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Interleukin-25 and mucosal T cells in noneosinophilic and eosinophilic chronic rhinosinusitis

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ABSTRACT

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is a heterogeneous disease of uncertain pathogenesis. Memory T cells acquire additional functions during the secondary response and play important roles in chronic inflammation.

Objective: To investigate characteristics of tissue memory CD4⁺ T cells obtained from patients with non-eosinophilic CRSwNP (NECRS) and eosinophilic CRSwNP (ECRS) by focusing on the influence of interleukin (IL)-25.

Methods: Pro-allergic cytokines in tissue homogenates were measured using enzyme-linked immunosorbent assays. NP mononuclear cells and CD4⁺ T cells were isolated from NPs from patients with CRSwNP. Cytokine expression and CD4⁺ T-cell subpopulations were analyzed using enzyme-linked immunosorbent assay, flow cytometry, and real-time polymerase chain reaction.

Results: The IL-25 level in NPs increased in patients with ECRS. IL-5 and IL-9 mRNA levels expressed by tissue CD4⁺ T cells were significantly elevated in patients with ECRS. Most infiltrating CD4⁺ T cells in ECRS and NECRS expressed CD45RO; however, regardless of the atopic status, high IL-17RB levels were detected in CD4⁺ T cells from patients with ECRS. IL-17RB mRNA levels expressed by tissue CD4⁺ T cells significantly correlated with the number of eosinophils in NPs. Elevation of IL-5 and IL-9 production was found in NP mononuclear cells from patients with ECRS, but not in those from patients with NECRS, by stimulation with IL-25 under T-cell receptor stimulation.

Conclusion: Interleukin-25 and a subpopulation of tissue T-helper type 2 and 9 cells that express increased IL-17RB levels could contribute to infiltration of eosinophils in NPs and could have produced the pathologic difference between NECRS and ECRS.

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Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous, commonly occurring, chronic inflammatory disease of the sinus. Clinically, CRS is classified into 2 types based on the presence of nasal polyps (NPs). CRS without NPs (CRSsNP) involves T-helper cell type (T_H) 1-skewed inflammation based on histologic and immunologic

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studies.^{1,2} The prognosis is generally satisfactory. In contrast, CRS with NPs (CRSwNP) is characterized by T_H2-skewed inflammation. Surgery is often required to treat NPs and relapse is expected. However, recent studies have described a type of CRSwNP in East Asia that is not characterized by eosinophilic inflammation and exhibits T_H1-skewed inflammation in NPs.^{3–5} Noneosinophilic CRSwNP (NECRS) differs from eosinophilic CRSwNP (ECRS) in the comorbidity rate of asthma and postoperative recurrence.^{3,6,7} In Western countries, approximately 80% of NPs are eosinophilic, but the mechanism underlying eosinophilic inflammation remains unclear.^{4,8} Therefore, comparing these types of CRSwNP in East Asia could be useful for determining the pathogenesis of CRSwNP.

Various types of T cells play important roles in the regulation of chronic inflammation. CD4⁺ T-cell lineages, such as T_H1, T_H2, and T_H17, and induced regulatory T cells, are defined by their cytokine

production patterns.⁹ Although immunologic 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. Interleukin (IL)-9–producing CD4⁺ T cells represent a distinct T-helper subset that has not been extensively studied in CRS.^{10,11} The authors previously published data showing an increase in T_H2 cells in NPs obtained from patients with ECRS compared with NPs from those with NECRS but did not study T_H9.¹² T_H9 cells might increase eosinophilic, allergic inflammation. These cells express the transcription factor PU.1, a factor required for T_H9 development, and mouse models have demonstrated that IL-9 enhances allergic airway inflammation and remodeling.¹¹

Memory T cells are localized in peripheral tissues and are activated in an antigen-specific manner, but memory T_H2 cells also contribute to innate immunity in allergy.¹³ IL-25 is an epithelium-derived cytokine that causes eosinophilic inflammation by T_H2 or T_H9 cells by its receptor IL-17RB.^{14–17} IL-25 production by airway epithelial cells is induced in response to various antigens and pathogens, and it causes T_H2-skewed inflammation in a T_H2-specific, antigen-independent manner. In nasal disease, allergic rhinitis is induced by specific allergen exposure and IgE-mediated inflammation. Local allergic reaction might be involved in the pathogenesis of allergic rhinitis and CRSwNP, but the putative allergen, if it exists, has not been discovered in most CRS cases, excluding superantigens or allergic fungal rhinosinusitis.^{5,18,19} In patients with asthma and 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, to investigate the mechanisms of eosinophilic inflammation in CRSwNP, the present study examined IL-25 expression and the characteristics of NP tissue CD4⁺ T cells including T_H9.

Methods

Patients

Patients with CRSwNP were recruited from Chiba University Hospital, Kimitsu Chuo Hospital, and the Chiba Otolaryngology Surgical Center (Chiba, Japan). All patients met the criteria for CRSwNP defined by the European position paper on rhinosinusitis and NPs.¹ Patients' characteristics are presented in Table 1. All patients signed informed consent forms, and the 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 tomographic (CT) scanning to confirm the diagnosis of CRS and were assigned scores according to

the CT scoring system of Lund and Mackay.²² 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 on a history of wheezing responsive to bronchodilator and confirmatory spirometry demonstrating reversibility. Aspirin-exacerbated respiratory disease was based on a history of respiratory symptoms after 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 house dust mite, Japanese cedar pollen, orchard grass pollen, ragweed pollen, and *Candida*, *Aspergillus*, *Alternaria*, *Penicillium*, and *Cladosporium* species detected using the ImmunoCAP method (Phadia AB, Uppsala, Sweden). CRSwNP was classified as ECRS when the tissue eosinophil count was higher than 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 2 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 minutes. The sections were treated with 0.3% H₂O₂, blocked, and then incubated overnight with an anti-IgE antibody diluted to 1:50 (Santa Cruz Biotechnology, Inc, Santa Cruz, California) or controls. This was followed by incubation with a secondary antibody, an antimouse immunoglobulin conjugated to a peroxidase-labeled polymer (En Vision; Dako, Carpinteria, California) for 30 minutes, visualization with 3,3'-diaminobenzidine tetrahydrochloride for 5 minutes, 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. 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 incubated overnight with anti-IL-25 (1:200; Lifespan Biosciences, Seattle, Washington), anti-IL-17RB (1:200; Lifespan Biosciences), anti-CD3 (1:100; Abcam, Cambridge, Massachusetts), anti-mast cell tryptase (1:200; Abcam), anti-eosinophil cationic protein (ECP; 1:100; Santa Cruz Biotechnology), or anti-platelet endothelial cell adhesion molecule-1 (PECAM-1; 1:100; Santa Cruz

Table 1
Characteristics of patients^a

	Control	CRSwNP (n = 69)			Noneosinophilic vs eosinophilic		
		Nonatopic vs atopic					
		Nonatopic	Atopic	P value	NECRS	ECRS	P value
Patients, n	16	24	45		33	36	
Age (y), median	37	60	52		54	53	
Men/women, n	11/5	13/11	33/12		22/11	24/12	
Asthma (early onset, age <16 y), n	2	1	4	.65	2	3	1.0
Asthma (late onset, age ≥16 y), n	1	7	11	.78	3	15	.0025
Aspirin intolerance, n	0	2	4	1.0	0	6	.026
Atopic status, n	14	0	45		20	25	.46
Blood total IgE level, IU/mL	160	267	516	.020	302	548	.12
Tissue IgE ⁺ cell count/10 HPFs	35	27	41	.44	7	63	<.001
Blood eosinophil count/mm ³	327	331	398	.25	222	514	<.001
Tissue eosinophil count/HPF	11	110	105	.67	14	186	<.001

Abbreviations: CRSwNP, chronic rhinosinusitis with nasal polyps; ECRS, eosinophilic chronic rhinosinusitis with nasal polyps; HPF, high-power field; NECRS, noneosinophilic chronic rhinosinusitis with nasal polyps.

^aInferior turbinates were used as control tissue. Statistical analysis was performed by the Mann-Whitney U test and the Fisher exact test.

Table 2

Polymerase chain reaction primers used

Primer set	Sense primer, 5'–3'	Antisense primer, 5'–3'
IFN- γ	GAGTGTGGAGACCATCAAGGAAG	TGCTTTGCGTTGGACATTCAAGTC
IL-5	AGCTGCCTACGTGTATGCCA	GCAGTGCCAAGGTCTCTTTCA
IL-9	GACCAGTTGTCTCTGTTGGGC	TTTACCCGACTGAAAATCAGTGG
IL-10	TCTCCGAGATGCCTTCAGCAGA	TCAGACAAGGCTTGGCAACCCA
IL-17	CGGACTGTGATGGTCAACCTGA	GAGCATTGATGCAGCCCAAGTTC
T-bet	ATTGCCGTGACTGCCTACCAGA	GGAATTGACAGTTGGGTCCAGG
GATA-3	GCGGGCTCTATCACAAAATGA	GCTCTCTGGCTGCAGACAGC
PU.1	GACACGGATCTATACCAACGCC	CCGTGAAGTTGTTCTCGCGCAA
Foxp3	GCCCTTGACAAGGACCCGATG	CATTGGCCAGCAGTGGGTAGGA
RORC	TTTTCGAGGATGAGATTGC	CTTTCCACATGCTGGCTACA
IL-17RB	ACAAACGCGAGCTTCAGTGTC	ATGCAGTCGCTGCCACAAGTAG

Abbreviations: IFN- γ , interferon- γ ; IL, interleukin.

Biotechnology). 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 TOPRO3 iodide (Invitrogen, Carlsbad, California). 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 described.¹² 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 DNaseI (Sigma-Aldrich, St Louis, Missouri). After incubation, NPMCs were separated using a Ficoll-Hypaque technique. Peripheral blood mononuclear cells (PBMCs) were purified from whole blood.

Nasal polyp tissues from patients with NECRS ($n = 18$) and ECRS ($n = 22$) and inferior turbinates from control subjects ($n = 16$) were weighed and minced. For every 100 mg of tissue, 1 mL of phosphate buffered saline 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 an enzyme-linked immunosorbent assay. IL-5, IL-13, and interferon- γ (IFN- γ) levels were measured as described previously.²³ IL-9 and IL-25 levels were measured using an enzyme-linked immunosorbent assay development kit (Pepro-tech, Rocky Hill, New Jersey).

Isolation of CD4⁺ T Cells

The 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 FACSria II (BD, Franklin Lakes, New Jersey).

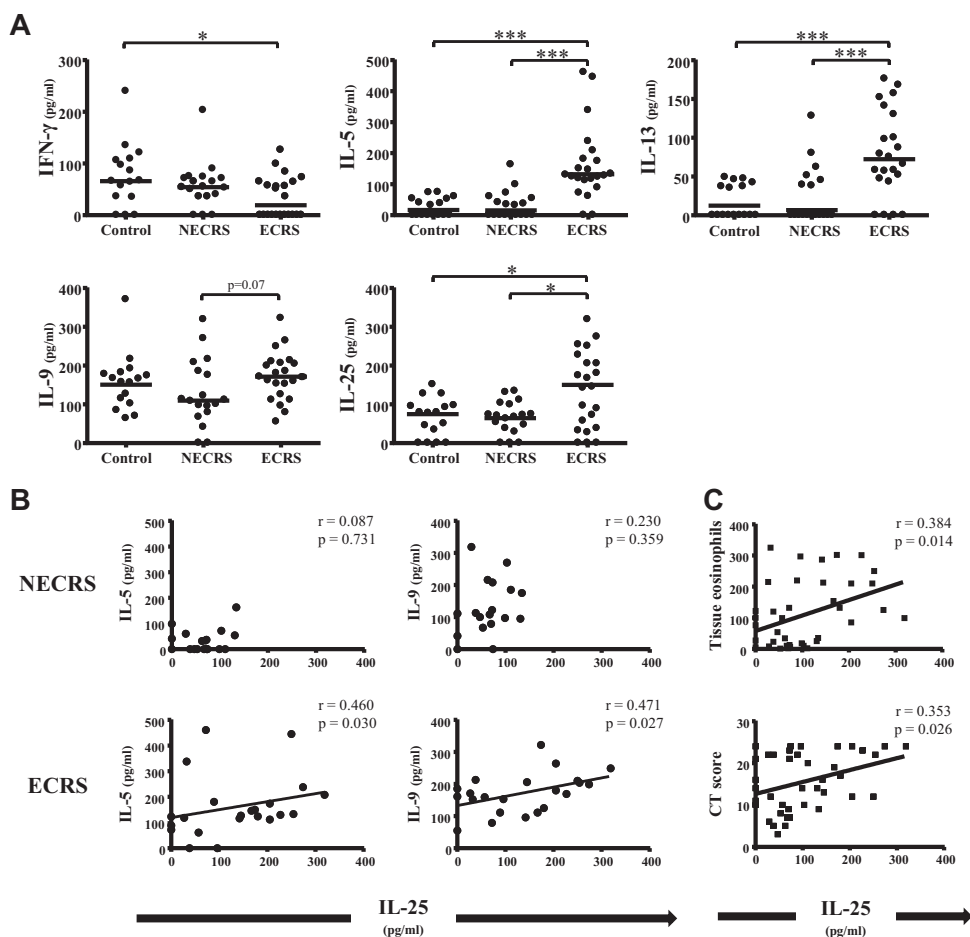


Figure 1. (A) Measurement of cytokines in tissue homogenates of inferior turbinates from controls and nasal polyps from patients with noneosinophilic (NECRS) and eosinophilic (ECRS) chronic rhinosinusitis with nasal polyps. (B) Correlation of expression levels of interleukin (IL)-25 with IL-5 and IL-9 in nasal polyps. (C) Correlation of expression levels of IL-25 in nasal polyps with the number of eosinophils and the Lund-Mackay computed tomographic score. $P < .05$, $***P < .001$. IFN- γ , interferon- γ .

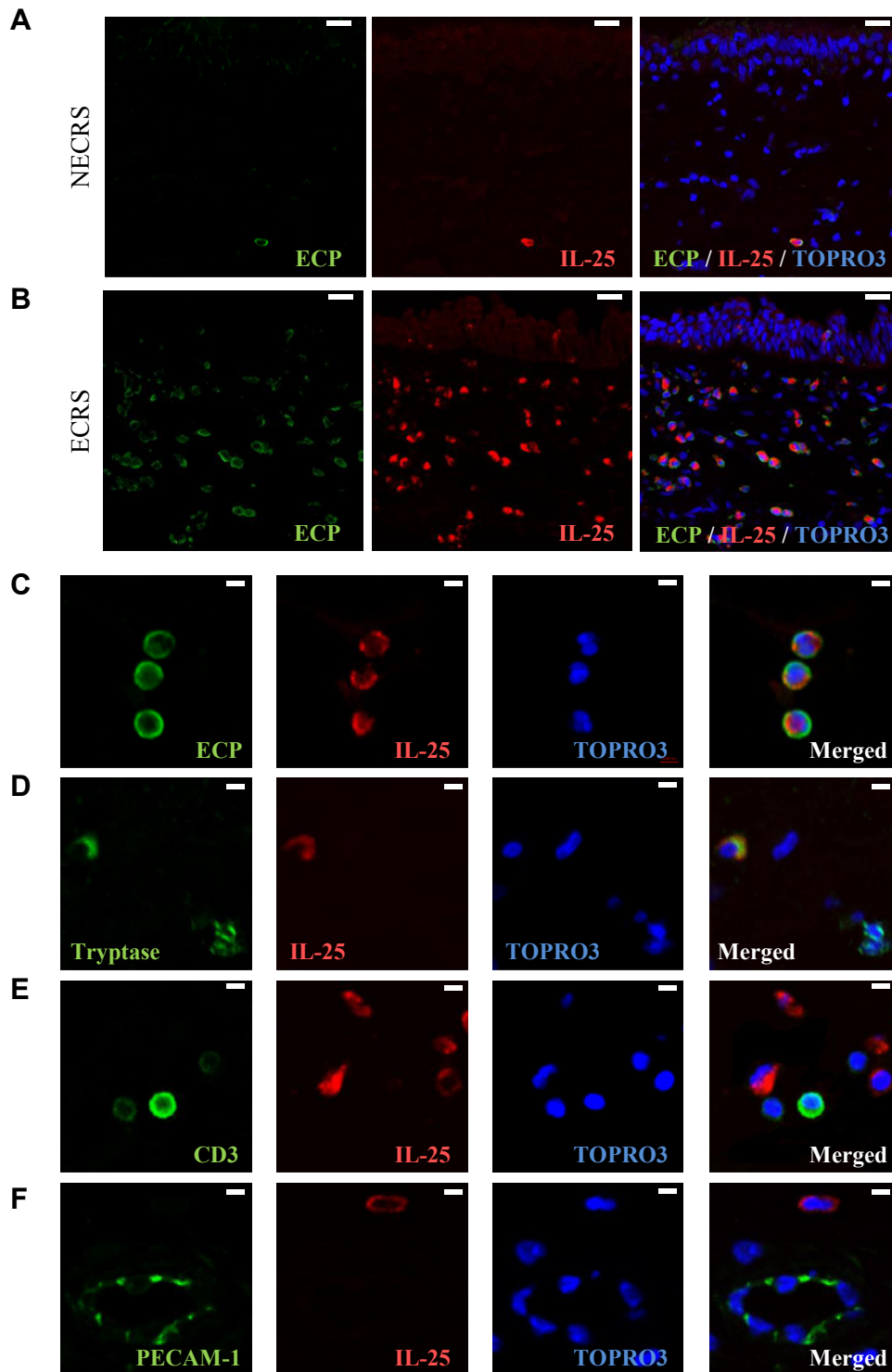


Figure 2. Immunofluorescence analysis of interleukin (IL)-25 expression in nasal polyps. (A, B) IL-25 and eosinophil cationic protein (ECP) staining of nasal polyps (original magnification $\times 40$). Scale bar = 20 μm . IL-25 and (C) ECP, (D) tryptase, (E) CD3, and (F) platelet endothelial cell adhesion molecule-1 (PECAM-1) (original magnification $\times 160$). Scale bar = 5 μm . ECRS, eosinophilic chronic rhinosinusitis with nasal polyps; NECRS, noneosinophilic chronic rhinosinusitis with nasal polyps.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from sorted fresh CD4⁺ T cells (Figures 3, 5A) and cultured NPMCs using TRIzol reagent (Invitrogen). The cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California). Semiquantitative real-time polymerase chain reaction 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

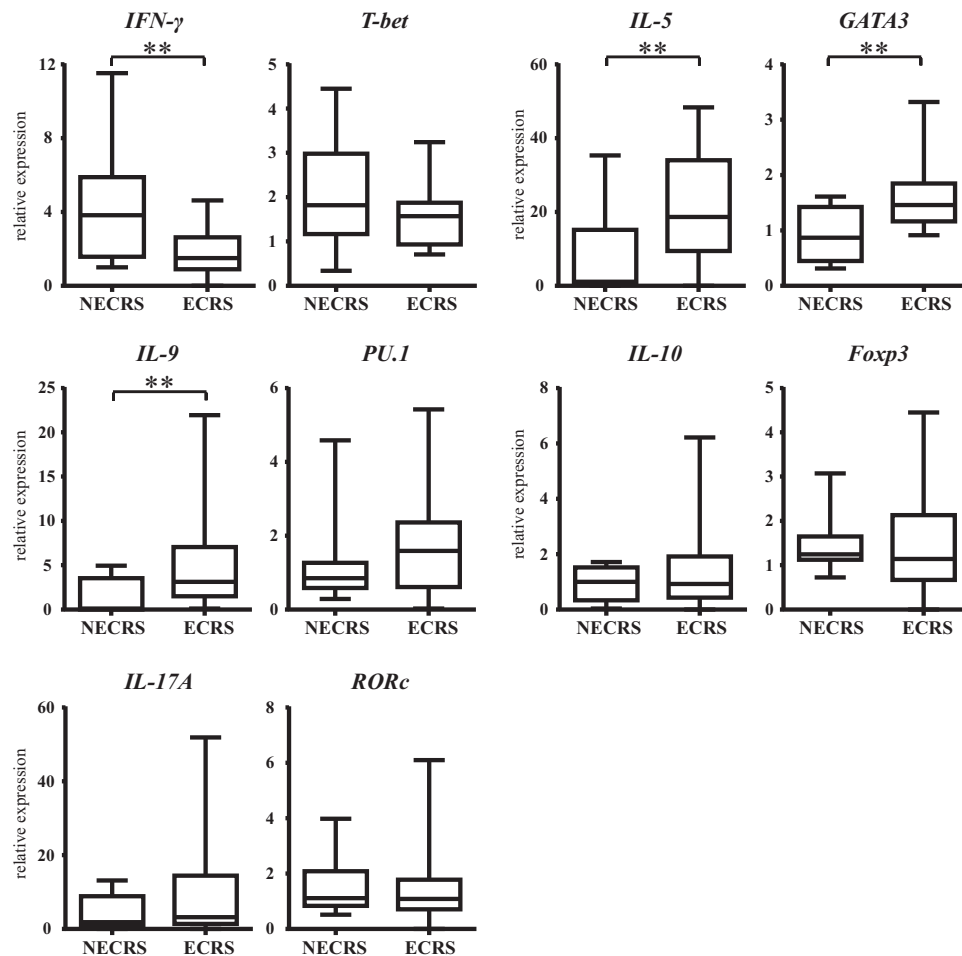


Figure 3. Levels of mRNAs encoding the cytokine and transcription factor in tissue CD4⁺ T cells isolated from nasal polyps. ***P* < .01. ECRS, eosinophilic chronic rhinosinusitis with nasal polyps; IFN- γ , interferon- γ ; NECRS, noneosinophilic chronic rhinosinusitis with nasal polyps.

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 minutes and stained on ice for 30 minutes. For intracellular staining, cells were fixed with 4% paraformaldehyde for 10 minutes, made permeable for 10 minutes, and blocked with 3% bovine serum albumin for 30 minutes. Samples were stained with a combination using antibodies as follows: fluorescein isothiocyanate and anti-CD3 (UCHT1; BD); phycoerythrin and anti-CD45RO (UCHL1; BD); anti-IL-9 (MH9D1; eBioscience, San Diego, California); peridinin chlorophyll protein complex, cyanine 5.5, and anti-CD4 (RPA-T4; BioLegend, San Diego, California); allophycocyanin and 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 Biosciences; final concentration 5 μ g/mL) or isotype control, followed by a secondary phycoerythrin and antimouse IgG1 antibody. Flow cytometry was performed using a FACScalibur flow cytometer (BD) and data were analyzed using FlowJo software (Tree Star, San Carlos, California).

Cell Culture

The NPMCs obtained from patients with NECRS (*n* = 8) and those with ECRS (*n* = 10) were cultured (1×10^6 cells/mL) at 37°C

for 60 hours. 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, Minnesota). For intracellular staining, cells were cultured with or without stimulation with anti-CD3–CD28 antibody-coated plates and re-stimulated with 25 ng/mL of phorbol-12-myristate-13-acetate and 1 μ g/mL of ionomycin in the presence of 2 μ mol/L of monensin for the final 4 hours. For IL-17RB staining, cells were first cultured on an anti-CD3 antibody-coated plate for 48 hours.

Statistics

Values are shown in dot plots as the median or in bar charts as mean $\pm$ standard deviation. 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 the 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 less than .05 were considered significant.

Results

Relations 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

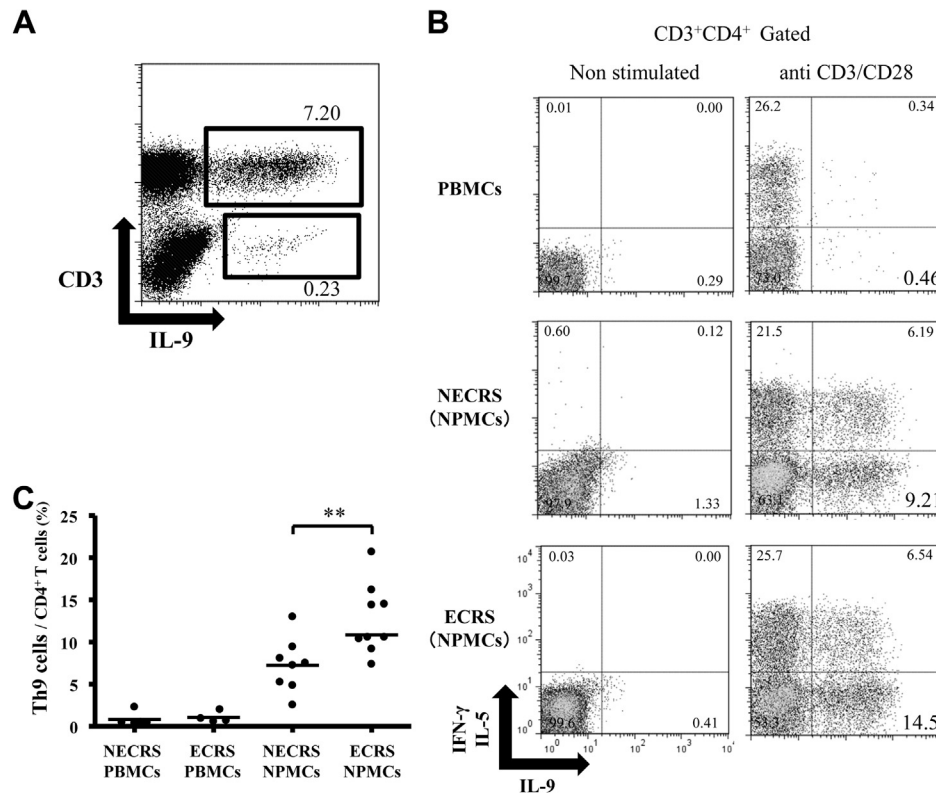


Figure 4. Flow cytometric analysis of interleukin (IL)-9-producing cells. (A) Intracellular staining of IL-9 in nasal polyp mononuclear cells (NPMCs) from patients with eosinophilic chronic rhinosinusitis with nasal polyps (ECRS). The percentage of positive cells is shown. (B) T-helper type 9 (Th9) cells were defined as IFN- γ ⁻ IL-5⁻ IL-9⁺ cells after gating on CD3⁺ CD4⁺ cells. (C) Proportion of Th9 cells in CD4⁺ T cells. ** $P < .01$. IFN- γ , interferon- γ ; NECRS, noneosinophilic chronic rhinosinusitis with nasal polyps; PBMC, peripheral blood mononuclear cell.

blood were higher in the atopic group than in the nonatopic group ($P = .02$), whereas the number of local IgE-positive cells in NPs was larger in patients with ECRS than in those with NECRS ($P < .001$). Late-onset asthma and aspirin-intolerant asthma (aspirin-exacerbated respiratory disease) were more prevalent in patients with ECRS compared with those with NECRS, whereas these comorbidities occurred similarly in patients with CRSwNP and nonatopic and atopic status.

Cytokine Levels in NP Tissue Homogenates

The IFN- γ , IL-5, IL-13, IL-9, and IL-25 levels in NPs were measured. The IFN- γ level significantly decreased in NP tissue homogenates from patients with ECRS compared with controls ($P < .05$; Fig 1A). In contrast, IL-5 and IL-13 levels were significantly elevated in patients with ECRS compared with controls ($P < .001$, $P < .001$) and NECRS ($P < .001$, $P < .001$). IL-9 levels were higher in patients with ECRS than in those with NECRS, but the difference was not significant ($P = .07$). IL-25 levels were significantly higher in patients with ECRS than in controls ($P < .05$) and those with NECRS ($P < .05$). IL-25 levels were significantly correlated with levels of IL-5 ($r = 0.460$, $P = .030$) and IL-9 ($r = 0.471$, $P = .027$) in ECRS samples (Fig 1B). IL-25 levels in NPs also were correlated with the numbers of eosinophils ($r = 0.384$, $P = .014$) and CT scores ($r = 0.353$, $P = .026$; $n = 40$; Fig 1C).

Source of IL-25 in NPs

Nasal polyp tissues were doubly 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 patients 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 polymerase chain reaction (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 ($P < .01$). In contrast, *IL5*, *GATA3*, and *IL9* mRNA levels were significantly upregulated in CD4⁺ T cells from patients with ECRS ($P < .01$). These mRNAs levels did not differ significantly between the atopic and nonatopic groups. The mRNA levels in CD4⁺ T cells isolated from inferior nasal turbinates could not be determined because the surgical samples were smaller than those for NPs and sufficient numbers of cells could not be obtained for sorting.

Th9 Cell Numbers in NPs

The authors previously reported that the numbers of Th2 cells in NPs were increased more in patients with ECRS than in patients with NECRS¹²; thus, NPs were 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 a significant number of Th9 cells was 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

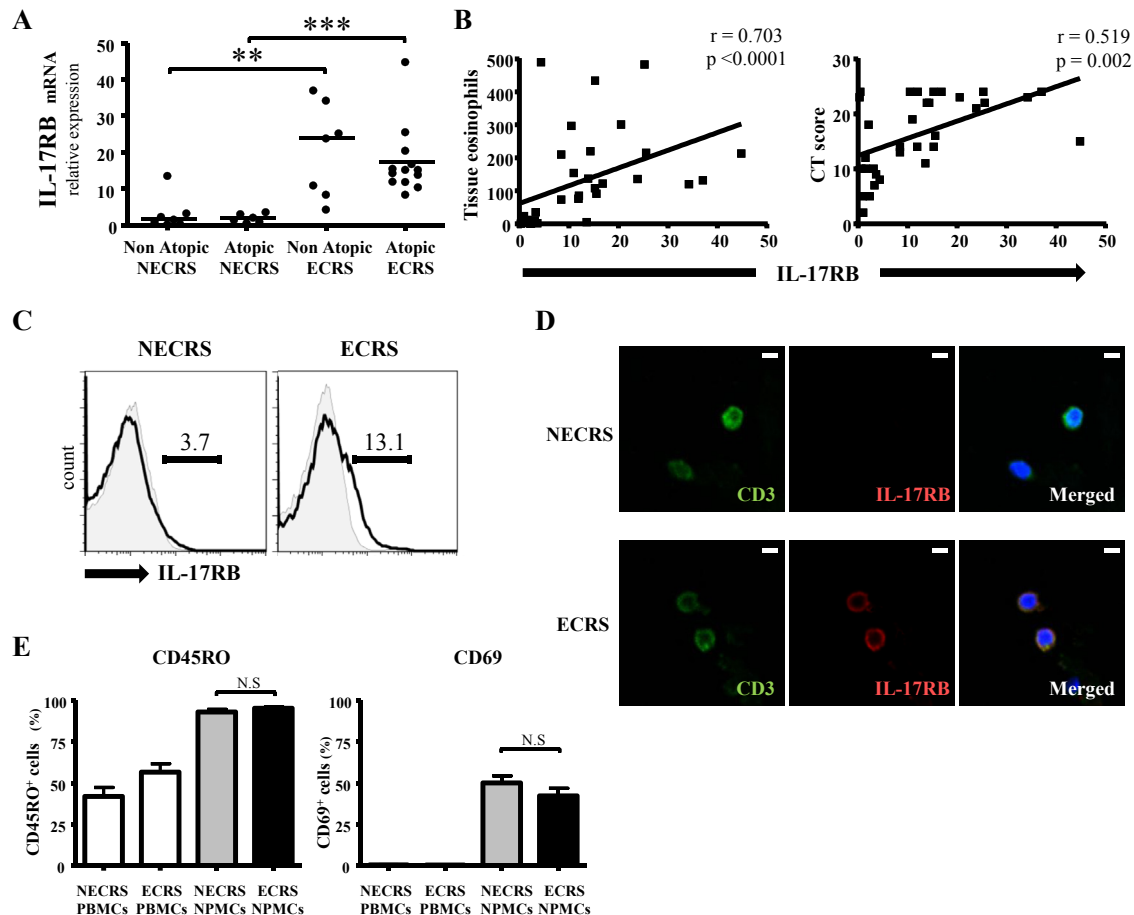


Figure 5. Expression of interleukin (IL)-17RB in tissue CD4⁺ T cells. (A) Real-time polymerase chain reaction analysis of IL-17RB expression in sorted tissue CD4⁺ T cells. (B) Correlation of the IL-17 RB mRNA level in tissue CD4⁺ T cells with the number of tissue eosinophils and computed tomographic (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 expression. Scale bar = 5 μ m. The results of flow cytometry and immunofluorescence represent 4 to 6 patients. (E) Flow cytometric analysis of CD45RO and CD69 expression by CD3⁺CD4⁺ T cells. ** $P < .01$, *** $P < .001$. ECRS, eosinophilic chronic rhinosinusitis with nasal polyps; IFN- γ , interferon- γ ; NECRS, non-eosinophilic chronic rhinosinusitis with nasal polyps; NPMC, nasal polyp mononuclear cell; NS, not significant; PBMC, peripheral blood mononuclear cell.

from patients with NECRS ($n = 8$) or ECRS ($n = 9$) were measured (Fig 4C). The T_H9 proportion in CD4⁺ T cells of NPMCs was significantly higher in patients with ECRS than in those with NECRS ($P < .01$).

Tissue Memory CD4⁺ T Cells from Patients with ECRS Express IL-17RB

The level of mRNA encoding IL-17RB was significantly higher in patients without atopy ($P < .01$) and with atopy ($P < .001$) and ECRS than in those with NECRS (Fig 5A). *IL5* ($r = 0.490$, $P = .004$), *GATA3* ($r = 0.482$, $P = .005$), and *IL9* ($r = 0.449$, $P = .009$) mRNAs in tissue CD4⁺ T cells significantly correlated with *IL17RB* mRNA levels. Moreover, *IL17RB* mRNA levels in tissue CD4⁺ T cells were significantly correlated with the number of eosinophils in NPs ($r = 0.703$, $P < .0001$) and CT scores ($r = 0.519$, $P = .002$; Fig 5B). There was no significant correlation with *IL17RB* levels and expression levels of 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 and those with 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 polymerase chain reaction (NECRS, $n = 6$; ECRS, $n = 8$; Fig 6A). *IL5* and *GATA3* mRNAs were significantly upregulated by IL-25 stimulation in patients with ECRS ($P < .05$ for the 2 comparisons) but not in patients with NECRS.

CD3–CD28 stimulation of NPMCs induced significant IFN- γ , IL-5, and IL-9 protein levels in the 2 groups (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 ($P < .01$; Fig 6B). IL-25 stimulation with anti-CD3–CD28 increased IL-5 ($P < .01$) and IL-9 ($P < .01$) 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 ($P < .01$). There was no significant difference in the proportion of CD4⁺ T cells in NPMCs between patients with ECRS ($11.4 \pm 3.1\%$) and those with NECRS ($10.5 \pm 3.6\%$).

Discussion

Most CD4⁺ T cells infiltrating NPs were memory T cells with different features in CRSwNP disease types classified according to

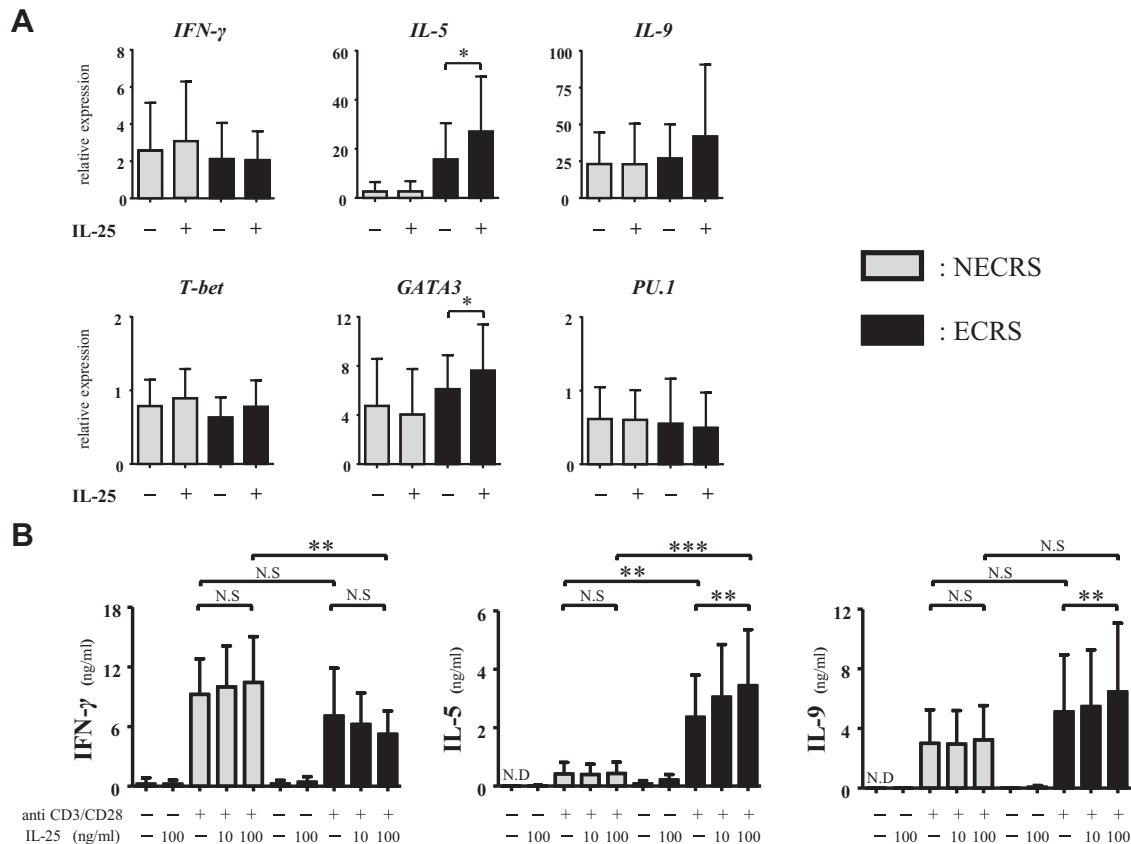


Figure 6. Influence of interleukin (IL)-25 on interferon- γ (IFN- γ), IL-5, and IL-9 production by nasal polyp mononuclear cells (NPMCs). (A) Expression levels of genes encoding IFN- γ , IL-5, IL-9, T-bet, GATA-3 and PU.1 in NPMCs. (B) IFN- γ , IL-5 and IL-9 protein levels in the supernatants. * $P < .05$, ** $P < .01$, *** $P < .001$. ECRS, eosinophilic chronic rhinosinusitis with nasal polyps; ND, not detected; NECRS, noneosinophilic chronic rhinosinusitis with nasal polyps; NS, not significant.

eosinophil infiltration. The *IFNG* mRNA level was elevated in CD4⁺ T cells obtained from tissues of patients with NECRS (Fig 3). In contrast, *IL5* and *IL9* mRNA levels were elevated in patients with ECRS, which also were characterized by increased IL-17RB expression (Fig 5). Moreover, IL-17RB expression in tissue CD4⁺ T cells significantly correlated with the number of eosinophils in NPs and CT score. IL-25 levels were increased in NPs obtained from patients with ECRS (Figs 1, 2) and enhanced the production of T_H2- and T_H9-specific cytokines induced by T-cell receptor stimulation of NPMCs obtained from patients with ECRS but not those from patients with NECRS (Fig 6). For tissue T_H2 and T_H9 cells, the functions mediated through IL-25 differed between cells obtained from patients with ECRS and those with NECRS. The present results indicated that IL-25 in NPs and IL-17RB expressing tissue memory CD4⁺ T cells were associated with eosinophil infiltration in CRSwNP.

The elevated *IL5* and *GATA3* mRNA levels in tissue CD4⁺ T cells and the IL-5 level in NPs of patients with ECRS indicate that T_H2 cells play important roles in infiltration of eosinophils in NPs. Interestingly, the proportion of T_H9 cells detected in NPMCs (10%) was larger than that in PBMCs (1%; Fig 4). Because some T_H9 cells might be reprogrammed cells from T_H2 cells by transforming growth factor- β for plasticity of the T-cell subset,²⁴ this result suggests that T_H9 cells were localized in the site as a consequence of a secondary immune response. In addition, the population of T_H9 cells in patients with ECRS was larger 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 upregulation.^{25–27} 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 regulatory T cells and T_H17 cells in tissue CD4⁺ T cells between patients with ECRS and those with 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 regulatory T cells was not established. Moreover, there were no differences in the populations of T_H17 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 T_H1-skewed inflammation and that tissue T_H1–T_H2 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} The authors propose that T_H2 cells with such an additional functions have pathogenic potential in eosinophilic inflammation.³⁶ In a study of mouse allergy models, Islam et al.³⁷ proposed that IL-5 derived from IL-17RB- and CCR8-expressing T_H2 cells amplified local secondary allergic immune responses. Endo et al.³⁸ showed that IL-5 produced by CD62L^{low}CXCR3^{low} T_H2 cells plays a critical role in allergic disease. In the present study, tissue memory CD4⁺ T cells from patients with ECRS were characterized by high IL-17RB expression levels. IL-5 and IL-9 production was observed in

NPMCs from patients with NECRS and those with ECRS; however, the 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). The present findings suggest that not only the numbers but also the reactivities to IL-25 differ in memory T_H2 and T_H9 cells between these cells from patients with NECRS and those with ECRS. A subpopulation of memory T_H2 and T_H9 cells expressing IL-17RB might 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).

Interleukin-25 was first described as a cytokine produced by mouse T_H2 cells.¹⁴ Epithelial cells, eosinophils, mast cells, and endothelial cells express IL-25 in patients with allergic diseases.^{20,21} In the present study, epithelial cells, mast cells, and eosinophils expressed 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 samples (Fig 1). Moreover, IL-25 levels in NPs were significantly correlated with the number of tissue eosinophils and CT scores. Accordingly, acting with IL-17RB-expressing T_H2 cells, IL-25 could 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 those with ECRS. In patients with asthma, although IL-25 was expressed by most epithelial cells in the bronchial mucosa, IL-25 expression by epithelial cells was not increased by allergen challenge.²¹ These effects might depend on an IL-25-releasing factor³⁹ or on the existence and sensitivity of IL-17RB-expressing cells, such as the T_H2 and T_H9 cells investigated in this study.

Atopic status might be involved in the development of eosinophilic inflammation in patients with CRSwNP.^{40,41} However, several clinical and pathologic features of CRSwNP are related to the classification of the grade of eosinophil infiltration (Table 1, Fig 5). The present results indicated that activating pathogenic tissue memory T_H2 and T_H9 cells in NPs could exacerbate chronic local eosinophilic inflammation regardless of atopic status. Clinically, a relation was found between ECRS and late-onset or aspirin-intolerant asthma (Table 1). Late-onset asthma is not related to atopy and its relation 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 T_H2 and T_H9 cells with similar properties could 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 asthma.⁴⁴

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 T_H2 and T_H9 cells that express IL-17RB could contribute to eosinophil infiltration in CRSwNP and could be the 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 T_H2 cells that express high IL-17RB levels, which could provide new treatment strategies for eosinophilic airway diseases.

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