

Expression of T helper cytokines associated with MUC5AC secretion in eosinophil-based endotypes of nasal polyps

To the Editor,

Endotype classification based on eosinophil expression patterns allows accurate diagnosis and targeted treatment development for chronic rhinosinusitis with nasal polyps (CRSwNP) patients.¹ Meanwhile, eosinophilic inflammatory patterns play a key role in mucin hypersecretion in CRSwNP.² Studying the mechanism of abnormal mucin secretion is of great significance to understand pathogenesis and clinical treatment of eosinophil-based endotypes of nasal polyps.

As key regulators of mucin secretion, expression patterns of Th cytokines were diverse in eosinophil-based endotypes of nasal polyps composed of eosinophilic (Eos) and non-Eos CRSwNP. An enhanced Th2/Th17 inflammatory pattern was identified in adult Chinese Eos CRSwNP patients.³ Dong et al⁴ identified IL-5 as one of the signature inflammatory mediators in Eos CRSwNP and IL-17A and IL-1 β as the main inflammatory markers in non-Eos CRSwNP. Related mechanisms of mucin hypersecretion that might be involved in Eos CRSwNP are likely to also be involved in non-Eos CRSwNP, and this can be attributed to the complex and diverse nature of the inflammatory patterns. Which kinds of differentially expressed Th cytokines mediate mucin hypersecretion in the eosinophil-based endotypes of CRSwNP is still unclear and needs further study. Hence, our study aimed to evaluate the effects of the different Th cytokine expression patterns on MUC5AC hypersecretion in both Eos and non-Eos CRSwNP.

First, we investigated correlation between MUC5AC and main Th1/Th2/Th17 cytokines (IL-1 β , interferon (IFN)- γ , IL-4, IL-5, IL-13, IL-6, IL-17A) or their associated mediators (eosinophil cationic protein (ECP), myeloperoxidase (MPO), immunoglobulin E (IgE)) in CRSwNP. We measured the MUC5AC and Th cytokine expression levels in the tissue homogenates obtained from the control and CRSwNP groups and found that MUC5AC, IL-4, IL-5, IL-6, IL-13, IL-17A, ECP, MPO, and IgE expression levels were significantly increased in the CRSwNP group, compared to the control group. However, no significant differences in IL-1 β and IFN- γ expression in the two groups were observed (Table S1). Correlation analysis indicated that the IL-6 ($r = 0.472$, $P = .004$), IL-13 ($r = 0.565$, $P = .000$), IL-17A ($r = 0.346$, $P = .039$), and IL-1 β ($r = 0.342$, $P = .041$) expression levels were highly correlated with MUC5AC expression in CRSwNP (Figure 1A).

Next, we observed the expression of IL-6, IL-13, IL-17A, and IL-1 β in the control and Eos and non-Eos CRSwNP groups to clarify the

main eosinophil-based endotype of CRSwNP in which these cytokines were distributed to regulate MUC5AC expression. We found that MUC5AC level was elevated in the non-Eos and Eos CRSwNP groups, compared with the control group, but no difference in the MUC5AC levels in the two CRSwNP groups was observed. IL-13 was significantly increased in the Eos CRSwNP group, but not in the non-Eos CRSwNP group, compared with the control group, while IL-17A expression was up-regulated in the non-Eos CRSwNP group, but not in the Eos CRSwNP group, compared with the control group. IL-6 expression, unlike IL-1 β expression, was elevated in both the CRSwNP groups (Figure 1B).

To verify the direct effects of IL-13, IL-17A, and IL-6 on MUC5AC expression, we observed the co-localization of MUC5AC and the corresponding cytokine receptors, including type 2 IL-4R (IL-4R α and IL-13R α 1), IL-17AR, and IL-6R, by staining the serial sections. We found that although IL-4R α , IL-13R α 1, IL-17AR, and IL-6R were widely expressed in the epithelium and subepithelium, they were all expressed in locations similar to those of MUC5AC-positive cells in the epithelium obtained from the control and CRSwNP groups (Figure 2A-D).

Our recent study showed that high IL-13 and IL-4 expression levels were responsible for MUC5AC and MUC5B secretion in Caucasian IL-5-positive CRSwNP patients.⁵ The effects of mixed Th inflammatory patterns on mucin secretion in Chinese CRSwNP patients, different from those in Western CRSwNP patients, are lesser known. In the current study, correlation analysis showed that IL-6, IL-13, IL-17A, and IL-1 β were closely associated with MUC5AC expression in Chinese CRSwNP patients. These cytokines represented specific inflammatory patterns that up-regulated MUC5AC expression in CRSwNP together. Previous studies, including ours, verified that IL-6,⁶ IL-13,⁵ IL-17A,⁷ and IL-1 β ⁸ could individually stimulate human nasal epithelial cells from tissue of patients with CRSwNP to induce MUC5AC expression in vitro.

To identify which Th cytokines, either alone or in combination, regulated MUC5AC secretion in the different eosinophil-based endotypes of CRSwNP, we analyzed the IL-6, IL-13, IL-17A, and IL-1 β levels in Eos and non-Eos CRSwNP. Elevated IL-13 and IL-17A levels observed in Eos and non-Eos CRSwNP, respectively, were mainly responsible for MUC5AC secretion. Elevated IL-6 levels were observed in both Eos and non-Eos CRSwNP groups, which indicated that IL-6 played a regulatory role in MUC5AC secretion in both groups. No

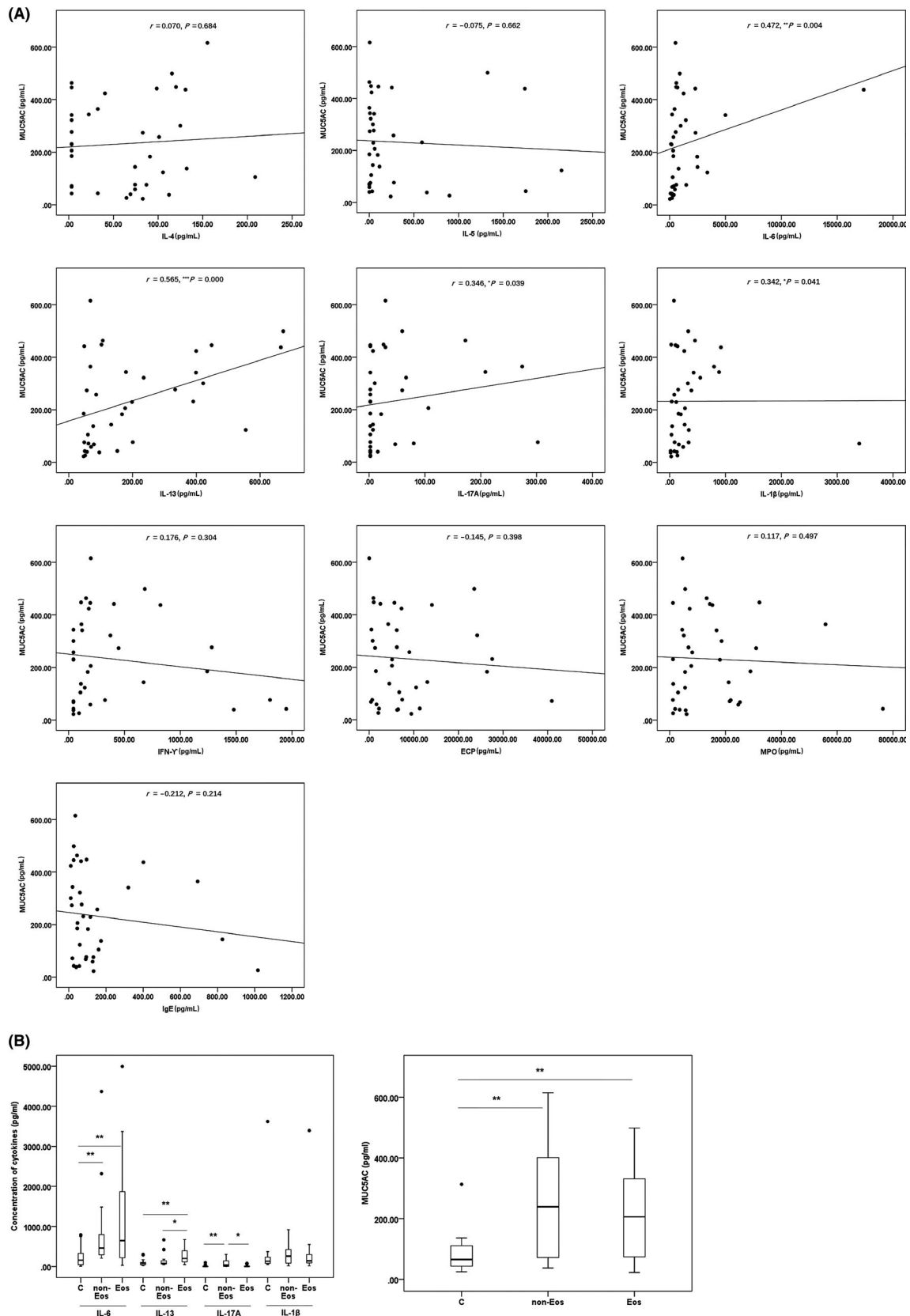


FIGURE 1 (A) Correlation analysis between MUC5AC and interleukin (IL)-4, IL-5, IL-6, IL-13, IL-17A, IL-1 β , interferon (IFN)- γ , eosinophil cationic protein (ECP), myeloperoxidase (MPO), and immunoglobulin E (IgE) in 36 patients with chronic rhinosinusitis with nasal polyps (CRSwNP). (B) IL-6, IL-13, IL-17A, IL-1 β , and MUC5AC concentrations in controls ($n = 20$) and patients with non-eosinophilic ($n = 16$) and eosinophilic ($n = 20$) CRSwNP r value represents the Spearman correlation coefficient. C, control group; non-Eos, non-eosinophilic CRSwNP group; Eos, eosinophilic CRSwNP group; and $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

differences in the IL-1 β levels in the two endotypes of CRSwNP, compared with the control group, were observed. We assumed that IL-1 β , unlike the other pro-inflammatory factors, possibly only maintained baseline mucin secretion and did not promote abnormal mucin hypersecretion in CRSwNP. The consistency of expression

sites between IL-6R, IL-4R α , IL-13R α 1, IL-17AR and MUC5AC in the nasal mucosa epithelium further indicated that IL-6, IL-13, and IL-17A could induce MUC5AC secretion directly in CRSwNP.

In our study, we for the first time revealed the relationship between Th cytokine expression patterns and MUC5AC in Eos and

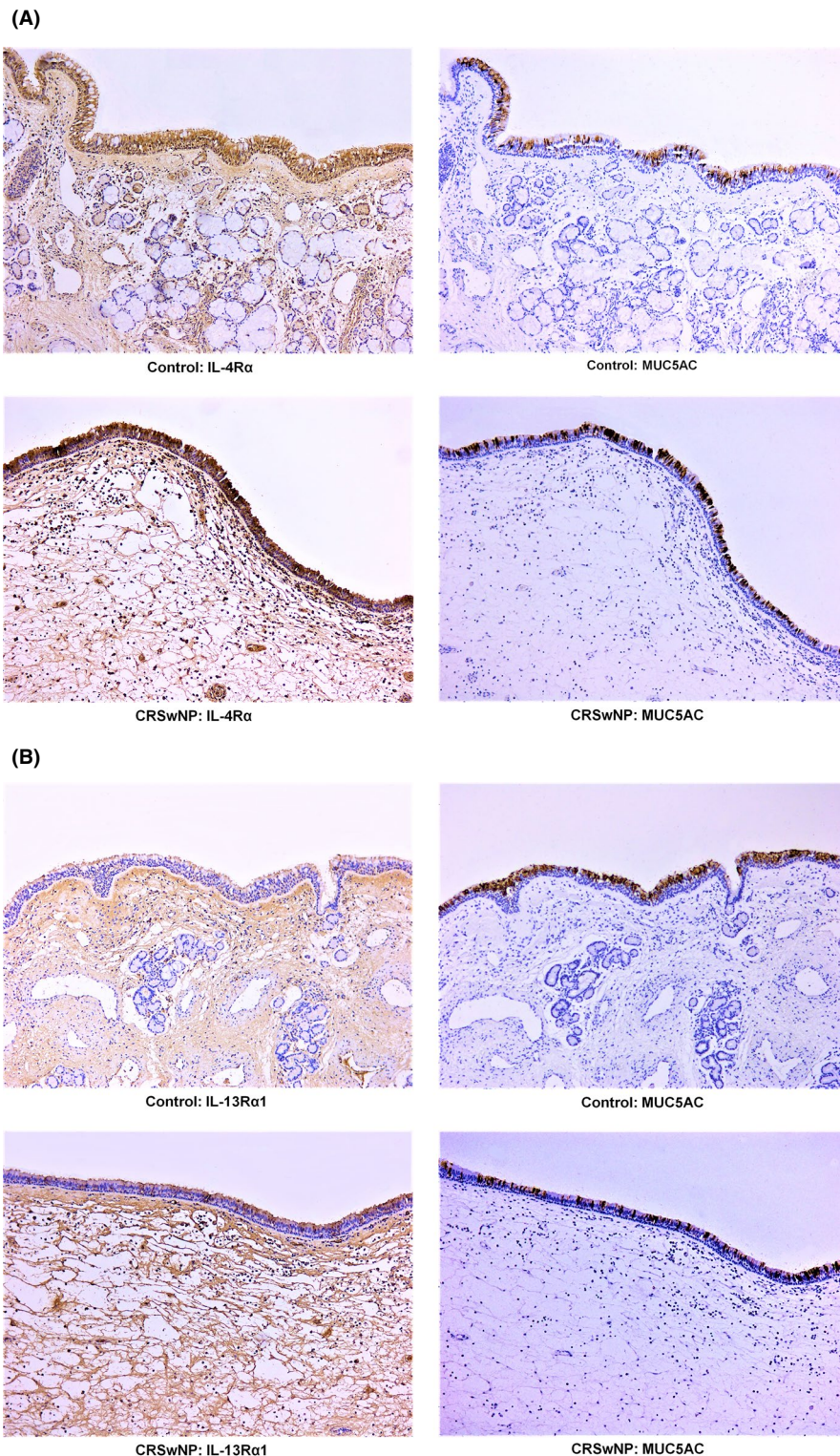
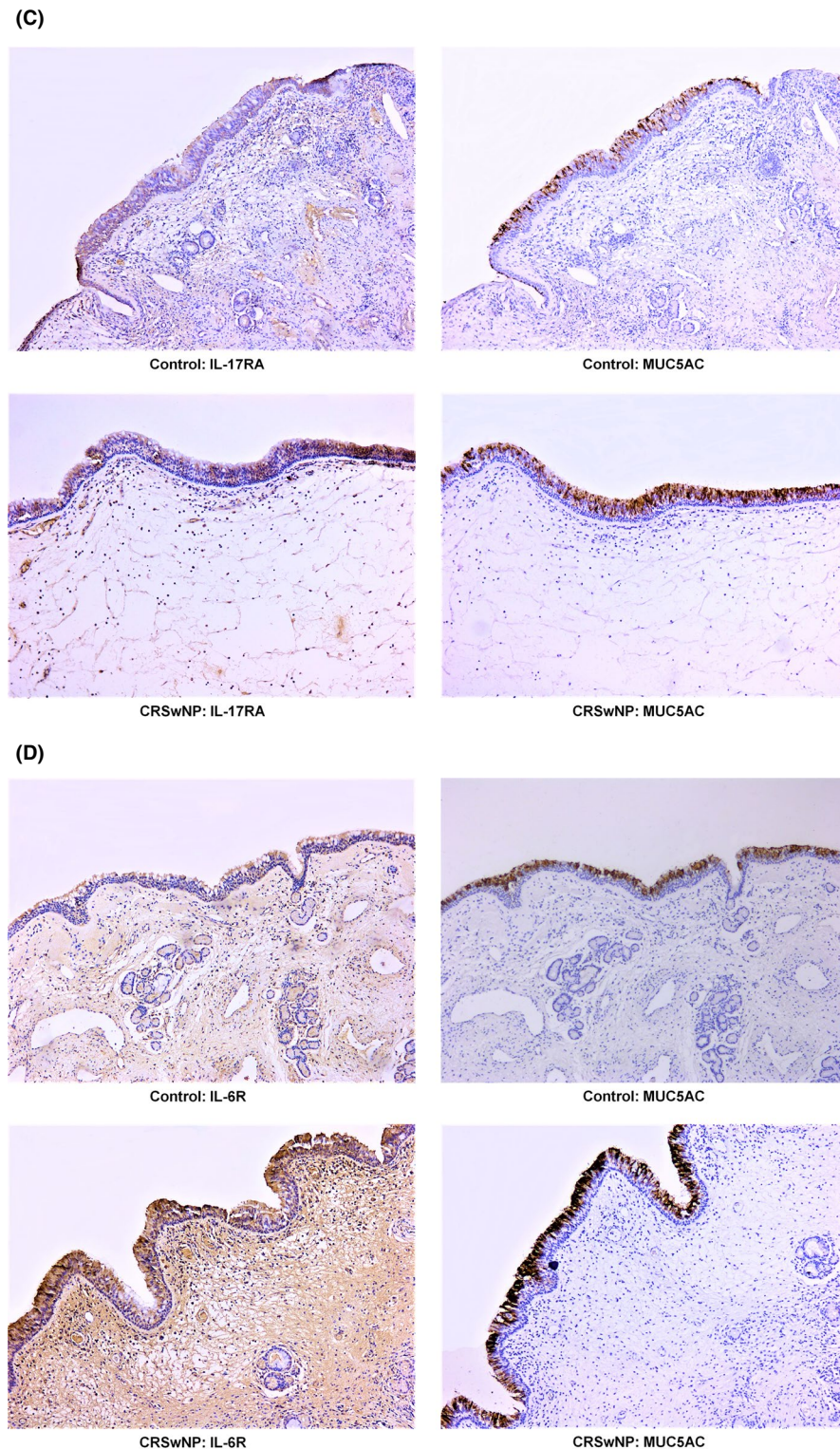


FIGURE 2 Co-localization of interleukin (IL)-4R α and MUC5AC (A), IL-13R α 1 and MUC5AC (B), IL-17AR and MUC5AC (C), IL-6R and MUC5AC (D) in the epithelium of normal nasal mucosa and nasal polyp observed by performing immunohistochemical staining of the serial tissue sections. The images were observed with a 10 $\times$ objective. CRSwNP, chronic rhinosinusitis with nasal polyp

**FIGURE 2** (Continued)

non-Eos CRSwNP and found that mixed Th cytokine expression patterns up-regulated MUC5AC expression in CRSwNP. IL-13 and IL-17A might play vital roles in Eos and non-Eos CRSwNP, respectively, while IL-6 might play an important role in both endotypes of CRSwNP. One limitation of our study was that the CRSwNP sample size in our study was not large enough to reflect the different Th

inflammatory patterns precisely. However, we found that diverse Th cytokines expressed in either Eos or non-Eos CRSwNP jointly played an important role in mucin secretion.

Excessive mucus obstruction and ciliary dysfunction triggered by abnormal mucin hypersecretion provide an environment for microbial colonization and aggravates nasal mucosa inflammation.

Identifying different intrinsic mechanisms of mucin secretion in Eos and non-Eos CRSwNP might contribute to individualized treatment. Meanwhile, with the discovery of more Th cell subsets, inflammatory patterns of Th cytokines associated with mucin secretion in eosinophil-based endotypes of nasal polyps need to be further explored.

KEYWORDS

chronic rhinosinusitis, endotype, MUC5AC, nasal polyp, Th cytokines

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Patients

In total 56 participants, including 20 controls and 16 non-Eos and 20 Eos CRSwNP patients, were enrolled in this study. General information of the patients is shown in Table S2. CRS was diagnosed based on medical history, relevant physical examination, nasal endoscopy, and computed tomography of the paranasal cavities, according to the European EPOS 2012 guidelines^[S1]. Eos CRSwNP was defined by a value of ≥ 10 eosinophils per high power field, as suggested by Solar^[S2]. Asthma was diagnosed based on the Global Initiative for Asthma guidelines^[S3]. Skin prick test for common airborne allergens was performed in all patients. We excluded those patients who had been treated with antibiotics and oral or intranasal glucocorticoids 2 weeks and 4 weeks before surgery, respectively. All study subjects provided written informed consent for participating in this study, and the study protocol was approved by the Ethics Committee of Beijing Tongren Hospital.

Measurement of MUC5AC, cytokine, and immunoglobulin E (IgE) levels in the tissue homogenates

We obtained nasal tissue homogenates following a previously described method^[S4] and detected the MUC5AC, IL-4, IL-5, IL-6, IL-13, IL-17A, IL-1 β , interferon (IFN)- γ , and myeloperoxidase (MPO) levels using ELISA kits (R&D Systems, Inc., Minneapolis, USA; MPO, OXIS International, Inc., Portland, Oregon, USA; MUC5AC, USCN Life Science, Wuhan, China; IL-13, Bio-Rad Laboratories, Inc., Philadelphia, USA). The UniCAP system (Pharmacia, Uppsala, Sweden) was used to detect IgE and eosinophil cationic protein (ECP).

Immunohistochemistry

Tissues from the normal inferior turbinate mucosa and nasal polyps were collected, fixed in 10% formalin, paraffin embedded, and sectioned. Serial sections were used to observe the co-localization of the cytokine receptors and MUC5AC. Sections were incubated with 3% hydrogen peroxide, rinsed in phosphate-buffered solution (PBS)

after autoclave pretreatment, and blocked with 5% skimmed milk. They were then incubated with anti-IL-4R α (R&D systems, Minneapolis, USA), anti-IL-13R α 1 (R&D systems, Minneapolis, USA), anti-IL-17AR (R&D systems, Minneapolis, USA), anti-IL-6R (Abcam, Cambridge, USA), or anti-MUC5AC (Abcam, Cambridge, USA) at 4°C overnight. The sections were then incubated with the secondary antibody at 37°C for 40 min. The stained slides were photographed using an Olympus BX51 microscope (Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using the IBM SPSS 22.0 statistical software (New York, USA). Continuous variables were compared using the two-tailed Student's t-test, while categorical variables were analyzed using Fisher's exact test or the chi-square test. Cytokine and MUC5AC levels in the control and CRSwNP groups were compared using the Mann-Whitney U-test. Data of the control and Eos and non-Eos CRSwNP groups were compared using the Kruskal-Wallis test. Spearman correlation coefficient (r) was used to represent the correlation between MUC5AC and Th cytokines. P-values <0.05 were considered statistically significant.

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SUPPLEMENTARY TABLES

Table S1. Th cytokine, ECP, MPO, IgE, and MUC5AC levels in the nasal tissues obtained from the controls and CRSwNP patients

Mediators/Mucin	Control (pg/mL) (n=20)	CRSwNP (pg/mL) (n=36)	P-value
IL-4	3.1 (3.1-3.1)	71.6 (3.1-104.4)**	0.001
IL-5	1.2 (1.2-1.2)	42.6 (5.5-266.6)***	0.000
IL-6	156.4 (40.8-344.2)	533.6 (236.9-1399.5)***	0.000
IL-13	77.0 (45.3-102.3)	142.5 (63.0-344.7)*	0.011
IL-17A	2.2 (2.2-10.3)	6.9 (2.2-56.2)*	0.035
IL-1 β	130.0 (70.5-242.2)	162.1 (83.0-342.1)	0.347
IFN- γ	88.6 (42.9-184.6)	163.3 (42.9-436.9)	0.148
ECP	156.5 (86.3-269.5)	5918.0 (1537.8-10246.5)***	0.000
MPO	2406.8 (1319.2-3098.2)	7445.4 (4523.8-21388.7)***	0.000
IgE	20.9 (10.4-51.6)	67.4 (35.8-132.8)***	0.000
MUC5AC	65.4 (43.8-116.1)	217.8 (72.9-359.1)**	0.001

Data are expressed as median and interquartile range. *P < 0.05, **P < 0.01, ***P < 0.001

Th, T helper; ECP, eosinophil cationic protein; MPO, myeloperoxidase; IgE, immunoglobulin E; CRSwNP, chronic rhinosinusitis with nasal polyps; IL, interleukin; IFN, interferon

Table S2. Patient characteristics

	Control (n=20)	Non-Eos CRSwNP (n=16)	Eos CRSwNP (n=20)	Control versus non-Eos CRSwNP (P-value)	Control versus Eos CRSwNP (P-value)	Non-Eos CRSwNP versus Eos CRSwNP (P-value)
Age (years), Mean±SD	40.4±13.9	40.8±15.3	45.8±12.1	0.933	0.201	0.287
Male, n (%)	12 (60.0%)	14 (87.5%)	17 (85.0%)	0.067	0.077	0.829
Smoking, n (%)	6 (30.0%)	5 (31.3%)	13 (65.0%)	0.936	0.027	0.044
Positive skin prick test, n (%)	1 (5.0%)	7 (43.8%)	8 (40%)	0.012	0.020	0.821
Asthma, n (%)	0	4 (25%)	4 (20%)	0.031	0.106	0.720

Chi-square test, Fisher's exact test, and Student's t-test were used for the control versus non-Eos CRSwNP, control versus Eos CRSwNP, and non-Eos CRSwNP versus Eos CRSwNP comparisons. Non-Eos CRSwNP, non-eosinophilic chronic rhinosinusitis with nasal polyps; Eos CRSwNP, eosinophilic chronic rhinosinusitis with nasal polyps; SD, standard deviation