

ORIGINAL ARTICLE

Role of fungal antigens in eosinophilia-associated cellular responses in nasal polyps: a comparison with enterotoxin

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Summary

Background Fungi and/or *Staphylococcus aureus* enterotoxins (SEs) may participate in the pathogenesis of eosinophilic inflammation in cases of chronic rhinosinusitis with nasal polyps (CRSwNP).

Objective We sought to determine the effects of fungal antigens on eosinophilia-associated cellular responses in nasal polyps.

Methods Dispersed nasal polyp cells (DNPCs) were prepared from 13 patients with CRSwNP. DNPCs were cultured with fungal extracts (*Aspergillus*, *Alternaria* and *Candida*) or SEB for 72 h, after which the levels of IL-5, IL-13 and RANTES were measured within the supernatant. Responses to β -D-glucan, mannan and chitin were also examined.

Results 38.5%, 69.2% and 30.8% of DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to 200 μ g/mL of *Aspergillus*. 53.8%, 53.8% and 7.7% of DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to 200 μ g/mL of *Alternaria*. 53.8%, 38.5% and 15.4% of DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to 200 μ g/mL of *Candida*. All DNPCs produced these cytokines in response to 0.1 μ g/mL of SEB. SEB induced significantly greater cytokine levels than the fungal extracts. No correlation between cytokine production following exposure to each of the fungal extracts or SEB and various clinical features, including nasal polyp eosinophilia and radiological severity of sinusitis was observed. Neither sensitization to fungus nor comorbidity with bronchial asthma was correlated with the fungal extract-induced cytokine production by DNPCs. β -D-glucan, mannan and chitin did not induce significant cytokine production.

Conclusions These results suggest that, although DNPCs produce IL-5, IL-13 and RANTES in response to fungal extracts, fungal antigens including major carbohydrates are less capable of inducing eosinophilia-associated cellular responses in nasal polyps than SEB.

Keywords cytokine, enterotoxin, eosinophil, fungi, nasal polyps

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Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a major eosinophilic airway disease often associated with asthma and aspirin sensitivity [1]. The aetiology and pathophysiology of nasal polyps remain poorly understood and appears to be multifactorial; however, there is recent evidence to suggest that *Staphylococcus aureus* enterotoxins (SEs) and/or ubiquitous airborne fungi may play a role in the pathogenesis of eosinophilic inflammation [2, 3].

In CRSwNP, SE and SE-producing *S. aureus* are detected at high levels in nasal polyp specimens [4]. Lym-

phocytes in nasal polyps have altered V β -domains that are strongly associated with SE [5]. Sensitization to SE is observed in local tissue samples and serum samples from patients with CRSwNP, especially in asthmatic patients [6]. Patients demonstrating local SE sensitization display severe pathophysiological features, such as marked eosinophilia and high levels of eosinophilic cationic protein (ECP), IL-5, LTC₄/D₄/E₄, LTB₄ and LXA₄, compared with patients without local sensitization [7]. In addition, SEB induces the release of Th2-mediated cytokines in nasal polyps [8]. For example, we have recently demonstrated that dispersed nasal polyp cells (DNPCs) produce comparable amounts of IL-5, IL-13 and RANTES, three

eosinophilia-associated cytokines, in response to SEB [8]. Peripheral blood mononuclear cells (PBMCs) from patients with CRS also display a modest increase in IL-5 mRNA expression after SEB stimulation [9]. *In vivo* we have demonstrated that nasal exposure to SEB augments eosinophilic inflammation, IgE production and Th2-skewed cytokine production [10].

Using a novel collection and culture method, fungi are also highly prevalent in nasal mucus from patients with CRS, as well as healthy subjects [11, 12]. Airborne fungi can induce Th2 responses and eosinophilic inflammation in the airway both *in vivo* and *in vitro* [13–15]. For example, we have demonstrated that nasal exposure to *Aspergillus* without an adjuvant induces specific IgE/IgG1 production and local eosinophilia in mice [13]. *Alternaria* displays potent Th2-like adjuvant effects within airways, possibly by regulating the activity of dendritic cells in mice [14]. Furthermore, fungi and *Alternaria* in particular can directly induce eosinophil activation and degranulation [15]. Although the clinical effectiveness of topical antifungal agents, such as amphotericin B, on CRS is controversial, fungi may participate in the pathogenesis of eosinophilic inflammation in CRS [16–19]. Shin et al. [20] have shown that PBMCs from patients with CRS, but not healthy controls, produce IL-5 and IL-13, as well as IFN- γ , in response to fungal extracts, particularly *Alternaria*. On the contrary, Douglas et al. [9] have demonstrated that extracts of *Aspergillus* and *Alternaria* result in minimal changes in IL-5 and IFN- γ mRNA expression in PBMC. However, little is known about the role of fungal antigens in local eosinophilia-associated cellular responses.

The present study was designed to compare the effect of fungal antigens and SEB on *ex vivo* cellular responses that are closely associated with eosinophilic inflammation in DNPCs. Because DNPCs contain not only constitutive cells such as epithelial cells and fibroblasts but also infiltrating inflammatory cells, such as eosinophils, lymphocytes, mast cells and macrophages, this culture system ensures an intensive interplay between the different cell populations in nasal polyps [8]. In addition, effects of the major fungal carbohydrates contained within the fungal extracts (β -D-glucan, mannan and chitin) on the overall inflammatory responses were examined because these carbohydrates can act as adjuvants to induce eosinophilia-associated cellular responses [13, 21]. We believe that the findings presented here provide a further insight into the pathogenic role of colonizing *Staphylococcal aureus* vs. ubiquitous airborne fungi in CRSwNP.

Methods

Patients

Thirteen Japanese patients (age range: 22–65 years; mean age: 42.8 years; eight men and five women) with CRSwNP

were studied. CRSwNP was defined using the diagnostic criteria of Benninger et al. [22]. All patients were refractory to medical treatment, including macrolide therapy, and thus had endonasal sinus surgery. Three patients were asthmatic, and none was thought to have aspirin sensitivity based on a history of asthma attacks precipitated by NSAIDs. None of the participants received systemic steroids for a period of at least 8 weeks before surgery, and none received pharmacotherapy for sinusitis, such as macrolide antibiotics or intranasal steroids, for a period of at least 3 weeks before surgery. Before surgery, each patient's blood eosinophil count was measured. A radiological assessment of the severity of sinusitis was also performed in each patient according to the Lund–Mackay system [23]. Serum samples were analysed for their IgE specificity to *Aspergillus*, *Alternaria* and *Candida* using the CAP system (Phadia, Uppsala, Sweden). Sensitization to the fungi was defined as positive when the titre for either the three antigens was more than 0.7 kU allergen/L. Informed consent for participation in the study was obtained from each patient, and the study was approved by the Human Research Committee of the Okayama University Graduate School of Medicine and Dentistry.

Antigen and reagents

We purchased the following study materials: SEB (Toxin Technology, Sarasota, FL, USA), β -D-glucan, mannan, chitin, RPMI-1640, L-glutamine–penicillin–streptomycin solution, protease, collagenase, hyaluronidase and DNase I (Sigma, St Louis, MO, USA), as well as diclofenac sodium (Wako Pure Chemicals, Osaka, Japan), FCS (Invitrogen, Carlsbad, CA, USA) and red blood cell lysis buffer (Roche, Indianapolis, IN, USA). The crude extracts of *Aspergillus fumigatus*, *Alternaria alternaria* and *Candida albicans* were provided by Torii Co. (Tokyo, Japan). In brief, stationary liquid cultures in center mold medium from the fungi were left to grow for 1 week at room temperature. After the incubation, the liquid medium was filtered, concentrated and dialysed by ultrafiltration. Afterwards, the extract was subjected to sterilizing filtration and lyophilization. All the antigens were dissolved with phosphate buffered saline (Sigma), and the solubility was confirmed at inspection.

Cell cultures

DNPC were prepared from nasal polyps by enzymatic digestion, as described previously [8]. Briefly, the minced nasal polyps were incubated for 2 h at 37 °C in RPMI 1640 (Sigma) (1 g tissue per 4 mL) containing 2.0 mg/mL protease, 1.5 mg/mL collagenase, 0.75 mg/mL hyaluronidase and 0.05 mg/mL DNase. The cell suspension was then filtered through a 70 μ m cell strainer (BD Falcon, Bedford, MA, USA) to remove any undigested tissue and washed

two times with washing medium (RPMI 1640 supplemented with 2% FCS, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin). The cell pellet was resuspended in erythrocyte lysis buffer and washed with washing medium. After washing, DNPC were suspended in culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin). $8.5 \pm 5.3\%$, $11.7 \pm 8.9\%$, $8.9 \pm 8.2\%$, $8.5 \pm 6.8\%$, $7.8 \pm 11.1\%$, $10.9 \pm 10.5\%$, $15.5 \pm 6.7\%$ and $21.6 \pm 7.7\%$ cells in DNPC express c-kit, ECP/EPX, CD79 α , CD68, CD4, CD8, cytokeratin and vimentin, respectively [8]. In flat-bottomed 48-well culture plates (Asahi Techno Glass, Tokyo, Japan), 500 µL of 1×10^6 /mL DNPC were stimulated with serial concentrations (2, 20 and 200 µg/mL) of *Aspergillus*, *Alternaria*, *Candida* or SEB at 0.001, 0.01 and 0.1 µg/mL in the presence of 10^{-5} M diclofenac at 37 °C in a 5% CO₂/air mixture. Alternatively, cells were stimulated with serial concentrations (1, 10 and 100 µg/mL) of β -D-glucan, mannan or chitin. As a control, DNPC were cultured without antigen stimulation. In order to abrogate the inhibitory effect of intrinsic PGE₂ on cytokine production, DNPC were pretreated with 10^{-5} M diclofenac because we have demonstrated previously that the treatment with diclofenac completely abolished spontaneous PGE₂ production by DNPC [8]. The culture supernatant was collected after 72 h and stored at -80 °C, after which the levels of IL-5, IL-13 and RANTES were determined by ELISA [8]. Our preparatory experiments revealed that fungal antigen-specific cytokine production was increased over 72 h incubation and maximal at 72 h in responders. Viability was assessed by the exclusion of trypan blue stain.

Histological examination

Sections from surgically excised nasal polyps were stained by haematoxylin/eosin solution to detect tissue eosinophilia. Eosinophils were counted at high power (10 $\times$ 40) in a blinded manner in the five fields with the greatest cellular infiltration, after which the average number of positive cells was determined [8].

Statistical analysis

Values are given as means $\pm$ standard errors. Nonparametric Mann-Whitney's *U*-test and Fisher's exact probability test were used for comparing data between groups, and the Wilcoxon's signed-ranks test was used for analysis within the group. If more than two groups were compared, the Kruskal Wallis test were examined before the use of Mann-Whitney's *U*-test. A correlation analysis was performed using Spearman's correlation coefficient by rank. *P*-values <0.05 were considered statistically significant. Statistical analyses were performed with Stat-View software (version 4.5, Abacus Concepts, Berkeley, CA, USA).

Results

Aspergillus extract induced eosinophilia-associated cytokine production by DNPCs

Aspergillus stimulation of DNPCs induced significant IL-5 ($P=0.013$ and $P=0.015$ at 20 and 200 µg/mL, respectively), IL-13 ($P=0.005$ and $P=0.002$ at 20 and 200 µg/mL, respectively) and RANTES ($P=0.041$ at 200 µg/mL) production (Fig. 1). Concentrations more than double the control values were tentatively considered significant, such that five (38.5%), nine (69.2%) and four (30.8%) out of 13 DNPCs demonstrated significant production of IL-5, IL-13 and RANTES, respectively, in response to 200 µg/mL *Aspergillus* (Fig. 2). The dose response to *Aspergillus* extract was relatively flat with peak production at either 20 or 200 µg/mL.

Alternaria extract induced eosinophilia-associated cytokine production by DNPCs

Seven (53.8%), seven (53.8%) and one (7.7%) out of 13 DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to 200 µg/mL of *Alternaria* (Fig. 2). Overall, *Alternaria* stimulation of DNPCs-induced significant IL-5 ($P=0.009$ at 200 µg/mL) and IL-13 ($P=0.003$ at 200 µg/mL) production. However, *Alternaria* did not induce RANTES production at 2 or 20 µg/mL, and inhibited RANTES production at 200 µg/mL ($P=0.019$) (Fig. 1).

Candida extract induced eosinophilia-associated cytokine production by DNPCs

Seven (53.8%), five (38.5%) and two (15.4%) out of 13 DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to *Candida* (Fig. 2). Overall, *Candida* stimulation of DNPCs-induced significant IL-5 ($P=0.023$ at 200 µg/mL) and IL-13 ($P=0.017$ at 200 µg/mL), but not RANTES, production (Fig. 1).

Pathophysiological significance of increased IL-5, IL-13 and RANTES production in nasal polyps in response to fungal extracts

Eosinophils were detected in all of the nasal polyps, ranging from 73 to 487 (mean 202.2) cells per field. The amount of IL-5, IL-13 or RANTES in response to *Aspergillus* did not correlate either with the degree of eosinophilia within nasal polyps or the radiological severity of sinusitis or blood eosinophil counts (Table 1). In addition, the degree of these pathophysiological parameters was similar between patients with or without positive responses to *Aspergillus* (Fig. 3). Similarly, significant correlations were not observed between cytokine productions following exposure to *Alternaria* or *Candida* and the

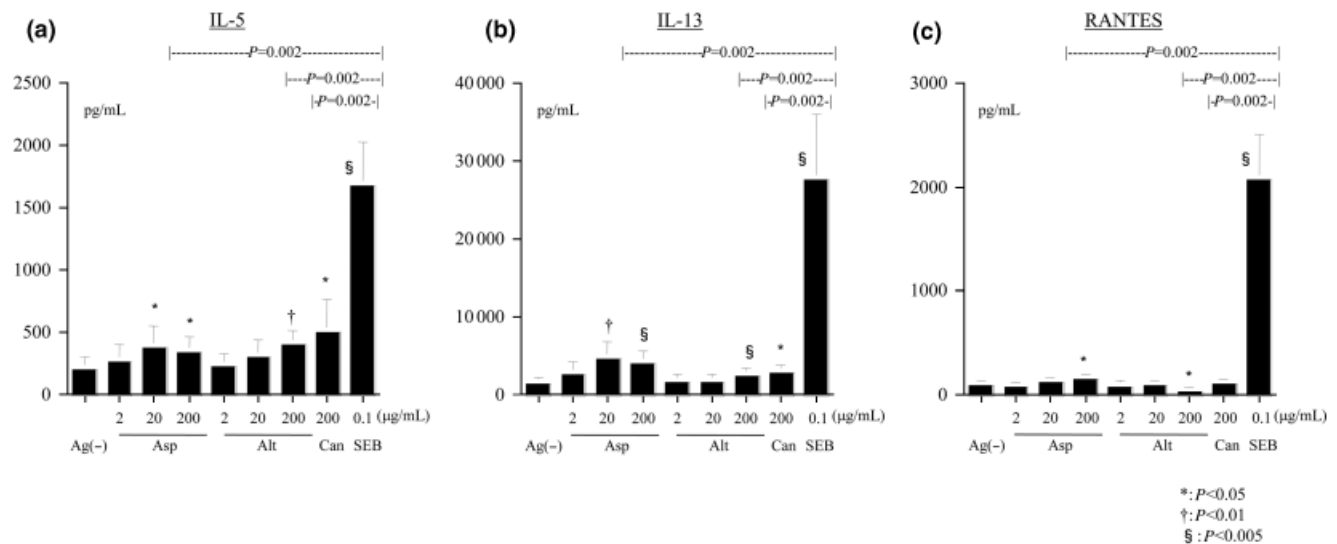


Fig. 1. Effect of fungal extracts on IL-5 (a), IL-13 (b) and RANTES (c) production by dispersed nasal polyp cells (DNPCs). Five hundred microlitres of 1×10^6 /mL DNPCs were stimulated with *Aspergillus*, *Alternaria*, *Candida* or SEB in the presence of diclofenac. The culture supernatant was collected after 72 h, and then levels of the cytokines were determined by ELISA. Results were shown as means $\pm$ standard errors of 13 cell cultures. P -values were determined by the Wilcoxon's signed-ranks test. * $P < 0.05$, † $P < 0.01$, § $P < 0.005$.

clinical characteristics are outlined in Table 1. Patients with positive and negative responses to *Alternaria/Candida* demonstrated similar pathophysiological features (data not shown). Four out of 13 patients (30.8%) showed a sensitization to fungus (*Aspergillus*, *Alternaria* and/or *Candida*). Presence or absence of sensitization to fungus did not affect the cytokine production by DNPC (data not shown). In addition, comorbidity with bronchial asthma did not affect the production (data not shown).

SEB induced eosinophilia-associated cytokine production by DNPCs

DNPCs produced substantial amounts of IL-5, IL-13 and RANTES in response to 0.001 µg/mL of SEB, and the productions were dose dependently increased. Peak productions were archived at 0.1 µg/mL of SEB. All of 13 DNPCs responded to 0.1 µg/mL of SEB to produce significant amounts of IL-5, IL-13 and RANTES. Kruskal-Wallis test ($P = 0.005$ for IL-5, $P = 0.002$ for IL-13 and $P < 0.001$ for RANTES) followed by the Mann-Whitney's U -test revealed that cytokine production was significantly greater in response to SEB compared with the fungal extracts (Figs 1 and 2). Like fungal antigens, responses to SEB did not correlate clinical parameters (data not shown).

Eosinophilia-associated cytokine production by DNPCs in response to carbohydrate on the fungal surface

Thirteen DNPCs were stimulated with serial concentrations (1, 10 and 100 µg/mL) of β -D-glucan, mannan and chitin. None of the carbohydrates induced a significant production of IL-5, IL-13 or RANTES. In fact, concentra-

tions of 1 µg/mL ($P = 0.019$) and 10 µg/mL ($P = 0.041$) of β -D-glucan inhibited RANTES production. Tentatively, taking concentrations more than double controls the levels to indicate significant production, only three (23.1%), two (15.4%) and one (7.7%) of the DNPCs-produced IL-5, IL-13 and RANTES, respectively, in response to β -D-glucan. Seven (53.8%), seven (53.8%) and two (15.4%) of the DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to mannan. Only five (38.5%), two (15.4%) and three (23.1%) of the DNPCs produced IL-5, IL-13 and RANTES, respectively, in response to chitin.

Discussion

In the present study, we examined the effect of fungal antigens and SEB, the major candidate antigens involved in the pathogenesis of CRS, on *ex vivo* cellular responses closely associated with eosinophilic inflammation in DNPCs. Although several groups have investigated the role of fungal antigens and/or SEB in the pathogenesis of CRS using PBMCs, this is the first report comparing the effect of fungal antigens and SEB on local immune responses [9, 20].

Aspergillus, *Alternaria* and *Candida* induced IL-5 and IL-13 production by DNPCs. Both cytokines are involved in eosinophilic inflammation by mediating eosinophil differentiation, recruitment and survival [24]. This result is consistent with a report by Shin et al. [20] showing that PBMCs from patients with CRS, but not healthy controls, produce IL-5 and IL-13 in response to *Alternaria*. In nasal polyps, the majority of IL-5 producing cells are T cells, and mast cells/eosinophils can also express IL-5 [25]. Mononuclear cells and eosinophils in nasal polyps express IL-13

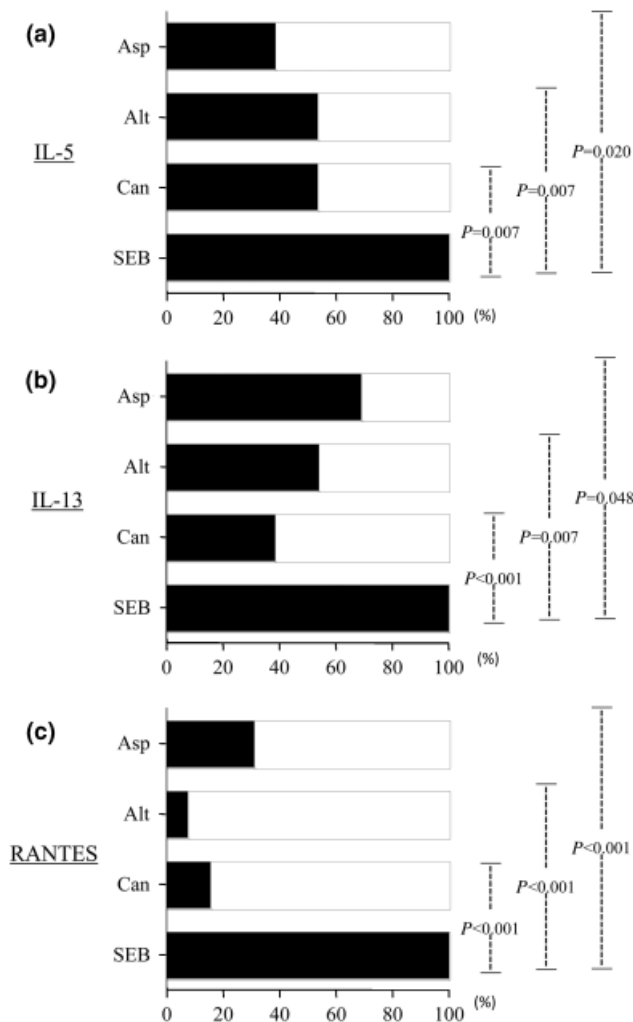


Fig. 2. Rates of positive responses of IL-5 (a), IL-13 (b) and RANTES (c) production to fungal extracts and SEB in DNPCs. Thirteen dispersed nasal polyp cells (DNPCs) were stimulated with 200 µg/mL of *Aspergillus*, 200 µg/mL of *Alternaria*, 200 µg/mL of *Candida* and 0.1 µg/mL of SEB. The response was considered as positive when the amount of cytokines produced by the antigens was over the twice of control. Black bars represent as a rate of positive response. Open bars represent as a rate of negative response. *P*-value was obtained through the use of Fisher's exact probability test.

[26]. Thus, fungal antigens may stimulate these inflammatory cells in nasal polyps directly and/or indirectly to produce IL-5 and IL-13.

On the other hand, *Aspergillus*, but not *Alternaria* or *Candida*, was observed to induce RANTES production by DNPCs. RANTES has also been associated with tissue eosinophilia in CRS [27]. In nasal polyps, RANTES is primarily detected in inflammatory cells and the epithelium [27]. *Aspergillus* contains both pan-allergens, including superoxide dismutase (Asp f6), and *Aspergillus*-specific allergens [28]. This suggests that molecules unique to *Aspergillus*, such as mitogillin (Asp f1), may participate in RANTES production by DNPCs [29].

As we demonstrated previously, DNPCs produced significant amounts of IL-5, IL-13 and RANTES in response to SEB [8]. In the present study, we compared cytokine production by DNPCs following exposure to fungal antigens and SEB. Fungal extracts at a concentration of 200 µg/mL produced significantly less IL-5, IL-13 and RANTES, than SEB at 0.1 µg/mL. This result is consistent with the results of a report by Douglas demonstrating that, in PBMCs from patients with CRS, stimulation with the fungal extracts *Aspergillus* and *Alternaria* at 100 PNU induced less expression of IL-5 mRNA than stimulation with SEB at a concentration of 0.1 µg/mL [9].

In addition, when concentrations more than double the control values were tentatively considered significant, a significant increase in cytokine production following exposure to each of the fungal extracts occurred significantly less frequently than following exposure to SEB, whereas SEB-induced cytokine production in 100% of DNPCs. These results suggest that fungal extracts are less potent at inducing eosinophilia-associated cytokine production by DNPCs compared with SEB. The major reason why we set double the control values as a cut-off point is that the spontaneous production of cytokines was observed in most diclofenac-treated DNPCs. In fact, DNPCs spontaneously produced 65.0 ± 39.3 (1–514) pg/mL, 398.8 ± 216.6 (0–2930) pg/mL and 58.3 ± 11.5 (0–122) pg/mL of IL-5, IL-13 and RANTES, respectively, without antigen stimulation. This criterion might be severe, and values just below this cut-off point are considered negative. However, we may overestimate the results with no biological significance when we just analyse the observed values.

There are several explanations why responses to fungi might be lower than SEB. The responses to fungal extracts are likely to be antigen-specific responses, whereas responses to SEB represent superantigen responses. Although antigen-specific T lymphocytes are expected to be enriched in nasal polyps, they are still likely to be at a lower frequency than the frequency of T cells with Vβ elements responding to SEB. It is known that there is a skewing towards Vβ elements that recognize staphylococcal superantigens in nasal polyp tissue [5]. It has recently been demonstrated that ICAM-1 responds to SEB in DNPCs to induce cytokine production, while the interaction between HLA-DR and SEB in DNPCs has a limited effect on cytokine production [8]. It is well known that ICAM-1 is widely expressed in nasal polyps, including epithelial cells [30]. ICAM-1 can provide costimulation for SEB-induced cellular responses [31]. Recently, we demonstrated that intrinsic PGE2 inhibits SEB-induced cytokine production via the EP2-mediated pathway in nasal polyps [8]. In addition, we demonstrated that the treatment of diclofenac completely abolished the production of PGE2 by DNPC [8]. Thus, treatment with diclofenac negated the inhibitory role of PGE2, resulting in more cytokine

Table 1. Correlation of fungal extracts-induced cytokine productions and pathophysiological characterizations including nasal polyp eosinophilia, radiological severity of sinusitis, and blood eosinophil count

		Eosinophilia into nasal polyps	Radiological severity	Blood eosinophil count
<i>Aspergillus</i>				
IL-5	Correlation coefficient (ρ)	-0.087	0.201	0.106
	<i>P</i> -value	0.760	0.502	0.717
IL-13	Correlation coefficient (ρ)	-0.549	0.504	0.143
	<i>P</i> -value	0.057	0.083	0.621
RANTES	Correlation coefficient (ρ)	-0.144	0.187	0.323
	<i>P</i> -value	0.614	0.533	0.265
IL-5	Correlation coefficient (ρ)	-0.011	-0.364	0.429
	<i>P</i> -value	0.970	0.196	0.138
<i>Alternaria</i>				
IL-13	correlation coefficient (ρ)	-0.473	0.202	0.264
	<i>P</i> -value	0.102	0.496	0.361
RANTES	correlation coefficient (ρ)	0.302	-0.188	-0.038
	<i>P</i> -value	0.295	0.496	0.894
<i>Candida</i>				
IL-5	Correlation coefficient (ρ)	0.005	0.087	0.044
	<i>P</i> -value	0.985	0.781	0.879
IL-13	Correlation coefficient (ρ)	-0.247	0.188	0.154
	<i>P</i> -value	0.379	0.537	0.605
RANTES	Correlation coefficient (ρ)	0.246	-0.214	0.221
	<i>P</i> -value	0.396	0.437	0.446

Correlation coefficient (upper column) and *P*-value (lower column) were determined by Spearman's correlation coefficient by rank.

production. One more possible reason for lower responses to fungal extracts than SEB is that the relevant antigens in the fungal extracts may be a less concentrated stimulus than purified SEB.

No significant correlations were detected with regard to the amount of IL-5, IL-13 or RANTES produced following exposure to fungal extracts and various pathophysiological features, including nasal polyp eosinophilia, peripheral blood eosinophilia or radiological severity of sinusitis. Moreover, patients in whom fungal extracts induced cytokine responses showed similar pathophysiological features compared with those with negative reactions. These results are consistent with recent clinical reports demonstrating that topical antifungal therapy has little effect on the pathophysiology of CRS [18, 19]. Together with the finding that fungi are detected in nasal mucus from almost all patients with CRS, as well as healthy subjects, these results suggest that fungi may not play as broad a role in the pathogenesis of CRS as suggested previously. However, our findings do not exclude the possibility that fungi may impact on the pathogenesis of particular subtypes of CRS, including allergic fungal rhinosinusitis in which fungi are known to cause type I and III allergic reactions, for which antifungal treatments may be more effective [32].

It is well known that fungal extracts contain abundant carbohydrate, including β -D-glucan, mannan and chitin [33]. Carbohydrates on the surface of fungi can participate in immune responses [12, 33–35]. For example, several

carbohydrate moieties from the *Aspergillus* extract react with IgE or IgG. [33, 34]. They can bind to carbohydrate-specific receptors, including the mannose receptor and CR3 on antigen-presenting cells, resulting in efficient antigen presentation [35]. In addition, we have demonstrated that carbohydrates on *A. fumigatus* act as internal adjuvants leading to Th2 allergic inflammation, including nasal eosinophilia, in a murine model of allergic rhinitis [13]. However, none of the carbohydrates tested (β -D-glucan, mannan or chitin) induced significant production of IL-5, IL-13 or RANTES in DNPCs. These results suggest that, although fungal carbohydrates can participate in immune responses, they are not major triggers of eosinophilia-associated cytokine production in nasal polyps.

The present study has several limitations. First, the results presented in this study may not be observed in nonrefractory nasal polyps because all samples were taken from patients refractory to standard medical treatment. Second, although we have reported that IL-5, IL-13 and RANTES were produced by DNPCs in response to SEB even when diclofenac was not added [8], we do not examine whether IL-5, IL-13 and RANTES are produced in response to fungal antigens in the absence of diclofenac. The addition of diclofenac influences the results in such a way that results may not be extrapolated to an *in vivo* situation. Third, there were no healthy controls in this study. However, we cannot excise normal sinonasal tissues from healthy individuals because of ethical reasons. Fourth, only one concentration (200 μ g/mL) of *Candida*

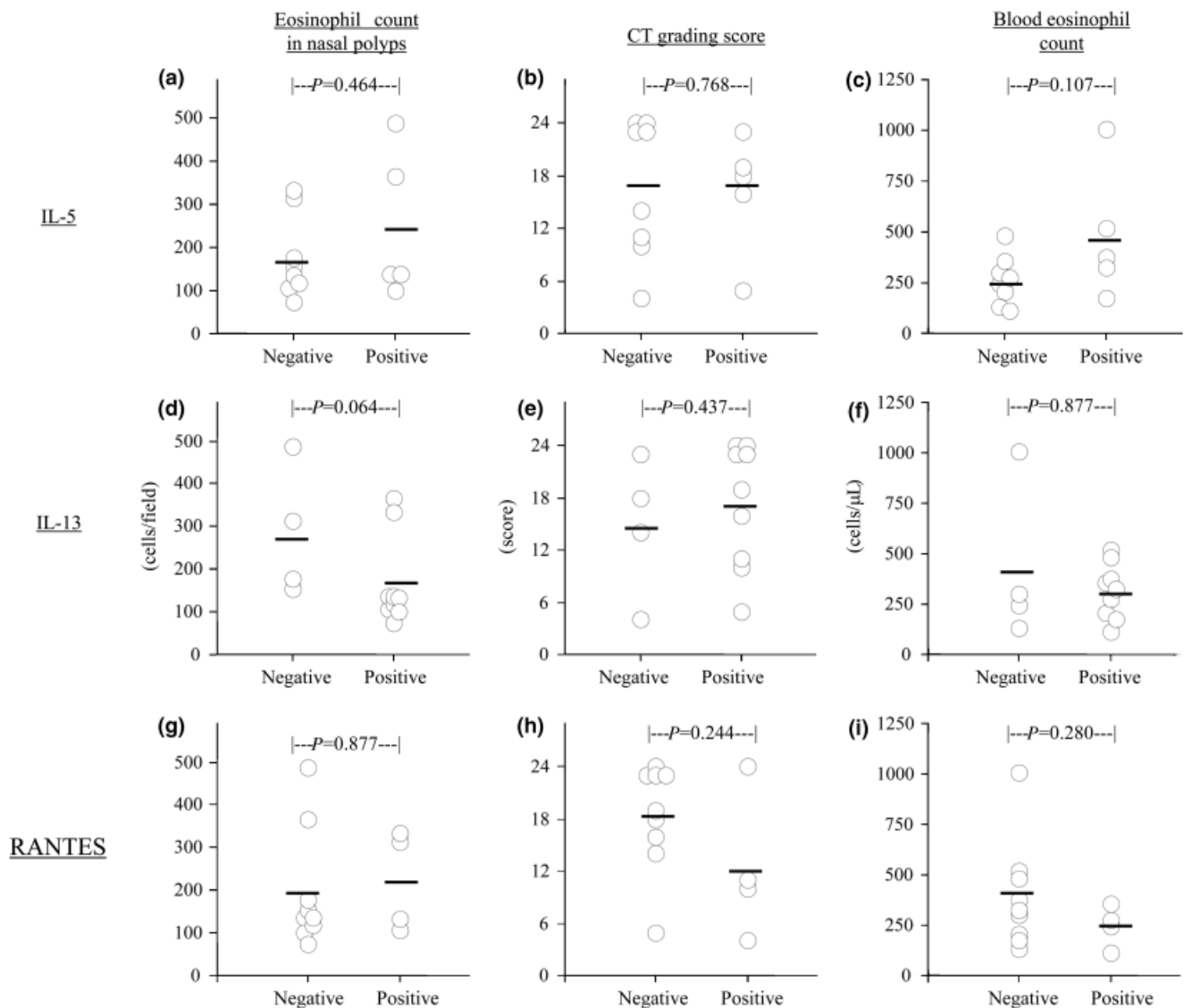


Fig. 3. Comparison of nasal polyp eosinophilia (a, d, g), radiological severity of sinusitis (b, e, h) and blood eosinophil count (c, f, i) between patients with positive and negative response of IL-5 (a–c), IL-13 (d–f) and RANTES (g–i) production in response to 200 μ g/mL of *Aspergillus* in dispersed nasal polyp cells. Bars represent as a mean. *P*-value was obtained through the use of Mann–Whitney's *U*-test.

was tested. Because little is known whether *Candida* plays a role in the cellular responses in CRSwNP as compared with *Aspergillus* and *Alternaria*, we firstly set *Candida* as a control for other fungi. Surprisingly, *Candida* evoked IL-5 and IL-13 production similar to *Aspergillus* and *Alternaria*.

In conclusion, we present here *ex vivo* functional evidence that, although DNPCs can respond to fungal extracts resulting in the production of eosinophilia-associated cytokines especially IL-5 and IL-13, these responses are significantly less than those induced by SEB. These observations suggest a role for SEB in promoting eosinophilic infiltration and may provide a basis for therapeutic approaches for targeting microorganisms in the management of CRSwNP.

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