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Original article

IL-22/IL-22R1 signaling regulates the pathophysiology of chronic rhinosinusitis with nasal polyps via alteration of MUC1 expression

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AIA, aspirin-intolerant asthma; AT, alpha-toxins; ATA, aspirin-tolerant asthma; CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps; DNPCs, dispersed nasal polyp cells; DUTCs, dispersed uncinate tissue cells; NP, nasal polyps; SEB, staphylococcal enterotoxin B; UT, uncinate tissues

ABSTRACT

Background: IL-22 is an IL-10-family cytokine that regulates chronic inflammation. We investigated the role of IL-22 and its receptor, IL-22R1, in the pathophysiology of chronic rhinosinusitis with nasal polyps (CRSwNP).
Methods: IL-22 and IL-22R1 protein and mRNA expression in NP and in uncinate tissues (UT) from CRS and non-CRS patients was examined using immunohistochemistry and real-time PCR, respectively. Dispersed NP and UT cells were cultured with the *Staphylococcus aureus* exotoxins, staphylococcal enterotoxin B and alpha-toxin, following which exotoxin-induced IL-22 levels and their association with clinicopathological factors were analyzed. Effects of IL-22 on MUC1 expression and cytokine release in NP cells were also determined.
Results: IL-22 and IL-22R1 in NP were mainly expressed in infiltrating inflammatory cells and in epithelial cells, respectively. IL-22 mRNA levels in NP were significantly higher than those in UTs from non-CRS patients whereas IL-22R1 levels were conversely lower in NPs. NP cells produced substantial amounts of IL-22 in response to exotoxins. Exotoxin-induced IL-22 production by NP cells significantly and negatively correlated with the degree of local eosinophilia and postoperative computed tomography (CT) score, whereas conversely it positively correlated with the forced expiratory volume in 1s (FEV_1)/forced vital capacity (FVC) ratio. IL-22 significantly enhanced MUC1 mRNA expression in NP cells. IL-22-induced MUC1 mRNA levels were significantly and positively correlated with IL-22R1 mRNA levels in NPs.
Conclusions: These data suggest that imbalance of IL-22/IL-22R1 signaling regulates the pathogenesis of CRSwNP, including local eosinophilia, via alteration of MUC1 expression.
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Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by mucosal inflammation and remodeling. The condition is often associated with asthma and substantially impairs quality of life due to longstanding symptoms including nasal congestion, headache, and loss of smell.[1,2] While the precise pathogenesis underlying this disease remains poorly understood, imbalances in expression of local cytokines including IL-5 and TGF-β appear to be involved.[3]

IL-22 is an IL-10-family cytokine produced by a variety of cells that include not only $CD4^+$ T cells of the Th0, Th17 and Th22 linage but also innate immune cells such as NK cells and type 3 innate

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lymphoid cells.[4,5] IL-22 has versatile effects on airway inflammation via binding to the IL-22 receptor, which consists of IL-22R1 and IL-10R2.[6] For example, IL-22 promotes the migration of airway smooth muscle cells and, in skin, IL-22 and TNF-α synergistically promote the production of chemokines including eotaxin-1 and eotaxin-2 by keratinocytes.[7,8] In contrast, IL-22 attenuates IL-25 production by airway epithelial cells and inhibits antigen-induced eosinophilic airway inflammation.[9] IL-22 also suppresses IFN-γ-induced expression of MHC class I, MHC class II, ICAM-1, RANTES and IP-10 in bronchial epithelial cells from asthmatic patients.[10] Gene delivery of IL-22 suppressed antigen-induced immune responses and eosinophilic airway inflammation via an IL-10-associated mechanism in a murine model of asthma.[11] In the intestine, IL-22 enhanced the expression of mucin genes including MUC1, which is known to display a regulatory role in mucosal immunity.[12,13] In addition, recent reports showed a dual role of IL-22 in airway inflammation in the mouse.[14,15] Thus, IL-22 is required for the onset of allergic inflammation but functions as a negative regulator of established allergic inflammation.[14] The pro-inflammatory and tissue-protective roles of IL-22 in bleomycin-induced airway inflammation are dependent on the presence or absence of IL-17A, respectively.[15]

To date, only a few reports have demonstrated an association between IL-22 and the severity of CRSwNP.[16,17] Ramanathan *et al.* reported that local mRNA expression of IL-22R1 but not of IL-22 was significantly lower in treatment-recalcitrant CRSwNP compared to responsive CRSwNP, suggesting that refractoriness of CRSwNP is associated with decreased expression of mucosal IL-22R1.[16] Endam *et al.* showed an association between three single nucleotide polymorphisms in IL-22R1 and severe CRS.[17] However, it remains unclear how IL-22 regulates the pathogenesis of this condition.

In the present study, we investigated the local production of IL-22 in NP using a recently developed *ex vivo* system.[18] Local expression of IL-22 and IL-22R1 was compared among various CRS phenotypes, and the role of IL-22/IL-22R1 signaling in the pathogenesis of CRS is discussed. The present findings provide novel insights into the pathogenesis of chronic eosinophilic airway diseases regulated by IL-22, in addition to providing a basis for the regulatory effect of IL-22 in airways via induction of MUC1 expression.

Methods

Patients

Sixty-six Japanese patients with CRS were enrolled for the quantification of IL-22 and IL-22R1 mRNA in sinonasal mucosa. Briefly, 53 of the 66 CRS patients exhibited NP (CRSwNP). The remainder of the CRS patients demonstrated no visible NP in the middle meatus (CRSsNP: $n = 13$). The diagnosis of CRSsNP and CRSwNP was defined using the criteria reported in a European position paper on rhinosinusitis and nasal polyps.[19] In order to eliminate the effect of macrolides and corticosteroids on the expression of IL-22 and IL-22R1, patients were excluded when they received systemic corticosteroids for at least eight weeks prior to surgery or they received pharmacotherapy for rhinosinusitis, such as macrolide antibiotics or intranasal glucocorticoids for at least three weeks prior to surgery. Thirty-seven patients were asthmatic and had NPs. Of these, 16 patients were considered to exhibit aspirin sensitivity based on their history of asthma attacks precipitated by non-steroidal anti-inflammatory drugs (aspirin-intolerant asthma: AIA). The remainder of the asthmatic patients were diagnosed as aspirin-tolerant asthma (ATA, $n = 21$). During surgery, the NP and uncinate process tissues (UT) were sampled from patients with CRSwNP and CRSsNP, respectively. In addition, 19 non-CRS patients (e.g. blowout fracture or sphenoidal cyst) with normal UT at inspection were enrolled as a control. The clinical characteristics of the patients are presented in Table 1. All patients provided informed consent prior to their participation, and the study was pre-approved by the Human Research Committee of the Okayama University Graduate School of Medicine and Dentistry.

Quantification of IL-22, IL-22R1 and MUC1 mRNA in sinonasal mucosa

Surgically excised NP and UT tissues were soaked in RNAlater™ RNA stabilization reagent (Qiagen, Hilden, Germany) and were stored at −30 °C until use. Extraction of total cellular RNA, reverse transcription to generate cDNA, and real-time quantitative PCR for IL-22 and IL-22R1 were then performed, as described previously.[18] Primers for analysis of GAPDH levels, which were used as an internal control, were purchased from Toyobo (Osaka, Japan). The absolute copy number was calculated for each sample, and samples are reported as copy numbers relative to GAPDH. The sequences and product size of the primers used for PCR were as follows: IL-22, forward 5′-GCTGCCTCCTTCTCTTGG-3′ and reverse 5′-GTGCGGTTGGTGATATAGG-3′ (112 bp); IL-22R1, forward 5′-TCTGCTCCAGCACGTGAAAT-3′ and reverse 5′-GTCCCTCTCTCCGTACGTCT-3′ (124 bp); MUC1, forward 5′-TTTCCAGCCCGGGATACCTA-3′ and reverse 5′-AGAGGCTGCTGCCACCATTA-3′ (PCR product size; 136 bp).

Immunohistochemistry

Immunohistochemical staining for IL-22 and IL-22R1 was performed according to a previously described protocol.[20] Briefly, 4-μm sections were collected from paraffin-embedded tissue blocks, deparaffinized and rehydrated. The sections were heated in sodium citrate buffer (pH 6.5) in a microwave oven for antigen retrieval and were incubated with primary antibodies including

Table 1
Subjects' characteristics.

Groups	Non-CRS	CRSsNP	CRSwNP without asthma	CRSwNP with ATA	CRSwNP with AIA
Number	19	13	16	21	16
Sex (male/Female)	6/13	8/5	11/5	15/6	9/7
Age (y)	54.7 ± 19.0	44.0 ± 17.7	41.1 ± 19.6	50.4 ± 16.7	52.3 ± 17.9
Age range (y)	14–81	14–74	13–65	21–77	21–79
Serum IgE (IU/mL)	204 ± 415	283 ± 291	243 ± 414	701 ± 1047	249 ± 252
Blood eosinophil count ($\times 10^2$/μL)	1.56 ± 1.24	2.94 ± 2.06	3.40 ± 2.52	7.17 ± 4.68	5.25 ± 3.19
CT grading score (Lund–Mackay)	0	5.8 ± 3.9	11.7 ± 6.7	18.0 ± 5.4	15.6 ± 6.7
FEV_1/FVC ratio (%)	79.9 ± 8.9	81.3 ± 5.3	80.4 ± 8.5	71.3 ± 12.9	64.0 ± 12.0

ATA, aspirin-tolerant asthma; AIA, aspirin-intolerant asthma; CT, computed tomography; FEV1, forced expiratory volume in one second per forced vital capacity ratio. Results were shown as a mean ± standard deviation.

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1:200 diluted rabbit anti-human IL-22 polyclonal antibodies (Gen Tex, Taipei, Taiwan), 1:200 diluted rabbit anti-human IL-22R1 polyclonal antibodies (Life Span Biosciences, Seattle, WA) or control serum (Universal Negative Control, Dako Japan, Tokyo, Japan) at 4 °C overnight. The avidin-biotin-immunoperoxidase system (VECTASTAIN Elite™, Vector Laboratories, Burlingame, CA, USA) was used according to the manufacturer's instructions to detect antibody–antigen interactions. The nuclei of the sections were then stained with hematoxylin and the sections were examined under a light microscope. A double immunostaining using the anti-IL-22 antibodies with anti-CD4 mAb (4B12), anti-CD68 (KP1), anti-ECP/EGX (EG2) or control Ab was performed to determine whether the IL-22 positive cells were $CD4^+$ T cells, macrophage or eosinophils in nasal polyps.[20]

Culture of dispersed nasal polyp cells and uncinate tissue cells

Dispersed nasal polyp cells (DNPCs) and uncinate tissue cells (DUTCs) were prepared from 22 patients with CRSwNP and 8 patients with CRSsNP, respectively, as described previously.[21] Because the difference in the numbers and characteristics of subjects in each experiment might have affected the results, the clinical characteristics of the patients enrolled in the experiments of cell culture are presented in Table 2. Nine and one out of 22

Table 2
Subjects' characteristics enrolled in the experiments of cell culture.

Groups	CRSsNP	CRSwNP without asthma	CRSwNP with ATA	CRSwNP with AIA
Number	8	12	9	1
Sex (male/Female)	5/3	12/0	7/2	1/0
Age (y)	52.5 ± 14.9	54.8 ± 13.8	63.7 ± 3.9	42
Age range (y)	32–73	26–71	57–69	42
Serum IgE (IU/mL)	257 ± 473	372 ± 658	299 ± 257	62
Blood eosinophil count (×102/μL)	3.10 ± 2.95	2.72 ± 1.33	5.26 ± 3.17	8.83
CT grading score (Lund–Mackay)	5.5 ± 3.5	15.0 ± 5.5	16.9 ± 4.6	24
FEV1/FVC ratio (%)	76.4 ± 13.9	74.1 ± 9.0	64.0 ± 13.1	65.5

ATA, aspirin-tolerant asthma; AIA, aspirin-intolerant asthma; CT, computed tomography; FEV1, forced expiratory volume in one second per forced vital capacity ratio. Results were shown as a mean ± standard deviation.

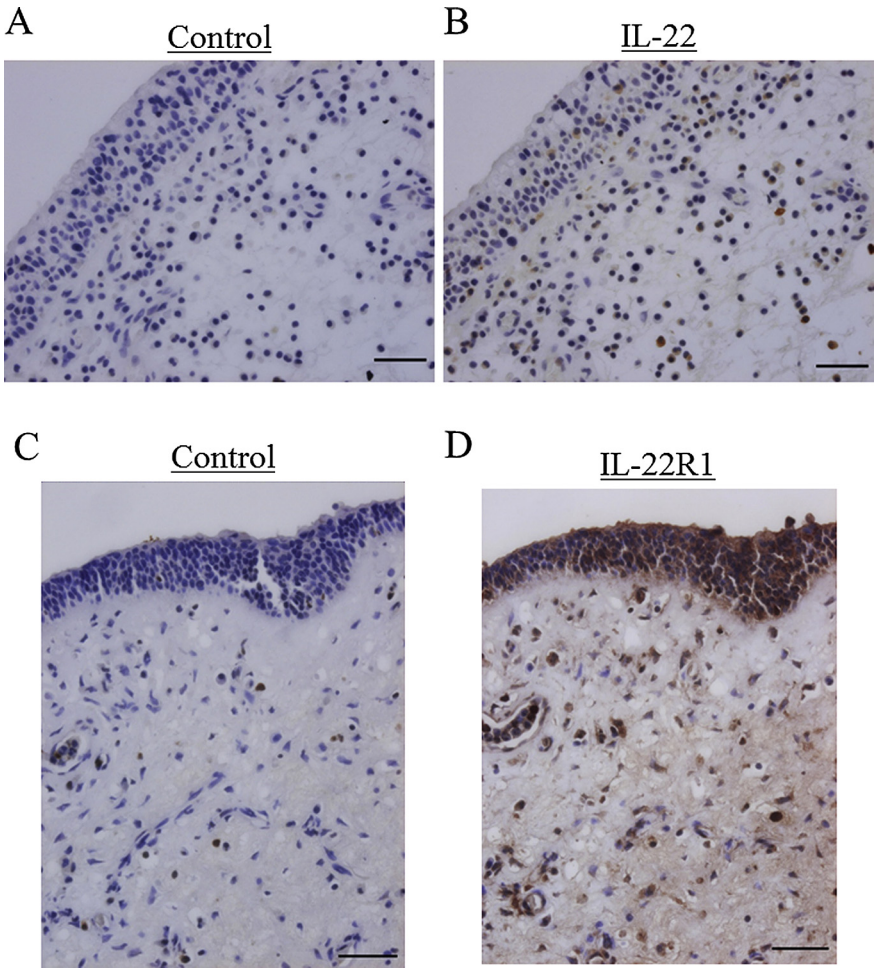

Fig. 1. Immunohistochemical staining of IL-22 (**A**, **B**) and IL-22R1 (**C**, **D**) in NPs. Sections were reacted with rabbit polyclonal antibody against IL-22 (**B**), IL-22R1 (**D**) or control (Universal Negative Control (Rabbit): **A**, **C**) after which they were stained using the avidin-biotin-immunoperoxidase system. Scale bar = 50 μm.

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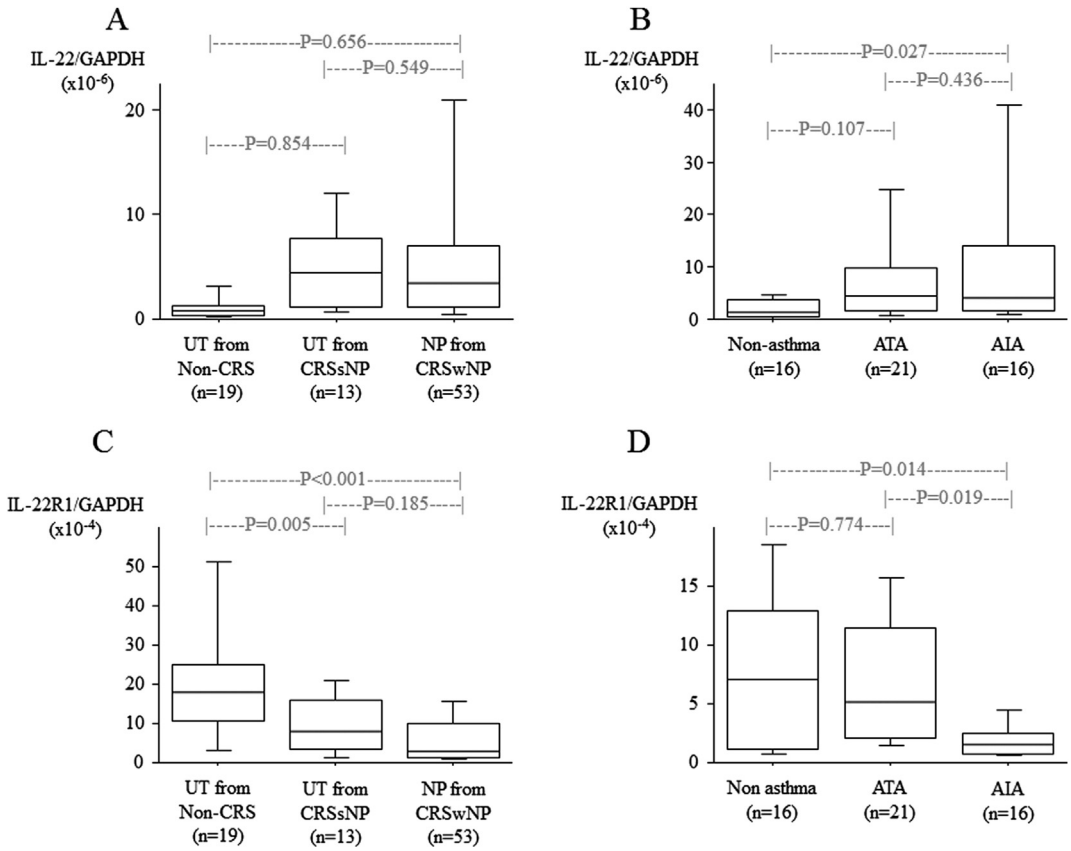

Fig. 2. Relative amounts of IL-22 (**A, B**) and IL-22R1 (**C, D**) mRNA in UTs and NPs. The rectangle includes the range from the 25th to 75th percentiles; the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. *P* values were determined by the Mann–Whitney's U-test.

patients with CRSwNP were complicated with ATA and AIA, respectively. Significant difference was seen between asthmatic (n = 10) and non-asthmatic (n = 12) patients in FEV_1/FVC ratio (P = 0.030 by Mann–Whitney U test) but not CT grading score (P = 0.275). DNPCs or DUTCs (1×10^6 cells per mL) were stimulated with 1 ng/mL of staphylococcal enterotoxin B (SEB; Toxin Technology, Sarasota, FL, USA) or alpha-toxin (AT; Sigma, St. Louis, MO, USA) and were incubated at 37 °C in a 5% CO_2 atmosphere. Alternatively, anti-human IL-22 Ab or control goat IgG (R&D systems, Minneapolis, MN, USA) at a concentration of 10 μg/mL was added into the culture of 10 different samples before SEB or AT stimulation in order to determine whether IL-22 affects exotoxin-induced IL-5 and IL-13 production. An aliquot of the culture supernatant was collected after 72 h and stored at −80 °C for subsequent cytokine analysis. The total serum IgE levels, blood eosinophil count and forced expiratory volume in 1s (FEV_1)/forced vital capacity (FVC) ratio was examined for each patient with CRSwNP before surgery. Computed tomography (CT) examination was performed to evaluate the radiological severity of sinusitis, and the severity was graded according to the Lund–Mackay system for each patient.[19] Sections from NP were stained with hematoxylin/eosin solution, and the average number of eosinophils per high power field (5 fields at 400× magnification) was then determined. Additionally, CT examinations were conducted six months after surgery for 20 of the 22 patients.

Effect of IL-22 on MUC1 expression and cytokine release by DNPCs

DNPCs of each of 18 patients were stimulated with 20 ng/ml human recombinant IL-22 (R&D Systems) for 72 h. Cells were then collected and the level of MUC1 mRNA was determined by real-time quantitative PCR as described above. The level of IL-22R1 mRNA in the respective NPs was also determined. Culture supernatants were collected from 9 samples and the concentration of TARC, RANTES, eotaxin, IL-8, IL-18, IL-25 and IL-33 in the supernatants was determined.

Cytokine measurement

The levels of IL-5, IL-13, TARC, RANTES, eotaxin, IL-8, IL-18, IL-25, IL-33, and IL-22 were determined using ELISA.[18] The levels of IL-5, IL-8 and eotaxin were measured using Opt™ EIA sets (BD Biosciences), according to the manufacturer's instructions. The levels of TARC, RANTES, IL-22 and IL-33 were measured using a DuoSet™ ELISA development kit (R&D Systems). IL-25 levels were measured using a kit from KOMA BIOTEC (Seoul, Korea). IL-13 levels were measured using paired capture and detection antibodies (BD Biosciences) and recombinant standards (R&D Systems). IL-18 concentrations were determined using anti-human IL-18 mAb (I25-2H, MBL, Nagoya, Japan), biotinylated anti-human IL-18 mAb (159-12B, MBL) and recombinant human IL-18 (MBL) as the capture antibody, detection antibody and standard, respectively. The detection limit was 4 pg/mL for IL-5, 2 pg/mL for IL-13, 4 pg/ml for TARC, 2 pg/ml for RANTES, 4 pg/ml for eotaxin, 8 pg/ml for IL-8, 2 pg/ml for IL-18, 4 pg/ml for IL-25, 8 pg/ml for IL-33, and 8 pg/mL for IL-22.

Statistical analysis

Values are given as the median value except for the values in Table 1, 2. 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. A Kruskal–Wallis test followed by a Dunn test was used for multiple comparisons. Correlation analyses were performed using Spearman's rank correlation. *P*-values less than 0.05 were considered statistically significant. Statistical analyses were performed with SPSS software (version 11.0, Chicago, IL, USA).

Results

Local expression of IL-22/IL-22R1 in nasal polyps

The expression and distribution of IL-22 and IL-22R1 in the NP of CRSwNP patients were immunohistochemically examined. Representative staining showed that the IL-22 protein was mainly expressed in infiltrating inflammatory cells ($n = 25$, Fig. 1A, B). In these cells, double immunohistochemical staining showed that IL-22 was expressed in $CD4^+$ cells and ECP/EPX^+ cell but not $CD68^+$ cells (data not shown). In contrast, the IL-22R1 protein was mainly expressed in epithelial cells and in some infiltrating cells (Fig. 1C, D).

Comparison of IL-22/IL-22R1 mRNA levels in nasal polyps and uncinated tissues

IL-22 and IL-22R1 mRNA levels in the NP of CRSwNP patients were compared with those in the UT of CRSsNP and non-CRS patients using real time quantitative-PCR. A Kruskal–Wallis test showed that the amounts of both IL-22 ($P = 0.002$) and IL-22R1 ($P < 0.001$) mRNA were significantly different among three groups including UT of non-CRS patients, UT of CRSsNP patients and NP of CRSwNP patients (Fig. 2A, C). A Dunn test further revealed that the amounts of IL-22R1 mRNA were significantly lower in UT of CRSsNP patients ($P = 0.005$) and NP of CRSwNP patients ($P < 0.001$) as compared with UT of non-CRS patients.

A Kruskal–Wallis test further showed that the amounts of both IL-22 ($P = 0.012$) and IL-22R1 ($P = 0.009$) mRNA were significantly different among three subgroups of NP (non-asthmatic patients, ATA patients and AIA patients) (Fig. 2B, D). A Dunn test further revealed that the amounts of IL-22 mRNA were significantly higher in NP from AIA patients as compared with NP from non-asthmatic patients ($P = 0.027$). Conversely, the amounts of IL-22R1 mRNA were significantly lower in NP from AIA patients as compared with NP from non-asthmatic patients ($P = 0.014$) and ATA patients ($P = 0.019$).

As a whole of sinonasal tissues ($n = 85$), a weak albeit significant negative correlation was found between IL-22 and IL-22R1 mRNA levels ($r = -0.223$, $P = 0.041$, Fig. 3). However, none of each group showed a significant correlation (UT of non-CRS patients: $r = 0.134$, $P = 0.569$; UT of CRSsNP patients: $r = -0.296$, $P = 0.304$; NP of non-asthmatic patients: $r = 0.313$, $P = 0.227$; NP of ATA patients: $r = -0.395$, $P = 0.077$; NP of AIA patients: $r = 0.032$, $P = 0.900$).

Production of IL-22 by nasal polyp and uncinated tissue cells in response to staphylococcal enterotoxin B and alpha-toxin

DNPCs and DUTCs from 22 to 8 patients respectively were stimulated with or without SEB or AT (1 ng/ml) for 72 h. A Kruskal–Wallis test showed that the amounts of IL-22 were significantly different among 3 groups stimulated with or without exotoxins in DNPCs ($P < 0.001$) but not in DUTCs ($P = 0.090$). A Dunn test further revealed that a significant production of IL-22 was seen in DNPCs in response to SEB ($P < 0.001$) and AT ($P < 0.001$). SEB- and AT-induced IL-22 production by DNPCs was significantly higher than that by DUTCs (SEB, $P = 0.013$; AT, $P = 0.037$) (Fig. 4A). In addition, IL-22 production by DNPCs in response to SEB was significantly and positively correlated with that induced in response to AT ($r = 0.927$, $P < 0.001$, Fig. 4B).

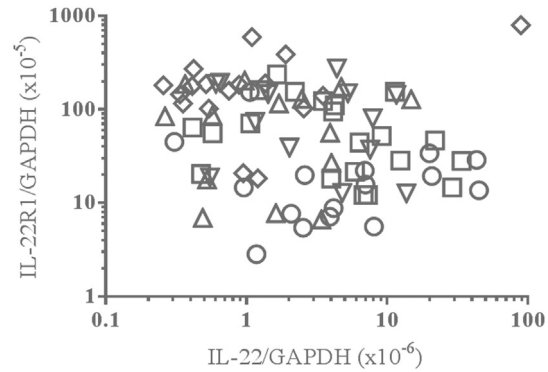

Fig. 3. Correlation between the relative amounts of IL-22 and IL-22R1 mRNA in sinonasal tissues from the following: ◇, UT of non-CRS patients; ▽, UT of CRSsNP patients; △, NP of non-asthmatic patients; □, NP of ATA patients; ○, NP of AIA patients.

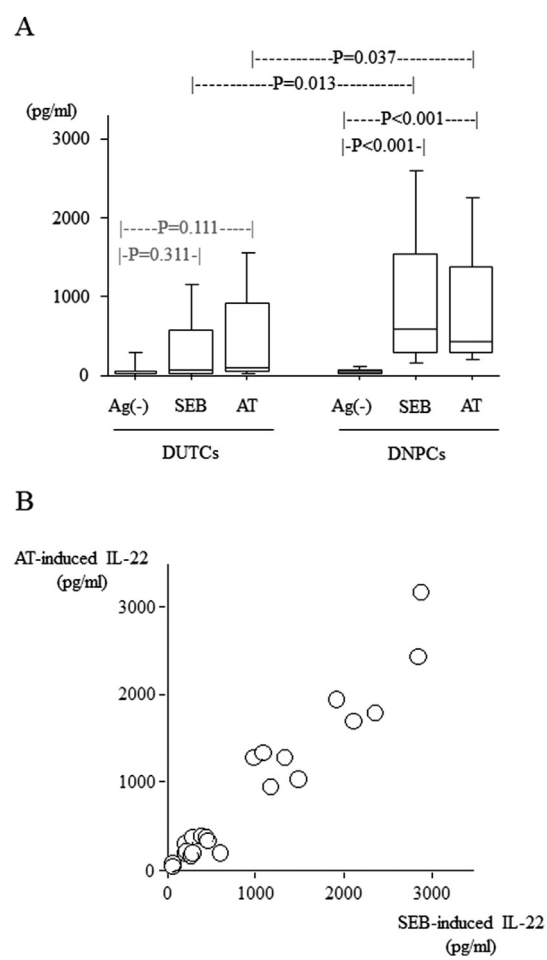

Fig. 4. (**A**) Effect of *S. aureus* SEB and AT on IL-22 production by DUTCs and DNPCs. The rectangle includes the range from the 25th to 75th percentiles; the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. P values were determined by the Wilcoxon's signed-ranks test. (**B**) Correlation of IL-22 production by DNPCs between SEB and AT stimulation.

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Pathophysiological significance of exotoxin-induced IL-22 production by nasal polyp cells

We next analyzed the correlation between SEB-induced IL-22 in DNPCs and the pathophysiological characteristics of the CRSwNP patients from whom the NP were derived. SEB-induced IL-22 production by DNPCs ($n = 22$) significantly and negatively correlated with the degree of eosinophilia in NP ($r = -0.479$, $P = 0.028$, Fig. 5A) and conversely positively correlated with the FEV_1/FVC ratio ($r = 0.610$, $P = 0.005$, Fig. 5E). Similar significant correlations were found when the cells were stimulated with AT ($r = -0.567$, $P = 0.009$ for the degree of eosinophilia in NP, $r = 0.538$, $P = 0.014$ for the FEV_1/FVC ratio). In addition, a trend towards an inverse correlation between SEB-induced IL-22 production and both the radiological severity of CRS as assessed by the pre-operative CT score ($r = -0.380$, $P = 0.078$, Fig. 5B) and blood eosinophil count ($r = -0.414$, $P = 0.058$, Fig. 5C) was observed. The postoperative CT score significantly and negatively correlated with SEB-induced IL-22 production by DNPCs ($r = -0.477$, $P = 0.032$, Fig. 5F). This score also showed a trend toward negative correlation with AT-induced IL-22 production ($r = -0.424$, $P = 0.056$). On the other hand, no significant correlation was seen between exotoxin-induced IL-22

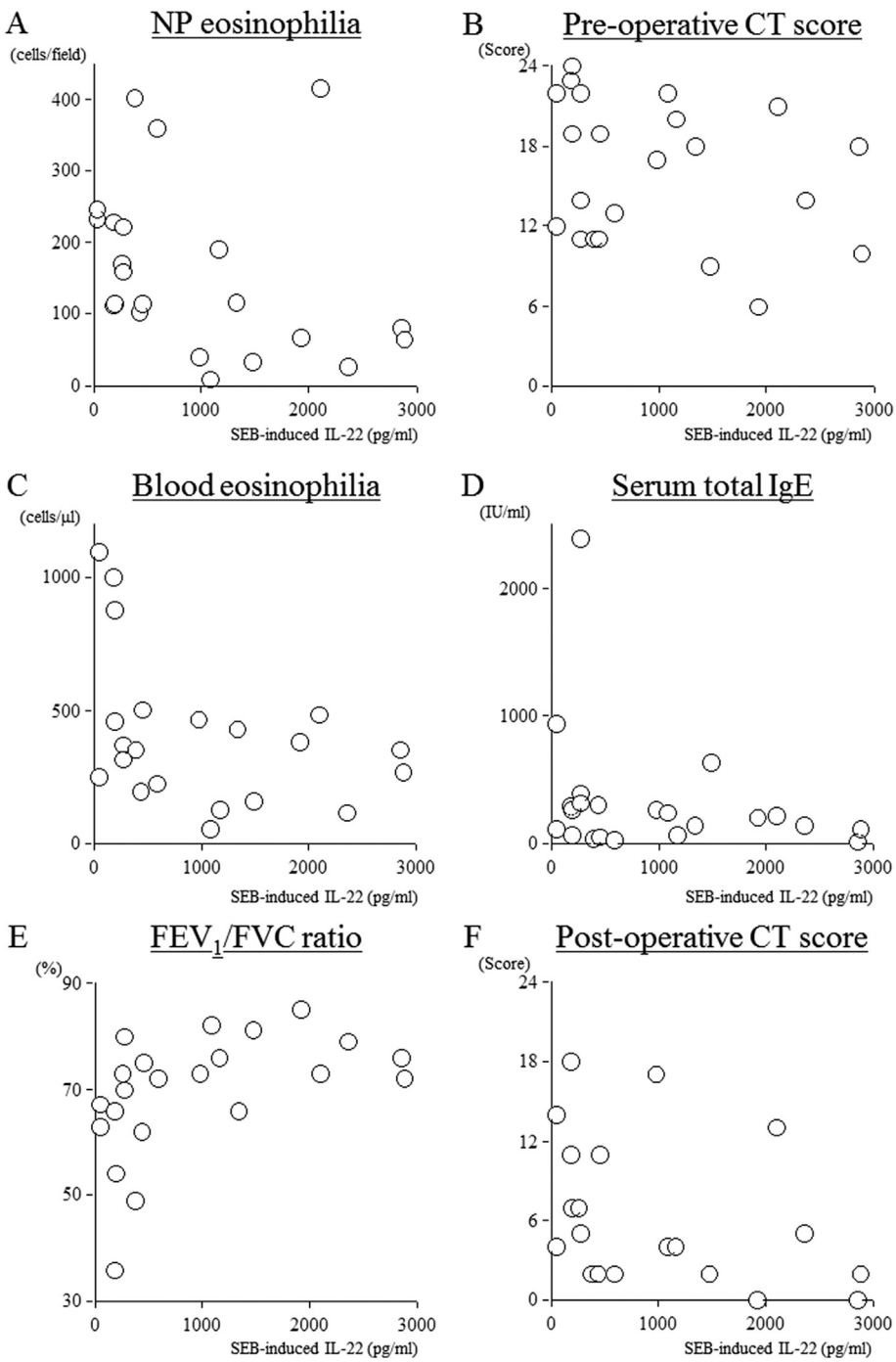

Fig. 5. Relationship between SEB-induced IL-22 production by DNPCs and pathophysiological characterizations including number of infiltrating eosinophils in nasal polyps (**A**), pre-operative CT score of rhinosinusitis (**B**), blood eosinophil count (**C**), serum total IgE levels (**D**), FEV_1/FVC ratio (**E**), and post-operative CT core (**F**).

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production and serum total IgE levels ($r = -0.335$, $P = 0.125$ for SEB (Fig. 5D) and $r = -0.250$, $P = 0.252$ for AT). A significant reduction of IL-22 production in response to SEB ($P = 0.004$) and AT ($P = 0.036$) was seen in DNPCs from asthmatic patients as compared to non-asthmatic patients. On the other hand, no significant associations were seen between SEB-induced IL-22 production by DUTCs from patients with CRSsNP and pre-operative CT score ($r = -0.101$, $P = 0.776$), blood eosinophil counts ($r = -0.452$, $P = 0.231$), serum total IgE levels ($r = 310$, $P = 0.412$) or FEV_1/FVC ratio ($r = -0.357$, $P = 0.344$). No significant associations were also seen between AT-induced IL-22 production by DUTCs and pre-operative CT score ($r = -0.113$, $P = 0.776$), blood eosinophil counts ($r = -0.381$, $P = 0.314$), serum total IgE levels ($r = -0.381$, $P = 0.314$) or FEV_1/FVC ratio ($r = -0.238$, $P = 0.529$).

Induction of MUC1 mRNA expression in DNPCs by IL-22

IL-22 is known to induce MUC1 expression in colonic epithelial cells.[12] We therefore sought to determine the effect of IL-22 on MUC1 mRNA expression in NP. MUC1 mRNA expression was significantly enhanced in DNPCs following their exposure to 20 ng/ml of recombinant IL-22 ($P = 0.048$) (Fig. 6A). MUC1 mRNA levels in response to IL-22 were significantly and positively correlated with IL-22R1 mRNA levels in DNPCs ($r = 0.538$, $P = 0.027$), suggesting that induction of MUC1 in response to IL-22 is dependent on the levels of IL-22 receptor in NP (Fig. 6B). DNPCs spontaneously produced TARC, RANTES, IL-8, IL-18 and IL-33 but not eotaxin or IL-25 without stimulation. The addition of IL-22 did not significantly alter the production of TARC, RANTES, eotaxin, IL-8, IL-18, IL-25 or IL-33 by DNPCs ($P > 0.05$ for all, Fig. 7).

We found no significant correlations between mRNA levels of MUC1 and those of IL-22 ($r = 0.181$, $P = 0.191$) or IL22R1 ($r = 0.097$, $P = 0.482$) in NP ($n = 53$). However, a subgroup analysis showed that a significant positive correlation was found between mRNA levels of MUC1 and those of IL-22R1 in AIA patients ($r = 0.550$, $P = 0.033$, $n = 16$). No significant correlation ($r = -0.176$, $P = 0.106$) was found between mRNA levels of MUC1 and IL-22/IL-22R1 ratio in sinonasal tissues ($n = 85$). In addition, none of each group showed a significant correlation (UT of non-CRS patients ($n = 19$): $r = 0.058$, $P = 0.806$, $n = 19$; UT of CRSsNP patients ($n = 13$): $r = -0.346$, $P = 0.230$; NP of non-asthmatic patients ($n = 16$): $r = 0.349$, $P = 0.177$; NP of ATA patients ($n = 21$): $r = -0.036$, $P = 0.871$; NP of AIA patients ($n = 16$): $r = -0.200$, $P = 0.439$).

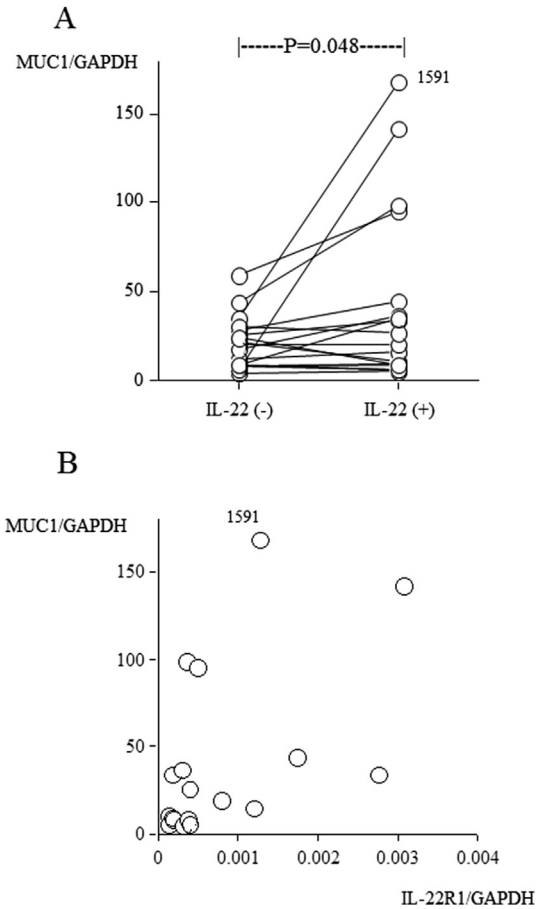

Fig. 6. Relationship between IL-22 signaling and MUC1 expression in NPs. (**A**) Effect of IL-22 on MUC1 mRNA expression in DNPCs. DNPCs were stimulated with 20 ng/ml of recombinant IL-22 for 72 h, then the levels of MUC1 mRNA in DNPCs were determined. P values were determined by the Wilcoxon's signed-ranks test. (**B**) Correlation of relative expression of IL-22R1 mRNA in nasal polyps and MUC1 expression in response to recombinant IL-22 at 20 ng/ml.

Effect of IL-22 blockade on exotoxin-induced IL-5 and IL-13 production by DNPCs

To determine the role of IL-22 in DNPC production of IL-5 and IL-13, the effect of blocking IL-22 action by treatment with the anti-human IL-22 Ab was assayed. Compared to control goat IgG, treatment with anti-human IL-22 Ab had no effect on either SEB-induced IL-5 ($P = 0.333$) or IL-13 ($P = 0.575$), or on AT-induced IL-5 ($P = 0.721$) or IL-13 ($P = 0.647$) production by DNPCs ($n = 10$, Fig. 8A, B). A significant reduction of IL-22 levels (94.3%) in culture supernatants of SEB-stimulated DNPCs was seen by the neutralization of anti-IL-22 antibody as compared to the control goat IgG ($n = 6$, $P = 0.028$), suggesting the successful neutralization of this anti-IL-22 antibody (Fig. 8C).

Discussion

In the present study, we characterized the expression of IL-22 and its receptor IL-22R1 in CRSwNP. Evidence is accumulating regarding the role of IL-22 in the pathogenesis of chronic airway diseases such as bronchial asthma. However, this is the first report of characterization of the production of IL-22 following exposure of NP to *Staphylococcus aureus* exotoxins, which are candidate toxins for facilitation of eosinophilic inflammation in airways.[21,22]

This study is the first demonstration that IL-22 and IL-22R1 are mainly expressed in infiltrating inflammatory cells and epithelial cells, respectively, in NP. These results are consistent with previous findings in other organs.[4,5,23] For example, IL-22R1 is expressed in several epithelial cells including keratinocytes.[4,23] Consistent with its original description as an IL-10-related T cell derived inducible factor (IL-TIF), IL-22 is known to be produced by lymphoid cells including $CD4^+$ T cells, NT cells and type 3 innate lymphoid cells.[4,5] The present result using double immunostaining suggests that $CD4^+$ T cells and eosinophils in nasal polyps have potential to produce IL-22. These results suggest that interaction between inflammatory cells and epithelial cells via IL-22 regulates the pathogenesis of CRSwNP.

The levels of IL-22 and IL-22R1 mRNA in NP were significantly higher and lower, respectively, than those in UT from non-CRS patients. In addition, the NP of asthmatic patients, especially of patients with AIA, showed high expression of IL-22 mRNA and conversely low expression of IL-22R1 mRNA. Since the presence of asthma, especially of AIA, has a negative impact on the pathogenesis of CRSwNP including on postoperative outcomes, the present results suggest that an imbalance in signaling via IL-22 affects the pathogenesis of CRSwNP.[19,24] Our results in terms of IL-22R1

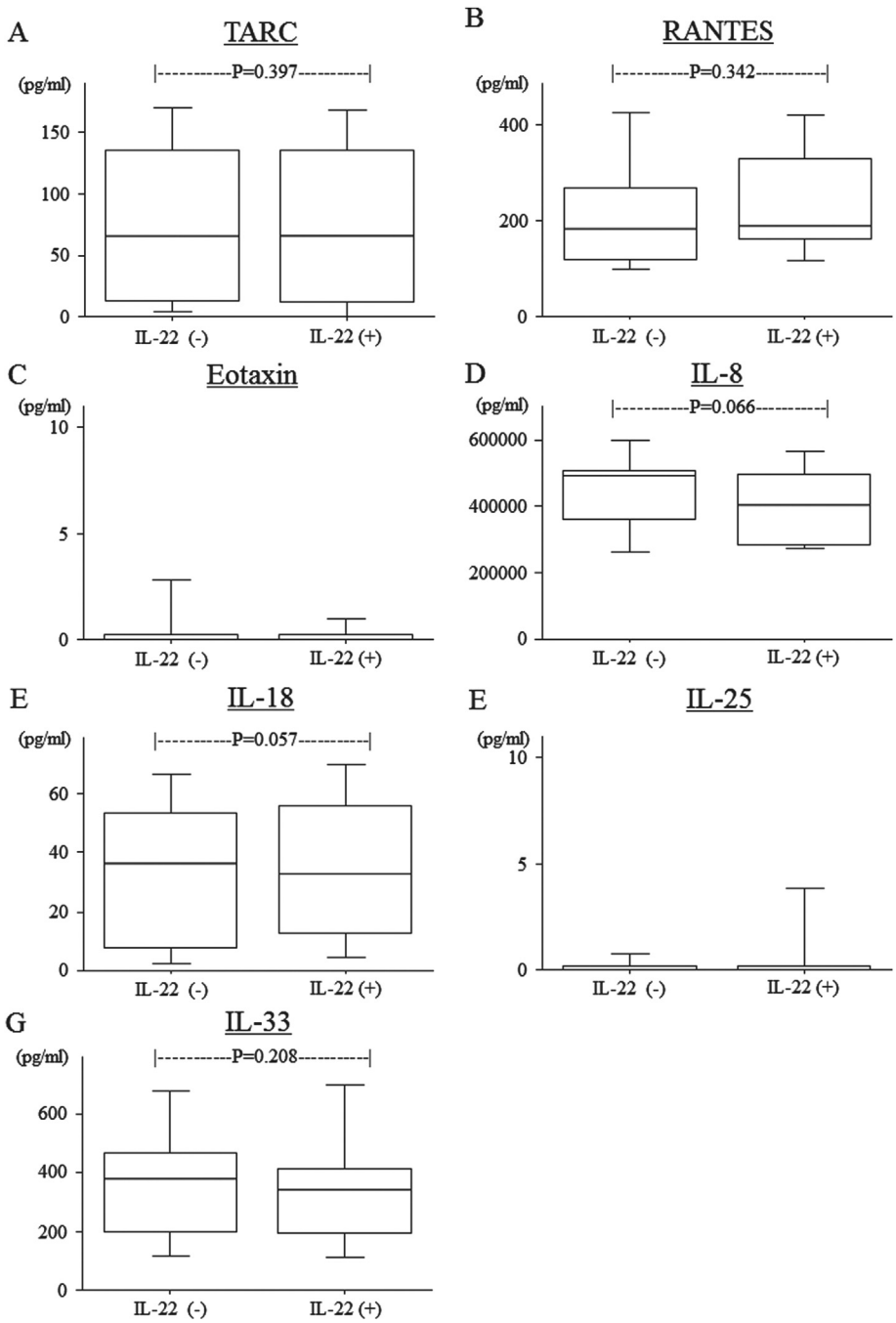

Fig. 7. Effect of IL-22 on epithelial cell-derived cytokine/chemokine production by DNPCs. DNPCs were stimulated with 5 or 20 ng/ml of recombinant IL-22 for 72 h, then the concentrations of TARC (**A**), RANTES (**B**), eotaxin (**C**), IL-8 (**D**), IL-25 (**E**), and IL-33 (**F**) were determined. *P* values were determined by the Wilcoxon's signed-ranks test.

expression are consistent with those of a previous report by Ramanthan; however, the expression of IL-22 differed between the two studies.[16] This discrepancy may be due to a difference in the control tissue used (uncinate tissue mucosa vs. sinonasal mucosal tissue). The difference in IL-22 mRNA levels in UT between non-CRS patients and CRS patients may be the differences in the numbers and/or activating status of infiltrating inflammatory cells.

Superantigenic enterotoxin and non-superantigenic AT, both of which are major exotoxins released by *S. aureus*, promote IL-22 production by NP cells. This finding is consistent with that of a previous report, which showed that both SEB and AT induced IL-22 production in PBMCs and in isolated $CD4^+$ T cells.[25] In that report, the AT-induced IL-22 production by PBMCs was significantly enhanced in patients with atopic dermatitis as compared with patients with psoriasis and healthy controls. Our finding that the SEB and AT-induced IL-22 production by NP cells was significantly higher than that of UT cells was similar to these previous reports, and suggests that enhancement of IL-22 production following exposure to *S. aureus* exotoxins is induced in inflamed tissues in CRS. This different response between DNPCs and DUTCs may result from different cell activation and/or different cell component. Our preliminary result showed that the proportion (mean ± standard deviation) of $CD4^+$, $CD8^+$, $CD19^+$, $CD68^+$ and $CD117^+$, ECP/EPX^+ cells in DUTCs was 4.8 ± 2.3%, 8.7 ± 3.8%, 11.8 ± 13.3%, 4.8 ± 3.6%,

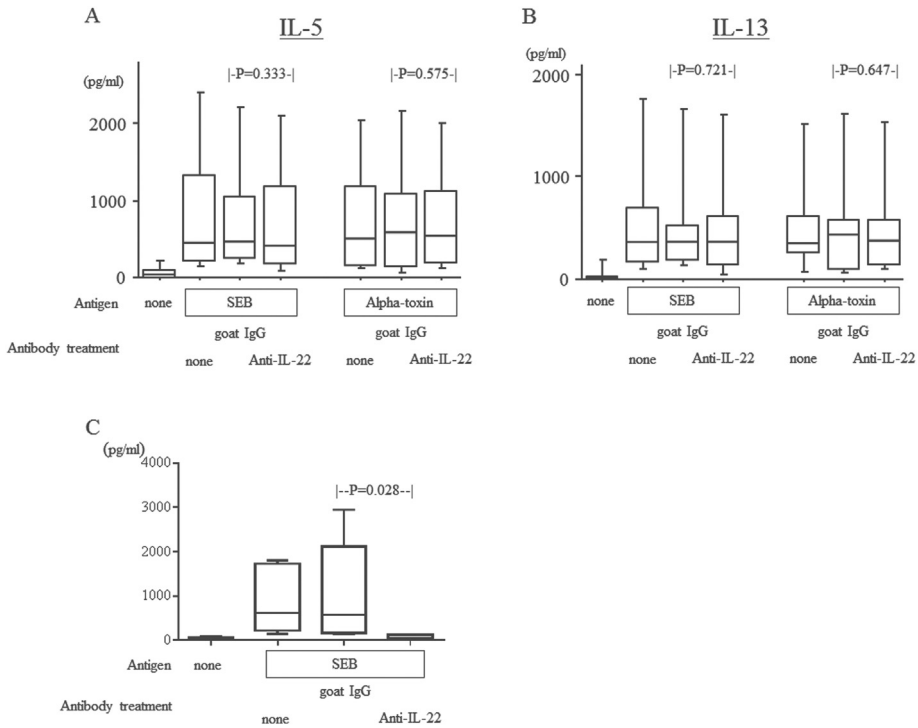

Fig. 8. Effect of IL-22 neutralization on *S. aureus* exotoxin-induced IL-5 (**A**) and IL-13 (**B**) and IL-22 (**C**) production by nasal polyp cells. DNPCs were stimulated with either SEB or AT in the presence or absence of anti-human IL-22 mAb or control goat IgG (20 μg/ml) for 72 h. *P*-values were determined using the Wilcoxon signed-rank test.

0.8 ± 0.4%, and 0.7 ± 0.7%, respectively (n = 3). As compared with our previous data on DNPCs ($CD4^+$ cell: 7.8 ± 11.1%, $CD8^+$ cells: 10.9 ± 10.5%, $CD79a^+$ cells: 8.5 ± 6.8%, $CD68^+$ cells: 8.9 ± 8.2%, $CD117^+$ cells: 8.5 ± 5.3%, ECP/EPX^+ cells: 11.7 ± 8.9%), the preliminary result suggests that the proportion of mast cells, eosinophils and $CD4^+$ T cells are lower DUTCs as compared with DNPCs.[26]

One of reasons why there was no significant difference in IL-22 mRNA levels between UT of CRSsNP and NP of CRSwNP whereas IL-22 production in response to SEB or AT was significantly higher in DNPCs than DUTCs may be that exotoxins are not the single elicitor to induce IL-22 production in sinonasal tissues. Other microbes such as fungi and viruses may also induce IL-22 production in CRS. For example, *Aspergillus-fumigatus* induces IL-22 production by human peripheral blood mononuclear cells.[27]

A significant inverse correlation was seen between the degree of eosinophilia in NP and exotoxin-induced IL-22 production by NP cells in patients with CRSwNP. In addition, the complication with asthma had a negative influence on exotoxin-induced IL-22 production by NP cells. This is the first report in which IL-22 was characterized in airways following exposure to *S. aureus* exotoxins. To date, the effect of IL-22 on eosinophilic airway inflammation in humans remains unclear. Pennino *et al.* recently showed that the expression of IL-22 in both bronchial mucosa and bronchoalveolar lavage fluid was higher in asthmatic patients as compared with healthy controls, and that IL-22 inhibited IFN-γ-mediated expression of MHC class I, MHC class II, ICAM-1, RANTES and IP-10 in bronchial epithelial cells.[10] In a murine model of asthma, delivery of IL-22 suppresses, whereas attenuation of IL-22 enhances eosinophilic inflammation.[9,11] Together with the finding that exotoxin-induced IL-22 production by DNPCs positively correlated with the FEV_1/FVC ratio whereas negatively correlated with postoperative CT score, the present results suggest that impairment in the synthesis of IL-22 following exposure to *S. aureus* exotoxins regulates the pathophysiology of CRSwNP including eosinophilic inflammation, lower respiratory function and persistent inflammation after surgery. On the other hand, impairment of IL-22 synthesis after exposure with *S. aureus* exotoxins is not associated with the pathophysiology of CRSsNP since no significant correlations were found between exotoxins-induced IL-22 production by DUTCs and pre-operative CT score, blood eosinophil counts, serum total IgE levels or FEV_1/FVC ratio.

It remains unclear how IL-22 regulates eosinophilic inflammation. Studies using mouse models of asthma suggest that the inhibitory effect of IL-22 on eosinophilic airway inflammation is associated with the suppression of Th2 cytokines such as IL-5 and IL-13.[9–11,28] However, this was not the case in the present study since treatment with an anti-human IL-22 Ab did not alter exotoxin-induced IL-5 or IL-13 production by NP cells. IL-22 is known to regulate the production of other epithelial-derived cytokines or chemokines including CCL17 (TARC), RANTES, IL-8 and IL-25.[9,10,26,29] However, in the present study the addition of recombinant IL-22 did not alter the production of these cytokines/chemokines by DNPCs.

On the other hand, IL-22 did significantly enhance the levels of MUC1 expression in NP cells. MUC1 is a membrane-tethered mucin that is located on sinonasal mucosa, and whose expression is significantly higher in NP as compared with healthy nasal mucosa.[30] MUC1 is known to exert an anti-inflammatory effect on airway inflammation through inhibition of TLR signaling including the signaling of TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9.[31] More recently, Milara *et al.* demonstrated that the cytoplasmic tail of MUC1 participates in the corticosteroid response that mediates GR alpha nuclear transcription in CRSwNP.[32] Our preliminary result showed that co-culture with unstimulated Beas-2B cells, an airway epithelial cell line, following treatment of MUC1-specific siRNA prolonged attached but not floating eosinophil survival (approximately 9% extension), compared with non-slicing siRNA, suggesting that MUC1 on epithelial cells displays an inhibitory effect on

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eosinophil survival by cell-to-cell contact (unpublished data). Although IL-22 is known to enhance MUC1 expression in the intestine, little is known about the effect of IL-22 on MUC1 expression in the airway.[12] Our results, together with the finding that MUC1 levels in response to IL-22 were significantly and positively correlated with IL-22RA mRNA levels in NP cells, suggest that one of the anti-inflammatory effects of IL-22 on eosinophilic airway inflammation is mediated by the enhancement of MUC1 expression, which is dependent on expression of the IL-22 receptor.

In conclusion, we show evidence that, following exposure of NP cells to *S. aureus* exotoxins, IL-22 plays a regulatory role in the pathogenesis of CRSwNP via enhancement of MUC1 expression by an IL-22 receptor-dependent pathway. The present observations might provide a basis for novel therapeutic approaches that target IL-22 and its receptor in the management of airway inflammatory diseases.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

MO, YN, KT and KN designed the study and wrote the manuscript. TF, SK, THa, SM and KK contributed to patient collection and *ex vivo* study. TK and JI contributed to immunohistochemistry. AK, YK and MA contributed to experiments regarding MUC1. THi and MT performed the statistical analysis and interpretation of the results.

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