# Comparative analysis of cytokine release from epithelial cell cultures of the upper airway*

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## Abstract

**Introduction:** Upper airway epithelial cells show a multi-potential ability to produce a variety of cytokines/chemokines in the steady-state and under external stimuli.

**Objective:** To compare various cytokines/chemokines released from primary cultures of human nasal epithelial cells (HNECs) derived from healthy controls and subjects with allergic rhinitis (AR), chronic rhinosinusitis with nasal polyps (CRSwNPs) in non-stimulated and IL-17A-stimulated conditions.

**Methods:** The supernatants derived from HNECs of healthy control, AR, CRSwNPs were used to measure 20 of cytokines/chemokines in the non-stimulated and IL-17A-stimulated conditions.

**Results:** AR and CRSwNPs showed significant up-regulation in the release of IL-6, IL-33, and thymic stromal lymphopoietin (TSLP), and the release of IL-6, TSLP, granulocyte macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor α (TNFα) in comparison with normal controls, respectively. Secretion of GM-CSF and TNFα were enhanced in patients with nasal polyps as compared with AR. Stimulation with IL-17A enhanced the secretion of IL-8 and granulocyte-colony stimulating factor (G-CSF) in the normal control, IL-6 and IL-8 in AR, and IL-6, TSLP, G-CSF, GM-CSF and TNFα in nasal polyps.

**Conclusion:** Epithelial cells derived from AR and CRSwNPs showed up-regulation of secretion of several cytokines/chemokines both in the steady state and after IL-17A stimulation, which may contribute to the inflammatory responses of AR and CRSwNPs.

**Keywords:** allergic rhinitis, nasal polyp, epithelial cells, IL-17A

## Introduction

Upper airway diseases, in particular allergic rhinitis (AR) and chronic rhinosinusitis with nasal polyps (CRSwNPs), are characterized by intense eosinophil infiltration, mucus hypersecretion, and airway remodeling [1]. The pathognomonic features of AR are well explained by antigen-specific Th2 cells and their cytokines, such as IL-4, IL-5, and IL-13 [1,2]. On the other hand, CRSwNPs shows heterogeneous pathogeneses and various underlying immunological and pathophysiological aspects, especially in Asian populations [3-7]. Both AR and CRSwNPs are associated with elevated numbers of infiltrated tissue eosinophils. A recent study demonstrated that eosinophil counts from nasal mucosa were significantly elevated in nasal polyps compared to AR and controls [8].

Airway epithelial cells are among the first responders to invading allergens and viruses, and mount an immediate innate response to counter an infection [9]. Moreover, the airway epithelium is an important source of inflammatory mediators and is believed to be involved in regulating airway inflammation related to eosinophil recruitment. The characteristics and distinct features of cytokine/chemokine profiles in human nasal

epithelial cells (HNECs) of AR and CRSwNPs may explain the differences in eosinophil recruitment between the two allergic upper airway diseases, but have rarely been elucidated.
Interleukin (IL)-17A is the cytokine produced by Th17 cells and has attracted attention recently. IL-17A appears to act primarily on non-hematopoietic cells such as endothelial cells, epithelial cells and fibroblasts, due to the restricted expression of one of its receptor subunits, IL-17 receptor C10. IL-17 signaling has been shown to be necessary for antigen-induced allergic inflammation of the airways and T cell activation, since the response was ablated in IL-17-deficient mice [11]. IL-17 plays a critical role in neutrophil and eosinophil recruitment to the lung by regulating the expression of various cytokine and chemokines [12]. IL-17A has been found to be associated with a variety of inflammatory conditions in the upper airway, such as AR [13-17] and CRS [3,6,7,18-21]. We previously revealed that IL-17A plays a significant role in both the recruitment of eosinophils and the remodeling of the nasal polyps of CRS [6,18]. Another study also demonstrated that IL-17A expression is associated with the eosinophilic inflammation of CRSwNPs [22]. We recently quantified simultaneous expression of 27 cytokines/chemokines in the culture supernatants of nasal polyp fibroblasts to identify their effect on nasal fibroblasts. The expression of some cytokines/chemokines significantly changed after IL-17A stimulation, which could contribute to the eosinophil recruitment in CRSwNPs [23]. Furthermore, we hypothesized that IL-17A plays a key role in eosinophil recruitment into the nasal polyps of CRS through chemo-attractive factors mediated by epithelial cells. However, there have been no studies regarding the effect of IL-17A on the cytokines/chemokines released from nasal epithelial cells.

To investigate the effect of IL-17A to pathological nasal mucosa epithelium, we report in the present study a comparative analysis of a variety of cytokines/chemokines released from primary cultures of human nasal epithelial cells (HNECs) derived from healthy controls and typical nasal disorders (AR or CRSwNPs) in non-stimulated and IL-17A-stimulated conditions.

## Methods

### Patients

The study population comprised 15 patients (9 males and 6 females, mean age 53.5 ± 11.5 years) with AR, 12 patients (6 males and 6 females, mean age 47.4 ± 10.9 years) with CRSwNPs and 14 healthy subjects (10 males and 4 females, mean age 57.7 ± 5.9 years).
The diagnosis of AR was established on the basis of the Japanese diagnostic criteria for AR [24], being symptoms such as nasal congestion, rhinorrhoea, sneezing and serum allergen-specific IgE. All patients were positive for IgE specific to house dust alone.
CRSwNPs was diagnosed based on the criteria of the European position paper [25], which require the presence of more than two of the following symptoms: blockage/congestion; discharge constituted of anterior/posterior drip; facial pain/pressure; reduction or loss of smell for at least 3 months; polyps or any other endoscopic signs; mucopurulent discharge from the middle meatus; oedema/mucosal obstruction primarily in the middle meatus; and mucosal changes within the ostiomeatal complex and/or sinuses. We selected patients with nasal polyps who had an eosinophil count of more than 100 per microscopic field (magnification, x400) using 3 fields located in the subepithelial area [7].
Patients with AR presenting with sensitization to seasonal allergens, a history of bronchial asthma, or aspirin sensitivity were excluded from the study. Patients with CRSwNPs associated with current signs of purulent nasal discharge, chronic obstructive pulmonary disease, diffuse panbronchiolitis, fungal sinus disease, congenital mucociliary diseases, cystic fibrosis, or aspirin intolerance were also excluded from this study.
None of the patients were treated previously with therapeutic interventions including surgical treatments, systemic corticosteroids or other immune-modulating drugs. The study was conducted at Juntendo University Hospital, Tokyo, Japan. All patients gave their written informed consent, and the study was approved by the ethics committee of Juntendo University Faculty of Medicine.

### Isolation and culture of nasal epithelial cells

HNECs collected from the inferior concha membrane or nasal polyps were used in this study. The cells were scraped by an endometrial smear, a sampling device for cytology, after topical anesthesia with nasal spray. Then, they were collected from the device by agitation and put into growth medium (Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12) containing 10% newborn calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, and 2.5 µg/ml amphotericin B. Epithelial cells were sedimented in tubes by 5-min centrifugation at 1000 g in a microhaematocrit centrifuge. After removing the supernatants, 1 ml of growth medium was added to the tube before agitation. Epithelial cells attached to the 24-well collagen-coated plates (Corning) and were then cultured until confluency, at a density of more than $5\times10^4$ per well. The culture was maintained for 3 weeks at 37°C and 5% $CO_2$, and the medium was changed every other day.

### Flow cytometry

The cell purity was analysed by indirect flow cytometry. A cell suspension was collected and centrifugated at 1500 g for 1 min. After discarding the supernatant, 1 µg of primary antibody (anti-cytokeratin monoclonal antibody) was added, vortexed gently and incubated for 20 min on ice in the dark. After cells were washed in a tube in 500 µl buffer (2% Fetal Calf Serum in

PBS, 0.1% sodium azide), 0.5 µg of secondary antibody (FITC anti-mouse Ig, multiple adsorption) was added to the sediment of the tube and incubated for 20 min on ice. Cells were washed in 500 µl buffer and centrifugated at 1500 g for 1 min. After discarding the supernatant, cells were resuspended in 500 µl buffer for flow cytometry. Indirect flow cytometry analysis was performed using a 200 µl aliquot of the buffer. More than 95% of the cultured cells were confirmed to be epithelial cells.

**IL-17A stimulation and in vitro multiplex immunoassay**

Cultured epithelial cells were plated at a density of $5\times10^4$ per well, then incubated for 24 hours in the absence (unstimulated group) or presence (stimulated group) of recombinant human IL-17A (HumanZyme Inc.) (IL-17A; 25 ng/ml) in growth medium containing 10% Newborn Calf Serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, and 2.5 µg/ml amphotericin B. Both the epithelial cells of the non-stimulated and stimulated group were stored at -80°C until use. At the end of the incubation, the supernatants were collected and simultaneous quantification of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-33, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), tumor necrosis factor α (TNFα), thymic stromal lymphopoietin (TSLP), Eotaxin-1 and regulated on activation normal T cell expressed and secreted (RANTES), which are typical cytokines/chemokines involved in the respiratory airway inflammation, was made with a human cytokine assay system (Bio-Plex; Bio-Rad). Each value was analysed comprehensively.

**Statistical analyses**

Statistical analyses were evaluated using StatMate IV for Windows. Pearson's chi-square test was used to compare the gender. One-way analysis of variance followed by Tukey test was used to compare the mean age of 3 groups, the supernatant concentrations and the IL-17A-induced increases in each cytokine/chemokine between the 3 groups. A paired-t test (or a Wilcoxon signed-rank test, if the results were of non-normal distribution) was used to compare the concentrations of each cytokine/chemokine between the non-stimulated and the stimulated group. Results were considered to be significant if the p values were less than 0.05.

## Results

The basal and IL-17A-induced secretion levels of each cytokine/chemokine are summarized in Table 1. The levels of IL-2, Il-4, IL-5, IL-7, IL-10, and IL-13 were below the detection limit. No significant differences among culture sources (normal nasal mucosa, nasal mucosa with allergic rhinitis and nasal polyp) or the presence and absence of IL-17A were observed for IL-1β, IL-12, IFN-γ,

Table 1. Basal and IL-17A-stimulated seretion of cytokines/chemokines in normal nasal mucosa, nasal mucosa with allergic rhinitis and nasal polyp.

| | Nasal mucosa, AR(-) (n = 14) | | Nasal mucosa, AR(+) (n = 15) | | Nasal polyp (n = 12) | |
|---|---|---|---|---|---|---|
| | Unstimulated | IL-17A stimulated | Unstimulated | IL-17A stimulated | Unstimulated | IL-17A stimulated |
| IL-1β | 0.44(0-2.09) | 0.99(0-5.48) | 1.30(0-13.12) | 2.97(0-30.3) | 1.03(0-5.1) | 2.59 (0-21.0) |
| IL-2 | below detection limit | | | | | |
| IL-4 | below detection limit | | | | | |
| IL-7 | below detection limit | | | | | |
| IL-10 | below detection limit | | | | | |
| IL-12 | 4.19(0.34-8.88) | 4.47(0.34-9.77) | 3.88(0.38-16.26) | 4.05(0.12-25.66) | 3.11(0.01-16.26) | 4.55 (0.18-25.48) |
| IL-13 | below detection limit | | | | | |
| IFN-γ | 21.34(10.5-50.1) | 16.33(2.2-50.5) | 19.60(2.1-66.52) | 13.91(2.33-56.55) | 10.47(0.20-66.52) | 7.21 (2.33-14.00) |
| MCP1 | 9.63(0.20-25.22) | 18.25(0.70-66.78) | 39.30(0-176.98) | 4.56(0-25.26) | 13.09(0-27.63) | 7.05 (0-19.96) |
| MIP-1β | 3.14(0-10.18) | 3.50(0-16.38) | 1.92(0-4.95) | 2.46(0-6.713) | 3.26(0-9.07) | 5.02 (0-16.50) |
| Eotaxin-1 | 10.26(0-33.3) | 13.91(0-88.7) | 26.36(0-77.2) | 27.90(0-91.2) | 33.82(0-102.5) | 30.37 (0-113.25) |
| RANTES | 16.05(0-85.3) | 15.63(0-88.9) | 17.06(0-65.6) | 21.79(0-58.8) | 38.96(0-112.1) | 41.58 (0-154.1) |

The results are presented as the mean level (range) pg/ml. AR, allergic rhinitis.

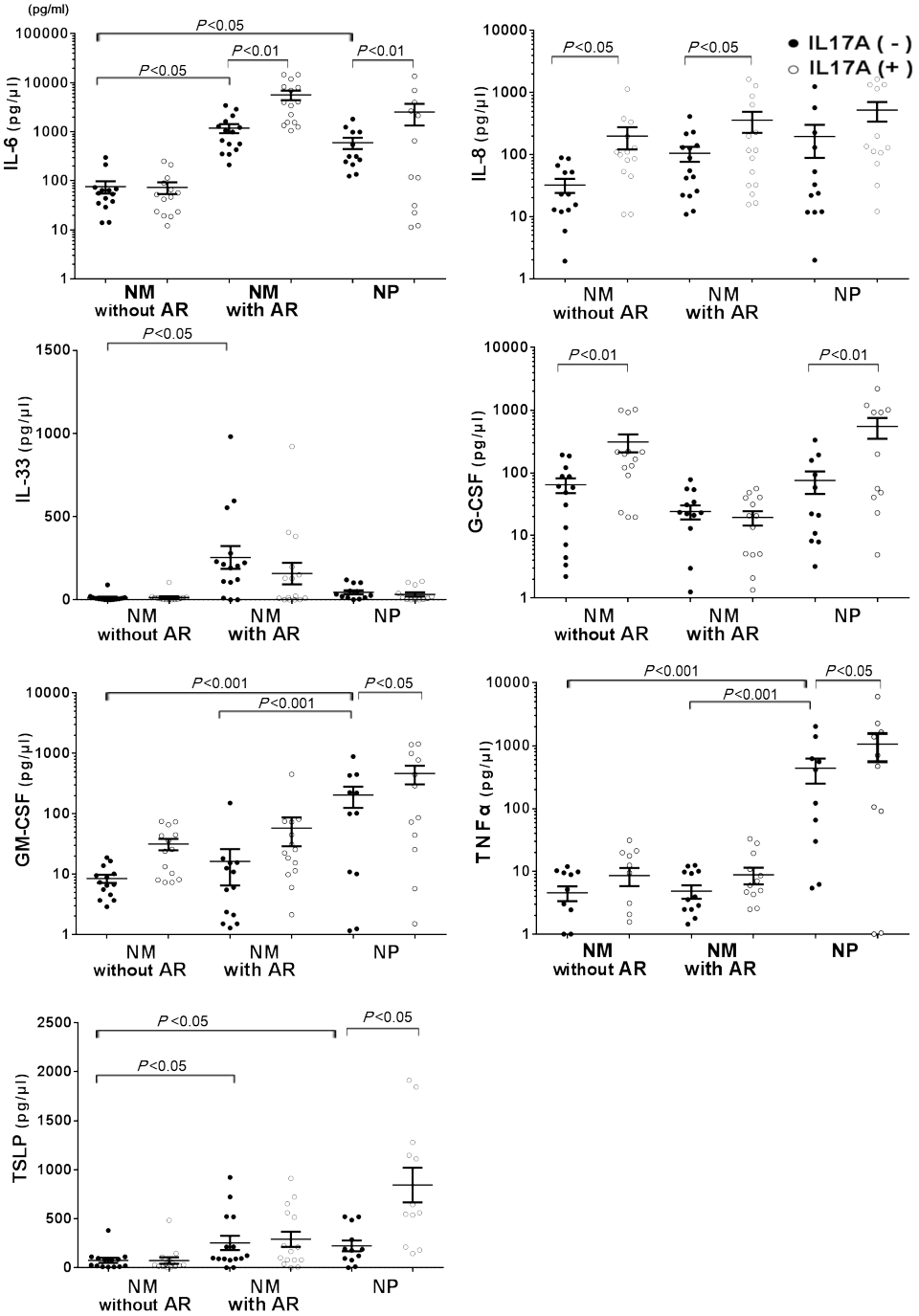


Figure 1. The comparison of the amount of the IL-6, IL-8, IL-33, G-CSF, GM-CSF, TNFα, and TSLP secretion in nasal mucosa without allergic rhinitis, nasal mucosa with allergic rhinitis, and nasal polyp in the IL-17A stimulated and unstimulated groups. The results are presented as the mean ± SD level (pg/ml). P values less than 0.05 are statistically significant. NM, nasal mucosa; AR, allergic rhinitis; NP, nasal polyp.

MCP-1, MIP-1β, Eotaxin-1 and RANTES. Figure 1 shows a scatter-plot of each finding and statistical analysis of the comparison of the basal secretion among the three culture sources, and of differences in the expression levels between the absence and presence of IL-17A stimulation in IL-6, IL-8, IL-33, G-CSF, GM-CSF, TNFα, and TSLP. For the IL-6 level, both AR and CRSwNPs showed a significant elevation of IL-17A-induced secretion in addition to the up-regulation of the basal secretion as compared with controls. IL-17A up-regulated IL-8 secretion in normal control and AR, but not in NP. The basal IL-33 level was significantly up-regulated in AR. The G-CSF secretion stimulated by IL-17A was enhanced in normal and CRSwNPs, but not in AR. In GM-CSF and

TNFα, CRSwNPs showed a significant up-regulation of the basal secretion as compared with control and AR in addition to IL-17A-induced enhancement of secretion. The basal secretion of TSLP was significantly up-regulated in AR and CRSwNPs as compared with control, and the secretion derived from NP was enhanced by IL-17A stimulation.

**Discussion**

The present study first compared the potential capacity to release a variety of cytokines/chemokines among three types of HNECs derived from healthy controls, AR, and CRSwNPs in non-stimulated and IL-17A-stimulated conditions. In AR, the release of IL-6, IL-33, and TSLP was significantly up-regulated as compared with normal controls. In nasal polyps associated with CRS, the release of IL-6, TSLP, GM-CSF, and TNFα was enhanced in comparison with normal controls. GM-CSF and TNFα secretions were enhanced in nasal polyps as compared with AR. The addition of IL-17A resulted in enhanced secretions for the following cytokines/chemokines: i) IL-8 and G-CSF in the normal controls, ii) IL-6 and IL-8 in AR, and iii) IL-6, TSLP, G-CSF, GM-CSF, and TNFα in nasal polyps.

Basal secretions of proinflammatory cytokines such as IL-6, GM-CSF, and TNFα were enhanced in the HNECs derived from AR and CRSwNPs. The studied proinflammatory cytokines/chemokines are known to be important in the pathogenesis of disorders characterized by upper airway inflammation [21,26-28]. IL-6 up-regulates the generation of naïve T cells into Th2 and Th17 cells [29]. GM-CSF prolongs eosinophil survival [30]. TNFα enhances IgE production and eosinophil migration [31]. The fact that basal secretions of GM-CSF and TNFα were greater in CRSwNPs than in AR may explain the abundance of eosinophils in the nasal polyps as compared with those in the nasal mucosa of AR. Thus, these proinflammtory cytokines may influence the growth, differentiation, proliferation and activation of inflammatory cells that contribute to the development of upper airway inflammation. More interestingly, epithelial cell-derived cytokines, TSLP and IL-33, showed an enhancement of basal secretion from HNECs of AR and CRSwNPs. Epithelial cell-derived cytokines, which are released during tissue damage, pathogen recognition or allergen exposure, have been found in AR [32-37] and CRSwNPs [38,39]. TSLP promotes the differentiation and cytokine secretion of Th2 cells [40]. IL-33 also has the capacity to induce Th2 cytokine production [41]. The up-regulation of the basal and constitutive secretion of a variety of cytokines related to Th2 cell-associated cytokines may explain the subclinical inflammatory state known as minimal persistent inflammation priming the nasal mucosa in CRS nasal polyps, as shown in AR patients [42].

Recent investigations revealed that IL-17A contributes to Th2-cell-mediated and eosinophilic lung inflammation [43]. IL-17A is known to play a critical role in neutrophil and eosinophil recruitment to the lung by regulating the expression of various inflammatory mediators [12]. In the present study, IL-8 and G-CSF were up-regulated in the HNECs derived from healthy controls after stimulation with IL-17A, which is consistent with the responses observed in primary human bronchial epithelial cells [44]. In addition, several studies demonstrated the up-regulation of IL-6, GM-CSF, and IL-19 induced by IL-17A in human bronchial epithelial cells [45-48], which was not observed in the healthy HNECs in this study. However, upper airway inflammation modified the profiles of cytokines/chemokines secretions from HNECs induced by IL-17A. The HNECs derived from AR enhanced both the release of IL-6 and IL-8. IL-6 promotes the specialization of Th17 cells from naive T cells [29]. The generated Th17 cytokines such as IL-17A might strongly induce the secretion of IL-6 from nasal epithelial cells, resulting in a vicious cycle of inflammation. Furthermore, TSLP can recruit eosinophils to sites of Th2 cytokine-associated inflammation by upregulating the cell surface expression of adhesion molecules on eosinophils. Thus, the release of various cytokines/chemokines induced by IL-17A in the HNECs derived from the nasal polyps may explain the intense accumulation of eosinophils and the resulting potent inflammation and tissue remodeling as in CRSwNPs [6,18] as compared with AR.

**Conclusion**

Epithelial cells derived from AR and CRSwNPs showed up-regulation in the secretion of several cytokines/chemokines both in the basal and IL-17A-stimulated states, which may contribute to the inflammatory responses of AR and CRSwNPs.

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## Authorship contribution

AS was involved in all stages of the study. MM, NO, HH, MH were involved in data collection and cell culture. KI was involved in drafting the manuscript. All authors gave final approval for the publication of this manuscript. All authors read and approved the final manuscript.

## Conflict of interest

None to report.

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