

***Staphylococcus aureus* enterotoxin B augments granulocyte migration and survival via airway epithelial cell activation**

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Abstract

Background: *Staphylococcus aureus* enterotoxin B (SEB) has recently been postulated to be involved in the pathology of granulocyte-dominated disease. Studying the immunologic interaction between SEB and airway epithelial cells in immortalized cell lines or long-term epithelial cell cultures has obvious disadvantages.

Methods: We used a novel technique of freshly isolated and purified human nasal epithelial cells (HNEC) from healthy, nonallergic individuals, which were incubated for 24 h without/with SEB at different concentrations. Chemokine production was evaluated in the supernatant using Cytometric Bead Array. The chemotactic activity of the supernatant was studied *in vitro* using a Boyden chamber. Survival was evaluated with flow cytometry, using propidium iodide to identify dead cells.

Results: *Staphylococcus aureus* enterotoxin B showed a dose-dependent induction of interferon-inducible protein-10, monokine induced by interferon- γ , regulated upon activation normal T cell expressed and secreted, monocyte chemoattractant protein 1 (MCP-1) and granulocyte colony stimulating factor production by epithelial cells *in vitro*. The supernatant of epithelial cells had chemotactic activity for granulocytes *in vitro*, which was enhanced in the supernatant of SEB-stimulated epithelial cells. Reduced number of propidium iodide positive granulocytes was found in the conditions where supernatant of SEB-stimulated epithelial cells was applied.

Conclusion: *Staphylococcus aureus* enterotoxin B exerts a direct pro-inflammatory effect on HNEC, with induction of chemokine and growth factor release, resulting in the migration and prolonged survival of granulocytes *in vitro*.

Staphylococcus aureus is a common human pathogen, which is often found as part of the normal microflora in the nasal cavity. The anterior nares of the nose are the most frequent carriage site for *S. aureus*, although multiple sites can be

colonized (e.g. skin, pharynx, and perineum) (1). Colonization with *S. aureus* may represent a major source of superantigens as a set of toxins are being produced including staphylococcal enterotoxins SAEs and toxic shock syndrome toxin-1 (TSST-1), which cause food poisoning and toxic shock syndrome, respectively (2). These toxins activate up to 20% of all T cells in the body by binding the human leukocyte antigen (HLA) class II molecules on antigen-presenting cells (APCs) and specific V beta regions of the T-cell receptor (3). Between 50 and 80% of *S. aureus* isolates are positive for at least one superantigen gene, and close to 50% of these isolates show superantigen production and toxin activity (4).

Abbreviations

SEB, *Staphylococcus aureus* enterotoxin B; SAEs, *Staphylococcus aureus* enterotoxins; IP-10, interferon-inducible protein-10; MIG, monokine induced by interferon- γ ; RANTES, regulated upon activation normal T cell expressed and secreted; MCP-1, monocyte chemoattractant protein 1; G-CSF, granulocyte colony stimulating factor.

The pathophysiologic role of enterotoxin producing *S. aureus* in human disease has recently been recognized. SAEs have immune-modulatory and pro-inflammatory effects in several granulocyte-dominated diseases like atopic dermatitis (5), allergic rhinitis (AR) (6) and asthma (7), nasal polyposis (8), or chronic obstructive pulmonary disease (COPD) (9).

Studies have shown a putative role for SAEs in patients suffering from the atopic eczema/dermatitis syndrome (AEDS), where colonization with *S. aureus* is found more frequently (80–100%) compared to healthy controls (5–30%) (10), and *S. aureus* isolates secrete identifiable enterotoxins like *Staphylococcus aureus* enterotoxin A (SEA), *Staphylococcus aureus* enterotoxin B (SEB), and TSST-1. IgE to these toxins was found in the serum of 57% of patients with AEDS (11), indicating immune responses against these bacterial products. Moreover, in 25% of patients with AR detectable serum IgE levels to SAE are found, whereas this is only found in a minority of nonallergic patients (6.3%). In AR, the presence of SAE-specific IgE was associated with the highest titer of total serum IgE (6, 12). Furthermore, SAEs are thought to play a major role in the pathogenesis of nasal polyp (NP) disease, as IgE against SEA and SEB has been demonstrated in NPs (13) and levels of SAE-specific IgE in NP correlated with markers of eosinophil activation and recruitment (14). Moreover, in patients with COPD, a significantly elevated IgE to SAE was found, pointing to a possible disease modifying role in COPD, similar to that in severe asthma (9).

In murine research, the role of SAEs as disease modifier has been demonstrated by in models of airway disease (15), atopic dermatitis (16), and food allergy (17). In addition, we have previously reported aggravation of experimental allergic asthma by application of SEB, characterized by higher IL-4 production and allergen-specific IgE (18). These findings highlight the important pathological consequences of SAE exposure, as these superantigens not only cause massive T-cell stimulation, but also lead to activation of B-cells and other pro-inflammatory cells like eosinophils, macrophages, and mast cells (19). Moreover, SAEs are known inducers of chemokine production in epithelial cells (20).

The involvement of the epithelium in the pathogenesis of airway disease is increasingly acknowledged. The airway epithelium not only is a physiological barrier, but also is actively involved in the immune response as a major source of inflammatory cytokines and mediators (21), relevant to the ongoing inflammatory responses dominated, among other features, by abundant granulocytes. Activated epithelial cells are potent sources of haematopoietic cytokines such as granulocyte (-macrophage) colony stimulating factor [G (M)-CSF], and chemokines like regulated upon activation normal T cell expressed and secreted (RANTES), eotaxin, monokine induced by interferon- γ (MIG), interferon-inducible protein-10 (IP-10), interleukin (IL)-8 (22), and monocyte chemoattractant protein 1 (MCP-1) (23).

Because of the earlier mentioned association between SAEs and granulocyte-dominated inflammatory disease, we have investigated the effect of SEB – a prototypic staphylococcal superantigen – on chemokine production of human nasal epithelial cells (HNEC). Until now, the immunologic interac-

tion between SEB and epithelial cells has mostly been studied in immortalized cell lines. Here, a novel technique of pure and freshly isolated epithelial cells is being elaborated and presented. Furthermore, we investigated the effects of these epithelial-derived mediators *in vitro* in a granulocytic chemotaxis and survival assay.

Methods

Human nasal epithelial cell isolation procedure

Nasal inferior turbinates were obtained from patients ($n = 10$) undergoing nasal surgery for nonmucosal anatomical abnormalities causing nasal obstruction. Exclusion criteria were smoking, occupational exposure to irritants, IgE-mediated hypersensitivity to a panel of frequent inhalant allergens demonstrated with a standardized skin prick test, or use of intranasal corticosteroid spray 6 weeks before the surgery. Inferior turbinates were harvested at the end of the septoplasty or rhinoplasty procedure, and immediately placed in a sterile saline solution, washed with saline and incubated for 24 h at 4°C with 0.1% sterile pronase solution for dissociation of the epithelial cell layer (Sigma, Bornem, Belgium, Fig. 1). After 24 h, large tissue pieces were removed with a sterile pincet. Fetal calf serum (FCS) (Sigma) was added to the solution to stop the pronase reaction. Cells were washed three times in culture medium (Hank's buffered salt solution supplemented with 0.05% bovine serum albumin). Next, supernatant was discarded, and the pellet was resolved in culture medium, transferred in a cell culture flask and incubated for 90 min at 37°C to let fibroblasts attach to the wall. To avoid contamination, all procedures were performed sterilely under a fume hood.

In a next step, nasal epithelial cells were negatively selected using magnetic activated cell sorting (MACS) cell separation

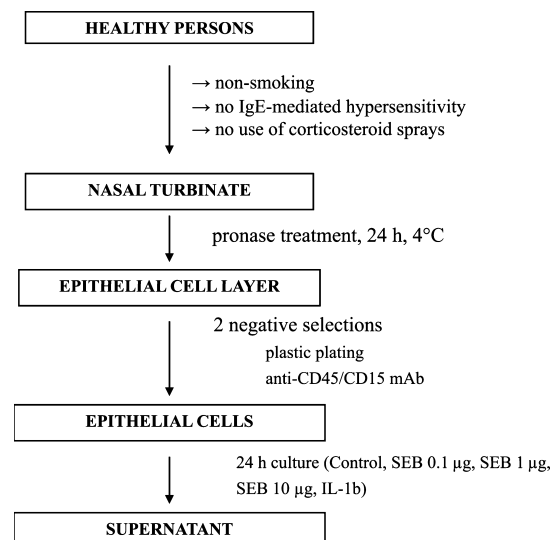


Figure 1 Human nasal epithelial cell isolation procedure. Using pronase treatment and negative selections, a nasal epithelial cell population with 98% purity and viability was obtained.

columns (Miltenyi, Utrecht, the Netherlands). Therefore, magnetic beads coated with anti-CD45 were added to the cell suspension, incubated for 20 min, and put in a column before transferring the supernatant. This procedure was repeated with anti-CD15-coated beads, and the supernatant was transferred into a cell culture flask. Viability was evaluated using Trypan blue, and May-Grünwald-Giemsa (MGG) staining of the cytopins was performed to confirm cell culture purity. This epithelial cell isolation procedure using two negative selections resulted in an epithelial cell population with 98% purity and viability.

Evaluation of SEB-induced chemokine secretion

Pilot studies showed that 3×10^5 HNEC were the optimal number of cells for stimulation studies and analyses of supernatants (data not shown). HNEC were incubated with 0.1, 1, or 10 μg of SEB (Sigma, LPS content: 34.82 pg/ml) in a total volume of 1 ml culture medium for 24 h. Incubation with IL-1 β was used as positive control condition. Supernatant was evaluated for IP-10, granulocyte colony stimulating factor (G-CSF), GM-CSF, MCP-1, MIG, RANTES, Eotaxin-1/2, IL-3, IL-4, and IL-8 using cytometric bead array Flex (BD Biosciences, Erembodegem, Belgium) according to the manufacturers' instructions. Samples were acquired with the FACS Array (BD Biosciences).

Stimulation index was calculated by dividing the values from the experimental conditions (SEB or IL-1 β) by the respective values from the control condition.

Granulocyte isolation

For migration and survival assays on granulocytes *in vitro*, blood cells were obtained from house dust mite allergic donors ($n = 5$) suffering from AR, which presented at the Outpatient clinic of the Department of Otorhinolaryngology of the University Hospital Leuven, Belgium. Patients with a positive skin prick test to house dust mite allergen (> 5 mm – GA²LEN pan-European skin prick test) were included in the study for blood sampling. All patients included in this study completed written informed consent, and the local ethical committee approved the study.

Blood was obtained and diluted with an equal volume of phosphate buffered saline (PBS). Then, 40 ml of this suspension was added to 10 ml Lymphoprep (Axis-Shield, Oslo, Norway), and tubes were centrifuged for 30 min at 800 g . Plasma and peripheral blood mononuclear cells (PBMCs) were discarded, and tubes were supplemented with an equal volume of Plasmasteril (Fresenius AG, Bad Homburg, Germany) and PBS. Tubes were incubated for 30 min at 37°C before centrifuging (10 min – 218 g). The pellet was washed with PBS, and residual red blood cells were lysed with hypotonic shock.

Granulocyte migration assay

To evaluate the chemotactic activity of SEB-stimulated HNEC supernatant, we used a Boyden chamber-based cell migration assay, as reported previously (24). Briefly, granu-

lyocytes (50 μl – 1×10^6 per ml) from house dust mite allergic donors were placed in the upper compartment and were allowed to migrate through 5 μm pore size poly (vinylpyrrolidone)-free (PVPF) polycarbonate filters (Nuclepore, Pleasanton, CA, USA) for 45 min at 37°C. The lower compartment contained supernatant (SN) of unstimulated HNEC (SN Medium), SEB-stimulated HNEC (SN SEB), or control medium. IL-8 (10 $\mu\text{g}/\text{ml}$) was used as positive control condition. After 45 min incubation, the membrane was subjected to MGG staining, and its lower side was evaluated for number of migrated granulocytes, by counting 10 high power fields per sample. According to standard morphological criteria of the cell nucleus and cytoplasmic granules, cells were further categorized into neutrophilic and eosinophilic granulocytes. The chemotactic index was calculated by dividing the number of cells migrated under the experimental condition (SN SEB) by the number of cells migrated under control condition (SN Medium). A chemotactic index of > 2 was considered to be a positive index of chemotaxis.

Granulocyte survival assay

Granulocytes (2×10^6 cells per ml) were incubated for 24 and 48 h with SN Medium, SN SEB or Medium. At time of analysis, cells were evaluated with flow cytometry (FACS Array, BD Biosciences), where propidium iodide was used to identify dead cells. Granulocytes were sorted with MACS into neutrophils and eosinophils using a CD16 monoclonal antibody (Miltenyi).

Statistical analysis

Statistical analysis was performed with MEDCALC software 9.2.0.1 (F. Schoonjans, Belgium; <http://www.medcalc.be>). All outcome variables were compared using nonparametrical tests. When comparisons were made between groups, the Kruskal–Wallis test was used to establish the significant inter-group variability. The Mann–Whitney- U test was then used for between-group comparison. The significance level was set at $\alpha = 0.05$. Data are expressed as mean with error bars expressing standard error of the mean.

Results

Effect of SEB on human nasal epithelial cells

Human nasal epithelial cells ($n = 6$) incubation for 24 h with increasing doses of SEB resulted in a significant increase in chemokine secretion of IP-10, G-CSF, MCP-1, MIG, and RANTES, showing a dose-dependent induction as depicted on Fig. 2A–F. Interestingly, IP-10 and MIG were already increased at the lowest (0.1 $\mu\text{g}/\text{ml}$) SEB concentration, whereas G-CSF only raised above control at SEB 1 $\mu\text{g}/\text{ml}$. Finally, RANTES and MCP-1 were significantly increased just at the highest SEB concentration (10 $\mu\text{g}/\text{ml}$). Incubation with IL-1 β as a general pro-inflammatory stimulus resulted in a significant increase in G-CSF and IL-8 secretion

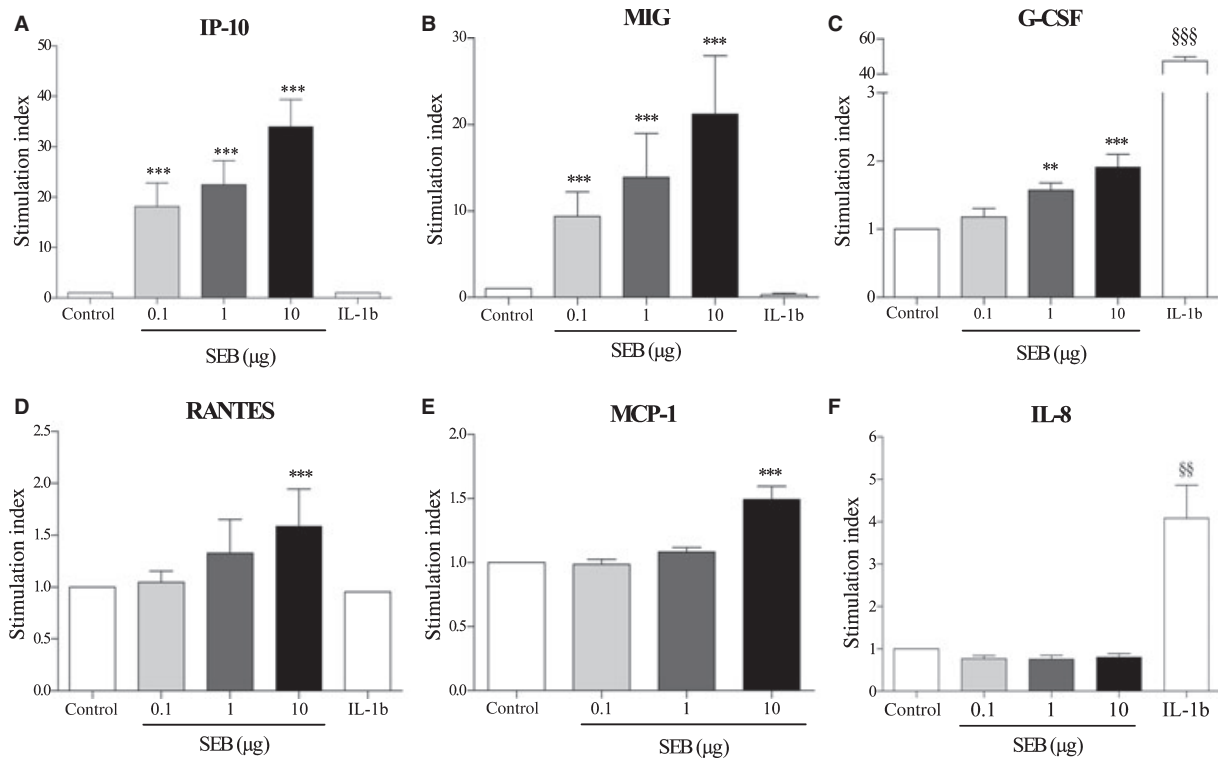


Figure 2 Human nasal epithelial cells incubation for 24 h with increasing doses of *Staphylococcus aureus* enterotoxin B (SEB) resulted in a significant increase in chemokine secretion of interferon-inducible protein-10 (IP-10), granulocyte colony stimulating factor (G-CSF), MCP-1, MIG, and regulated upon activation normal T cell expressed and secreted (RANTES), showing a dose-dependent induction. Interferon-inducible protein-10 (A) and MIG (B) were already increased at the lowest (0.1 µg) SEB concentra-

tion, whereas G-CSF (C) only raised above control at SEB 1 µg. Finally, RANTES (D) and MCP-1 (E) were significantly increased just at the highest SEB concentration (10 µg). Incubation with IL-1beta as a general pro-inflammatory stimulus resulted in a significant increase in G-CSF (C) and interleukin 8 (F) secretion by nasal epithelial cells. (** $P < 0.01$ vs control, *** $P < 0.001$ vs control, §§ $P < 0.01$ vs control, §§§ $P < 0.001$ vs control).

by nasal epithelial cells. Levels of eotaxin-1/2 and GM-CSF were below detection limit (data not shown).

Because the purity of the epithelial cells was 98%, we believe that the relative contribution of remaining nonepithelial cells to the observed induction of chemokines, is negligible.

Effects of SEB-stimulated epithelial cell mediators on the granulocyte migration *in vitro*

A Boyden chamber was utilized for the evaluation of migration of granulocytes *in vitro* through a semipermeable membrane (Fig. 3). We evaluated the level of granulocyte migration after 45 min incubation with supernatant from medium-stimulated (SN Medium) or SEB-stimulated HNEC (SN SEB, $n = 4$) and demonstrate that the chemotactic index of SN SEB is significantly higher (2.62 ± 0.30) compared to SN Medium, which was set to 1 ($P < 0.001$).

The supernatant from SEB-stimulated HNEC (SN SEB) appeared to be particularly chemotactically active for neutrophils. As shown in Fig. 4A, the number of migrated neutrophils was significantly higher upon SN SEB incubation (10.0 ± 0.6 cells per field) compared to SN Medium

(7.4 ± 0.6 cells per field, $P < 0.001$) and Medium (3.6 ± 0.4 , $P < 0.001$). Interestingly, the supernatant of epithelial cells itself (SN Medium) had chemotactic activity for neutrophils (Medium, $P < 0.05$, Fig. 4A). As expected, IL-8 was the most potent chemoattractant in this assay (18.0 ± 1.8 , $P < 0.001$ vs Medium).

Looking to the chemoattraction of eosinophils, we could demonstrate a significant decrease in chemotactic activity in SN SEB (4.8 ± 0.4) compared to SN Medium (6.9 ± 0.6 , $P < 0.01$) and Medium (9.3 ± 1.1 , $P < 0.001$, Fig. 4B).

Effects of SEB-stimulated epithelial cell-derived mediators on granulocyte survival *in vitro*

Modulation of granulocyte survival by supernatant from SEB-stimulated HNEC (SN SEB) was evaluated after 1 and 2 days of co-incubation. As can be appreciated from Fig. 5A, neutrophil survival was not significantly altered upon SN SEB incubation after 24 or 48 h. However, SN SEB caused a significant increase in eosinophil survival after 24 h ($62.76 \pm 9.70\%$), compared to medium ($30.63 \pm 3.83\%$ living eosinophils, $P < 0.001$, Fig. 5B). After 48 h incubation with SN SEB, $27.99 \pm 4.14\%$ of eosinophils were alive, vs

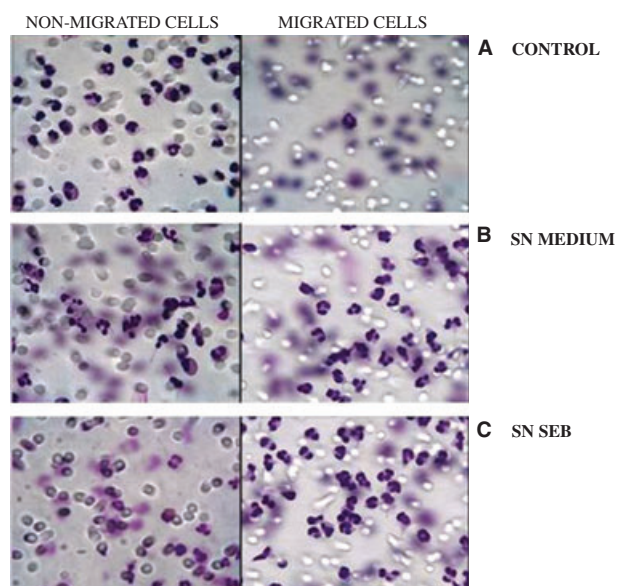


Figure 3 Boyden chamber-based cell migration assay. Granulocytes from house dust mite allergic donors were allowed to migrate through 5 μ m pore size poly (vinylpyrrolidone)-free (PVPP) polycarbonate filters for 45 min at 37°C. At 45 min after incubation, the membrane was subjected to May-Grünwald-Giemsa staining, and its lower side was evaluated for number of migrated granulocytes, by counting 10 high power fields per sample. (A) control: granulocytes incubated with medium. (B) SN Medium: granulocytes incubated with supernatant from medium-stimulated epithelial cells. (C) SN SEB: granulocytes incubated with supernatant from SEB-stimulated epithelial cells. (magnification $\times 400$).

$9.90 \pm 4.22\%$ in the Medium group ($P < 0.05$). Interestingly, at that time point SN Medium incubation resulted only in $14.16 \pm 1.97\%$ living eosinophils, which is significantly lower compared to SN SEB incubation ($P < 0.01$, Fig. 5B).

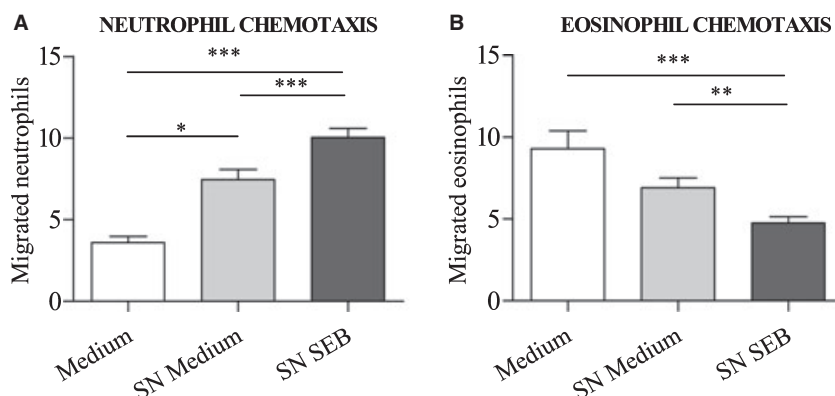


Figure 4 (A) Effects of *Staphylococcus aureus* enterotoxin B (SEB)-stimulated epithelial cell mediators on the granulocyte migration *in vitro*. The number of migrated neutrophils was significantly higher upon SN SEB incubation compared to SN Medium. Interestingly, control experiments revealed a significantly increased number

Discussion

The experimental approach of using freshly isolated and purified epithelial cells from healthy donors allowed us to study epithelial cell immunology more accurately compared to the usage of cell lines. By means of magnetic cell sorting, we obtained a cell population consisting of uncontaminated, naïve nasal epithelial cells, in which we could study the effect of exogenous signals. Specifically, the role of SEB in the activation of HNEC for chemokine secretion with subsequent granulocyte migration and survival was evaluated.

Stimulation of HNEC with SEB has resulted in significantly increased, dose-dependent chemokine production of IP-10, G-CSF, MCP-1, MIG, and RANTES, as was measured in the supernatant. Interestingly, compared to control medium this supernatant was significantly more chemotactically active for granulocytes, in particular for neutrophils. Moreover, granulocyte survival analysis revealed a significantly prolonged survival of eosinophilic granulocytes, when incubated with supernatant from SEB-stimulated HNEC. These results indicate the importance of the epithelium in the orchestration of granulocyte-dominated inflammation and demonstrate the significant role of SEB as disease modifier.

Previous studies using either SEB or *S. aureus* itself confirm the role of the innate immune activation through epithelial cells secreting cytokines and chemokines, which lead to a secondary influx of inflammatory cells (23, 25–27). However, this is the first study evaluating the effect of the prototypic staphylococcal superantigen SEB on a highly purified human nasal epithelial cell population. Interestingly, HNEC are capable of secreting IFN γ -inducible protein-10 (IP-10) and monokine induced by IFN γ (MIG) upon SEB stimulation. Both chemokines have been shown to be functional agonists of CXC chemokine receptor 3 (CXCR3), and they largely act on natural killer (NK) cells and activated T cells. However, CXCR3-expression is also found on eosinophils (28) and neutrophils (29), in particular in inflamma-

of migrated neutrophils upon SEB incubation, compared to control medium. (B) The chemoattraction of eosinophils did not differ upon incubation with SN Medium or SN SEB. Surprisingly, a significant lower number of eosinophils were found upon SEB incubation. (** $P < 0.01$, *** $P < 0.001$).

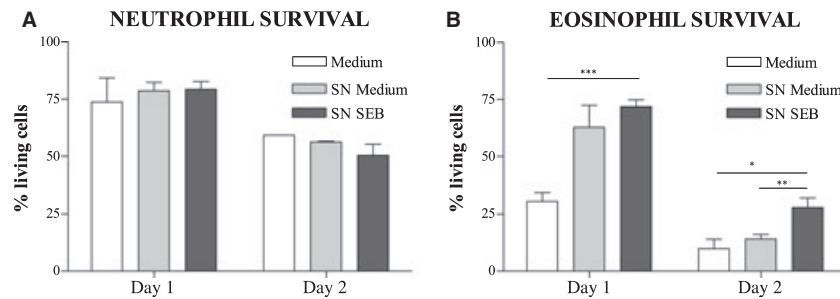


Figure 5 Effects of *Staphylococcus aureus* enterotoxin B (SEB)-stimulated epithelial cell-derived mediators on granulocyte survival *in vitro*. (A) Neutrophil survival was not significantly altered upon

SN SEB incubation after 24 or 48 h. (B) However, SN SEB caused a significant increase in eosinophil survival after 24 and 48 h. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

tory microenvironments. Furthermore, increased levels of RANTES and MCP-1 – both chemoattractant for mononuclear cells – after SEB stimulation are in line with previous data (23), and increased levels of G-CSF – causing granulocyte proliferation and differentiation – are reported after *S. aureus* stimulation of epithelial cells (30). Pretreatment of HNEC with IFN γ has resulted in increased levels of IL-8 upon SEB stimulation (20). However, we and others (25) did not find increased IL8 levels in untreated SEB-stimulated HNEC. Altogether, these data stress the major role of airway epithelial cells in the immune response to SEB, therefore actively participating in the pathogenesis of granulocyte-dominated diseases linked to SAEs like asthma, nasal polyposis, or AR (31). A potential drawback of the used approach, however, might be the loss of epithelial cell polarization, allowing SEB to reach the apical and basal side of epithelial cells equally. In addition, we had to limit the number of analyzed chemokines in the supernatant for practical purposes, although other inflammation-related genes and proteins are most likely also induced by SEB incubation.

The mechanism via which SEB activates HNEC to produce chemokines has been linked to major histocompatibility complex (MHC) class II binding and crosslinking (20), although involvement of non-MHC class II receptors has also been demonstrated (26). Moreover, SEB is known to activate APCs like dendritic cells via Toll-like receptor (TLR)2, a receptor, which plays an important role in pathogen recognition and innate immunity (32). Interestingly, these TLRs are also present on nonprofessional APCs like epithelial cells (33). We have performed pilot experiments using anti-TLR2 and anti-TLR4 mAbs to block epithelial cell activation by SEB via TLR2/4. Incubation with anti-TLR2, anti-TLR4, or both did not reduce the epithelial production of IP-10, MCP-1, or MIG upon SEB stimulation (data not shown), pointing to other mechanisms of epithelial cell activation. The typing and contribution of receptors involved in the SEB-induced chemokine secretion therefore clearly merits further investigation.

Chemokines present in the supernatant of SEB-stimulated HNEC (SN SEB) were able to increase granulocyte chemotaxis, in particular neutrophilic chemotaxis (Fig. 4A), probably because of the increase in G-CSF (34). Interestingly,

eosinophilic chemotaxis was downregulated by SN SEB, a finding possibly related to raised levels of MIG, which has a known negative regulatory effect on eosinophil recruitment (35).

Granulocyte survival has been evaluated upon incubation with SN SEB. Interestingly, only eosinophilic survival was significantly increased after 1 and 2 days. Although we could not demonstrate an increase in individual factors directly linked to eosinophil survival like GM-CSF (36) or eotaxin1/2 (37), it is tempting to speculate that their synergistic effect has contributed to the observed increase in survival, as was shown by others (37). Alternatively, other yet unidentified factors, which are also under the tight control of the nuclear factor (NF)- κ B complex, could be responsible for the observed effects (38). Surprisingly, neutrophilic survival was not altered by SN SEB, although epithelial-derived G-CSF has been described to prolong neutrophil survival in cystic fibrosis (CF) airways (30). Altogether, we could speculate that the presence of neutrophils in SEB-mediated granulocyte-dominated disease might be because of increased chemotaxis, whereas increased number of eosinophilic granulocytes could be linked to augmented survival. However, this hypothesis needs to be confirmed in further studies.

Altogether, we hereby demonstrate that stimulation of HNEC with the superantigen SEB leads to production of cytokines and chemokines, important in the chemotaxis of T cells, monocytes, and granulocytes. Moreover, *in vitro* analysis of these factors confirmed their involvement in the pathogenesis of granulocyte-dominated disease, as they significantly increased granulocyte migration and survival. These findings contribute to the understanding of SAE modulation of airway disease and stress the opportunity to target epithelial cells for therapeutic intervention.

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