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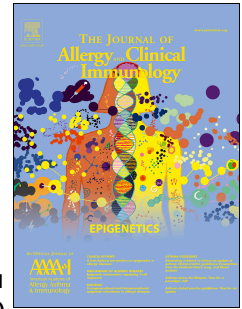
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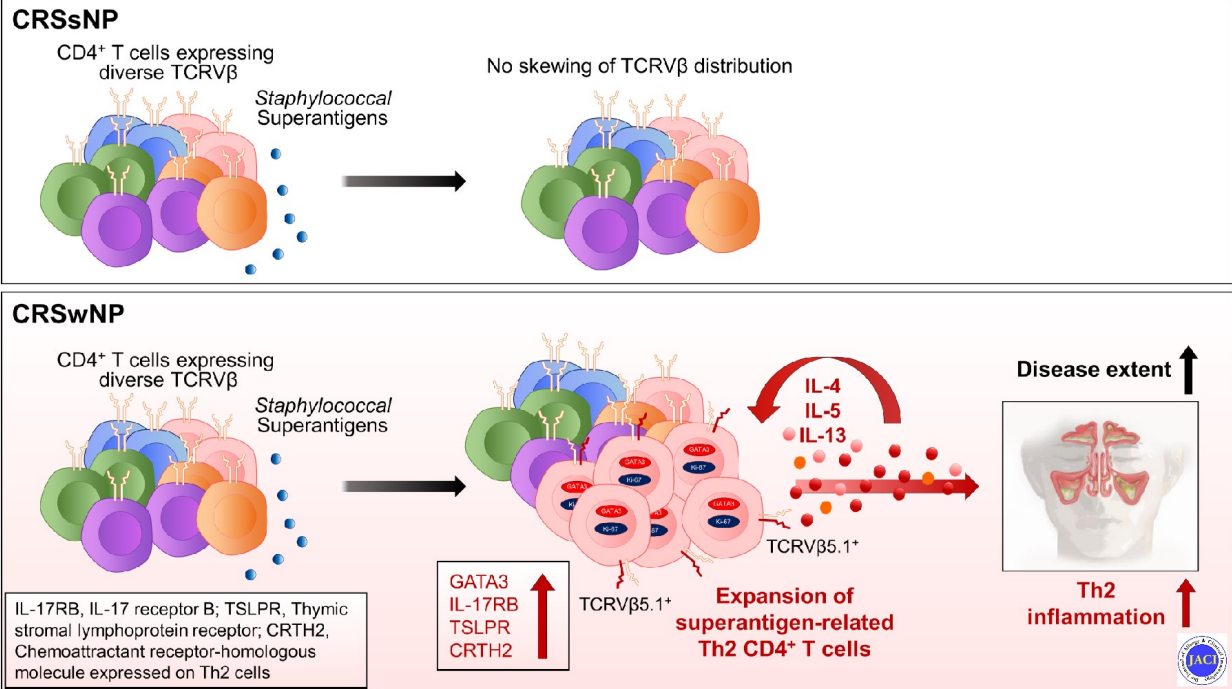
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Superantigen-related Th2 CD4⁺ T Cells in Non-asthmatic Chronic Rhinosinusitis



Superantigen-related Th2 CD4⁺ T Cells in Non-asthmatic Chronic Rhinosinusitis with Nasal Polyps

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40 **Conflict of interest**

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Abstract

Background: *Staphylococcus aureus* enterotoxin (SAE) superantigens are detected in nasal polyps (NPs), and SAE-specific immunoglobulin E predicts asthma comorbidity in patients with NPs. However, roles of SAE superantigens and superantigen-related T-cell responses remain to be elucidated in non-asthmatic patients.

Objective: We investigated the presence of SAEs and SAE-related T cell receptor (TCR) V β in non-asthmatic NPs, the phenotypes and functions of SAE-related T cells, and the clinical implication of SAE-related T-cell expansion.

Methods: Sinonasal tissues were obtained from patients with non-asthmatic chronic rhinosinusitis (CRS) with (CRSwNP) or without NP (CRSsNP) and control subjects. SAE genes were detected by polymerase chain reaction and the TCRV β distribution and T-cell phenotypes examined by flow cytometry.

Results: Various SAE genes were detected not only in NPs but also in sinonasal tissues of CRSsNP patients and controls. The *S. aureus* enterotoxin I (SEI) gene was detected in all NPs. The fraction of SEI-responsive TCRV β ⁺ (TCRV β 1⁺ and V β 5.1⁺) CD4⁺ T cells was significantly increased only in NPs and ethmoidal mucosal tissues from CRSwNP patients, indicating superantigen-induced expansion. The expanded TCRV β 5.1⁺ CD4⁺ T cells expressed proliferation marker Ki-67 and the Th2 transcription factor GATA3. Furthermore, TCRV β 5.1⁺ CD4⁺ T cells in NPs highly expressed Th2 markers, including IL-17RB, TSLPR, and CRTH2, with a potent Th2 cytokine-producing ability. Moreover, the expansion of TCRV β 1⁺ or V β 5.1⁺ CD4⁺ T cells was associated with the Lund-Mackay CT score, indicating disease extent.

Conclusion: In non-asthmatic CRSwNP patients, superantigen-related expansion of CD4⁺ T cells with Th2 differentiation was associated with the disease extent.

Clinical Implications

Superantigen-related expansion of CD4⁺ T cells with Th2 differentiation is associated with the extent of disease in non-asthmatic CRSwNP patients.

Capsule summary

Superantigen-related CD4⁺ T cells were expanded in NPs and ethmoidal mucosal tissues from non-asthmatic CRSwNP patients and exhibited a potent Th2 cytokine-producing ability. In addition, their expansion was associated with disease extent in CRSwNP.

Key words: Chronic rhinosinusitis; nasal polyp; T cells; superantigen; *Staphylococcus aureus* enterotoxin; Th2

Abbreviations

CRS: Chronic rhinosinusitis

NP: Nasal polyp

CRS_wNP: Chronic rhinosinusitis with nasal polyps

CRS_sNP: Chronic rhinosinusitis without nasal polyps

EM: Ethmoidal mucosa

SAE: *Staphylococcus aureus* enterotoxin

SAE-IgE: *Staphylococcus aureus* enterotoxin-specific immunoglobulin E

SEB: *Staphylococcus aureus* enterotoxin B

SEI: *Staphylococcus aureus* enterotoxin I

TCRV β : T cell receptor variant beta chain

Th1: T helper 1

Th2: T helper 2

TSLP: Thymic stromal lymphoprotein

TSLPR: Thymic stromal lymphoprotein receptor

CRT_H2: Chemoattractant receptor-homologous molecule expressed on Th2 cells

Introduction

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nose and paranasal sinuses with symptoms of nasal obstruction, nasal discharge, facial pressure, and/or reduction of smell persisting more than 12 weeks.^{1, 2} Nasal polyps (NPs) are frequently associated with CRS and present as the most severe form of CRS, affecting quality of life. Approximately 2-3% of the general population have NPs.^{3, 4} However, the exact pathogenesis remains unclear.

CRS is characterized by a dysfunctional host-environment interaction at the nasal mucosa.^{1, 5} As a host factor, epithelial damage or genetic defects (barrier defect hypothesis) may lead to increased colonization of bacteria; subsequently, bacterial components invade the mucosa and aggravate the inflammatory reaction.^{6, 7} As an environmental factor, an increase in colonization with *Staphylococcus aureus* has been demonstrated in the nasal mucosa of patients with CRS with NPs (CRSwNP) compared to control subjects or patients with CRS without NPs (CRSsNP).⁸ In addition, *S. aureus*-derived enterotoxins can act as superantigens, inducing polyclonal stimulation. *S. aureus* enterotoxin (SAE)-specific immunoglobulin E (SAE-IgE) is detected in almost half of the nasal tissue homogenates from NPs.⁹

Staphylococcal superantigens induce nasal polyposis by affecting a variety of cell types, including epithelial cells, lymphocytes, eosinophils, basophils, mast cells, and dendritic cells.¹⁰⁻¹⁶ The net effect of superantigens in NPs is the production of T helper 2 (Th2) cytokines, regulatory T cell inhibition, and accentuated eosinophil and mast cell activity, resulting in tissue damage and remodeling.^{14, 16-18} Skewed distribution of T cell receptor V β chain (TCRV β) has also been reported in NPs as evidence of the pathogenicity of staphylococcal superantigens.¹⁹⁻²¹ Moreover, as a biomarker, SAE-IgE in NPs has been reported to be associated with comorbidities of CRSwNP, such as asthma and aspirin intolerance.^{8, 9, 22}

However, several issues remain to be answered in the study of staphylococcal superantigens in the pathogenesis of CRSwNP. First, the roles of staphylococcal superantigens remain to be elucidated in non-asthmatic NPs. Second, although several studies have suggested skewed TCRV β distribution as evidence of the superantigen hypothesis, the association of the presence of SAEs and expansion of the relevant TCRV β has not been clarified. Third, the phenotypes and functions of superantigen-related expanded T cells and their roles in the extent of disease in NPs are unknown.

In the present study, we comprehensively examined the presence of various types of staphylococcal superantigens and the expansion of related TCRV β ⁺ T cells in the NPs of non-asthmatic CRSwNP patients. In addition, we investigated the immunological phenotypes and functions of the expanded T cells in detail in the NPs and analyzed their clinical implications in non-asthmatic CRSwNP patients.

Methods

Study subjects

Twenty patients with CRSwNP and 20 patients with CRSsNP were initially enrolled in this study (CRS Cohort 1). The diagnosis of CRS and NP was confirmed by nasal endoscopy and computed tomography (CT) scans. Each CRS patient met the criteria for CRS as defined by “EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012” guidelines.¹ Patients who used systemic or nasal topical corticosteroids within a month prior to tissue collection, had undergone a previous sinonasal surgery, or had aspirin-exacerbated respiratory disease or asthma were excluded. The diagnosis of asthma and aspirin intolerance was based on history and physician diagnosis, including the bronchial provocation test. The Lund-Mackay CT scoring system was used to determine disease severity.²³ Ethmoidal mucosa (EM) and/or NP tissues were obtained during the functional endoscopic sinus surgery. We also obtained NP tissues from an independent cohort of patients with CRSwNP (CRS Cohort 2) at Boramae Medical Center. This cohort included 30 patients with non-asthmatic CRSwNP. Details of the patients’ characteristics are presented in Table E1. Inferior turbinal mucosal tissues were also obtained from control subjects. Control subjects were patients who underwent septoturbineoplasty to correct a deviated nasal septum and inferior turbinal hypertrophy with no evidence of paranasal sinus problems. This study was reviewed and approved by the Institutional Review Board (26-2016-57) and conducted according to the principles of the Declaration of Helsinki. Informed consent was obtained from all study participants.

Polymerization chain reaction (PCR) for staphylococcal superantigens

DNA was extracted from the NPs of CRSwNP patients (n=20), EM tissues of CRSsNP patients (n=20), and inferior turbinal mucosal tissues of the control subjects (n=10)

using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was quantified using NanoDrop2000 (Thermo Scientific, Waltham, MA, USA). PCR was performed to amplify the staphylococcal superantigen genes using 10 ng of DNA and 35 cycles of denaturation for 2 min at 94°C, annealing for 2 min at various temperatures, and extension for 1 min at 72°C. The oligonucleotide primers for each staphylococcal superantigen gene are summarized in Table E2.

Multi-color flow cytometry

Sinonasal cells were stained with fluorochrome-conjugated antibodies for specific surface markers for 10 minutes at room temperature. Dead cells were excluded using LIVE/DEAD red fluorescent reactive dye or near-IR fluorescent reactive dye (Invitrogen, Carlsbad, CA, USA). For intracellular staining, cells were fixed and permeabilized using the Fixation/Permeabilization Buffer Set (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions after cell surface staining, and then stained with intracellular markers for 30 minutes at 4°C. Multi-color flow cytometry was performed using a BD LSR II instrument (BD Biosciences, San Jose, CA, USA) and FACSDiva software (BD Biosciences). Data were analyzed with FlowJo software (FlowJo LLC, Ashland, Oregon, USA).

TCRV β typing

Isolated sinonasal cells from the NPs and EM tissues of CRSwNP patients, EM tissues of CRSsNP patients, and inferior turbinal mucosal tissues of the control subjects were used for TCRV β analysis. Dead cells were stained using the LIVE/DEAD[®] Fixable Red Dead Cell Stain Kit (Invitrogen) and excluded when flow cytometry data were analyzed. FITC-,

PE-, or FITC/PE-conjugated antibodies from the IOTest[®] Beta Mark Kit (Beckman Coulter, Inc., Brea, CA, USA) were used for staining 24 different TCRV β s.

Calculation of TCRV β skewing in T cells

We calculated the average percentage (and standard deviation [SD]) of each TCRV β fraction in CD4⁺ or CD8⁺ T cells when we analyzed sinonasal cells isolated from the inferior turbinal mucosal tissues of the control subjects (Table E3). The skewing degree of each TCRV β was calculated using following equation^{20, 24-26}:

$$\frac{(\% \text{ of TCRV}\beta \text{ in patients} - \text{average of } \% \text{ of TCRV}\beta \text{ in the controls})}{\text{SD of } \% \text{ of TCRV}\beta \text{ in the controls}}$$

Statistical analysis

The chi-square test or Fisher's exact test was used to analyze differences in the detection rate of superantigen genes. The Mann-Whitney *U* test was used to compare data between two unpaired groups. The Wilcoxon signed-rank test was used to compare data between two paired groups. To assess the significance of the correlation, the Pearson correlation test was performed. Statistical analyses were performed using GraphPad Prism Version 7 (GraphPad Software, Carlsbad, CA, USA) or SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). *P*<0.05 was considered significant. Data are expressed as the mean $\pm$ SD.

Results

Detection of staphylococcal superantigen genes in sinonasal tissues

First, we performed PCR for detection of Staphylococcal superantigen genes in sinonasal tissues. Various staphylococcal superantigen genes were detected in the NPs of CRSwNP patients (Table I). However, they were also detected in EM tissues from CRSsNP patients and inferior turbinal mucosal tissues from control subjects. Among the staphylococcal superantigen genes, *S. aureus* enterotoxin I (SEI) was detected in all of the tested NPs.

Skewed TCRV β distribution in sinonasal CD4⁺ T cells from CRSwNP patients

Next, we examined the distribution of TCRV β s in sinonasal tissues. The fraction of TCRV β 1⁺ or V β 5.1⁺ cells, which are known to be expanded by SEI,^{19, 27} was significantly increased in the CD4⁺ T cell population of NPs in CRSwNP patients, although such skewing of the TCRV β distribution was not found in the CD8⁺ T cell population (Fig 1A). The same was observed in EM tissues from CRSwNP patients (Fig 1B). However, the increased fraction of TCRV β 1⁺ or V β 5.1⁺ cells was not observed in the EM tissues of CRSsNP patients (Fig 1C). Taken together, we conclude from these results that CD4⁺ T cells carrying TCRV β 1 and V β 5.1 are expanded only in the NPs and EM tissues of CRSwNP patients.

Expression of proliferation marker Ki-67 and anti-apoptotic protein Bcl-2 in

TCRV β 5.1⁺ CD4⁺ T cells from the sinonasal tissues of CRSwNP patients

We further analyzed TCRV β 5.1⁺ CD4⁺ T cells to explore how they expanded in the sinonasal tissues of CRSwNP patients. Therefore, we examined the expression of Ki-67, a marker of proliferation, and Bcl-2, an anti-apoptotic protein,^{28, 29, 30} in TCRV β 5.1⁺ CD4⁺ T

cells from the NPs and EM tissues of CRSwNP patients and EM tissues of CRSsNP patients. Representative FACS plots for gating TCRV β 5.1⁺ CD4⁺ T cells are presented in Fig 2A, and representative FACS histograms for Ki-67 and Bcl-2 are presented in Fig 2B. The frequency of Ki-67⁺Bcl-2⁺ cells in the gate of TCRV β 5.1⁺ CD4⁺ T cells was significantly higher in the NPs of CRSwNP patients than the EM tissues of CRSwNP patients or CRSsNP patients (Fig 2C). We also found that the frequency of Ki-67⁺Bcl-2⁺ cells was significantly higher among TCRV β 5.1⁺ CD4⁺ T cells than among TCRV β 5.1⁻ CD4⁺ T cells from the NPs of CRSwNP patients (Fig 2C). Furthermore, the EM tissues of CRSwNP patients had a higher frequency of Ki-67⁺Bcl-2⁺ cells in the gate of TCRV β 5.1⁺ CD4⁺ T cells than the EM tissues of CRSsNP patients (Fig 2C). As expected, the frequency of Ki-67⁺Bcl-2⁺ cells among TCRV β 5.1⁺ CD4⁺ T cells significantly correlated with the frequency of TCRV β 5.1⁺ cells among total CD4⁺ T cells in the NPs of CRSwNP patients (Fig 2D).

Taken together, these data indicate that TCRV β 5.1⁺ CD4⁺ T cells undergo cellular proliferation in the sinonasal tissues of CRSwNP patients, particularly NPs, and that the expansion of TCRV β 5.1⁺ CD4⁺ T cells might be derived from cellular proliferation caused by superantigens.

Th2 phenotypes of TCRV β 5.1⁺ CD4⁺ T cells from NPs

Th2 inflammation is recognized as being mainly involved in CRSwNP.^{31, 32} When we analyzed total CD4⁺ T cells, the expression of the Th2 transcription factor, GATA3, was significantly higher in NPs from CRSwNP patients than the EM tissues of CRSsNP patients (Fig 3A). As TCRV β 5.1⁺ CD4⁺ T cells were expanded in the tissue samples from NPs, we asked whether TCRV β 5.1⁺ CD4⁺ T cells deviate to Th2 phenotypes. In NPs from CRSwNP patients, TCRV β 5.1⁺ CD4⁺ T cells had higher expression of GATA3 than TCRV β 5.1⁻ CD4⁺ T

cells (Fig 3B and C). In addition, the frequency of GATA3⁺ cells among the TCRV β 5.1⁺ CD4⁺ T cells was significantly higher in the NPs of CRSwNP patients than the EM tissues of CRSsNP patients (Fig 3C). However, the expression of T-bet, the Th1 transcription factor, was not different between TCRV β 5.1⁺ and TCRV β 5.1⁻ CD4⁺ T cells in NPs from CRSwNP patients (Fig E1).

Recent studies have suggested that the IL-25 receptor IL-17RB and thymic stromal lymphoprotein (TSLP) receptor are phenotypic markers of Th2 cells.³³⁻³⁵ In addition, human Th2 cells can be identified by the expression of the prostaglandin D2 receptor chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2).^{36, 37} As TCRV β 5.1⁺ CD4⁺ T cells exhibited higher expression of GATA3, we investigated the expression of IL-17RB, TSLP receptor (TSLPR), and CRTH2 on TCRV β 5.1⁺ CD4⁺ T cells from NPs. The TCRV β 5.1⁺ CD4⁺ T cells expressed significantly higher levels of IL-17RB, TSLPR, and CRTH2 than TCRV β 5.1⁻ CD4⁺ T cells (Fig 3D and E), indicating that superantigen-expanded CD4⁺ T cells are Th2 cells. We also found that TCRV β 5.1⁺ CD4⁺ GATA3⁺ T cells expressed higher levels of IL-17RB, TSLPR, and CRTH2 compared to TCRV β 5.1⁺ CD4⁺ GATA3⁻ T cells (Fig 3F). Altogether, we conclude that CD4⁺ T cells in NPs, particularly superantigen-expanded TCRV β 5.1⁺ CD4⁺ T cells in NPs, deviate toward Th2 status.

Production of Th2 cytokines by TCRV β 5.1⁺ CD4⁺ T cells from NPs

We further examined the production of Th2 cytokines by CD4⁺ T cells following anti-CD3 stimulation. The frequency of IL-4⁺ cells in total CD4⁺ T cells was significantly higher in NPs from CRSwNP patients than in EM tissues from CRSsNP patients (Fig 4A and B). Moreover, we found that TCRV β 5.1⁺ CD4⁺ T cells produced significantly higher levels of Th2 cytokines, including IL-4, IL-5, and IL-13, than TCRV β 5.1⁻ CD4⁺ cells from the NPs of

CRSwNP patients (Fig 4C, D, Fig E2A). The same results were observed when sinonasal cells from NPs were stimulated with anti-CD3/CD28 antibodies (Fig E2B) or SEB (Fig E2C). Interestingly, the percentage of TCRV β 5.1⁺ cells among CD4⁺ T cells positively correlated with the frequency of IL-4⁺ or IL-13⁺ cells among TCRV β 5.1⁺ CD4⁺ T cells (Fig E3), indicating that TCRV β 5.1⁺ CD4⁺ T cells from patients with a higher TCRV β skewing produce higher levels of Th2 cytokines.

To investigate the role of Th2 cytokines in the regulation of sinonasal CD4⁺ T cells, we stimulated CD4⁺ T cells obtained from the nasal mucosa of control subjects with anti-CD3 in the presence or absence of IL-4 or IL-13. Ki-67 expression increased in the presence of IL-4 or IL-13 (Fig 4E and Fig E4), suggesting that IL-4 or IL-13 secreted from GATA3⁺ CD4⁺ T cells enhances the superantigen-induced proliferation of CD4⁺ T cells in nasal mucosa. In addition, the frequency of IL-4⁺ cells among total CD4⁺ T cells positively correlated with both the frequency of TCRV β 5.1⁺ cells among total CD4⁺ T cells and the percentage of GATA3⁺ cells among TCRV β 5.1⁺ CD4⁺ cells in the NPs of CRSwNP patients (Fig 4F). These findings suggest positive feedback from Th2 cytokine production to the superantigen-induced proliferation of Th2 cells.

Recently, dupilumab, a monoclonal antibody that inhibits signaling by IL-4 and IL-13,^{38, 39} was approved by the US Food and Drug Administration (FDA) for the treatment of patients with inadequately controlled CRSwNP. Therefore, we investigated the effect of dupilumab on sinonasal CD4⁺ T cells. Dupilumab inhibited IL-4-induced proliferation and GATA3 upregulation of sinonasal CD4⁺ T cells (Fig E5). These results indicate that dupilumab may inhibit superantigen-related expansion of pathogenic Th2 cells in patients with non-asthmatic CRSwNP.

Correlation between the expanded CD4⁺ T cells and disease extent of CRS

Finally, we investigated the clinical implication of the expanded TCRV β 1⁺ and V β 5.1⁺ CD4⁺ T cells. The frequency of TCRV β 5.1⁺ cells among the total CD4⁺ T cells from NPs and EM tissues significantly correlated with the Lund-Mackay CT score in CRSwNP patients (Fig 5A). This correlation was also observed when TCRV β 1⁺ cells among CD4⁺ T cells from NPs were analyzed (Fig 5B). However, we found no correlation between TCRV β 1⁺ or V β 5.1⁺ cells among total CD4⁺ T cells from EM tissues and Lund-Mackay CT score in CRSsNP patients (Fig 5A and B). In addition, the frequency of TCRV β 2⁺ cells, which were not expanded in NPs, among total CD4⁺ T cells did not correlate with Lund-Mackay CT score in CRSwNP or CRSsNP patients (Fig 5C). Taken together, these data indicate that the expansion of superantigen-related CD4⁺ T cells is directly related to advanced CRS.

Next, we analyzed the relationship between the function of TCRV β 5.1⁺ CD4⁺ T cells and eosinophilic inflammation as evaluated by the number of infiltrated eosinophils in NP tissue. We found a positive correlation between IL-4 production by TCRV β 5.1⁺ CD4⁺ T cells and the number of infiltrated eosinophils (Fig E6), suggesting that TCRV β 5.1⁺ CD4⁺ T cells directly contribute to eosinophilic inflammation by producing Th2 cytokines.

Discussion

S. aureus and staphylococcal superantigens are etiological agents for the development of CRS. Several previous studies have suggested a role of superantigens in CRSwNP by demonstrating a skewed TCRV β distribution of T cells.¹⁹⁻²¹ In addition, SEB stimulation of nasal tissue explants induces the production of Th2 cytokines.^{17, 40} However, the exact mechanism underlying the effect of staphylococcal superantigens on Th2 inflammation in CRSwNP is unclear. Here, we demonstrated that CD4⁺ T cells with superantigen-related expansion exhibit Th2 phenotypes with proliferative features in non-asthmatic CRSwNP. Moreover, given that the expansion of TCRV β 5.1⁺ CD4⁺ T cells significantly correlated with disease extent in CRSwNP, we conclude that these expanded CD4⁺ T cells play a critical role in type 2 inflammation and may be regulatory targets for the management of CRSwNP.

In the present study, we examined TCRV β distribution not only in NPs from CRSwNP patients, but also in EM tissues from CRSwNP and CRSsNP patients. To the best of our knowledge, this is the first study to comprehensively analyze TCRV β distribution by examining both NPs and non-polyp EM tissues. Interestingly, superantigen-related expansion of T cells occurred not only in NPs, but also in the EM tissues of CRSwNP patients, and not in the EM tissues of CRSsNP patients. These data indicate that superantigen-related expansion of T cells is only observed in CRSwNP patients, but NPs do not necessarily develop in sinonasal tissues with superantigen-related expansion of T cells. Factors driving polypogenesis in the presence of superantigen-related T-cell expansion need to be investigated in future studies.

Consistent with several prior studies,^{8, 24, 41, 42} we found that various SAE genes were detected not only in NPs, but also in the sinonasal tissues of CRSsNP patients and control subjects. Intriguingly, SEI gene was detected in all NPs we studied. Among diverse

superantigen-associated TCRV β types, TCRV β 1⁺ and V β 5.1⁺ CD4⁺ T cells are expanded by SED and SEI.^{19, 27} Considering that SED and SEI were detected in 45% and 100% of the tested NPs, respectively, we conclude that SEI could be a major superantigen driving the expansion of TCRV β 1⁺ or V β 5.1⁺ CD4⁺ T cells. However, skewed TCRV β distribution was not observed in the sinonasal mucosa of control subjects and CRSsNP patients, although superantigen genes were present in all subjects. Further studies are required to clarify which factors determine superantigen-related T-cell expansion and subsequent skewing of the TCRV β distribution in the presence of superantigens in the sinonasal mucosa.

In the current study, the expanded TCRV β 5.1⁺ CD4⁺ T cells in NPs expressed GATA3 rather than T-bet. In addition, TCRV β 5.1⁺ CD4⁺ T cells expressed higher levels of several phenotypic markers, including IL-17RB, TSLPR, and CRTH2, which have been proposed to identify Th2 cells. There is mounting evidence that epithelial-derived cytokines, including IL-25 and TSLP, play critical roles in the pathogenesis of CRSwNP.^{43, 44} IL-25 has been suggested to enhance Th2 cytokine production by activated Th2 memory cells,⁴⁵ and TSLP signaling in T cells drives pathogenic Th2 responses.⁴⁶ The IL-17RB⁺ CD4⁺ T cells represented Th2 cells in CRSwNP,³³ and TSLPR was highly expressed on activated Th2 cells.^{34, 35} Our current findings indicate that superantigen-related expanded CD4⁺ T cells in NPs are Th2 cells and can further respond to epithelial-derived cytokines that intensify Th2 inflammation.

TCRV β 5.1⁺ CD4⁺ T cells in NPs have a potent ability to produce Th2 cytokines in the functional analysis, consistent with high expression of GATA3. Previous studies have shown superantigen-induced production of Th2 cytokines, such as IL-4 and IL-5,^{12, 14, 15, 17, 47} which augment the activation and proliferation of Th2 cells. In the present study, IL-4 or IL-13 treatment in addition to anti-CD3 stimulation resulted in stronger expression of Ki-67 in sinonasal CD4⁺ T cells from control subjects than anti-CD3 stimulation alone. In addition, IL-

4 is known to inhibit activation-induced cell death of superantigen-activated CD4⁺ T cells.⁴⁸,
Furthermore, we found that the percentage of TCRVβ5.1⁺ cells among CD4⁺ T cells
positively correlated with the production of IL-4 or IL-13 by TCRVβ5.1⁺ CD4⁺ T cells. Thus,
superantigen-induced T-cell proliferation with Th2 differentiation results in the production of
Th2 cytokines, and Th2 cytokines further contributes to the proliferation and survival of
superantigen-expanded T cells.

In a previous randomized clinical trial, dupilumab improved the Lund-Mackay CT
score, UPSIT score, and SNOT-22 total score in CRSwNP patients without comorbid
asthma.³⁸ In line with the previous report, we found that dupilumab inhibited IL-4-induced
proliferation and GATA3 upregulation of sinonasal CD4⁺ T cells. These results suggest that
dupilumab may inhibit superantigen-related expansion of pathogenic Th2 cells in non-
asthmatic CRSwNP, potentially explaining one of the possible mechanisms of the beneficial
effect of dupilumab on patients with non-asthmatic CRSwNP.

In the current study, we included only subjects without asthma and characterized
superantigen-related expanded CD4⁺ T cells in patients with non-asthmatic CRSwNP, which
explains 67~99% of total CRSwNP in East Asia.^{4, 18, 31} Staphylococcal superantigens are
considered to be closely associated with asthmatic CRSwNP because an increased level of
SAE-IgE is associated with asthma comorbidity in CRSwNP,⁸ and SAE-IgE positivity is
significantly greater in patients with severe asthma than in control subjects.⁵⁰ However, the
immunological characteristics of CD4⁺ T cells from asthmatic NPs have not been well
defined. The TCRVβ distribution and phenotypes of superantigen-related expanded T cells in
asthmatic NPs may differ from our current results in non-asthmatic NPs. Thus, it would be
interesting to investigate the differences in superantigen-related pathogenesis between
asthmatic and non-asthmatic CRSwNP.

In summary, although the presence of SAE genes was not different between groups, the TCRV β distribution was skewed in sinonasal tissues from non-asthmatic CRSwNP patients. In particular, TCRV β 1⁺ and V β 5.1⁺ CD4⁺ T cells were significantly increased in sinonasal tissues from CRSwNP patients. Furthermore, they had significantly higher proliferative and Th2-deviated features. The frequency of these expanded CD4⁺ T cells significantly correlated with the Lund-Mackay CT score, indicating disease extent, in non-asthmatic CRSwNP patients. These findings significantly advance our understanding of superantigen-related pathogenesis of non-asthmatic CRSwNP and suggest opportunities for novel therapeutic intervention.

Acknowledgements

None.

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Figure legends

Figure 1. TCRV β distribution of T cells from the sinonasal tissues of CRSwNP and CRSsNP patients. The fraction of each TCRV β -expressing T cells among CD4 $^{+}$ or CD8 $^{+}$ T cell population was measured by flow cytometry using cells from NP tissues (A; n=20) or EM tissues (B; n=15) of CRSwNP patients or EM tissues of CRSsNP patients (C; n=20). In addition, inferior turbinal mucosa from control subjects (n=23) was analyzed for the fraction of TCRV β -expressing T cells among the CD4 $^{+}$ or CD8 $^{+}$ T cell population. The skewing degree of each TCRV β was calculated as (% of TCRV β in patients - average of % of TCRV β in the controls) / (SD of % of TCRV β in the controls). The mean value for each TCRV β is presented as a short, blue, horizontal bar. If the skewing degree of each TCRV β was higher than 2 (dashed red lines), the skewing was considered significant. The data for the significantly skewed TCRV β are presented as red dots.

Figure 2. Expression of Ki-67 and Bcl-2 in TCRV β 5.1 $^{+}$ CD4 $^{+}$ T cells from the sinonasal tissues of CRSwNP and CRSsNP patients. (A) Representative FACS plots showing the percentage of TCRV β 5.1 $^{+}$ cells among total CD4 $^{+}$ T cells in an NP from a CRSwNP patient and EM tissue from a CRSsNP patient are presented. (B and C) Representative FACS histograms showing the expression of Ki-67 and Bcl-2 in TCRV β 5.1 $^{+}$ and V β 5.1 $^{-}$ CD4 $^{+}$ T cells from NPs are presented (B). The expression of Ki-67 and Bcl-2 was analyzed in TCRV β 5.1 $^{+}$ or V β 5.1 $^{-}$ CD4 $^{+}$ T cells from the NPs (n=18) and EM tissues (n=15) of CRSwNP patients and EM tissues of CRSsNP patients (n=18) (C). Horizontal bars and error bars represent mean and SD, respectively. The comparison between TCRV β 5.1 $^{+}$ and V β 5.1 $^{-}$ CD4 $^{+}$ T cells from NPs was performed using the Wilcoxon signed rank test, and the other comparisons were performed using the Mann-Whitney *U* test. (D) The frequency of Ki-

67⁺Bcl-2⁻ cells in TCRVβ5.1⁺ CD4⁺ T cells was plotted against the frequency of TCRVβ5.1⁺ cells among total CD4⁺ T cells in NPs from CRSwNP patients (n=18). The significance of the correlation was tested using the Pearson correlation test.

Figure 3. Th2 phenotypes of TCRVβ 5.1⁺ CD4⁺ T cells from NPs. (A) The expression of GATA3 in total CD4⁺ T cells was compared between the NPs of CRSwNP patients (n=9) and EM tissues of CRSsNP patients (n=9). (B and C) Representative FACS histogram showing the expression of GATA3 in TCRVβ5.1⁺ and Vβ5.1⁻ CD4⁺ T cells from NPs is presented (B). The expression of GATA3 was analyzed in TCRVβ5.1⁺ or Vβ5.1⁻ CD4⁺ T cells from the NPs of CRSwNP patients (n=9) and EM tissues of CRSsNP patients (n=9) (C). (D and E) Representative FACS plots showing the expression of IL-17RB, TSLPR, and CRTH2 in TCRVβ5.1⁺ and Vβ5.1⁻ CD4⁺ T cells from NPs are presented (D). The expression of IL-17RB (n=19), TSLPR (n=18), and CRTH2 (n=13) was analyzed in TCRVβ 5.1⁺ and Vβ 5.1⁻ CD4⁺ T cells from the NPs of CRSwNP patients (E). (F) The expression of IL-17RB, TSLPR, and CRTH2 was analyzed in GATA3⁺ and GATA3⁻ TCRVβ 5.1⁺ CD4⁺ T cells from the NPs of CRSwNP patients (n=14). All horizontal bars and error bars represent mean and SD, respectively. Statistical analysis was performed using the Mann-Whitney U test (A and C) or the Wilcoxon signed-rank test (E and F).

Figure 4. Production of Th2 cytokines by TCRVβ 5.1⁺ CD4⁺ T cells from NPs. (A–D) Sinonasal cells were stimulated with anti-CD3 antibody for 12 hours. (A and B) The percentage of IL-4⁺ cells in total CD4⁺ T cells was analyzed in NPs from CRSwNP patients (n=7) and EM tissues from CRSsNP patients (n=7). Representative FACS plots (A) and summary data (B) are presented. (C and D) The percentage of IL-4⁺, IL-5⁺, and IL-13⁺ cells was analyzed in TCRVβ5.1⁺ and Vβ5.1⁻ CD4⁺ T cells from the NPs of CRSwNP patients

(n=12). Representative FACS plots (C) and summary data (D) are presented. (E) The expression of Ki-67 and Bcl-2 was examined in CD4⁺ T cells from the nasal mucosa of control subjects after stimulation with anti-CD3 antibody for 72 hours in the presence or absence of IL-4. Representative FACS histograms are presented. (F) The frequency of TCRV β 5.1⁺ CD4⁺ T cells among total CD4⁺ T cells (left) and the frequency of GATA3⁺ cells among TCRV β 5.1⁺ CD4⁺ T cells (right) were plotted against the frequency of IL-4⁺ cells among total CD4⁺ T cells in NPs from CRSwNP patients (n=7). All horizontal bars and error bars represent mean and SD, respectively. Statistical analysis was performed using the Mann-Whitney *U* test (B), the Wilcoxon signed-rank test (D), or the Pearson correlation test (F).

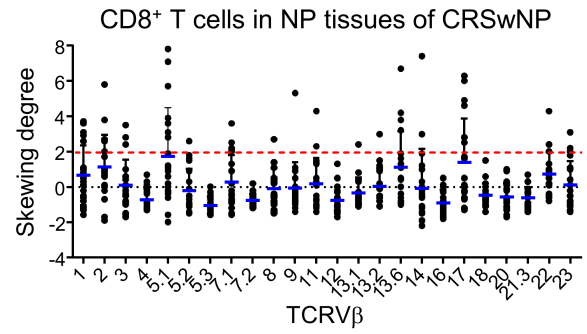
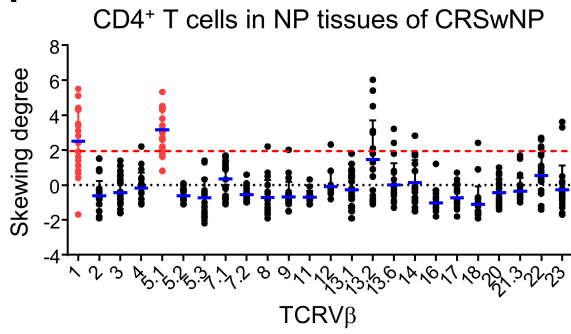
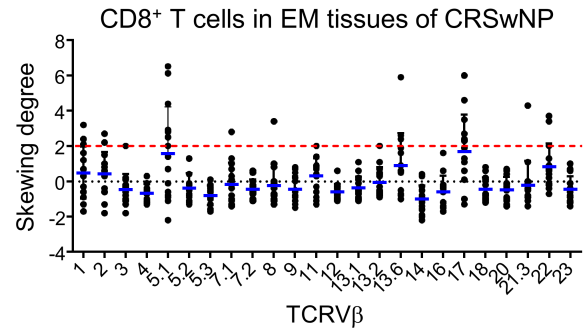
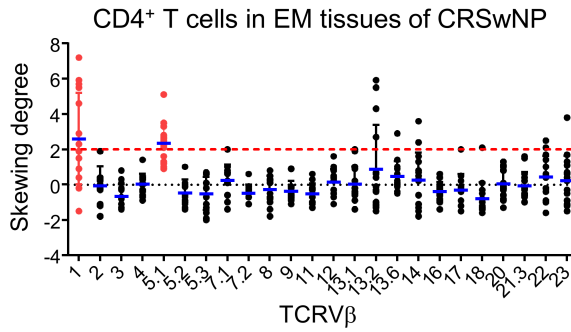
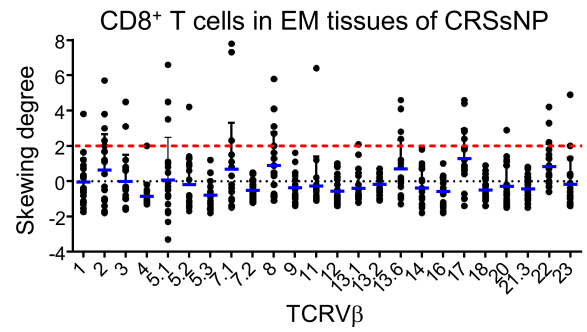
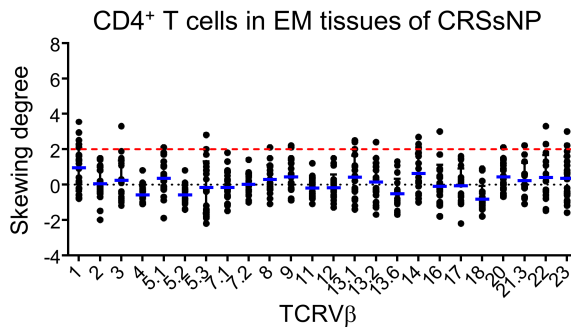
Figure 5. Correlation between the frequency of TCRV β 5.1⁺ or V β 1⁺ CD4⁺ T cells and Lund-Mackay CT score. (A–C) Lund-Mackay CT score was plotted against the frequency of TCRV β 5.1⁺ (A), V β 1⁺ (B), or V β 2⁺ (C) cells among total CD4⁺ T cells from the NPs of CRSwNP patients (n=20), and the EM tissues of CRSwNP (n=15) and CRSsNP patients (n=20). The significance of the correlation was tested using the Pearson correlation test.

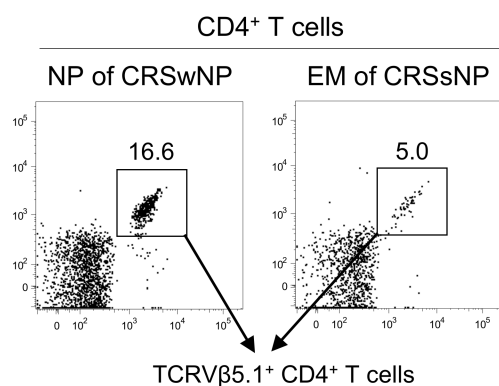
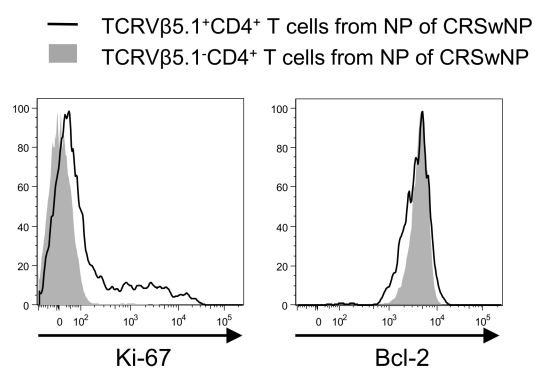
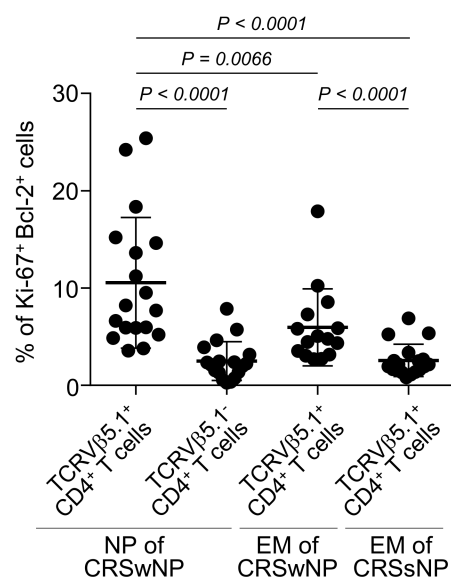
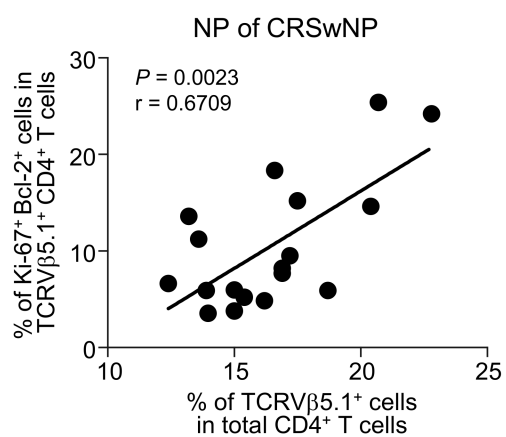
611 **Table I. Superantigens detected in the subjects' nasal mucosa**

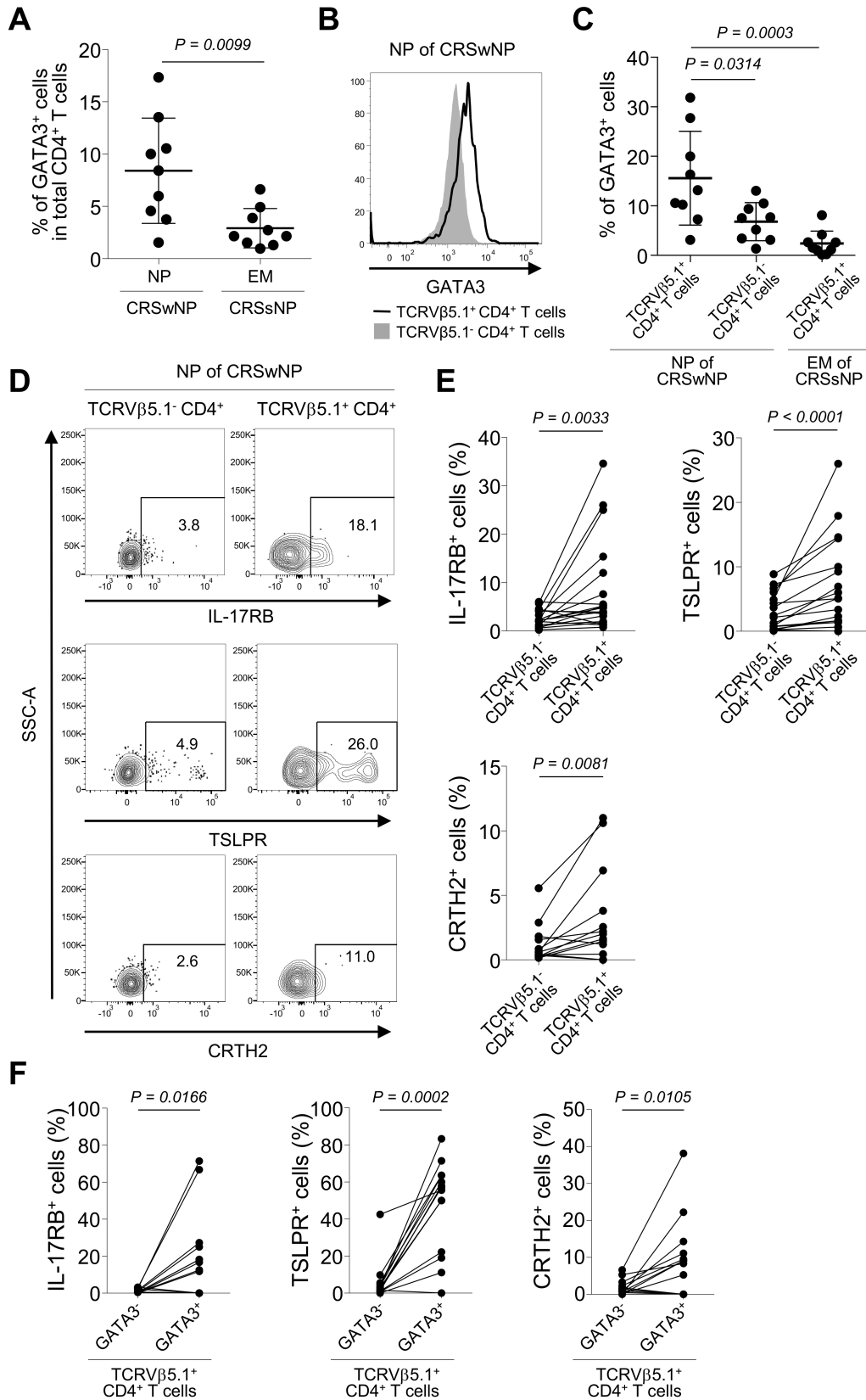
	Group			<i>P</i> -value ^a
	CRS _w NP (n = 20)	CRS _s NP (n = 20)	Control (n = 10)	
SEA	13 (65%)	15 (75%)	6 (60%)	n.s.
SEB	16 (80%)	17 (85%)	7 (70%)	n.s.
SEC	15 (75%)	14 (70%)	6 (60%)	n.s.
SED	9 (45%)	10 (50%)	5 (50%)	n.s.
SEE	8 (40%)	12 (60%)	5 (50%)	n.s.
SEG	18 (90%)	15 (75%)	7 (70%)	n.s.
SEH	8 (40%)	11 (55%)	5 (50%)	n.s.
SEI	20 (100%)	19 (95%)	9 (90%)	n.s.
TSST-1	13 (65%)	11 (55%)	5 (50%)	n.s.

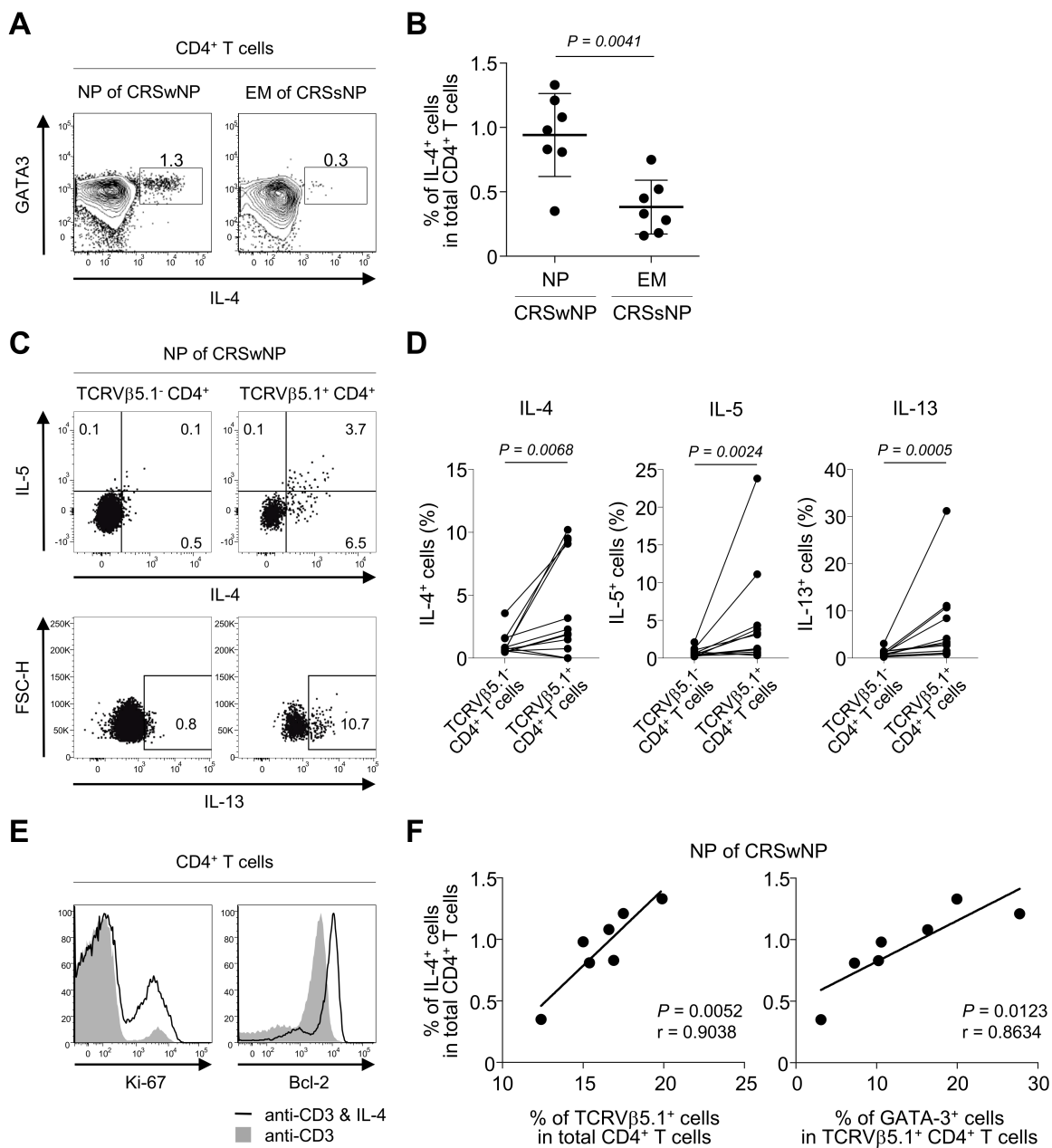
612 ^aChi-square test or Fisher's exact test.

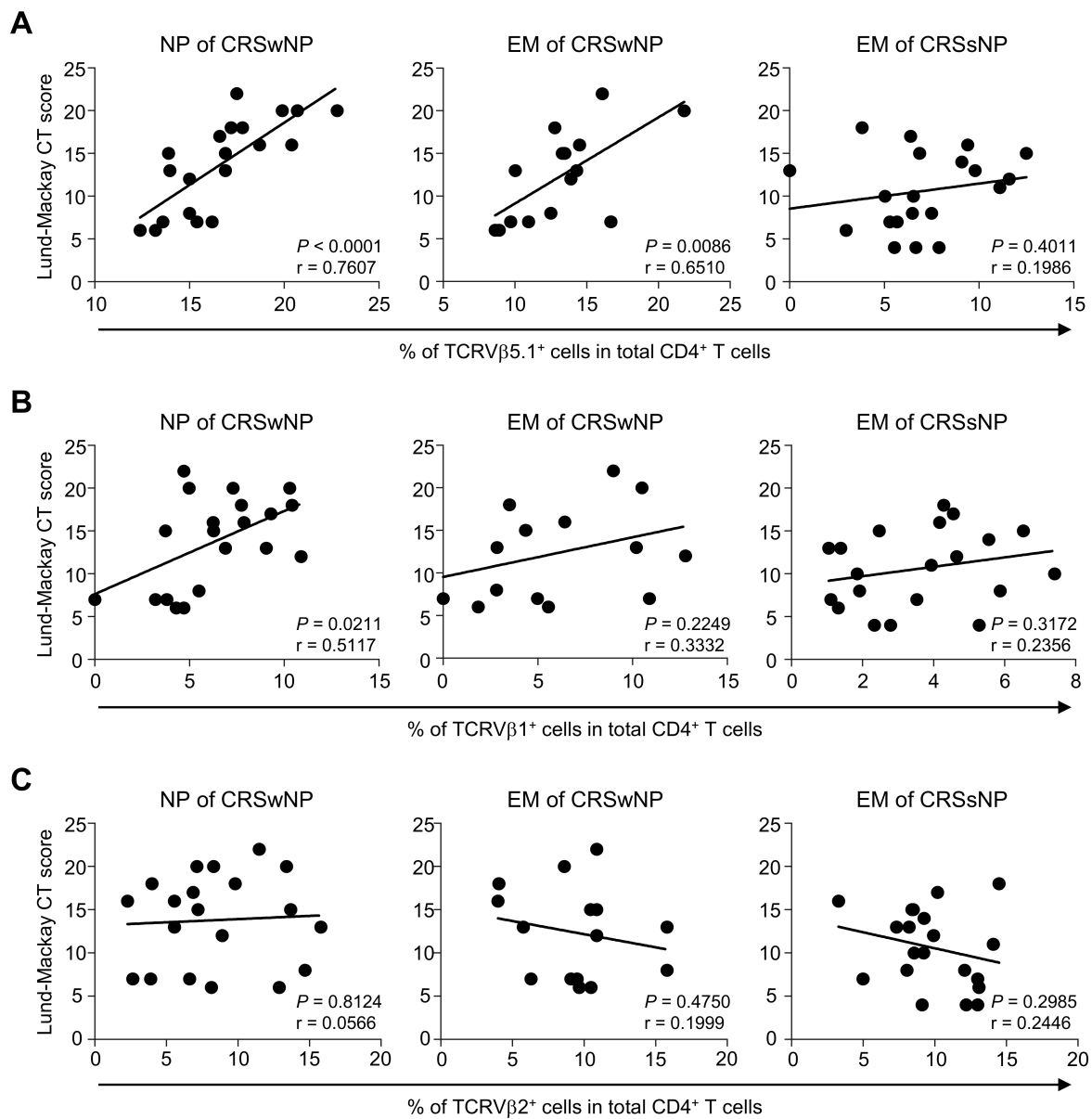
613 n.s.: not significant.

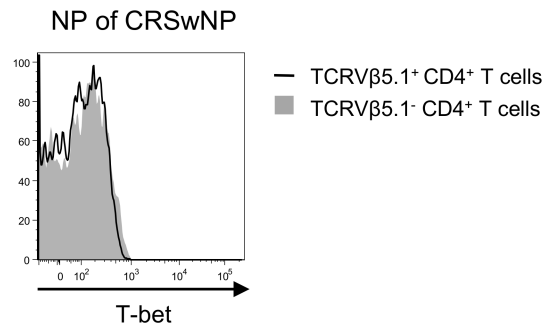
A**B****C**

A**B****C****D**

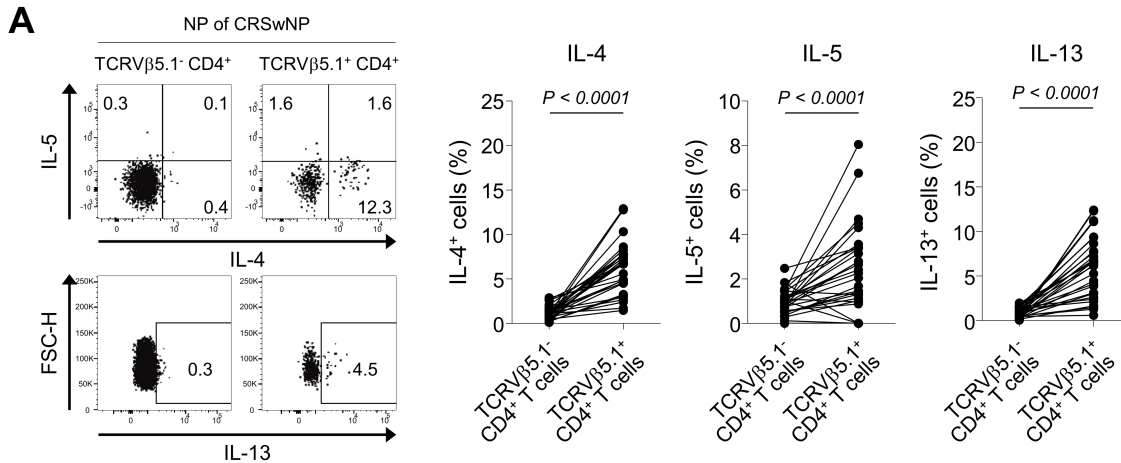




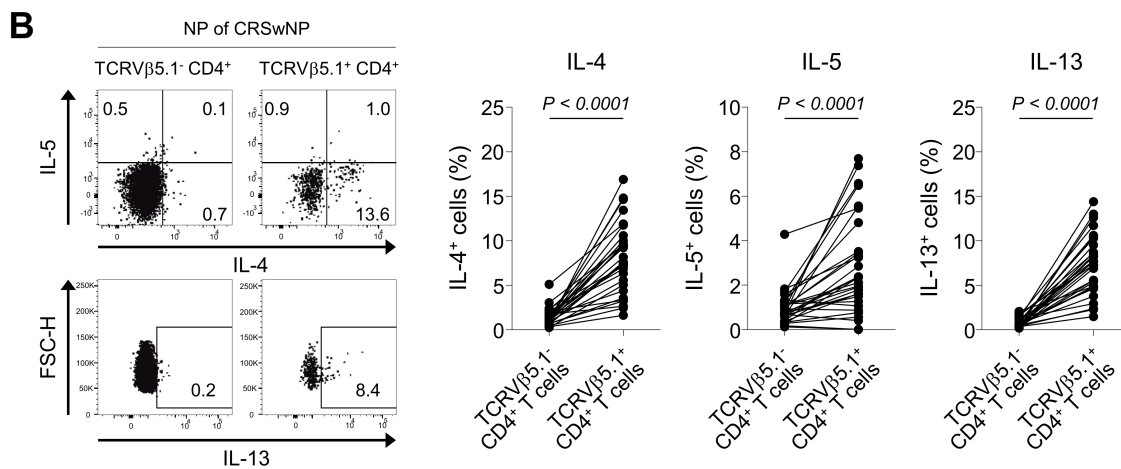




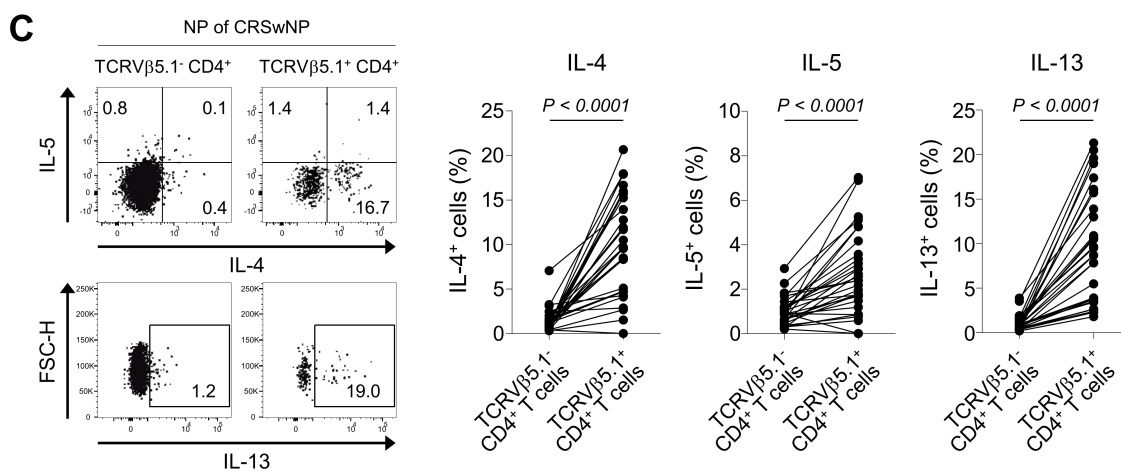
anti-CD3 stimulation



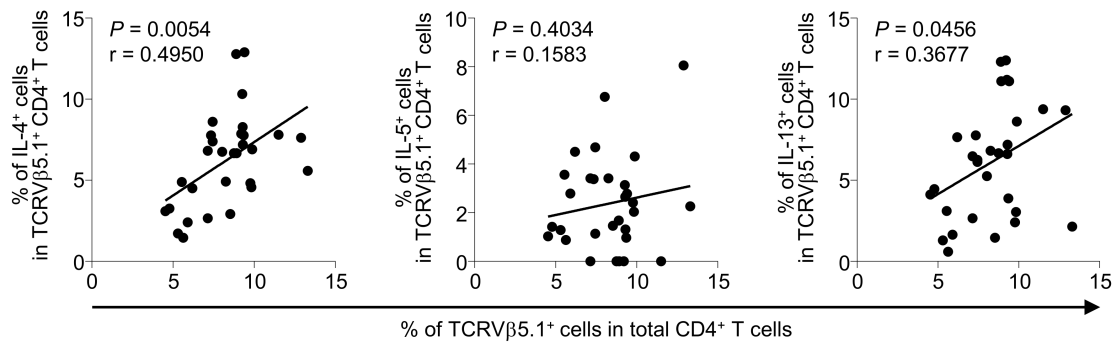
anti-CD3/CD28 stimulation

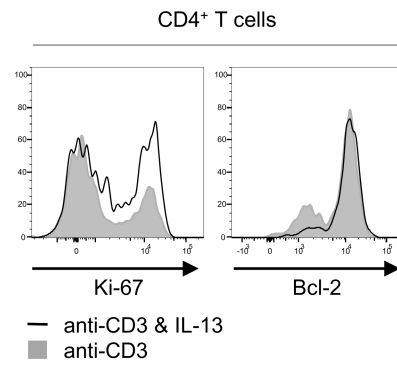


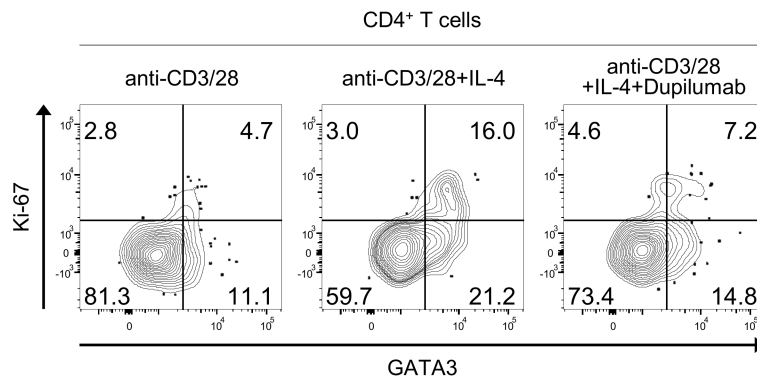
SEB stimulation

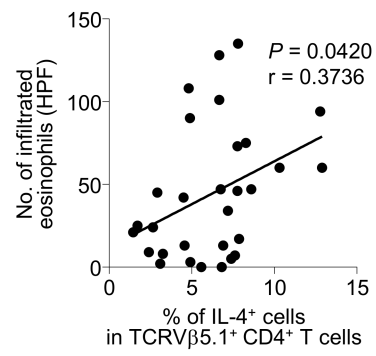


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Supplementary Methods

Sinonasal cell isolation

Harvested tissues from the NPs or EM during functional endoscopic sinus surgery were minced into small pieces, and then mechanically homogenized. The homogenates were filtered using a 70- μ m cell strainer (SPL Lifesciences, Pocheon, South Korea) and filtered cells collected.

Flow cytometry antibodies

Multi-color flow cytometry was performed using the following fluorochrome-conjugated antibodies: V500-conjugated anti-CD3 (UCHT1), APC-H7-conjugated anti-CD4 (RPA-T4), APC-H7-conjugated anti-CD8 (SK1), Alexa-Fluor 647-conjugated anti-Ki-67 (B56), PE or V450-conjugated anti-Bcl-2 (Bcl-2/100), BV421 or PE-Cy7-conjugated anti-GATA3 (L50-823), Alexa-Fluor 647-conjugated anti-T-bet (4B10), APC-conjugated anti-IFN- γ (B27), APC-conjugated anti-IL-5 (TRFK5), and BV510-conjugated anti-TSLPR (1F11/TSLPR) from BD Biosciences; PE-conjugated anti-IL-4 (7A3-3) and PE-Vio770-conjugated anti-TCRV β 5.1 (REA1062) from Miltenyi Biotec (Bergisch Gladbach, Germany); Alexa-Fluor 488-conjugated anti-IL-13 (eBio13A) from eBioscience; PerCP-Cy5.5-conjugated anti-CRTH2 (BM16) from BioLegend (San Diego, CA, USA); and PE-conjugated IL-17RB (FAB1207P) from R&D Systems (Minneapolis, MN, USA).

In vitro stimulation of sinonasal T cells

For intracellular cytokine staining (ICS), cells were stimulated with anti-CD3 antibody (0.1 μ g/mL; OKT3, BD Biosciences) or anti-CD3 (0.1 μ g/mL)/CD28 (1 mg/mL; CD28.2, BD Biosciences) antibodies for 12 hours. One hour after stimulation, brefeldin A

(BD Biosciences) was added and cells incubated another 11 hours. In some experiments, sinonasal cells were stimulated with *Staphylococcus aureus* enterotoxin B (SEB; 100 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) for 24 hours and brefeldin A added for the last 5 hours. After incubation, cells were harvested and subjected to ICS. When ICS was performed in combination with TCRV β 5.1 staining, PE-Vio770-conjugated anti-TCRV β 5.1 antibody (REA1062; Miltenyi Biotec) was used for staining compatibility. To examine the effect of IL-4 and IL-13 on Ki-67 expression, cells from the nasal mucosa of control subjects were cultured in the presence or absence of recombinant human IL-4 (10 ng/mL; PeproTech, Rocky Hill, NJ, USA) or IL-13 (10 ng/mL; PeproTech) for 24 hours. Next, anti-CD3 antibody (0.1 μ g/mL) was added, the culture incubated 72 hours, and Ki-67 stained as described above. To investigate the effect of dupilumab, sinonasal cells were treated with dupilumab (100 nM; Creative Biolabs, Shirley, NY, USA) in combination with anti-CD3/CD28 antibodies and IL-4.

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Table E1. Characteristics of the study subjects

Characteristics	CRS cohort 1		CRS Cohort 2
	CRSwNP	CRSsNP	CRSwNP
No. of subjects	20	20	30
Age, years	46.0 ± 10.8	41.5 ± 12.9	47.0 ± 14.9
Male/female	15/5	16/4	22/8
CT score ^a	13.8 ± 5.3	10.6 ± 4.5	15.4 ± 5.6
Atopy ^b	6	5	10
Asthma ^c	0	0	0
Eosinophil, % ^d	6.8 ± 1.2	5.1 ± 1.0	5.4 ± 3.5

Data are presented as mean ± SD unless otherwise noted.

^aEvaluated by the Lund-Mackay scoring system.

^bNumber of subjects with positive atopy results.

^cNumber of subjects with asthma.

^dFraction of eosinophil among peripheral white blood cells.

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44 **Table E2. PCR primers for superantigen detection**

Gene ^a	Primer	Oligonucleotide sequence (5'-3')	Location within gene ^b	Size of amplified product (bp)	Annealing temperature (°C)
SEA	Forward	TTGGAAACGGTTAAAACGAA	490-509	120	57
	Reverse	GAACCTTCCCATCAAAAACA	591-610		
SEB	Forward	TCGCATCAAACGACAAACG	634-653	478	60
	Reverse	GCAGGTACTCTATAAGTGCC	1091-1110		
SEC	Forward	GACATAAAAGCTAGGAATTT	676-695	257	57
	Reverse	AAATCGGATTAACATTATCC	913-932		
SED	Forward	CTAGTTTGGTAATATCTCCT	354-373	317	57
	Reverse	TAATGCTATATCTTATAGGG	652-671		
SEE	Forward	TAGATAAAGTTAAAACAAGC	491-510	170	57
	Reverse	TAACTTACCGTGGACCCTTC	640-659		
TSST-1	Forward	ATGGCAGCATCAGCTTGATA	251-270	350	57
	Reverse	TTTCCAATAACCACCCGTTT	581-600		
SEG	Forward	GCTATCGACACACTACAACC	62-81	583	60
	Reverse	CCAAGTGATTGTCTATTGTCG	44-624		
SEH	Forward	CACATCATATGCGAAAGC	261-278	548	60
	Reverse	CGAATGAGTAATCTCTAGG	808-790		
SEI	Forward	GATACTGGAACAGGACAAGC	6-25	789	62
	Reverse	CTTACAG GCAGTCCATCTCC	794-775		

^aThe sequences and locations are from the published data for SEA, SEB, SEC, SED, SEE, and TSST-1¹ and for SEG, SEH, and SEI.²

^bGiven in nucleotide numbers.

48 **Table E3. TCRV β chain usage in CD4⁺ T cells from the nasal mucosa of control subjects**

Subjects	Vβ (%)																							
	1	2	3	4	5.1	5.2	5.3	7.1	7.2	8	9	11	12	13.1	13.2	13.6	14	16	17	18	20	21.3	22	23
C01	3.8	12.1	4.6	0.0	8.8	5.2	2.8	0.0	0.0	7.5	1.8	0.0	0.0	6.0	1.5	1.5	2.7	3.5	7.0	6.6	5.5	8.3	1.4	3.0
C02	5.7	4.2	3.1	5.3	9.1	1.7	3.1	7.6	1.4	2.6	3.1	1.3	1.9	6.3	2.2	7.4	1.2	2.7	8.3	3.0	1.1	1.2	7.2	3.3
C03	1.3	18.8	10.8	1.5	4.7	0.0	0.4	0.8	1.5	1.7	2.1	0.8	1.3	5.5	6.6	1.1	1.5	2.1	2.5	2.4	2.4	4.0	2.3	2.7
C04	0.0	11.1	8.3	1.5	5.3	5.6	1.7	3.3	7.4	2.2	6.1	3.7	5.6	2.0	2.7	1.6	0.3	6.1	3.0	1.4	3.1	6.7	7.4	0.0
C05	1.8	9.2	4.7	4.2	12.8	3.3	2.3	5.7	3.0	8.5	0.5	5.3	0.0	1.0	1.0	2.2	6.1	3.1	9.0	2.8	1.8	0.0	1.8	0.0
C06	2.9	8.8	1.3	1.8	9.3	2.2	1.7	8.2	5.4	4.8	0.8	6.7	1.2	3.3	3.1	2.2	3.4	2.0	8.3	4.3	2.0	1.2	1.2	3.2
C07	1.9	6.7	3.0	1.5	6.8	0.4	0.9	1.2	0.9	3.4	4.2	0.4	1.5	5.0	5.4	2.9	4.1	3.2	15.5	2.0	1.6	1.1	5.5	2.4
C08	3.1	6.2	6.5	2.1	8.3	0.5	1.5	1.0	0.0	5.9	2.8	0.4	1.6	2.7	1.4	1.6	2.9	3.2	5.1	1.4	8.3	1.0	3.7	2.5
C09	2.1	10.4	5.2	6.7	2.8	1.2	2.2	1.4	2.3	8.2	3.2	3.1	1.0	1.4	2.9	2.6	1.1	1.9	8.8	3.3	7.7	0.9	1.9	1.5
C10	3.1	11.3	1.4	0.0	9.0	0.9	1.7	2.4	1.4	3.8	1.1	2.3	8.7	3.8	2.7	7.6	5.4	0.0	2.8	5.5	3.9	4.6	1.5	3.1
C11	1.0	7.9	2.6	0.2	5.1	1.7	1.3	5.3	0.5	3.0	0.8	3.5	5.6	5.6	5.7	5.7	1.6	2.0	6.2	1.6	2.1	3.4	4.6	1.8
C12	1.5	11.4	1.6	1.7	7.0	3.3	3.2	3.8	1.1	2.8	2.2	1.8	2.6	3.0	7.2	3.7	2.2	0.8	8.9	0.9	1.0	4.1	2.2	3.2
C13	2.7	6.8	4.0	3.9	2.6	4.2	4.2	7.8	2.2	1.1	0.3	3.4	4.4	1.9	3.3	2.0	3.7	4.6	3.6	1.2	2.6	1.9	6.1	0.2
C14	0.2	10.1	4.3	8.7	6.7	1.1	1.2	2.9	1.2	2.7	1.9	1.3	5.6	2.4	3.0	2.5	4.2	3.3	7.4	3.7	1.3	2.2	2.4	0.9
C15	1.9	5.8	9.2	3.9	8.3	0.6	1.8	3.9	0.3	1.8	4.5	0.9	1.1	5.0	1.5	1.2	5.1	1.3	10.6	1.2	4.1	1.8	1.1	1.2
C16	2.3	9.6	8.2	1.6	3.4	4.0	2.2	0.4	3.4	3.0	0.7	1.3	2.9	3.8	3.2	3.8	2.3	0.7	4.6	6.0	3.1	0.8	2.1	3.4
C17	1.9	10.3	4.5	1.2	5.4	1.8	3.2	2.1	0.9	5.9	2.0	3.4	7.8	2.0	4.4	2.8	1.2	3.8	3.8	4.8	1.9	0.9	1.9	1.0
C18	3.0	9.5	3.1	0.9	3.3	2.2	2.3	4.9	1.1	7.6	4.7	4.2	2.3	3.6	2.2	3.5	3.6	2.4	5.2	7.2	0.7	2.4	3.4	0.9
C19	2.6	15.4	6.8	0.6	1.1	3.2	1.9	2.2	3.6	4.6	2.9	1.8	1.6	1.9	4.7	2.3	1.2	2.8	8.1	3.2	1.9	3.7	2.6	2.1
C20	5.6	7.3	5.6	2.2	2.7	0.8	3.0	4.5	2.3	2.0	1.3	0.6	2.2	1.0	5.1	3.6	3.4	2.6	13.3	2.0	5.8	1.4	0.8	1.1
C21	1.7	9.6	3.8	1.2	1.2	1.9	1.2	5.6	2.1	3.5	2.7	1.1	3.4	2.1	3.1	1.9	3.5	6.1	10.0	4.9	2.3	0.3	2.4	1.8
C22	0.5	8.1	7.2	5.2	3.1	4.8	0.9	5.0	3.2	2.0	1.7	2.3	4.6	4.5	2.8	3.3	2.9	5.3	5.8	2.2	1.3	0.7	3.3	4.5
C23	0.6	12.4	1.9	1.3	8.8	1.9	2.6	3.6	0.6	2.5	5.6	1.1	2.2	5.2	4.5	2.3	1.4	3.4	7.7	3.6	3.4	2.1	2.2	1.9
Mean	2.2	9.7	4.8	2.5	5.9	2.3	2.0	3.6	2.0	4.0	2.5	2.2	3.0	3.4	3.5	3.0	2.8	2.9	7.2	3.3	3.0	2.4	3.0	2.0
SD	1.5	3.2	2.6	2.3	3.1	1.6	0.9	2.4	1.8	2.2	1.6	1.7	2.3	1.7	1.7	1.8	1.5	1.6	3.3	1.9	2.1	2.1	1.9	1.2

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Supplementary Figure Legends

Figure E1. T-bet expression in TCRV β 5.1⁺ and V β 5.1⁻ CD4⁺ T cells from NPs.

Representative FACS histogram showing the expression of T-bet in TCRV β 5.1⁺ and V β 5.1⁻ CD4⁺ T cells from the NPs of CRSwNP patients is presented.

Figure E2. Production of Th2 cytokines by TCRV β 5.1⁺ CD4⁺ T cells from NPs. (A–C)

Sinonasal cells from the NPs of CRSwNP patients (n=30) in CRS cohort 2 were stimulated with anti-CD3 antibody (A), anti-CD3/CD28 antibodies (B), or SEB (C). (A and B) Following stimulation, sinonasal cells were incubated for 12 hours and ICS performed to examine the percentage of IL-4⁺, IL-5⁺, and IL-13⁺ cells among TCRV β 5.1⁺ and V β 5.1⁻ CD4⁺ T cells. (C) Following stimulation, sinonasal cells were incubated for 24 hours and ICS performed to examine the percentage of IL-4⁺, IL-5⁺, and IL-13⁺ cells in TCRV β 5.1⁺ and V β 5.1⁻ CD4⁺ T cells. Representative FACS plots and summary data are presented. Statistical analysis was performed using the Wilcoxon signed-rank test.

Figure E3. Correlation between the frequency of TCRV β 5.1⁺ CD4⁺ T cells and production of Th2 cytokines by TCRV β 5.1⁺ CD4⁺ T cells. Sinonasal cells from the NPs of CRSwNP patients (n=30) in CRS cohort 2 were stimulated with anti-CD3 antibody for 12 hours. The percentages of IL-4⁺, IL-5⁺, and IL-13⁺ cells among TCRV β 5.1⁺ CD4⁺ T cells were plotted against the frequency of TCRV β 5.1⁺ cells among total CD4⁺ T cells from the NPs of CRSwNP patients. The significance of the correlation was tested using the Pearson correlation test.

Figure E4. The effect of IL-13 on the expression of Ki-67 and Bcl-2 in sinonasal CD4⁺ T cells. The expression of Ki-67 and Bcl-2 was examined in CD4⁺ T cells from the nasal

mucosa of control subjects (n=3) after stimulation with anti-CD3 antibody for 72 hours in the presence or absence of IL-13. Representative FACS histograms are presented.

Figure E5. The effect of dupilumab on IL-4-induced proliferation and GATA3

upregulation of sinonasal CD4⁺ T cells. The expression of Ki-67 and GATA3 was examined in CD4⁺ T cells from the nasal mucosa of control subjects (n=3) after stimulation with anti-CD3/CD28 antibodies for 72 hours in the presence or absence of IL-4 or dupilumab. Representative FACS plots are presented.

Figure E6. Correlation between the production of IL-4 by TCRV β 5.1⁺ CD4⁺ T cells and the number of eosinophils infiltrating NPs. Sinonasal cells from the NPs of CRSwNP patients (n=30) in CRS cohort 2 were stimulated with anti-CD3 antibody for 12 hours. The number of infiltrated eosinophils was plotted against the percentage of IL-4⁺ cells among TCRV β 5.1⁺ CD4⁺ T cells from the NPs of CRSwNP patients. The significance of the correlation was tested using the Pearson correlation test.

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