Cellular Responses to *Staphylococcus aureus* Alpha-Toxin in Chronic Rhinosinusitis with Nasal Polyps

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**ABSTRACT**

**Background:** In contrast to *Staphylococcus aureus*-derived superantigenic exotoxins, the role of non-superantigenic exotoxins in the pathogenesis of eosinophilic airway diseases remains obscure. We sought to characterize *S. aureus* alpha-toxin-induced cellular responses in chronic rhinosinusitis with nasal polyps (CRSwNP).

**Methods:** Dispersed nasal polyp cells and uncinate tissue cells were prepared from patients with CRS with and without nasal polyps, respectively. Cells were incubated with various concentrations of alpha-toxin or staphylococcal enterotoxin B and then the levels of IL-5, IL-13, IFN-γ, IL-17A, and IL-10 in the cell supernatants were determined. The pathophysiological significance of alpha-toxin-induced cytokine production was also determined including radiological severity of rhinosinusitis, tissue and blood eosinophilia, serum total IgE level, and 1-s forced expiratory volume/forced vital capacity ratio (FEV₁/FVC).

**Results:** Nasal polyp cells produced substantial amounts of IL-5, IL-13, IFN-γ, IL-17A, and IL-10 in response to alpha-toxin. Cytokine production was higher in nasal polyp cells than in uncinate tissue cells. The potency of alpha-toxin in stimulating IL-5, IL-13, and IL-10 production was comparable to that of enterotoxin. Alpha-toxin-induced IFN-γ, IL-17A, and IL-10 production significantly and negatively correlated with the degree of eosinophil infiltration into nasal polyps. Conversely, alpha-toxin-induced IFN-γ and IL-10 production significantly and positively correlated with FEV₁/FVC. IL-10 production was significantly lower in asthmatic patients compared to non-asthmatics.

**Conclusions:** *S. aureus*-derived alpha-toxin can provoke cellular responses in nasal polyps. These responses, especially failure to synthesize IL-10, may play a role in the pathophysiology of CRSwNP.

**KEY WORDS**

alpha-toxin, chronic rhinosinusitis, eosinophil, IL-10, nasal polyps

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**INTRODUCTION**

Chronic rhinosinusitis with nasal polyps (CRSwNPs) is characterized by eosinophilic inflammation, and is often associated with asthma and aspirin sensitivity.¹ While the precise etiology and pathophysiology underlying this disease remains poorly understood, imbalances in local Th1, Th2, Th17, and Treg responses appear to be involved.² ³ Components and products derived from microbes such as viruses, fungi, and bacteria can elicit cellular responses in patients with CRSwNP.⁴ ⁵ ⁷ *Staphylococcus aureus* exotoxins are among the best characterized elicitors of cellular responses and are thought to be

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heavily involved in the pathogenesis of CRSwNP through their behavior as superantigens.4,8,9

In addition to superantigenic exotoxins, S. aureus cells also contain other immunogenic components, such as the capsule, enzymes, and non-superantigenic toxins.10,11 Since S. aureus per se induces an inflammatory reaction in airway cells, these components may also play a role in the pathogenesis of CRSwNP.12

Alpha-toxin is one of the non-superantigenic exotoxins produced by S. aureus.13 Alpha-toxin is a basic protein consisting of 293 amino acids. Almost all S. aureus strains possess the gene that encodes alpha-toxin, hla. Also known as alpha-hemolysin, alpha-toxin binds to phospholipids on the surface of erythrocytes and then forms a cylindrical heptamer in the cell membrane, which induces hemolysis.14

Alpha-toxin can regulate immune responses in humans, including IL-1β release by monocytes, IL-8 secretion by monocytic or epithelial cell lines, LTB4 release from neutrophils, t-bet expression and IFN-γ production by CD4+ T cells, and IL-17A and IL-22 production by cytokine-activated DNPCs or DUTCs per mL was stimulated with 0.01, 0.1, or 1.0 ng/mL of alpha-toxin (Sigma, St. Louis, MO, USA) and incubated at 37°C in a 5% CO2 atmosphere. An aliquot of the culture supernatant was collected after 24 and 72 hours and stored at -80°C for subsequent cytokine analysis. Alternatively, cells were stimulated with 1.0 ng/mL of staphylococcal enterotoxin B (SEB; Toxin Technology, Sarasota, FL, USA).

CULTURE OF DISPERSED NASAL POLYP CELLS AND UNCINATE TISSUE CELLS
Nasal polyps and uncinate tissues were sampled from patients with CRSwNP and CRSSNP, respectively. Dispersed nasal polyp cells (DNPCs) and dispersed uncinate tissue cells (DUTCs) were prepared from nasal polyps and uncinate tissues, respectively, by enzymatic digestion, as previously described.5 1×10⁶ DNPCs or DUTCs per mL was stimulated with 0.01, 0.1, or 1.0 ng/mL of alpha-toxin (Sigma, St. Louis, MO, USA) and incubated at 37°C in a 5% CO2 atmosphere. An aliquot of the culture supernatant was collected after 24 and 72 hours and stored at -80°C for subsequent cytokine analysis. Alternatively, cells were stimulated with 1.0 ng/mL of staphylococcal enterotoxin B (SEB; Toxin Technology, Sarasota, FL, USA).

STATISTICAL ANALYSIS
Values are given as the median. The nonparametric Mann-Whitney U test was used to compare data between groups, and Wilcoxon’s signed rank test was used to analyze data within each group. Correlation analyses were performed using the Spearman rank correlation. P-values less than 0.05 were considered statistically significant. Statistical analyses were per-
formed with SPSS software (version 11.0, Chicago, IL, USA).

RESULTS

ALPHA-TOXIN-INDUCED CYTOKINE PRODUCTION BY DNPCs
24 h-stimulation of DNPCs with 1 ng/mL of alpha-toxin induced significant increases in the production of IL-5 (P = 0.005), IL-13 (P = 0.003), IFN-γ (P = 0.003), IL-17A (P = 0.021), and IL-10 (P < 0.001) compared to unstimulated DNPCs. Stimulation with 0.01 ng/mL of alpha-toxin did not induce production of any of the cytokines examined, while stimulation with 0.1 ng/mL did induce significant production of IL-10 (P = 0.003). In addition, 72 h-stimulation of DNPCs with 0.01 ng/mL alpha-toxin resulted in a significant production of all the cytokines tested, and these productions were substantially increased in a dose-dependent manner (Fig. 1; P < 0.001).

COMPARISON OF ALPHA-TOXIN-INDUCED CYTOKINE PRODUCTION BY NASAL POLYP CELLS AND UNCINATE TISSUE CELLS
Stimulation of 1 × 10^6/mL of uncinate tissue cells derived from CRSsNP patients with 1 ng/mL of alpha-toxin for 72 h induced a modest but significant increase in production of IL-5 (P = 0.043), IL-13 (P = 0.006), IFN-γ (P = 0.012), IL-17A (P = 0.012), and IL-10 (P = 0.018). However, as shown in Figure 2, stimulating the same number of nasal polyp cells derived from CRSwNP patients in the same manner induced production of significantly higher levels of these cytokines (IL-5: P = 0.001, IL-13: P = 0.017, IFN-γ: P = 0.006, IL-17A: P = 0.004, IL-10: P = 0.001).

COMPARISON OF CYTOKINE PRODUCTION FOLLOWING STIMULATION WITH ALPHA-TOXIN OR SEB
Next, we compared the potency of alpha-toxin and SEB, with respect to inducing cytokine production by DNPCs. Stimulation with alpha-toxin induced production of a similar amount of IL-5 (P = 0.230), IL-13 (P = 0.516), and IL-10 (P = 0.263) as did stimulation with SEB (Fig. 3A, B, E). In contrast, production of IFN-γ (P = 0.002) and IL-17A (P < 0.001) was significantly lower in DNPCs stimulated with alpha-toxin than in DNPCs stimulated with SEB (Fig. 3C, D).

PATHOPHYSIOLOGICAL SIGNIFICANCE OF ALPHA-TOXIN-INDUCED CYTOKINE PRODUCTION IN CRSwNP
As shown in Table 1, the production of IL-5 (ρ = 0.146, P = 0.503) and IL-13 (ρ = 0.108, P = 0.621) following a 72-h stimulation with 1 ng/mL of alpha-toxin did not correlate with eosinophil infiltration into nasal polyps. However, there was a significant negative correlation between local eosinophilia and production of IFN-γ (ρ = -0.432, P = 0.048), IL-17A (ρ = -0.566, P = 0.009), and IL-10 (ρ = -0.567, P = 0.009) (Fig. 4). Alpha-toxin-induced cytokine production did not correlate with other pathophysiological parameters, including radiological severity of rhinosinusitis, blood eosinophil count, or serum total IgE or FEV1/FVC, except for positive correlations between IFN-γ production and FEV1/FVC (ρ = 0.456, P = 0.037) and between IL-10 production and FEV1/FVC (ρ = 0.538, P = 0.014). Production of IL-10 was significantly lower in asthmatic patients compared to non-asthmatics (P = 0.035); however, the presence of asthma did not affect the production of other cytokines (Fig. 5).

DISCUSSION
In the present study, we characterized the alpha-toxin-induced cytokine productions from patients with CRSwNP and CRSsNP. Our results suggest that antigens other than SEB, namely alpha-toxin in this study, are also likely targets for stimulatory molecules. Both the innate and the adaptive immune systems clearly respond to bacterial structural components as well as their secreted exotoxins.4 In this case, alpha-toxin can stimulate a robust synthesis of cytokines known to be associated with eosinophilic airway inflammation.

In response to alpha-toxin stimulation, DNPCs produce substantial amounts of IL-5, IL-13, IFN-γ, IL-17A, and IL-10, cytokines known to regulate the pathogenesis of CRSwNP.2,3 In nasal polyps, a majority of the IL-5-producing cells is T cells, and mast cells and eosinophils can express IL-5.23 Mononuclear cells and eosinophils in nasal polyps express IL-13.24 We have previously reported that macrophages, CD4+ T cells and eosinophils express IL-17A in nasal polyps.3 T cells are the principal source of allergen-induced IL-10 production in nasal polyps.26 Thus, alpha-toxin may stimulate these inflammatory cells directly and/or indirectly to produce cytokines. Additional experiments, e.g., whether adherent cell or non-adherent cell produce cytokines in response to alpha-toxin, should be performed in order to identify which cells are the main sources for cytokine production.

High concentrations of alpha-toxin induce cell death due to the formation of hexameric transmembrane pores.13 For example, about two-thirds of Jurkat T cells treated with 100 ng/mL of alpha-toxin will undergo subsequent apoptosis.27 In contrast, exposure to lower concentrations of alpha-toxin can induce pro-inflammatory effects in a variety of cells without causing cell lysis, presumably via alteration of the cellular ion balance following pore formation, particularly through calcium influx.16 For example, exposure of human CD4+ T cells to alpha-toxin at concentrations less than 100 ng/mL does not lead to cell death.16 Alpha-toxin concentrations less than 1,000 ng/mL were found to be sublytic to human macrophages.19 In the present study, we exposed DNPCs and DUTCs to alpha-toxin at concentrations of 0.01,
Effect of alpha-toxin of *S. aureus* on IL-5 (A), IL-13 (B), IFN-γ (C), IL-17A (D) and IL-10 (E) production by DNPCs (*n* = 22). DNPCs were stimulated with 0.01, 0.1, or 1.0 ng/mL of alpha-toxin and incubated for either 24 or 72 hours. The rectangle includes the range from the 25th to the 75th percentiles, the horizontal line indicates the median, and the vertical line indicates the range from the 10th to the 90th percentiles. *P* values were determined by Wilcoxon’s signed-ranks test.

0.1, and 1.0 ng/mL, concentrations which were lower than those used in previous studies.15-19 Our preliminary study revealed that 1 ng/mL of alpha-toxin does not induce a substantial number of cells to undergo cell death, as determined by trypan blue dye exclusion tests (data not shown). These results suggest that in CRSwNP alpha-toxin induces active production of cytokines rather than their release due to cell lysis.

It seems to be difficult to determine the actual concentration of alpha-toxin in the nose because viable *S. aureus* may consistently produce the toxin. In addition, kits to determine the concentration is not commercially available at present. Since hemolysis of erythrocytes is not generally detected in nasal polyps, we think that the concentration of alpha-toxin in the
mucus adjacent to the polyps is below 200 nM.\textsuperscript{14} 1 ng/ml (approximately 30 pM) of alpha-toxin could induce cytokine production including IL-5, IL-13, IFN-γ and IL-17A by nasal polyp cells, suggesting that alpha-toxin is being produced in sufficient concentrations that would produce a pro-inflammatory effect in the polyp tissue.

In response to alpha-toxin stimulation, nasal polyps cells from CRSwNP produced significantly higher amounts of IL-5, IL-13, IFN-γ, IL-17A, and IL-10 than did uncinate tissue cells from CRSsNP. This result was similar to the previous report by Patou \textit{et al.}, who demonstrated that SEB stimulated significantly higher production of IL-2, IL-4, IL-5, IL-10, IL-13, and IFN-γ in nasal polyps than in inferior turbinates.\textsuperscript{4} Our results suggest that, as is the case with SEB, alpha-toxin induces enhanced immune responses that are associated with nasal polyp formation in CRSwNP.

\textbf{Fig. 2} Comparison of alpha-toxin-induced IL-5 (A), IL-13 (B), IFN-γ (C), IL-17A (D) and IL-10 (E) production between DNPCs from patients with CRSwNP (n = 22) and DUTCs from patients with CRSsNP (n = 9). The rectangle includes the range from the 25th to the 75th percentiles, the horizontal line indicates the median, and the vertical line indicates the range from the 10th to the 90th percentiles. \(P\) values were determined by Mann-Whitney’s U test.
The introduction of a healthy control cohort would bring more clarity into the project, and our preliminary results showed that the dispersed cells from non-inflamed concha bullosa (n = 3) produced less amount of IL-5 (73.3 pg/ml), IL-13 (160.3 pg/ml), IFN-γ (766.7 pg/ml), IL-17A (84.3 pg/ml), and IL-10 (147.7 pg/ml) as compared with DNPCs. Although substantial cases of nasal polyps are grown from uninated tissue, future examination about the comparison of alpha-toxin-induced cytokine production between uninated tissue cells and nasal polyp cells in identical patients with CRSwNP or AERD will provide valuable information for understanding the pathogenesis of this disease such as the mechanisms of nasal polyp formation.

We previously reported that exposure to SEB at a concentration of 1 ng/mL induces IL-5, IL-13, IFN-γ, and IL-17A production in DNPCs.\(^3\) In the present
study, we also demonstrated that exposing DNPCs to SEB induces IL-10 production. This result was in agreement with the previous report by Patou et al., which showed that 500 ng/mL of SEB induces IL-10 production in nasal polyp explants. We found that at 1 ng/mL, both alpha-toxin and SEB induce production of similar amounts of IL-5, IL-13, and IL-10 in DNPCs. Our preliminary experiment showed that simultaneous exposure to alpha-toxin and SEB did not display a synergistic effect on cytokine production by DNPCs as compared with the single exposure. Since the molecular weight of SEB (approximately 27 kDa) is similar to that of alpha-toxin (28-30 kDa), these results suggest that both the superantigenic and non-superantigenic exotoxins of *S. aureus* equally induce Th2 and regulatory immune responses in CRSwNP. However, we found that production of IFN-γ and IL-17A is significantly lower in DNPCs exposed to alpha-toxin than in cells exposed to SEB. Other studies have shown that IFN-γ and IL-17A play roles in the pathogenesis of CRS. For example, IFN-γ is abundantly expressed in CRSsNP as compared with CRSwNP. These results suggest that SEB affects cytokine production more broadly than does alpha-toxin, probable due to its superantigenic profile.

There was a significant negative correlation between the degree of eosinophil infiltration into nasal polyps and alpha-toxin-induced production of IFN-γ, IL-17A, and IL-10 by DNPCs. Studies involving mice have shown that IFN-γ inhibits airway eosinophilia by blocking Th2 cell cytokine production, CD4+ T cell recruitment into airways, and/or by suppressing the function of antigen-presenting cells. In humans, 80% of mild asthmatics that received IFN-γ via inhalation exhibited a reduction in the number of airway eosinophils. IFN-γ causes an opening of tight junctions in CRS which may lead to shedding and drainage of inflammation. The present results consist with the reports, and suggest that IFN-γ has a regulatory effect on eosinophilic inflammation in CRSwNP. In contrast, alpha-toxin-induced production of IL-5 and IL-13 did not correlate with local eosinophilia. This result was similar to those of our previous report, which found no correlation between SEB-induced production of IL-5 and IL-13 and local eosinophilia. However, as shown in Table 1, the levels of IL-5 production showed a tendency to positively correlate with blood eosinophilia (ρ = 0.411, P = 0.060). IL-5 is involved in the differentiation and maturation of eosinophils in the bone marrow, migration to tissue sites, and prevention of eosinophil apoptosis. Our results suggest that alpha-toxin-induced IL-5 production is involved in the pathophysiology of CRSwNP by increasing blood eosinophils.

It is known that IL-10 plays a critical role in controlling eosinophilic airway inflammation. In CRS patients, IL-10 suppresses IL-6 and TNF-α production by upper airway dendritic cells. Additionally, IL-10 suppresses allergen-induced IL-5 and IFN-γ production by DNPCs. In the present study, the negative correlation between the degree of eosinophil infiltration into nasal polyps and alpha-toxin-induced cytokine production was strongest for IL-10. These results suggest that alpha-toxin-induced IL-10 production plays an important role in protecting eosinophilic inflammation in CRSwNP.

It remains unclear why IL-10 production is suppressed in DNPCs derived from asthmatic patients. However, a recent meta-analysis demonstrated the association between IL-10 promoter gene polymorphism and susceptibility to asthma. Another study showed a lower serum concentration of IL-10 in patients with early-onset current asthma. Thus asthmatic patients may genetically show the impairment in IL-10 production even in the upper airway.

Alpha-toxin-induced IL-10 production was positively correlated with FEV1/FVC. This may be due to the inhibitory effect of IL-10 on eosinophil inflammation by suppression of IL-5 and GM-SCF through

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**Table 1** Correlation between pathophysiological characterizations and alpha-toxin-induced cytokine production by DNPCs

<table>
<thead>
<tr>
<th>Nasal polyp eosinophilia (cells/field)</th>
<th>Radiological severity (score)</th>
<th>Blood eosinophilia (cells/µl)</th>
<th>Serum total IgE (IU/ml)</th>
<th>FEV1/FVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-5</td>
<td>ρ 0.146</td>
<td>0.103</td>
<td>0.411</td>
<td>-0.089</td>
</tr>
<tr>
<td></td>
<td>P 0.503</td>
<td>0.646</td>
<td>0.060</td>
<td>0.685</td>
</tr>
<tr>
<td>IL-13</td>
<td>ρ 0.108</td>
<td>0.111</td>
<td>0.278</td>
<td>-0.307</td>
</tr>
<tr>
<td></td>
<td>P 0.621</td>
<td>0.620</td>
<td>0.202</td>
<td>0.160</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>ρ -0.432</td>
<td>-0.097</td>
<td>-0.263</td>
<td>-0.408</td>
</tr>
<tr>
<td></td>
<td>P 0.048</td>
<td>0.644</td>
<td>0.229</td>
<td>0.061</td>
</tr>
<tr>
<td>IL-17A</td>
<td>ρ -0.566</td>
<td>-0.014</td>
<td>-0.372</td>
<td>-0.192</td>
</tr>
<tr>
<td></td>
<td>P 0.009</td>
<td>0.937</td>
<td>0.088</td>
<td>0.377</td>
</tr>
<tr>
<td>IL-10</td>
<td>ρ -0.567</td>
<td>-0.334</td>
<td>-0.295</td>
<td>-0.250</td>
</tr>
<tr>
<td></td>
<td>P 0.009</td>
<td>0.121</td>
<td>0.176</td>
<td>0.252</td>
</tr>
</tbody>
</table>

ρ and P values were determined by Spearman’s rank correlation coefficient.
upregulation of suppressor of cytokine signaling (SOCS)-3. Alternatively, suppression of airway nitric oxide production by IL-10 may be involved the function.

AERD is one of endotypes in CRSwNP. Amount of alpha-toxin (1 ng/ml)-induced IL-5 (883 pg/ml), IL-13 (849 pg/ml), IFN-γ (2,951 pg/ml), IL-17A (627 pg/ml) and IL-10 (200 pg/ml) by DNPC from a patient with AERD was not an outlier of the average +/- standard deviation of IL-5 (752 +/- 640 pg/ml), IL-13 (670 +/- 615 pg/ml), IFN-γ (5,249 +/- 4,583 pg/ml), IL-17A (1,305 +/- 1,312 pg/ml) and IL-10 (905 +/- 871) of 22 DNPCs, respectively, as determined by Grubbs test (P > 0.05). This suggests that the data from a patient with AERD had little effect on skewing the results.

In conclusion, not only superantigenic but also non-superantigenic exotoxins derived from S. aureus modulate local immune reactions and affect the

**Fig. 4** Relationship between the number of eosinophils in nasal polyps and alpha-toxin-induced IL-5 (A), IL-13 (B), IFN-γ (C), IL-17A (D) and IL-10 (E) production by DNPCs (n = 22).
pathophysiology of CRSwNP. It is known that the endoscopic sinus surgery can improve pulmonary function in asthmatic patients. In addition, Proimos et al. showed a clear improvement in the use of bronchodilators, oral steroids, and need for hospitalization for asthma after the surgery in asthmatic CRS patients. The present findings may provide new insight into the role of alpha-toxin in the pathogenesis of eosinophilic airway diseases, including allergic rhinitis and bronchial asthma, and provide a basis for the development of novel therapeutic approaches that target S. aureus in order to limit eosinophilic airway inflammation.
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