

# Characterization of IL-18 Expression and Release in the Pathogenesis of Chronic Rhinosinusitis

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## Key Words

Eosinophilic inflammation · IFN- $\gamma$  · Interleukin-5 ·  
Interleukin-13 · Interleukin-18 · Nasal polyps · Protease ·  
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## Abstract

**Background:** Interleukin-18 (IL-18) is a member of the IL-1 cytokine family that affects chronic inflammation. We sought to characterize IL-18 expression and investigate its release during chronic rhinosinusitis (CRS). **Methods:** The expression of IL-18 in nasal polyps (NPs) and uncinatate tissues (UTs) from both CRS and non-CRS patients was examined via immunohistochemistry. After culturing dispersed NP cells (DNPCs) with or without various stimulations, IL-18 levels were measured in the culture supernatants. Furthermore, the effect of IL-18 neutralization on staphylococcus enterotoxin B (SEB)-induced cytokine production was also examined. **Results:** Similar expression of IL-18 in the epithelial layers was observed between the NPs and UTs. However, there was a significantly higher number of IL-18<sup>+</sup> cells in the lamina propria from NPs compared to UTs without CRS. This increased number was significantly correlated with the radiological severity of sinusitis and local eosinophilia. After the dispersion, IL-18 was spontaneously released by NP cells in a phase-dependent manner. While SEB, fungal antigens, and TLR ago-

nists did not enhance the release, exposure to protease or one cycle of a freeze-and-thaw treatment did induce release of IL-18 from rested DNPCs. In addition, neutralization of IL-18 significantly suppressed SEB-induced IL-5, IL-13, and IFN- $\gamma$ , but not IL-17A production. **Conclusions:** These results suggest that the pro-inflammatory effect of IL-18 released by danger signals may be involved in the pathogenesis of CRS, which includes eosinophilic inflammation and NP formation, via the augmentation of both Th2- and Th1-associated cytokine production.

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## Introduction

Chronic rhinosinusitis (CRS), especially CRS with nasal polyps (CRSwNPs), is characterized by eosinophilic inflammation that is often associated with asthma and aspirin sensitivity [1, 2]. Recent epidemiological studies have shown that CRS prevalence is 11 and 14% in Europe and the USA, respectively [1, 3]. It is currently thought that NP formation affects between 1 and 4% of the population [2]. While the precise etiology and pathophysiology underlying CRS remains poorly understood, it has been proposed that networks of Th1, Th2, Th17, and Treg-associated cytokines might be involved [4–7].

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Interleukin-18 (IL-18), which is a member of the IL-1 cytokine family, has pluripotent activity bridging between innate and acquired immunity [8–10]. Since IL-18 together with IL-12 induces a large amount of IFN- $\gamma$  production along with being able to inhibit IgE production, this originally led to the characterization of IL-18 as a Th1-associated cytokine [11]. However, later studies determined that IL-18 could induce Th2-associated cytokine production, which includes IL-13 via T cells (the so-called ‘super Th1 cells’), NK cells, NKT cells, and mast cells/basophils [9, 12, 13]. For example, it has been shown that when CD4<sup>+</sup> T cells are cultured with IL-2 and IL-18 without any TCR engagement, this induces IL-4 and IL-13 [14]. In humans, IL-18 is expressed not only in structural cells that include the epithelial cells and synovial fibroblasts but also in infiltrating inflammatory cells that include macrophages and dendritic cells [9, 15]. IL-18 is intracellularly stored as pro-IL-18, with maturation occurring due to the action of the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin-domain-containing 3 (NLRP3) inflammasome, which is then followed by activation of caspase 1 [13]. Pathogen-associated molecular patterns, which include lipopolysaccharide (LPS), microbial toxins, live bacteria, viruses, and damage-associated molecular patterns (the so-called ‘danger signals’), can activate NLRP3 [16].

Accumulating evidence has implicated IL-18 in the initiation and progression of eosinophilic airway inflammation, especially in the lower airways [13, 17–23]. Compared to that seen in healthy subjects, some studies have shown increased serum levels of IL-18 in asthmatics, while other studies have reported contradictory results [13]. Asthma patients have significantly higher IL-18 secretion from mononuclear cells compared to controls [17]. In an experimental model of asthma, intranasal challenge with IL-18 together with allergen or IL-2 evoked substantial lung eosinophilia in BALB/c and CBA/J mice [18–21]. In contrast, IL-18 gene-deficient mice of the C57BL/6 strain exhibited either no difference or exacerbation of the local eosinophilia compared to their wild-type counterparts [22, 23]. Thus, the studies in mice suggest that the effects of IL-18 on allergic inflammation are complex and not easily regarded as either promoting or suppressing Th2 inflammation.

Presently, it remains unclear whether IL-18 is involved in the overall pathogenesis of CRS or in eosinophilic inflammation. One previous report demonstrated that spontaneous IL-18 release by sinus lavage cells was found in the majority of the treatment-resistant pediatric patients with CRS [24]. However, currently, little is known

regarding the cells that express IL-18, or the mechanisms underlying the release of IL-18 and its regulatory effect on the immune responses in the upper airway.

In this study, we framed a hypothesis of whether the expression of IL-18 in sinonasal tissues plays a substantial role in the pathogenesis of CRS. We compared the local expression of IL-18 among the various CRS phenotypes and examined the characterization of IL-18 expression in the pathogenesis of CRS. We further investigated not only the regulation of the local IL-18 release but also the regulatory effect of IL-18 on Th1/Th2/Th17-associated cytokine production in NPs using a recently developed ex vivo system [5, 7]. The present findings provide novel insights into the pathogenesis of chronic eosinophilic airway diseases regulated by IL-18, in addition to providing a basis for the role of danger signals in IL-18 release in the airways [25].

## Materials and Methods

### *Patients and Tissue Sampling*

Of the 73 Japanese subjects who were enrolled into the immunohistochemical experiment, a total of 62 patients with CRS were included. Eleven non-CRS patients with normal uncinat tissues (UTs) at their initial examination and blow-out fracture, optic canal fracture, and sphenoidal/posterior ethmoidal cyst were enrolled as controls. Of the 62 CRS patients, 46 patients exhibited NPs (CRSwNPs group), while the remaining 16 CRS patients exhibited no visible polyps in the middle meatus (CRSsNPs group). The criteria for the CRSwNPs and CRSsNPs groups were based on the diagnostic criteria reported by Benninger et al. [28]. Thirty patients were determined to be asthmatic and have NPs. There were no asthmatic patients in the CRSsNPs group. In the asthmatic group, 17 were thought to have aspirin sensitivity based on their history of asthma attacks precipitated by nonsteroidal anti-inflammatory drugs (aspirin-intolerant asthma group; AIA), while the remaining 13 patients were grouped into the aspirin-tolerant asthma group (ATA). As all of these CRS patients proved to be resistant to medical treatment, which included macrolide therapy, they underwent endoscopic sinus surgery. The samples of NPs and UTs were collected during the surgeries on the patients with CRSwNPs and CRSsNPs, respectively. None of the participants who underwent the surgery had received any systemic steroid treatment during at least an 8-week period prior to the procedure. In addition, none of the patients had received any pharmacotherapy for sinusitis, such as macrolide antibiotics or intranasal steroids, for a period of at least 3 weeks prior to the surgery. In addition, we examined the total serum IgE levels, blood eosinophil count, and forced expiratory volume in 1 s (FEV<sub>1</sub>)/forced vital capacity (FVC) ratio in each of the patients before the surgical procedure. To evaluate the radiological severity of the sinusitis, a computed tomography (CT) examination was performed prior to surgery, with the severity graded in accordance with the Lund-Mackay system [29]. Table 1 lists the clinical characteristics of the patients.

**Table 1.** Subject characteristics

|                                          | Non-CRS     | CRSsNPs     | CRSsNPs without BA | CRSsNPs with ATA | CRSsNPs with AIA |
|------------------------------------------|-------------|-------------|--------------------|------------------|------------------|
| Patients, n                              | 11          | 16          | 16                 | 13               | 17               |
| Sex (male:female)                        | 4:8         | 8:8         | 12:4               | 9:4              | 9:8              |
| Age, years                               | 49.4 ± 18.2 | 52.1 ± 17.1 | 41.1 ± 18.1        | 56.2 ± 14.5      | 50.9 ± 14.6      |
| Age range, years                         | 15–81       | 14–74       | 14–73              | 21–77            | 22–77            |
| Serum IgE, IU/ml                         | 144 ± 153   | 193 ± 184   | 448 ± 729          | 740 ± 622        | 533 ± 710        |
| Blood eosinophils, × 10 <sup>2</sup> /ml | 1.43 ± 1.12 | 3.04 ± 4.18 | 2.24 ± 3.78        | 6.76 ± 4.15      | 6.08 ± 4.68      |
| CT grading score                         | 0           | 4.0 ± 2.9   | 11.28 ± 6.9        | 17.6 ± 6.0       | 18.2 ± 4.9       |
| FEV <sub>1</sub> /FVC ratio              | 80.6 ± 10.5 | 81.0 ± 5.7  | 80.2 ± 9.1         | 66.2 ± 10.0      | 66.2 ± 12.6      |

Results are presented as means ± SD. BA = Bronchial asthma.

**Table 2.** Anti-human antibodies used for immunohistochemistry

| Cell type                            | Molecule    | Clone   | Isotype    | Dilution | Manufacturer |
|--------------------------------------|-------------|---------|------------|----------|--------------|
| Mast cell                            | c-kit       | AA1     | mouse IgG1 | 1:100    | MBL          |
| Eosinophil                           | ECP/EPX     | EG2     | mouse IgG1 | 1:100    | Pharmacia    |
| Neutrophil                           | elastase    | NP57    | mouse IgG1 | 1:100    | Dako Japan   |
| B cell                               | CD79a       | JCB117  | mouse IgG1 | 1:50     | Dako Japan   |
| Macrophage                           | CD68        | KP1     | mouse IgG1 | 1:50     | Dako Japan   |
| T cell                               | CD4         | 4B12    | mouse IgG1 | 1:50     | MBL          |
| Epithelial cell                      | cytokeratin | AE1/AE3 | mouse IgG1 | 1:50     | Dako Japan   |
| Vascular endothelial cell/fibroblast | vimentin    | V9      | mouse IgG1 | 1:50     | Dako Japan   |

Dispersed NP cells (DNPCs) were prepared from a subset of 16 patients with CRSsNPs. NPs were sampled during the endoscopic sinus surgery and then treated by enzymatic digestion using 2.0 mg/ml protease, 1.5 mg/ml collagenase, 0.75 mg/ml hyaluronidase, and 0.05 mg/ml DNase (all from Sigma, St. Louis, Mo., USA), as per a previously described method [5]. The study was preapproved by the Human Research Committee of the Okayama University Graduate School of Medicine and Dentistry, and all patients provided informed consent prior to their participation.

#### Immunohistochemistry

We performed immunohistochemical staining for IL-18, eosinophils, and neutrophils in accordance with a previously described protocol [2]. Briefly, 4-μm sections were collected from paraffin-embedded tissue blocks, deparaffinized, and then rehydrated. Antigen retrieval was performed using a microwave treatment for 20 min, followed by incubation of the sections with primary antibody that included 1:200 diluted rabbit anti-human IL-18 polyclonal antibody (MBL, Nagoya, Japan) or control antibody (normal rabbit immunoglobulin fraction; Dako Japan, Tokyo, Japan) at 4°C overnight. A peroxidase-labeled secondary anti-rabbit antibody (Histofine MAX-PO (R); Nichirei Bioscience, Tokyo, Japan) with diaminobenzidine substrate was used according to the

manufacturer's instructions for detecting antibody-antigen interactions. After nuclear staining using hematoxylin, sections were then examined under a light microscope. Since it is difficult to choose fields randomly, positive stained cells were counted in 5 fields at high power (4 × 100) where the highest cellular infiltration in the lamina propria was observed, with the mean number of positive cells then determined. Eosinophils and neutrophils were stained by 1:100 diluted anti-ECP/EPX monoclonal antibody (EG2, mouse IgG1; Pharmacia, Uppsala, Sweden) and anti-elastase monoclonal antibody (NP57, mouse IgG1; Dako), respectively.

#### Double Immunofluorescence

To determine IL-18 expression, double immunohistochemistry was performed using a previously described method [7]. Briefly, sections were incubated with murine antibodies directed against markers of mast cells, eosinophils, neutrophils, B cells, macrophages, T cells, epithelial cells, vascular endothelial cells and fibroblasts, or against control mouse IgG1 (Dako), together with either rabbit anti-human IL-18 antibody or normal rabbit immunoglobulin fraction (Dako) overnight at 4°C (table 2). After washing, the sections were further incubated with Alexa-Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, Eugene, Oreg., USA) diluted 1:200 and Alexa-Fluor 546-conjugated goat anti-

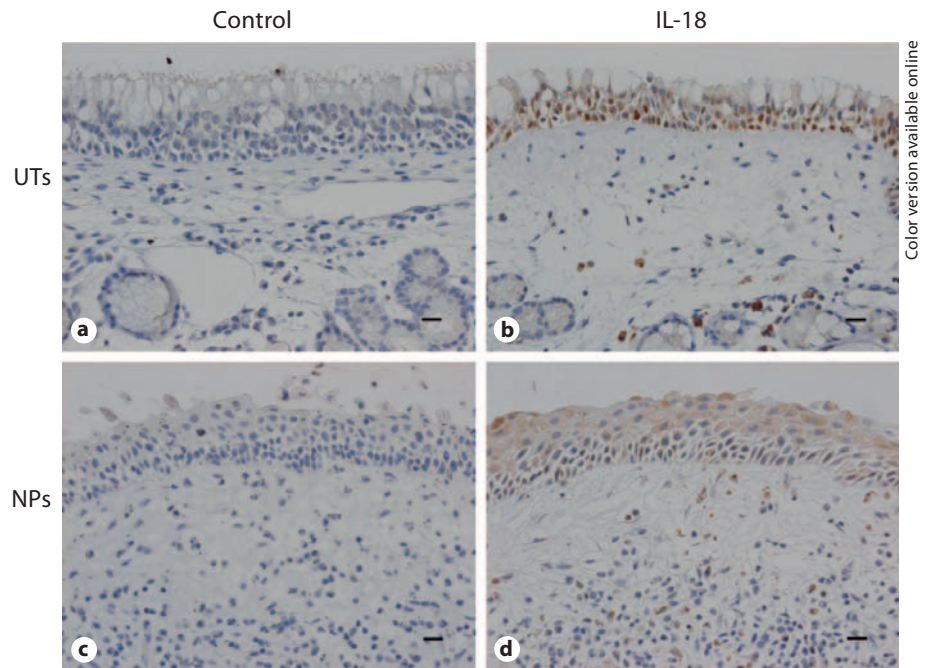


**Fig. 1.** Immunohistochemical staining of IL-18 in UTs (**a, b**) and NPs (**c, d**) from a patient with CRSsNP and a patient with AIA, respectively. Sections were reacted with rabbit polyclonal antibody against IL-18 (**b, d**) or the control [universal negative control (rabbit); **a, c**] after which they were stained using a Histofine MAX-PO (R) with a diaminobenzidine substrate. Scale bar = 50  $\mu$ m.

rabbit IgG (Invitrogen) diluted 1:200 for 30 min at room temperature. The sections were then observed using a confocal laser scanning microscope (Zeiss, Hallberg, Germany).

#### *Release of IL-18 by DNPCs in Response to Various Stimulations*

To determine whether IL-18 was released by the DNPCs, 8 DNPCs (500  $\mu$ l;  $2 \times 10^6$ /ml) were cultured in the presence or absence of the following stimulants at 37°C in a 5% CO<sub>2</sub>/air mixture: staphylococcal enterotoxin B (SEB) (1 ng/ml; Toxin Technology, Sarasota, Fla., USA), fungal extracts of *Aspergillus fumigatus* (200  $\mu$ g/ml; Torii Co., Tokyo, Japan), *Alternaria alternata* (200  $\mu$ g/ml; Torii) and *Candida albicans* (200  $\mu$ g/ml; Torii), and TLR agonists that included Pam<sub>3</sub>Cys-Ser-(Lys)<sub>4</sub> (10  $\mu$ g/ml; Alexis Corp., Lausen, Switzerland), LPS (0.2  $\mu$ g/ml; Sigma), poly(I).poly(C) (100  $\mu$ g/ml; Imgenex, San Diego, Calif., USA), and type B CpG ODN (5  $\mu$ g/ml; Imgenex). The culture supernatant was harvested at 15 min, and 1, 12, 48, and 72 h. Concentrations of IL-18 were determined by ELISA using anti-human IL-18 mAb (125-2H, 1  $\mu$ g/ml; MBL), biotinylated anti-human IL-18 mAb (159-12B, 0.5  $\mu$ g/ml; MBL), and recombinant human IL-18 (MBL) as the capture antibody, detection antibody, and standard, respectively. The detection limit of the assays was 2 pg/ml. Alternatively, isolated DNPCs were allowed to rest in culture medium for 48 h and then subjected to one cycle of freeze and thaw, or sham treatment. After this step, cells were cultured for 2 h, followed by measurement of IL-18 concentrations in the supernatants. Due to the small sample volume, similar ex vivo studies of UTs could not be performed.

#### *Effect of IL-18 Blockade on SEB-Induced Cytokine Production by DNPCs*

In flat-bottom, 48-well culture plates (AGC Techno Glass, Tokyo, Japan), 8 DNPCs were treated with anti-human IL-18 mAb

(125-2H, mouse IgG1; MBL) or control mouse IgG1 (R&D Systems, Minneapolis, Minn., USA). Cells were then stimulated with or without 1 ng/ml SEB at 37°C in a 5% CO<sub>2</sub>/air mixture. The culture supernatant was collected after 72 h, with the concentrations of IL-5, IL-13, IFN- $\gamma$ , and IL-17A then determined. The levels of IL-5, IL-13, and IFN- $\gamma$  were measured using Opt EIA sets (BD Biosciences, San Jose, Calif., USA) as per the manufacturer's instructions. The level of IL-17A was measured using a DuoSet ELISA development kit (R&D). The detection limits were 2 pg/ml for the IL-13 assay, and 4 pg/ml for the IL-5, IFN- $\gamma$ , and IL-17A assays.

#### *Statistical Analysis*

Values are presented as medians. A nonparametric Mann-Whitney U test was used to compare the data between the groups, while a Wilcoxon signed-rank test was used for analysis within the groups. A correlation analysis was performed using Spearman's correlation coefficient by rank.  $p < 0.05$  was considered statistically significant. Statistical analyses were performed using StatView software (version 4.5; Abacus Concepts, Berkeley, Calif., USA).

## **Results**

### *Local Expression of IL-18 in CRS*

We immunohistochemically examined the expression and distribution of IL-18 in NPs and UTs. IL-18 was expressed in both the epithelial layers and the lamina propria mucosae (fig. 1). A similar expression of IL-18 in the epithelial layers was observed between the NPs and UTs

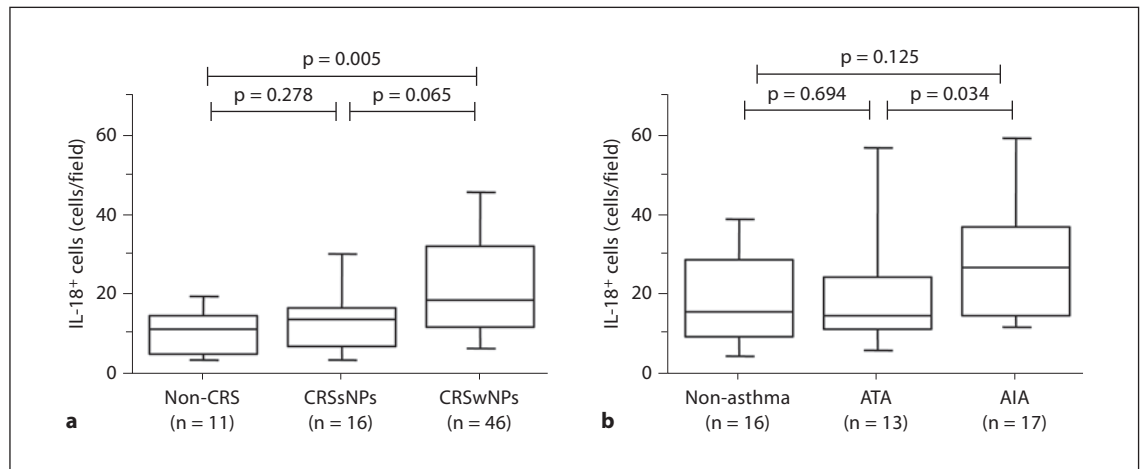


**Fig. 2.** Comparison of numbers of IL-18<sup>+</sup> cells in the lamina propria among non-CRS UTs, CRS UTs, and NPs (a), and NPs from non-asthmatic, ATA, and AIA patients (b). The rectangle includes the range from the 25th to the 75th percentiles, the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. p values were determined by the Mann-Whitney U test.

(fig. 1). On the other hand, the number of IL-18<sup>+</sup> cells in the lamina propria was significantly higher than that seen in the UTs without CRS ( $p = 0.005$ ) (fig. 2a). In NPs, the number of IL-18<sup>+</sup> cells was higher in the AIA patients compared to the ATA patients ( $p = 0.034$ ) (fig. 2b).

Double immunofluorescence staining revealed that the majority of the cytokeratin<sup>+</sup> cells (epithelial cells) expressed IL-18. CD68<sup>+</sup> cells (macrophages) also clearly expressed IL-18. It was not obvious, but a substantial proportion of the CD79a<sup>+</sup> cells (B cells and plasma cells) were also found to express IL-18. While a small subset of the CD4<sup>+</sup> cells and vimentin<sup>+</sup> cells (fibroblasts/vascular endothelial cells) expressed IL-18, the CD8<sup>+</sup> cells, tryptase<sup>+</sup> cells (mast cells), EG2<sup>+</sup> cells (eosinophils), and elastase<sup>+</sup> cells (neutrophils) showed no expression (fig. 3).

#### *Pathophysiological Significance of IL-18 Expression in CRS*

Total number of IL-18<sup>+</sup> cells in whole tissues including the epithelial layer did not correlate with the pathophysiological parameters due to a constant expression of IL-18 in the majority of epithelial cells irrespective of inflammatory conditions. However, a significant positive correlation was observed between the number of IL-18<sup>+</sup> cells in the lamina propria and the radiological severity of the sinusitis ( $\rho = 0.391$ ,  $p < 0.001$ ; fig. 4a). In contrast, the number of IL-18<sup>+</sup> cells was not correlated with the FEV<sub>1</sub>/FVC ratio ( $\rho = -0.090$ ,  $p = 0.450$ ; fig. 4b), the total serum IgE levels ( $\rho = 0.043$ ,  $p = 0.744$ ; fig. 4c), or the blood eo-

sinophil counts ( $\rho = 0.199$ ,  $p = 0.092$ ; fig. 4d). Subsequently, we attempted to determine whether the number of IL-18<sup>+</sup> cells was associated with the local accumulation of eosinophils and neutrophils. The degree of EG2<sup>+</sup> cell infiltration into the lamina propria was both significantly and positively correlated with the number of IL-18<sup>+</sup> cells ( $\rho = 0.246$ ,  $p = 0.047$ ; fig. 4e). However, no correlation was observed between the degree of elastase<sup>+</sup> cell infiltration and the number of IL-18<sup>+</sup> cells ( $\rho = 0.214$ ,  $p = 0.147$ ; fig. 4f). Especially, the number of IL-18<sup>+</sup> cells in NPs was significantly and positively correlated with the radiological severity of the sinusitis ( $\rho = 0.295$ ,  $p = 0.049$ ). This number also correlated positively with the degree of EG2<sup>+</sup> cell infiltration into NPs, although the difference did not reach statistical significance ( $\rho = 0.280$ ,  $p = 0.067$ ). On the other hand, this number did not show correlation with other parameters including the FEV<sub>1</sub>/FVC ratio ( $\rho = -0.035$ ,  $p = 0.814$ ), total serum IgE levels ( $\rho = 0.025$ ,  $p = 0.870$ ), blood eosinophil counts ( $\rho = 0.069$ ,  $p = 0.645$ ), or the degree of the elastase<sup>+</sup> cell infiltration into NPs ( $\rho = -0.046$ ,  $p = 0.638$ ).

#### *Release of IL-18 Protein by DNPCs*

After enzymatic dispersion, NP cells spontaneously released IL-18 protein. The highest release was seen 15 min after placing the DNPCs into the culture well (median 226 pg/ml). This release gradually decreased in line with the length of the culture period, with levels near the detection limit at the 48-hour point (median 11.4 pg/ml).

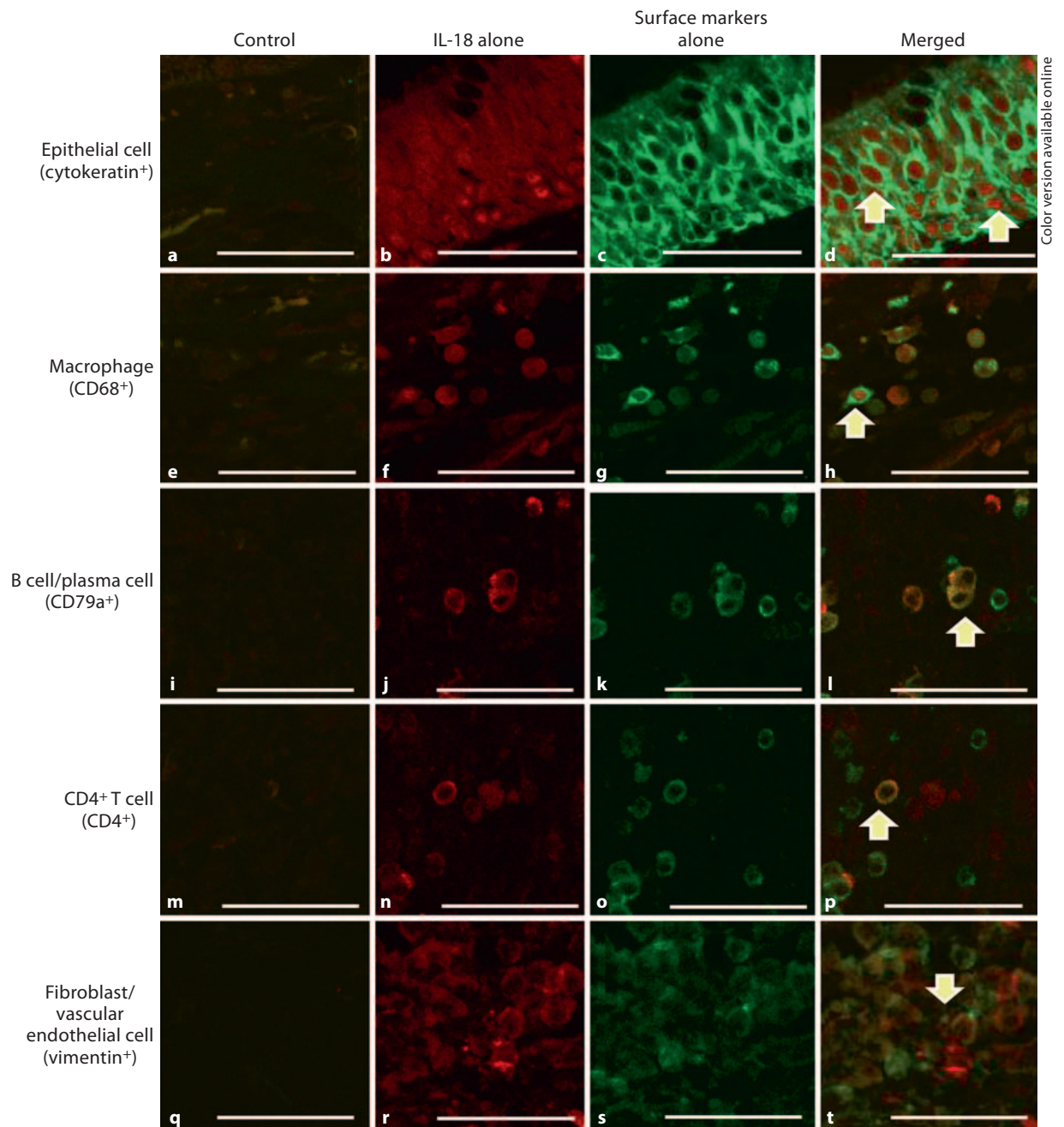


**Fig. 3.** Expression of IL-18 in cytokeratin<sup>+</sup> (d), CD68<sup>+</sup> (h), CD79a<sup>+</sup> (l), CD4<sup>+</sup> (p), and vimentin<sup>+</sup> (t) cells in NP. As controls, sections were stained with anti-IL-18 polyclonal Ab alone (b, f, j, n, r), anti-cytokeratin (c), CD68 (g), CD79a (k), CD4 (o), and vimentin (s) mAb alone, or control Abs. Arrows indicate double-immunostained cells.

(fig. 5a). IL-18 release at 15 min or 1 h was not enhanced in response to SEB, fungal extracts that included *Aspergillus*, *Alternaria*, and *Candida*, or the TLR ligands that included Pam<sub>3</sub>Cys-Ser-(Lys)<sub>4</sub>, poly(I).poly(C), LPS, and CpG ODN (fig. 5b).

Since the DNPCs were under chemical and/or physical stress (e.g. protease digestion) during preparation, we sought to determine whether these factors could affect IL-18 release by DNPCs. Thus, after the DNPCs were rested for 48 h, they were treated with protease

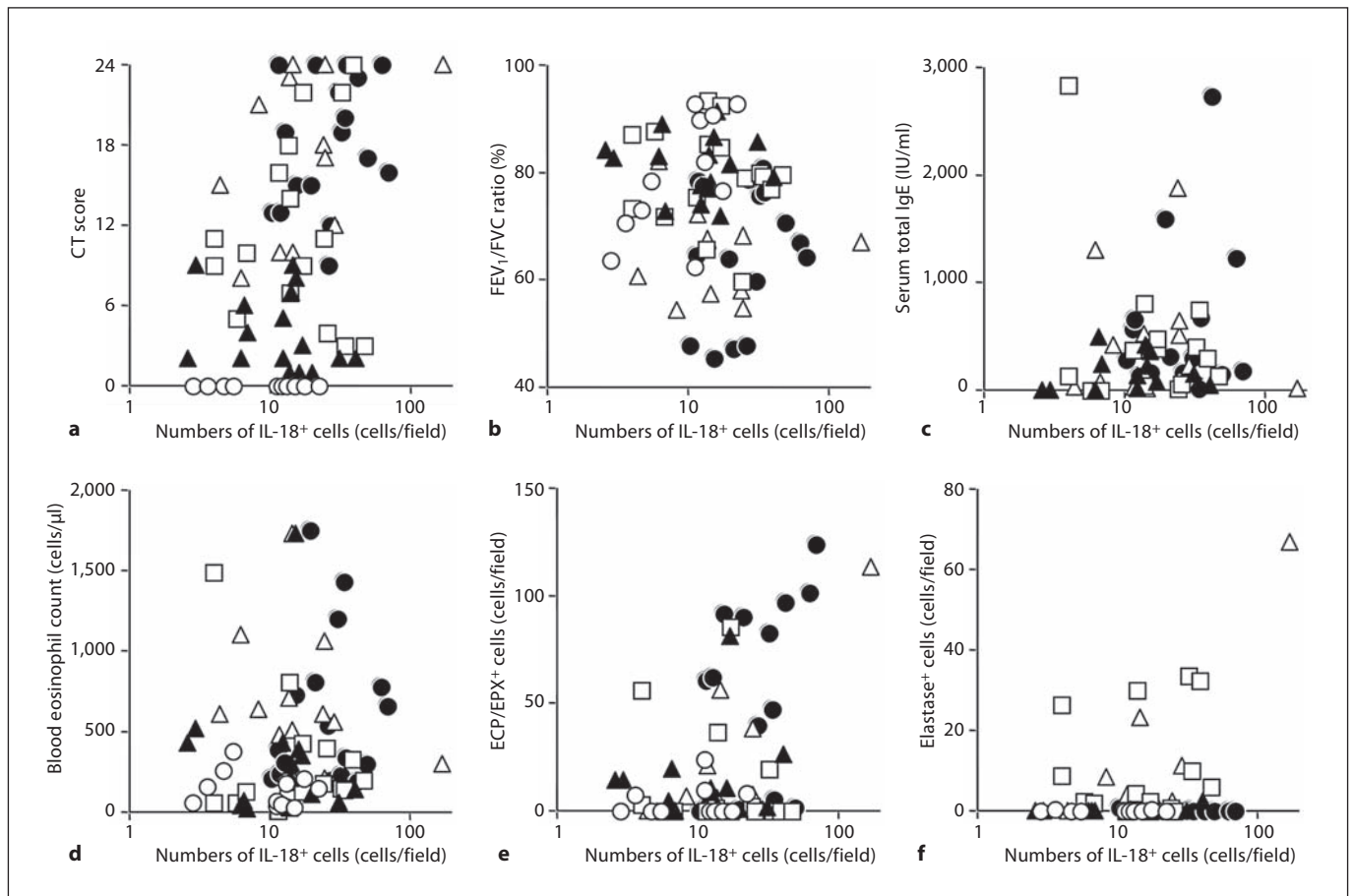


**Fig. 4.** Relationship between numbers of IL-18<sup>+</sup> cells in the lamina propria and pathophysiological characterizations including radiological severity of sinusitis (a), the FEV<sub>1</sub>/FVC ratio (b), serum total IgE levels (c), the blood eosinophil count (d), the number of infiltrating ECP/EPX<sup>+</sup> cells in sinonasal tissues (e), and the num-

ber of elastase<sup>+</sup> cells in sinonasal tissues (f). Sinonasal tissues were sampled from non-CRS patients (○), CRSsNPs (▲), CRSwNPs without asthma (□), CRSwNPs with ATA (△), and CRSwNPs with AIA (●).

(0.02, 0.2, 2.0, and 20 mg/ml), collagenase (1.5 mg/ml), hyaluronidase (0.75 mg/ml), and DNase (0.05 mg/ml), with the enzymes for dispersion of the NP cells used individually or in combination for 2 h. Alternatively, the rested DNPCs were also treated with or without one cycle of freeze and thaw, followed by a 2-hour culture. Treatment with collagenase ( $p = 0.311$ ), hyaluronidase ( $p = 0.946$ ), or DNase ( $p = 0.845$ ) did not induce a significant release of IL-18 by the rested DNPCs. On the other hand, treatment with protease did induce a dose-dependent release ( $p = 0.019$  at 20 mg/ml). In addition, treatment with one cycle of freeze and thaw also significantly induced IL-18 release compared to the sham treatment ( $p = 0.023$ ) (fig. 5c).

#### Effect of IL-18 Blockade on SEB-Induced Cytokine Production

As we previously reported, DNPCs produced substantial amounts of IL-5, IL-13, IFN- $\gamma$ , and IL-17A in response to 1 ng/ml SEB. While neutralization of IL-18 significantly suppressed SEB-induced IL-5 ( $p = 0.012$ ), IL-13 ( $p = 0.012$ ), and IFN- $\gamma$  ( $p = 0.012$ ), it did not alter the production of IL-17A ( $p = 0.327$ ) (fig. 6).

#### Discussion

In the present study, we examined the characterization of IL-18 expression during CRS pathogenesis. Immunohistochemical examinations demonstrated that the local

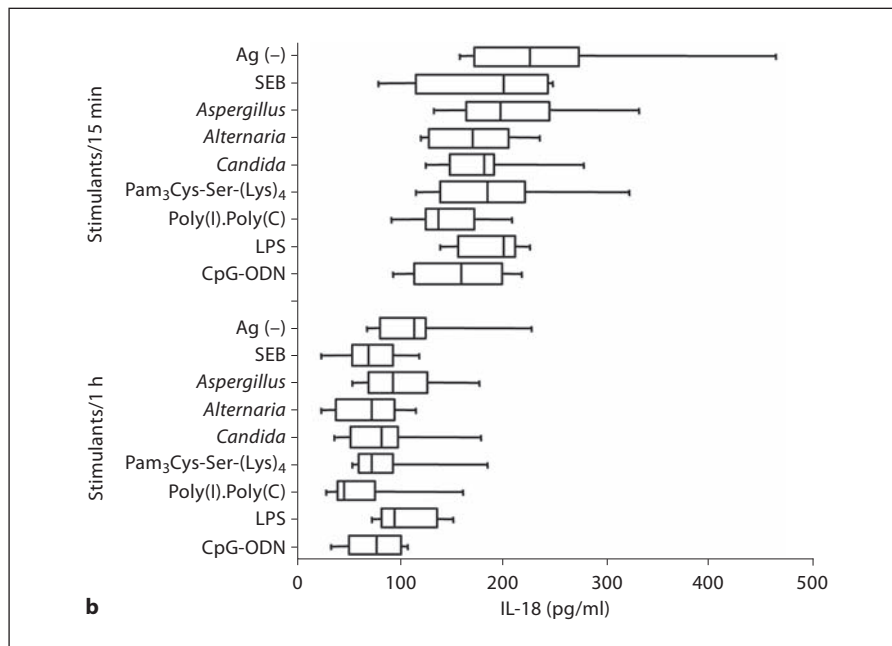
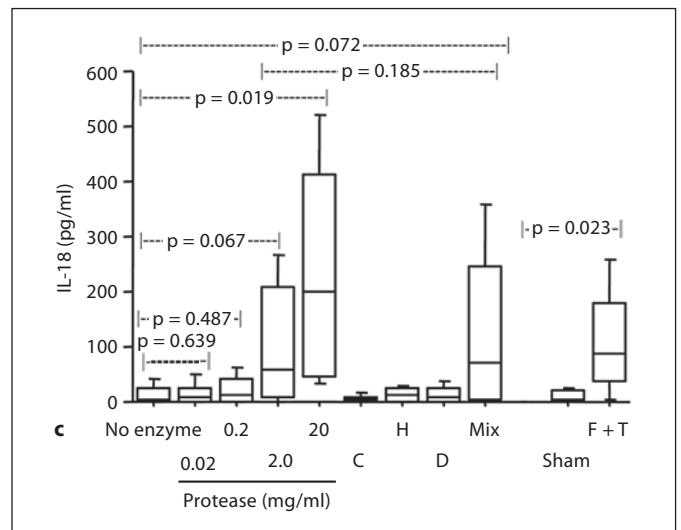
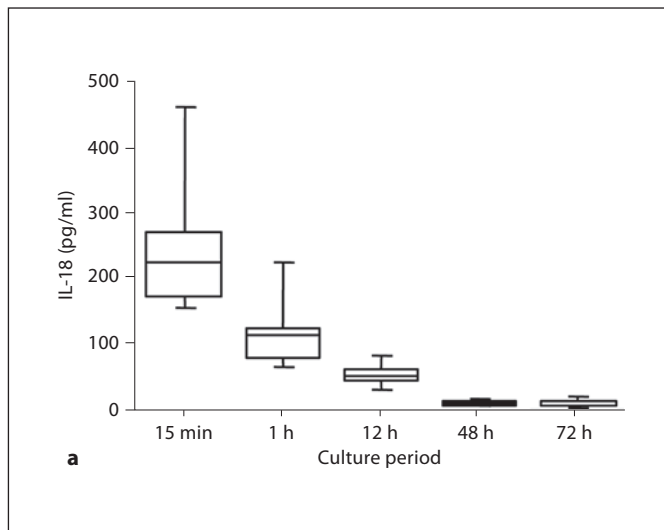
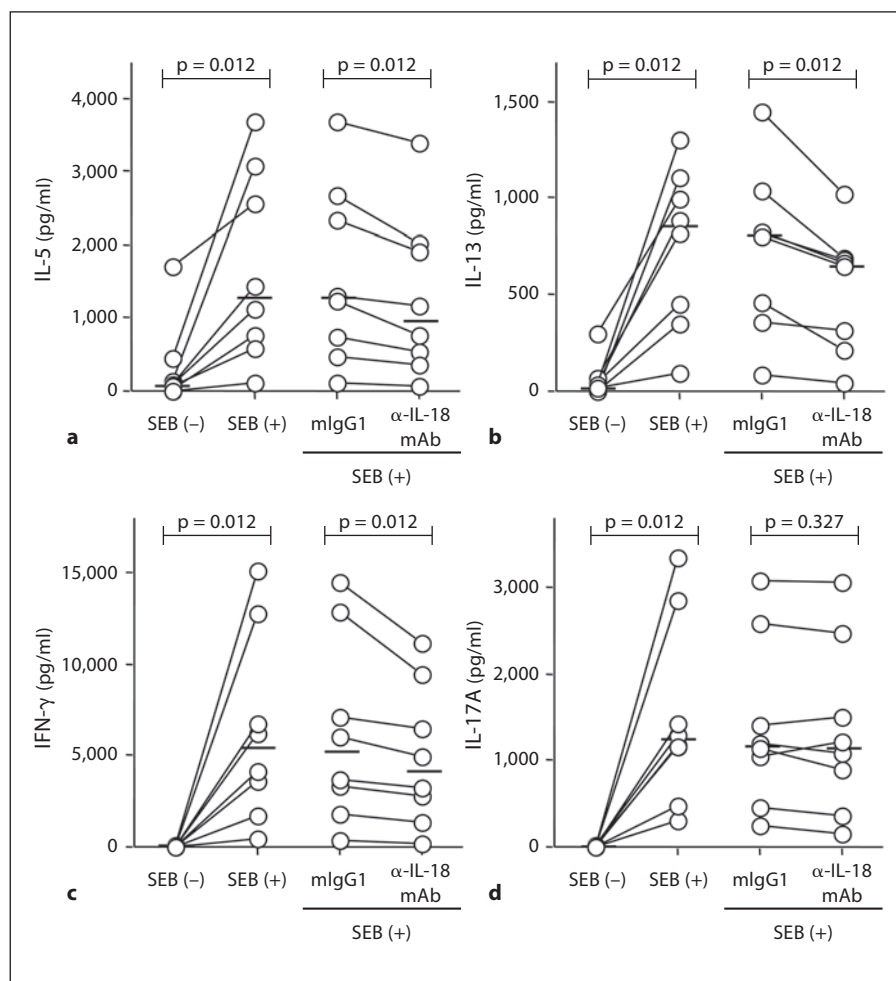


**Fig. 5.** Release of IL-18 by DNPCs. **a** Isolated DNPCs were cultured from 15 min to 72 h, and then the IL-18 concentration was determined. **b** Isolated DNPCs were stimulated with SEB, fungal antigens, and TLR agonists for 15 min or 1 h. **c** Rested DNPCs were cultured with protease, collagenase (C), hyaluronidase (H), DNase (D), and protease or a mixture of these four enzymes (Mix) for 2 h. Alternatively, rested DNPCs were exposed to one cycle of freeze-and-thaw (F + T) or sham treatment and then cultured for 2 h.

expression of IL-18 in the lamina propria was correlated with the radiological severity of sinusitis and local eosinophilia. The present findings also suggest that there is an association between the IL-18 expression and NP formation. Ex vivo analysis showed that DNPCs release IL-18 in response to both protease and a necrotic signal. In addition, neutralization of IL-18 significantly suppressed the production of SEB-induced IL-5, IL-13, and IFN- $\gamma$ . These results suggest that IL-18 exerts its pro-inflammatory effects on the CRS pathogenesis including NP formation by enhancing both the Th1 and Th2 responses.

It is well documented that IL-18 is expressed in respiratory epithelial cells, macrophages, and dendritic cells [8–10]. As our results are consistent with these findings, this suggests that epithelial cells and macrophages are major producers of IL-18 in the upper airway. In addition, double immunofluorescence staining revealed that IL-18 was also expressed on cells positive for CD79a which recognizes pan B cell lineage as well as plasma cells [30]. As it has been reported that human tonsillar B cells can express and release IL-18 [31], the present findings suggest that sinonasal B cells are also potent producers of IL-18.

**Fig. 6.** Effect of IL-18 neutralization on SEB-induced cytokine production by DNPCs. DNPCs ( $n = 8$ ) were cultured in the presence or absence of SEB. Anti-IL-18 mAb or control mouse IgG1 was added to the culture. After a 72-hour incubation, levels of IL-5 (**a**), IL-13 (**b**), IFN- $\gamma$  (**c**), and IL-17A (**d**) in the culture supernatants were measured. The horizontal line indicates the median.  $p$  values were determined by using the Wilcoxon signed-rank test.



We revealed that the number of IL-18<sup>+</sup> cells in the lamina propria was significantly higher in NPs compared to UTs without CRS. Determination of IL-18 levels in the homogenates from the tissue samples will facilitate the acquisition of more quantitative data. Recently, Zhang et al. [32] demonstrated that the expression of IL-18 was up-regulated significantly in NP tissues compared with control tissues by Western blot.

DNPCs spontaneously release IL-18 protein in a phase-dependent manner; the highest release was seen 15 min after placing the DNPCs into the culture well. These results are consistent with a previous report that demonstrated spontaneous IL-18 release by the sinus lavage cells in CRS [26]. Conversely, SEB, fungal extracts, and the TLR agonists that included Pam<sub>3</sub>Cys-Ser-(Lys)<sub>4</sub>, poly(I), poly(C), LPS, and CpG ODN did not stimulate IL-18 release by the DNPCs, which suggests that these microbial components only have a limited stimulatory effect on IL-

18 release in CRS. Although TLR agonists such as LPS have been shown to induce IL-18 release, this release appears to be dependent on the cell type and/or the micro-environment [8, 13, 17]. For example, in the human airways, alveolar macrophages from patients with interstitial lung disease release IL-18 after stimulation with LPS [33]. However, LPS is not able to alter the expression of IL-18 by bronchial epithelial cells, fibroblasts, or smooth muscle cells [17]. When a TLR2 or TLR4 agonist stimulates human dendritic cells and peripheral blood mononuclear cells, these cells only release trace amounts of IL-18. However, when aluminum hydroxide is present, these TLR agonists do cause the cells to release IL-18 [8].

IL-18 concentration was the highest at 15 min and lower in the later time points. Little is known about the kinetics of IL-18 release by human cells. This kinetics seems to be dependent on the cell types and/or stimulations. For example, Piccini et al. [34] demonstrated that LPS plus

ATP induced IL-18 release by human monocytes at 3 h, but the release was diminished at 15 h following the stimulation. Silliman et al. [35] also reported that TNF- $\alpha$  caused rapid release of IL-18 by human neutrophils at 3 min, but the release returned to baseline at 10 min. On the other hand, Foster et al. [36] showed that LPS induced IL-18 release by human monocytic THP-1 cells at 6 h, and this release was still seen 24 h after the stimulation. The lower concentration of IL-18 in the later time points may be due to the breakdown of released IL-18.

Exposure to protease and one cycle of freeze-and-thaw treatment induced release of IL-18 by rested DNPCs, which suggests that chemical and physical stresses can induce sinonasal tissues to release IL-18. NLRP3 inflammasome activates caspase 1, which leads to the processing and secretion of IL-18 [13]. Microorganisms, endogenous danger signals, and environmental irritants are also known to activate the NLRP3 inflammasome [37]. While little is known about whether protease can induce IL-18 release, a recent report demonstrated that the mite allergen Der p 1, which contains cysteine protease activity, causes caspase-1 activation and the release of IL-18 in human keratinocytes [38]. Therefore, it may be possible that this protease can trigger NLRP3 activation in the airways. As determined by the trypan blue dye exclusion test, treatment with protease at 2 mg/ml did not induce significant cell death compared with the control (22.0 vs. 24.6% for 2 h if incubation,  $p = 0.344$  by Wilcoxon's signed-rank test,  $n = 7$ ). However, the number of dead cells was significantly higher in DNPCs treated with protease at 20 mg/ml (63.4%,  $p = 0.018$ ). Alternatively, IL-18 captured by a receptor on the membrane may be cleaved off by protease treatment.

In addition, it has been reported that the NLRP3 inflammasome is activated in necrotic cells, leading to the release of IL-18 [27]. A further study also reported that necrosis-inducible stimulations such as cigarette smoke can induce IL-18 release in the airways [39, 40]. Our current findings confirmed these previous studies, as we showed that cells exposed to one cycle of freeze and thaw, which causes necrosis, led to a release of IL-18 by the cells. For clinical implications, tissue-damaged operation during the sinus surgery and smoking should be avoided in order to prevent a release of IL-18.

Murai et al. [41] demonstrated that *Alternaria* extracts could induce a rapid release of IL-18 on normal human bronchial epithelial cells (for 15 min of exposure) and in the BAL fluid of mice treated with *Alternaria* extracts (for 60 min of exposure), whereas we could not confirm the release by DNPCs in response to *Alternaria* extracts. This

discrepancy may be due to a different content of components, especially protease, between the extracts. Therefore, protease can be a positive control for IL-18 release.

Neutralization of IL-18 significantly suppressed 1 ng/ml SEB-induced IL-5, IL-13, and IFN- $\gamma$  productions by DNPCs, suggesting that IL-18 can enhance both Th1- and Th2-type cytokine production in NPs. This is consistent with the consensus that IL-18 enhances both Th1 and Th2 responses and that these responses are dependent upon the specific circumstances under which the IL-18 is presented [10]. Since both IL-5 and IL-13 are involved in eosinophilic inflammation due to their mediating eosinophil differentiation, recruitment, and survival, this may explain why the enhancing effect of IL-18 on the production of these cytokines was associated with a positive correlation between the number of IL-18<sup>+</sup> cells in the sinonasal tissues and the radiological severity of sinusitis and the degree of local eosinophilia [42]. On the other hand, the present results are not consistent with the reports that show that IL-18 can enhance IL-17A production both in vivo and in vitro, which suggests there might be a unique regulatory effect of IL-18 on the upper airways [10].

The argument may be made that 72 h of stimulation is long and the concentration of SEB is low [43]. However, we have preliminary data that 72 h of stimulation with SEB at 1 ng/ml, 10 ng/ml, and 100 ng/ml induced a similar amount of IL-5 by DNPCs. Twenty-four hours of incubation with 1 ng/ml SEB did not induce a substantial amount of IL-5 compared with 72 h of incubation. Therefore, we think that 72 h of stimulation with SEB at 1 ng/ml is optimal. In addition, we examined whether IL-18 blockade can affect cytokine production by DNPCs in response to high-dose (0.5  $\mu$ g/ml) SEB for a duration of 24 h. DNPCs produced a substantial amount of IL-13 ( $p = 0.027$  by Wilcoxon's signed-rank test,  $n = 8$ ), IFN- $\gamma$  ( $p = 0.012$ ), and IL-17A ( $p = 0.028$ ) but not IL-5 ( $p = 0.109$ ) in this condition. Similar to the result in which DNPCs were stimulated with 1 ng/ml SEB for 72 h, neutralization of IL-18 significantly suppressed 0.5  $\mu$ g/ml SEB-induced IL-13 ( $p = 0.034$ ) and IFN- $\gamma$  ( $p = 0.012$ ) but not IL-17A ( $p = 0.753$ ) for a duration of 24 h.

In conclusion, the present study provides evidence of a pro-inflammatory role for IL-18 in the pathogenesis of CRS both in vivo and ex vivo. These observations may provide a basis for the development of future therapeutic approaches that either target IL-18 or modulate danger signals involved in severe eosinophilic airway diseases, including CRSwNPs, allergic rhinitis, and bronchial asthma.

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## Disclosure Statement

The authors have no conflict of interest.

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