strains:			
		<i>S. aureus</i> Newman		<i>S. aureus</i> COL	
		Parental Strain	<i>hemB</i> Mutant III33	Parental Strain	<i>hemB</i> Mutant IA48
Total protease		590	2769* <i>p</i> = 0.026	571	1410 NS
Serine protease	AEBSF	289	1847* <i>p</i> = 0.041	356	1000 NS
Metalloprotease	EDTA	94	953* <i>p</i> = 0.009	214	20 NS
Thiol protease	DTT, EDTA	248	615* <i>p</i> = 0.041	179	705 NS
	DTT, EDTA, E64 (10 μ M)	82	230 NS	0	101 NS

^a 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 DTT (1 mM), EDTA, and the cysteine inhibitor E64 (1 mM). **p*, Displays significance niveau of medians 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).

Face ERK1/2

Face ERK1/2 (Active Motif) was tested for sensitivity and specificity in detecting phosphorylation of ERK1/2 MAPK. The phospho-ERK Ab is specific for phosphorylated ERK1 and ERK2. This Ab was raised against a synthetic phospho-peptide corresponding to residues Thr²⁰² and Tyr²⁰⁴ of human ERK1 and Thr¹⁸⁵ and Tyr¹⁸⁷ of human ERK2. This Ab recognizes both phosphorylated ERK1 and ERK2. It does not recognize unphosphorylated ERK1 or ERK2, nor does it recognize other phosphorylated proteins. The total ERK Ab recognizes ERK1 and ERK2 regardless of the phosphorylation state.

The phospho-ERK and total ERK signals were assessed after 15 min and, according to the manufacturer's recommendations, the results were corrected for cell number by use of the Crystal Violet protocol. Before stimulation with bacterial supernatants or trypsin, primary human epithelial cells were seeded overnight in wells and incubated at 37°C, 5% CO₂. As in the other experiments, the medium was replaced by serum-free medium 24 h before the study.

NF- κ B-binding assay and EMSA in nuclear epithelial cell extracts

After incubation of primary nasal epithelial cells with trypsin (10⁻⁷ M), TNF- α (50 ng/ml), and supernatants of the four different *S. aureus* strains and genetically defined mutants for 60 min, cells were washed with PBS and lysed in 60 μ l of low salt buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, and 0.1 mM EGTA (pH 7.9)) and protease inhibitors (1 mM DTT, 0.1 mM PMSF, and protease complete mixture). For inhibitor experiments, primary nasal epithelial cells were incubated with cortisone (10⁻⁵ M) and AEBSF (1 mM) before exposure to the different stimuli.

The lysate was cleared by centrifugation, and the supernatant (cytosol extraction) was pipetted away. The cell pellet was then lysed in high salt buffer (20 mM HEPES, 400 mM NaCl, 1.0 mM EDTA, and 1.0 mM EGTA (pH 7.9)) and protease inhibitors. Again, the lysate was cleared by centrifugation, and the supernatant containing the nuclear extracts was stored at -80°C after determining the protein concentration. Protein concentrations were determined by the Bradford method (Bradford Protein Assay; Bio-Rad).

For EMSA experiments, the binding reaction was conducted in 20 μ l of the mixture containing 4 μ l of cell extract (10 μ g protein), 4 μ l of 5 $\times$ binding buffer (20 mM HEPES (pH 7.7), 50 mM KCl, 1 mM DTT, 2.5 mM MgCl₂, and 20% Ficoll), 2 μ g of poly(dI-dC) (Roche Diagnostics) as a nonspecific competitor DNA, 2 μ g of BSA, and 80,000 cpm of the labeled oligonucleotide. After 20 min of incubation at room temperature, the samples were electrophoresed through a 4% nondenaturing polyacrylamide gel. The gel was then dried and exposed to Kodak BioMax XAR film. To verify specific NF- κ B activity, competitive EMSA with a 30-fold excess of unlabeled oligonucleotide was used in the binding reaction and allowed to react for 20 min before adding ³²P-end-labeled oligonucleotide.

Activation of NF- κ B transcription factor was quantified using the TransAM NF- κ B kit (Active Motif) in nuclear extracts. Nuclear extracts (5 μ g) were added to each well of a 96-well plate that had been coated with the immobilized oligonucleotide containing the NF- κ B consensus site (5'-GGGACTTCC-3') in 20-fold excess. The active form of NF- κ B contained in the nuclear extract specifically binds to this oligonucleotide. The primary Abs used to detect NF- κ B recognize the epitope on p50 and p65. An HRP-conjugated secondary Ab provides a sensitive calorimetric read-out at 450 nm. Each Ab was tested in nuclear cell extracts alone, with wild-type and mutated competitor oligonucleotides.

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).

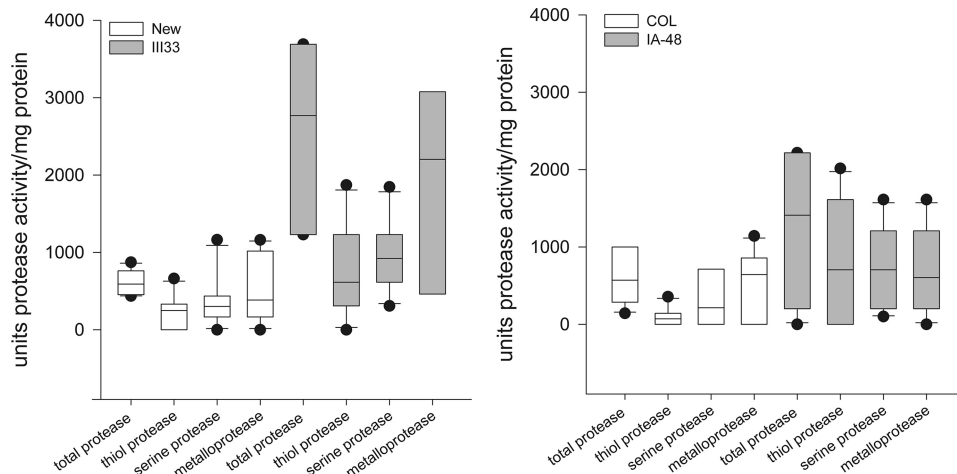
In fact, total extracellular proteolytic activity significantly differed in the *hemB* mutant derived from *S. aureus* Newman compared with its parent strain (Table II). Noteworthy, the same phenomenon was observed comparing *S. aureus* COL proteolytic activity with its *hemB* mutant IA48, but reached 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 III33 compared with the parental strain (Fig. 1; Table II). Metalloprotease activity was also mostly detected in III33. 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

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 AEBF (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.



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).

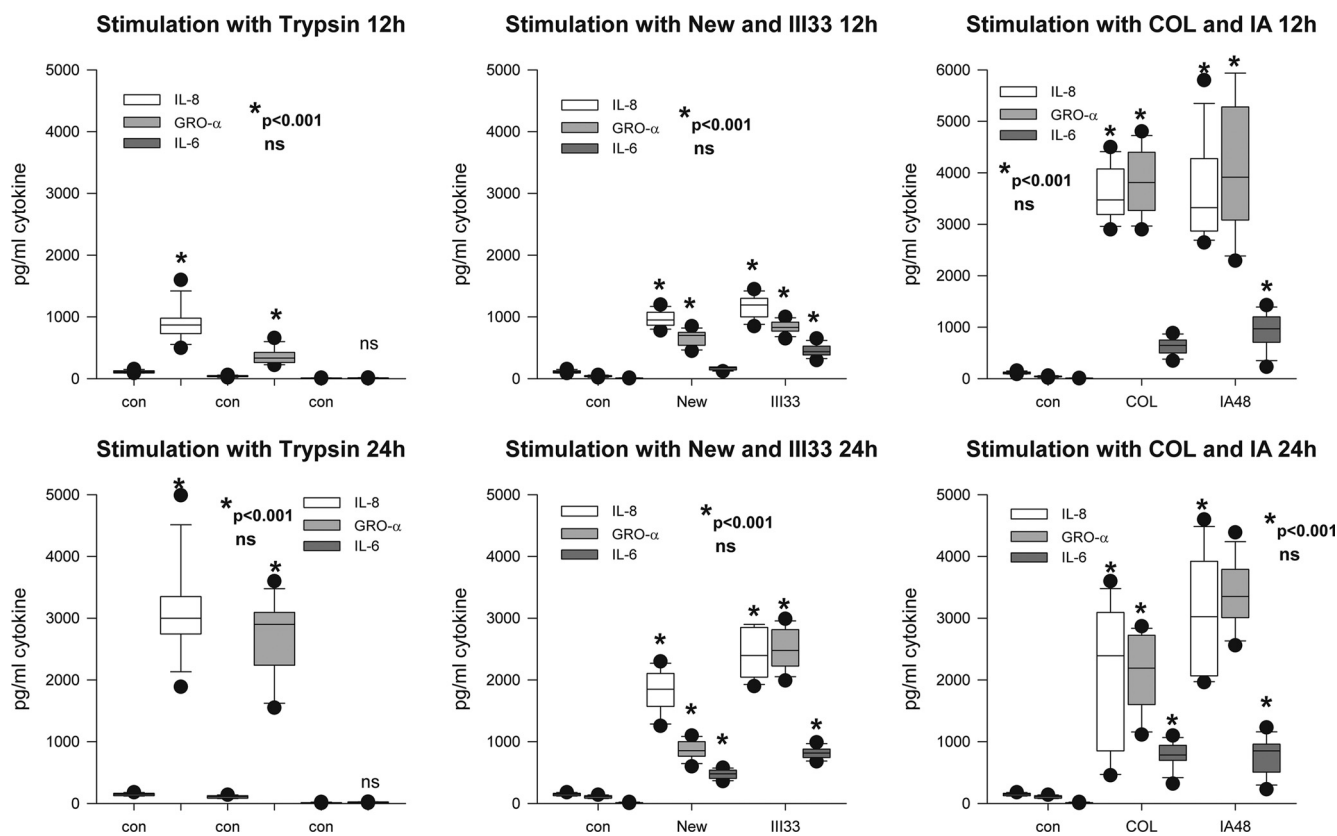


FIGURE 2. Production of IL-8, GRO- α , and IL-6 by primary nasal epithelial cells stimulated with trypsin or with bacterial supernatants after 12 and 24 h. Epithelial cell cultures were obtained from six different inferior turbinates taken from sinuses of subjects undergoing conchotomy ($n = 6$ for each 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 inducers 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.

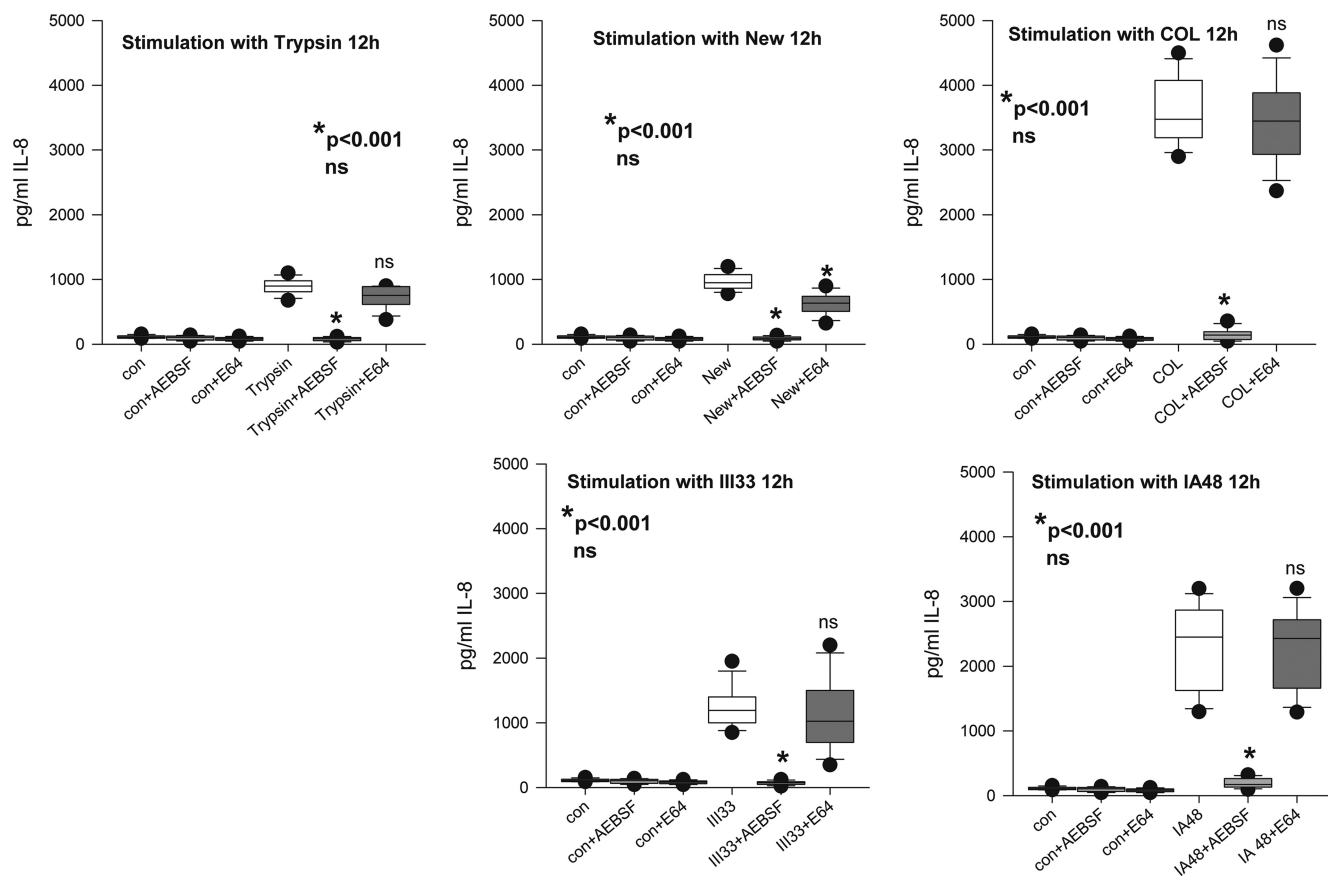


FIGURE 3. Inhibitory effects of AEBSF and E64 on IL-8 production by primary nasal epithelial cells. Epithelial cell cultures were obtained from eight different inferior turbinates taken from sinuses of subjects undergoing conchotomy ($n = 8$ for each series). The box plots represent IL-8 levels of nonstimulated cells after 12 h and stimulated cells after 12 h with and without pretreatment using specific protease inhibitors. Value of $p < 0.05$ (*) was considered statistically significant according to the Mann-Whitney U rank sum test comparing nonstimulated cells with stimulated cells pretreated with AEBSF and E64 cells ($n = 8$). All significances calculated by Mann-Whitney U rank sum test are $p < 0.001$. 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.

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 μ M 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

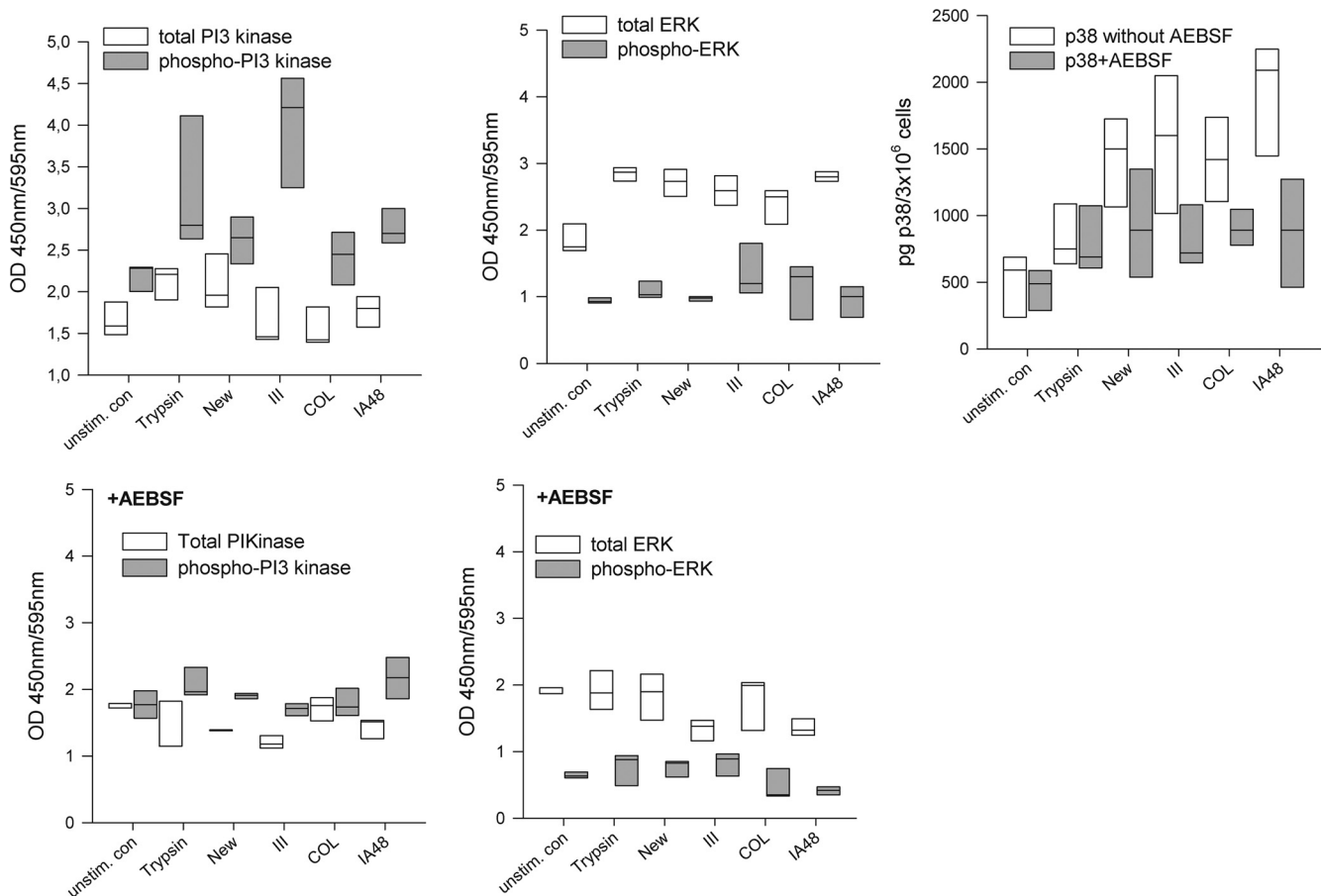


FIGURE 4. Induction with bacterial supernatants and inhibitory effects of AEBSF on PI3K, ERK, or p38 expression by primary nasal epithelial cells. Epithelial cell cultures were obtained from 12 different inferior turbinates taken from sinuses of subjects undergoing conchotomy. The box plots represent phosphorylated kinases after 30 min of stimulation with trypsin and bacterial supernatants ($n = 3$). Induction experiments were repeated after pretreatment with AEBSF ($n = 3$). These results of the inhibition series are displayed in the lower part of the figure corresponding to each kinase with exception of p38. Both groups are displayed in one figure. Value of $p < 0.05$ (*) is considered statistically significant according to the Mann-Whitney U rank sum test comparing nonstimulated cells with stimulated cells. Furthermore, Mann-Whitney U rank sum test was objected to compare stimulated cells without AEBSF with stimulated cells with AEBSF treatment ($n = 3$). Abbreviations: neg., negative; 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.

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- κ B

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

The activity of the NF- κ B 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- κ B 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- κ B activation, although controls treated with AEBSF showed slight increase in NF- κ B activation.

To determine whether prednisolone inhibits the activation of NF- κ B 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

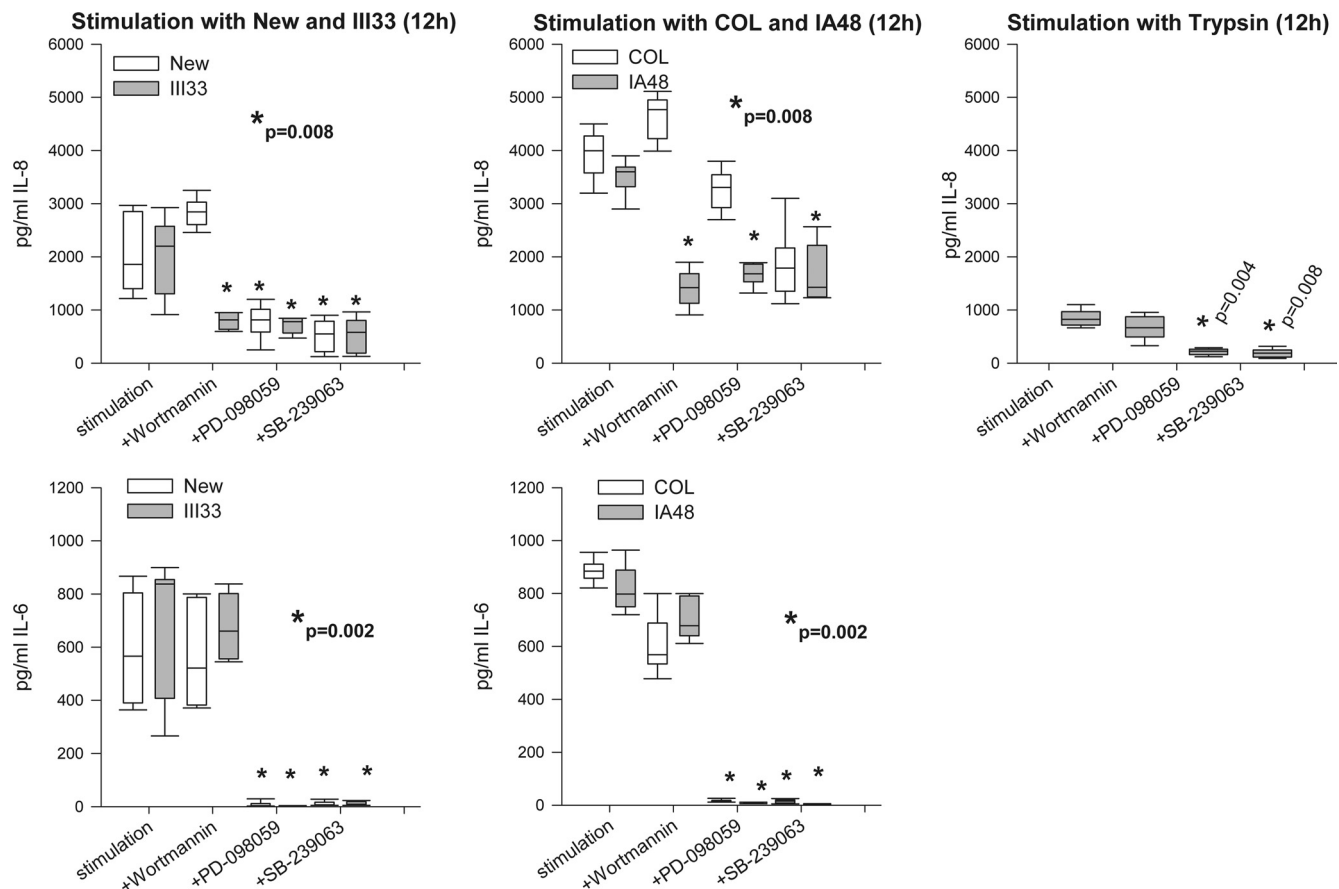


FIGURE 5. Production of IL-8 and of IL-6 by primary nasal epithelial cells stimulated with trypsin or bacterial supernatants after 12 h and inhibition by kinase inhibitors. Primary nasal epithelial cells were generated from six different inferior turbinates taken from sinuses of subjects undergoing conchotomy ($n = 5$ for each series). These were pretreated with either PD98059 at a concentration of $50 \mu\text{M}$ and wortmannin at a concentration of 100 nM or with the selective p38 inhibitor SB-239063 at a concentration of $30 \mu\text{M}$ for 30 min. Thereafter, the preparations were challenged with *S. aureus* supernatants ($n = 5$). After 12 h of stimulation, de novo cytokine synthesis of IL-8 and IL-6 was measured. Data are represented in box plots indicating the 25th and 75th percentiles. The whiskers above and below the box indicate the 90th and 10th percentiles. Value of $p < 0.05$ (*) was considered statistically significant according to the Mann-Whitney U rank sum test comparing nonstimulated epithelial cells with stimulated ($n = 5$ repeats) for each *S. aureus* strain and phenotype and for trypsin. 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.

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- κ B-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- κ B

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 ~ 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- κ B 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

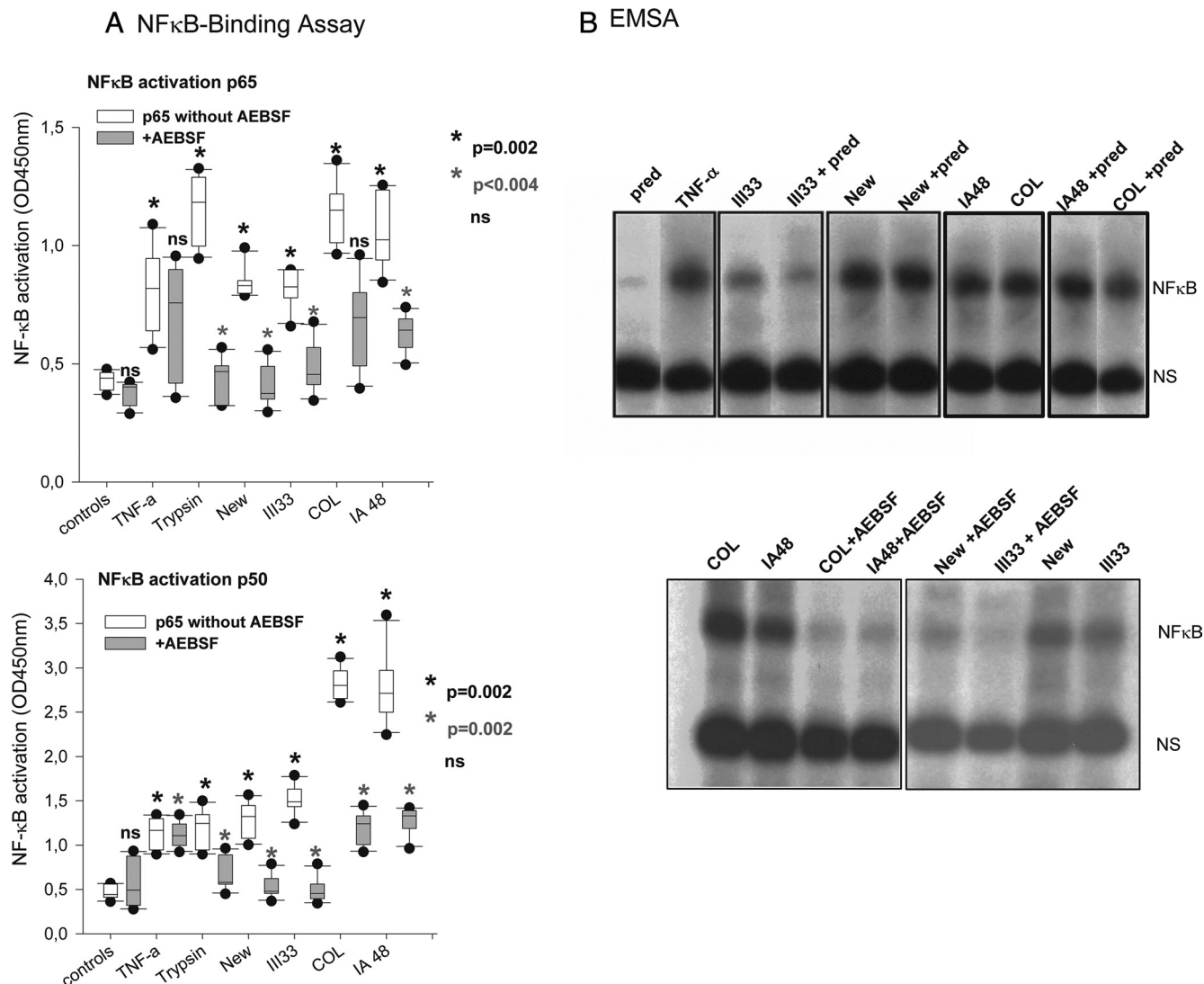


FIGURE 6. Activation of NF- κ B induction in human nasal epithelial cells. **A**, Nuclear cell extracts ($n = 6$) from nasal epithelial cells stimulated with TNF- α , trypsin, and supernatants from different *S. aureus* strains, and isogenic *hemB* mutants were analyzed by NF- κ B-binding assay. The goal was to determine activation of NF- κ B 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 $p < 0.05$ (*) 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 AEBF with stimulated cells pretreated with AEBF (*, 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- κ B-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.

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 AEBF in all experiments. Inhibition of the cysteine protease activity resulted in significant cytokine reduction applying *S. aureus* Newman.

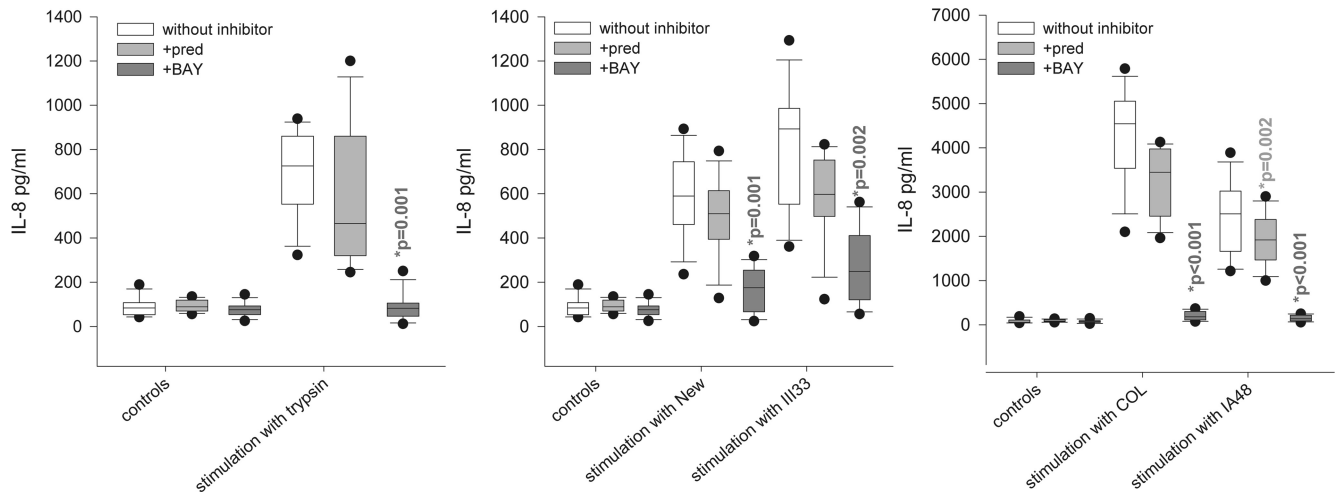


FIGURE 7. Inhibitory effects of prednisolone and BAY11-7085 on IL-8 production by primary nasal epithelial cells. Epithelial cell cultures were obtained from eight inferior turbinates taken from sinuses of subjects undergoing conchotomy ($n = 6$ repeats for each series). IL-8 levels are represented in box plots indicating the 25th and 75th percentiles. The whiskers above and below the box indicate the 90th and 10th percentiles. Value of $p < 0.05$ (*) was considered statistically significant according to the Mann-Whitney U rank sum test comparing nonstimulated cells with stimulated cells; both cell groups were pretreated with BAY and steroids. Abbreviations: pred, prednisolone; BAY, BAY11-7085; 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- κ B. Based on our findings, we propose a NF- κ B translocation-dependent pathway in inflammatory responses through serine proteases, because AEBSF dramatically represses activation of NF- κ B 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 I κ B- α (24) by AEBSF, stimulation of nasal epithelial cells with TNF- α facilitating NF- κ B expression was investigated. Block-

ing with AEBSF resulted not in a significant reduction of NF- κ B expression. One possible explanation for the AEBSF-induced NF- κ B 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- κ B-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- κ B/I κ B- 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- κ B 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 down-regulate 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 secondary signaling processes on p38 and on ERK pathway by means of secreted cytokines have to be taken into account when considering the mechanisms of signaling pathways. NF- κ B 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 hydrocortisone nor cysteine protease inhibitors weakened the cytokine production.

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

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Disclosures

The authors have no financial conflict of interest.

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