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Local IL-25 contributes to Th2-biased inflammatory profiles in nasal polyps

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Short title: Local IL-25 contributes to Th2-biased inflammatory profiles in NP

Summary

Background IL-25 has been proposed to play a key role in the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP). This study aims to evaluate the association of IL-25 with the Th2-biased inflammatory profiles in CRSwNP.

Methods Nasal polyp (NP) tissues and control uncinata process tissues were collected from 92 patients with CRSwNP, 20 patients with chronic rhinosinusitis without nasal polyps (CRSsNP), and 16 normal control subjects. IL-25 expression was examined using immunohistochemistry and immunofluorescence staining, flow cytometry, RT-qPCR, and ELISA. The inflammatory profiles and clinical characteristics of 2 NP subtypes (IL-25^{high} and IL-25^{low}) were evaluated, and the effects of IL-25 on Th2 cytokine production in cultured dispersed polyp cells were examined *in vitro*.

Results The mRNA and protein levels of IL-25 were significantly increased in the polyp tissues compared with the control uncinata process tissues. The IL-25^{high} subtype showed greater computed tomography scores, endoscopic scores, and Th2 response. Exposure to IL-25 activated type 2 innate lymphoid cells and Th2 cells in NP simultaneously which further increased Th2 cytokine production *in vitro*.

Conclusions Local IL-25 plays a crucial role in promoting Th2-biased inflammatory profiles in NP, and may serve as a promising therapeutic target in CRSwNP patients.

Key words

chronic rhinosinusitis; interleukin 25; nasal polyps; Th2 response

Abbreviations

CRS, chronic rhinosinusitis; CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps; CT, computed tomography; DPCs, dispersed polyp cells; ELISA, enzyme-linked immunosorbent assay; EPCAM, epithelial cell adhesion molecule; IHC, immunohistochemistry; ILC2, type 2 innate lymphoid cells; Th2, type 2 T-helper cell.

Introduction

Chronic rhinosinusitis (CRS) is one of the most common diseases in the upper respiratory tract, with an estimated prevalence of 8% in China, affecting more than 100 million people (1, 2). It is defined as an inflammatory condition of the paranasal sinuses lasting at least 12 weeks in duration. According to endoscopic examination of visible nasal polyp (NP) in the nasal meatus, CRS can be subdivided into CRS with NP (CRSwNP) and CRS without NP (CRSsNP) (1). CRSwNP is usually characterized by type 2 T-helper (Th2) response and tissue eosinophilia, in contrast to CRSsNP that shows Th1 cell predominant response (3). Although emerging evidence identified numerous cytokines and chemokines involved in the interaction of host and environmental factors during polypogenesis, the key pathogenic mechanisms are not completely elucidated (4).

CRSwNP is a heterogeneous disease that can be further subclassified into distinct phenotypes based on pathogenic and molecular features (5). The tissue eosinophilia is likely to promote NP formation; however, the extent of eosinophilia in polyp tissues can be quite variable. Recent studies show that a subset of CRSwNP patients are presented with less tissue eosinophilia or non-eosinophilia in East Asian regions (e.g. China and Japan) and have distinct disease characteristics and corticosteroid sensitivity (6-8). The immunological phenotypes (or endotypes) of CRSwNP can be based on the expression of cytokines or infiltration of inflammatory cells in polyp tissues (9, 10).

Among them, a major endotype is the Th2-biased, eosinophilic inflammation (10), where the Th2 cytokines (e.g. IL-4, IL-5 and IL-13) produced by airway Th2 cells and type 2 innate lymphoid cells (ILC2) promote eosinophilic inflammation and tissue remodeling (5, 11). However, the key factors orchestrating Th2 response have not been fully understood.

Sinonasal epithelium plays crucial roles in the host defense besides constituting the physical barrier (9, 12). Airway epithelial cells are now found to license innate and adaptive immunity by producing a series of pro-inflammatory cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 that orchestrate the type 2 responses at the mucosa site (13). IL-25 (also known as IL-17E) is a member of the IL-17 cytokine family and plays a variety of roles in different inflammatory murine models, such as asthma, atopic dermatitis, and pulmonary fibrosis (14). Intraperitoneal or intranasal administration of IL-25 protein results in the accumulation of eosinophils or production of Th2 cytokines in the bronchoalveolar lavage fluid and the lung tissues (15, 16). Conversely, blocking IL-25 decreases Th2 cytokine production in an animal model of asthma (17). In a recent study, Shin et al. (18) demonstrated that the anti-IL-25 treatment reduced the number of polyps, mucosal edema, collagen deposition, and infiltration of inflammatory cells, such as eosinophils and neutrophils in a murine model of NP. These studies suggest that IL-25 may be a therapeutic target for the treatment of CRSwNP. This study thus aims to characterize the inflammatory profiles of IL-25^{high} and IL-25^{low} subtypes of the polyp tissues, and to evaluate the association of IL-25 with Th2-biased inflammatory profiles.

Materials and methods

This study was approved by the Ethical Committee of the first affiliated hospital of Sun Yat-sen University. An informed consent was obtained from each subject.

Patients and tissue samples

Ninety-two CRSwNP patients and 20 CRSsNP patients were recruited from the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). The diagnosis of CRSwNP was carried out

based on the disease history, nasal endoscopy, and computed tomography scan of the paranasal cavities, according to the current European position paper on rhinosinusitis and nasal polyps (EPOS 2012) (1). NP size on both sides was recorded by nasal endoscopy (using the Lund-Kennedy score system). The visual analogue scale (VAS) was used to evaluate the 4 main symptoms of CRS, wherein 0 cm represented “no complaints whatsoever” and 10 cm represented “the worst imaginable complaints”. Concurrently, the total nasal symptom score (TNSS) was calculated as the sum of scores for the 4 main individual symptoms (1, 2). The atopic status was evaluated by skin prick test and the levels of serum IgE (Phadia, Uppsala, Sweden) specific to common inhalant allergens (e.g. pollens, house dust mites, pets, molds, and cockroaches). None of the patients included used oral or nasal steroids or other immunomodulatory drugs within 4 weeks prior to surgery. As normal controls, 16 patients with traumatic optic neuropathy were enrolled, and the uncinate process tissues were sampled during endoscopic optic nerve decompression. Blood samples were collected before surgery, and the peripheral blood eosinophil counts were performed using an automated analyzer. More information on the sinonasal tissues sampling during endoscopic surgery can be found in the online supplement.

Immunohistochemistry (IHC) and Immunofluorescence (IF) staining

Sinonasal tissues embedded in paraffin were cut into 5- μ m sections and placed onto glass slides. 2×10^4 cells were prepared for per cytopsin slide at 500 rpm for 5 min with mild acceleration according to the manufacturer’s instructions (ThermoScientific, Waltham, MA). Hematoxylin-eosin (HE) staining was routinely performed for histological examination, and tissue eosinophilia and basement membrane thickness were evaluated as well by referring to the study reported by Bassiouni et al. (19). IHC staining was performed using peroxidase-labeled streptavidin-biotin technique as previously described (20), and IF staining was performed as previously described (20). More information on IHC and IF staining can be found in the online supplement.

Real time quantitative PCR (RT-qPCR)

The mRNA expression levels of target genes were detected by RT-qPCR analysis as previously described (21). The relative genes expression was analyzed using the $2^{-\Delta\Delta C_t}$ method. More information can be found in online supplement.

Luminex-based multiplex assay

Protein extracted from polyp and control uncinata process tissues was analyzed by Luminex-based multiplex assay (Millipore) according to manufacturer's protocol (details can be found in online supplement).

Dispersed polyp cells (DPCs) isolation and culture

The isolation of DPCs was performed as previously described (22), and can be found in online supplement. DPCs were cultured on a 12-well plate for 5 days in the presence of IL-25 (50 ng/mL, R&D systems) and IL-2 (10 ng/mL, R&D systems) with or without IL-25 blocking antibody (10 μ g/mL, R&D systems, Catalog no. AF1258), or dexamethasone (1 μ g/mL, Sigma-Aldrich). After 5 days, cells and supernatants were harvested for flow cytometry and ELISA respectively.

Flow cytometric analysis

For flow cytometry, living DPCs of 10^6 or more were suspended in 400 μ L PBS with 1% bovine serum albumin to block unspecific staining according to the manufacturer's protocol. The detailed information of antibodies used in this study can be found in online supplement. Similar cell aliquots were incubated with species- and subtype-matched antibodies (BD Bioscience) to determine nonspecific fluorescence, as negative controls. Data compensation and analysis were performed using the accompanying software (FACSDiva, BD and FlowJo, LLC, Ashland, OR).

ELISA

Levels of IL-4, IL-5, IL-13, IFN- γ , and IL-17A in the supernatants of cultured DPCs were measured by ELISA (R&D Systems) according to the manufacturer's instructions. Detailed information is provided in online supplement.

Statistical analysis

Data was expressed as the medians and interquartile ranges (IQRs) and analyzed using the Kruskal-Wallis *H* test and nonparametric Mann-Whitney *U* test unless otherwise stated. For *in vitro* assays, the data was expressed as mean and standard errors of the mean (SEM) of three independent experiments, and one-way Analysis of Variance (ANOVA) and paired or unpaired Student's *t*-test were used for the statistical analysis. A *P* value less than 0.05 was considered as statistically significant.

Results

Expression of IL-25 in CRSwNP patients

We enrolled 92 CRSwNP patients, 20 CRSsNP patients and 16 normal controls to evaluate the significance of IL-25 in determining their inflammatory profile. The demographic data and atopic status of all subjects are listed in **Table 1** and **S1**. Our results showed that the mRNA and protein levels of IL-25 were significantly elevated in polyp tissues as compared to the control uncinate process tissues ($P < 0.001$, **Fig. 1A and B**). As for IL-33 and TSLP, we observed increased mRNA levels but not protein levels in polyp tissues as compared to the control uncinate process tissues ($P < 0.001$, **Fig. 1A and B**). IHC staining revealed increased immunoreactivity of IL-25 and its receptor, IL-17RB in polyp tissues as compared to the control uncinate process tissues ($P < 0.001$, **Fig. 1F-H**). In polyp tissues, IL-25 was distributed in both epithelial layer and the inflammatory cells of the subepithelial area, whereas IL-17RB was mainly located in the inflammatory cells of the subepithelial area (**Fig. 1F-L**). IF staining highlighted that epithelial cell adhesion molecule positive (EpCAM⁺) cells also

expressed IL-25 (**Fig. 2A**). The percentage of IL-25⁺EpCAM⁺ cells was significantly higher in polyp tissues than in the control uncinata process tissues by means of flow cytometry ($P=0.006$, **Fig. 2B-D**). IL-17RB mRNA expression was significantly elevated in polyp tissues as compared to the control uncinata process tissues. Furthermore, the mRNA expression of IL-17RB was positively correlated with IL-25 mRNA expression in polyp tissues ($r=0.893$, $P<0.001$, **Fig. 1L**).

To highlight the critical role of IL-25 in the polypogenesis, we next divided the CRSwNP patients into IL-25^{high} and IL-25^{low} subgroups, according to the IL-25 transcript levels in the polyp tissues (above or below the 95th percentile for normal control subjects) by referring to the study reported by Cheng et al. (23). We found that the CT scores, as well as endoscopy scores, were significantly higher in IL-25^{high} subgroup than those of IL-25^{low} subgroup ($P<0.001$), but not those of IL-33 and TSLP (**Fig. 1C and D**). The immunoreactivity of IL-25 and IL-17RB was significantly increased in the IL-25^{high} subgroup compared with the IL-25^{low} subgroup (**Fig. 1F, I and J**).

Inflammatory profiles and histological characteristics in IL-25^{high} and IL-25^{low} subgroups

Next, we performed the luminex-based multiplex assay to determine the cytokine levels in IL-25^{high} and IL-25^{low} polyp tissues. As shown by **Fig. 3A**, we found that the levels of Th2-relevant cytokines, including IL-4, IL-5, IL-13, IL-25, IL-9, eotaxin, eotaxin-2, and eotaxin-3, were significantly increased in the IL-25^{high} subgroup as compared to the IL-25^{low} subgroup. The mRNA levels of transcription factors GATA-3 and RORc (**Fig. 3B and D**), as well as Th2 cytokines IL-4, IL-5 and IL-13 (**Fig. 3E-G**) were also significantly increased in the IL-25^{high} subgroup than those in the IL-25^{low} subgroup.

Moreover, we observed that the basement membrane thickness (the indicator of subepithelial fibrosis) was greater in the IL-25^{high} subgroup than that in the IL-25^{low} subgroup ($P<0.001$). The IL-25^{high} subgroup showed more severe eosinophilia in the tissue ($P<0.001$) and blood ($P=0.012$) than those in the IL-25^{low} subgroup, but not neutrophilia in the tissue. We also found that the transcript levels of MUC5AC ($P=0.008$) and MUC5B ($P<0.001$) were significantly higher in the IL-25^{high} subgroup than those in the IL-25^{low} subgroup. These results suggested that the epithelial IL-25 expression is

associated with more severe Th2-biased, eosinophilic inflammation and tissue remodeling in CRSwNP subjects (**Fig.4**).

Stimulatory effects of recombinant IL-25 on cultured DPCs *in vitro*

To characterize the Th2 and ILC2 cell compartments in CRSwNP patients, we performed flow cytometry analysis of Th2 cells (CD45⁺CD4⁺CRTH2⁺ cells) and ILC2 cells (CD45⁺lineage⁻ CD127⁺ CD161⁺CRTH2⁺ CD123⁻ cells) in 12 NP samples (gates setting were shown in **Fig. S1**). The representative histogram of IL-4, IL-5, IL-13 and IL-17RB in NP ILC2 and Th2 cells in the presence of IL-25, IL-25 blocking antibody and dexamethasone, as indicated by flow cytometry, was shown in **Fig. 5**. A significantly elevated expressions of IL-5 and IL-13 were observed in both Th2 and ILC2 cells ($P<0.05$), while the increased expressions of IL-4 and IL-17RB were only observed in Th2 cells. The increased expressions of IL-5 and IL-13 were significantly inhibited by the presence of IL-25 blocking antibody and/or dexamethasone in both Th2 and ILC2 cells.

Consistently, in the supernatants of cultured DPCs, we observed a significant increase in IL-4 ($P<0.001$), IL-5 ($P=0.030$) and IL-13 ($P=0.032$) production in the presence of IL-25, but not IL-17A and IFN- γ . Similarly, the increase in the expression of IL-4, IL-5 and IL-13 was significantly inhibited by the presence of IL-25 blocking antibody and/or dexamethasone in the supernatants (**Fig. 5**). In both settings, the treatments and their effects did not result in detrimental effects to the cells, as there was no significant difference in cell death across treatment and control groups (**Fig. S2**).

Discussion

The epithelial cell-derived cytokine IL-25, acting through the receptor IL-17RB, has been implicated in promoting Th2 responses in airway inflammation (13, 24). However, the clinical relevance of local IL-25 in CRSwNP patients remains relatively uncharacterized. In the present study, we identified a link between the local IL-25 expression and the Th2-biased inflammatory profiles in polyp tissues of CRSwNP patients. These novel findings might expand our understanding on the pathophysiology of CRSwNP and contribute to the treatment of CRSwNP.

CRSwNP is a Th2-biased, eosinophilic mucosal inflammation. Th2 cytokines, in particular IL-5 and IL-13, are implicated in the pathogenesis of the eosinophilic airway inflammation such as asthma, allergic rhinitis and CRSwNP (5, 25). The triad of epithelial derived cytokines, IL-25, IL-33 and TSLP, has provoked considerable interest as potential therapeutic targets, as they initiate the Th2-biased inflammation profiles by activating Th2 and ILC2 cells (13). Our findings revealed that the expression of IL-25 and its receptor IL-17RB was significantly increased in patients with CRSwNP, and IL-25 was distributed in both epithelium and the inflammatory cells of the subepithelial area. Additionally, while the IL-25 expression in our study showed a significant increasing; for the first time it is observed that, at the sample size of our study, there is a stratification of IL-25 expressions in CRSwNP patients (IL-25^{high} and IL-25^{low}). Low expression of IL-25 was previously reported from similar studies such as Miljkovic et al. (26), where they noted a reduced expression of IL-25 in a small scale of sampling of Australian patients (12 CRSsNP and 13 CRSwNP). On the other hand, increased expression of IL-25 was also observed and recently proposed to be associated with the pathogenesis of eosinophilic CRSwNP (27, 28). For example, Linuma T et al. (27) reported that the IL-25 level in polyps was increased in patients with eosinophilic CRS, and elevation of IL-5 and IL-9 production was found in polyp mononuclear cells from patients with eosinophilic CRS by stimulation with IL-25 under T-cell receptor stimulation. Liao et al. (29) found that IL-25 and IL-17RB were enhanced in epithelial cells in both eosinophilic and noneosinophilic CRSwNP compared to the control. These studies highlighted the presence of both reduced and increased IL-25 expression in CRSwNP patients which prompted us to focus on the differences between IL-25^{high} and IL-25^{low} subgroups, where IL-25 may serve as a factor affecting the pathogenesis of CRSwNP. Consistently, we found the CT scores and endoscopy scores were greater in IL-25^{high} subgroup than that in IL-25^{low} subgroup in the CRSwNP patients, which highlights the expression of IL-25 as a risk factor in CRSwNP pathogenesis.

IL-25, as an epithelial cytokine, can be secreted by barrier epithelial cells in response to allergens (30), helminth and viral infection (31, 32). Von Moltke et al. (31) found that IL-25 was co-expressional in EpCAM⁺ cells in a mouse helminth infection model, which is consistent with our results. IL-25 is traditionally considered as the primary mediator of airway inflammation in asthmatic patients by promoting airway dysfunction and tissue remodeling. Several recent studies addressed the role of IL-25 in promoting the inflammatory response in CRSwNP patients (18, 28). Lam M et al.

(28) reported excessive IL-25 and IL-33 production but not TSLP in eosinophilic CRS, and suggested that the release of these 2 cytokines by dysfunctional endothelium may perpetuate the eosinophilic inflammation in CRS. Our results demonstrate a potential contributor in epithelial IL-25 expression which can lead to more severe Th2 response and eosinophilic inflammation.

Additionally, IL-25 was shown to promote tissue remodeling by acting directly on human fibroblasts to induce collagen secretion in asthmatic models. Moreover, the recruitment of endothelial progenitor cells to the lung and subsequent neovascularisation was also IL-25 dependent, demonstrating a direct role of IL-25 during angiogenesis *in vivo* (33). Strikingly, in our study, we observed that the basement membrane thickness (the indicator of subepithelial fibrosis) was greater in IL-25^{high} subgroup than that in IL-25^{low} subgroup. IL-25^{high} subgroup showed more severe eosinophilia in the tissue and blood, as well as an increase in MUC5AC and MUC5B mRNA expression. This is consistent with the study by Shin et al. (18), which demonstrated that IL-25 was significantly increased in both human polyp tissues and murine model, and anti-IL-25 treatment reduced the number of polyps, mucosal edema thickness, collagen deposition, and infiltration of inflammatory cells. Our findings, together with other studies, suggest that IL-25 could contribute to the infiltration of eosinophils in polyp tissues resulting in the pathologic difference between eosinophilic and non-eosinophilic subsets. Our findings may be beneficial for designing a potential strategy for the optimal prevention and management of eosinophilic CRSwNP patients.

In summary, we have shown the increase in the expression of IL-25 was associated with Th2-biased inflammation and tissues eosinophilia in CRSwNP patient, and demonstrated the role of IL-25 in the activation of Th2 cells and ILC2s *in vitro*. Thus IL-25 may be a promising therapeutic target and an indicator for the management of eosinophilic CRSwNP patients.

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Competing interests

None.

Authors contributions

H.-Y. Hong, F.-H. Chen and Y.-Q. Sun performed RT-qPCR, immunohistochemical staining, cell culture, flow cytometry, cytospin preparation, ELISA, luminex-based multiplex assay, data analysis, and prepared manuscript; X.-T. Hu, and Y. Wei performed RT-qPCR, IHC and IF staining, and data analysis; Y.-P. Fan, J. Zhang, D.-H. Wang, and R. Xu participated sample collection; H.-B. Li and J.-B. Shi designed the study and prepared the manuscript.

Supporting Information

File S1. Supplementary materials and methods

Table S1. Prevalence of common aeroallergen sensitization in the 92 NP patients

Fig S1. Gates setting of Th2 cells (CD45⁺CD4⁺CRTH2⁺ cells) and ILC2 cells (CD45⁺lineage⁻CD127⁺CD161⁺CRTH2⁺CD123⁻ cells)

Fig S2. Live/apoptotic cells in DPCs culture

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Figure legends

Fig 1. Increased mRNA and protein level of IL-25 and its receptor IL-17RB in nasal polyps (NP) tissues

(A) The mRNA levels of IL-25, IL-33 and TSLP in NP and non-NP tissues; (B) The protein levels of IL-25, IL-33 and TSLP in NP and non-NP tissues; (C-E) The association of IL-25, IL-33 and TSLP protein levels with CT score, endoscopic score and serum IgE levels in CRSwNP patients. (F) The immunoreactivity of IL-25 and IL-17RB in NP and non-NP tissues (magnification, 400×); (G and H) The protein levels of IL-25 and IL-17RB in NP and non-NP tissues; (I and J) The protein levels of IL-25 and IL-17RB in the IL-25^{high} and IL-25^{low} subgroups (K); The mRNA level of IL-17RB in NP and non-NP tissues; (L) The correlation of IL-25 and IL-17RB mRNA expression in NP tissues. Data are expressed as median (IQRs).

Fig 2. Increased percentages of IL-25⁺EpCAM⁺ cells in NP

(A) Co-expression of IL-25 and nasal epithelial markers in cytopsin slides of NP tissues;

Representative flow plots of IL-25⁺EpCAM⁺ cells in NP tissue from the CRSwNP patient (C) and

uncinate process tissue from the CRSsNP patient (B) ; (D) Percentages of IL-25⁺EpCAM⁺ cells in

CRSwNP and CRSsNP patients (n=6).

Fig 3. The distinct protein and gene expression profiles in IL-25^{high} and IL-25^{low} NP tissues

(A) Shown is the heatmap of cytokines upregulated or downregulated in IL-25^{high} and IL-25^{low} NP

tissues, as determined by Luminex-based multiplex assay. (B-D) The mRNA levels of transcription

factors GATA-3, T-bet and RORc in IL-25^{high} and IL-25^{low} NP tissues; (E-G) The mRNA levels of Th2

cytokines IL-4, IL-5 and IL-13 in IL-25^{high} and IL-25^{low} NP tissues. Data are expressed as median (IQRs).

Fig 4. The distinct histological characteristics in IL-25^{high} and IL-25^{low} NP tissues

(A) Representative HE-stained histological sections of IL-25^{high} and IL-25^{low} NP tissues (magnification,

400×); (B) The immunoreactivity of MPO in IL-25^{high} and IL-25^{low} NP tissues (magnification, 400×);

(C-E) The counts and ratio of tissue eosinophils and neutrophils in IL-25^{high} and IL-25^{low} NP tissues; (F)

The basement membrane thickness in IL-25^{high} and IL-25^{low} NP tissues; (G and H) The mRNA levels of

MUC5AC and MUC5B in IL-25^{high} and IL-25^{low} NP tissues. Data are expressed as median (IQRs).

Fig 5. Cytokines production in cultured dispersed polyp cells (DPCs) and the supernatants *in vitro*

(A) Representative histogram of IL-4, IL-5, IL-13 and IL-17RB in NP ILC2 and Th2 cells in the presence

of IL-25, anti-IL-25, and dexamethasone, as indicated by flow cytometry; (B) Positive cells of IL-4,

IL-5, IL-13 and IL-17RB in NP ILC2 and Th2 cells in the presence of IL-25, anti-IL-25, and

dexamethasone, as indicated by flow cytometry (n=6); (C) Protein levels of IL-4, IL-5, IL-13, IL-17A,

and IFN-γ in the supernatants of cultured DPCs in the presence of IL-25, anti-IL-25 blocking antibody

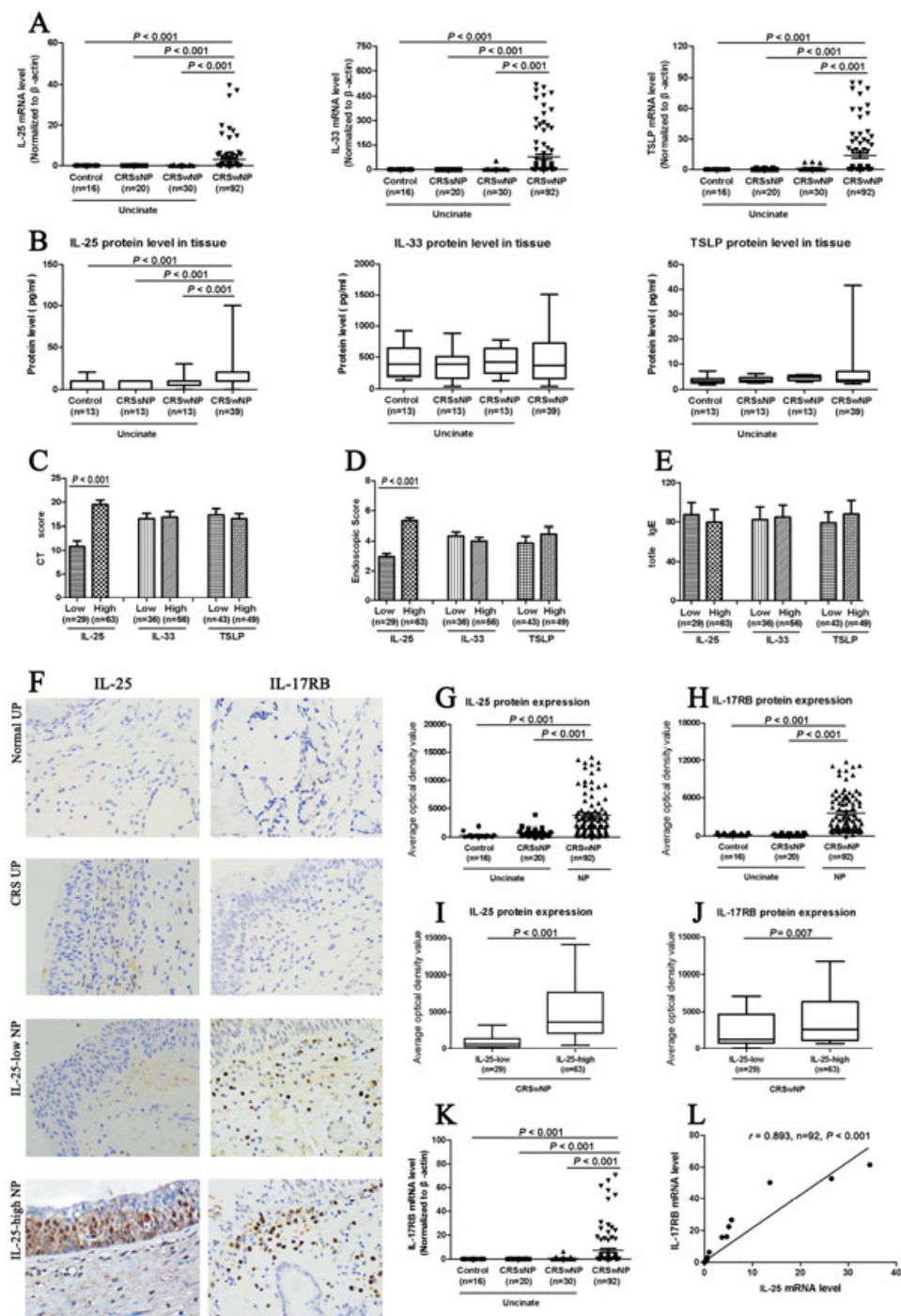
and dexamethasone, as determined by ELISA (n=6).

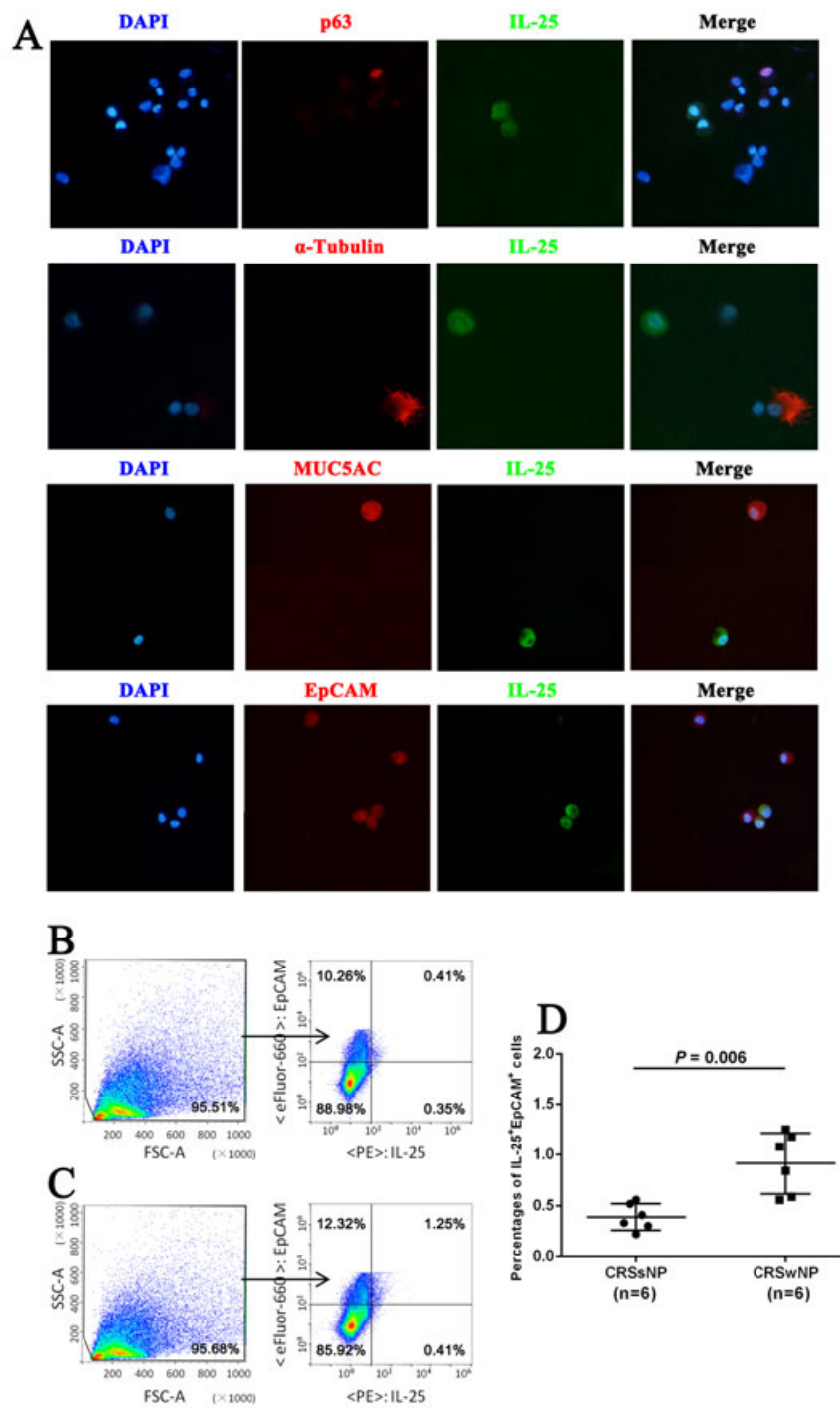
Table 1. Demographics of subjects

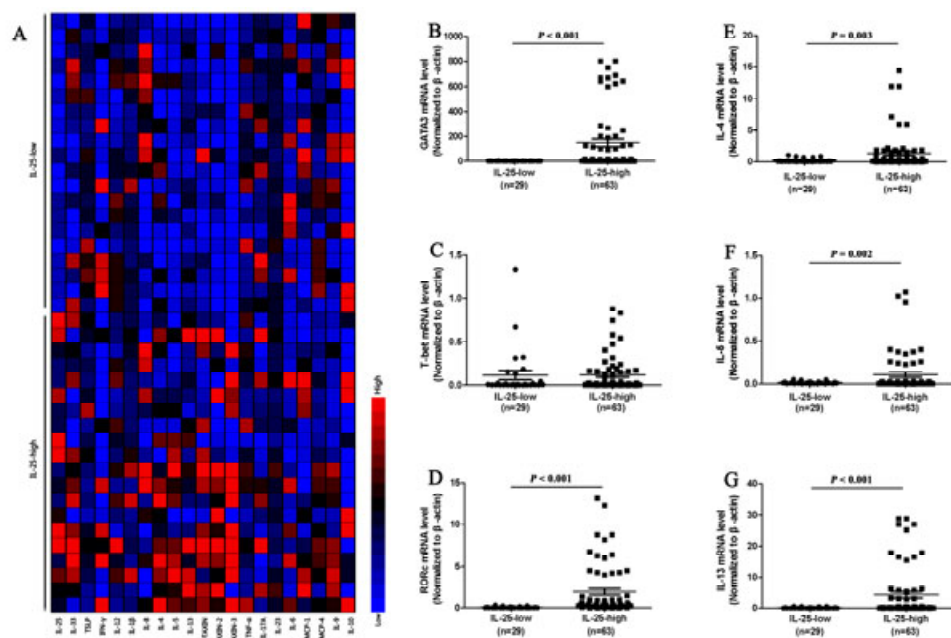
	Control Subjects	Patients with CRSsNP	Patients with CRSwNP
No.	16	20	92
Tissue sampled	UP	UP	UP, NP
Age, mean(SD), (y)	33.5(11.09)	35(9.43)	35.17(9.87)
Atopy, no. (%)	3(18.75)	4(20)	21(21.88)*
Asthma, no. (%)	0	0	0
Aspirin sensitivity, no.	0	0	0
Sex (male/female)	10/6	12/8	63/29
NP Endoscopic Scores, Mean(SD)	N/A	N/A	4.17(1.78)
CT Scores, mean(SD)	N/A	7.1(2.49)	16.36(9.28)
Total IgE, mean(SD), (IU/mL)	11.11(5.20)	78.88(34.18)*	82.30(47.21)*
Blood eosinophilis, mean(SD), (10⁹/L)	0.03(0.05)	0.22(0.33)*	0.31(0.30)*
Methodologies used			
Tissue IHC	16	20	92
Tissue mRNA	16	20	92
Flow cytometry	0	0	12
Luminex	13	13	UP13; NP39

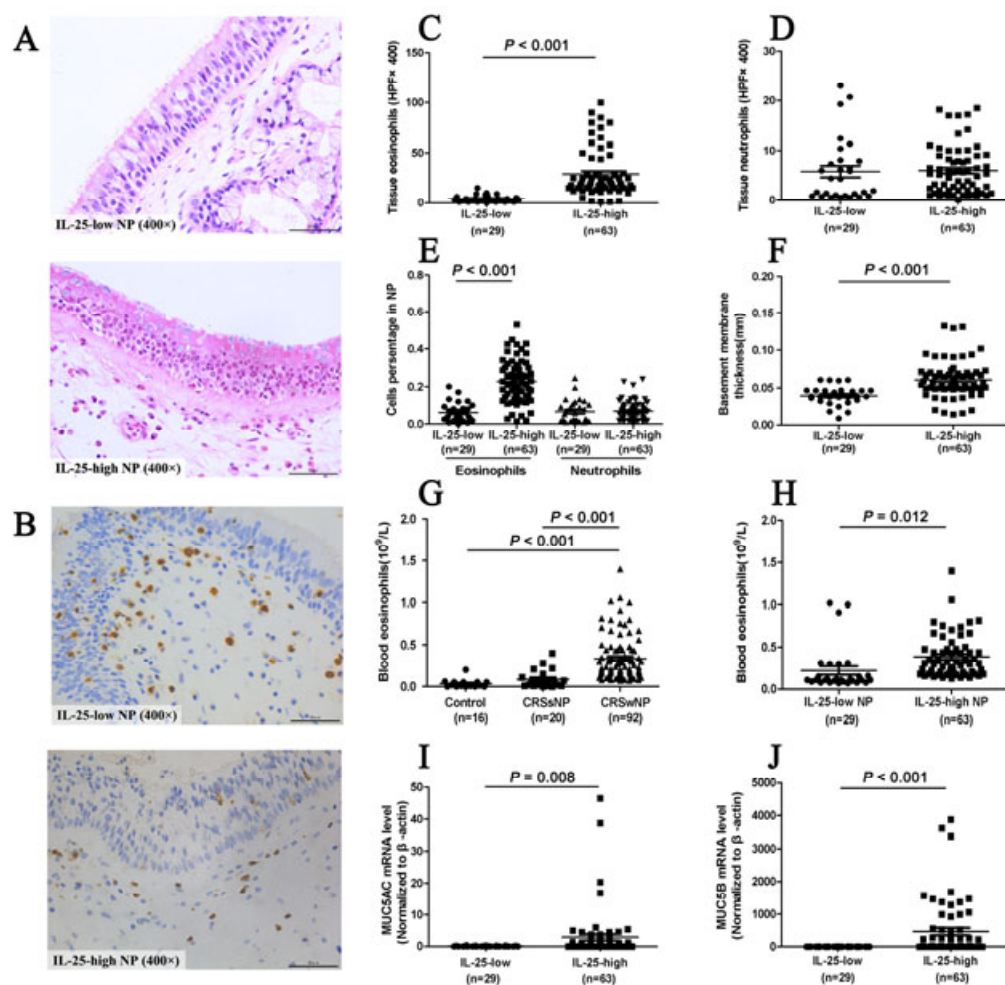
Definition of abbreviations: CRSsNP=chronic rhinosinusitis without nasal polyps; CRSwNP=chronic rhinosinusitis with nasal polyps; NP=nasal polyps; UP= unciate process; N/A= not applicable.

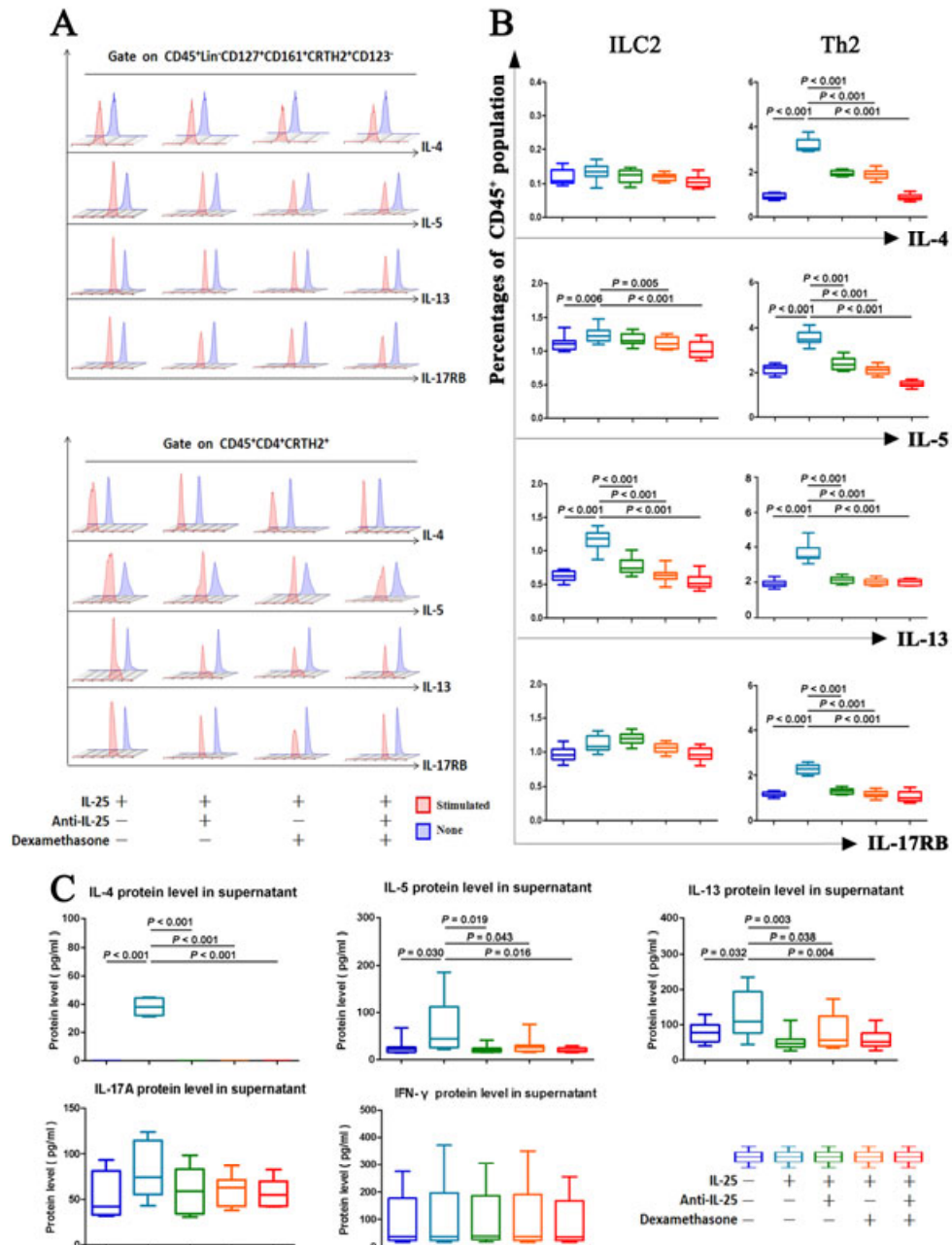
* $P<0.05$, compared with control subjects.



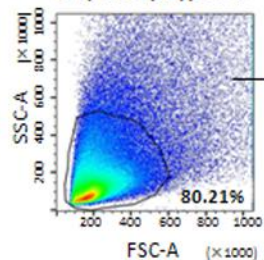




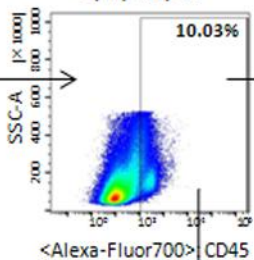
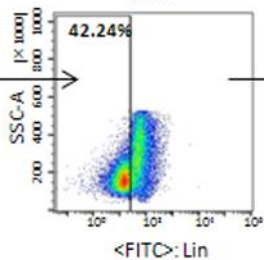
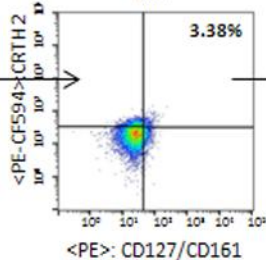
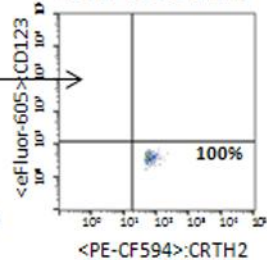
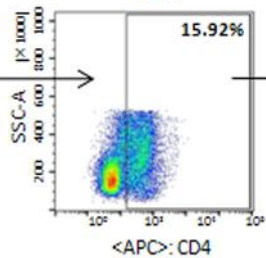
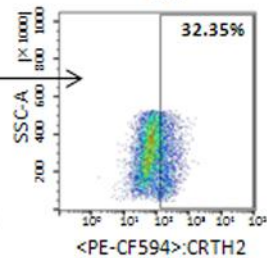




Dispersed polyp cells



Lymphocytes

CD45⁺Lin⁻CD127⁺CD161⁺CRTH2⁺CD45⁺CD4⁺

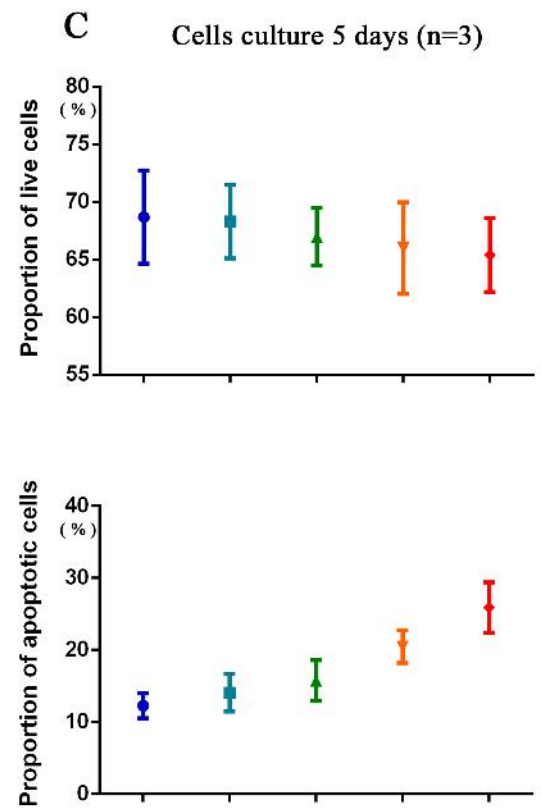
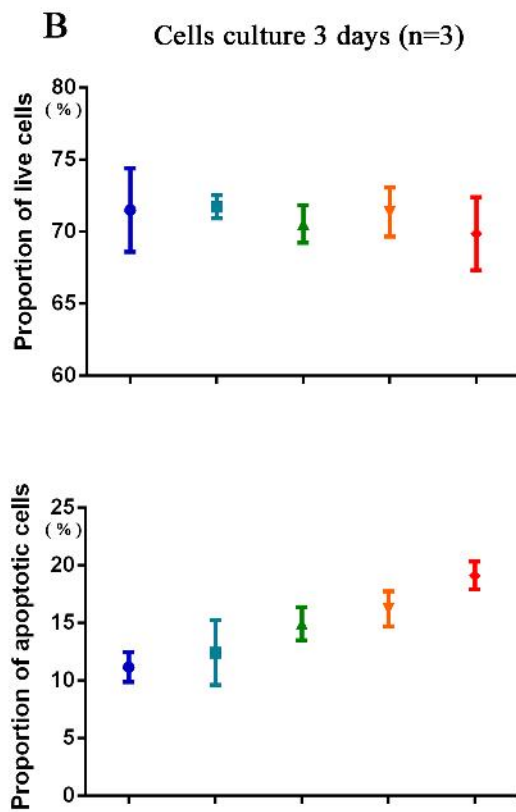
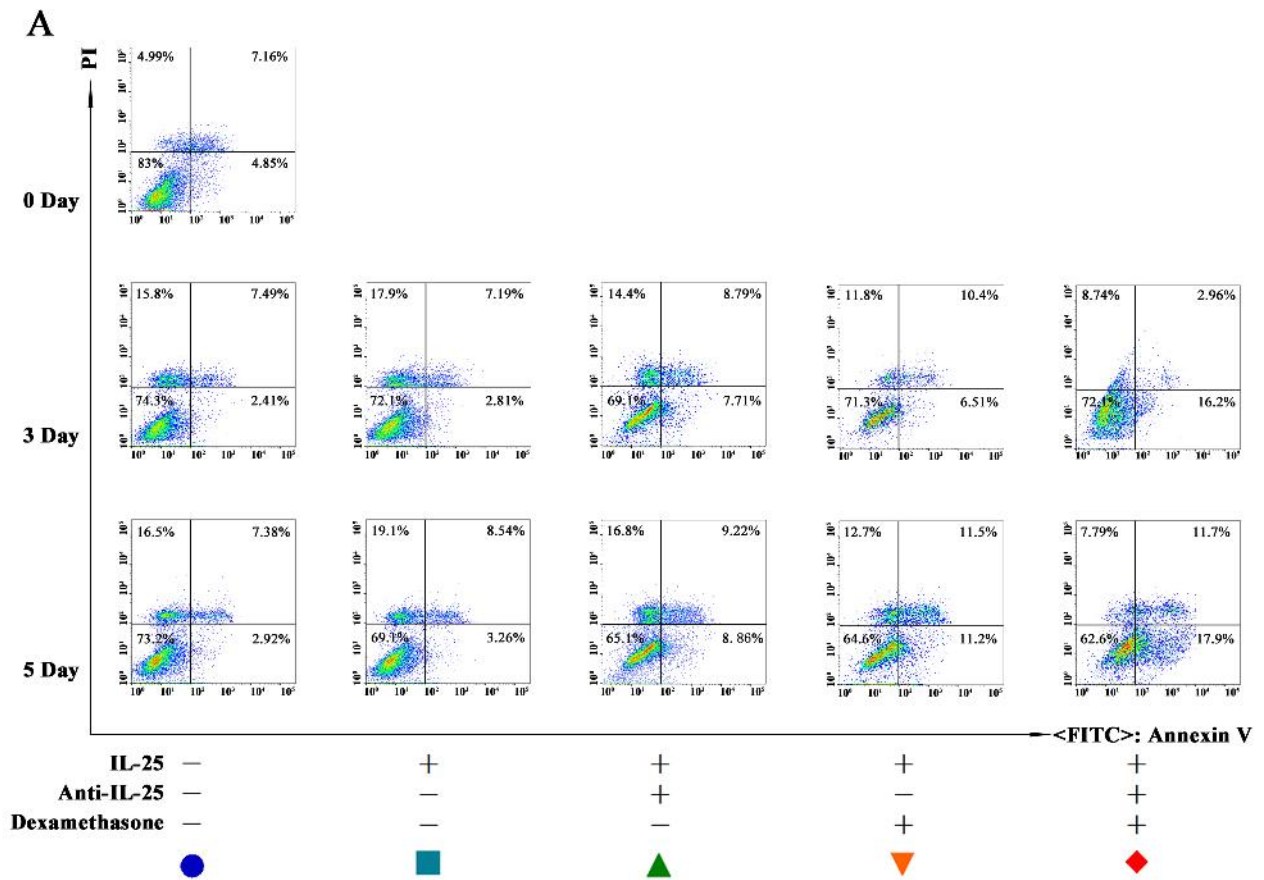


Table S1. Prevalence of common aeroallergen sensitization in the 92 NP patients

	IL-25-low NP	IL-25-high NP
No.	29	63
Sex (male/female)	17/12	44/19
Age, mean(SD), (y)	34.10(11.01)	35.67(9.35)
Atopy, no. (%)	5(17.24)	16(25.40)
Dermatophagoides pteronyssinus, no.	5	15
Dermatophagoides farinae, no.	4	16
Blomia tropicalis, no.	3	12
Canis familiaris, no.	1	3
Felis domesticus, no.	0	2
Blattella germanica, no.	2	5
Alternaria alternata, no.	1	1
Artemisia vulgaris, no.	0	1
Ragweed, no.	0	1
Timothy grass, no.	0	2
Birch, no.	1	1

Definition of abbreviations: NP=chronic rhinosinusitis with nasal polyps. * $P<0.05$, compared with each other.

ORIGINAL ARTICLE

Local IL-25 contributes to Th2-biased inflammatory profiles in nasal polyps

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Online Supplementary

Materials and methods

Patients and tissue samples

During endoscopic surgery, the sinonasal tissues were sampled as follows: the polyp and uncinate process tissue in the CRSwNP group and the uncinate process tissue in the CRSsNP group. As normal controls, 16 patients with traumatic optic neuropathy were enrolled, and the uncinate process tissue were sampled during endoscopic optic nerve decompression. Tissue samples were divided into 3 portions; the first portion was frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation, the second portion was used for protein isolation, the third portion was fixed overnight in a freshly prepared fixative containing 4% paraformaldehyde in PBS (pH 7.4) and was embedded in paraffin wax for histological staining. In addition, a portion of fresh polyp was further used for activation assay in dispersed polyp cells (DPCs) *in vitro*.

Immunohistochemistry (IHC) staining

IHC staining was performed by peroxidase-labeled streptavidin-biotin technique as previously described (1). Briefly, after blocking the endogenous peroxidase in 3% hydrogen peroxide and with 1% bovine serum albumin, the sections were incubated overnight at 4°C in the presence of the primary antibodies (rabbit anti-IL-25 antibody, Abcam, Cambridge, MA, 1:250; rabbit anti-IL-17RB antibody, Millipore, Danvers, MA, 1:250; rabbit anti-MPO antibody, Abcam, 1:200). Thereafter, each section was incubated with biotinylated goat anti-rabbit secondary antibody and then with horseradish peroxidase-labeled streptavidin complex (Zhongshanjinqiao, Beijing, China). Distribution of peroxidase was revealed by incubating the sections in a solution containing 3% 3,3-diaminobenzidine tetrahydrochloride before being counterstained with hematoxylin and mounted with coverslips. Negative control studies were performed by replacing the primary antibodies with normal IgG in appropriate concentrations.

The sections were blindly examined and coded with no awareness of the clinical data. The immunoreactivity of five randomly selected subepithelial areas (400×) on each section of the subjects were quantitatively scored by using Image-Pro Plus 6.0 software and the average optical density (AOD) value was calculated.

Cytospin preparation

1.5×10^5 dispersed polyp cells (DPCs) were fixed with 4% formaldehyde for 10 minutes at room temperature, followed by two rounds of wash with 1X PBS and spun down. About 2×10^4 cells were prepared for per cytospin slide at 500 rpm for 5 min with mild acceleration according to the manufacturer's instructions (ThermoScientific, Waltham, MA). Cytospin slides were stored until staining in -20°C freeze.

Immunofluorescence (IF) staining

Immunofluorescence (IF) staining was performed by the technique as previously described (2). Briefly, after being incubated with 0.1% Triton X-100 and 1% Goat serum (Sigma, USA), the slides were incubated overnight at 4°C in the presence of primary antibodies (rabbit anti-IL-25 antibody, Abcam, 1:250; mouse anti-P63 antibody, Abcam, 1:250; mouse anti- α -Tubulin, Abcam, 1:800; mouse anti-Mucin5AC antibody, Biosciences, 1:800; mouse anti-EpCAM antibody, Abcam, 1:250). Thereafter, each slide was incubated with goat anti-rabbit (Alexa Fluor 488) or mouse (Alexa Fluor 594) secondary antibodies (Life Technologies, 1:500) are then mounted with ProLong AntiFade mounting medium with DAPI (Life Technologies). The stained slides were then visualized using an Olympus IX51 fluorescence microscope with 40 $\times$ objective lens.

Real time quantitative PCR (RT-qPCR)

The mRNA expression level of target genes was detected by RT-qPCR analysis as previously described (1). Briefly, total RNA was extracted from the samples with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed and the cDNA was synthesized from 2 μ g of total RNA by using an oligo (dT) 18 primer and M-MLV reverse transcriptase (TAKARA, Syuzou, Shiga, Japan) for quantitative PCR. RNA integrity and the success of the reverse transcription reaction were monitored by PCR amplification of β -actin transcripts. Expression of mRNA was determined using ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Taq™ (TAKARA). The primer sequences were as follows: IL-25 forward: 5'-CCA GGT GGT TGC ATT CTT GG-3'; IL-25 reverse: 5'-TGG CTG TAG GTG TGG GTT CC-3'; IL-33 forward: 5'-AGC CTA GAT GAG ACA CCG AAT T-3'; IL-33 reverse: 5'-GGT CAG AAG GGA TGG TAG GC-3'; TSLP forward: 5'-ACC GAG TTC AAC AAC ACC GT-3'; TSLP reverse: 5'-TCC GAA TAG CCT GGG CAC-3'; IL-17RB forward: 5'-GGC TTC CTA TCC CAC CAA TT-3'; IL-17RB reverse: 5'-AGG CTG TTT GTA GGC TGC A-3'; T-bet forward: 5'-GTC AAT TCC TTG GGG GAG AT-3'; T-bet reverse: 5'-TCA TGC TGA CTG CTC GAA AC-3'; GATA-3 forward: 5'-ACC ACA ACC ACA CTC TGG AGG A-3'; GATA-3 reverse: 5'-TCG GTT TCT GGT CTG GAT GCC T-3'; RORC forward: 5'-GCT GTG ATC TTG CCC AGA ACC-3'; RORC reverse: 5'-CTG CCC ATC ATT GCT GTT AAT CC-3'; IL-4 forward: 5'-GCA GTT CTA CAG CCA CCA TG-3'; IL-4 reverse: 5'-ATG ATC GTC TTT AGC CTT TCC-3'; IL-5 forward: 5'-ATC TTT CAG GGA ATA GGC ACA C-3'; IL-5 reverse: 5'-CCG TCT TTC TTC TCC ACA CTT T-3'; IL-13 forward: 5'-AGC ATG GTA TGG AGC ATC AA-3'; IL-13 reverse: 5'-CAG CAT CCT CTG GGT CTT CT-3'; MUC5AC forward: 5'-TGA TCA TCC AGC AGC AGG GCT-3'; MUC5AC reverse: 5'-CCG AGC TCA GAG GAC ATA TGG G-3'; MUC5B forward: 5'-GTC AAC AGC CAT GTG GAC AAC-3'; MUC5B reverse: 5'-CTC CTC ACA GGA GTA GCA GCA-3'; β -actin forward: 5'-GTC ACC AAC TGG GAC GAC A-3'; β -actin reverse: 5'-CAC AGC CTG GAT AGC AAC G-3'. PRISM samples contained 1 $\times$ SYBR Green Master Mix, 1.5 μ L 5 μ M primers, and 25 ng synthesized cDNA in a 25- μ L volume. Reactions were heated to 95°C for 10min followed by 40 cycles of denaturation at 95°C for 10 s, annealing extension at 60°C for 60 s. Melting curve analysis was used to control for amplification specificity. The

mean threshold cycle (Ct) values were normalized to β -actin, and the relative mRNA levels of target genes were analyzed with the $2^{-\Delta\Delta Ct}$. Experiments were performed in triplicate for each data point.

Luminex-based multiplex assay

Protein extracted from polyp and uncinated process tissues was measured by luminex-based multiplex assay. Briefly, 30 mg of each sample was homogenized in 0.5 mL lysis buffer supplemented with protease inhibitor (Millipore) for 1 min on ice and centrifuged for 20 min at 4000 rpm at 4°C, the supernatants were collected and stored at -80°C for further analysis. Total protein concentration in the supernatants that determined by using a standard BCA assay was normalized to a concentration of 50 μ g total protein per sample. All samples were analyzed for IFN- γ , IL-12, IL-1 β , IL-8, IL-17E(IL-25), TSLP, IL-33, IL-4, IL-5, IL-13, EOTAXIN, EOTAXIN-2, EOTAXIN-3, TNF- α , IL-17A, IL-23, IL-6, MCP-1, MCP-4, IL-9, IL-10 using Luminex-based Multiplex kits (Millipore) according to manufacturer's protocol.

Dispersed polyp cells (DPCs) isolation and culture

The isolation of DPCs was performed as previously described (3). Briefly, fresh polyp tissues were washed and then cut into small pieces of 1×1 mm in size, digested in incomplete RPMI-1640 medium with endotoxin-free collagenase I (2 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) and DNase (0.1 mg/mL, Sigma-Aldrich) for 60 min at 37°C. The digested fragments were filtered through a 100- μ m cell nylon mesh (BD Biosciences) to disperse the cells. The mononuclear cells were then obtained from the dispersed cells by Ficoll-Hypaque (Haoyang Bio., Tianjin, China) density gradient centrifugation at 2200 rpm for 20 min and then washed twice with Hank's solution. DPCs were suspended at a concentration of 1×10^6 /mL in complete RPMI-1640 medium containing 10% FCS (Sijiqing, Hangzhou, China), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mmol/L L-glutamine (all from Gibco, Grand Island, NY, USA), and were cultured at a 12-well plate for 5 days in the presence of IL-25 (50 ng/mL, R&D systems) and IL-2 (10 ng/mL, R&D systems) with or without anti-IL-25 blocking antibody (10 μ g/mL, polyclonal goat IgG, R&D systems, Catalog no. AF1258), or dexamethasone (1 μ g/mL, Sigma-Aldrich). After 5 days, cells and supernatants were harvested for flow cytometry and ELISA, respectively.

Flow cytometric analysis

For flow cytometry, living DPCs of 10^6 or more were suspended in 400 μ L PBS with 1% bovine serum albumin for blocking unspecific staining. They were stained with the following anti-human monoclonal antibodies: anti-CD45-Alexa-Fluor700, anti-Lineage-FITC, a cocktail of lineage-specific antibodies including: CD2, CD3, CD14, CD16, CD19, CD56, and CD235a, anti-CD127-PE-Cy7, anti-CD161-PE-Cy7, anti-CD123-eFluor-605, anti-CRTH2-PE-CF594, anti-CD4-APC, anti-IL-17RB-PE, and anti-EpCAM-eFluor-660 for surface staining; anti-IL-4-APC-Cy7, anti-IL-5-PerCP-Cy5.5, anti-IL-13-BV421, and anti-IL-25-PE for intracellular staining. All antibodies and isotype controls were purchased from eBioscience (eBioscience,

San Diego, CA, USA). DPCs were first stained for surface antigens. Following fixation and permeabilization with Permeabilization/Fixation buffer (BD Biosciences), these cells were stained with conjugated mAbs for cytokines according to the protocol of the Permeabilization/Fixation Kit. Samples were analyzed using a flow cytometer (Gallios, Beckman Coulter, Hercules, CA, USA). Fluorescence was determined for all cells in each sample after debris, dead cells, and aggregates were excluded by forward angle and side scatter gating. Similar cell aliquots were incubated with species- and subtype-matched antibodies (BD Bioscience) to determine nonspecific fluorescence, as negative controls. Data compensation and analysis was performed using the accompanying software (FACSDiva, BD).

ELISA

The levels of IL-4, IL-5, IL-13, IFN- γ , and IL-17A in the supernatants of cultured DPCs and the level of IL-4, IL-5, IL-13, IFN- γ , IL-17A and IL-25 in blood samples were measured by ELISA using a series of commercially available ELISA kits (all were purchased from R&D Systems) according to the manufacturer's instructions. The detection limit for the assays were as follows: 3.9 pg/mL for IL-5, 57.4 pg/mL for IL-13, 11.7 pg/mL for IL-25, 15.6 pg/mL for IFN- γ , and 31.2 pg/mL for IL-4 and IL-17A. For convenient analysis, all values below the detectable limit were considered zero.

Annexin V/apoptosis assay

Annexin V/apoptosis assay (Annexin-V-FITC Apoptosis Detection Kit I, BD Biosciences) was used to detect the apoptotic and live cells by flow cytometry analysis. DPCs were cultured, stained, and analyzed as described above. The live cells (Annexin-V-FITC- PI-) and apoptotic cells, including early apoptotic cells (Annexin-V-FITC+ PI-) and late apoptotic cells (Annexin-V-FITC+ PI+) were presented as percentage of total cells.

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