Abstract

Role of Innate Immunity in Chronic Rhinosinusitis with Nasal Polyp

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Background: Currently, growing evidences suggest that chronic rhinosinusitis with nasal polyp (CRSwNP) in Western patients is characterized by a Th2-based immune response with abundant eosinophilic infiltration, whereas studies of CRSwNP in Asian patients predominantly show non-eosinophilic inflammation with a mixed T cell immune response. However, the precise pathogenesis of CRSwNP in Asian still remains unclear.

Objective: I thought to investigate the role of innate immunity in CRSwNP in Asian population.

Methods: Tissues from uncinate process (UP) were obtained from controls and CRS without nasal polyps (CRSsNP). NP and UP were obtained from CRSwNP. The innate immune cells, interleukin (IL)-25, 33 were evaluated by immunohistochemistry (IHC), quantitative reverse transcription PCR (qRT-PCR), and enzyme-linked immunosorbent assay **(**ELISA). To confirm the function of IL-25, 33 during nasal polypogenesis, anti-IL-25, -33 neutralizing antibody was administered in a murine model of CRS with polypoid lesions.

Results: In the innate immune cell study, the MBP^+ and $CD11c^+$cells were continuously increased during progression of CRS and positively correlated with disease extent of CRS. The distribution of

innate immune cells was remarkably different depending on the allergic status of CRSwNP patients. In the IL-25 study, I found that epithelial cells and infiltrating mast cells of NPs showed prominent IL-25 expression, which positively correlated with the expression of multiple inflammatory markers, such as T-bet, RORC, GATA3. In addition, I observed the anti-polyp effect obtained by inhibiting IL-25-responsive innate lymphoid cells and by suppressing the recruitment of effector cells, such as eosinophils and neutrophils in a murine model of CRS with polypoid lesions. In the IL-33 study, I revealed that CRSwNP is characterized by elevated expression of IL-33 in UP tissues, and these levels correlated with levels of Th1/Th17 cytokines, and multiple remodeling markers. In addition, the expression of IL-33 was upregulated and associated with inflammatory markers, which related with tissue remodeling and neutrophil recruitment, in non-eosinophilic NPs. Moreover, anti-IL-33 antibody showed an inhibitory effect on nasal polypogenesis by reducing the recruitment of neutrophils in a murine model of CRS with polypoid lesions.

Conclusions: The infiltration of MBP^+ and $CD11c^+$ innate immune cells show a significant association with phenotype of CRS and disease extent of CRSwNP, and allergic status also may influences cellular phenotype in Asian CRSwNP. Moreover, IL-25 and IL-33 expressions are elevated in patients with CRSwNP and the expression of those is associated with other inflammatory markers. Neutralizing those (IL-25 and IL-33) reduce the nasal polypogenesis in an animal model. Therefore, I propose that the innate immunity is one of the crucial roles of nasal polypogenesis as well as a promising target for the treatment of nasal polyposis in the Asian population.

Key Words: Chronic rhinosinusitis, nasal polyp, innate immunity, interleukin-25, interleukin-33.

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Introduction

Chronic rhinosinusitis (CRS) affects is characterized by accumulation of inflammatory cells with marked tissue remodeling. This disease is currently divided into two categories: CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP) (1). In the past several decades, numerous studies have been performed to investigate nasal polypogenesis in patients from Western countries. These studies suggest that CRSwNP in Western countries is characterized by a Th2-based immune response with abundant eosinophilic infiltration, high levels of interleukin (IL-5), and low levels of transforming growth factor (TGF)-β1 (2-4). To date, several studies for CRS have been conducted in Asian. In contrast to the findings in Western patients, these studies described a Th1/Th2/Th17 mixed polarization and less eosinophilic inflammation (5-7). Thus, upstream mechanisms that incite the T cell immune response are crucial for understanding the pathogenesis of CRSwNP (8). However, these mechanisms are not thoroughly explored, especially in Asian patients. Recent some studies describe an important role of the innate immunity on the initiation of adaptive immunity and thus, it seems to play an important role in pathogenesis of CRS (9-11). Specifically, the type 2 innate lymphoid cells (ILC2s) which produce IL-5 and IL-13 are responded to exposure of innate cytokines, such as IL-25 and IL-33 (9).

IL-25 (also known as IL-17E), a member of the IL-17 cytokine family, were introduced to play an important function in promoting Th2-mediated inflammation (12). In animal study, intraperitoneal or intranasal administration of IL-25 protein resulted in the production of eosinophils, eotaxin, and Th2 cytokines in the bronchoalveolar lavage and lung tissue (13, 14). Conversely, blocking of IL-25 decreases the production of Th2 cytokines in an asthma animal model (14-16). In addition, IL-25 enhances thymic stromal lymphopoietin-induced Th2 immune response (17). Elevated expression of IL-25 and IL-25R also has been observed in patients with asthma and atopic dermatitis, possibly linking their functions with the exacerbation of allergic disorders (17). Moreover, one study described that an increased IL-25 expression associated with disease severity and increased serum eosinophils

levels in patients with CRS (18). Despite the relationship between IL-25 and Th2-dominant diseases, the specific role of IL-25 in CRSwNP remains unclear.

Meanwhile, IL-33 is a novel member of the IL-1 superfamily and is constitutively expressed at high levels in epithelial cells from human and mouse tissues during homeostasis (19). Biologically active full-length IL-33 can be released extracellularly by necrotic cells after tissue damage (20, 21). After release, IL-33 subsequently serves as an "alarmin" by triggering innate immunity by activating various types of immune cells, including mast cells, basophils, eosinophils, and ILC2s (22-25). In addition, endogenous IL-33 induces IL-5 and IL-13 by ILC2s for the initiation of Th2-mediated inflammatory responses in allergic diseases, such as asthma, allergic rhinitis, and atopic dermatitis (26-30). More recently, expression of IL-33 and its receptor ST2 was investigated in CRSwNP patients (31-33), and two of these studies reported elevated expression of ST2 (31, 32), implying that the IL-33/ST2 pathway may function in nasal polypogenesis. Despite these findings, the specific role of IL-33 in the pathophysiology of CRSwNP in Asian patients has not been fully understood.

Furthermore, these innate cytokines are produced by innate immune cells including, eosinophils, mast cells, macrophages, dendrite cells, basophils, and neutrophils (10-12). However, the relationship between innate immune cells and pathogenesis of CRS in Asian patients has not yet been investigated. Therefore, in this study, I firstly evaluated the distribution of innate immune cells and investigated any differences of its clinical relevance according to the phenotype of CRS in Asian patients. Secondly, I investigated the expression of innate cytokines (IL-25 and IL-33) and the relationship between each innate cytokine and inflammatory markers in sinonasal tissues according to the different phenotypes of Asian patients with CRS. I also evaluated the therapeutic potential of IL-25 or IL-33 blockade on the nasal polypoid mucosa in a murine model.

Materials and Methods

Patients and tissue samples

This study was approved by the internal review board of Seoul National University Hospital, Boramae Medical Center (No. 06-2012-109). CRS was diagnosed according to the 2012 European position paper on rhinosinusitis and nasal polyps (EPOS) guidelines (1). Exclusion criteria were as follows: younger than 18 years of age, prior treatment with antibiotics, systemic or topical corticosteroids, or other immune-modulating drugs for 4 weeks before surgery, and unilateral rhinosinusitis, antrochoanal polyp, allergic fungal sinusitis, cystic fibrosis, or immotile ciliary disease. In this study, I obtained in uncinate process (UP) tissue from control and CRS patients, including those with CRSsNP or CRSwNP. I also evaluated NP tissue in patients with CRSwNP. When NPs originated from UPs, only NPs were obtained during routine functional endoscopic sinus surgery in CRSwNP patients. Each sample obtained was divided into three parts: one third was fixed in 10% formaldehyde and embedded in paraffin for histological analysis, another third was immediately frozen and stored at -80ºC for subsequent isolation of mRNA and proteins, and the final third of the tissue was submersed in 1 mL phosphate-buffered saline (PBS) supplemented with 0.05% Tween-20 (Sigma-Aldrich, St Louis, MO) and 1% PIC (Sigma-Aldrich) per 0.1g of tissue. This tissue was homogenized with a mechanical homogenizer at 1,000 rpm for 5 min on ice. After homogenization, the suspensions were centrifuged at 3,000 rpm for 10 min at 4ºC. The supernatants were separated and stored at -80ºC for further analysis of cytokines and other inflammatory mediators. The atopic status of study patients was evaluated using the ImmunoCAP® assay (Phadia, Uppsala, Sweden). NPs were classified into two groups: eosinophilic NP, which was defined as having an eosinophil count of more than 10% of the inflammatory cell population, and non-eosinophilic NP, which did not fulfill this criterion (34). Allergic CRSwNP were defined as NP tissues from patients who had allergic symptoms and positive results on the ImmunoCAP assay (35).

Immunohistochemistry (IHC)

IHC staining was performed with Polink-2, polymerized horseradish peroxidase (HRP), and broad DAB Detection System (Golden Bridge International Labs., WA, USA). To identify cellular sources of IL-25, sequential IHC was employed using polymer-HRP and alkaline phosphatase kits to detect mouse and rabbit primary antibodies for human tissue with permanent-Red and Emerald (Polink DS-MR-Hu C2 Kit; Golden Bridge International Labs). The primary antibodies were mouse anti-human eosinophil major basic protein (MBP) (1:50; Santa Cruz Biotech., California, USA), mouse anti-human CD11c (1:5; BD Pharmingen, California, USA), mouse anti-mast-cell tryptase (1:500; Abcam, Cambridge, UK), mouse anti-CD68 (1:250; Abcam), mouse anti-CD163 (1:25; Abcam), mouse anti-basophils (2D7) (1:10; Abcam), anti-human neutrophil elastase (HNE) (1:100; Abcam), rabbit anti-human IL-25 (1:500; Abcam), goat anti-human IL-17RB (1:50; R&D systems, MN, USA), rabbit anti-human IL-33 (1:250; Abcam), rabbit anti-human ST2 (1:100; Millipore, Darmstadt, Germany), and rabbit anti-human collagen (1:1000; Abcam). For quantification of positive collagen content, image analysis was performed using ImageJ software. The total collagen amount was calculated for each image after subtraction of background, and this value was expressed as a percentage of the total area.

Quantitative real-time PCR (qRT-PCR)

The qRT-PCR was performed with a LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany). Total RNA was extracted from tissue samples using the TRI reagent (Invitrogen, Carlsbad, CA). One microgram total RNA was reverse transcribed to cDNA using the cDNA Synthesis Kit (*amfiRivert Platinum* cDNA Synthesis Master Mix, GenDEPOT). Primer sequences are as follows: GAPDH, 5′-CATGGGTGTGAACCATGAGAA-3′ for the forward primer and 5′-GGTCATGAGTCCTTCCACGAT-3′ for the reverse primer; T-bet, 5′-GTCAATTCCTTGGGGGAGAT-3′ for the forward primer and 5′-TCATGCTGACTGCTCGAAAC-3′ for the reverse primer; GATA3, 5′-ACCACAACC ACACTCTGGAGGA-3′ for the forward primer and 5′-TCGGTTTCTGGTCTGGATGCCT-3′ for the reverse primer; RORC, 5′-

GCTGTGATCTTGCCCAGAACC-3′ for the forward primer and 5′-CTGCCCATCATTGCTGTTAATCC-3′ for the reverse primer; TGF-β1, 5′-TGAACCGGCCTTTCCTGCTTCTCATG-3′ for the forward primer and 5′-GCGGAAGTCAATGTACAGCTGCCGC-3′ for the reverse primer; TGF-β2, 5′-TGGATGCGGCCTATTGCT TTA-3′ for the forward primer and 5′-GCGGAAGTCAATGTACAGCTGCCGC-3′ for the reverse primer; TGF-β3, 5′-GTGAGTGGCTGTTGAGAAGAGA-3′ for the forward primer and 5′-GAGGATTAGAGGGTTGTGG-3′ for the reverse primer; and ECP, 5′-TCGGAGTAGATTCCGGGTG-3′ for the forward primer and 5′-GAACCA CAGGATACCGTGGAG-3′ for the reverse primer. In addition, primer for IL-25 and COL1A1 (#QT00037793) were purchased from Qiagen (Qiagen Korea Ltd., Seoul, Korea). TaqMan® Gene Expression Assay kits (Life Technologies Korea, Seoul, Korea) were also purchased and used for measuring mRNA levels from human and animal samples (Table 1). Pre-developed assay reagent kits containing primers and probes were purchased from Applied Biosystems (Foster City, CA). Expression of GAPDH (Human; Hs02758991_g1 and Mouse; Mm99999915_g1) was used as an internal control for normalization. Cycling conditions were as follows: 95°C for 5 min followed by 60 cycles at 95°C for 15 sec, 60°C for 20 sec, and 72°C for 20 sec. To analyze the data, I used Sequence Detection Software version 1.9.1 (Applied Biosystems). Relative gene expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Before ELISA, the protein concentrations for tissue extracts were determined using the Quick Start Bradford Protein Assay Kit (BIO-RAD, Seoul, Korea). Samples were thawed at room temperature and vortexed to ensure well-mixed sample. And then, tissue homogenates were assayed for IL-25, IL-17RB, and IL-33 proteins by using commercially available ELISA kits (R&D systems) in accordance with the manufacturer's instructions. The minimal detection limit for these kits is 62.5, 156, and

23.4pg/mL, respectively. All procedures followed the information of the manufacturer. Concentrations of IL-25, IL-17RB, and IL-33 were normalized to the concentration of total protein.

Murine model of allergic chronic rhinosinusitis with polypoid lesions

All animal experiments were approved by the IACUC of Boramae Medical Center (No.2013-0001) and were performed under strict governmental and international guidelines on animal experimentation. The murine models of allergic CRS with polypoid lesions was induced according to the previously established protocol (36), which has been confirmed by multiple studies (37-39). Thirty-two female BALB/c mice (4 weeks of age, 20-25 g) were purchased from Koatech Laboratory Animals, Inc. (Pyeongtaek, Korea). The mice were maintained under specific pathogen-free conditions with a 12/12-hour light/dark cycle. Briefly, mice in the experimental groups were systemically sensitized with 25 μg of ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 300 μl of PBS in the presence of 2 mg of aluminum hydroxide gel as an adjuvant by intraperitoneal injection on days 0 and 5. One week after the second intraperitoneal injection, mice were challenged intranasally with 3% OVA diluted in 40 μl of PBS daily for 1 week. Thereafter, continual local stimulation was maintained by the same procedure three times per week for 12 consecutive weeks. During the last eight consecutive weeks, 20 ng of *Staphylococcal enterotoxin B* (SEB) diluted in 20 mL of PBS was used as a challenge immediately after the administration of 3% OVA weekly. In the IL-25 study, mice were categorized into one control and three experimental groups: PBS-instilled (PBS, n=10), NP model group (POLYP, n=10), NP model group treated with anti-IL-25 (R&D systems, Minneapolis, MN; POLYP+IL-25, n=8), and NP model group treated with dexamethasone (POLYP+steroid, n=8). POLYP group, POLYP+αIL-25 group and POLYP+steroid group were weekly administered intraperitoneal isotype IgG (300 μg per a mouse), anti-IL-25 (300 μg per a mouse) and dexamethasone (1mg/kg) from day 49 through day 102 before OVA instillation, respectively. Mice in control (PBS) were not sensitized but weekly administered intraperitoneal isotype IgG (300 μg per a mouse) from day 49 through day 102 before OVA instillation. Mice were sacrificed on day 103. Mice

were sacrificed 24 hours after the last OVA challenge. In the IL-33 study, mice were categorized into one control and two experimental groups: PBS control group (n=10), SEB-induced nasal polyp model group (POLYP, n=12), and polyp model group treated with anti-IL-33 (R&D systems, Minneapolis, MN, USA; POLYP+αIL-33, n=10). The POLYP group and POLYP+αIL-33 group were administered isotype IgG (purified normal rabbit IgG; R&D Systems; 30 μg per mouse) and anti-IL-33 (R&D Systems; 30 μg per a mouse) intraperitoneally each week from 5 to 12 weeks before OVA and SEB administration, respectively. Mice in the control group (PBS) were not sensitized but were given intraperitoneal isotype IgG (30 μg per a mouse) weekly from day 49 through day 102 before OVA instillation. Mice were sacrificed 24 hours after the last OVA challenge.

Histopathological analysis of animal tissues

Twenty-four hours after the final OVA challenge, the heads of 5 mice from the PBS and POLYP+αIL-33 groups and those of 7 mice from the POLYP group were removed *en bloc* and then fixed in 4% paraformaldehyde for histopathology analysis. After exposing the nasal cavity from the heads of the other mice, the nasal mucosa was removed meticulously using a small curette and micro-forceps under the microscopic (n=5 for each group). For evaluation of nasal histopathology, nasal tissues were decalcified, embedded in paraffin, and sectioned coronally (4μm thickness) approximately 5 mm from the nasal vestibule. Samples were stained with the following stains to compare the characteristics between groups: hematoxylin and eosin (H&E) for polyp-like lesions, Sirius red for eosinophils, anti-neutrophilic antibody (1:50; Abcam) for neutrophils, Alcian blue for goblet cells, and Masson's trichrome staining for collagen fibers in the subepithelial layer. Ten areas from nasal mucosal sections were chosen randomly for evaluation under high-power magnification (x400). Polyp-like lesions were defined as distinct mucosal elevations with eosinophilic infiltration and microcavity formation. The thickness of the subepithelial collagen was measure using an image analysis system (DP2-BSW software; Olympus, Tokyo, Japan). The mucosal thickness was measured as the distance between the apex of the epithelial cells and the upper border of the subepithelial glands

zone using an image analysis system. For the assessment of mucosal thickness, at least three measurements at random points with a minimum distance of 20 μm between the points were made in the appropriate area of each high power field (HPF), and the mean from four different HPFs was recorded for comparison.

Cytokines from nasal lavage fluid in murine model

After partial tracheal resection under deep anesthesia, a micropipette was inserted into the posterior choana through the tracheal opening in the direction of the upper airway. Each nasal cavity was gently perfused with 200μL PBS, and the fluid from the nostril was collected and centrifuged. Supernatants were stored at -80ºC. Levels of cytokines in the nasal lavage fluids were measured using ELISA kits purchased from BioLegend (San Diego, CA). The lower detection limits of these ELISA kits were 8pg/mL for IFN-γ, 0.5pg/mL for IL-4, 2.7pg/mL for IL-17A, and 2.3pg/mL for TGF-β1.

Statistical analysis

Statistical analyses were performed using IBM SPSS 21 (SPSS, Inc., Chicago, IL) and GraphPad Prism software 6.0 (GraphPad software Inc, La Jolla, CA). In this study, the Kruskal-Wallis test and the Mann-Whitney *U*-test with a 2-tailed test for unpaired comparisons were used. The Pearson correlation test was also used to determine variable relationships. If the data was not normally distributed, the Spearman correlation coefficient was utilized (**P*<0.05, ***P*<0.010, and ****P*<0.001).

Table 1. TaqMan® Gene Expression Assays.

Gene of Interest	Sample	Primer
IL-33	Human	Hs00369211_m1
IL1RL1 (ST2)	Human	Hs00545033_M1
IL-4	Human	Hs00174122_m1
IL-5	Human	Hs01548712_g1
IL-13	Human	Hs00174379_m1
IL-17A	Human	Hs00174383_m1
IL-22	Human	Hs01574154_m1
IL-23p19	Human	Hs00900828_g1
IFN-γ	Human	Hs00989291_m1
TNF-α	Human	Hs01113624_g1
CXCL1	Human	Hs00236937_m1
CXCL2	Human	Hs00601975_m1
MPO	Human	Hs00924296_m1
IL-4	Mouse	Mm00445259_m1
IL-5	Mouse	Mm00439646_m1
IL-17A	Mouse	Mm00439618_m1
IFN-γ	Mouse	Mm01168134_m1
ICAM 1	Mouse	Mm00516023_m1
VCAM 1	Mouse	Mm01320970_m1
CCL11	Mouse	Mm00441238_m1
CCL24	Mouse	Mm00444701_m1
CXCL1	Mouse	Mm04207460_m1
CXCL2	Mouse	Mm00436450_m1

Results

Role of Innate immune in Chronic Rhinosinusitis

1. Distribution of innate immune cell in different types of CRS

Patient characteristics and type of method in this study was presented in Table 2. To investigate the cellular distribution of innate immune cells, I performed IHC on UP tissues from controls, CRSsNP, and CRSwNP, and on NPs from CRSwNP (Fig 1). Figure 2 shows the comparison of cellular distribution in nasal mucosal tissues according to different types of CRS. UP tissues from those with CRSsNP showed a significantly greater number of tryptase$^+$ cells, CD68$^+$ cells, CD163$^+$ cells, 2D7$^+$ cells, and HNE$^+$ cells compared to those from the controls. In contrast, UP tissues from patients with CRSwNP had increased numbers of MBP$^+$ cells, CD68$^+$ cells, and CD11c$^+$ cells compared to the UP tissues from the CRSsNP group. Examination of the cell populations in UP and NP tissues from the CRSwNP group showed that greater numbers of MBP$^+$ cells, CD11c$^+$ cells, 2D7$^+$ cells, and HNE$^+$ cells were present in NP tissues, whereas the number of CD68$^+$ cells were decreased in NP tissues. In addition, the MBP$^+$ and CD11c$^+$ cells were increased from UP of CRSsNP, to UP of CRSwNP, and to NP of CRSwNP (Fig 2A and 2E). On analyzing the relationship between the distribution of innate immune cells (Fig. 2H and 2I), I found that the number of MBP$^+$ cells was strongly correlated with other immune cells (r=0.417***, MBP vs. CD68; r=0.618***, MBP vs. CD11c; r=0.543***, MBP vs. tryptase; r=0.557**, MBP vs. 2D7; r=0.380*, MBP vs. CD163). In addition, the number of CD11c$^+$ cells also showed strong correlation with several immune cells (r=0.618***, CD11c vs. MBP; r=0.357*, CD11c vs. tryptase; r=0.490***, CD11c vs. 2D7; r=0.427**, CD11c vs. CD163).

2. Correlation of innate immune cells and clinical implications in CRSwNP

The relationships between innate immune cells and clinical parameters of CRSwNP are shown in Table 3. Interestingly, the disease extent of CRSwNP was positively correlated with the number of

MBP^+ cells (r=0.516, $P<.05$) and $CD11c^+$ cells (r=0.449, $P<.05$) in UPs from CRSwNP. Meanwhile, polyp grade and postoperative findings was not significantly correlated with all innate immune cells.

3. Cellular pattern of innate immune cells according to clinicohistologic parameters in CRSwNP

I evaluated the distribution patterns of innate immune cells in different types of CRSwNP. As illustrated in Figure 3, there was a significant higher count of MBP^+, $tryptase^+$, $CD163^+$, and $CD11c^+$ cells in eosinophilic NP compared with non-eosinophilic NP. Compared with non-allergic NP, the numbers of MBP^+, $tryptase^+$, $CD163^+$, and $CD11c^+$ cells were also significantly increased in allergic NP. Meanwhile, in non-eosinophilic NP, overall innate immune cells were decreased compared with eosinophilic or allergic NP. Furthermore, in comparison between UP from CRSwNP and NP from non-eosinophilic NP, the number of HNE^+ cells and $CD11c^+$ cells were significantly higher in NP tissues and UP tissues. However, I found that less number of $CD68^+$ cells was present in NP tissues. Based on the above results, I classified NP samples into four groups: non-eosinophilic non-allergic, non-eosinophilic allergic, eosinophilic non-allergic or eosinophilic allergic (Fig 4). The frequency of MBP^+ cell infiltration was higher in eosinophilic allergic NP than in non-eosinophilic non-allergic NP (Fig 4A). Moreover, eosinophilic allergic NP had more $tryptase^+$ cells, $CD11c^+$ cells, and $2D7^+$ cells than observed in other types of CRSwNP (Fig 4B, 4E, and 4F). In addition, compared with non-eosinophilic non-allergic NP and eosinophilic non-allergic NP, significantly increased numbers of $2D7^+$ cells and HNE^+ cells were found in non-eosinophilic allergic NP (Fig 4F and 4G). These data imply that allergic status influence cellular distribution in eosinophilic or non-eosinophilic NP.

4. T cell differentiation in different types of non-asthmatic CRS

I investigated the expression levels of mRNAs for T-bet, GATA-3, and RORC in UP tissues from non-asthmatic patients with CRSsNP, patients with CRSwNP, and controls. Interestingly, the mRNA levels of T-bet, GATA-3, and RORC were significantly up-regulated in CRSwNP compared with those in controls (P=.020, P=.029, P=.041). Moreover, T-bet and RORC mRNA expression levels were significantly increased in CRSwNP compared with those in CRSsNP (P=.038, P=.030). However,

there were no differences in the levels of T-bet, GATA-3, and RORC3 in the CRSsNP group and control group, although differences in T-bet expression approached significance (*P*=.065).

Table 2. Patient characteristics and type of method in the innate immue cell study

	Control	**CRSsNP**	**CRSwNP**	
Total no. of subjects	**N = 18 (5 male)**	**N = 45 (23 male)**	**N = 56 (35 male)**	
Tissue used	UP	UP	UP	NP
Age (yr), mean (SD)	41 (19)	49 (12)	49 (13)	46 (16)
Allergic rhinitis, N (%)	0 (0%)	16 (35%)	7 (36%)	16 (37%)
Asthma, N	0	0	0	0
Aspirin sensitivity, N	0	0	0	0
Lund-Mackay CT score	0 (0)	8.8 (5.0)	14.0 (6.5)	15.2 (6.6)
Lund-Kennedy score	NA	0.9 (1.6)	3.2 (2.5)	3.4 (2.8)
Blood eosinophil (%)	3.6 (2.3)	5.4 (3.8)	4.1 (3.6)	3.6 (3.4)
Polyp characteristics (Eosinophilic/Non-eosinophilic)	NA	NA	NA	22/34
Methodologies used				
Tissue IHC	18	45	56	56
FACS	0	10	0	11
Tissue mRNA	18	25	19	25

Lund-Kennedy score was evaluated at postoperative 6 months.

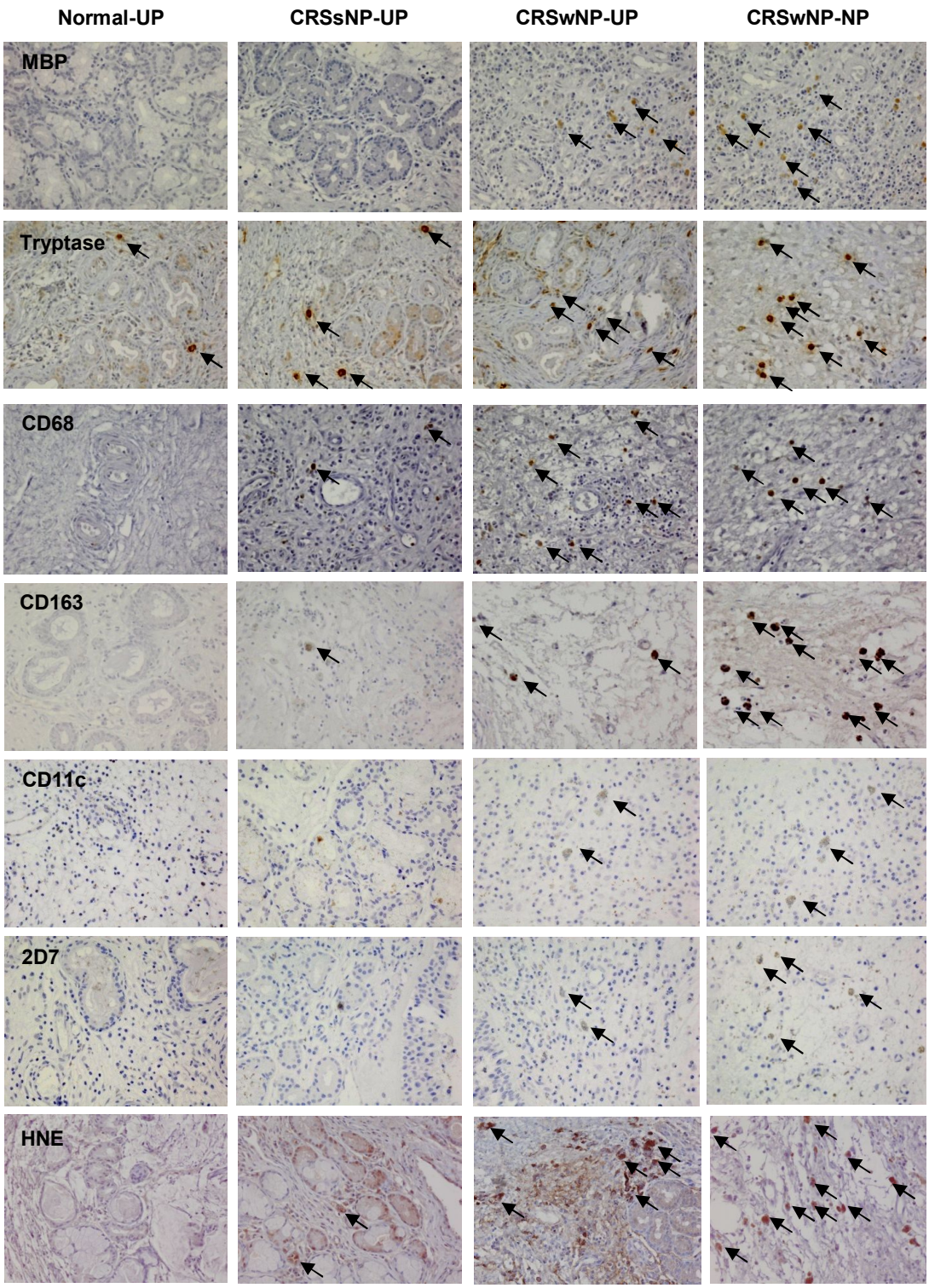

Figure 1. Representative photographs of the immunohistochemical detection of innate immune cells. UP, uncinate process tissue; NP, nasal polyp tissue; CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps; MBP, anti-human eosinophil major basic protein; HNE, anti-human neutrophil elastase (magnification x400).

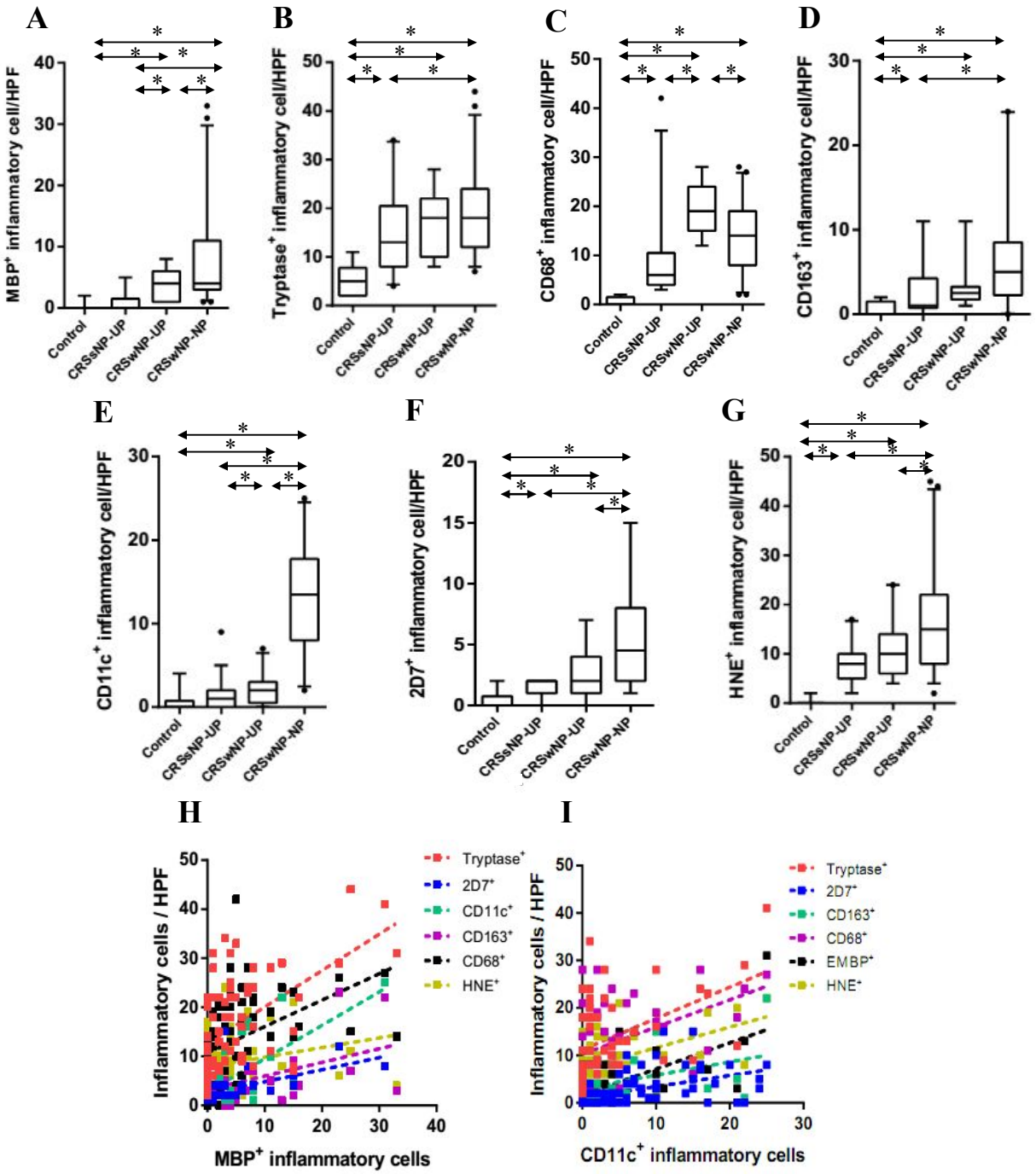

Figure 2. Distribution of innate immune cells in different type of CRS. The number of **(A)** MBP^+ **(B)** $tryptase^+$ **(C)** $CD68^+$ **(D)** $CD163^+$ **(E)** $CD11c^+$ **(F)**, $2D7^+$ **(G)** HNE^+ cells in nasal mucosal tissues was compared according to different types of chronic rhinosinusitis. Correlation between innate immune cells was analyzed; in terms of **(H)** MBP^+ cell and **(I)** $CD11c^+$ cell. UP, uncinate process tissue; NP, nasal polyp tissue; CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps (*P <.05).

Table 3. Relationship between immunohistochemistry analysis and clinical parameters

Tissue	Positive cell	Clinical parameters		
		CT score	**Polyp grade**	**LK score at 6 months**
UP in CRSwNP	MBP^+	**0.516***	0.371	0.471
	$Tryptase^+$	0.228	0.204	0.066
	$CD68^+$	0.128	-0.151	0.224
	$CD163^+$	-0.044	-0.122	0.354
	$CD11c^+$	**0.449***	0.358	0.458
	$2D7^+$	0.112	-0.120	0.125
	$Elastase^+$	-0.192	-0.298	-0.259

LK score means Lund-Kennedy score. *P<.05

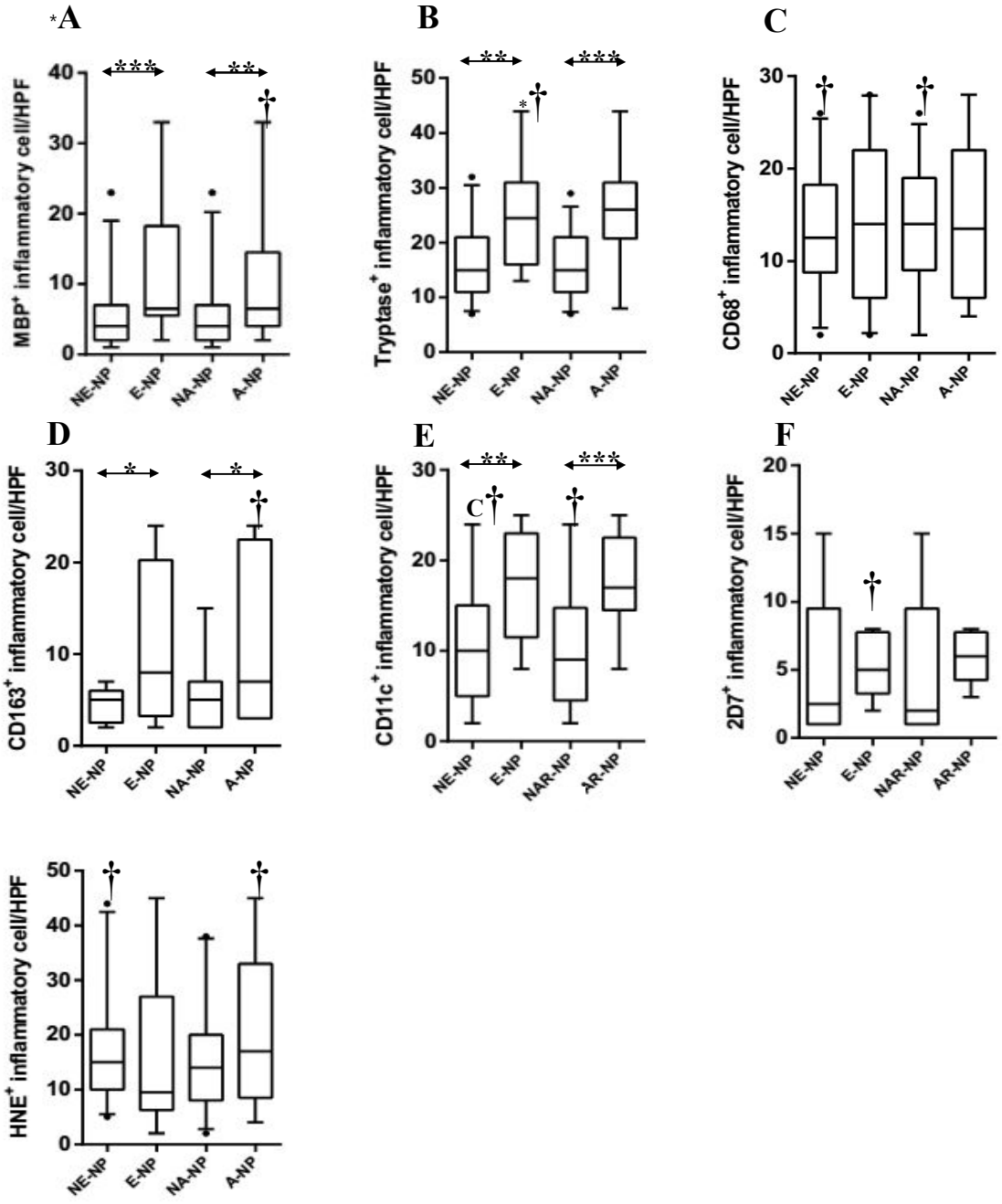

Figure 3. Distribution of innate immune cells according to clinicohistologic parameter. The number of **(A)** MBP$^+$ **(B)** tryptase$^+$ **(C)** CD68$^+$ **(D)** CD163$^+$ **(E)** CD11c$^+$ **(F)** 2D7$^+$ **(G)** HNE$^+$ cells in nasal mucosal tissues was compared according to clinicohistologic parameter: NE-NP, non-eosinophilic CRSwNP; E-NP, eosinophilic CRSwNP; NA-NP, non-allergic CRSwNP; A-NP, allergic CRSwNP (*P<.05, **P<.01, ***P<.001). †symbol means statistical significance when it compared with uncinate process tissue from CRSwNP.

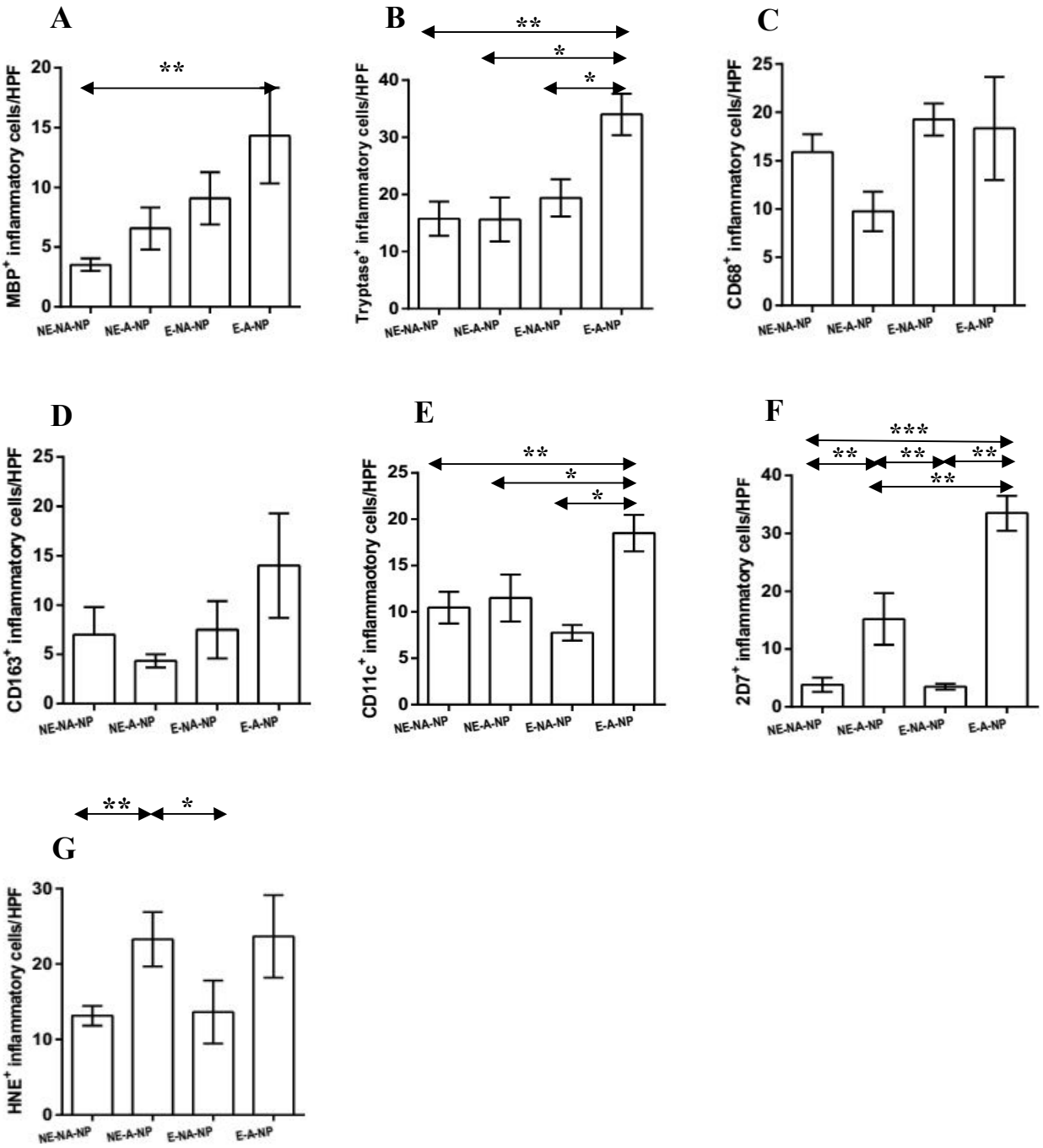

Figure 4. Expression of innate immune cells between subgroup analyses. Comparison of **(A)** MBP$^+$ **(B)** tryptase$^+$ **(C)** CD68$^+$ **(D)** CD163$^+$ **(E)** CD11c$^+$ **(F)** 2D7$^+$ **(G)** HNE$^+$ cells were performed in nasal polyp tissues among four groups: non-eosinophilic non-allergic (NE-NA-NP), non-eosinophilic allergic (NE-A-NP), eosinophilic allergic (E-NA-NP), eosinophilic allergic NP (E-A-NP) (*P<.05, **P<.01, ***P<.001).

Role of Interleukin-25 in Chronic Rhinosinusitis

1. Interleukin-25 expression and cellular origin in CRSwNP

Patient characteristics and type of method in this study was presented in Table 4. To measure expression of IL-25 in patients with CRSwNP, tissues were collected from patients with CRSsNP (UP), patients with CRSwNP (NP and UP), and control subjects (UP). Expression was higher in epithelial cells of NPs than in those of UPs in CRSsNP patients and controls (Fig. 5A and 5B). I documented a significant increase in IL-25-positive inflammatory cells in NPs and UPs of CRSwNP patients compared with UPs from control and CRSsNP subjects (Fig. 5C). I also examined the expression of IL-25 mRNA in each tissue and found that IL-25 mRNAs levels were significantly higher in NP and UP tissues from patients with CRSwNP than UP tissues from other patient groups (Fig. 5D). To examine this observation at the protein level, the concentration of IL-25 was measured using ELISA. These data demonstrated that IL-25 protein levels were significantly increased in NP tissue homogenates from patients with CRSwNP compared with control tissues (Fig. 5E). I used double IHC staining to identify IL-25 positive cells in the subepithelial layer, (Fig. 5F), and the number of double-positive IL-25 and tryptase cells ranged from 1 to 41/HPF (median 19/HPF, n=7) in NPs, whereas the number of double-positive cells for IL-25 and other immune cells, such as MBP^+, $CD68^+$, $CD11c^+$ and $2D7^+$ cells, was 0-23 (median 5/HPF, n=7), 0-12 (median 3/HPF, n=7), 3-13 (median 4/HPF, n=7), and 0-13 (median 6/HPF, n=7), respectively.

2. Interleukin-17RB expression in CRSwNP

IL-25 has been reported to bind and signal through IL-17RB (also known as IL-17BR or IL-17Rh1), a member of the IL-17R family of cytokine receptors. Therefore, I measured expression levels of IL-17RB in nasal tissues from control, CRSsNP, and CRSwNP patients. IL-17RB-positive inflammatory cells were significantly increased in both CRSsNP and CRSwNP patients compared with controls (Fig. 5G and 5H), and IL-17RB protein levels were significantly higher in NP tissue homogenates from patients with CRSwNP compared with controls (Fig. 5I).

3. Correlations between interleukin-25 mRNA expression and other inflammatory markers

To investigate the implication of upregulated IL-25 in CRSwNP, I examined if IL-25 expression correlated with inflammatory markers, such as T-bet, RORC, GATA3, ECP, TGF-β1, and TGF-β2. Figure 6 shows IL-25 expression positively correlated with all inflammatory markers tested: T-bet (r=0.805, *P*=0.001), RORC (r=0.970, *P*<0.001), GATA3 (r=0.508, *P*=0.007), ECP (r=0.696, *P*=0.010), TGF-β1 (r=0.447, *P*<0.001), and TGF-β2 (r=0.473, *P*=0.009).

4. Anti-polyp effect of interleukin-25 neutralizing antibody in polyp animal models

To investigate the role of IL-25 in nasal polypogenesis, I used an NP mouse model and confirmed its IL-25 expression. Nasal polyp models showed higher IL-25 expression in NP mice compared to control mice (Fig. 7A and 7B). Both anti-IL-25 (POLYP+αIL-25) and steroid (POLYP+steroid) treatment reduced the number of nasal polyps, and mucosal thickness in NP mice (Fig. 7C-E). I also observed the decreased numbers of eosinophils, neutrophils and the thickness of subepithelium in the POLYP+αIL-25 group compared to the untreated POLYP group (Fig. 7F-H). However, the anti-IL-25 antibody had no inhibitory effect on goblet cell hyperplasia (Fig. 7I).

5. Changes in cytokines, chemokines, and adhesion molecules after interleukin-25 inhibition

Both anti-IL-25 and steroid therapy suppressed IL-25 expression in the mouse model (Fig. 8A), and also cytokine profiles of nasal lavage fluid samples reflected the histological findings. Anti-IL-25 treatment reduced levels of IL-4, INF-γ, and TGF-β1 in POLYP+αIL-25 mice were suppressed but similar to control mice (Fig. 8B-D). However, I observed no significant difference of IL-17A between POLYP and POLYP+αIL-25 groups (Fig. 8E). To verify inflammatory cell recruitment was inhibited by anti-IL-25 treatment, eosinophil chemotactic cytokines (CCL11 and CCL24), neutrophil-recruiting chemokines (CXCL1 and CXCL2), and recruitment adhesion molecules (E-selectin, ICAM-1, and VCAM-1) were assessed in NP mice. These chemokines and adhesion molecules were upregulated when NPs were induced in mice (Fig. 8F-9L), but IL-25 inhibition led to the downregulation of CCL11, CXCL2, ICAM-1 and VCAM-1.

Table 4. Patient characteristics and type of method in the IL-25 study

	Control	**CRSsNP**	**CRSwNP**	
Total no. of subjects	**N = 18**	**N = 45**	**N = 64**	
Tissue used	UP	UP	UP	NP
Age (yr), mean (SD)	41 (19)	49 (12)	49 (13)	46 (16)
Atopy, N (%)	4 (22%)	13 (36%)	7 (36%)	16 (37%)
Asthma, N	0	0	0	0
Aspirin sensitivity, N	0	0	0	0
Lund-Mackay CT score	0 (0)	8.8 (5.0)	15.9 (5.9)	15.9 (6.2)
Lund-Kennedy score	NA	0.9 (1.6)	3.3 (2.6)	3.5 (2.8)
Blood eosinophil (%)	3.6 (2.3)	5.4 (3.8)	4.1 (3.6)	3.6 (3.4)
Methodologies used				
Tissue IHC (Double)	8	25	19	43 (7)
Tissue mRNA	16	25	19	46
Homogenate ELISA	10	15	15	15

Lund-Kennedy score was evaluated at postoperative 6 months.

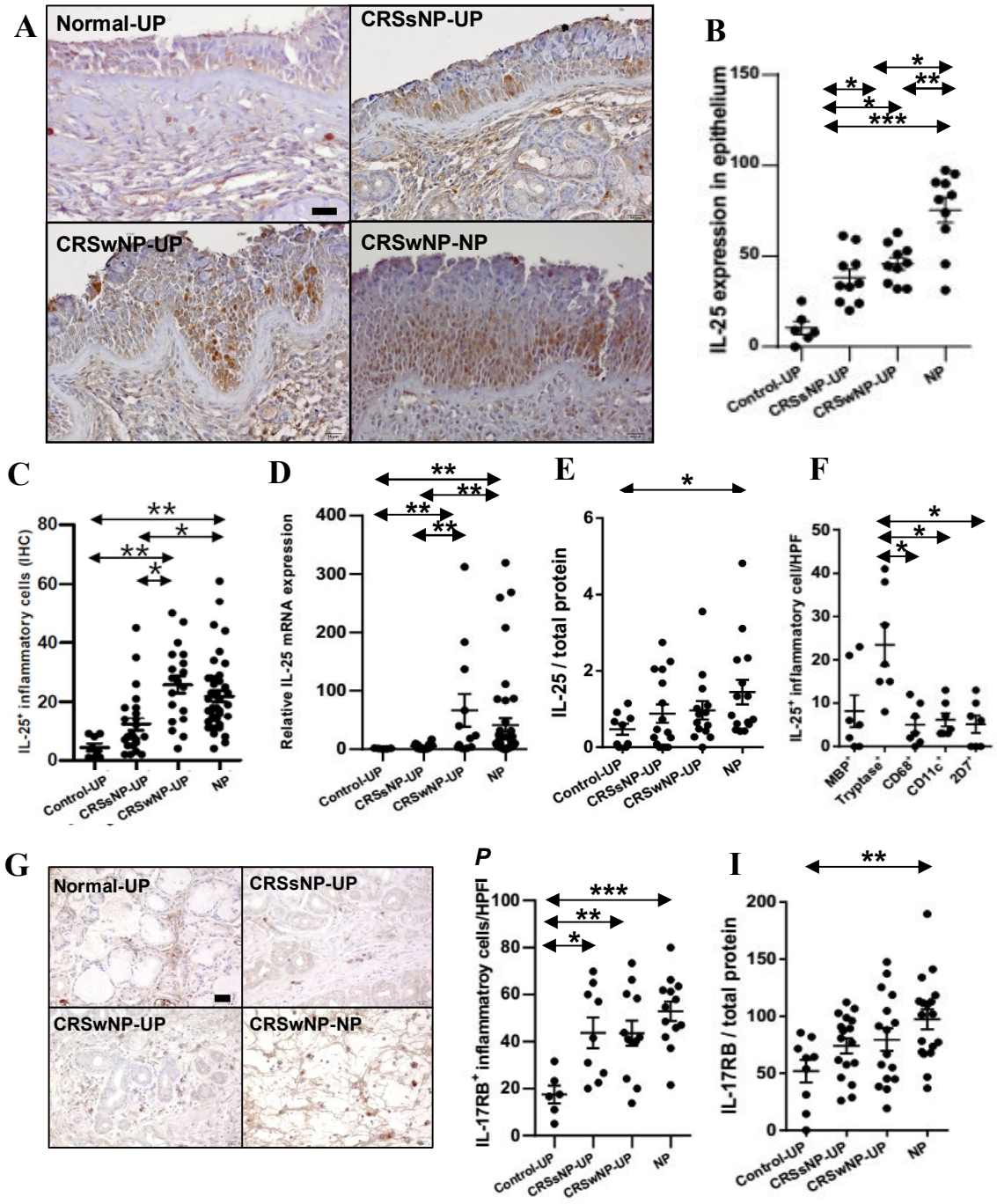

Figure 5. Expression of IL-25 and IL-17RB in CRS. (A) Representative immunostaining for IL-25 **(B)** Number of IL-25^{+} epithelial cells per 100 cells **(C)** Number of IL-25^{+} inflammatory cells (HPF; x400) **(D)** Relative IL-25 mRNA expression **(E)** Protein levels of IL-25 **(F)** Double immunohistochemical stain for MBP, Tryptase, CD68, CD11c or 2D7, and IL-25 **(G)** Representative photos for IL-17RB immunostaining **(H)** Number of IL-17RB^{+} inflammatory cells **(I)** Protein level of IL-17RB (*P<.05, **P<.01, ***P<.001).

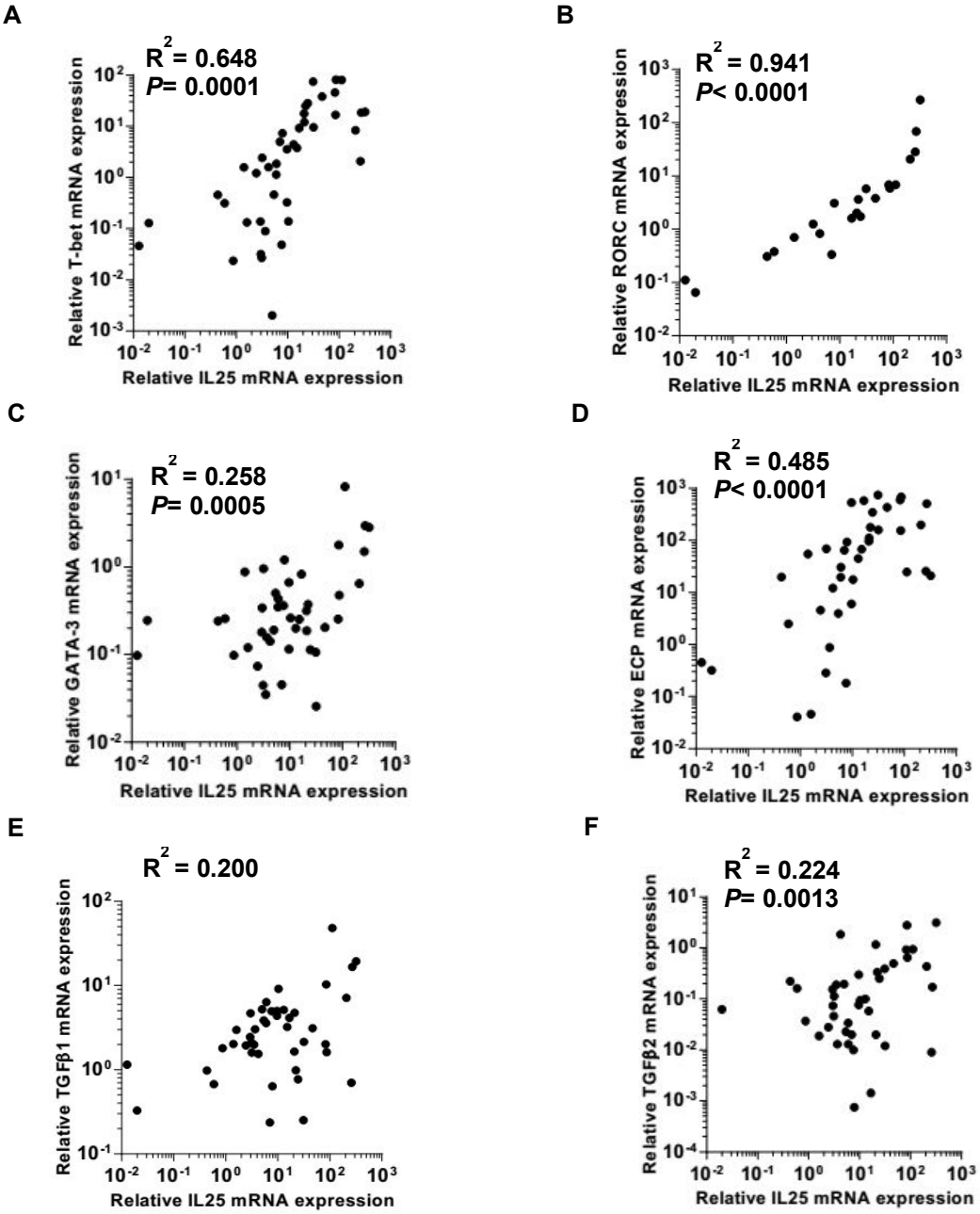

Figure 6. Correlation between mRNA expression of IL-25 and inflammatory markers. The mRNA expression levels of T-bet, RORC, GATA-3, ECP, TGF-β1, and TGF-β2 were measured in nasal polyps (n=43), and correlations between IL-25 and each inflammatory markers were investigated. Pearson's correlation test was used and R-square values indicate the coefficient of determination.

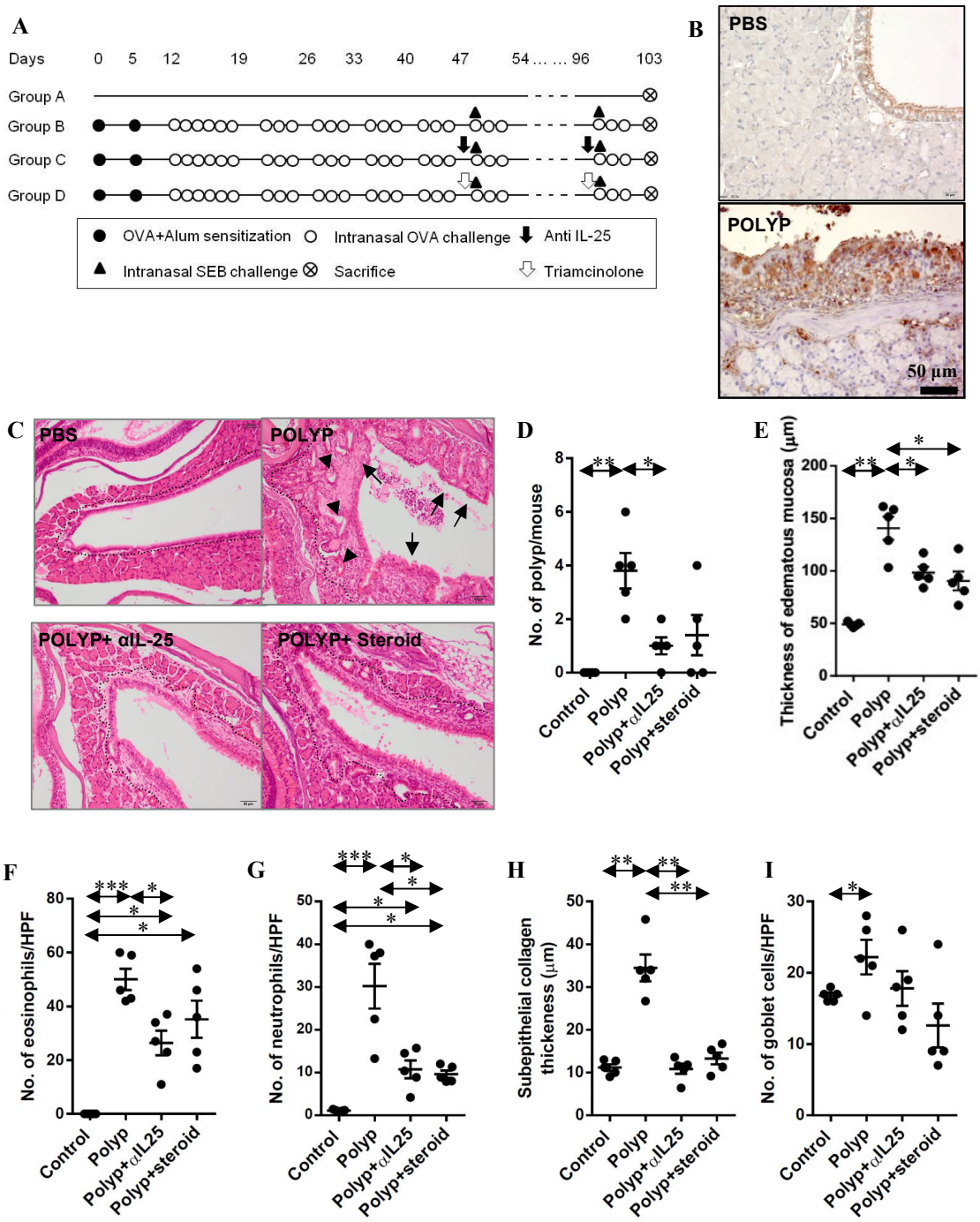

Figure 7. Anti-polyp effect of IL-25 neutralizing antibody in animal models. (A) Protocol for the murine model of allergic CRS with polypoid lesions. **(B)** Representative images in control (PBS) and polyp model (POLYP). **(C)** Dotted lines represent the border between glandular structure and maxillary sinus mucosa. Arrows and arrowheads indicate polypoid lesions and epithelial ingrowth. **(D)** The number of polypoid lesion **(E)** mucosal thickness **(F)** number of eosinophils **(G)** number of neutrophils **(H)** subepithelial collagen deposition **(I)** number of goblet cells were counted.

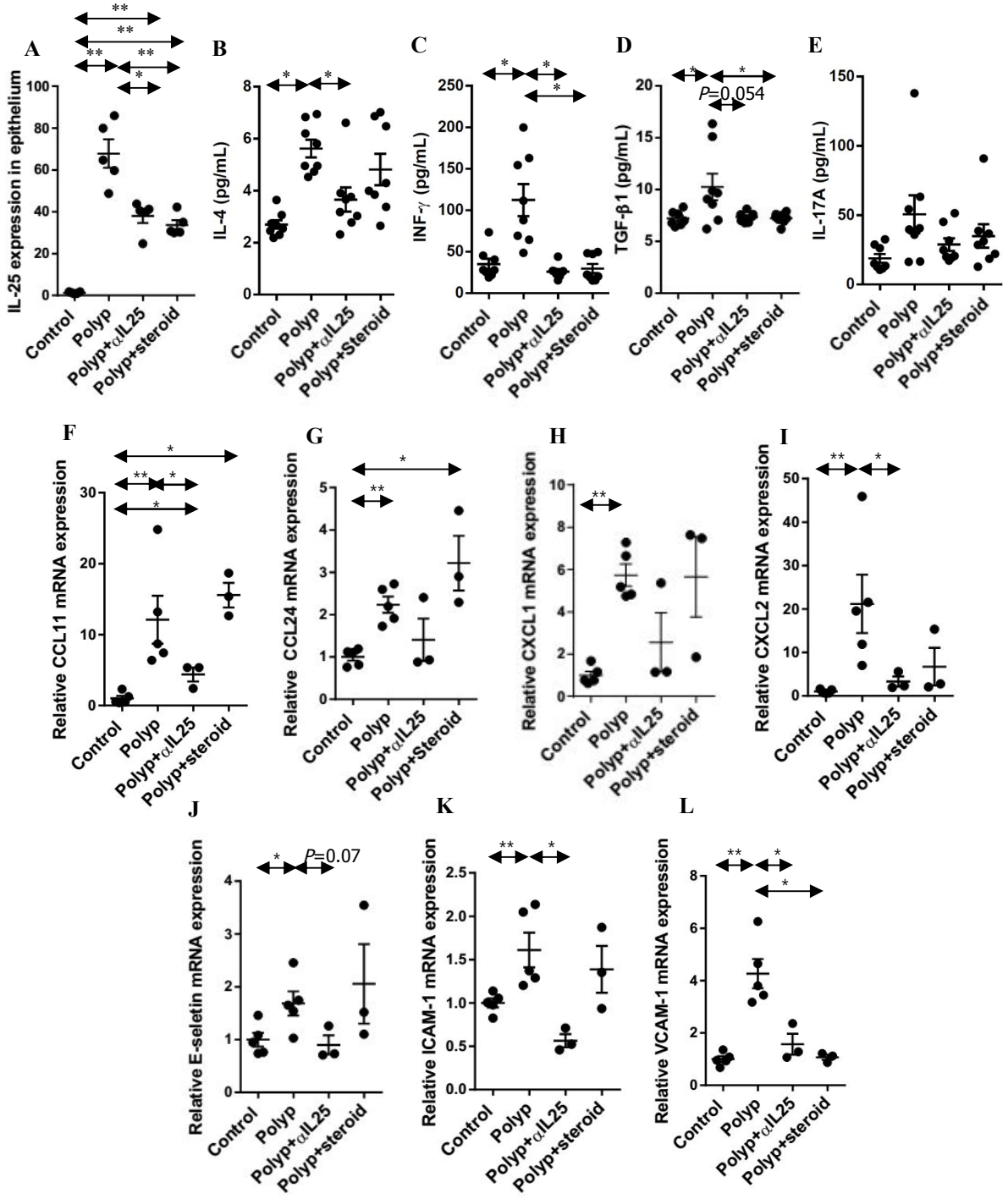

Figure 8. Changes in cytokine profiles, chemokine and adhesion molecule expression after anti-IL-25 therapy. (A) The number of IL-25-positive epithelial cells was counted. **(B-E)** Cytokine profile from nasal lavage fluid (n=8 for each group). **(F-I)** Relative mRNA expression levels of leukocyte-recruiting cytokines from each group were compared. **(J-L)** Relative mRNA expression levels of intercellular adhesion molecules from each group were compared.

Role of Interleukin-33 in Chronic Rhinosinusitis

1. Interleukin-33 expression and ST2 expression in CRS

Patient characteristics and type of method in this study was presented in Table 5. To examine expression of IL-33, UP tissue was collected from control subjects (Control-UP), patients with CRSsNP (C-UP), and patients with CRSwNP (P-UP) and NP tissues were obtained from patients with CRSwNP (NP). IHC revealed that the expression of IL-33 was higher in epithelial cells of C-UP and P-UP than in Control-UP or NP (Fig. 9A). IL-33-positive inflammatory cells were significantly increased in P-UP compared with Control-UP or C-UP (Fig. 9B). I also examined the expression of IL-33 mRNA in each tissue and found that IL-33 mRNAs levels were significantly increased in P-UP compared to Control-UP (Fig. 9C). Moreover, UP tissues from CRSwNP patients had greater expression of IL-33 mRNA than NP tissues from CRSwNP patients (Fig. 9C). To confirm these findings at the protein level, the concentration of IL-33 was measured using ELISA and normalized to total protein concentration. These data demonstrated that IL-33 protein levels were significantly increased in C-UP and P-UP homogenates compared to those in Control-UP and NP homogenates (Fig. 9D). IL-33 has been reported to function via binding to the ST2 receptor complex. Therefore, I measured expression levels of ST2 in nasal tissues from control, CRSsNP, and CRSwNP patients. By IHC and qRT-PCR, the number of ST2-positive cells and the expression level of ST2 were significantly higher in C-UP and P-UP tissues than in Control-UP and NP tissues (Fig. 9E-G).

2. Correlations between IL-33 mRNA expression and inflammatory markers in UP from CRS

Because I previously identified elevated expression of IL-33 in UPs but not in NPs from patients with CRSwNP, I next sought to elucidate the role of IL-33 in the development of CRS. I examined the relationship between IL-33 expression and inflammatory markers, namely IL-4, IL-5, IL-13, IFN-γ, TNF-α, IL-17A, IL-22, IL-23p19, ECP, and MPO in UP from CRS patients. Expression of mRNA of IFN-γ ($r=0.4892^{**}$), IL-17A ($r=0.4298^{**}$), IL-22 ($r=0.3020^{*}$), and IL-23p19 ($r=0.3237^{*}$) were positively correlated with IL-33 mRNA expression (Fig. 10A-D); however, the level of IL-33 mRNA

was not correlated with that of IL-4, IL-5, IL-13, TNF-α, ECP, or MPO mRNA. Interestingly, in UP from CRS, a significant correlation between CXCL1 and IL-33 mRNA expression was observed (Fig. 10E). I also investigated whether IL-33 expression was associated with remodeling of inflammatory markers such as TGF-β1,-2,-3, and COL1A. Expression of IL-33 mRNA was correlated with the remodeling markers TGF-β1 (r=0.3739*; Fig 10F) and COL1A1 (r=0.3215*; Fig. 10G). In addition, using a semi-quantitative method, I found that total collagen protein levels were positively correlated with the numbers of IL-33-positive inflammatory cells (r=0.4401*; Fig. 10H).

3. Differences in IL-33 expression between eosinophilic and non-eosinophilic CRSwNP

Previous studies demonstrated that non-eosinophilic NPs is usually characterized by glandular hypertrophy and fibrosis rather than edema, similarly to UPs, as well as increased neutrophilic infiltration compared to eosinophilic NPs (6, 7, 40). Thus, I investigated the difference in IL-33 expression between patients with eosinophilic and non-eosinophilic NPs (Fig 11A-C). The expression of IL-33 in epithelial cells was significantly higher in non-eosinophilic NPs than in eosinophilic NPs. IL-33 protein levels and IL-33 mRNA expression were also significantly higher in non-eosinophilic NPs. Next line, to analyze the role of IL-33 in non-eosinophilic NPs, I investigated the relationship between IL-33 expression and expression of other inflammatory markers according to the endotypes of CRSwNP. Using qRT-PCR, I found that the expression of IL-33 mRNA in non-eosinophilic NPs was correlated with mRNA expression of IFN-γ, TNF-α, and TGF-β1 (r=0.4780**, r=0.4118*, and r=0.4231*, respectively; Fig. 11D-F). To determine whether IL-33 was associated with recruitment of neutrophils, human neutrophil marker (MPO) and neutrophil-recruiting chemokines (CXCL1 and CXCL2) were assessed in NP tissues. In non-eosinophilic NPs, MPO and CXCL1 and CXCL2 mRNA levels were correlated with IL-33 expression (r=0.4826*, r=0.5421**, and r=0.5186**, respectively; Fig. 11G-I). In addition, mRNA expression of IFN-γ, TNF-α, CXCL1 and CXCL2 in eosinophilic NPs, which was though expressed at lower levels, also associated with IL-33 expression (r=0.6217*, r=0.5239*, r=0.4521* and r=0.6121**, respectively). Collectively, these findings indicate that non-eosinophilic NPs exhibits increased IL-33 expression and that IL-33 is involved in remodeling in non-

eosinophilic NPs, depending on recruitment of neutrophils.

4. Anti-polyp effect of IL-33 neutralizing antibody in murine models

To investigate the therapeutic potential of IL-33 blockade in nasal polypogenesis, I used a murine model of CRS with polypoid lesions (Fig. 12A). This murine model showed higher expression of IL-33 in the epithelial layer and in inflammatory cells than the control mice (Fig. 12B). The mucosal thickness was apparently more severe in NP mice than in control mice. Significantly, treatment with anti-IL-33 (POLYP+αIL-33 group) reduced the number of NP-like lesions, edematous mucosal thickness, thickness of the subepithelial collagen layer, and number of goblet cells in the NP models (Fig. 12C-F). The cytokine profiles of the nasal lavage fluid from treatment samples were also in accordance with histological findings (Fig. 12G-J). After treatment with anti-IL-33 antibody, the protein levels of IFN-γ, IL-17A, and TGF-β1 were significantly decreased compared with those of the POLYP group; however, no statistical differences in the titers of IL-4 between two groups.

5. Changes in neutrophil recruitment after IL-33 inhibition

To verify the change of cytokines IL-33 inhibition, I investigated the infiltration of inflammatory cells. The degree of neutrophilic infiltration in POLYP+αIL-33 group was decreased compared to that in POLYP group, whereas the anti-IL-33 treatment had no inhibitory effect on the number of eosinophils in these mice (Fig. 13A and 13B). To verify that the inflammatory cell recruitment was inhibited by anti-IL-33 treatment, qRT-PCR was used to assess the levels of recruitment adhesion molecules (ICAM-1 and VCAM-1), eosinophil chemotactic cytokines (CCL11 and CCL24), and neutrophil-recruiting chemokines (CXCL1 and CXCL2) in this experimental model (Fig. 13C-H). These adhesion molecules and chemokines were upregulated in POLYP group compared with control mice, while the anti-IL-33 treatment led to the downregulation of ICAM-1, VCAM-1, and CXCL2.

Table 5. Patient characteristics and study methodology in the IL-33 study

	Control	**CRSsNP**	**CRSwNP**	
Total no. of subjects	**N = 19**	**N = 46**	**N = 41**	**N = 68**
Tissue used	UP	UP	UP	NP
Age (yr), mean (SD)	45 (19)	49 (13)	48 (14)	49 (14)
Atopy, N (%)	6 (31%)	18 (39%)	14 (34%)	26 (38%)
Asthma, N	0	3	5	5
Lund-Mackay CT score	0 (0)	8.9 (5.3)	15.3 (6.1)	15.6 (5.1)
Blood eosinophil number (/mm^3)	86.9 (53.7)	172.9 (112.3)	162.8 (132.9)	139.1 (118.5)
Methodologies used				
Tissue IHC	9	25	24	58
Tissue mRNA	19	42	41	68
Homogenate ELISA	9	18	16	31

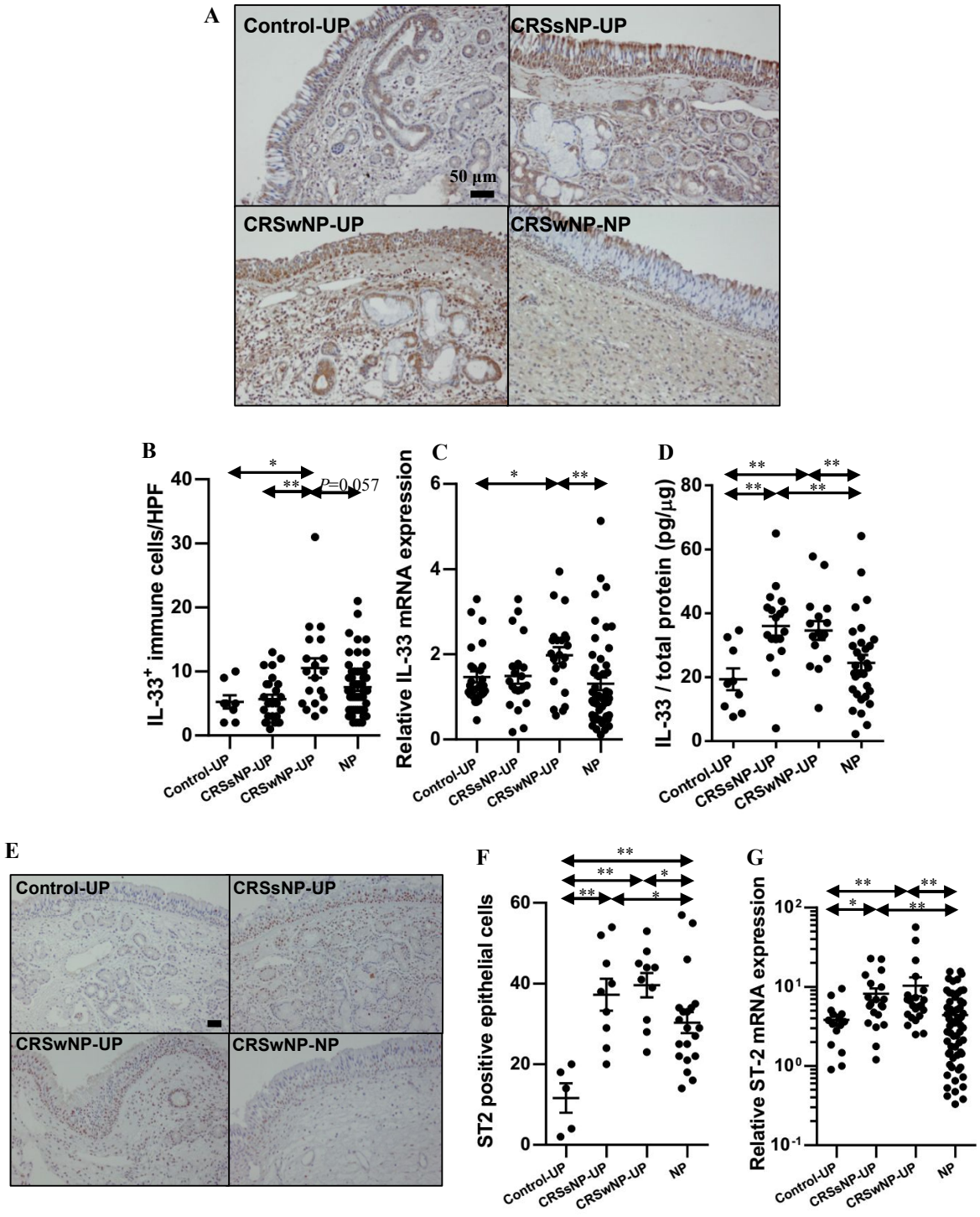

Figure 9. Expression of IL-33 and ST2 in different types of CRS. (A) IHC detection of IL-33 **(B)** Number of IL-33-positive inflammatory cells (HPF; x400) **(C)** Relative IL-33 mRNA expression **(D)** Protein levels of IL-33 **(E)** IHC detection of ST2 **(F)** Number of ST2-positive inflammatory cells (HPF; x400) **(G)** Relative ST-2 mRNA expression (*P<0.05, **P<0.01, and ***P<0.001).

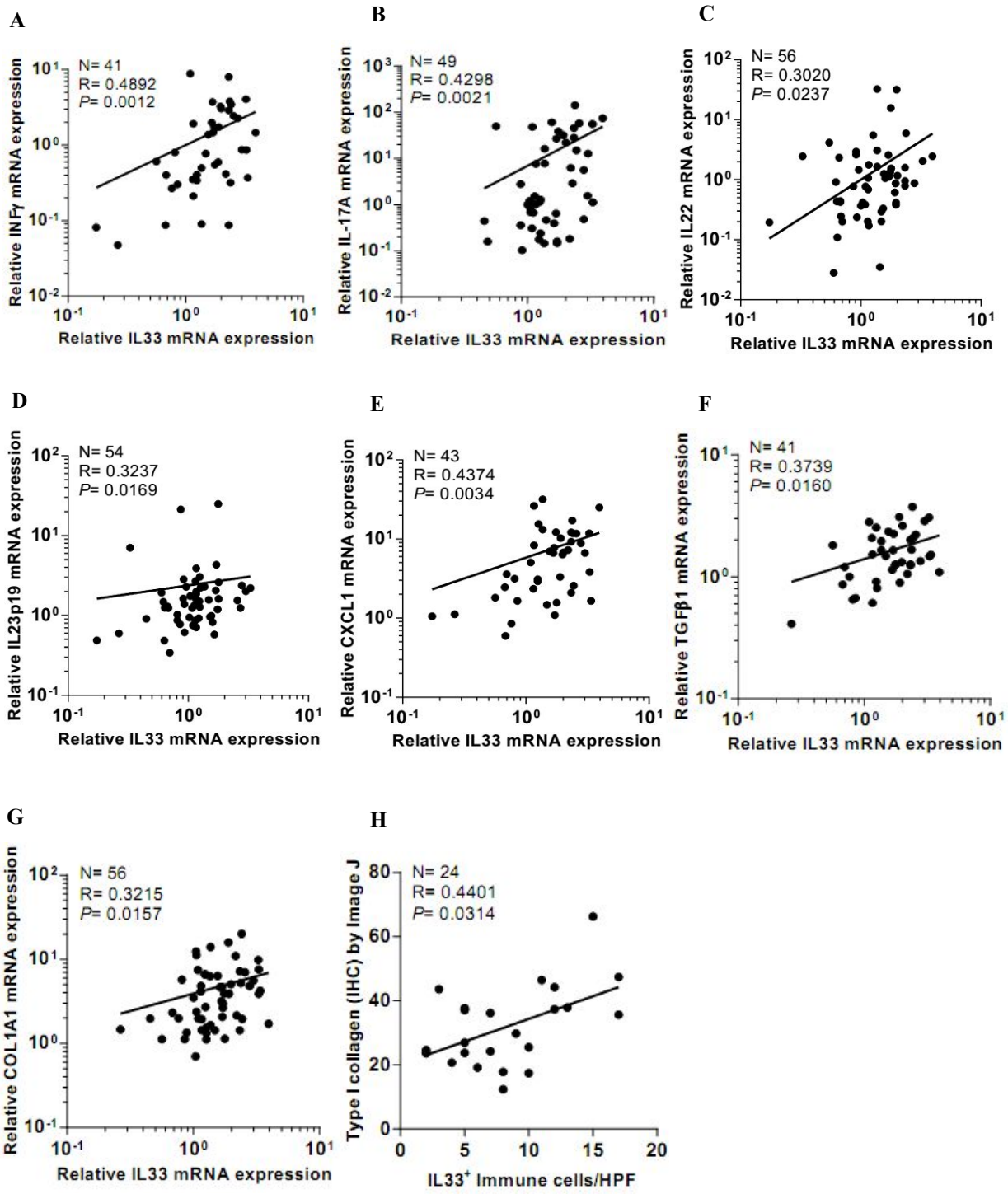

Figure 10. Correlation between IL-33 expression and inflammatory markers in UP tissues from CRS patients. The correlation of mRNA expression of IL-33 and inflammatory markers: **(A)** IFN-γ (n=41) **(B)** IL-17A (n=49) **(C)** IL-22 (n=56) **(D)** IL-23p19 (n=54) **(E)** CXCL1 (n=43) **(F)** TGF-β-1 (n=41) **(G)** COL1A1 (n=56). **(H)** The correlation between the number of IL-33-positive cells and quantification of collagen using image analysis were investigated in UPs from patients with CRS.

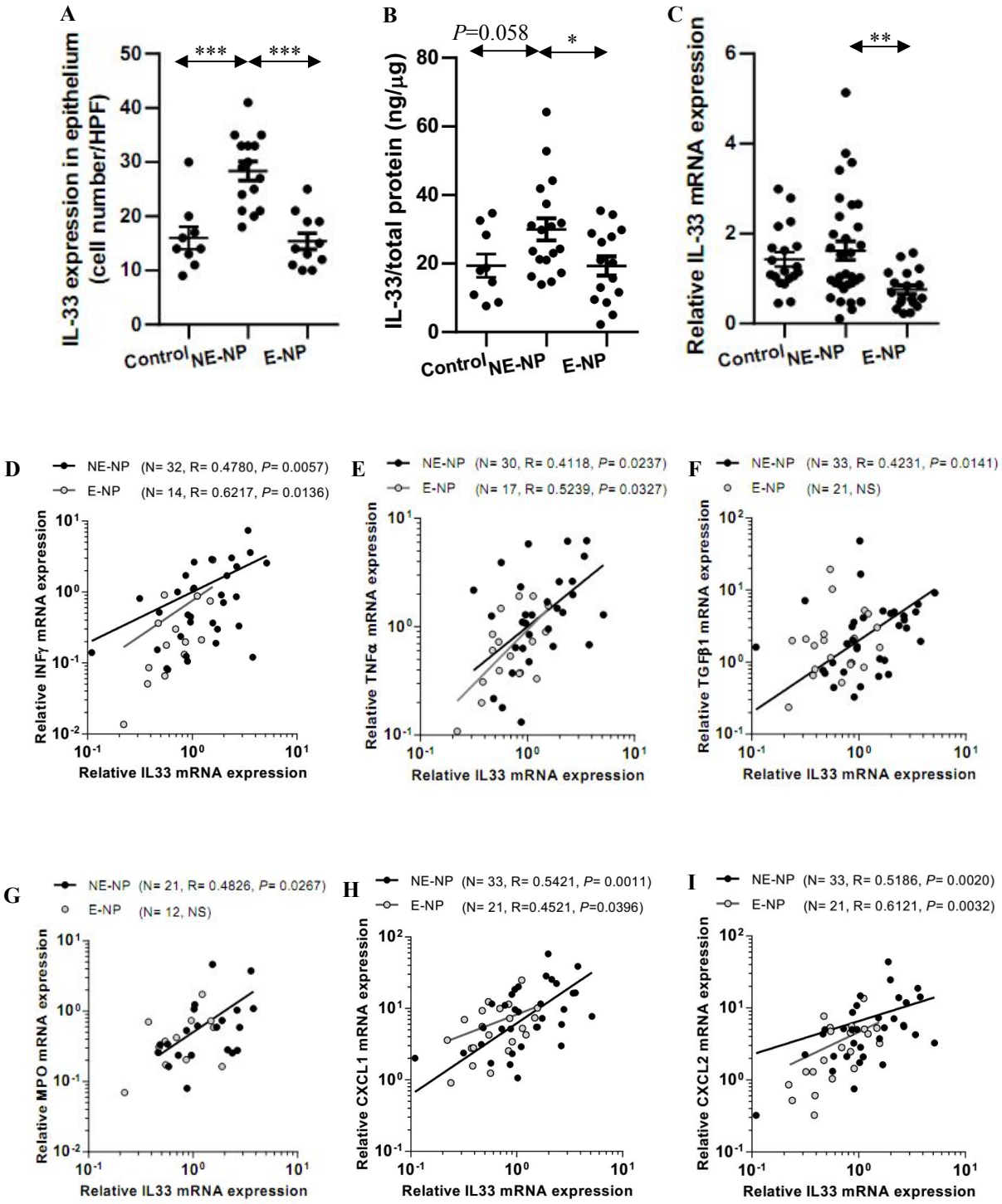

Figure 11. Expression of IL-33 and correlation between IL-33 expression and inflammatory markers according to the endotype of CRSwNP. (A) Number of IL-33-positive epithelial cells per 100 cells **(B)** Protein levels of IL-33 **(C)** Relative IL-33 mRNA expression The correlations of mRNA expression levels between IL-33 and inflammatory markers, including **(D)** IFN-γ **(E)** TNF-α **(F)** TGF-β-1 **(G)** MPO **(H)** CXCL1,and **(I)** CXCL2 were investigated in NPs.

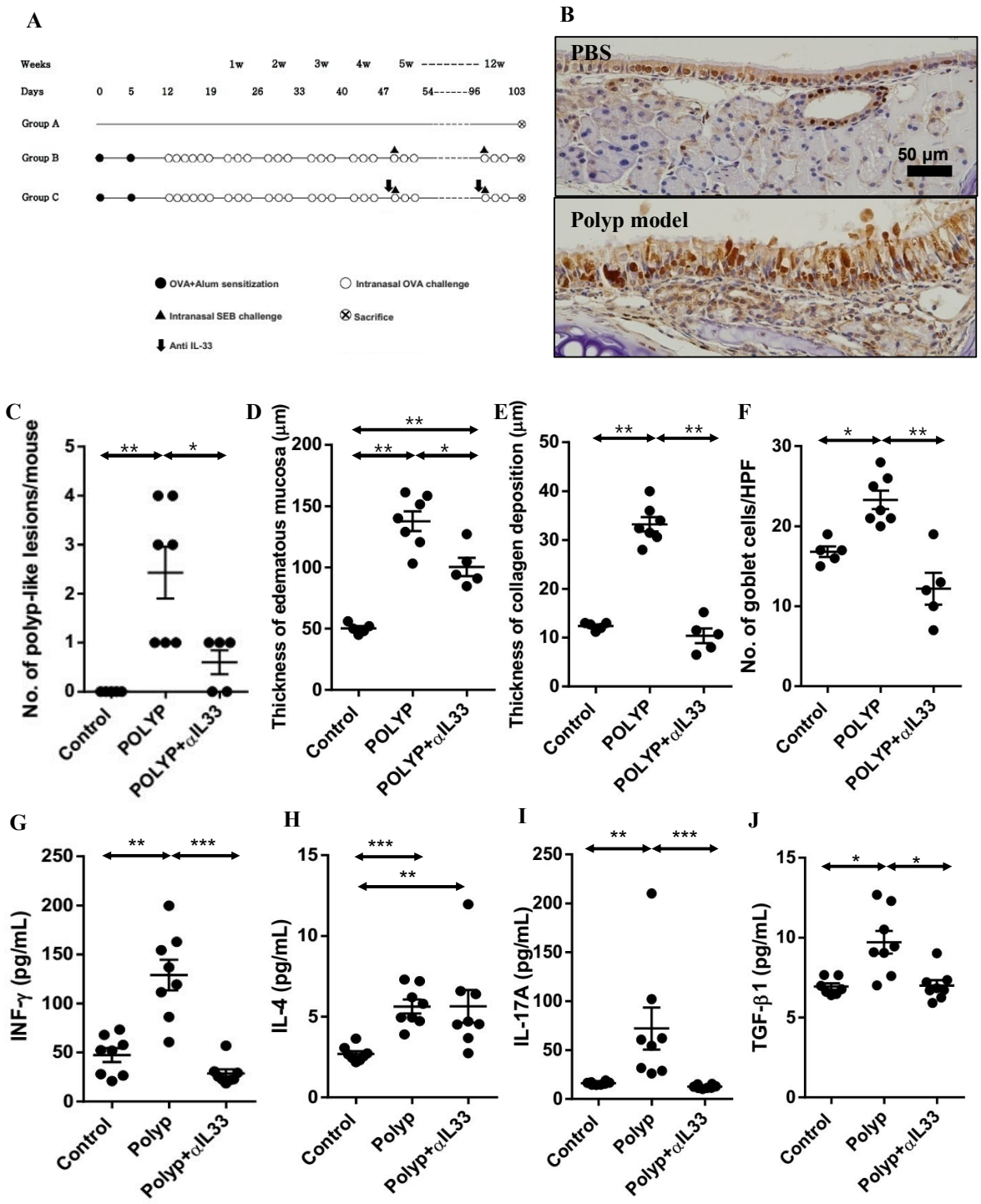

Figure 12. Anti-polyp effect of IL-33 neutralizing antibody in polyp animal models. (A) Protocol for the murine model of allergic CRS with polypoid lesions **(B)** IL-33 was strongly expressed in the nuclei of epithelial cells from NP model. **(C)** The number of NP lesions **(D)** edematous mucosal thickness **(E)** subepithelial collagen deposition **(F)** the number of goblet cells were compared. **(G-J)** Cytokine profile from nasal lavage fluids were compared (**P*<0.05, ***P*<0.01, and ****P*<0.001).

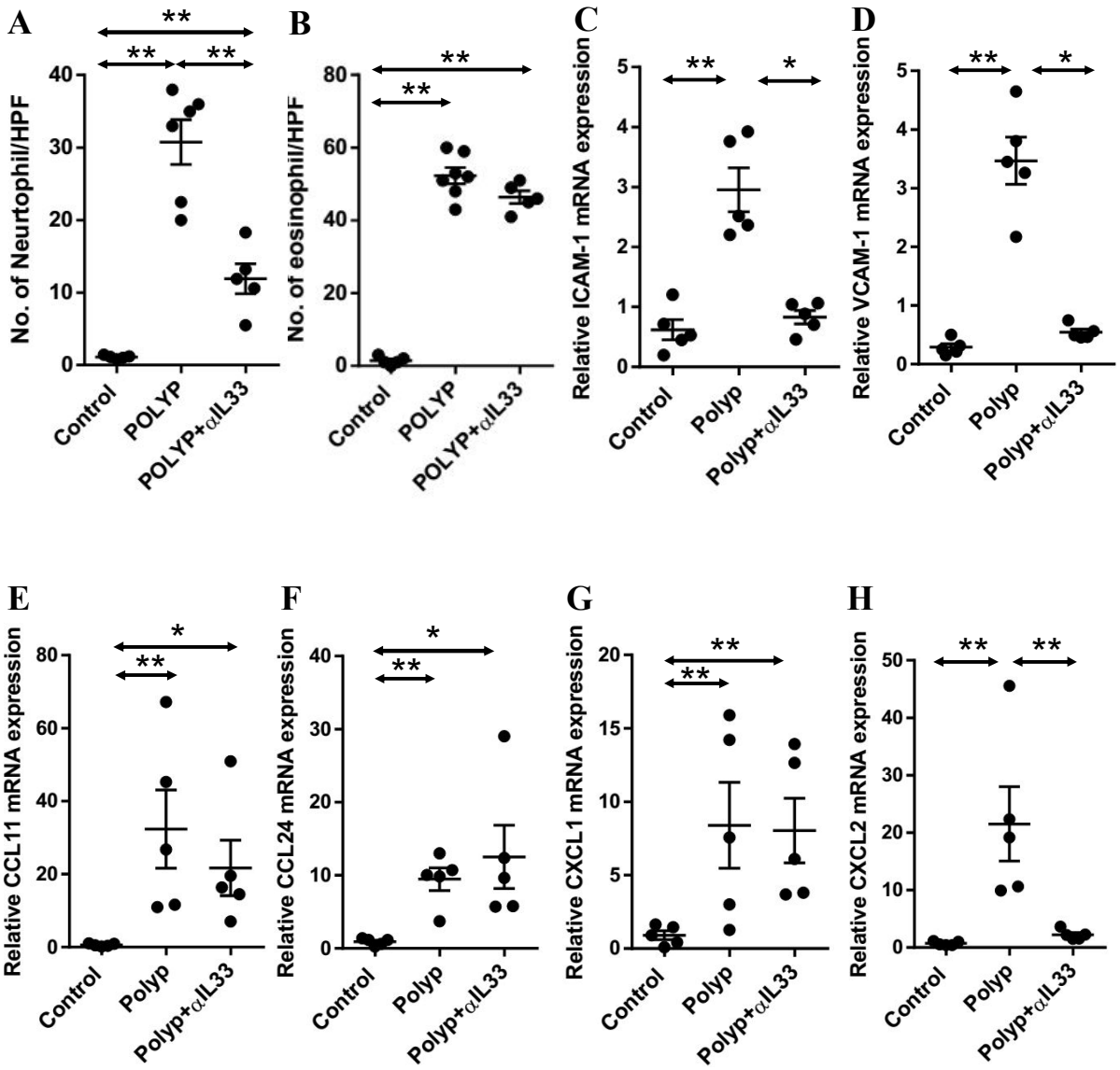

Figure 13. Anti-IL-33 therapy suppresses neutrophil infiltration and expression of neutrophil chemotactic cytokines and intercellular adhesion molecules in a murine model. (A) The number of infiltrated neutrophils and **(B)** eosinophils were counted and compared among groups. Relative mRNA expression levels of **(C)** ICAM-1 **(D)** VCAM-1 **(E)** CCL11 **(F)** CCL24 **(G)** CXCL1 **(H)** CXCL2 were compared among groups (**P*<0.05, ***P*<0.01, and ****P*<0.001).

DISCUSSION

In the present study, firstly, I evaluated the role of innate immune cells in different type of CRS in Asian patients. Several stains were conducted in order to compare the innate immune cells according to the phenotype of CRS: MBP for eosinophils, tryptase for mast cells, CD68 for M1 polarized macrophages, CD163 for M2 polarized macrophages, CD11c for dendritic cells, 2D7 for basophils, and HNE for neutrophils. To my knowledge, this is the first study to investigate immunohistologic features of innate immunity according to the phenotype of CRS in Asian patients. Importantly, in the immunohistochemical study of UP tissues, I found that patients with CRSwNP had a higher infiltrate of all kinds of innate immune cells in UP tissues than that of normal controls, whereas other cells except for MBP^+ and $CD11c^+$ cells were increased in UP tissues from CRSsNP compared to controls. In addition, CRSwNP showed more severe MBP^+ cells, $CD68^+$ cells, and $CD11c^+$ cells infiltration than CRSsNP. This is consistent with evidence that various innate immune cells are associated with the pathogenesis of CRS (11, 35). In this study, I also evaluated the effector cells that considered to contribute for nasal polypogenesis using comparison of UP and NP from CRSwNP. I found that NP tissues showed excessive accumulation of MBP^+ cells, $CD11c^+$ cells, $2D7^+$ cells, and HNE^+ cells compared to UP tissues from CRSsNP or CRSwNP. Although CD11c is not exclusively as marker for dendritic cell, these data are in agreement with previously reported elevated eosinophils, dendritic cells, basophils, and neutrophils in CRSwNP (41-45). In addition, the infiltration of MBP^+ and $CD11c^+$ cells increased gradually with the disease progression and I found a positive correlation between these and disease extent in UP tissues from CRSwNP. Thus, these results suggest that MBP^+ cells and $CD11c^+$ cells may be major effector cells for nasal polypogenesis in Asian CRSwNP.

Secondly, I evaluated the role of innate cytokines (IL-25 and IL-33) in different type of CRS in Asian patients. Innate cytokines are produced and secreted from epithelial cells in response to external stimuli, such as pollutants, allergens, and microbes (46). IL-25 and IL-33 are both produced in sinonasal epithelial cells and may play an important role in promoting Th2 inflammation in CRSwNP

(8, 12). Although innate cytokine-responsive ILC2s have been identified in NPs (12), the specific role of IL-25 in NP pathogenesis has not been fully understood. Interestingly, the present study showed IL-25 expression levels increased in NP tissues and correlated with T-bet, RORC and GATA3 upregulation in CRSwNP, suggesting IL-25 may be involved in diverse inflammatory pathways. The mixed phenotypes of Th1/Th2/Th17 pathways observed in Asian NP patients indicates a causal factor superior to final effector cells or cytokines in the inflammatory hierarchy may exist, such as the innate cytokine IL-25. NP tissues from this study were characterized by non-asthmatic and lower eosinophilic patient features, in contrast with NP tissues often found in Western groups. The expression of typical transcriptional factors involved in Th1/Th2/Th17 cell responses was elevated simultaneously in NP tissues from this study. Thus, the significant correlations between IL-25, T-bet, RORC, and GATA3 expression implies IL-25 may affect NP pathogenesis in Asian individuals by inducing mixed inflammatory reactions. I also observed mast cells were more abundant among infiltrated inflammatory IL-25^{+} cells than in eosinophils. Considering NPs of Asian patients are often less eosinophilic, mast cells may play a protective role in mucosal maintenance when the epithelium is injured or inflamed. Mast cells can produce diverse cytokines related to Th1 and Th2, which could contribute to the heterogeneous inflammatory responses, observed in Asian patients with CRSwNP. However, my study shows only the potential engagement of mast cells in NP pathogenesis, and their exact role during nasal polypogenesis should be further explored. Moreover, treatment with anti-IL-25 neutralizing antibody or steroid therapy equally suppressed polyp formation in the animal model. Anti-IL-25 treatment also suppressed IL-4 and IFN-γ expression in nasal lavage fluids from mice and downregulated mRNA expression of CCL11, CXCL2, ICAM-1, and VCAM-1. I concluded the anti-polyp effect could be obtained by suppressing the recruitment of effector cells.

Apart from IL-25, I also investigated the role of IL-33 in the pathophysiology of CRS in Asian patients and report differences in IL-33 expression according to the phenotype and endotype of CRS patients. These data indicated that the concentration of IL-33 expression in NP tissues was not significantly different than that in Control-UP tissues; however, I found a significant difference in IL-33 expression in UP tissues according to the phenotype of CRS. My results showed elevated

expression of IL-33 in UP tissues, but not in NP tissues, from CRS patients compared with healthy control subjects. Moreover, analysis of the correlation between IL-33 and inflammatory markers indicated that expression of IL-33 in UPs correlated with expression of the Th1 (IFN-γ) and Th17 (IL-17A, IL-22, and IL-23p19) cytokines as well as with the multiple remodeling markers, such as TGF-β1 and collagen type I. These results suggest that IL-33 may play a crucial role in nasal mucosa inflammation and tissue remodeling in CRS in Asian patients. To date, the non-eosinophilic type has been distinguished as the predominant endotype of CRSwNP in Asians. Interestingly, I found a difference in IL-33 expression between eosinophilic and non-eosinophilic NPs. The expression of IL-33 levels was significantly increased in non-eosinophilic NPs compared to those in eosinophilic NPs. Non-eosinophilic NPs also exhibited increased numbers of HNE^+ cells, and the expression of typical cytokines (IFN-γ/TNF-α) involved in the Th1 immune response was elevated in those. In addition, we observed that in non-eosinophilic NPs, IL-33 levels were correlated with IFN-γ and TNF-α, which serve as markers for Th1 cytokines, with TGF-β1, which serves as a marker for tissue remodeling, and MPO, which serves as a marker for neutrophil infiltration, and with CXCL1/CXCL2, which serve as chemokines for neutrophil recruitment. Collectively, these data suggest a role of IL-33 in non-eosinophilic NPs and indicate an association between inflammation and remodeling via neutrophil recruitment. Moreover, in animal study, I detected the expression of IL-33 in epithelial and inflammatory cells in nasal polypoid mice. In addition, I found that the formation of nasal polypoid lesions and the degree of average mucosal thickness were diminished by administration of anti-IL-33. Similarly, the POLYP+αIL-33 group showed a significant decrease in the thickness of the subepithelial collagen and in the number of goblet cells compared to the POLYP group. Interestingly, I observed that the infiltration of neutrophils was decreased in the POLYP+αIL-33 group compared with levels in the POLYP group; yet, no significant differences in the infiltration of eosinophils were observed between these groups. Furthermore, I demonstrated decreased expression of cytokines (IFN-γ/IL-17A/TGF-β1), adhesion molecules (ICAM-1/VCAM-1), and neutrophil-recruiting chemokine (CXCL2) in the POLYP+αIL-33 group compared to that in the POLYP group. Considering these results, the anti-polyp effect of IL-33 inhibition may stem from suppression of neutrophil recruitment.

Conclusion

In the present study, firstly, I found that infiltration of MBP^+ and $CD11c^+$ innate immune cells show a significant association with phenotype and disease extent of CRS and allergic status also may influences cellular phenotype in Asian patients with CRSwNP. Secondly, IL-25 and IL-33, secreted from the sinonasal epithelia, plays a crucial role in the pathogenesis of Asian patients with CRSwNP. In addition, these results demonstrate the novel possibility of treating nasal polyposis with anti-IL-25 or IL-33 therapy.

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국문 초록

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이비인후과학 전공

서론: 기존 연구 결과에 따르면 서양인에서 비용종을 동반한 만성 부비동염은 호산구 침윤을 특징으로 하는 Th2 세포 면역반응을 보인다. 반면에 동양인의 비용종의 경우 비호산구성 염증세포 침윤이 주로 나타나며 Th1/Th2/Th17 세포 면역반응이 복합적으로 나타난다고 보고되고 있으나, 아직까지 동양인에서 명확한 비용종 발생과정의 병태생리는 규명되어 있지 않다.

목적: 동양인의 비용종을 동반한 만성 부비동염에서 선천성 면역체계의 역할을 살펴본다,

방법: 정상인, 비용종을 동반하지 않은 만성 부비동염 환자, 비용종을 동반한 만성 부비동염 환자에서 구상돌기 조직을 채취하였고, 비용종을 동반한 만성 부비동염 환자에서 비용종 조직을 채취하여 총 4개의 그룹으로 비교연구를 진행하였다. 선천성 면역세포와 선천성 사이토카인 (인터루킨-25 및 -33)의 발현을 면역화학염색, RT-PCR, ELISA를 통해 평가하였고, 다른 염증반응 매개체들과의 상관성을 분석하였다. 또한, 선천성 사이토카인의 비용종 형성과정의 역할을 규명하기 위해 항 인터루킨-25와 -33을 투여한 질환 동물모델 실험을 시행하였다.

결과: 동양인의 비용종에서 선천성 면역세포들의 분포는 MBP+ 세포와 CD11c+ 세포 침윤이 다른 선천성 면역세포들의 침윤과 양의 상관관계를 보였으며 질환의 중증도와 의미 있는 연관성을 나타냈었다. 또한, 환자의 알러지 보유 유무에 따라 선천성

면역세포들의 침윤 정도가 다르게 나타났다. 인터루킨-25의 발현은 동양인의 비용종에서 증가되어 있으며, 다수의 염증반응 매개체들의 발현과 양의 상관관계를 나타냈다. 그리고, 질환 동물모델 실험을 통해 인터루킨-25 항체를 투여하였을 때 호산구 화학주성인자, 호중구 화학주성인자, 세포부착분자 감소로 인한 호산구, 호중구의 침윤 감소로 비용종 발생이 억제되는 것을 관찰하였다. 한편, 인터루킨-33 발현은 비용종을 동반한 만성 부비동염 환자의 비용종이 아닌 구상돌기에서 증가되어 있었으며, Th1과 Th2 면역반응 및 조직재형성과 관련된 염증반응 매개체들의 발현과 양의 상관관계를 보였다. 게다가, 비호산구성 비용종에서 호산구성 비용종에 비해 인터루킨-33 발현이 증가되어 있으며, 조직재형성과 호중구 동원과 관련 있음을 검증하였다. 그리고 질환 동물모델 실험을 통해 인터루킨-33 항체를 투여하였을 때 호중구 화학주성인자, 세포부착분자 감소로 인한 호중구 침윤 및 동원 감소를 확인하였다.

결론: 동양인의 비용종을 동반한 만성 부비동염에서 MBP+ 세포와 CD11c+ 세포 침윤이 다른 선천성 면역세포 침윤 정도 및 질환의 중증도와 관련 있으며, 알러지 유무가 침윤되는 선천성 면역세포 종류에 영향을 미치는 것을 확인하였다. 또한, 인터루킨-25, -33은 비용종을 동반한 만성 부비동염에서 발현이 증가되어 있으며 여러 종류의 염증반응 매개체들의 발현과 밀접하게 연관되어 있어 동양인의 비용종 형성 과정에 중요한 역할을 수행할 것으로 생각된다. 또한, 동물실험을 통해 인터루킨-25, -33 항체가 비용종 발생을 효과적으로 억제함을 관찰하여 향후 치료제 개발을 위한 타겟으로 가능성을 제시하였다.

주요어: 만성 부비동염, 비용종, 선천성 면역체계, 인터루킨-25, 인터루킨-33

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