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Original Article

Inflammatory Endotypes and Tissue Remodeling Features in Antrochoanal Polyps

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ABSTRACT

Purpose: The pathogenic mechanisms of antrochoanal polyps (ACPs) remain largely unknown. This study aimed to characterize inflammatory patterns and tissue remodeling features in ACPs.
Methods: Inflammatory cell infiltration and tissue edema severity as well as fibrin deposition in ACPs and bilateral eosinophilic and noneosinophilic nasal polyps (NPs) were studied with immunohistochemical and immunofluorescence staining. Cytokine levels in sinonasal tissues were detected with the Bio-Plex assay. The expression of coagulation and fibrinolytic markers was measured using reverse-transcription polymerase chain reaction and enzyme-linked immunosorbent assays.
Results: Compared to control tissues and bilateral eosinophilic and noneosinophilic NPs, ACPs had higher levels of neutrophil infiltration and expression of myeloperoxidase (MPO), interleukin (IL)-8 and interferon (IFN)-γ. In total, 94.4% of ACPs demonstrated an eosinophil cationic protein/MPO ratio of < 1, compared to 79.0% of noneosinophilic and 26% of eosinophilic NPs. Principle component and multiple correspondence analyses revealed a neutrophilic and type 1 inflammation pattern in ACPs. Compared to control tissues, edema scores and fibrin deposition were increased, whereas d-dimer and tissue plasminogen activator (tPA) levels were decreased in ACPs and bilateral NPs, with more prominent changes in ACPs even than in eosinophilic NPs. The tPA levels were negatively correlated with IFN-γ, IL-8, and MPO levels in ACPs. Neutrophils were the major cellular source of IFN-γ in ACPs, and the number of IFN-γ^+ neutrophils was elevated in ACPs than in control tissues and bilateral eosinophilic and noneosinophilic NPs.
Conclusions: ACPs are characterized by the neutrophilic and type 1 inflammation endotype. Neutrophil-derived IFN-γ is associated with reduced tPA production in ACPs.

Keywords: Nasal polyps; tissue plasminogen activator; interferon; neutrophils; inflammation; edema

INTRODUCTION

Antrochoanal polyps (ACPs) are benign sinonasal polyps in the maxillary sinus, growing through the sinus ostium and posterior nasal cavity, and extending into the choana and

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Disclosure

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nasopharynx.[1-3] The vast majority of ACPs are unilateral and more common in children than in adults. ACPs account for 4%–6% of all types of nasal polyps (NPs) and up to 33% in children.[4-6] Clinical treatment strategies for patients with ACPs are limited.[6,7] Although intranasal glucocorticoids are considered the first-line treatment for bilateral NPs, the lack of well-designed prospective studies has led to a poor understanding of the therapeutic effects of systemic and local glucocorticoids in patients with ACPs. Endoscopic sinus surgery is commonly accepted as a treatment for ACPs; however, the recurrence rate of ACPs can be up to 21% after surgery.[8-11] These problems arise partly because the underlying pathogenic mechanisms of ACPs are poorly understood. With an increasing understanding of the immunological characteristics of bilateral NPs, novel biologics targeting interleukin (IL)-4, IL-5, and immunoglobulin E (IgE) have been developed and shown promising efficacy in relieving symptoms and reducing NPs in patients with bilateral chronic rhinosinusitis with NPs (CRSwNP).[12,13] Therefore, a better understanding of the pathogenesis of ACPs may ultimately aid in the discovery of novel therapeutic strategies to improve treatment outcomes of ACPs.

Based on the extent of eosinophilic inflammation, CRSwNP can be classified as eosinophilic or noneosinophilic, particularly in East Asians.[14,15] Eosinophilic CRSwNP is dominated by type 2 inflammation, whereas the noneosinophilic type is characterized by neutrophil-, and type 1 and type 3 response-biased inflammation.[14] Several recent studies have shown that ACPs are likely associated with increased infiltration of neutrophils.[7,16,17] However, only limited cellular and molecular biomarkers have been investigated in those studies, and the comprehensive and integrated analysis of endotypes of ACPs is still lacking.

Tissue remodeling, particularly edema formation, plays a direct and critical role in the development of NPs.[18-25] Takabayashi *et al.*[20] reported that type 2 cytokines down-regulate tissue plasminogen activator (tPA), leading to excessive fibrin deposition and edema formation in bilateral eosinophilic NPs. Recently, we have found that, in addition to type 2 cytokines, interferon (IFN)-γ also reduced tPA expression and contributed to fibrin deposition and edema formation in bilateral noneosinophilic NPs.[18] Nevertheless, the tissue remodeling features and the underlying mechanisms in ACPs remain unexplored.

Here, to provide novel insights into the pathogenesis of ACPs, we comprehensively compared inflammatory cell infiltration, cytokine expression, edema severity, and coagulation and fibrinolytic system disturbance in ACPs, bilateral eosinophilic and noneosinophilic NPs, and control tissues. It was found that ACPs are characterized by neutrophilic and type 1 inflammation endotype. Neutrophil-derived IFN-γ associates with reduced tPA production in ACPs.

MATERIALS AND METHODS

Subjects and specimens

This study was approved by the Ethics Committee of Tongji Hospital (permit number 20160301) and conducted with written informed consent from all adult participants or parents of patients who are less than 18 years old. A total of 153 patients, including 44 with bilateral eosinophilic CRSwNP, 42 with bilateral noneosinophilic CRSwNP, 32 with unilateral ACPs, and 35 control subjects, were enrolled in this study. The diagnosis of CRSwNP was made according to European and American guidelines.[26,27] CRSwNP was defined as eosinophilic when the percentage of tissue eosinophils exceeded 10% of the total infiltrating cells as previously reported.[14] Control subjects were those undergoing septoplasty because

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of anatomic variations and without other sinonasal diseases. Atopic status was evaluated by using skin prick test with a panel of 19 common inhalant allergens in our region (Macro-Union Pharmaceutical Co., Beijing, China) and/or specific IgE against common inhalant allergens detected by using the ImmunoCAP (Phadia, Uppsala, Sweden).[28] The diagnosis of allergic rhinitis was made based on the concordance between atopic status and typical allergic symptoms. The diagnosis of asthma was made according to the Global Initiative for Asthma guideline.[29] Symptoms, including nasal obstruction, rhinorrhea, facial pain/pressure, and loss of smell, were scored on the visual analog scale from 0 to 10, with 0 for no symptom and 10 for the worst.[26] Endoscopic physical findings, including polyp size, edema, and discharge, were scored according to the Lund-Kennedy scoring system.[26] Computed tomography (CT) scans were graded based on the Lund-Mackay scoring system.[26] Oral glucocorticoid and intranasal steroid spray were discontinued at least 3 months and 1 month before surgery, respectively. Patients with an acute upper respiratory tract infection or acute asthma episode within 4 weeks of entering the study, and patients under immunotherapy were excluded. In addition, patients who had fungal sinusitis, cystic fibrosis, primary ciliary immobility syndrome, immunodeficiency, or systemic vasculitis were excluded from the study.

Inferior turbinate mucosal tissues from control subjects, NP tissues from patients with CRSwNP, and ACP tissues from patients with ACPs were collected during surgery. Nasal epithelial cells were scraped from the middle meatus of control subjects and polyp tissues of patients with CRSwNP or ACPs.[30] Not all samples were included in each experiment protocol because of limited quantity. The number of samples for each experiment was indicated in figures or figure legends.

Histology, immunohistochemistry, and immunofluorescence staining

Fresh tissue samples were fixed in formaldehyde solution and embedded in paraffin. After deparaffinization and rehydration, tissue sections (4 μm) were stained with haematoxylin-eosin. For immunohistochemistry, sections were subjected to heat-induced antigen retrieval using Target Retrieval Solution (Dako, Carpinteria, CA, USA). After blocking, sections were incubated with specific primary antibodies (**Supplementary Table S1**) at 4°C overnight. All antigens were detected by using the poly-horseradish peroxidase complex (Boster Biotechnology, Wuhan, China) method, and color development was achieved with 3′, 3′-diaminobenzidine. Tissue sections were finally counterstained with hematoxylin. For immunofluorescence staining, after blocking, tissue sections were incubated with primary antibodies (**Supplementary Table S1**) overnight at 4°C and then incubated with fluorescence-conjugated secondary antibodies (**Supplementary Table S2**) for 1 hour at room temperature. Species- and subtype-matched antibodies were used as negative controls.

The number of cells was counted at × 400 magnification. Edema was scored on a 3-point scale, with 0 representing the lowest and 2 representing the highest score at × 200 magnification.[18,31] Ten fields per section were randomly selected for analysis analyzed by 2 independent physicians who were blinded to the clinical data as previously described.[18] Fibrin and tPA staining intensity were automatically quantified by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA), and 10 fields at × 400 magnification per section were randomly selected for analysis.

Quantitative reverse-transcription polymerase chain reaction (RT-PCR)

RNA was extracted from the samples by using TRIzol reagent as previously described.[18] Single-strand cDNA was synthesized by reverse transcription. RT-PCR was performed with

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SYBR fluorescence reagent and specific primers (**Supplementary Table S3**). Glyceraldehyde-3-phosphate dehydrogenase was used as a housekeeping gene for normalization and relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ method.[32]

Measurement of mediators in nasal tissues

Snap-frozen sinonasal tissue samples were weighed and homogenized, and then the supernatants were harvested as previously described.[33,34] The levels of tPA (Abcam, Cambridge, MA, USA), thrombin-antithrombin (TAT) complex (Abcam), d-dimer (Abcam), and myeloperoxidase (MPO; R&D Systems, Minneapolis, MN, USA) in tissue homogenates were measured by using the commercial enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. Eosinophilic cationic protein (ECP) was detected by using UniCAP system (Pharmacia, Uppsala, Sweden) as previously described.[35] The protein levels of 35 inflammatory mediators (**Supplementary Table S4**) were measured by using the Bio-Plex suspension chip method (Bio-Rad, Hercules, CA, USA).[36] The lower detection limits are shown in **Supplementary Tables S4** and **S5**. The activity of tPA in tissue homogenates was analyzed with an activity assay kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions.

Classification of ACPs and bilateral NPs based on inflammatory cytokines

ACPs and bilateral NPs were stratified into endotypes 1 (T1), 2 (T2), and 3 (T3) when the protein levels of IFN-γ, IL-5, and IL-17A were higher than the corresponding cutoff value.[34] The cutoff value is the 95th percentile of cytokine levels in control tissues.[37] When a sample showed expression levels of 2 or 3 cytokines above the cutoff value, it was considered the double or triple mixed type. The sample was defined as all negative when a sample with the expression levels of all 3 cytokines below the cutoff value.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) and SPSS 23.0 statistical software (IBM SPSS, Armonk, NY, USA). Data distribution was tested for normality using the Kolmogorov-Smirnov test. For continuous variables, results are represented in dot plots. Symbols represent individual samples, horizontal bars represent medians, and error bars show interquartile ranges. The Kruskal-Wallis *H* test was used to assess significant intergroup variability, and the Mann-Whitney *U2* test was used for between-group comparison. For multiple comparisons, Bonferroni correction was used to adjust the significance levels by using a value of 0.017 and 0.008 for 3 and 4 study groups, respectively. For categorical variables, a χ^2 test was applied to determine differences between groups. The Spearman test was performed for correlation analysis. Principle component analysis (PCA) and multiple correspondence analyses (MCAs) were performed using the R package “devtools” and “MASS” (R Foundation, Vienna, Austria), respectively.

RESULTS

Clinical characteristics of patients with ACPs

As shown in **Table 1**, patients with ACPs were significantly younger than those with bilateral eosinophilic and noneosinophilic CRSwNP, and control subjects. Although there were no significant differences in the frequencies of comorbidities among different groups of subjects, patients with ACPs had low prevalence of concomitant atopy (21.8%), allergic rhinitis (9.4%), or asthma (0%), similar to those with noneosinophilic CRSwNP, but unlike those with eosinophilic CRSwNP (**Table 1**).

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Table 1. Demographic of study subjects

Characteristic	Control (n = 35)	ACP (n = 32)	Non-Eos NP (n = 42)	Eos NP (n = 44)	Overall *P* value
Sex, male	20 (57.2)	19 (59.0)	28 (66.7)	28 (63.6)	0.834
Age (yr)	35.0 (25.1–46.0)[§]	19.0 (12.1–31.8)[†,‡]	32.0 (20.0–47.0)	42.0 (27.5–51.5)	< **0.001**
Patients with atopy	2 (5.7)[†]	7 (21.8)	10 (23.8)	16 (36.4)	**0.015**
Patients with AR	0 (0)[†]	3 (9.4)	6 (14.3)	9 (20.5)	**0.041**
Patients with asthma	0 (0)	0 (0)	3 (7.1)	5 (11.4)	0.061
Patients with aspirin	0 (0)	0 (0)	0 (0)	1 (2.3)	0.483
Smoker*	8 (22.8)	3 (9.4)	11 (26.2)	14 (31.8)	0.145

Values are presented as number (%) or median (interquartile range). Kruskal-Wallis *H* test were used to assess significant intergroup variability among the 4 groups, followed by Mann-Whitney *U2* test with Bonferroni correction for between group comparisons. The overall *P* values < 0.05 indicate that at least 1 of the 4 groups was different from other groups. The overall *P* values in bold are less than 0.05. For the comparison between 4 groups, the differences are considered statistically significant if *P* values < 0.008 after Bonferroni correction.
ACP, antrochoanal polyp; AR, allergic rhinitis; Eos NP, eosinophilic nasal polyp; Non-Eos NP, noneosinophilic nasal polyp.
*Smokers were defined as current cigarette smoker who consumed one or more packs of cigarettes a day, averaged over one year, in the last 12 months.
[†]Significant difference vs. Eos NP; [‡]Significant difference vs. Non-Eos NP; [§]Significant difference vs. ACP.

Compared to patients with bilateral eosinophilic and noneosinophilic CRSwNP, those with ACPs had significantly less impairment of smell (**Table 2**). Patients with ACPs had the lowest polyp scores due to the unilateral feature of ACPs (**Table 2**). CT scanning revealed that, in contrast to eosinophilic and noneosinophilic CRSwNPs, ACPs mainly involved the maxillary sinus, while other sinuses were rarely affected (**Table 2**).

ACPs display neutrophilic inflammation

We first evaluated inflammatory cell infiltration in ACPs. Similar to noneosinophilic NPs, ACPs had a lower number of ECP$^+$ eosinophils than those in eosinophilic NPs (**Fig. 1A**). Although MPO$^+$ neutrophils were increased in all types of polyp tissues compared to those in control tissues, ACPs had a higher number of neutrophils than those in bilateral eosinophilic and noneosinophilic NPs (**Fig. 1A**). The numbers of CD20$^+$ B cells and CD68$^+$ macrophages were

Table 2. Clinical characteristics of patients with different types of NPs

Characteristic	ACP (n = 32)	Non-Eos NP (n = 42)	Eos NP (n = 44)	Overall *P* value
Symptom VAS score				
Nasal obstruction	8.0 (7.0–10.0)[†]	5.0 (3.8–7.5)	8.0 (5.0–9.8)	**0.007**
Rhinorrhea	4.5 (2.0–7.0)	4.5 (2.0–6.0)	5.0 (1.3–8.0)	0.989
Facial pain	0 (0)	0 (0–4.0)	0 (0–3.5)	0.171
Loss of smell	0 (0–4.3)*,[†]	5.0 (1.8–8.3)*	9.0 (3.3–10.0)	< **0.001**
Total score	16.5 (10.0–20.0)*	14.5 (13.8–22.3)[†]	21 (18.3–28.8)	**0.007**
Endoscopic score				
Polyp	2.0 (1.5–3.0)*	3.0 (2.0–5.0)	4.0 (2.0–6.0)	**0.010**
Edema	2.0 (1.0–2.0)	2.0 (0–2.3)	2.0 (2.0–2.0)	0.804
Discharge	2.0 (0.5–2.0)	2.0 (0.8–2.0)	2.0 (2.0–2.0)	0.582
CT score				
Frontal sinuses	0 (0–1.0)*	1.0 (0–4.0)[†]	2.0 (0.25–4.0)	**0.002**
Anterior ethmoidal sinuses	0.5 (0–2.0)*,[†]	3.0 (2.0–4.0)	4.0 (2.0–4.0)	< **0.001**
Posterior ethmoidal sinuses	1.0 (0–2.0)*,[†]	2.0 (1.8–4.0)*	4.0 (2.0–4.0)	< **0.001**
Maxillary sinus	2.0 (0.3–2.0)*	2.5 (2.0–4.0)	2.5 (2.0–4.0)	**0.003**
Sphenoidal sinus	0 (0)*,[†]	0.5 (0–3.0)	2.0 (0–3.0)	**0.002**
OMC	0 (0–2.0)*,[†]	3.5 (2.0–4.0)	4.0 (2.5–4.0)	< **0.001**
Total CT score	4.0 (2.0–8.8)*,[†]	11.0 (7.8–21.0)	19.0 (0–20.8)	< **0.001**

For continuous variables, data are expressed by medians and interquartile ranges. Kruskal-Wallis *H* test were used to assess significant intergroup variability among the 3 groups, followed by Mann-Whitney *U2* test with Bonferroni correction for between group comparisons. The overall *P* values < 0.05 indicate that at least 1 of the 3 groups was different from other groups. The overall *P* values in bold are less than 0.05. For the comparison between 3 groups, the differences are considered statistically significant if *P* values < 0.017 after Bonferroni correction.
ACP, antrochoanal polyp; CT, computed tomography; Eos NP, eosinophilic nasal polyp; Non-Eos NP, noneosinophilic nasal polyp; NP, nasal polyp; OMC, ostiomeatal complex; VAS, visual analog scale.
*Significant difference vs. Eos NP; [†]Significant difference vs. Non-Eos NP.

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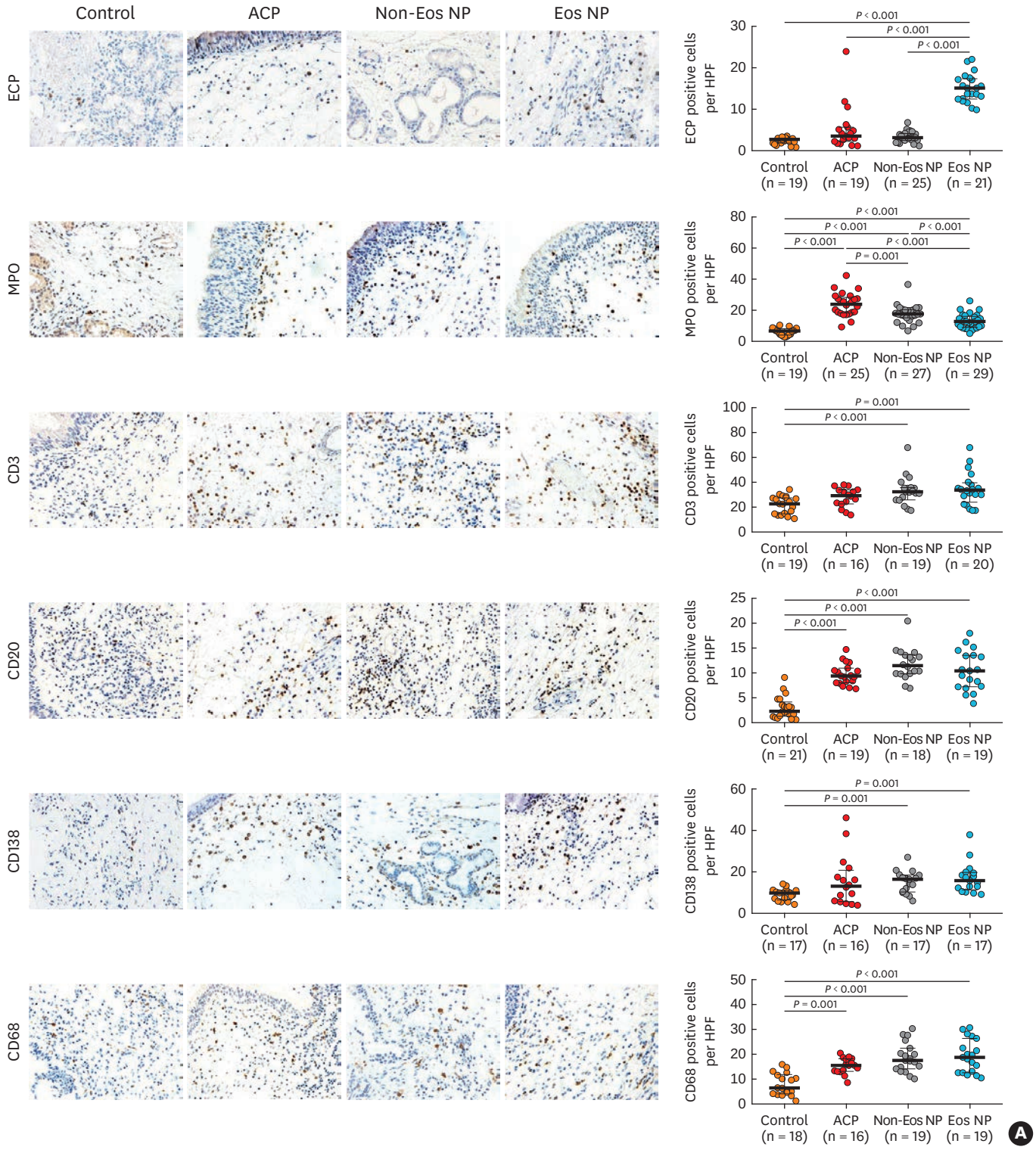

Fig. 1. Inflammatory cell infiltration in ACPs. (A) Representative immunostaining photomicrographs showing ECP, MPO, CD3, CD20, CD138, and CD68 positive cells and quantification of these cells in control nasal tissues and different types of polyp tissues (original magnification × 400). (B) The protein levels of ECP and MPO in tissue homogenates. (C) The percentages of the samples with eosinophilic (ECP/MPO ratio > 1) and neutrophilic (ECP/MPO ratio < 1) phenotypes.
ACP, antrochoanal polyp; ECP, eosinophilic cationic protein; Eos NP, eosinophilic nasal polyp; MPO, myeloperoxidase; Non-Eos NP, noneosinophilic nasal polyp.
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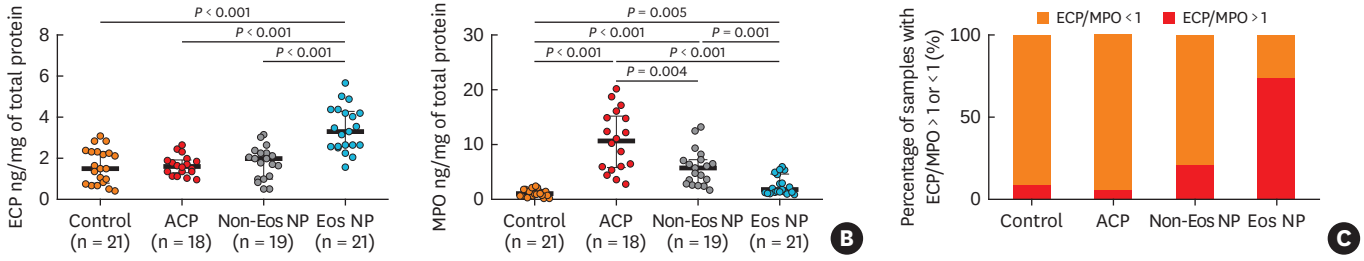

Fig. 1. (Continued) Inflammatory cell infiltration in ACPs. (A) Representative immunostaining photomicrographs showing ECP, MPO, CD3, CD20, CD138, and CD68 positive cells and quantification of these cells in control nasal tissues and different types of polyp tissues (original magnification × 400). (B) The protein levels of ECP and MPO in tissue homogenates. (C) The percentages of the samples with eosinophilic (ECP/MPO ratio > 1) and neutrophilic (ECP/MPO ratio < 1) phenotypes. ACP, antrochoanal polyp; ECP, eosinophilic cationic protein; Eos NP, eosinophilic nasal polyp; MPO, myeloperoxidase; Non-Eos NP, noneosinophilic nasal polyp.

increased in eosinophilic and noneosinophilic NPs as well as in ACPs compared to those in control tissues (**Fig. 1A**). Nevertheless, $CD138^+$ plasma cells and $CD3^+$ T cells were not significantly increased in ACPs as compared to those in control tissues (**Fig. 1A**). We consistently, found that ECP levels were elevated in eosinophilic NPs, but not in noneosinophilic NPs and ACPs, whereas ACPs had the highest levels of MPO (**Fig. 1B**). We further found that 94.4% of patients with ACPs demonstrated an ECP/MPO ratio of < 1 compared to 79.0% in patients with noneosinophilic NPs and 26% in patients with eosinophilic NPs (**Fig. 1C**).

ACPs demonstrate predominant type 1 response

Using the Bio-Plex suspension chip method, we measured the protein levels of 35 biomarkers in sinonasal tissues (**Supplementary Table S6**). Those having different expression in at least 1 of the 4 subject groups compared to other groups are shown in the heat map in **Fig. 2A**. The expression levels of selected biomarkers are shown in **Fig. 2B**. Consistent with previous reports,[38,39] eosinophilic NPs demonstrated higher levels of IL-5, IL-9, IL-13, and IgE than those in control tissues and other types of polyp tissues (**Fig. 2A and B**). We found that ACPs displayed the highest levels of IL-8 and IFN-γ among all types of nasal tissues (**Fig. 2A and B**).

We further classified ACPs and NPs into several inflammatory endotypes based on the tissue levels of T-cell–related cytokines. We found that T1, T2, and T3 endotypes accounted for 81.1%, 22.6% and 63.6% of ACPs, respectively, which was close to the feature in noneosinophilic NPs (58.5%, 20.6%, and 55.1% for T1, T2, and T3 endotype, respectively), but distinct from that in eosinophilic NPs (48.3%, 79.3%, and 41.3% for T1, T2, and T3 endotype, respectively) (**Fig. 2C**). To explore the similarity of inflammation endotype between ACPs and eosinophilic and noneosinophilic NPs, we performed MCA based on IFN-γ, IL-5, and IL-17A expressions. We found that the inflammation endotype of ACPs and noneosinophilic NPs were located near non-T2 and T1 and T3, whereas that of eosinophilic NPs was situated near T2 (**Fig. 2D**). We next conducted PCA to further characterize the endotypes of patients with different types of polyps based on the 17 biomarkers shown in **Fig. 1** together with ECP and MPO (**Fig. 2E**). Patients with ACPs were clearly segregated from patients with eosinophilic CRSwNP and controls, but largely overlapped with patients with noneosinophilic CRSwNP (**Fig. 2E**). These comprehensive data suggest a neutrophilic and T1 response-dominated endotype of ACPs.

Edema formation in ACPs

Edema is a key feature of tissue remodeling in NPs. Not surprisingly, a significant increase in edema scores was found in bilateral eosinophilic and noneosinophilic NPs and ACPs compared to those of control tissues (**Fig. 3A**). Eosinophilic NPs and ACPs demonstrated

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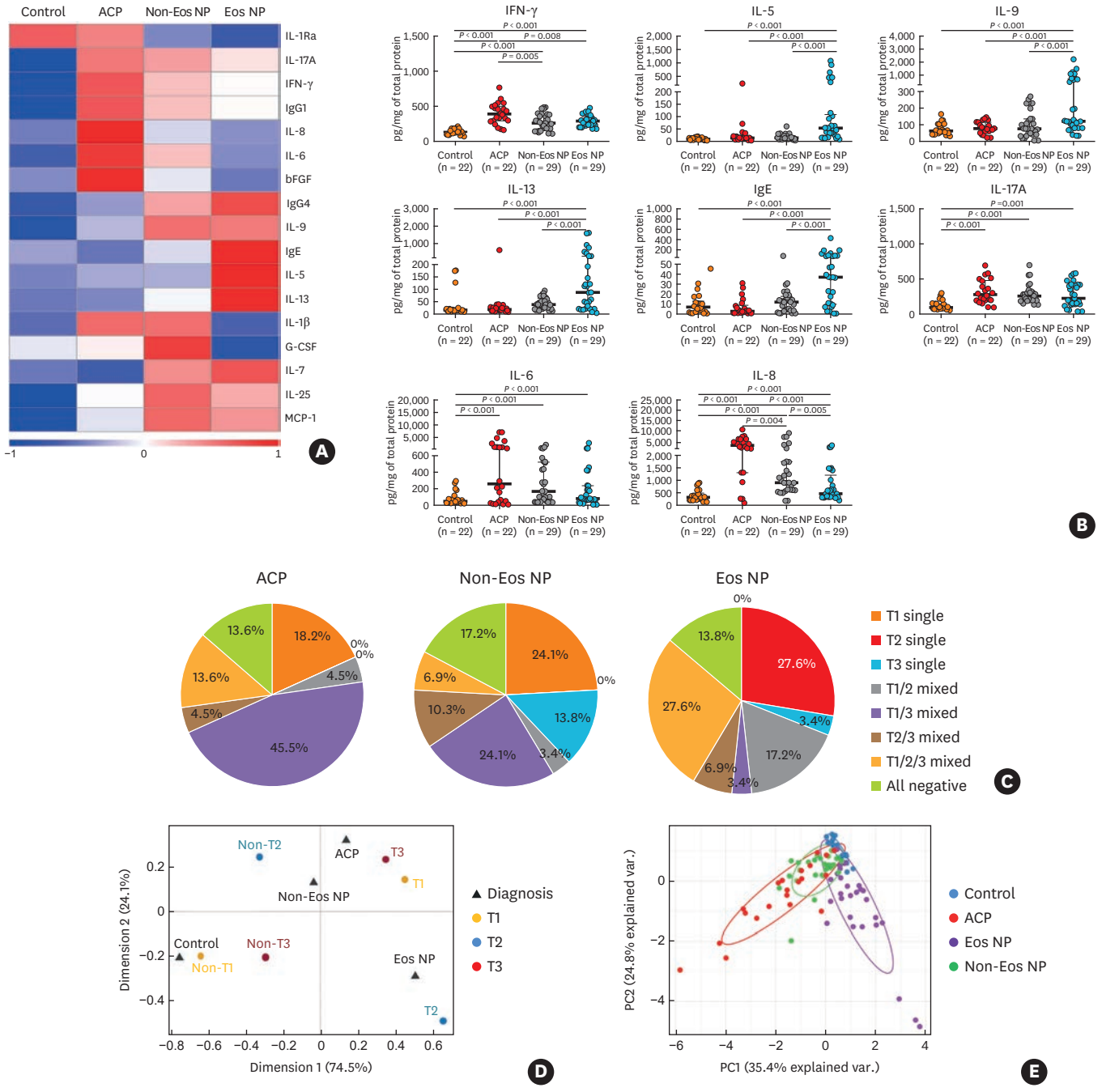

Fig. 2. Immunological endotype of ACPs. (A) Heat map showing the relative expression levels of inflammatory cytokines, chemokines, and Igs in tissue homogenates as detected by Bio-Plex assay, which have different expression in at least 1 of the 4 groups as compared to other groups. (B) The levels of selected inflammatory mediators in tissues in different groups. (C) Patterns of T1, T2, and T3 endotype in different types of NPs. (D) MCAs plot for the interrelationships between ACP, Eos NP, Non-Eos NP, control phenotype, and endotypes T1/T2/T3. (E) Principal component analysis based on inflammatory mediators indicated in heat map together with ECP and MPO.
ACP, antrochoanal polyp; bFGF, basic fibroblast growth factor; ECP, eosinophilic cationic protein; Eos NP, eosinophilic nasal polyp; G-CSF, granulocyte colony-stimulating factor; IFN-γ, interferon-γ; Ig, immunoglobulin; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; Non-Eos NP, noneosinophilic nasal polyp; MCA, Multiple correspondence analysis.

higher edema scores than those of noneosinophilic NPs (**Fig. 3A**). Although there was no statistical significance in edema scores between ACPs and eosinophilic NPs, 62.5% of ACPs had edema scores greater than 1, in contrast to 36.4% of eosinophilic NPs ($P = 0.036$).

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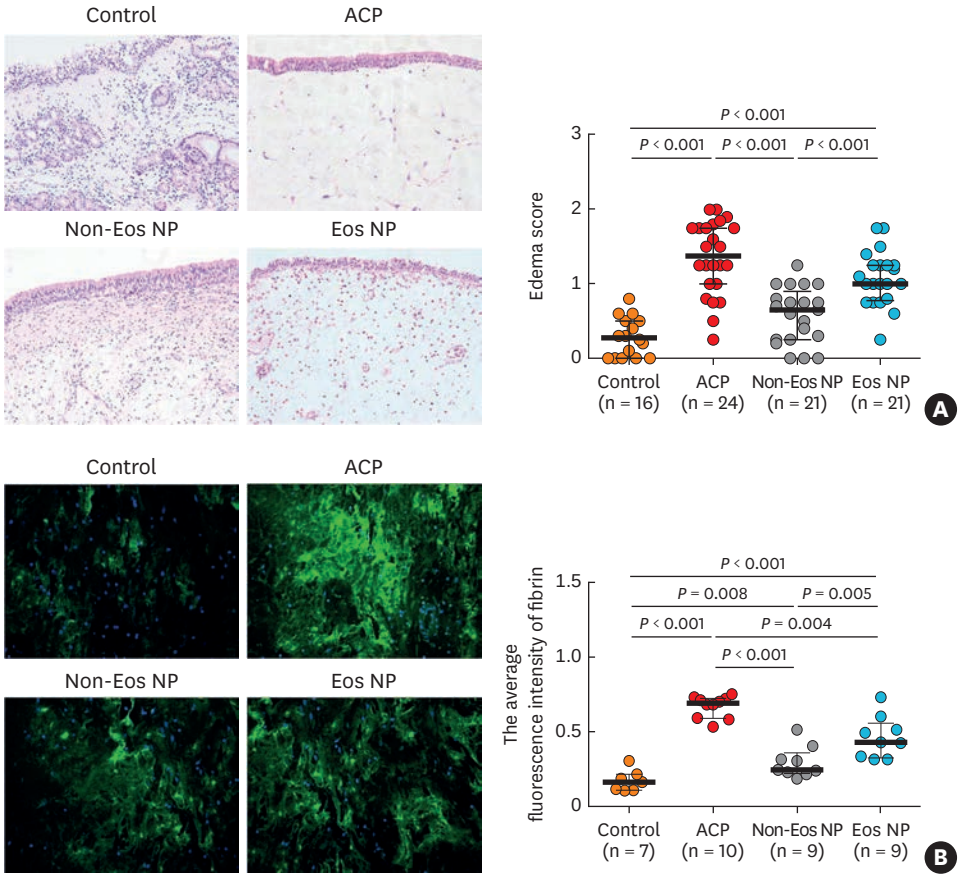

Fig. 3. Edema severity in ACPs. (A) Representative photomicrographs of hematoxylin and eosin staining (original magnification × 200) and quantitative evaluation for edema severity in control nasal tissues and different types of polyp tissues. (B) Representative immunostaining photomicrographs showing the fibrin deposition (original magnification × 400) and quantification of fibrin deposition as detected by immunofluorescence staining in control nasal tissues and different types of polyp tissues.
ACP, antrochoanal polyp; Eos NP, eosinophilic nasal polyp; Non-Eos NP, noneosinophilic nasal polyp.

Increased fibrin deposition is a critical step for retaining plasma proteins and facilitating edema formation in both eosinophilic and noneosinophilic NPs.[20] Immunofluorescence staining revealed significantly upregulated fibrin deposition in the lamina propria in all types of polyps compared with that in control tissues, and ACPs demonstrated notably more excessive fibrin deposition than that in eosinophilic and noneosinophilic NPs (**Fig. 3B**).

Impaired fibrin degradation in ACPs

Excessive fibrin deposition may result from overproduction or reduced degradation of fibrin in polyp tissues.[20] Thrombin-antithrombin (TAT) complex is an evanescent marker of thrombin activation and fibrin production.[22] Although both eosinophilic and noneosinophilic NPs and ACPs had markedly increased protein levels of TAT complex compared with those in the control tissues, there was no significance difference among ACPs, bilateral eosinophilic and noneosinophilic NPs (**Fig. 4A**). D-dimer is an important degradation product of fibrin.[40] A significant reduction in d-dimer levels was observed in ACPs compared with those in eosinophilic and noneosinophilic NPs and control tissues (**Fig. 4B**). These data indicate that the downregulation of fibrin degradation may contribute to the excessive deposition of fibrin in ACPs in comparison to bilateral eosinophilic and noneosinophilic NPs.

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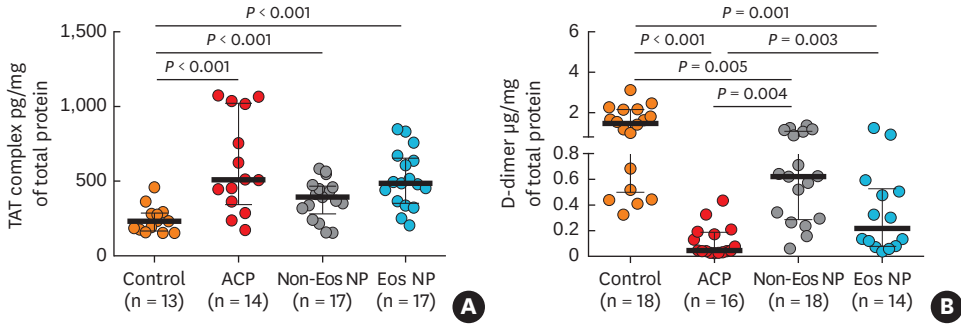

Fig. 4. Dysregulation of coagulation and fibrinolytic cascades in ACPs. The protein levels of TAT complex (A), and d-dimer (B) in control nasal tissues and different types of polyp tissues as detected by enzyme-linked immunosorbent assay.
ACP, antrochoanal polyp; Eos NP, eosinophilic nasal polyp; Non-Eos NP, noneosinophilic nasal polyp; TAT, thrombin-antithrombin.

Reduced production of tPA in ACPs

Fibrin degradation is facilitated by plasmin, which is generated from plasminogen under cleavage by urokinase plasminogen activator (uPA) and tPA.[41] We previously demonstrated that there was no change in uPA mRNA levels in eosinophilic and noneosinophilic NPs compared to those in control tissues.[18] Here, we found that there was no change of uPA mRNA levels in ACPs in comparison to those in control tissues (**Supplementary Fig. S1**). Consistent with our previous report,[18] we found that tPA production and activity were significantly impaired in both eosinophilic and noneosinophilic NPs compared to those in control tissues, with a more prominent decrease in eosinophilic NPs (**Fig. 5A-C**). We further found that the mRNA and protein levels and activity of tPA were even lower in ACPs than those in eosinophilic NPs (**Fig. 5A-C**). Immunohistochemistry demonstrated that tPA was mainly expressed in nasal epithelial cells in nasal tissues (**Fig. 5D**). The staining intensity of tPA in epithelial cells was reduced in ACPs compared to eosinophilic and noneosinophilic NPs, and control tissues (**Fig. 5E**). We consistently found that tPA mRNA levels were significantly downregulated in scraped nasal epithelial cells in patients with ACPs and eosinophilic and noneosinophilic NPs compared to those in control subjects, with the lowest levels found in nasal epithelial cells in patients with ACPs (**Fig. 5F**).

tPA levels are associated with neutrophilia and type 1 inflammation in ACPs

Previous studies have demonstrated that both type 2 (IL-4 and IL-13) and type 1 (IFN-γ) cytokines suppressed tPA production in nasal epithelial cells.[18,20] We found that tPA protein levels negatively correlated with the protein levels of IFN-γ, IL-6, IL-8, and MPO, but not those of IL-13 or ECP in ACPs (**Fig. 6**), suggesting a role for neutrophilia and type 1 inflammation, but not eosinophilia or type 2 inflammation, in the regulation of tPA production in ACPs. In addition, we found that tPA protein levels negatively correlated with IFN-γ, IL-6, IL-8 and MPO levels in noneosinophilic NPs, and IFN-γ, IL-6, IL-8, MPO, IL-13 and ECP levels in eosinophilic NPs (**Supplementary Fig. S2**), suggesting a role of both type 1 and type 2 inflammation in the regulation of tPA production in eosinophilic NPs.

Neutrophils are the main source of IFN-γ in ACPs

Next, we investigated the tissue-specific cellular source of IFN-γ in ACPs by immunofluorescence staining. Consistent with the IFN-γ protein levels in tissue homogenates, we found that the numbers of IFN-γ^+ cells were significantly increased in ACPs compared to those in eosinophilic and noneosinophilic NPs and control tissues (**Fig. 7A**). Double immunofluorescence staining

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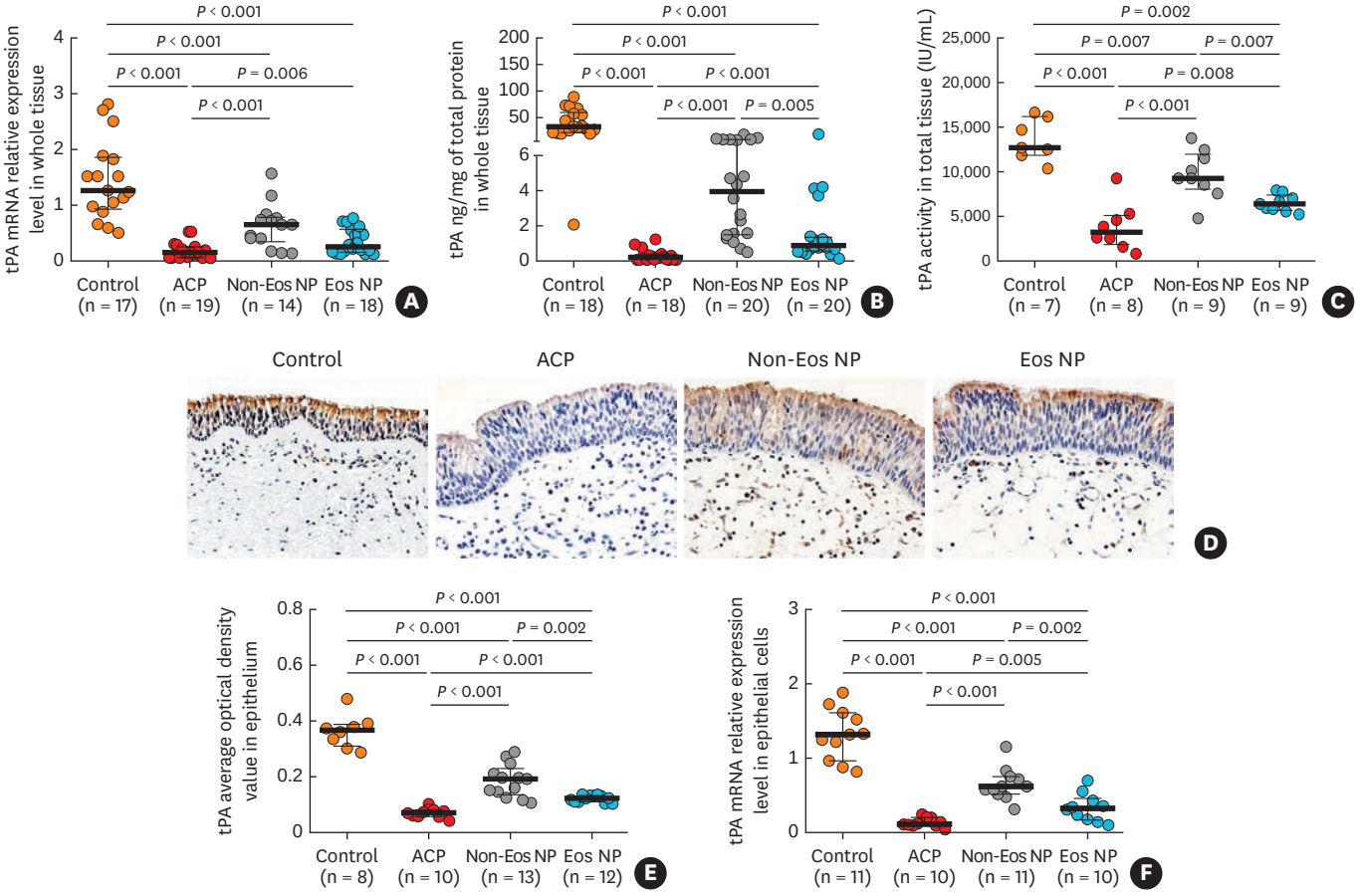

Fig. 5. Reduction of tPA production and activity in ACPs. (A) Quantitative analysis of tPA mRNA expression in control nasal tissues and different types of polyp tissues as detected by quantitative RT-PCR. (B) The protein levels of tPA in tissue homogenates detected by enzyme-linked immunosorbent assay. (C) The activity of tPA in tissue homogenates measured by using a tPA activity kit. (D) Representative immunostaining photomicrographs showing the immunoreactivity of tPA in control nasal tissues and different types of polyp tissues (original magnification × 400). (E) Quantification of staining intensity of tPA in epithelial cells. (F) The relative mRNA levels of tPA in scraped human nasal epithelial cells from the middle meatus of control subjects and different types of polyp tissues as detected by quantitative RT-PCR.
ACP, antrochoanal polyp; Eos NP, eosinophilic nasal polyp; Non-Eos NP, noneosinophilic nasal polyp; tPA, tissue plasminogen activator; RT-PCR, reverse-transcription polymerase chain reaction.

revealed that MPO^+ neutrophils and $CD3^+$ T cells were the principal cell types expressing IFN-γ in eosinophilic and noneosinophilic NPs and ACPs (**Fig. 7B and C**). IFN-γ^+ neutrophils accounted for 62.7% (mean) of the total IFN-γ^+ cells in ACPs (**Fig 7C**). In addition, the numbers of IFN-γ^+ neutrophils were significantly increased in ACPs compared to those in eosinophilic and noneosinophilic NPs and control tissues (**Fig. 7D**).

DISCUSSION

Although ACPs account for 4%–6% of all types of NPs and have a lower recurrence rate than bilateral NPs,[4-6,11,42] they mainly occur in children and the symptoms, such as nasal congestion, and affect children more significantly than adults. In addition, once ACPs relapse, we have a few treatment options besides repeated surgery. Considerable efforts have been made to understand the molecular and cellular bases of bilateral NPs.[43,44] However, little is known about the etiology and pathogenesis of unilateral ACPs. Here, we established several important clinical, histological, and immunological features of ACPs and provided novel

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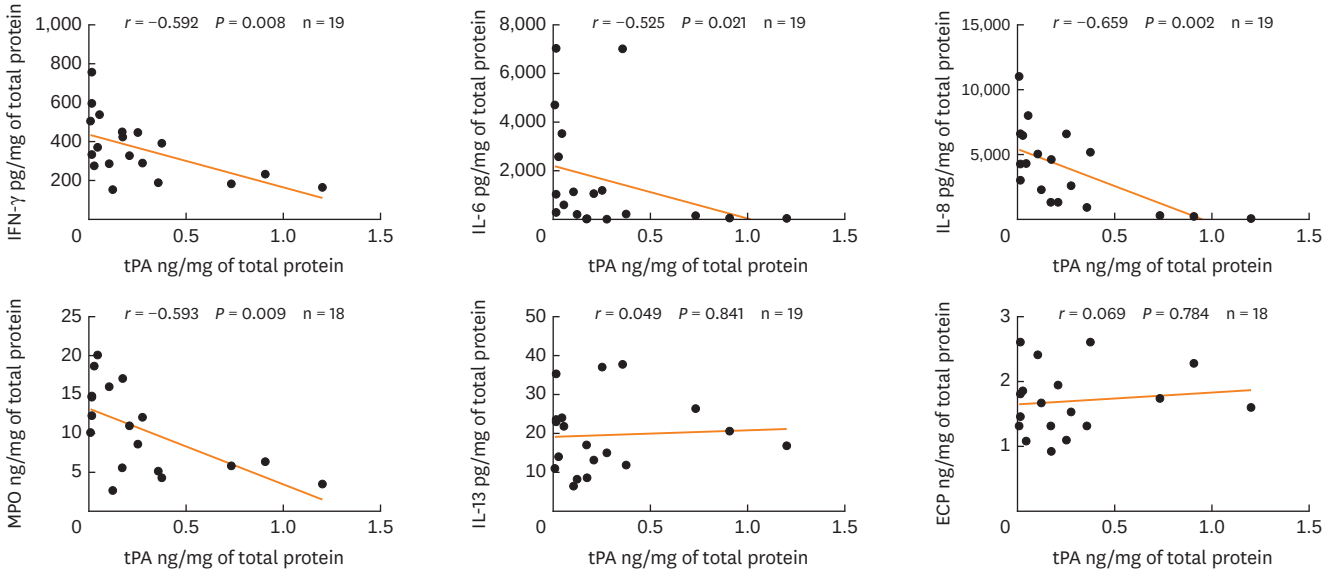

Fig. 6. Correlations of tPA levels with IFN-γ, IL-6, IL-8, MPO, IL-13, and ECP levels in ACPs.
ACP, antrochoanal polyp; ECP, eosinophilic cationic protein; IFN-γ, interferon-γ; IL, interleukin; MPO, myeloperoxidase; tPA, tissue plasminogen activator.

evidence for the involvement of neutrophilic and type 1 inflammation, and dysregulation of coagulation and fibrinolytic cascades in ACP pathogenesis.

ACPs occur most frequently in young individuals, with a male predominance.[6,7] Consistently in our study, 59.0% of patients with ACPs were male, and the median age of those patients were 19 years, being significantly younger than patients with eosinophilic and noneosinophilic bilateral NPs. We found that patients with ACPs presented with considerable nasal obstruction, but exhibited no or mild impairment of olfactory function, consistent with major involvement of the maxillary sinus but no other sinuses in those patients.

Previous studies have suggested the involvement of neutrophilic inflammation in ACPs.[6,7,16,45,46] Zheng *et al.*[16] reported increased infiltration of neutrophils and elevated IL-6 and IL-8 levels in ACPs. Jin *et al.*[7] observed that 87.9% of ACP tissues demonstrated neutrophilia. However, limited immune cell types and inflammatory cytokines have been investigated, and the inflammatory endotype of ACPs remains to be clarified. Among the inflammatory cells studied, we found that eosinophil and neutrophil infiltration demonstrated significant variations among different types of polyps. The numbers of eosinophils were significantly increased in eosinophilic NPs compared to those in noneosinophilic NPs and ACPs, which showed no difference from those in control tissues. In line with previous reports,[7,45] we found that the numbers of MPO^+ neutrophils in ACPs were higher than those in control tissues. Moreover, ACPs demonstrated increased neutrophil infiltration compared to that in eosinophilic and noneosinophilic NPs. These data suggested marked neutrophil-biased inflammation in ACPs, which was further supported by the highest MPO levels and the lowest ECP/MPO ratio in ACPs. A comprehensive evaluation of the inflammatory mediators in ACPs and NPs revealed the highest levels of IL-8 and IFN-γ in ACPs, but no change in IL-5, IL-9, IL-13, ECP, and IgE protein levels in ACPs compared to those in control tissues. Through MCA analysis, we found that ACPs were closer to T1 and T3, which was similar to noneosinophilic NPs, and the PCA also showed that ACPs and noneosinophilic NPs were considerable overlapped. However, in addition to higher numbers of neutrophils, ACPs demonstrated

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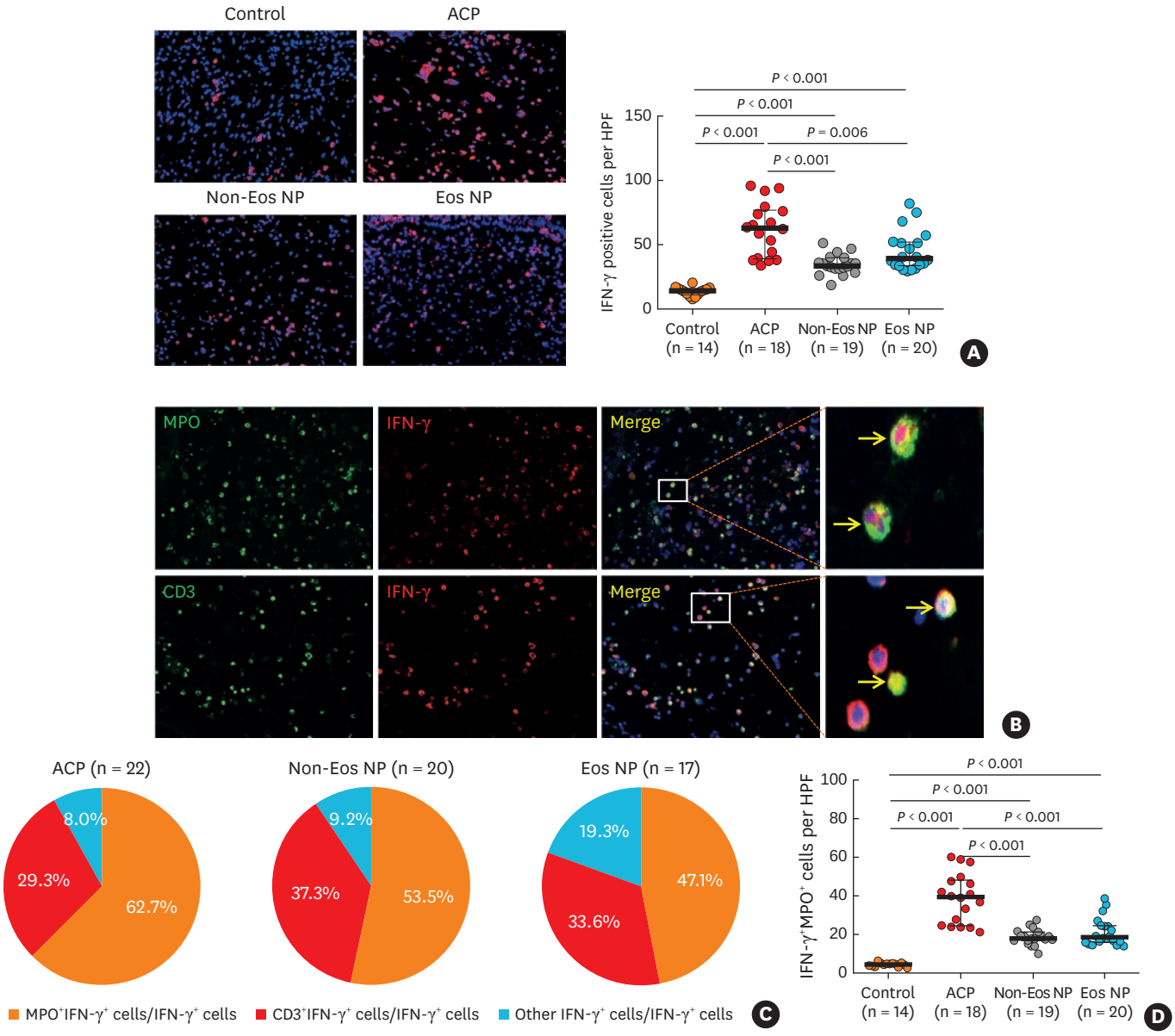

Fig. 7. Increased IFN-γ-positive neutrophils in ACPs. (A) Representative photomicrographs showing IFN-γ+ cells (original magnification × 400) and quantification of IFN-γ+ cells in control nasal tissues and different types of polyp tissues. (B) Representative photomicrographs showing IFN-γ+MPO+ neutrophils and IFN-γ+CD3+ T cells in ACPs tissues (original magnification × 400). (C) Mean percentages of MPO+ neutrophils and CD3+ T cells accounting for total IFN-γ+ cells in different types of polyp tissues. (D) Quantification of MPO+IFN-γ+ neutrophils in control nasal tissues and different types of polyp tissues.
ACP, antrochoanal polyp; Eos NP, eosinophilic nasal polyp; IFN-γ, interferon-γ; MPO, myeloperoxidase; Non-Eos NP, noneosinophilic nasal polyp.

more prominent T1 inflammation in comparison to noneosinophilic NPs. In addition, unlike noneosinophilic NPs, ACPs have no single T3 endotype. Collectively, using several molecular or cellular biology methods, we clearly revealed neutrophilic and T1 endotype of ACPs.

Tissue remodeling involved in NP development includes epithelial cell damage and regeneration, basement membrane thickening, fibrosis, and edema.[47] We found that ACPs were highly edematous in histology. Both eosinophilic NPs and ACPs demonstrated more severe edema than that in noneosinophilic NPs. Although there was no statistically significant difference in edema scores between ACPs and eosinophilic NPs possibly due to

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the limited sensitivity of the 3 scales of the edema score, we found that there were more ACPs with 1 < edema scores than eosinophilic NPs, suggesting that edema in ACP tissues was more prominent than that in eosinophilic NP tissues.

Dysregulation of the coagulation and fibrinolytic cascades has recently been implicated in edema development in bilateral NPs.[18-20,24,25] Fibrin, as the final product of the coagulation cascade, plays a major role in blood clotting. Recent studies have shown that excessive fibrin deposition causes eosinophilic NP tissue edema.[20] We recently showed that fibrin deposition was increased not only in eosinophilic NPs but also in noneosinophilic NPs; nevertheless, fibrin deposition was significantly increased in eosinophilic NPs compared to noneosinophilic NPs.[18] Here, we found for the first time that fibrin deposition was significantly increased in ACPs even compared to that in eosinophilic NPs. Excessive fibrin deposition may result from increased fibrin production or reduced fibrin degradation. We found that protein levels of the TAT complex, a marker reflecting fibrin production, were increased comparably in eosinophilic and noneosinophilic NPs and ACPs compared to those in control tissues, suggesting that the generation of fibrin is similar in several types of NPs. Subsequently, we assessed the degradation of fibrin in ACPs. D-dimer is an important degradation product of fibrin. We observed a significant reduction of d-dimer levels in ACPs compared to those in control tissues and eosinophilic and noneosinophilic NPs, suggesting that excessive deposition of fibrin in ACPs in comparison with bilateral NPs is largely caused by defective degradation. Fibrin degradation is facilitated by plasmin, which is generated through the cleavage of plasminogen by uPA and tPA.[41] In this study, we found that tPA, but not uPA, was significantly downregulated in ACPs, even compared to that those in eosinophilic NPs, which is in line with the fibrin deposition levels in different types of polyp tissues. We and others have found that both T1 (IFN-γ) and T2 (IL-4 and IL-13) cytokines downregulated tPA production in the nasal epithelial cells.[18,20] In this study, correlational analysis showed that T1 cytokines and neutrophil-related indicators, but not T2 cytokines, were negatively correlated with protein levels of tPA in ACPs. Furthermore, neutrophils have been revealed as the main source of IFN-γ in ACPs and the number of IFN-γ^+ neutrophils were increased in patients with ACPs compared to those with eosinophilic and noneosinophilic CRSwNP and controls. These results suggest that neutrophil-derived IFN-γ may contribute to tPA downregulation and edema formation in ACPs. However, this conclusion should be verified by further mechanistic studies.

A limitation of our study was the use of inferior turbinate mucosal samples as controls. Nevertheless, we did not identify obvious differences in tPA expression between the inferior turbinate mucosa and the normal ethmoid mucosal samples (**Supplementary Fig. S3**), and clear difference was observed between ACPs and bilateral eosinophilic or noneosinophilic NPs. ACPs sometimes occur in adults. It is interesting to explore whether there is any difference in immunopathological characteristics of ACPs between young and adult patients. However, due to the limited number of adult patients with ACPs in our study, we were unable to make this comparison, and further studies with a larger sample size are warranted. Unilateral NPs arising from ethmoid sinus or maxillary sinus without extending to choana more likely affect adults. This kind of unilateral NPs are also characterized by neutrophilic inflammation.[48] Including this kind of unilateral NPs as a control besides bilateral NPs would provide us more comprehensive view of inflammatory and immune features of different types of NPs and is also worth further investigations. In this study, we found that there were more prominent reductions in tPA in ACPs than in eosinophilic NPs. The underlying reason is currently unclear. However, it seems that there are additional mechanisms regulating tPA production in nasal epithelial cells. We found that, in NPs with no elevation of IFN-γ, IL-13, or

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IL-17A, there was also a reduced expression of tPA in comparison with control tissues.[18] Here, different endotypes were revealed for ACPs, and eosinophilic and noneosinophilic NPs. Why different inflammatory endotypes occur in similar edematous polypoid tissues? Whether they are related to anatomical structure, environmental factors (such as allergens, microorganisms and air pollutants), or genetic and epigenetic factors awaits future explorations.

In conclusion, ACPs demonstrate significant neutrophilic and type 1 inflammation. Neutrophil-derived IFN-γ is associated with reduced tPA production and edema formation in ACPs. These data extend our understanding of the mechanisms of ACPs and offer potential therapeutic options for ACPs by targeting neutrophilic and type 1 inflammation and tPA.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1
Primary antibodies used in immunohistochemistry and immunofluorescence

Click here to view

Supplementary Table S2
Secondary antibodies used immunofluorescence

Click here to view

Supplementary Table S3
Primers used for quantitative polymerase chain reaction analysis

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Supplementary Table S4
Detection limits for Bio-Plex suspension chip assay

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Supplementary Table S5
Detection limits for ELISA and UniCAP assay

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Supplementary Table S6
The tissue levels of inflammatory mediators detected by using the Bio-Plex assay

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Supplementary Fig. S1

The relative mRNA levels of uPA in scraped human nasal epithelial cells from the middle meatus of control subjects and different types of polyp tissues.

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Supplementary Fig. S2

Correlations of tPA levels with IFN-γ, IL-6, IL-8, MPO, IL-13 and ECP levels in Eos NPs (A) and Non-Eos NPs (B).

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Supplementary Fig. S3

The protein levels of tPA in tissue homogenates in normal UT and IT. Normal UT samples were obtained from subjects suffering from sinus cyst, nasal tumor or maxillofacial trauma and without rhinosinusitis or rhinitis (medians and interquartile ranges, 33.5 [24.3–46.0] years; 3 females and 3 males).

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