**IL-25 and mucosal T cells in noneosinophilic and eosinophilic chronic
rhinosinusitis**

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56 **Introduction**

57 Chronic rhinosinusitis (CRS) is a heterogeneous, commonly occurring, chronic
58 inflammatory disease of the sinus. Clinically, CRS is classified into two types based on
59 the presence of nasal polyps (NPs). Chronic rhinosinusitis without nasal polyps
60 (CRSsNP) involves Th1-skewed inflammation based on histological and immunological
61 studies ^{1,2}. The prognosis is generally satisfactory. In contrast, chronic rhinosinusitis
62 with nasal polyps (CRSwNP) is characterized by Th2-skewed inflammation. Surgery is
63 often required to treat NPs and relapse is expected. However, recent studies have
64 revealed a type of CRSwNP in East Asia that is not characterized by eosinophilic
65 inflammation and exhibits Th1-skewed inflammation in NPs ³⁻⁵. Noneosinophilic
66 chronic rhinosinusitis with nasal polyps (NECRS) differs from eosinophilic chronic
67 rhinosinusitis with nasal polyps (ECRS) in the comorbidity rate of asthma and
68 postoperative recurrence ^{3,6,7}. In Western countries, approximately 80% of NPs are
69 eosinophilic, but the mechanism underlying eosinophilic inflammation remains unclear
70 ^{4,8}. Therefore, comparing these types of CRSwNP in East Asia may be useful for
71 determining the pathogenesis of CRSwNP.

72 Various types of T cells play important roles in regulation of chronic inflammation.

73 CD4⁺ T cell lineages, such as Th1, Th2, Th17, and induced regulatory T cells (iTregs),

are defined by their cytokine production patterns⁹. Although immunological analyses of T helper-cytokines present in NPs have been performed, detailed analyses of tissue CD4⁺ T cell populations in patients with ECRS or NECRS are lacking. IL-9 producing CD4⁺ T cells represent a distinct T helper subset that has not been extensively studied in CRS^{10,11}. Our group has previously published data showing an increase in Th2 cells in NPs obtained from ECRS patients compared to NPs from NECRS, but did not study about Th9¹². Th9 cells may increase eosinophilic, allergic inflammation. These cells express the transcription factor PU.1, a factor required for Th9 development, and mouse models have demonstrated that IL-9 enhances allergic airway inflammation and remodeling¹¹.

Memory T cells are localized in peripheral tissues and are activated in an antigen-specific manner, but memory Th2 cells also contribute to innate immunity in allergy¹³. IL-25 is an epithelium-derived cytokine that causes eosinophilic inflammation by Th2 or Th9 cells via its receptor IL-17RB¹⁴⁻¹⁷. IL-25 production by airway epithelial cells is induced in response to various antigens and pathogens, and it causes Th2-skewed inflammation in a Th2-specific, antigen-independent manner. In nasal disease, allergic rhinitis is induced by specific allergen exposure and IgE-mediated inflammation. Local allergic reaction may be involved in the pathogenesis of allergic

rhinitis and CRSwNP, but the putative allergen, if it exists, has not been discovered in the majority of CRS cases, excluding superantigens or allergic fungal rhinosinusitis^{5, 18, 19}. In patients with asthma or those with eosinophilic granulomatosis with polyangiitis, IL-25 levels are elevated in the respiratory tract and peripheral blood, respectively^{20, 21}. However, the role of IL-25 in CRS has not been established.

Thus, in the current study, to investigate the mechanisms of eosinophilic inflammation in CRSwNP, we examined IL-25 expression and the characteristics of NP tissue CD4⁺ T cells including Th9.

Methods

Patients

Patients with CRSwNP were recruited from Chiba University Hospital, Kimitsu Chuo Hospital, and Chiba Otolaryngology Surgical Center. All patients met the criteria for CRSwNP defined in the European position paper on rhinosinusitis and NPs¹. The characteristics of the subjects are summarized in Table 1. All patients signed informed consent forms, and study was approved by the Ethics Committees of the Chiba University Graduate School of Medicine and each of the participating hospitals.

All patients underwent computed tomography (CT) for confirming the diagnosis of CRS and were assigned scores according to the Lund–Mackay CT scoring system²². NP tissues were obtained during endoscopic sinus surgery. Inferior nasal turbinates for use as controls were obtained during turbinectomy from patients with no sinusitis based on CT scan. The diagnosis of asthma was based upon a history of wheezing responsive to bronchodilator and confirmatory spirometry demonstrating reversibility. Aspirin exacerbated respiratory disease was based upon a history of respiratory symptoms following ingestion of aspirin or other nonsteroidal anti-inflammatory drugs. Patients were not administered systemic corticosteroids for at least 4 weeks before surgery. Atopic status was evaluated based on clinical history and the presence of IgE specific

for the house dust mite, Japanese cedar pollen, orchard grass pollen, ragweed pollen, *Candida*, *Aspergillus*, *Alternaria*, *Penicillium*, and *Cladosporium* detected using the immunoCAP method (Phadia AB, Uppsala, Sweden). CRSwNP was classified as ECRS when tissue eosinophils were >70 per high-power field (HPF), with reference to the results of Nakayama et al.⁶. The number of tissue eosinophils in an HPF ×400 was counted in a blinded manner by two observers.

Immunohistochemistry

Formaldehyde-fixed NPs and control samples were embedded in paraffin. Sections were rehydrated, and antigen retrieval was performed using protease digestion for 5 min. The sections were treated with 0.3% H₂O₂, blocked, and then incubated overnight with an anti-IgE antibody diluted 1:50 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or controls. This was followed by incubation with a secondary antibody, anti-mouse immunoglobulin conjugated to peroxidase labeled-polymer (En Vision; Dako, Carpinteria, CA, USA) for 30 min, visualization with 3,3'-diaminobenzidine tetrahydrochloride for 5 min, and counterstaining with hematoxylin. The number of tissue IgE⁺ cells per HPF was counted in a blinded manner and is presented as the sum of 10 HPFs. For immunofluorescence, samples were fixed in 4% paraformaldehyde and

equilibrated in a final concentration of 20% sucrose in phosphate-buffered saline (PBS).

Frozen sections were boiled in Histo VT One (Nacalai Tesque, Kyoto, Japan) for

antigen retrieval and blocked by 3% bovine serum albumin and human FcR Blocking

Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). The sections were then

incubated overnight with anti-IL-25 (1:200; Lifespan Biosciences, Seattle, WA, USA),

anti-IL-17RB (1:200; Lifespan Bio), anti-CD3 (1:100; Abcam, Cambridge, MA, USA),

anti-mast cell tryptase (1:200; Abcam), anti-eosinophil cationic protein (ECP) (1:100;

Santa Cruz), or anti-PECAM-1 (1:100; Santa Cruz). This was followed by incubation

with Alexa Fluor® 488- or 555-labeled secondary antibodies. The same concentrations

of isotype controls were used. Nuclei were counterstained with TO-PRO3 iodide

(Invitrogen, Carlsbad, CA, USA). Analyses were conducted using a confocal laser

microscope (LSM710; Carl Zeiss Inc., Oberkochen, Germany).

Preparation of nasal polyp mononuclear cells and tissue homogenates

Nasal polyp mononuclear cells (NPMCs) were obtained as previously described¹². In

brief, freshly obtained NPs were immediately minced and incubated in RPMI 1640

medium that contained 1 mg/ml of collagenase, 0.5 mg/ml of hyaluronidase, and 0.2

mg/ml of DNase I (Sigma-Aldrich, St Louis, MO, USA). After incubation, NPMCs

were separated using a Ficoll–Hypaque technique. Peripheral blood mononuclear cells (PBMCs) were purified from whole blood.

NP tissues from NECRS (n = 18) and ECRS (n = 22), and inferior turbinates from control (n = 16) were weighed and minced. For every 100 mg of tissue, 1 ml of PBS supplemented with aprotinin and leupeptin (Roche, Mannheim, Germany) was added. Tissues were homogenized on ice and centrifuged. The supernatants were stored at –20°C.

Cytokine assays

Cytokine levels in tissue homogenates and culture supernatants were measured using ELISA. IL-5, IL-13, and IFN- γ levels were measured as described previously²³. IL-9 and IL-25 levels were measured using an ELISA development kit (Peprotech, Rocky Hill, NJ, USA).

Isolation of CD4⁺ T cells

NECRS (nonatopic, n = 7; atopic, n = 6) and ECRS (nonatopic, n = 7; atopic, n = 13) samples from which sufficient NPMCs could be obtained ($>2 \times 10^7$) were used for cell sorting. CD3⁺CD4⁺ T cells were sorted using a FACS Aria II (BD, Franklin Lakes, NJ,

USA).

Real-time PCR

Total RNA was isolated from sorted fresh CD4⁺ T cells (Fig3 and 5A) and cultured NPMCs (Fig6A) using TRIzol reagent (Invitrogen). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Semiquantitative real-time PCR was performed using the SYBR® Green method. The mRNA levels encoding IFN- γ , IL-5, IL-9, IL-10, IL-17, T-bet, GATA-3, PU.1, FOXP3, RORC, and IL-17RB were determined, and relative gene expression was calculated using the comparative CT method. The mRNA isolated from CD4⁺ T cells of sinus mucosal tissues from a CRSsNP sample and NPMCs stimulated with CD3/CD28 and without IL-25 were used for calibration. The mRNA levels for β -actin (*ACTB*) were used to normalize the mRNA levels of the other transcripts. Primer and probe sets (Table 2) were purchased from Eurofins Operon MWG (Ebersberg, Germany).

Flow cytometry

Samples were incubated with human FcR Blocking Reagent (Miltenyi Biotec) for 30 min and stained on ice for 30 min. For intracellular staining, cells were fixed with 4%

paraformaldehyde for 10 min, permeabilized for 10 min, and blocked with 3% BSA for 30 min. Samples were stained with a combination using antibodies as follows: FITC-anti-CD3 (UCHT1; BD), PE-anti-CD45RO (UCHL1; BD), and anti-IL-9 (MH9D1; eBioscience, San Diego, CA, USA), PerCP-Cy5.5-anti-CD4 (RPA-T4; BioLegend, San Diego, CA, USA), APC-anti-CD3 (UCHT1; BD), anti-CD69 (FN50; eBioscience), anti-IFN- γ (4S.B3; BioLegend), and anti-IL-5 (TRFK5; BioLegend). Staining with these antibodies was performed according to the manufacturers' protocols. For IL-17RB staining, NPMCs were stained with anti-IL-17RB (97C691; LifeSpan Bio; final concentration 5 μ g/ml) or isotype control, followed by a secondary PE-anti-mouse IgG1 antibody. Flow cytometry was performed using a FACScalibur flow cytometer (BD) and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Cell culture

NPMCs obtained from patients with NECRS (n = 8) or ECRS (n = 10) were cultured (1×10^6 cells/ml) at 37°C for 60 h. For cytokine production, cells were stimulated with or without plate-bound anti-CD3 (OKT3) (2 μ g/ml) plus anti-CD28 (CD28.2) (10 μ g/ml) (eBioscience) antibodies in the presence or absence of IL-25 (10 or 100 ng/ml) (R&D

Systems, Minneapolis, MN, USA). For intracellular staining, cells were cultured with or without stimulation with anti-CD3/CD28 antibody-coated plates and restimulated with 25 ng/ml of phorbol-12-myristate-13-acetate and 1 μ g/ml of ionomycin in the presence of 2 μ M monensin for the final 4 h. For IL-17RB staining, cells were first cultured on an anti-CD3 antibody-coated plate for 48 h.

Statistics

Values are shown in dot plots as the median or in bar charts as mean $\pm$ standard deviation (SD). In box and whiskers plots, the central line of the box represents the median. Minimum $\pm$ maximum (whisker) and 25th $\pm$ 75th percentile (box) are presented. Statistical analysis was performed using the Mann–Whitney U test and Fisher exact test for comparing data between groups and the Wilcoxon signed-rank test for data within a group. Correlation analysis was performed using the Spearman rank correlation coefficient. P values <0.05 were considered significant.

Results

Relationships between atopic status and tissue eosinophil infiltration in patients with CRSwNP

Patients with CRSwNP were divided into groups based on atopic status and tissue eosinophil infiltration (Table 1). Total IgE levels in blood were higher in the atopic group than in the nonatopic group ($P = 0.02$), while the number of local IgE-positive cells in NPs was higher in patients with ECRS than in those with NECRS ($P < 0.001$).

Late onset asthma and aspirin-intolerant asthma (aspirin exacerbated respiratory disease) were more prevalent in subjects with ECRS compared to NECRS; whereas, these comorbidities occurred similarly in CRSwNP subjects with nonatopic and atopic status.

Cytokine levels in NP tissue homogenates

IFN- γ , IL-5, IL-13, IL-9, and IL-25 levels in NPs were measured. The IFN- γ level significantly decreased in NPs tissue homogenates from patients with ECRS compared with controls ($P < 0.05$) (Fig. 1A). In contrast, IL-5 and IL-13 levels were significantly elevated in patients with ECRS compared with controls ($P < 0.001$, $P < 0.001$) and NECRS ($P < 0.001$, $P < 0.001$). IL-9 levels were higher in patients with ECRS than in

those with NECRS, but the difference was not significant ($P = 0.07$). IL-25 levels were significantly higher in patients with ECRS than in controls ($P < 0.05$) and those with NECRS ($P < 0.05$). IL-25 levels were significantly correlated with the levels of IL-5 ($r = 0.460$, $P = 0.030$) and IL-9 ($r = 0.471$, $P = 0.027$) in ECRS samples (Fig. 1B). IL-25 levels in NPs were also correlated with the numbers of eosinophils ($r = 0.384$, $P = 0.014$) and CT scores ($r = 0.353$, $P = 0.026$) ($n = 40$) (Fig. 1C).

Source of IL-25 in NPs

NP tissues were double-stained with antibodies against IL-25 and ECP, mast cell tryptase, CD3, or PECAM-1. IL-25 was moderately expressed in epithelial cells obtained from patients with NECRS (Fig. 2A) and those with ECRS (Fig. 2B). There were a few IL-25⁺ tryptase⁺ cells, but most IL-25⁺ cells were IL-25⁺ ECP⁺ in the submucosa (Fig. 2B, C, D). IL-25⁺ CD3⁺ and IL-25⁺ PECAM-1⁺ cells were not detected (Fig. 2E, F). In these figures, the epithelium is shown in the upper region.

Representative results for 10 subjects are shown.

Characteristics of tissue CD4⁺ T cells

The levels of mRNAs encoding cytokines and major transcription factors of tissue CD4⁺

T cells from patients with NECRS (n = 13) and ECRS (n = 20) were determined using real-time PCR (Fig. 3). There was no significant difference among groups in the expression levels of mRNAs encoding T-bet, PU.1, IL-10, FOXP3, IL-17, and RORC. *IFNG* mRNA was significantly upregulated in patients with NECRS ($P < 0.01$). In contrast, *IL5*, *GATA3*, and *IL9* mRNA levels were significantly upregulated in CD4⁺ T cells from patients with ECRS ($P < 0.01$). These mRNAs levels did not differ significantly between atopic and nonatopic patients. We could not determine mRNA levels in CD4⁺ T cells isolated from inferior nasal turbinates because the surgical sample sizes were smaller than those for NPs and we could not obtain sufficient numbers of cells for sorting.

Th9 cell numbers in NPs

We previously reported that the numbers of Th2 cells in NPs were increased more in patients with ECRS as compared to patients with NECRS¹²; thus, we examined for the presence of Th9 cells. By flow cytometric analysis, most IL-9-producing cells in NPMCs expressed CD3 (Fig. 4A). Th9 cells are defined as IFN- γ ⁻IL-5⁻IL-9⁺ CD3⁺CD4⁺ cells and only a few Th9 cells were detected in PBMCs, whereas the significant number of Th9 cells were observed in NPMCs and their rate to CD4⁺ T cells

was high (Fig. 4B). Th9 cells in PBMCs from patients with NECRS (n = 5) or ECRS (n = 4), or NPMCs from patients with NECRS (n = 8) or ECRS (n = 9) were measured (Fig. 4C). The Th9 proportion in CD4⁺ T cells of NPMCs was significantly higher in patients with ECRS than in those with NECRS ($P < 0.01$).

Tissue memory CD4⁺ T cells from patients with ECRS express IL-17RB

The level of mRNA encoding IL-17RB was significantly higher in nonatopic ($P < 0.01$) and atopic ($P < 0.001$) patients with ECRS than those with NECRS (Fig 5A). *IL5* ($r = 0.490$, $P = 0.004$), *GATA3* ($r = 0.482$, $P = 0.005$), and *IL9* ($r = 0.449$, $P = 0.009$) mRNAs in tissue CD4⁺ T cells significantly correlated with the *IL17RB* mRNA levels. Moreover, the *IL17RB* mRNA levels in tissue CD4⁺ T cells were significantly correlated with the numbers of eosinophils in NPs ($r = 0.703$, $P < 0.0001$) and CT scores ($r = 0.519$, $P = 0.002$) (Fig. 5B). There was no significant correlation with *IL17RB* levels and the expression levels of the mRNAs encoding IFN- γ , T-bet, PU.1, IL-10, FOXP3, IL-17, and RORC.

Flow cytometric (Fig. 5C) and immunofluorescence (Fig. 5D) analyses detected increased IL-17RB expression in patients with ECRS. Approximately 95% of CD4⁺ T cells in NPMCs expressed CD45RO (PBMCs; NECRS n = 9, ECRS, n = 7) NPMCs;

NECRS n = 15, ECRS n = 13), and approximately 50% of CD4⁺ T cells expressed CD69 (Fig. 5E). Neither of these expression levels significantly differed between patients with ECRS or NECRS.

Influence of IL-25 on cytokine production by NPMCs

The levels of mRNAs encoding cytokines and major transcription factors of NPMCs stimulated with CD3/CD28 in the presence or absence of IL-25 (100 ng/ml) were determined using real-time PCR (NECRS, n = 6; ECRS, n = 8) (Fig. 6A). *IL5* and *GATA3* mRNAs were significantly upregulated by IL-25 stimulation in patients with ECRS (both $P < 0.05$) but not in patients with NECRS. CD3/CD28 stimulation of NPMCs induced significant IFN- γ , IL-5, and IL-9 protein levels in both group (NECRS, n = 8; ECRS, n = 10), however, the IL-5 level was higher in NPMCs from patients with ECRS than in those from patients with NECRS ($P < 0.01$) (Fig. 6B). IL-25 stimulation with anti-CD3/CD28 increased IL-5 ($P < 0.01$) and IL-9 ($P < 0.01$) production dose-dependently in NPMCs from patients with ECRS, but the same enhancement was not observed in NPMCs from patients with NECRS. IL-25 stimulation (100 ng/ml) with anti-CD3/CD28 increased IL-9 production in NPMCs from patients with ECRS ($P < 0.01$). There was no significant difference in the

314 proportion of CD4⁺ T cells in NPMCs between patients with ECRS ($11.4 \pm 3.1\%$) and
315 NECRS (10.5 ± 3.6).
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317

318 **Discussion**

319 Most CD4⁺ T cells infiltrating NPs were memory T cells with different features in
320 CRSwNP disease types classified according to eosinophils infiltration. The *IFNG*
321 mRNA level was elevated in CD4⁺ T cells obtained from tissues of patients with
322 NECRS (Fig. 3). In contrast, the *IL5* and *IL9* mRNA levels were elevated in patients
323 with ECRS, which were also characterized by increased IL-17RB expression (Fig. 5).
324 Moreover, IL-17RB expression in tissue CD4⁺ T cells significantly correlated with the
325 number of eosinophils in NPs and CT score. IL-25 levels were increased in NPs
326 obtained from patients with ECRS (Figs. 1, 2) and enhanced the production of Th2- and
327 Th9-specific cytokines induced by T cell receptor (TCR) stimulation of NPMCs
328 obtained from patients with ECRS but not NECRS (Fig. 6). For tissue Th2 and Th9
329 cells, the functions mediated through IL-25 differed between cells obtained from
330 patients with ECRS and NECRS. Our results indicated that IL-25 in NPs and IL-17RB
331 expressing tissue memory CD4⁺ T cells were associated with eosinophil infiltration in
332 CRSwNP.

333 The elevated *IL5* and *GATA3* mRNA levels in tissue CD4⁺ T cells and the IL-5 level in
334 NPs of patients with ECRS indicate that Th2 cells play important roles in infiltration of

eosinophils in NPs. Interestingly, The proportion of Th9 cells detected in NPMCs (10%) was higher than those in PBMCs (1%) (Fig. 4). Because some Th9 cells may be reprogrammed cells from Th2 cells by TGF- β for plasticity of T cell subset²⁴, this result suggests that Th9 cells were localized in the site as a consequence of a secondary immune response. We also found that the population of Th9 cells in patients with ECRS was higher than that in patients with NECRS, which was consistent with the *IL9* mRNA levels in CD4⁺ T cells. IL-9 was first purified and characterized as a T cell growth factor and is a potent stimulus for mast cell development and its Fc ϵ RI upregulation²⁵⁻²⁷. IL-9 induces goblet cell hyperplasia and upregulates mucus expression in airway epithelial cells^{28, 29}, although further studies will be required to determine the role of IL-9 in NPs.

In contrast, there was no difference in the numbers of Tregs and Th17 cells in tissue CD4⁺ T cells between patients with ECRS or NECRS (Fig. 3). FOXP3 expression decreased in patients with CRSwNP compared with the expression of factors associated with regulatory T cells in patients with CRSsNP or CRSwNP^{4, 30}. However, because the source of IL-10 or FOXP3 was not addressed in those studies, a role for Tregs was not established. Moreover, there were no differences in the populations of Th17 cells in patients with NECRS or ECRS, which agrees with another study by Jiang et al.³¹. In contrast, an increased *IFNG* mRNA level characterizes tissue CD4⁺ T cells in patients

with NECRS. These results indicate that NECRS involves Th1-skewed inflammation and that tissue Th1/Th2 balance is linked to the pathogenesis of the CRSwNP subtype.

Memory T cells are localized in peripheral tissue and defend against secondary infection^{32, 33}. Memory CD4⁺ T cells also acquire additional functions during the secondary response and are subdivided based on a homing function or expression of surface molecules in addition to subsets of cytokine production^{34, 35}. We propose that Th2 cells with such an additional functions have pathogenic potential in eosinophilic inflammation³⁶. In a study of mouse allergy models, Islam et al.³⁷ proposed that IL-5 derived from IL-17RB- and CCR8-expressing Th2 cells amplified local secondary allergic immune responses. Endo et al.³⁸ showed that IL-5 produced by CD62L^{low}CXCR3^{low} Th2 cells plays a critical role in allergic disease. In the present study, tissue memory CD4⁺ T cells from ECRS were characterized by high IL-17RB expression levels. IL-5 and IL-9 production was observed in NPMCs from patients with NECRS and ECRS, however, elevated IL-5 and IL-9 production that was induced by treatment with IL-25 occurred only in NPMCs from patients with ECRS (Fig 6B). Our findings suggest that not only the numbers but also the reactivities to IL-25 differ in memory Th2 and Th9 cells between these cells from patients with NECRS and ECRS. A subpopulation of memory Th2 and Th9 cells expressing IL-17RB may promote chronic

eosinophilic inflammation in patients with CRSwNP and cause differences in pathogenesis between ECRS and NECRS, because the IL-17RB level of tissue memory CD4⁺ T cells correlated with the number of infiltrating eosinophils and CT score respectively (Fig. 5B).

IL-25 was first described as a cytokine produced by mouse Th2 cells¹⁴. Epithelial cells, eosinophils, mast cells, and endothelial cells express IL-25 in patients with allergic diseases^{20, 21}. In the present study, we show that epithelial cells, mast cells and eosinophils express IL-25 in NPs (Fig. 2). A few mast cells in NPs expressed IL-25, although the major source of IL-25 in ECRS was most likely eosinophils based on the large numbers of IL-25⁺ ECP⁺ cells. IL-25 levels were significantly increased and were correlated with IL-5 and IL-9 levels in ECRS samples but not in NECRS (Fig. 1). Moreover, IL-25 levels in NPs were significantly correlated with the numbers of tissue eosinophils and CT scores. Accordingly, acting with IL-17RB-expressing Th2 cells, IL-25 may induce a vicious cycle that accelerates eosinophil infiltration. However, immunofluorescence analyses did not show any significant difference in IL-25 expression by epithelial cells between patients with NECRS and ECRS. In asthmatics, although IL-25 was expressed by a majority of epithelial cells in the bronchial mucosa, IL-25 expression by epithelial cells was not increased by allergen challenge²¹. These

effects may depend on an IL-25 releasing factor³⁹ or on the existence and sensitivity of IL-17RB-expressing cells, such as the Th2 and Th9 cells shown here.

Atopic status may be involved in the development of eosinophilic inflammation in patients with CRSwNP^{40, 41}. However, several clinical and pathological features of CRSwNP are related to the classification of the grade of eosinophil infiltration (Table 1, Fig. 5). Our results indicated that activating pathogenic tissue memory Th2 and Th9 cells in NPs may exacerbate chronic local eosinophilic inflammation regardless of atopic status. Clinically, we found a relationship between ECRS and late onset or aspirin-intolerant asthma (Table 1). Late onset asthma is not related to atopy and its relationship with ECRS is consistent with the features of CRSwNP observed in a large European study^{42, 43}. From the perspective of eosinophilic airway comorbidity, pathogenic memory Th2 and Th9 cells with similar properties may contribute to the onset and exacerbation of late onset asthma. This possibility is supported by findings that *IL-17RB* gene polymorphisms affected the sensitivity of individuals with asthma⁴⁴.

In conclusion, this study provides new information on a subpopulation of tissue memory CD4⁺ T cells and on the functional significance of local T cells and IL-25 in human NPs. IL-25 and memory Th2 and Th9 cells that express IL-17RB may contribute to eosinophil infiltration in CRSwNP and may make difference between NECRS and

ECRS. Further studies will be required to clarify the detailed roles of IL-25 in eosinophilic inflammation and the mechanisms of differentiation and development of pathogenic memory Th2 cells that express high IL-17RB levels, which may provide for new treatment strategies for eosinophilic airway diseases.

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571 Tables

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573 **Table 1.** Characteristics of patients

	CRSwNP (n = 69)						
	control	nonatopic versus atopic			noneosinophilic versus eosinophilic		
		nonatopic	atopic	p value	NECRS	ECRS	p value
patients, n	16	24	45		33	36	
age, median	37	60	52		54	53	
sex male/female, n	11/5	13/11	33/12		22/11	24/12	
asthma (early onset, age<16), n	2	1	4	0.65	2	3	1.0
asthma (late onset, age≥16), n	1	7	11	0.78	3	15	0.0025
aspirin intolerance, n	0	2	4	1.0	0	6	0.026
atopic status, n	14	0	45		20	25	0.46
blood total IgE level, IU/ml	160	267	516	0.020	302	548	0.12
tissue IgE ⁺ cells count, /10HPFs	35	27	41	0.44	7	63	<0.001
blood eosinophil count, /mm ³	327	331	398	0.25	222	514	<0.001
tissue eosinophil count, /HPF	11	110	105	0.67	14	186	<0.001

574 CRSwNP, Chronic rhinosinusitis with nasal polyps

575 NECRS, non eosinophilic chronic rhinosinusitis with nasal polyps; ECRS, eosinophilic chronic

576 rhinosinusitis with nasal polyps; HPF, high power field

577 Statistical analysis was performed by Mann-Whitney-U test and Fisher exact test.

578 **Inferior turbinates were used as control tissue.**

579

580 **Table 2**

581 PCR primers used

Primer set	Sense primer, 5'-3'	Antisense primer, 5'-3'
IFN- γ	GAGTGTGGAGACCATCAAGGAAG	TGCTTTGCGTTGGACATTCAAGTC
IL-5	AGCTGCCTACGTGTATGCCA	GCAGTGCCAAGGTCTCTTTCA
IL-9	GACCAGTTGTCTCTGTTTGGGC	TTTCACCCGACTGAAAATCAGTGG
IL-10	TCTCCGAGATGCCTTCAGCAGA	TCAGACAAGGCTTGGCAACCCA
IL-17	CGGACTGTGATGGTCAACCTGA	GAGCATTGATGCAGCCCAAGTTC
T-bet	ATTGCCGTGACTGCCTACCAGA	GGAATTGACAGTTGGGTCCAGG
GATA-3	GCGGGCTCTATCACAAAATGA	GCTCTCCTGGCTGCAGACAGC
PU.1	GACACGGATCTATACCAACGCC	CCGTGAAGTTGTTCTCGGCGAA
Foxp3	GCCCTTGGACAAGGACCCGATG	CATTTGCCAGCAGTGGGTAGGA
RORc	TTTTCCGAGGATGAGATTGC	CTTTCCACATGCTGGCTACA
IL-17RB	ACAAACGCGAGCTTCAGTGGTG	ATGCAGTCGCTGCCACAAGTAG

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Figure legends

Fig. 1. (A) Measurement of cytokines in tissue homogenates of inferior turbinates from controls and NPs from NECRS and ECRS. (B) Correlation of the expression levels of IL-25 with IL-5 and IL-9 in nasal polyps. (C) Correlation of the expression levels of IL-25 in nasal polyps with the number of eosinophils and the Lund–Mackay computed tomography (CT) score. $*P < 0.05$, $***P < 0.001$.

Fig. 2. Immunofluorescence analysis of IL-25 expression in NPs. (A, B) IL-25 and ECP-staining of NPs (Original magnification, $\times 40$). Scale bar = 20 μm . (C–F) IL-25 and ECP (C), tryptase (D), CD3 (E), and PECAM-1 (F) (Original magnification, $\times 160$). Scale bar = 5 μm .

Fig. 3. The levels of mRNAs encoding cytokine and transcription factor in tissue CD4^+ T cells isolated from nasal polyps. $**P < 0.01$.

Fig. 4. Flow cytometric analysis of IL-9-producing cells. (A) Intracellular staining of IL-9 in nasal polyp mononuclear cells (NPMCs) from patients with ECRS. The

percentage of positive cells is shown. (B) Th9 cells were defined as IFN- γ ⁻ IL-5⁻ IL-9⁺ cells, after gating on CD3⁺ CD4⁺ cells. (C) The proportion of Th9 cells in CD4⁺ T cells. $**P < 0.01$.

Fig. 5. Expression of IL-17RB in tissue CD4⁺ T cells. (A) Real-time PCR analysis of IL-17RB expression in sorted tissue CD4⁺ T cells. (B) Correlation of the IL-17 RB mRNA level in tissue CD4⁺ T cells with the number of tissue eosinophils and computed tomography (CT) score. (C) Flow cytometric analysis of IL-17RB expression. The figure presents the percentage of IL-17RB-expressing cells gated on CD3⁺CD4⁺ cells. The gray histogram represents isotype control staining. (D) Immunofluorescence analysis of IL-17RB. Scale bar = 5 μ m. The results of flow cytometry and immunofluorescence represent of 4–6 patients. (E) Flow cytometric analysis of CD45RO and CD69 expression by CD3⁺CD4⁺ T cells. $**P < 0.01$, $***P < 0.001$. N.S., not significant.

Fig. 6. Influence of IL-25 on IFN- γ , IL-5, and IL-9 production by nasal polyp mononuclear cells (NPMCs). (A) Expression levels of genes encoding IFN- γ , IL-5, IL-9, T-bet, GATA-3 and PU.1 in NPMCs. (B) IFN- γ , IL-5 and IL-9 protein levels in the

629 supernatants. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N.D, not detected. N.S, not

630 significant.

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Figure 1

Figure 2

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Figure 3

Figure 4

Figure 5

Figure 6

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