

ORIGINAL ARTICLE

Interleukin-6 is essential for Staphylococcal exotoxin B-induced T regulatory cell insufficiency in nasal polyps

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Summary

Background The pathogenesis of nasal polyps is still unclear. There is increasing evidence indicating that *Staphylococcal aureus* (*S. aureus*) is associated with the formation of nasal polyps, but the mechanism has not been well documented to date.**Methods** We stimulated cultured nasal polyps and turbinate tissues with *Staphylococcal exotoxin B* (SEB), detected the expression of pro-inflammatory cytokines (IL-2, IL-6, and IL-8) and T cell cytokines (IFN- γ , IL-4, IL-5, IL-10, and IL-17) in the supernatants, and evaluated mRNA expression (T-bet, GATA-3, Foxp3, and ROR γ t) and frequencies of CD4⁺CD25⁺ T regulatory cells (Tregs) in nasal tissues. We also evaluated the effects of blocking IL-6 with monoclonal antibodies to T cell profiles in cultured nasal tissues stimulated by SEB.**Results** Levels of IL-6, IFN- γ and IL-4 increased significantly in SEB-stimulated nasal polyps. Meanwhile, mRNA expressions of T-bet and GATA-3 were significantly up-regulated, while Foxp3 was inhibited and the frequencies of CD4⁺CD25⁺ Tregs were decreased after SEB stimulation. After blocking IL-6, the levels of IL-10 and Foxp3 mRNA, as well as the frequencies of CD4⁺CD25⁺ Tregs, were significantly increased, while IFN- γ and IL-4production and the mRNA expression of T-bet and GATA-3 were significantly inhibited. **Conclusions** SEB is able to modulate pro-inflammatory factors, T-helper type 1/Th2 profiles and suppress Treg activity in cultured nasal polyps, which were rescued by blocking IL-6 activity. Therefore, IL-6 is essential for SEB-induced Treg insufficiency in nasal polyps.**Keywords** cytokine, nasal polyps, *Staphylococcal aureus*, T regulatory cells

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Introduction

The human nasal passage is the most common site for *Staphylococcal aureus* (*S. aureus*) colonization [1]. The *Staphylococcal* exotoxins (SEs) are able to activate T cells via the T cell receptor – major histocompatibility complex class II complex, by binding to the variable β -chain of the T cell receptor, leading to activation of abundant T cells in a given tissue [2]. Up to now, it has been well established that SEs, including *Staphylococcal exotoxin A* (SEA) and *Staphylococcal exotoxin B* (SEB), are tightly linked with atopic diseases such as rhinitis, asthma, and dermatitis, etc. [3].

Although nasal polyps, including chronic rhinosinusitis with nasal polyps, are not related to atopy [4], there is increasing evidence indicating that *S. aureus* is also

associated with the formation of nasal polyps. Studies have shown increased *S. aureus* colonization in nasal polyps compared with control individuals and patients with chronic rhinosinusitis without nasal polyps [5]. Moreover, an increased number of T cells expressing the T cell receptor variable β -chain, which is known to be induced by superantigens, were detected in nasal polyps [6]. These studies confirm that *S. aureus* plays an important role in the development of nasal polyps. However, the mechanism of *S. aureus* in this process has not been fully understood.

SEB have demonstrated extensive effects on numerous cells types in nasal mucosa. Recent studies suggest that SEB can increase pro-inflammatory cytokines, such as IL-1, IL-6, IL-8 in nasal epithelial cells [7, 8]. In addition, SEB is able to influence the activity of immunomodulatory

cells, such as dendritic cells and T regulatory cells (Tregs). For example, it has been recently demonstrated that human dendritic cells conditioned with SEB promote Th2 cell polarization and SEB stimulation leads Tregs to lose their immunosuppressive activity [9, 10]. Therefore, SEB may have a potentially important role in the pathogenesis of nasal polyps by modulating pro-inflammatory cytokines and Tregs.

In a previous study, we showed that Foxp3^+ CD4^+ CD25^+ Tregs have decreased in nasal polyps [11]. To further understand the role of SEB and Tregs in the pathogenesis of nasal polyps, we aimed to elucidate whether SEB has an impact on IL-6 production and whether IL-6 participates in suppressing Tregs activity in cultured nasal polyps in this study. Our results have demonstrated that IL-6 is up-regulated significantly by SEB and plays an essential in SEB-induced Treg insufficiency in cultured nasal polyps. Our findings therefore suggest IL-6 might be regarded as a potential therapeutic target for preventing the development of nasal polyps.

Materials and methods

Study subjects

Seventeen patients with nasal polyps were included in this study, for which there were 10 men and seven women (22–57 years of age, mean age 36.9 years). The diagnosis of chronic rhinosinusitis with nasal polyps in this study was based on clinical history, anterior rhinoscopy, nasal endoscopy, and paranasal computed tomography (CT) scan. The patients met criteria for nasal polyps according to the American Academy of Otolaryngology-Head and Neck Surgery Chronic Rhinosinusitis Task Force [12]. CT scans were graded as per Lund-Mackay, and polyps were graded by size and extent in both the left and right nasal fossa on a scale of 0–3 [13]. The atopic status was evaluated by skin prick tests (SPTs) to common inhalant allergens, such as house dust mite and pollen. Subjects had no history of asthma and other diseases, were refractory to medical treatment (oral antibiotics, topical steroids, decongestants, and mucolytic agents for longer than 6 weeks), and therefore planned to undergo endoscopic sinus surgery. Patients with a single polyp (antrochoanal, sphenochoanal) or with other diseases correlated with nasal polyps such as cystic fibrosis, primary ciliary dyskinesia, and fungal rhinosinusitis were excluded. The use of local or systemic corticosteroids or other medicine was stopped at least 4 weeks before endoscopic sinus surgery. During the sinus surgery, polyps and the inferior turbinate were sampled. As a control group, 14 patients (eight men and six women, 28–49 years of age, mean age 31.7 years) with septum deviation were recruited. These subjects had no history of other respiratory pathology or

Table 1. Characteristics of nasal polyps patients and control subjects

Groups	Controls (n = 14)	Nasal polyps (n = 17)
Sex (male : female)	8 : 6	10 : 7
Age (years)	28–49	22–57
Skin prick test-positive	0/14	3/17
Asthma in history and present	0/14	0/17
Smoking	3/14	4/17
Duration of disease (years)	0	4.4
CT score (Lund-MacKay)	0	13.6
Total polyps scores (two sides)	0	4.3

CT, computed tomography.

allergy, and demonstrated a negative SPT. During the septum surgery, the inferior turbinate was sampled as the normal control mucosa. The details of the subjects' characteristics are included in Table 1. This study was approved by our Institutional Ethics Committee, and informed consent was obtained from all subjects.

Nasal explants culture

Nasal tissue was rinsed three times with PBS containing antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin; Invitrogen, Carlsbad, CA, USA), then sectioned into multiple samples according to a method described elsewhere [14]. Briefly, sections of tissue (150–180 mg) were weighed and cut into 1–2-mm-large specimens for tissue culture, and 60 mg tissue was placed in 1 mL of RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen). For this study, we randomly divided 17 nasal polyps patients (polyps and turbinates) and 14 controls into two groups: seven nasal polyp patients and five controls were used for evaluating the effect of SEB with different concentrations (50 and 500 ng/mL; Sigma, St. Louis, MO, USA) for 24 h on pro-inflammatory factor production, while the rest was used for further study.

We preliminarily found that maximal IL-6 production was obtained at the concentration of 500 ng/mL of SEB for 24 h, and that more than 50% suppression of IL-6 bioactivity was obtained by 100 ng/mL IL-6 mAb in the cultured nasal epithelial cells by MTT [tetrazolium salt-(4,5 dimethylthiazol-2yl)-2,5-triphenyl tetrazodibromide] (data not shown). Therefore, in the following experiment, nasal tissues from 10 nasal polyps patient (polyps and turbinates) and nine controls were cultured in the presence of SEB (500 ng/mL; Sigma) with or without IL-6 mAb (100 ng/mL, R&D Systems, Minneapolis, MN, USA) for 24 h.

The supernatants and tissues were separated by centrifugation. All supernatants were collected and stored at -80°C for ELISA, and fresh tissues in the second experiment were immediately divided into two parts for real-time RT-PCR and flow cytometry.

Reverse transcriptase polymerase chain reaction

Real-time RT-PCR was performed as in our previous report [15]. Briefly, total RNA was extracted from nasal tissue with TRIzol reagent (Invitrogen) following the manufacturer's instructions. RT was performed and the cDNA was synthesized from 2 µg of total RNA using an oligo (dT)18 primer and M-MLV RT (Takara, Syuzou, Shiga, Japan) for quantitative PCR. Expression of mRNA was determined using an ABI PRISM 7300 Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Taq™ (Takara). The sequences of the primer were as follows: T-bet (NM_013351) forward: 5'-GAT GTT TGT GGA CGT GGT CTT G-3'; T-bet reverse: 5'-CTT TCC ACA CTG CAC CCA CTT-3'; GATA-3 (NM_002051) forward: 5'-GCG GGC TCT ATC ACA AAA TGA-3'; GATA-3 reverse: 5'-GCT CTC CTG GCT GCA GAC AGC-3'; Foxp3 (NM_014009) forward: 5'-GAG AAG CTG AGT GCC ATG CA-3'; Foxp3 reverse: 5'-AGG AGC CCT TGT CGG ATG AT-3'; RORγt (NM_001001523) forward: 5'-TGA GAA GGA CAG GGA GCC AA-3'; RORγt reverse 5'-CCA CAG ATT TTG CAA GGG ATC A-3'; β-actin (NM_001101) forward: 5'-AAG ATG ACC CAG ATC ATG TTT GAG ACC-3'; β-actin reverse: 5'-AGC CAG GTC CAG ACG CAG GAT-3'. PRISM samples contained 1×SYBR Green Master Mix, 1.5 µL of 5 µM primers, and 25 ng synthesized cDNA in a 25 µL volume. Reactions were heated to 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 10 s, and annealing extension at 60 °C for 60 s. All PCR reactions were performed in duplicate. Melting curve analysis was used to control for amplification specificity. Expression of the target gene was expressed as a fold increase or decrease, relative to the expression of β-actin. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_t). The level of gene expression was then calculated as the difference (ΔC_t) between the C_t value of the target gene and the C_t value of β-actin. Fold changes in mRNA from nasal polyps and turbinates normalized to controls (normal or unstimulated controls) were determined as $2^{-\Delta\Delta C_t}$.

Enzyme-linked immunosorbent assay

Cytokine concentration in the supernatants was determined by ELISA. Cytokine concentrations (IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-17) were determined by using a commercially available solid-phase sandwich ELISA kit (Bioss Co., Beijing, China) and performed according to the manufacturer's instructions. Pro-inflammatory factors (IL-2, IL-6, and IL-8) were measured in the first study, while IL-6 and T cell cytokines (IFN-γ, IL-4, IL-5, IL-10, and IL-17) were measured in the second study. Assays were performed in duplicate, and the detection limit for cytokines was as follows: IFN-γ, 15.6 pg/mL; IL-2, 15.6 pg/mL; IL-4, 3.2 pg/mL; IL-5, 3.2 pg/mL; IL-6,

31.2 pg/mL; IL-8, 31.2 pg/mL; IL-10, 7.8 pg/mL; IL-17, 7.8 pg/mL; TGF-β, 7.8 pg/mL.

Flow cytometric analysis

Nasal tissues from 10 nasal polyps patients (polyps and inferior turbinates) and nine controls in the second experiment were washed and cut into small fragments and teased apart to allow dispersion of the nasal cells into RPMI 1640 media (Life Technologies Inc., Rockville, MD, USA). After incubation with 0.5% collagenase (Sigma) at 37 °C for 15 min, the cells were passed through a 40 µm-mesh screen to achieve a single cell suspension. Cell viability per the control was >80% (Trypan blue staining). Following washing, cells were adjusted maximally to 2×10^6 cells/mL and were labelled with the following anti-human mAbs: PerCP conjugated CD4, FITC-conjugated CD25 (eBioscience, San Diego, CA, USA). Cell fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA), and data were analysed using CellQuest software (BD Biosciences).

Statistical analysis

Data were expressed as the means±SEM, and a one-way ANOVA test was used to test the statistical significance of multi-group comparisons. Then, the statistical significance of the differences was assessed with a paired Student's *t*-test for intragroup comparisons and the unpaired Student's *t*-test for inter-group comparisons. A *P*-value of <0.05 was considered statistically significant.

Results

Levels of pro-inflammatory factors interleukin-2, interleukin-6, and interleukin-8 in the supernatants of cultured nasal polyps after Staphylococcal exotoxin B stimulation

In order to evaluate the possible roles of pro-inflammatory factors in the formation of nasal polyps, we examined the levels of IL-2, IL-6, and IL-8 in the supernatants of cultured nasal tissues in the presence or absence of SEB at 24 h. As illustrated in Fig. 1, IL-2, IL-6, and IL-8 were detected in the supernatants of cultured nasal tissues without SEB stimulation, indicating a spontaneous release from nasal tissues. By ANOVA analysis and Student's *t*-test, we found the levels of IL-6 and IL-8 were elevated significantly in the supernatants of cultured nasal polyps compared with that of cultured turbinates from patients and normal controls. Our results thus indicate the importance of IL-6 and IL-8 in the development of nasal polyps.

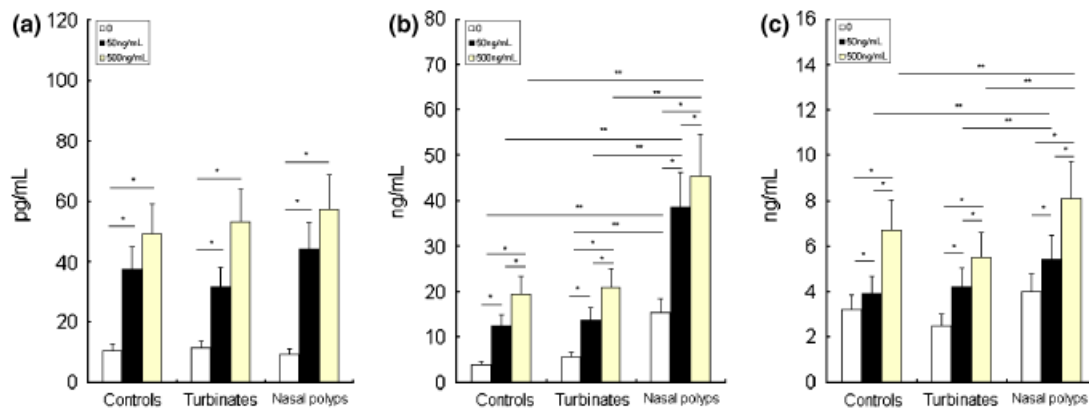


Fig. 1. The levels of pro-inflammatory factors IL-2 (a), IL-6 (b), and IL-8 (c) in the supernatants of cultured nasal tissues after *Staphylococcal exotoxin B* (SEB) stimulation. The levels of IL-2, IL-6, and IL-8 were elevated in the supernatants of cultured nasal tissues after SEB stimulation (50 and 500 ng/mL) for 24 h in a dose-dependent manner. The levels of IL-6 and IL-8 were elevated significantly in the supernatants of cultured nasal polyps compared with that of cultured turbinates from patients and controls. * $P < 0.05$, intra-group analysis by paired t -test; ** $P < 0.05$, inter-group analysis by unpaired t -test.

Furthermore, we found the levels of IL-2, IL-6, and IL-8 were elevated in the supernatants of cultured nasal tissues due to SEB stimulation (50 and 500 ng/mL) for 24 h. By paired Student's t -test, we found the levels of IL-6 and IL-8 increased significantly in the supernatants of cultured nasal polyps in the presence of SEB (50 and 500 ng/mL), compared with that released spontaneously. In addition, we found the levels of IL-6 and IL-8 in the presence of 500 ng/mL of SEB were significantly higher than those in the presence of 50 ng/mL of SEB, and presented in a dose-dependent manner. By ANOVA analysis and paired and unpaired t -test, we also found the levels of IL-6 and IL-8 were elevated significantly in the supernatants of cultured nasal polyps compared with that of cultured turbinates from patients and controls in the presence of SEB (50 and 500 ng/mL) for 24 h.

Levels of T cell cytokines interferon- γ , interleukin-4, interleukin-5, interleukin-10, and interleukin-17 in supernatants of cultured nasal polyps after *Staphylococcal exotoxin B* stimulation

It has been suggested that nasal polyps present a mixed T-helper type 1 Th1/Th2 cytokine profile [16]. In order to evaluate the effects of SEB stimulation (500 ng/mL) on T cell subsets in cultured nasal polyps, we detected the indicative T cell cytokines IFN- γ for Th1 subset, IL-4 and IL-5 for Th2 subset, IL-10 for Tregs subset, and IL-17 for Th17 subset, in the supernatants of cultured nasal tissues by ELISA assay. As illustrated in Fig. 2, we found, by ANOVA analysis, that the levels of IFN- γ and IL-4 changed significantly in the supernatants of three nasal tissues after SEB stimulation for 24 h, while the levels of IL-5, IL-10, and IL-17 had no significant changes.

To further understand the effect of SEB stimulation on nasal polyps, we analysed the differences of five cytokines in the supernatants of three nasal tissues by paired and unpaired t -test. As illustrated in Fig. 2, we found the levels of IFN- γ and IL-4 in the supernatants of nasal polyps were significantly higher than those in turbinate and control tissues. On the other hand, IFN- γ increased from 35.9 pg/mL to 127.8 pg/mL and IL-4 increased from 6.0 to 21.8 pg/mL in the supernatants of nasal polyps after SEB stimulation, where the differences were significant ($P < 0.01$). However, there were no significant differences in the levels of IL-5, IL-10, and IL-17 in the supernatants of nasal polyps without or with SEB stimulation. Our results therefore suggest that SEB stimulation was able to increase both Th1 and Th2 cytokines (IFN- γ and IL-4) in nasal polyps.

Gene expressions of transcription factors in cultured nasal polyps after *Staphylococcal exotoxin B* stimulation

The differentiation of the T cell subset is controlled by specific transcription factor. We consistently detected transcription factors underlying differentiation of T cell subsets in cultured nasal tissues by real-time PCR analysis: T-bet for Th1 subset, GATA-3 for Th2 subset, Foxp3 for the Treg subset, and ROR γ t for Th17 subset. We found that the mRNA levels of T-bet and GATA-3 had significant differences in three nasal tissues after SEB stimulation (500 ng/mL) for 24 h by ANOVA analysis and paired t -test. In this study, T-bet mRNA increased 2.6-fold in controls, 3.4-fold in turbinates, and 3.1-fold in nasal polyps compared with that in unstimulated tissues ($P < 0.05$). Correspondingly, GATA-3 mRNA was elevated by 3.4-fold, 3.7-fold and 3.9-fold, respectively. However, there were no significant changes in Foxp3 and ROR γ t mRNA in the three

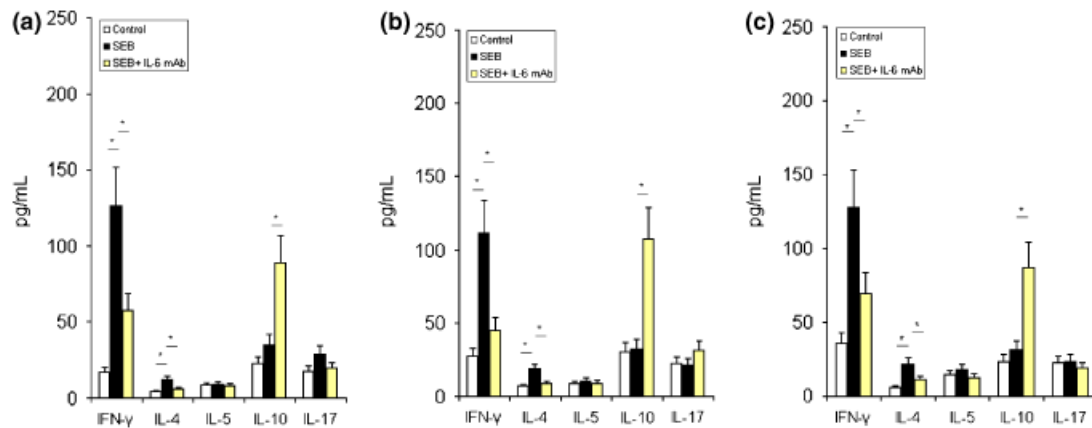


Fig. 2. The levels of T cell cytokines in the supernatants of cultured control mucosa (a), turbinate tissue (b), and nasal polyps after *Staphylococcal exotoxin B* (SEB) stimulation (500 ng/mL) for 24 h, without or with IL-6 mAb blockage (100 ng/mL). The levels of IFN- γ and IL-4 in the supernatants of nasal polyps were significantly higher than that in turbinate and control tissues. After blocking IL-6, the level of IL-10 in the supernatants of SEB-stimulated nasal tissues increased significantly compared with unstimulated nasal tissues, and the levels IFN- γ and IL-4 decreased significantly. * $P < 0.05$, intra-group analysis by paired t -test.

nasal tissues after SEB stimulation. On the other hand, by paired and unpaired t -test, we found the mRNA levels of T-bet and GATA-3 in nasal polyps was significantly higher than that in turbinate and control tissues (4.1-fold and 4.7-fold, respectively, $P < 0.05$), and that the mRNA levels of Foxp3 in nasal polyps was significantly lower than those in turbinate and control tissues (0.61-fold and 0.49-fold, respectively, $P < 0.05$). However, ROR γ t mRNA had no significant changes. Our results therefore suggest that SEB stimulation was able to modulate Tregs by inhibiting the expression of Foxp3 mRNA and shift the balance of Th1/Th2 subsets in nasal polyps.

Blockage of interleukin-6 on interleukin-10 production and Foxp3 messenger ribonucleic acid expression and the frequencies of CD4⁺CD25⁺ T regulatory cells in Staphylococcal exotoxin B-stimulated nasal polyps

Because IL-6 can be elevated in nasal polyps by SEB stimulation, and IL-6 is able to suppress the Treg activity, we evaluated the effect of blocking IL-6 by neutralized monoclonal antibody to Treg subsets in nasal polyps. As illustrated in Fig. 2, we found the level of IL-10 in the supernatants of SEB-stimulated nasal tissues increased significantly after neutralization by IL-6 mAb compared with un-stimulated nasal tissues by ANOVA analysis and paired and unpaired t -test. Meanwhile, we found that the levels IFN- γ and IL-4 decreased significantly ($P < 0.05$). However, the levels of IL-5 and IL-17 had no significant changes. Correspondingly, we also found significant elevation in Foxp3 mRNA (5.1-fold, $P < 0.01$) in cultured nasal polyps after blockage by IL-6 mAb. Yet, T-bet and GATA-3 mRNA decreased significantly (0.44-fold and 0.57-fold, respectively) ($P < 0.05$). Similar results were observed in the turbinate tissues and in controls.

To further understand the effect of IL-6 on Tregs in nasal polyps, we detected the frequencies of CD4⁺CD25⁺ Tregs by flow cytometric analysis. As illustrated in Figs 3 and 4, our results showed that the frequencies of CD4⁺CD25⁺ Tregs in nasal polyps decreased significantly compared with that in turbinate and control tissues by ANOVA analysis and paired and unpaired t -test. Furthermore, we found SEB stimulation decreased the frequencies of CD4⁺CD25⁺ Tregs significantly in nasal polyps, as well as in turbinate tissues. However, after neutralization by IL-6 mAb in this study, we found the frequencies of CD4⁺CD25⁺ Tregs increased significantly by paired t -test (control mucosa, from 7.2% to 9.7%; turbinate tissues, from 7.9% to 10.1%; nasal polyps, from 3.5% to 6.9%). These findings, therefore, were consistent with the changes in Foxp3 mRNA expression and IL-10 production, suggesting that blockage of IL-6 is essential for Treg activity in nasal polyps (Fig. 5).

Discussion

In the present study, we demonstrated that SEB stimulation was able to increase various pro-inflammatory factors, including IL-2, IL-6, and IL-8 in cultured nasal polyps and turbinate. Furthermore, we found that SEB stimulation skewed the balance among T cell subsets by inhibiting Treg activity. Mixed Th1/Th2 infiltrations and Treg insufficiency in nasal polyps have been reported in previous studies [16, 17, 11]. In this study, we found that it was proven to be associated with IL-6 production and resulted in elevated IFN- γ , IL-5, and IL-4 production, an immune imbalance that may underlie the pathogenesis of nasal polyps. Our findings therefore demonstrate that IL-6 plays a central role in suppressing Treg activity in nasal polyps. To our knowledge, this is the first report focused

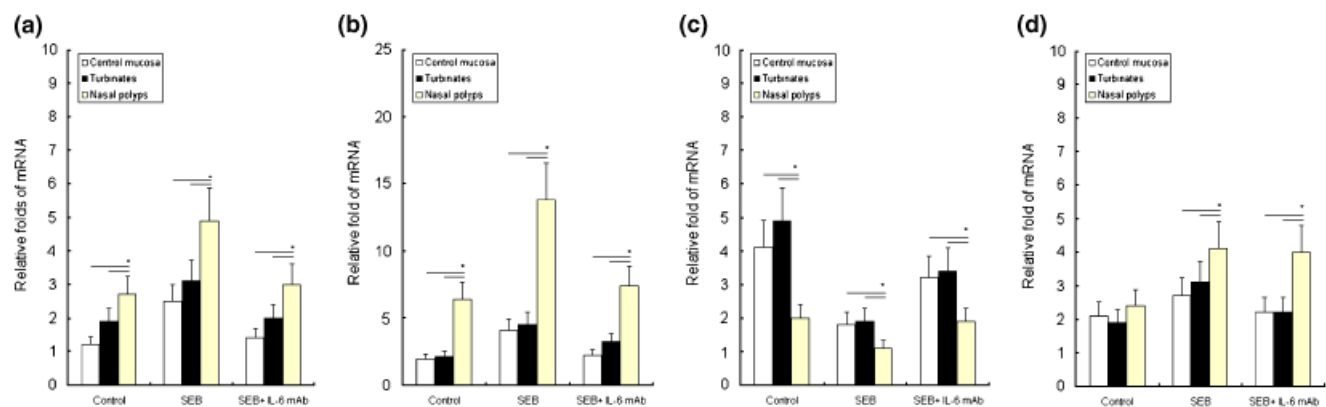


Fig. 3. The levels of T-bet (a), GATA-3 (b), Foxp3 (c), and RORγt mRNA (d) in cultured control mucosa, turbinate tissue and nasal polyps after *Staphylococcal exotoxin B* (SEB) stimulation (500 ng/mL) for 24 h, without or with IL-6 mAb blockage (100 ng/mL). The mRNA levels of T-bet and GATA-3 in nasal polyps were significantly higher than those in turbinate and control tissues ($P < 0.05$). And the mRNA levels of Foxp3 in nasal polyps was significantly lower than those in turbinate and control tissues ($P < 0.05$). The mRNA levels of T-bet and GATA-3 had significant differences in three nasal tissues after stimulation ($P < 0.05$). Significant elevation in Foxp3 mRNA ($P < 0.01$) in cultured nasal polyps after blockage. And T-bet and GATA-3 mRNA decreased significantly ($P < 0.05$). Similar results were observed in the turbinate tissues and in controls. * $P < 0.05$, inter-group analysis by paired t -test.

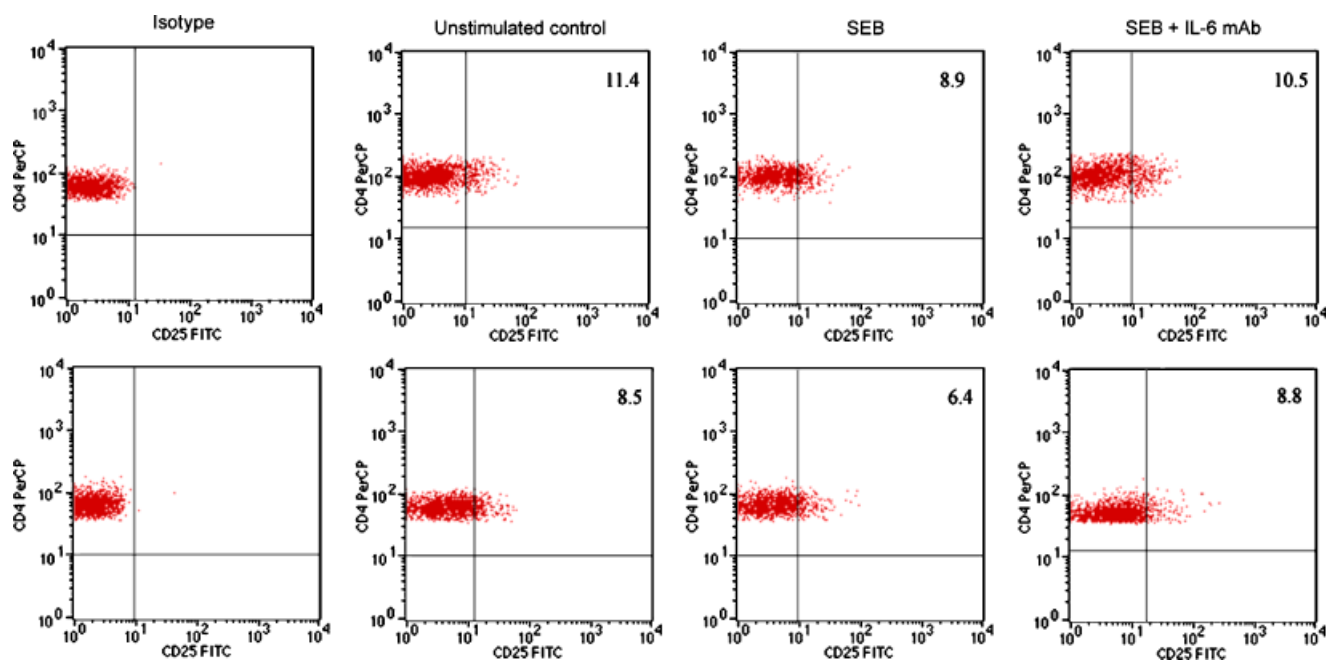


Fig. 4. The frequencies of CD4⁺CD25⁺ Tregs in cultured nasal tissues after *Staphylococcal exotoxin B* (SEB) stimulation (500 ng/mL) for 24 h, without or with IL-6 mAb blockage (100 ng/mL). Representative 2D images of CD4⁺CD25⁺ Tregs in cultured nasal tissues are presented by flow cytometric analysis. The upper lane represents results from control mucosa, while the lower lane represents results from nasal polyps.

on the role of IL-6 in SEB-stimulated Treg insufficiency in nasal polyps.

Currently, the linkage between *S. aureus* and nasal polyps has been well established, but the mechanism requires further understanding. Nasal polyps generally represent a subset of chronic inflammation of the mucous membrane in the paranasal sinus, which is distinct from chronic rhinosinusitis without nasal polyps [12]. Histological features of nasal polyps include increased accumulation

of inflammatory cells, up-regulated Th1/Th2 cytokines and excessive tissue hyperplasia [18]. Numerous studies have shown that SEs are able to exert extensive effects on nasal tissues. For example, Wang et al. [19] reported that SEA and SEB can up-regulate IL-6 and IL-8 production in epithelial cell lines. O'Brien et al. [8] reported that isolated nasal epithelial cells secreted high amounts of IL-8, but not TNF- α or eotaxin following stimulation with SEA and SEB. In the present study, we also found prominently elevated

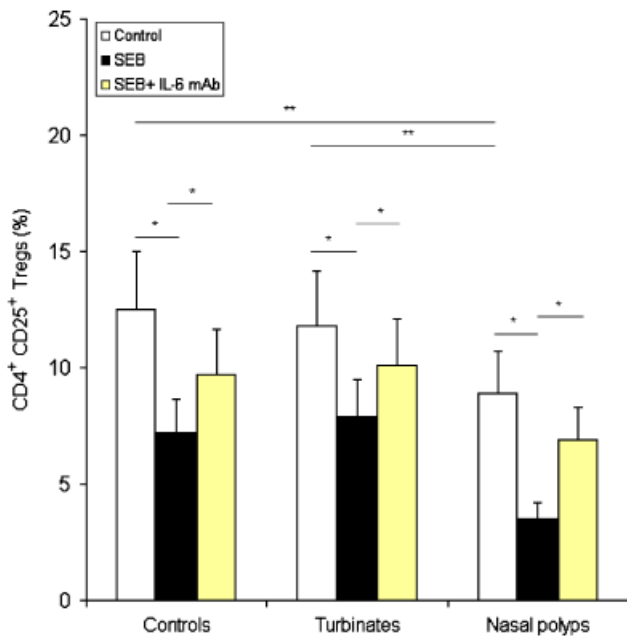


Fig. 5. The frequencies of $CD4^+CD25^+$ Tregs in cultured nasal tissues after *Staphylococcal exotoxin B* (SEB) stimulation (500 ng/mL) for 24 h, without or with IL-6 mAb blockage (100 ng/mL). Data were expressed as means $\pm$ SEM. The frequencies of $CD4^+CD25^+$ Tregs in nasal polyps decreased significantly compared with those in turbinate and control tissues, and SEB stimulation decreased the frequencies of $CD4^+CD25^+$ Tregs significantly in nasal polyps, as well as in turbinate tissues. Blocking IL-6 increased the frequencies of $CD4^+CD25^+$ Tregs significantly in SEB-stimulated nasal polyps, as well as in turbinate tissues. * $P < 0.05$, intra-group analysis by paired *t*-test.

production of IL-6, as well as other pro-inflammatory cytokines such as IL-2 and IL-8, in cultured nasal tissues after stimulation with SEB (50 and 500 ng/mL) for 24 h. We found that the levels of IL-6 and IL-8 increased significantly in the supernatants of cultured nasal polyps in the presence of SEB (50 and 500 ng/mL), compared with those released spontaneously. In addition, the levels of IL-6 and IL-8 were elevated significantly in the supernatants of cultured nasal polyps compared with that of cultured turbinates from patients and controls in the presence of 500 ng/mL of SEB. Our finding thus provided evidence that IL-6 plays an important role in a SEB-associated disease-modifying mechanism.

Nasal polyps are a multifactorial inflammatory disease with a high incidence of 2–5% worldwide [12]. It is a disease entity consisting of various inflammatory cells such as eosinophils, lymphocytes, neutrophils, and the like. Previous studies have demonstrated that nasal polyps were characterized by imbalanced T cell subsets and excessive Th1/Th2 cytokines [16], and Treg insufficiency in nasal polyps has been reported in both western and eastern populations [11, 17]. However, little is known about the modulation of SEB in Treg subsets in nasal polyps.

Recently, Patou et al. [20] evaluated the effects of SEB on cultured nasal polyp fragments and found higher levels of the Th2 cytokines IL-4, IL-5, but not IL-10 and TGF- β , which are considered to be the active markers of Treg subsets, thus indicating SEB was able to affect Treg activity. Because Th1/Th2 activity is modulated by Treg subsets [21], and T cell phenotypes in nasal polyps showed a mixed redundant Th1/Th2 cytokine pattern [16], we therefore speculate that SEB probably exert their effects on formation of nasal polyps by suppressing Treg activity to some degree. To prove this possibility, we examined the indicative T cell cytokines and mRNA expression of transcription factors underlying differentiation of T cell subsets in SEB-stimulated nasal tissues. Consistent with previous reports [21], our results demonstrate that SEB stimulation increases the levels of IFN- γ and IL-4, but not IL-10 in cultured nasal polyps, and up-regulates the mRNA expressions of T-bet and GATA-3, but not that of Foxp3 or ROR γ t. In addition, we found the mRNA levels of T-bet and GATA-3 in nasal polyps was significantly higher than in turbinate and control tissues, and that the mRNA levels of Foxp3 in nasal polyps was significantly lower than in turbinate and control tissues. We also showed that the frequencies of $CD4^+CD25^+$ Tregs were decreased in SEB-stimulated nasal tissues by flow cytometric analysis. Our findings thus prove that SEB stimulation results in dysregulated Th1/Th2 subsets and Treg insufficiency in nasal polyps, which may be crucial for the development of nasal polyps.

Additionally, other authors have provided evidence that SEB causes Treg insufficiency in a study on atopic dermatitis [10]. They have shown that SEB stimulation leads Tregs to lose their immunosuppressive activity, which was ascribed to the induction of glucocorticoid-induced TNF on monocytes in atopic dermatitis [22]. To further understand the mechanisms underlying Treg insufficiency in nasal polyps resulting from SEB stimulation, we evaluated the role of IL-6 in bridging SEB stimulation and Treg insufficiency by means of IL-6 mAb in this study. The reason that we focused on this is because the IL-6 that is produced by dendritic cells has been reported to overcome Treg activity *in vitro*. IL-6 is well known to be a pro-inflammatory cytokine that plays a central role in host defense [23]. Recently, it has been shown that IL-6 is able to render pathogen-specific T cells refractory to the suppressive activity of $CD4^+CD25^+$ Tregs and induce Th17 cell differentiation *in vitro*, as well as controlling lung $CD4^+CD25^+$ Treg development and function *in vivo* [24–26]. In the present study, we adopted IL-6 mAb to suppress the bioactivity of SEB-induced IL-6 to evaluate its role in suppressing Treg activity in cultured nasal polyps. Interestingly, our findings demonstrated that blocking IL-6 leads to significant elevation of Foxp3 mRNA and IL-10 production, as well as increased frequencies of $CD4^+CD25^+$ Tregs in nasal polyps. IL-6 has

been found to be elevated in nasal polyps by many authors [27, 28] and IL-6 can be induced in nasal epithelial cells by various allergens, fungi, bacteria, and viruses [19, 29, 30], but its significance has not been characterized. Our findings thus demonstrate that IL-6 plays an important role in Treg insufficiency and the development of nasal polyps. In addition to IL-10 and Foxp3, we also investigated TGF- β in this study, as it is well known to play an essential role in the development of Tregs. However, we did not find significant and consistent changes in TGF- β production in different samples in this study (data not shown). The broad cell source of TGF- β might lead to confusion regarding its relevance in Tregs in nasal polyps. We did not find elevated IL-17 production in this study, either. This is somewhat conflicting, because IL-6 contributes to the differentiation of Th17 cells combined with TGF- β . We attributed this to the insufficiency of TGF- β in nasal polyps [31]. Additionally, our findings contribute to explaining how nasal polyps develop in some parts of the world, such as China and other Asian countries, where *S. aureus* is uncommon in patients with nasal polyps [31].

The mechanisms underlying IL-6 bioactivity in inhibiting peripheral tolerance have not been fully understood. It has been demonstrated that IL-6 can bind to the soluble IL-6R, through a mechanism called trans-signaling, inducing proliferation of cells expressing the cognate receptor gp130. This mechanism appears to be used for proliferation by developed Th2 cells in the airways. Additionally, through the membrane-bound IL-6R, IL-6 controls CD4⁺CD25⁺ survival in the airway [26]. Considering the importance of IL-6 in SEB-stimulated Treg insufficiency in nasal polyps, this study thus has been helpful for us to better understand the pathogenesis of nasal polyps. Therefore, blockade of IL-6 or the soluble IL-6R through the fusion protein gp130, or blocking the membrane-bound IL-6R, may be able to alleviate the impact that SEB exerts on nasal polyps.

In conclusion, our findings support the hypothesis that *S. aureus* may contribute to the formation of nasal polyps and provide evidence that IL-6 may play an important role in Th1/Th2 dysregulation in nasal polyps by inhibiting Foxp3⁺CD4⁺CD25⁺ Treg activity. In this respect, IL-6 bridges *S. aureus* infection and Treg insufficiency in the development of nasal polyps and represents a novel direction for further study.

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