

IL-25 as a novel therapeutic target in nasal polyps of patients with chronic rhinosinusitis

Hyun-Woo Shin, MD, PhD,^{a,b,*} Dong-Kyu Kim, MD,^{c,*} Min-Hyun Park, MD, PhD,^d Kyoung Mi Eun, BSc,^d Mingyu Lee, BSc,^a Daeho So, BSc,^a Il Gyu Kong, MD, PhD,^e Ji-Hun Mo, MD, PhD,^f Min-Suk Yang, MD,^g Hong Ryul Jin, MD, PhD,^d Jong-Wan Park, MD, PhD,^a and Dae Woo Kim, MD, PhD^d *Seoul, Chuncheon, and Chonan, Korea*

Background: Chronic rhinosinusitis (CRS) with nasal polyps (NPs) in Western populations is associated with T_H2 cytokine polarization. IL-25, an IL-17 family cytokine, was recently reported to induce T_H2-type immune responses and to contribute to several allergic diseases, such as atopic dermatitis and asthma. However, the role of IL-25 in Asian patients with nasal polyposis remains unclear.

Objective: We sought to determine the role of IL-25 in Asian patients with nasal polyposis and CRS.

Methods: We investigated IL-25 expression and its cellular origins in NPs of human subjects using immunohistochemistry (IHC), quantitative RT-PCR, and ELISA of NP tissues.

Correlations between IL-25 expression and expression of other inflammatory markers in NP tissues were also explored. Anti-IL-25 neutralizing antibody was administered in an ovalbumin- and staphylococcal enterotoxin B-induced murine NP model to confirm the function of IL-25 during nasal polyposis.

Results: IL-25 expression was upregulated in NP mucosa from patients with CRS with NPs compared with uncinate process tissue from control subjects and those with CRS without NPs. Overexpression of epithelial IL-25 was confirmed by using IHC, and double IHC staining showed that tryptase-positive cells were one of the main sources of IL-25 among immune cells. Furthermore, IL-17 receptor B levels were also increased in immune cells of patients with NPs compared with those in control subjects. In NPs IL-25 mRNA expression positively

correlated with the expression of several inflammatory markers, including T-box transcription factor, RAR-related orphan receptor C, GATA3, eosinophil cationic protein, TGF- β 1, and TGF- β 2. IL-25 was more abundant in the murine NP model compared with control mice, and similar correlations between IL-25 and inflammatory markers were observed in murine models. Anti-IL-25 treatment reduced the number of polyps, mucosal edema thickness, collagen deposition, and infiltration of inflammatory cells, such as eosinophils and neutrophils. This treatment also inhibited expression of local inflammatory cytokines, such as IL-4 and IFN- γ .

Furthermore, expression of CCL11, CXCL2, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1 in the nasal mucosa was suppressed in the anti-IL-25-treated group.

Conclusion: Our results suggest that IL-25 secreted from the sinonasal epithelia and infiltrating mast cells plays a crucial role in the pathogenesis of CRS with NPs in Asian patients. In addition, our results suggest the novel possibility of treating nasal polyposis with anti-IL-25 therapy. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Nasal polyp, IL-25, sinusitis, allergy, animal models

Chronic rhinosinusitis (CRS) is a common upper airway disease that affects 5% to 16% of the population worldwide.¹⁻³ CRS is characterized by chronic inflammation of the sinonasal mucosa that persists for at least 12 weeks despite medical treatment.² Nasal polyps (NPs) frequently accompany CRS, and their occurrence indicates a more serious illness with recurrent clinical phenotypes.^{4,5} Chronic rhinosinusitis with nasal polyps (CRSwNP) in Western populations is associated with T_H2 cytokine polarization and prominent eosinophilic infiltration.⁶ Thus upstream mechanisms that incite the T_H2 response are crucial for understanding the pathogenesis of CRSwNP⁷; however, these mechanisms are not fully understood.

IL-25 (also known as IL-17E) is a member of the IL-17 cytokine family and has been reported to play a variety of roles in different inflammatory murine models, such as asthma, atopic dermatitis, and pulmonary fibrosis. Intraperitoneal or intranasal administration of IL-25 protein resulted in the production of eosinophils or T_H2 cytokines, such as IL-4, IL-5, IL-13, and eotaxin, in bronchoalveolar lavage fluid and lung tissue.^{8,9} Conversely, blocking IL-25 decreases T_H2 cytokine production in an animal model of asthma.¹⁰⁻¹² In addition, IL-25 could function in patients with allergic dermatitis by inducing the T_H2 response, as well as by inhibiting flaggrin synthesis, consequently affecting skin barrier function.^{13,14} Apart from allergic diseases, IL-25 also plays an important role in different

From ^athe Department of Pharmacology, Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine; ^bthe Department of Otorhinolaryngology–Head and Neck Surgery and ^cHealthcare System Gangnam Center, Seoul National University Hospital; ^dthe Department of Otorhinolaryngology, Chuncheon Sacred Heart Hospital, Hallym University College of Medicine, Chuncheon; ^ethe Department of Otorhinolaryngology–Head and Neck Surgery and ^fthe Department of Internal Medicine, Boramae Medical Center, Seoul National University College of Medicine; and ^gthe Department of Otorhinolaryngology, Dankook University College of Medicine, Chonan.

*These authors contributed equally to this work.

Supported by a clinical research grant-in-aid from the Seoul Metropolitan Government Seoul National University (SMG-SNU) Boramae Medical Center (03-2013-7), by SK Telecom Research Fund (06-2012-109), and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (0411-20130037).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication June 12, 2014; revised December 30, 2014; accepted for publication January 12, 2015.

Corresponding author: Dae Woo Kim, MD, PhD, Department of Otorhinolaryngology–Head and Neck Surgery, Boramae Medical Center, Seoul National University College of Medicine, 425 Shindaeabang 2-dong, Dongjak-gu, Seoul 156-707, Korea. E-mail: kicubi@daum.net.

0091-6749/\$36.00

© 2015 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2015.01.003>

Abbreviations used

CRS:	Chronic rhinosinusitis
CRSsNP:	Chronic rhinosinusitis without nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
CT:	Computed tomography
ECP:	Eosinophil cationic protein
hpf:	High-power field
ICAM:	Intercellular adhesion molecule
IHC:	Immunohistochemistry
IL-17R:	IL-17 receptor
NP:	Nasal polyp
OVA:	Ovalbumin
RORC:	RAR-related orphan receptor C
SEB:	Staphylococcal enterotoxin B
T-bet:	T-box transcription factor
UP:	Uncinate process
VCAM:	Vascular cell adhesion molecule

inflammatory conditions. One recent study showed that expression of IL-25 was increased and correlated with levels of periostin, an extracellular matrix protein, in patients with idiopathic pulmonary fibrosis.¹⁵ Moreover, this study demonstrated that IL-25 can drive fibrosis, which was confirmed by a decrease in collagen deposition in IL-25-deficient animal models.¹⁵ Collectively, these data indicate that IL-25 is a potent cytokine that acts in diverse inflammatory conditions.

Recently, IL-25 and IL-33, which are both produced by sinonasal epithelial cells, were reported to have critical roles in promoting T_H2-mediated inflammation.¹⁶ Induction of IL-33, a member of the IL-1 cytokine family, has been observed in epithelial cells from patients with CRSwNP. IL-33 induction also stimulates IL-13 production in ST2⁺ innate lymphoid cells from NPs.¹⁷ IL-25 enhances thymic stromal lymphopoietin-induced T_H2 cell expansion and function.¹⁸ Although one study reported that increased IL-25 levels correlated with poorer computed tomographic (CT) scores and increased serum eosinophil numbers in sinus mucosal tissues in patients with CRS, thus suggesting a relationship between IL-25 and T_H2-dominant diseases,¹⁹ the specific role of IL-25 in Asian patients with CRSwNP has not been thoroughly explored. In this study we investigated the expression and cellular origin of IL-25, as well as correlations between IL-25 and inflammatory surrogates in sinonasal tissues from patients with CRSwNP. We also evaluated the effects of anti-IL-25 therapy on nasal polyp formation in an NP-induced murine model. Some results of this study have been previously reported in the form of an abstract.²⁰

METHODS

Patients and tissue samples

Sinonasal and polyp tissues were obtained from routine functional endoscopic sinus surgery in patients with CRS. CRS diagnoses were based on personal medical history, physical examination, nasal endoscopy, and CT findings of the sinuses according to the "EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012" guidelines.²¹ Patients were excluded if they were (1) younger than 18 years old; (2) asthmatic or aspirin sensitive; (3) previously treated with antibiotics, systemic or topical corticosteroids, or other immune-modulating drugs up to 4 weeks before surgery; and (4) afflicted with conditions, such as unilateral rhinosinusitis, antrochoanal polyps, allergic fungal rhinosinusitis, cystic fibrosis, or immotile ciliary disease. Control tissues were obtained from patients without any sinonasal

disease during other rhinologic surgeries, such as skull base, lacrimal duct, or orbital decompression surgery. We also obtained uncinate process (UP) tissue from control subjects and patients with CRS, including those with chronic rhinosinusitis without nasal polyps (CRSsNP) and those with CRSwNP. We also evaluated NPs in patients with CRSwNP. Each sample obtained was divided into 3 parts: one part was fixed in 10% formaldehyde and embedded in paraffin for histologic analyses, another part was immediately frozen and stored at -80°C for subsequent isolation of mRNA and proteins, and the third part was submersed in 1 mL of PBS supplemented with 0.05% Tween 20 (Sigma-Aldrich, St Louis, Mo) and 1% PIC (Sigma-Aldrich) per 0.1 g of tissue. This tissue was homogenized with a mechanical homogenizer at 1000 rpm for 5 minutes on ice. After homogenization, the suspensions were centrifuged at 3000 rpm for 10 minutes at 4°C . Supernatants were separated and stored at -80°C for further analysis of cytokines and other inflammatory mediators.²²

The atopic status of study patients was evaluated by using the ImmunoCAP assay (Phadia, Uppsala, Sweden), which detects IgE antibodies against 6 mixtures of common aeroallergens (house dust mites, molds, trees, weeds, grass, and animal dander). Patients were considered atopic if the allergen-specific IgE level was greater than 3.51 kU/L. The diagnosis of asthma and aspirin sensitivity was performed by an allergist based on lung function and challenge tests. Lund-Mackay CT scores and Lund-Kennedy endoscopic scores were obtained before surgery and 6 months after surgery, respectively (Table 1).

All patients provided written informed consent for study participation. This study was approved by the Internal Review Board of Seoul National University Hospital, Boramae Medical Center (no. 06-2012-109).

Immunohistochemistry

Paraffin sections were treated with 3% hydrogen peroxide (H_2O_2) and then incubated with primary antibodies and biotinylated secondary antibodies. Immune complexes were visualized with the Vectastain ABC Kit (Vector Laboratories, Burlingame, Calif). The numbers of positive cells in epithelia, glands, and submucosa were counted in the densest tissue region in 5 high-power fields (hpf; $\times 400$ magnification) by 2 independent observers, and average values were scored. Detailed immunohistochemistry (IHC) procedures are described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Quantitative real-time RT-PCR for inflammatory markers

The mRNA levels of IL-25, T-box transcription factor (T-bet), GATA3, RAR-related orphan receptor C (RORC), eosinophil cationic protein (ECP), TGF- β 1, TGF- β 2, and several cytokines and chemokines in human NP tissues, mouse nasal tissues, or both were evaluated by using semiquantitative real-time PCR analysis, as previously described.²³ Detailed semiquantitative real-time PCR conditions are described in the [Methods](#) section in this article's Online Repository.

ELISA for IL-25 and IL-17 receptor B in human tissue homogenates

IL-25 (R&D Systems, Minneapolis, Minn) and IL-17 receptor (IL-17R) B (R&D Systems) levels were measured with commercially available ELISA kits. The minimal detection limits for these kits are 62.5 and 156 pg/mL, respectively. All procedures followed the manufacturer's recommendations. Concentrations of IL-25 and IL-17RB in the tissue homogenate were normalized to the concentration of total protein. Detailed methods are described in the [Methods](#) section in this article's Online Repository.

Murine NP model

All animal experiments were approved by the Institutional Animal Care and Use Committee of Boramae Medical Center (No. 2013-0001) and performed under strict governmental and international guidelines on animal experimentation. Thirty-six female BALB/c mice (age, 4 weeks; weight,

TABLE I. Characteristics and methods

	Control subjects	Patients with CRSsNP	Patients with CRSwNP	
Total no. of subjects	27	65	50	72
Tissue used	UP	UP	UP	NP
Age (y), mean (SD)	42 (18)	48 (14)	48 (13)	49 (14)
Atopy, no. (%)	6 (22)	19 (29)	15 (30)	21 (29)
Asthma, no.	0	0	0	0
Aspirin sensitivity, no.	0	0	0	0
Lund-Mackay CT score	0 (0)	8.6 (4.9)	16.2 (4.7)	15.2 (5.2)
Lund-Kennedy score*	NA	0.8 (1.4)	2.8 (2.4)	3.0 (2.1)
Blood eosinophil number (/mm ³)	89.5 (50.1)	149.3 (102.1)	150.9 (127.4)	145.8 (120.0)
Methodologies used				
Tissue IHC (double)	8	25	19	43 (7)
Tissue mRNA	25	45	35	55
Homogenate ELISA	10	15	15	15

*The Lund-Kennedy score was evaluated at 6 months postoperatively.

20–25 g) were divided into 4 groups: the PBS-instilled group (PBS, $n = 10$), the NP model group (POLYP, $n = 10$), the NP model group treated with anti-IL-25 (R&D Systems; POLYP+ α IL-25, $n = 8$), and the NP model group treated with dexamethasone (POLYP+steroid, $n = 8$). Detailed animal experiments are described in the [Methods](#) section in this article's Online Repository.

Cytokines from nasal lavage fluid

Nasal lavage was performed, as previously described.²⁴ After partial tracheal resection during 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 200 μ L of PBS, and fluid from the nostril was collected and centrifuged. Supernatants were stored at -80°C . IL-4, IL-17A, IFN- γ , IL-10, and TGF- β 1 levels in nasal lavage fluid were measured with ELISA kits purchased from BioLegend (San Diego, Calif). The lower detection limits of these ELISA kits were 0.5 pg/mL for IL-4, 2.7 pg/mL for IL-17A, 8 pg/mL for IFN- γ , 2.7 pg/mL for IL-10, and 2.3 pg/mL for TGF- β 1.

Immunoblotting assays

Proteins were electrophoresed on SDS-PAGE and transferred to Immobilon-P membranes. Membranes were incubated with primary antibodies, incubated with horseradish peroxidase-conjugated secondary antibodies, and visualized by using the ECL reaction (GE Healthcare, Hatfield, United Kingdom). The primary antibodies against IL-25 and β -tubulin were purchased from Abcam (Cambridge, United Kingdom) and Santa Cruz Biotechnology (Dallas, Tex), respectively.

Statistical analysis

Statistical analyses used in this study include the Kruskal-Wallis test and the Mann-Whitney U test with the 2-tailed test for unpaired comparisons and were performed with IBM SPSS 20 (SPSS, Chicago, Ill) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, Calif) software. When comparisons were made between groups, Kruskal-Wallis tests were used to establish significant intergroup variability. Mann-Whitney U tests (2-tailed) were then used for between-group comparisons. Pearson correlations were used to determine variable relationships. If not normally distributed, the Spearman correlation coefficient was selected. A P value of less than .05 was considered statistically significant.

RESULTS

IL-25 expression and cellular origin in patients with CRSwNP

Tissues were collected from patients with CRSsNP (UP tissues), patients with CRSwNP (NP and UP tissues), and control subjects (UP tissues) to measure IL-25 expression in patients with

CRSwNP. IL-25 expression was greater in epithelial cells of NPs compared with those of UPs in patients with CRSsNP and control subjects ([Fig 1, A and B](#)). We documented a significant increase in IL-25⁺ inflammatory cell numbers in NPs and UPs of patients with CRSwNP compared with those in UPs from control subjects and patients with CRSsNP ([Fig 1, C](#), and see [Fig E1](#) in this article's Online Repository at www.jacionline.org). We 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 the other patient groups ([Fig 1, D](#)). IL-25 concentrations were measured by using ELISA to examine this observation at the protein level. These data demonstrated that IL-25 protein levels were significantly increased in NP tissue homogenates from patients with CRSwNP compared with those in control tissues ([Fig 1, E](#)). We used double IHC staining to identify IL-25⁺ cells in the subepithelial layer ([Fig 1, F and G](#)). 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 major basic protein-positive, CD68⁺, CD11c⁺, and 2D7⁺ cells, was 0 to 23 (median, 5/hpf; $n = 7$), 0 to 12 (median, 3/hpf; $n = 7$), 3 to 13 (median, 4/hpf; $n = 7$), and 0 to 13 (median, 6/hpf; $n = 7$), respectively. Collectively, our data show that human NPs had increased IL-25 expression in epithelial cells and partly in infiltrating inflammatory cells, including mast cells and eosinophils.

IL-17RB expression in patients with CRSwNP

IL-25 was previously 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.^{25,26} Therefore we measured IL-17RB expression in nasal tissues from control subjects, patients with CRSsNP, and patients with CRSwNP. IL-17RB⁺ inflammatory cell counts were significantly increased in both patients with CRSsNP and those with CRSwNP compared with those in control subjects ([Fig 2, A and B](#)). IL-17RB protein levels were significantly greater in NP tissue homogenates from patients with CRSwNP compared with those in control subjects ([Fig 2, C](#)).

Correlations between IL-25 mRNA expression and other inflammatory markers in human NP tissues

To investigate the implication of upregulated IL-25 expression in patients with CRSwNP, we examined whether IL-25

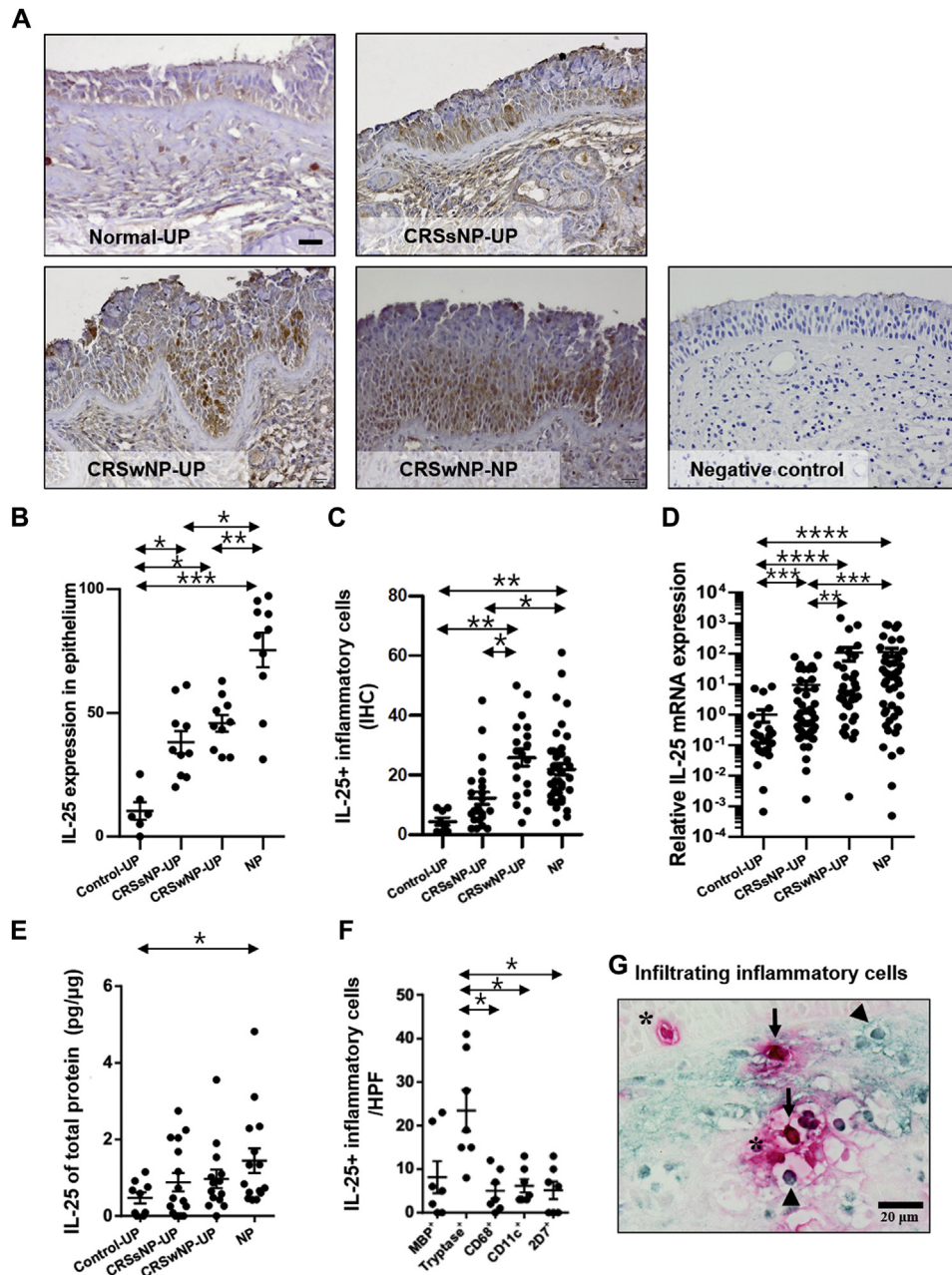


FIG 1. Expression of IL-25 in patients with CRSwNP or those with CRSsNP. **A**, Control UP mucosa from patients without nasal diseases, UPs from patients with CRSsNP and CRSwNP, and NP tissues from patients with CRSwNP were immunostained with IL-25 antibody. The negative control was immunostained with isotype IgG. **B**, Comparison of IL-25 expression levels in each tissue (n = 6 for control UP, n = 10 for CRSsNP-UP, n = 10 for CRSwNP-UP, and n = 10 for CRSwNP-NP). Numbers of IL-25⁺ epithelial cells per 100 cells were counted and averaged from 3 different areas of epithelium. **C**, Numbers of IL-25⁺ inflammatory cells were counted from the 5 densest areas (hpfs; magnification ×400) and averaged in each group (n = 8 for control-UP, n = 25 for CRSsNP-UP, n = 19 for CRSwNP-UP, and n = 43 for CRSwNP-NP). **D**, Relative IL-25 mRNA expression of whole tissues from each group were compared (n = 17 for control-UP, n = 40 for CRSsNP-UP, n = 35 for CRSwNP-UP, and n = 48 for CRSwNP-NP). **E**, Protein levels of IL-25 were measured by means of ELISA and compared (n = 9 for control-UP, n = 15 for CRSsNP-UP, n = 14 for CRSwNP-UP, and n = 15 for CRSwNP-NP). **F**, Double immunohistochemical staining for major basic protein, tryptase, CD68, CD11c or 2D7, and IL-25 was performed, and double-positive cells were counted (n = 7 for each group). **G**, Immunostaining of representative cells with both tryptase (red, asterisks) and IL-25 (green, arrowheads) in NP tissue. Arrows indicate double-positive immune cells (magnification ×1000). Scale bar = 20 μm. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001, Mann-Whitney *U* test.

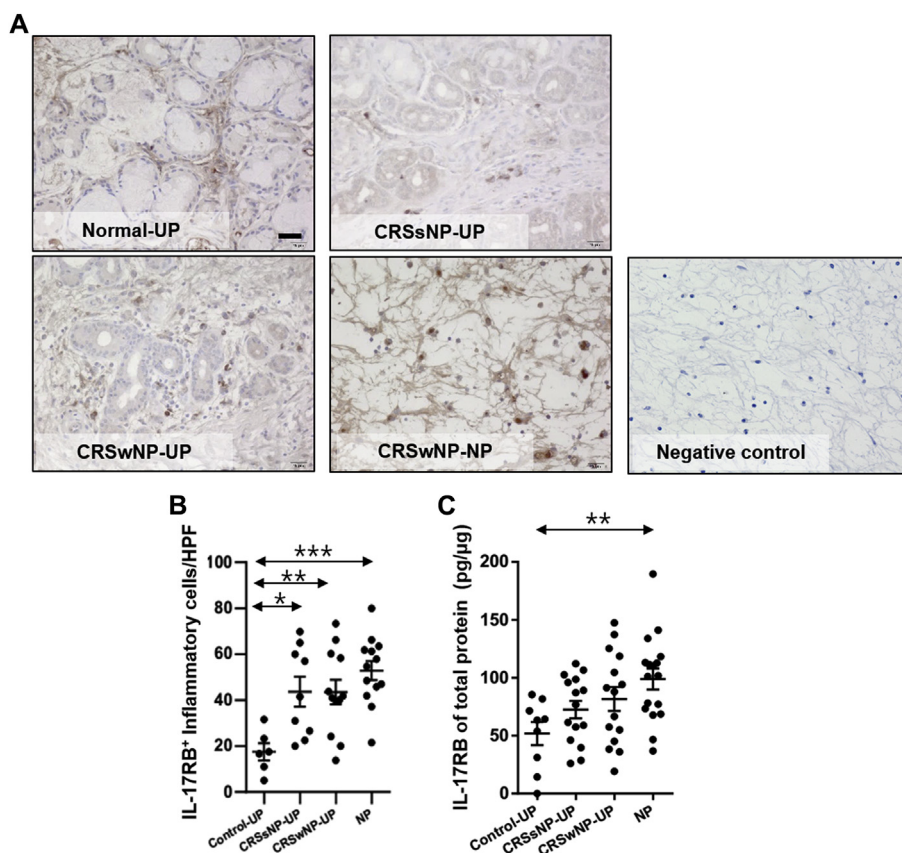


FIG 2. Expression of IL-17RB in patients with CRSwNP or those with CRSsNP. **A**, Control UP mucosa from patients without nasal diseases, UPs from patients with CRSsNP, and NP tissues from patients with CRSwNP were immunostained with IL-17RB antibody. Negative controls were immunostained with isotype IgG. **B**, Comparison of IL-17RB expression in each tissue ($n = 6$ for control-UP, $n = 9$ for CRSsNP-UP, $n = 11$ for CRSwNP-UP, and $n = 13$ for CRSwNP-NP). Expression levels of IL-17RB were reviewed under the hpf (magnification $\times 400$). The final score of each sample is presented as the average of scores from the 5 densest areas (hpf; magnification $\times 400$). **C**, Protein levels of IL-17RB were measured by means of ELISA and compared ($n = 9$ for control-UP, $n = 15$ for CRSsNP-UP, $n = 15$ for CRSwNP-UP, and $n = 15$ for CRSwNP-NP). * $P < .05$, ** $P < .01$, and *** $P < .001$, Mann-Whitney U test.

expression correlated with other inflammatory markers, such as T-bet (a major transcription factor in T_H1 responses), RORC (a major transcription factor in T_H17 responses), GATA3 (a major transcription factor in T_H2 responses), ECP, TGF- $\beta 1$, and TGF- $\beta 2$ (see Fig E2 in this article's Online Repository at www.jacionline.org). Fig 3 shows IL-25 expression positively correlated with all inflammatory markers tested: T-bet ($r = 0.806$, $P = .0001$), RORC ($r = 0.970$, $P < .0001$), GATA3 ($r = 0.430$, $P = .0005$), ECP ($r = 0.697$, $P < .0001$), TGF- $\beta 1$ ($r = 0.360$, $P = .0026$), and TGF- $\beta 2$ ($r = 0.423$, $P = .0013$).

Anti-polyp effect of IL-25 neutralizing antibody in animal models of polyyps

To investigate the role of IL-25 in nasal polypogenesis, we used an NP mouse model and confirmed IL-25 expression. NP models showed higher IL-25 expression in epithelial layers and inflammatory cells compared with that seen in control mice (see Fig E3 in this article's Online Repository at www.jacionline.org). Both anti-IL-25 (POLYP+ α IL-25) and steroid (POLYP+steroid) treatment reduced the number of nasal polypoid lesions, mucosal thickness, and collagen deposition in NP mice (Fig 4, B-D and G,

and see Fig E4, A and D, in this article's Online Repository at www.jacionline.org). We also observed decreased numbers of eosinophils and neutrophils in the POLYP+ α IL-25 group compared with the untreated POLYP group (Fig 4, E and F, and see Fig E4, B and C). Although steroid treatment had anti-inflammatory effects on the nasal mucosa, the anti-IL-25 antibody exerted a stronger effect on eosinophilic recruitment than the steroid treatment (Fig 4, E). However, the anti-IL-25 antibody had a minor inhibitory effect on goblet cell hyperplasia compared with steroid therapy (Fig 4, H, and see Fig E4, E).

Alterations in cytokine profiles after IL-25 inhibition

Both anti-IL-25 and steroid therapy suppressed IL-25 expression in the mouse model (Fig 5, A and B), and cytokine profiles of nasal lavage fluid samples reflected the histologic findings (Fig 5, C). Anti-IL-25 treatment also reduced IL-4, IFN- γ , and TGF- $\beta 1$ levels in nasal lavage fluid from mice. Level of IL-10, an anti-inflammatory cytokine, were increased by steroid therapy but not by anti-IL-25 treatment. These data suggest that the anti-polyp effect of anti-IL-25 treatment might have a different mechanism from the anti-inflammatory effect of the steroid.

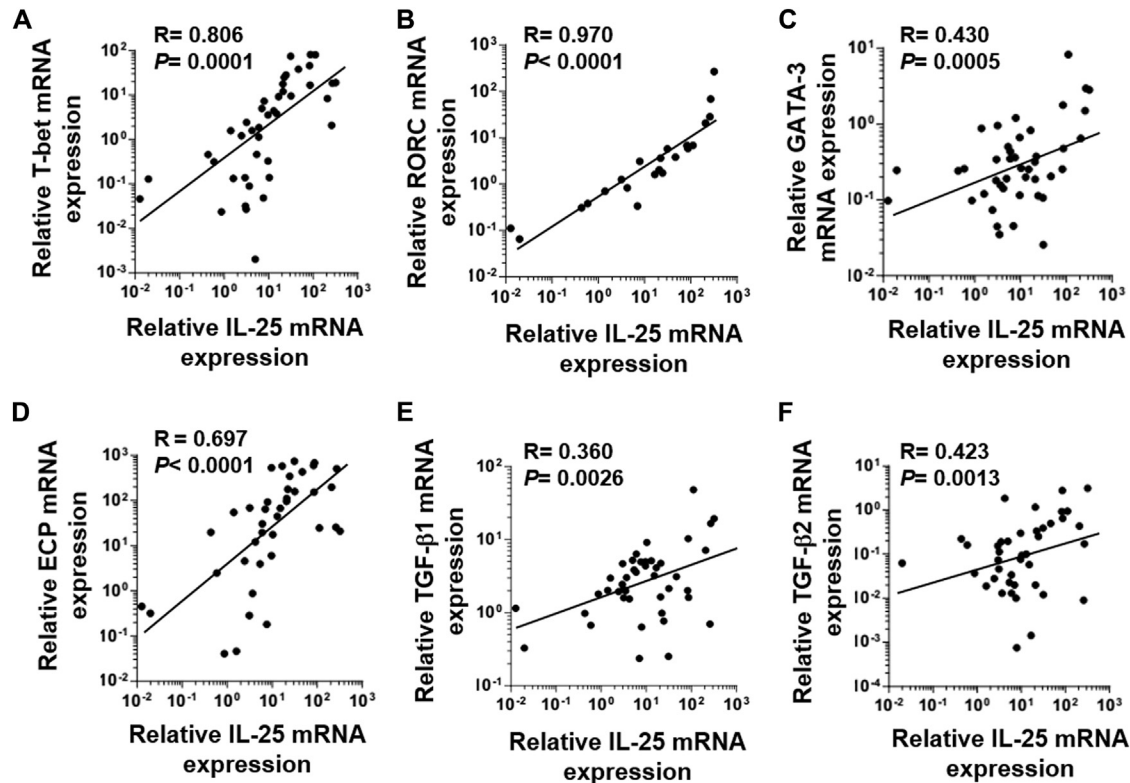


FIG 3. Correlation between mRNA expression of IL-25 and inflammatory markers. **A-F**, mRNA expression levels of T-bet, RORC, GATA-3, ECP, TGF- β 1, and TGF- β 2 were measured in NPs ($n = 43$), and correlations between IL-25 and each inflammatory marker were investigated. The Pearson correlation test was used, and R values indicate Pearson correlation coefficients.

Alterations in chemokine and adhesion molecule expression after IL-25 inhibition

Eosinophil chemotactic chemokines (CCL11 and CCL24), neutrophil-recruiting chemokines (CXCL1 and CXCL2), and recruitment adhesion molecules (E-selectin, intercellular adhesion molecule [ICAM] 1, and vascular cell adhesion molecule [VCAM] 1) were assessed in mice with NPs to verify that inflammatory cell recruitment was inhibited by anti-IL-25 treatment. These chemokines and adhesion molecules were upregulated when NPs were induced in mice (Fig 6); however, IL-25 inhibition led to downregulation of chemotactic factors (CCL11 and CXCL2) and adhesion molecule expression (ICAM-1 and VCAM-1).

DISCUSSION

IL-25 production by bronchial and nasal epithelial cells is regulated by transcription and protein expression, and allergen proteases can play pivotal roles in both of these biological processes.²⁷ NPs represent a disease the development of which is predisposed by the presence of allergen.²¹ Thus our findings that epithelial cells from both human and mouse NPs induced by ovalbumin (OVA) and staphylococcal enterotoxin B (SEB) have quite high expression of IL-25 are reasonable. Regarding the cellular source of IL-25, we also observed that tryptase-positive cells were one of the abundant cell types among the infiltrating inflammatory IL-25⁺ cells in human NP tissues. Several types of cells, including mast cells, secrete IL-25 and

upregulate the IL-25 receptor IL-17RB. Various studies have demonstrated that IL-25 is mainly produced by T_H2 cells and mast cells.^{9,28,29}

Because NPs of Asian patients are less eosinophilic compared with those of Western populations, mast cells might be involved as much as eosinophils in the mucosal pathogenesis in NPs from Asian patients. Recently, it was reported that local IgE induced by common aeroallergens might mediate mast cell activation and contribute to subsequent eosinophilic inflammation in Chinese patients with CRSwNP.³⁰ In Western patients with NPs, glandular mast cells and other diverse subsets of mast cells were detected more frequently in NP tissues than in UP tissues from control subjects and patients with CRS.³¹ Mast cells can produce diverse cytokines related to T_H1 and T_H2, which could thus contribute to the heterogeneous inflammatory responses observed in Asian patients with CRSwNP. However, our study only demonstrates the potential engagement of mast cells in the pathogenesis of NPs in Asian patients. Therefore their exact role during nasal polypogenesis should be explored in future studies.

Our study showed increased IL-25 expression levels in human NP tissues that correlated with T-bet, RORC, and GATA-3 upregulation in patients with CRSwNP, as well as in patients with CRSsNP (data not shown). In addition, expression of transcription factors involved in T_H1/T_H2/T_H17 T-cell responses was simultaneously increased in our NP tissues (see Fig E2). Because IL-25 is involved in diverse T_H2-mediated diseases, these mixed phenotypes and correlations between IL-25 and

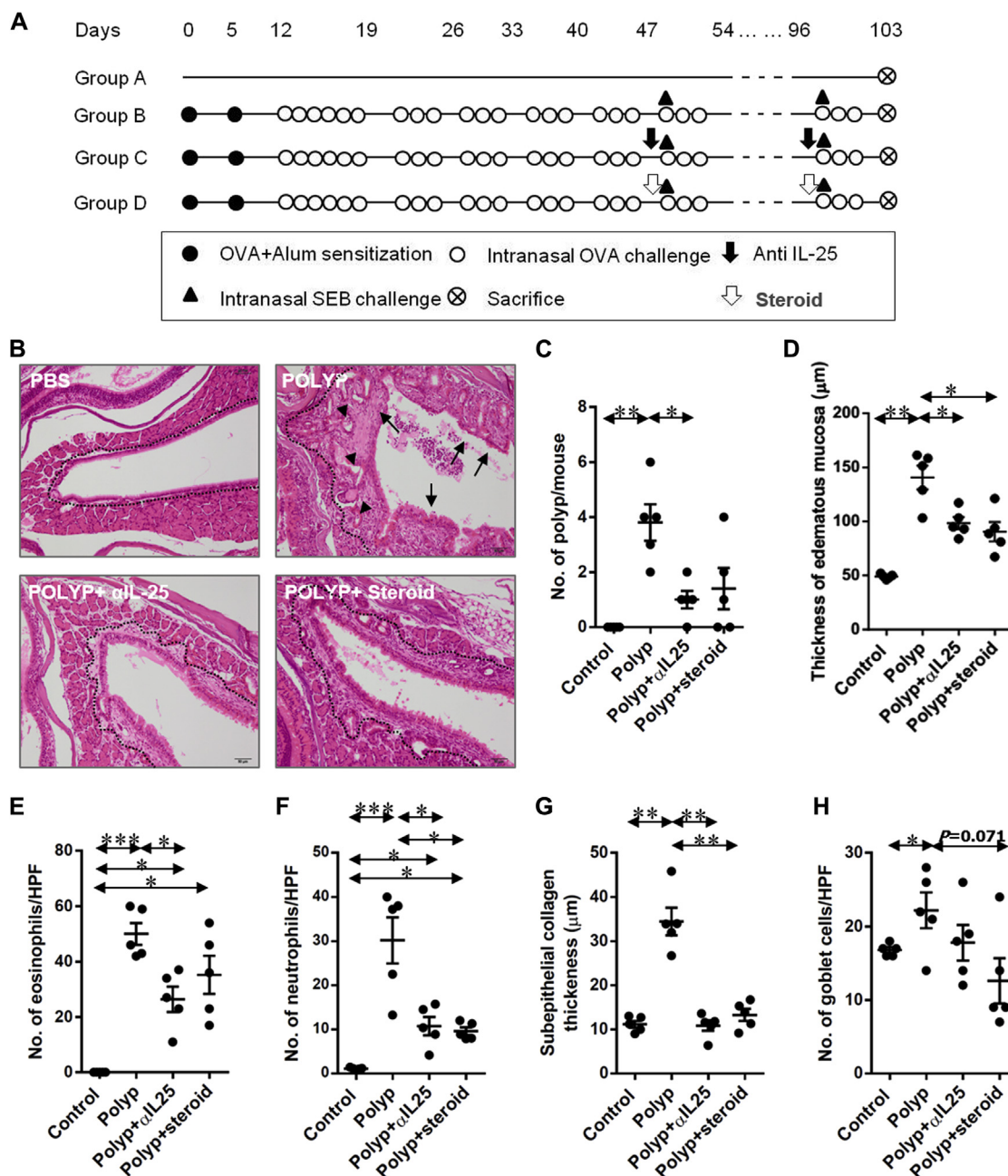


FIG 4. Effect of anti-IL-25 on NP formation in the mouse model. **A**, Protocol for generating the murine NP model. OVA and SEB were instilled into the nasal cavity to induce nasal polyp formation. Anti-IL-25 or dexamethasone (1 mg/kg) was administered intraperitoneally to investigate their effects on nasal inflammation and polyp formation. **B**, Photographs of representative maxillary sinus mucosa in each group of mice. Dotted lines represent the border between glandular structure and maxillary sinus mucosa. Arrows and arrowheads indicate polypoid lesions and epithelial ingrowth, respectively. **C-H**, Numbers of nasal polypoid lesions (Fig 4, C), mucosal edema thickness (Fig 4, D), numbers of infiltrated eosinophils (Fig 4, E), numbers of infiltrated neutrophils (Fig 4, F), subepithelial collagen deposition (Fig 4, G), and numbers of goblet cells (Fig 4, H) were counted from 10 different hpfs (magnification $\times 400$) and compared among each group ($n = 5$). The Mann-Whitney U test was used to analyze the results of these experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$, Mann-Whitney U test.

T_H1 and T_H17 activation markers were unexpected in our study. These mixed phenotypes of $T_H1/T_H2/T_H17$ pathways observed in Asian patients with NPs might be attributed to an upstream causal factor that induces diverse inflammatory cytokines, including IL-25. Alternatively, a spectrum might exist between

CRSwNP and CRSsNP in Asian patients with CRS, whereas CRSwNP in Western patients is regarded as a disease entity distinct from CRSsNP. Specifically, Asian CRS subtypes can be explained by differences of severity rather than inflammatory skewing. Therefore the upregulation of IL-25 in patients with

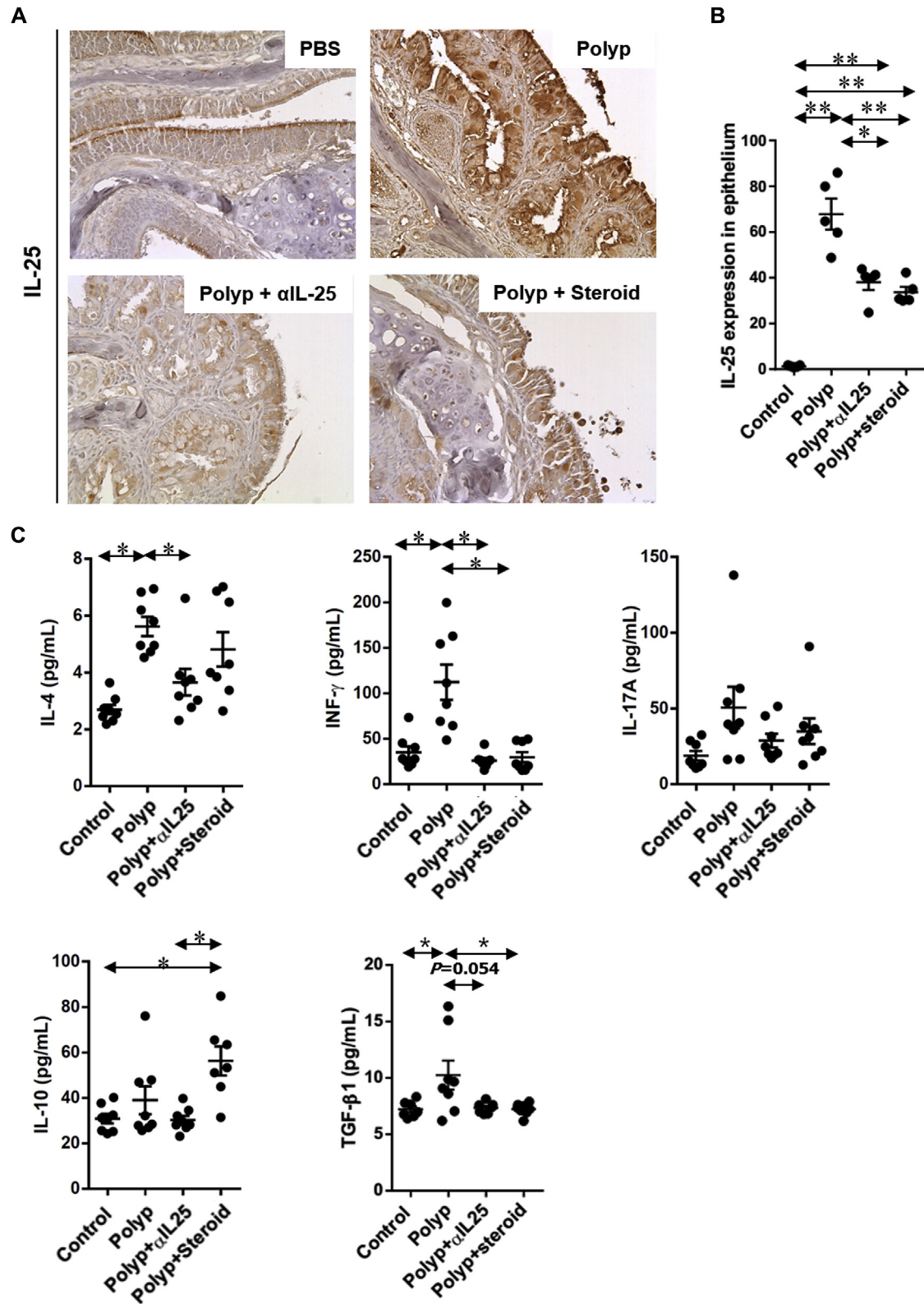


FIG 5. IL-25 expression and cytokine profiles in the murine NP model. **A**, Representative photographs of IL-25 expressions in epithelia from each group of mice. **B**, Comparison of IL-25 expression among the indicated groups. The number of IL-25⁺ epithelial cells was counted in hpfs (magnification $\times 400$). The final score of each sample is presented as the average score from 5 different hpfs. **C**, Cytokine profile from nasal lavage fluid ($n = 8$ for each group). *Control*, PBS-instilled group; *Polyp*, mouse polyp model; *POLYP*+ α IL-25, mouse polyp model treated with anti-IL-25; *POLYP*+*Steroid*, mouse polyp model treated with dexamethasone. * $P < .05$ and ** $P < .01$, Mann-Whitney U test.

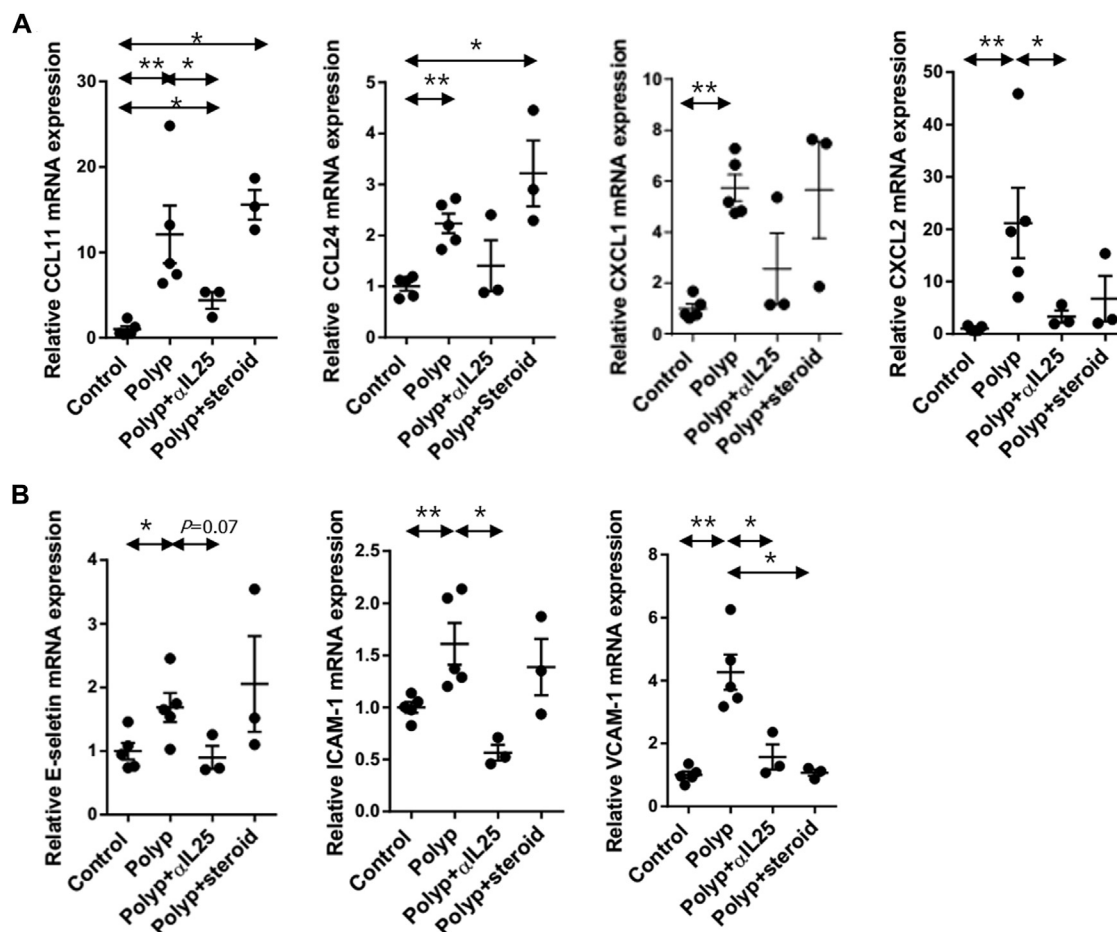


FIG 6. Anti-IL-25 therapy suppresses expression of both leukocyte chemotactic cytokines and ICAMs in a murine nasal polyp model. **A**, Relative mRNA expression levels of leukocyte-recruiting cytokines from each group were compared. **B**, Relative mRNA expression levels of ICAMs from each group were compared. * $P < .05$ and ** $P < .01$.

CRSsNP and even more in patients with CRSwNP might reflect the advanced status of mixed mucosal inflammation. Interestingly, the relevance of IL-25 to the mixed inflammation pattern has been elucidated in recent reports in which patients with uncontrolled or obesity-associated asthma had mixed cytokine profiles, such as high IL-25/high IL-17A/high IL-5 levels.^{32,33} In addition, this mingled pattern was associated with neutrophilic inflammation.³³ These clinical phenotypes are similar to NPs in Asian patients in which neutrophilic infiltration was commonly observed, indicating IL-25 induction was accompanied by diverse inflammatory regulators. Lastly, a relatively weak correlation between IL-25 and TGF- β suggests that they influence each other in an indirect manner (Fig 3, E and F).

We also used a murine NP model developed in a previous study.³⁴ In this model NPs were generated by means of intranasal instillation of OVA and SEB after OVA sensitization. Recent studies have used this model to evaluate epithelial remodeling, the therapeutic benefits of anti-polyp treatment, and various immunologic host characteristics.^{23,34-37} NPs in this animal model showed mixed inflammation involving both eosinophilic and neutrophilic activity, which is consistent with phenotypes of NPs from Asian patients. The collagen deposition found in mouse NPs also resembled the epithelial remodeling observed in Asian patients.³⁸ Furthermore, the mucosal tissues in these

mice showed prominent IL-25 expression similar to that observed in Korean patients with NPs. IL-25 mRNA levels were also correlated with diverse inflammatory markers in the mouse polyp model (see Fig E5 in this article's Online Repository at www.jacionline.org). Thus we concluded that this murine NP model could be useful for evaluating the role of IL-25 in the pathogenesis of disease in Asian patients with NPs.

Although the role of IL-10 or regulatory T cells in patients with CRS is controversial,^{39,40} IL-10 is an important cytokine that is produced by inducible regulatory T cells, which regulate immune responses. In addition, IL-10 from other cell types, such as CD8⁺ T cells, B cells, macrophages, and epithelial cells, might have a negative feedback role in the inflammatory process in patients with CRS. However, changes in cytokine profiles with anti-IL-25 treatment indicated that the anti-polyp effect of the anti-IL-25 antibody could not be attributed to IL-10 activity in contrast to the effects of steroid therapy. In our animal experiments anti-IL-25 treatment suppressed IL-4 and IFN- γ expression in nasal lavage fluid and downregulated mRNA expression of CCL11, CXCL2, ICAM-1, and VCAM-1. Therefore we propose that the anti-polyp effect results from inhibiting IL-25-responsive innate lymphoid cells in NPs and suppressing the recruitment of effector cells, including eosinophils and neutrophils.

In summary, we show that epithelial cells and infiltrating mast cells of human NPs showed prominent IL-25 expression, which positively correlated with expression of multiple inflammatory markers: T-bet, RORC, GATA3, ECP, and TGF- β . IL-25 expression was more abundant in the NP murine model compared with tissues from control mice, and anti-IL-25 treatment reduced polyp formation, mucosal thickness, collagen deposition, and infiltration of inflammatory cells, including eosinophils and neutrophils. Taken together, these findings suggest IL-25 plays a crucial role in the pathogenesis of disease in Asian patients with NPs, and blocking IL-25 activity could be a novel therapeutic strategy to improve clinical outcomes of patients with nasal polyposis.

Clinical implications: IL-25 expression is increased in NPs and correlates with principal inflammatory markers. Neutralizing IL-25 reduces nasal polypogenesis in an animal model and might represent a novel therapeutic target.

REFERENCES

- Hastan D, Fokkens WJ, Bachert C, Newson RB, Bislimovska J, Bockelbrink A, et al. Chronic rhinosinusitis in Europe—an underestimated disease. A GA(2) LEN study. *Allergy* 2011;66:1216-23.
- Hamilos DL. Chronic rhinosinusitis: epidemiology and medical management. *J Allergy Clin Immunol* 2011;128:693-709.
- Soler ZM, Mace JC, Litvack JR, Smith TL. Chronic rhinosinusitis, race, and ethnicity. *Am J Rhinol Allergy* 2012;26:110-6.
- Banerji A, Piccirillo JF, Thawley SE, Levitt RG, Schechtman KB, Kramper MA, et al. Chronic rhinosinusitis patients with polyps or polypoid mucosa have a greater burden of illness. *Am J Rhinol* 2007;21:19-26.
- Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, et al. Rhinosinusitis: developing guidance for clinical trials. *J Allergy Clin Immunol* 2006;118(suppl):S17-61.
- Van Zele T, Claeys S, Gevaert P, Van Maele G, Holtappels G, Van Cauwenberge P, et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. *Allergy* 2006;61:1280-9.
- Chin D, Harvey RJ. Nasal polyposis: an inflammatory condition requiring effective anti-inflammatory treatment. *Curr Opin Otolaryngol Head Neck Surg* 2013;21:23-30.
- Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S, et al. New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. *J Immunol* 2002;169:443-53.
- Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* 2001;15:985-95.
- Ballantyne SJ, Barlow JL, Jolin HE, Nath P, Williams AS, Chung KF, et al. Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. *J Allergy Clin Immunol* 2007;120:1324-31.
- Gregory LG, Jones CP, Walker SA, Sawant D, Gowers KH, Campbell GA, et al. IL-25 drives remodelling in allergic airways disease induced by house dust mite. *Thorax* 2013;68:82-90.
- Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB, et al. Interleukin 25 promotes the initiation of proallergic type 2 responses. *J Exp Med* 2007;204:1509-17.
- Hvid M, Vestergaard C, Kemp K, Christensen GB, Deleuran B, Deleuran M. IL-25 in atopic dermatitis: a possible link between inflammation and skin barrier dysfunction? *J Invest Dermatol* 2011;131:150-7.
- Deleuran M, Hvid M, Kemp K, Christensen GB, Deleuran B, Vestergaard C. IL-25 induces both inflammation and skin barrier dysfunction in atopic dermatitis. *Chem Immunol Allergy* 2012;96:45-9.
- Hams E, Armstrong ME, Barlow JL, Saunders SP, Schwartz C, Cooke G, et al. IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis. *Proc Natl Acad Sci U S A* 2014;111:367-72.
- Mjosberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol* 2011;12:1055-62.
- Shaw JL, Fakhri S, Citardi MJ, Porter PC, Corry DB, Kheradmand F, et al. IL-33-responsive innate lymphoid cells are an important source of IL-13 in chronic rhinosinusitis with nasal polyps. *Am J Respir Crit Care Med* 2013;188:432-9.
- Wang YH, Angkasekwinai P, Lu N, Voo KS, Arima K, Hanabuchi S, et al. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. *J Exp Med* 2007;204:1837-47.
- Lam M, Hull L, McLachlan R, Snidvongs K, Chin D, Pratt E, et al. Clinical severity and epithelial endotypes in chronic rhinosinusitis. *Int Forum Allergy Rhinol* 2013;3:121-8.
- Kim DK, Shin HW, Park JW, Yang MS, Kim DW. The role of innate cytokine in non-asthmatic, non-eosinophilic nasal polyps: IL-25, IL-33 and TSLP. *J Allergy Clin Immunol* 2013(suppl):AB169.
- Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 2012;50:1-12.
- Nagarkar DR, Poposki JA, Tan BK, Comeau MR, Peters AT, Hulse KE, et al. Thymic stromal lymphopoietin activity is increased in nasal polyps of patients with chronic rhinosinusitis. *J Allergy Clin Immunol* 2013;132:593-600.e12.
- Shin HW, Cho K, Kim DW, Han DH, Khalmuratova R, Kim SW, et al. Hypoxia-inducible factor 1 mediates nasal polypogenesis by inducing epithelial-to-mesenchymal transition. *Am J Respir Crit Care Med* 2012;185:944-54.
- Rhee CS, Libet L, Chisholm D, Takabayashi K, Baird S, Bigby TD, et al. Allergen-independent immunostimulatory sequence oligodeoxynucleotide therapy attenuates experimental allergic rhinitis. *Immunology* 2004;113:106-13.
- Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity* 2004;21:467-76.
- Moseley TA, Haudenschild DR, Rose L, Reddi AH. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev* 2003;14:155-74.
- Kouzaki H, Tojima I, Kita H, Shimizu T. Transcription of interleukin-25 and extracellular release of the protein is regulated by allergen proteases in airway epithelial cells. *Am J Respir Cell Mol Biol* 2013;49:741-50.
- Dolgachev V, Petersen BC, Budelsky AL, Berlin AA, Lukacs NW. Pulmonary IL-17E (IL-25) production and IL-17RB+ myeloid cell-derived Th2 cytokine production are dependent upon stem cell factor-induced responses during chronic allergic pulmonary disease. *J Immunol* 2009;183:5705-15.
- Ikedo K, Nakajima H, Suzuki K, Kagami S, Hirose K, Suto A, et al. Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. *Blood* 2003;101:3594-6.
- Cao PP, Zhang YN, Liao B, Ma J, Wang BF, Wang H, et al. Increased local IgE production induced by common aeroallergens and phenotypic alteration of mast cells in Chinese eosinophilic, but not non-eosinophilic, chronic rhinosinusitis with nasal polyps. *Clin Exp Allergy* 2014;44:690-700.
- Takabayashi T, Kato A, Peters AT, Suh LA, Carter R, Norton J, et al. Glandular mast cells with distinct phenotype are highly elevated in chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2012;130:410-20.e5.
- Seys SF, Grabowski M, Adriaenssens W, Decraene A, Dilissen E, Vanoirbeek JA, et al. Sputum cytokine mapping reveals an 'IL-5, IL-17A, IL-25-high' pattern associated with poorly controlled asthma. *Clin Exp Allergy* 2013;43:1009-17.
- Marijse GS, Seys SF, Schelpe AS, Dilissen E, Goeminne P, Dupont LJ, et al. Obese individuals with asthma preferentially have a high IL-5/IL-17A/IL-25 sputum inflammatory pattern. *Am J Respir Crit Care Med* 2014;189:1284-5.
- Kim DW, Khalmuratova R, Hur DG, Jeon SY, Kim SW, Shin HW, et al. *Staphylococcus aureus* enterotoxin B contributes to induction of nasal polypoid lesions in an allergic rhinosinusitis murine model. *Am J Rhinol Allergy* 2011;25:e255-61.
- Kim SW, Kim JH, Jung MH, Hur DG, Lee HK, Jeon SY, et al. Periostin may play a protective role in the development of eosinophilic chronic rhinosinusitis with nasal polyps in a mouse model. *Laryngoscope* 2013;123:1075-81.
- Kim SW, Kim DW, Khalmuratova R, Kim JH, Jung MH, Chang DY, et al. Resveratrol prevents development of eosinophilic rhinosinusitis with nasal polyps in a mouse model. *Allergy* 2013;68:862-9.
- Lee KI, Kim DW, Kim EH, Kim JH, Samivel R, Kwon JE, et al. Cigarette smoke promotes eosinophilic inflammation, airway remodeling, and nasal polyps in a murine polyp model. *Am J Rhinol Allergy* 2014;28:208-14.
- Van Crombruggen K, Zhang N, Gevaert P, Tomassen P, Bachert C. Pathogenesis of chronic rhinosinusitis: inflammation. *J Allergy Clin Immunol* 2011;128:728-32.
- Cao PP, Li HB, Wang BF, Wang SB, You XJ, Cui YH, et al. Distinct immunopathologic characteristics of various types of chronic rhinosinusitis in adult Chinese. *J Allergy Clin Immunol* 2009;124:478-84, e1-2.
- Zhang N, Van Zele T, Perez-Novo C, Van Bruene N, Holtappels G, DeRuyck N, et al. Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus disease. *J Allergy Clin Immunol* 2008;122:961-8.

METHODS

IHC

Single IHC staining was performed by using the Polink-2 HRP Plus Broad DAB Detection System (Golden Bridge International Labs, Bothell, Wash). Briefly, after deparaffinization, the sections were incubated in 3% hydrogen peroxide for endogenous peroxidase inhibition and microwave treated in 10 mmol/L citrate buffer (pH 6.0) for heat-induced epitope retrieval. Then sections were incubated for 60 minutes at room temperature with each primary antibody, which included rabbit anti-human IL-25 (1:500, Abcam) or goat anti-human IL-17RB (1:50, R&D systems). Incubation was done in a broad antibody enhancer and polymer-horseradish peroxidase, and then sections were stained with the DAB Detection System. Finally, slides were counterstained with hematoxylin. Sequential IHC was used with polymer-horseradish peroxidase 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) to identify cellular sources of IL-25. Primary antibodies against cellular phenotypic markers included mouse anti-human eosinophil major basic protein (1:50, Santa Cruz Biotechnology), mouse anti-human CD11c (1:10; BD PharMingen, San Jose, Calif), mouse anti-mast cell tryptase (1:500, Abcam), mouse anti-CD68 (1:250, Abcam), mouse anti-basophils (2D7, 1:50; Abcam), and mouse anti-human neutrophil elastase (1:100, Abcam). These antibodies were mixed with the other primary antibody, rabbit anti-human IL-25 (1:500, Abcam) was applied to the tissue, and incubation was done for 30 to 60 minutes. Polymer mixtures were made by adding alkaline phosphatase polymer anti-mouse IgG and polymer-horseradish peroxidase anti-rabbit IgG at a 1:1 ratio and applied to cover each section. Unless noted otherwise, all manufacturer's instruction was followed.

Quantitative real-time RT-PCR for inflammatory markers, chemokines, and adhesion molecules

Total RNA was extracted from tissue samples by using the TRI reagent (Invitrogen, Carlsbad, Calif). One microgram of total RNA was reverse transcribed to cDNA by using the cDNA Synthesis Kit (*amfiRivert Platinum* cDNA Synthesis Master Mix; GenDEPOT, Katy, Tex). Quantitative real-time PCR was performed with the LightCycler 480 SYBR Green I Master (Roche, Mannheim, Germany) and primers that specifically amplify IL-25, T-bet, GATA3, RORC, ECP, TGF- β 1, and TGF- β 2. Primer sequences are as follows: IL-25 primers were purchased from Qiagen (Hilden, Germany); T-bet, 5'-GTCAATTCTTGGGGGAGAT-3' for the forward primer and 5'-TCATGCTGACTGCTCGAAAC-3' for the reverse primer; GATA3, 5'-ACCACAACC ACCTCTGGAGGA-3' for the forward primer and 5'-TCGGTTTCTGGTCTGGATGCCT-3' for the reverse primer; RORC, 5'-GCTGTGATCTTGCCAGAAC-3' for the forward primer and 5'-CTGCCATCATTGCTGTTAATCC-3' for the reverse primer; ECP, 5'-TCGGAGTAGATCCGGGTG-3' for the forward primer and 5'-GAAC CACAGATACCTGGAG-3' for the reverse primer; TGF- β 1, 5'-TGAA CCGGCCTTCTCTCTCATG-3' for the forward primer and 5'-GCG GAAGTCAATGTACAGCTGCCGC-3' for the reverse primer; and TGF- β 2, 5'-TGGATGCGGCCTATTGCTTTA-3' for the forward primer and 5'-GCGGAAGTCAATGTACAGCTGCCGC-3' for the reverse primer. TaqMan Gene Expression Assay kits (Applied Biosystems, Foster city, Calif) were purchased and used for measuring mRNA levels of CCL11 (Mm00441238_m1), CCL24 (Mm00444701_m1), CCL26 (Mm02763057_u1), CXCL1 (Mm04207460_m1), CXCL2 (Mm00436450_m1), CXCL5 (Mm00436451_g1), ICAM-1 (Mm01187466_m1), VCAM-1 (Mm01320970_m1), E-selectin (Mm00441238_m1), TGF- β 1 (Mm01178820_m1), TGF- β 2 (Mm00436955_m1), IL-25 (Mm00499822_m1), IL-4 (Mm00445259_m1), IL-5 (Mm00439646_m1), IL-17A (Mm00439618_m1), IFN- γ (Mm01168134_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mm03302249_g1). Predeveloped assay reagent kits of primers and probes were purchased from Applied Biosystems. Expression of glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for normalization. Cycling conditions were 95°C for 5 minutes, followed by 60 cycles at 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 20 seconds.

To analyze the data, we used Sequence Detection Software (version 1.9.1, Applied Biosystems). Relative gene expression was calculated by using the comparative $2^{-\Delta\Delta CT}$ method.

Murine NP model and tissue preparations

The detailed experimental protocol for generating the mouse NP model was described in a previous article.^{E1} In brief, mice (POLYP, POLYP+ α IL-25, and POLYP+steroid groups) were injected with 25 μ g of OVA (Sigma, St Louis, Mo) in 2 mg of aluminum hydroxide gel administered intraperitoneally on days 0 and 5, followed by daily intranasal instillation with 3% OVA diluted in 40 μ L of PBS from day 12 to day 17. Thereafter, the same amount of 3% OVA was instilled 3 times a week from day 21 to day 102. Intranasal instillation was performed in the head-down position, with the mouse's head kept down for 30 seconds after instillation to prevent pulmonary provocation. In addition, mice were challenged weekly with 10 ng of SEB (List Biological laboratories, Campbell, Calif) from day 49 through day 102 after OVA instillation. The POLYP, POLYP+ α IL-25, and POLYP+steroid groups were administered weekly intraperitoneal isotype IgG (300 μ g per a mouse), anti-IL-25 (300 μ g per a mouse), and dexamethasone (1 mg/kg) from day 49 through day 102 before OVA instillation, respectively. Control mice (PBS) were not sensitized but administered weekly intraperitoneal isotype IgG (300 μ g per a mouse) from day 49 through day 102 before OVA instillation. Mice were killed on day 103. Death occurred 24 hours after the last OVA challenge. The heads of 5 mice from each group were removed *en bloc* and then fixed in 4% paraformaldehyde for histopathologic analysis. After exposing the nasal cavities of the other mice, the nasal mucosa was taken out meticulously with a small curette and microforceps under microscopic vision.

Histopathologic analysis of animal tissues

For evaluation of nasal histopathology, nasal tissues were decalcified, embedded in paraffin, and sectioned coronally (4 μ m thickness) approximately 5 mm from the nasal vestibule. Several stains were conducted to compare characteristics between groups: hematoxylin and eosin for polyp-like lesions, Sirius red for eosinophils, anti-neutrophilic antibody (1:50, Abcam) for neutrophils, Alcian blue for goblet cells, and Masson trichrome stain for collagen fiber in the subepithelial layer. Ten areas from nasal mucosal sections were chosen randomly for evaluation under hpfs (magnification $\times$ 400) and measured by 2 examiners who were blind to group assignment. Polyp-like lesions were defined as distinct mucosal elevations with eosinophilic infiltration and microcavity formation. Three consecutive slides were reviewed to exclude processing errors. Mucosal thickness was measured as the distance between the apex of the epithelial cells and the upper border of the subepithelial glands zone by using an image analysis system. For assessment of mucosal thickness, at least 3 measurements at random points with a minimum distance of 20 μ m between the points were made in the appropriate area of each hpfs, and the mean from 4 different hpfs was recorded for comparison.

Measurement of IL-25 and IL-17RB in tissue homogenates

Before ELISA, protein concentrations for tissue extracts were determined by using the Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, Calif). For ELISA analysis of nasal polyp tissues, 1 mL of saline solution or PBS per every 0.1 g of tissue was added to make homogenates, and homogenates were used without diluting (dilution factor = 1). Samples were thawed at room temperature and vortexed to ensure a well-mixed sample. IL-25 (R&D Systems) and IL-17RB (R&D Systems) levels were assayed with commercially available assay kits. The minimal detection limits for these kits are 62.5 and 156 pg/mL, respectively. All procedures followed the manufacturer's recommendations. IL-25 and IL-17RB concentrations in the tissue homogenate were normalized to the concentration of total protein, as described previously.^{E2}

REFERENCES

- E1. Kim DW, Khalmuratova R, Hur DG, Jeon SY, Kim SW, Shin HW, et al. *Staphylococcus aureus* enterotoxin B contributes to induction of nasal polypoid lesions in an allergic rhinosinusitis murine model. *Am J Rhinol Allergy* 2011; 25:e255-61.
- E2. Takabayashi T, Kato A, Peters AT, Suh LA, Carter R, Norton J, et al. Glandular mast cells with distinct phenotype are highly elevated in chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2012;130:410-20.e5.

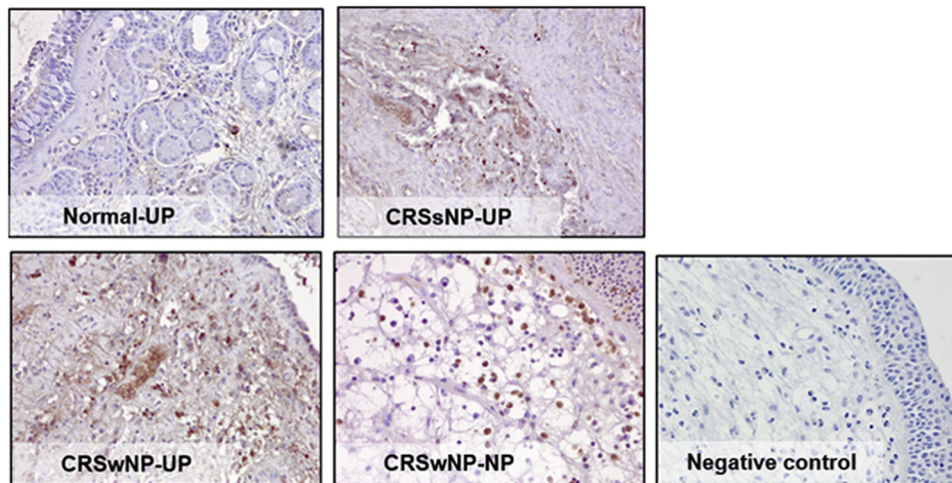


FIG E1. Expression of IL-25 in infiltrated inflammatory cells. Control UP mucosa from patients without nasal diseases, UPs from patients with CRSsNP and CRSwNP, and NP tissues from patients with CRSwNP were immunostained with IL-25 antibody. Negative controls were immunostained with isotype IgG.

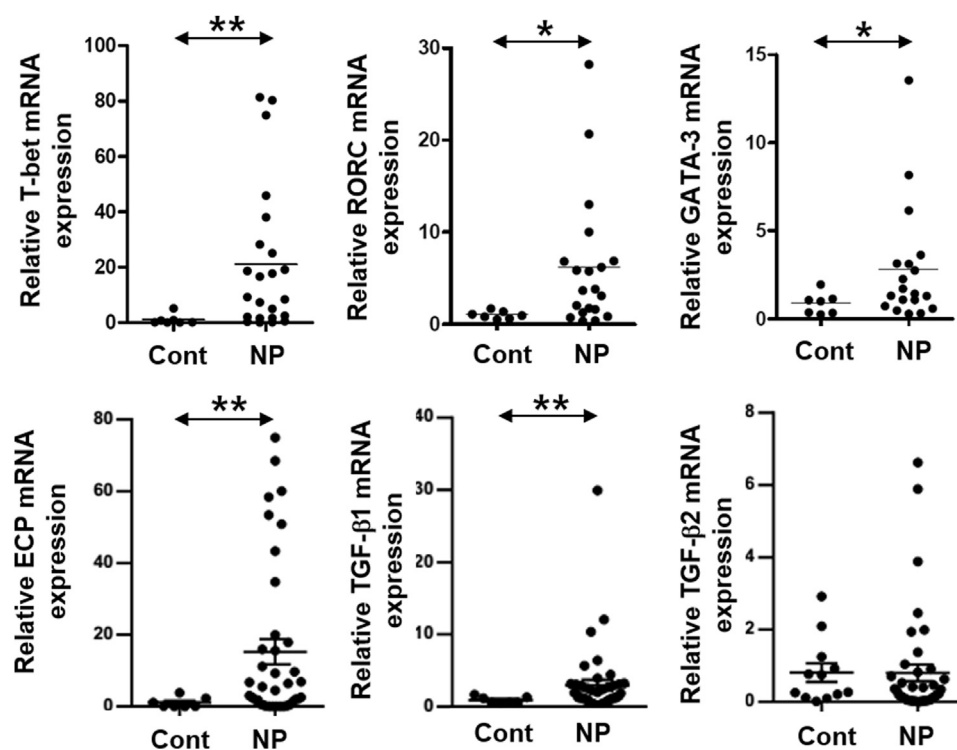


FIG E2. Immunologic characteristics of NPs in this study. mRNA expression of T-bet, RORC, GATA-3, ECP, TGF-β1, and TGF-β2 were measured. * $P < .05$ and ** $P < .01$, Mann-Whitney U test.

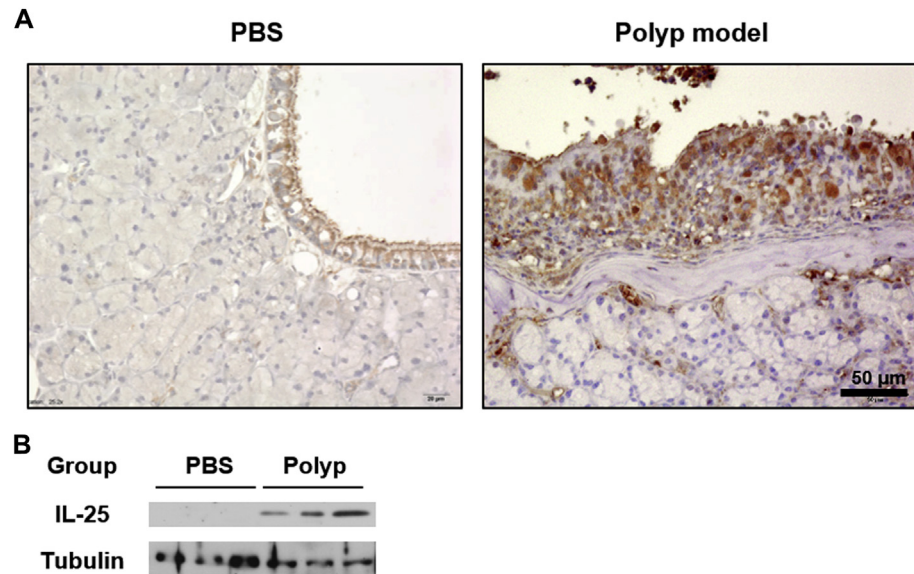


FIG E3. IL-25 expression in epithelium from a murine polyp model. **A**, Representative photographs in control mice (PBS) and the mouse polyp model. **B**, Expression of IL-25 in epithelium from murine nasal mucosa was compared with that in control mucosa by means of Western blotting (n = 3).

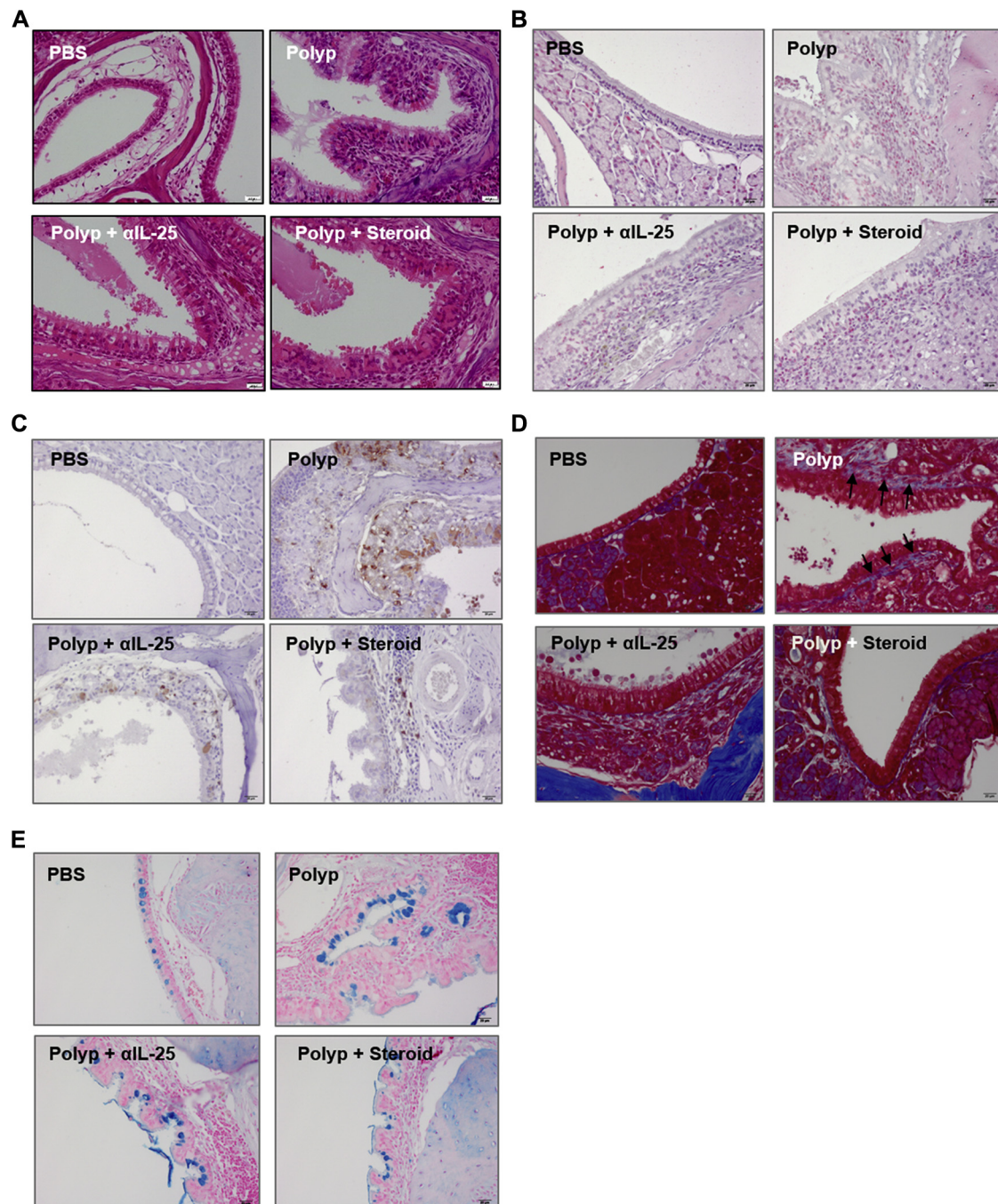


FIG E4. Representative photographs for polyp lesions, eosinophils, neutrophils, collagen deposition, and goblet cells in each group. **A**, Sinonasal mucosa with or without polyps stained with hematoxylin and eosin. **B**, Eosinophils stained with Sirius red. **C**, Neutrophils immunostained with neutrophilic antibody. **D**, Masson trichrome stain for collagen deposition. Arrows indicate thickening of collagen deposition. **E**, Alcian blue stain for goblet cells. *PBS*, Control group; *Polyp*, mouse polyp model; *Polyp*+ α IL-25, mouse polyp model treated with anti-IL-25; *Polyp*+Steroid, mouse polyp model treated with dexamethasone.

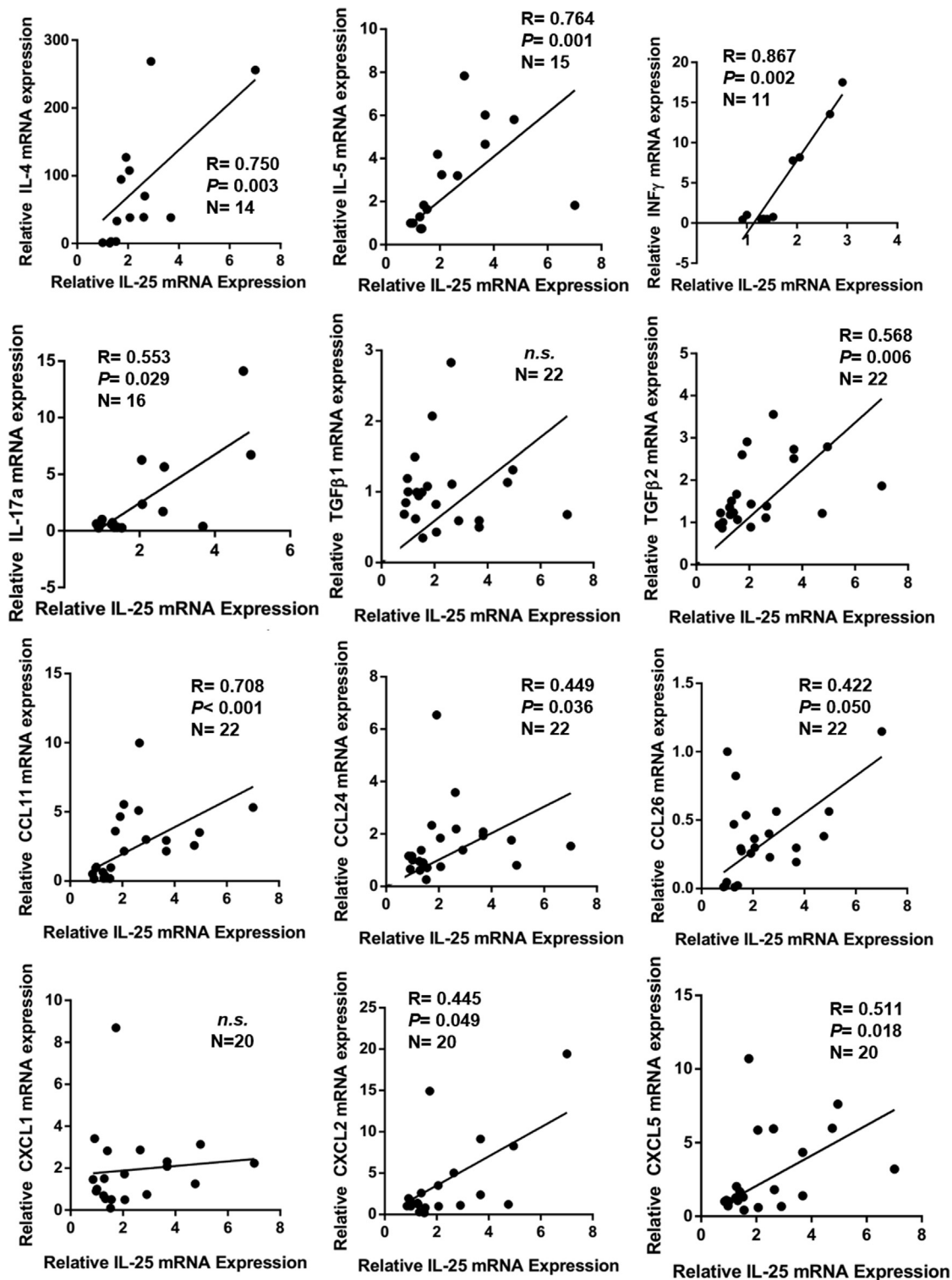


FIG E5. Correlation between mRNA expression of IL-25 and inflammatory markers in the mouse polyp model. mRNA expression levels of IL-4, IL-5, IFN- γ , IL-17a, TGF- β 1, TGF- β 2, CCL11, CCL24, CCL26, CXCL1, CXCL2, and CXCL5 were measured in nasal tissues from the mouse polyp model, and correlations between IL-25 and each inflammatory marker were investigated. Total RNA was isolated from whole sinonasal tissues from the control ($n = 10$) and polyp ($n = 10$) models. If the mRNA level was not detected on quantitative RT-PCR, that pair was deleted in a list-wise manner from the correlation analysis. Spearman correlation test was used, and R values indicate Spearman correlation coefficients.