

## Original Article

# Inflammatory infiltration and tissue remodeling in nasal polyps and adjacent mucosa of unaffected sinus

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**Abstract:** Background: This study was designed to explore the characteristics of inflammatory infiltration and tissue remodeling in the adjacent unaffected sinus mucosa of the polyp tissue (ANP). Methods: Nasal polyps (NP) and ANP were obtained from 24 CRSwNP patients who received endoscopic sinus surgery. The frequency and distribution of Eosinophils, T lymphocytes (CD4+ T cells and CD8+ T cells), B lymphocytes, native macrophages, regulatory T cells and the total of inflammatory cells were detected by immunohistochemistry and hematoxylin-eosin stain. The thickness of the basal membrane was evaluated. Results: Multivariate analysis of Variance (MANOVA) and F-tests were conducted for each independent variable between two groups. With test criterion alpha = 0.05, significant differences were observed between NP and ANP groups in terms of CD3 (F-Value = 10.47, P-value = 0.0120), CD4 (F-Value = 9.03, P-value = 0.0169), CD8 (F-Value = 17.03, P-value = 0.0033) and regulatory T cells (F-Value = 60.42, P-value <0.0001). Wilks' Lambda test (F-Value = 25.74, P-value = 0.1513) was conducted and no significant difference was observed between the NP group and the ANP group. The percentage of regulatory T cells in ANP was significantly higher than that in NP (3.7110±0.2395 vs 14.6300±1.8360). Conclusion: ANP and NP may be one disease entity. Treg cells have impacts on the morphology of the tissues and might be a key factor in the further development of ANP.

**Keywords:** Chronic rhinosinusitis, regulatory T cell, nasal polyp, eosinophils

## Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a common disease of the nasal cavity and sinuses that affects millions of people worldwide [1]. An intense edematous stroma with albumin deposition, the formation of pseudocysts, and inflammatory cells infiltration are the special features. Bacteria, viruses, and fungi have been implicated in the establishment of the inflammatory process [2, 3]. In addition, a variety of cytokines may play a part in the inflammatory process. However, the underlying mechanisms of CRSwNP formation remain unclear [4]. CRSwNP is generally considered as a heterogeneous collection of inflammatory diseases involving the nose and paranasal sinuses, suggesting that sinus inflammation is usually accompanied by adjacent mucosal inflammation. NP is thought to be a typical characteristic of the sinus mucosa

inflammation [5]. Several studies have been performed to determine immune effector cells in the NPs of CRSwNP patients [6, 7]. Data about the pattern of inflammatory cells and remodeling in ANP is still lacking. NP and ANP were regarded as one entity by some researchers. Some studies showed no major histological difference between ANP and polyps obtained from CRSwNP patients. The ANP specimens obtained from CRSwNP patients were regarded as a diseased group, while most studies selected NP as the diseased group. Therefore, whether the ANP can be regarded as one entity remains unclear.

Recent studies demonstrated that these pathologies can be immunologically separated into distinct groups or regarded as one entity based on the expression of inflammatory and tissue remodeling [8, 9]. Thus, our study was designed to compare the inflammatory and tis-

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**Table 1.** General clinical data of all patients

Item	
Gender (male/female)	12/12
Mean age (years)	41
Mean bilateral CT score	14 (2-23)
Mean total symptom score	23 (9-39)

Data presented as means  $\pm$  SD, or *n* patients.

sue remodeling within NP and ANP and provide further insight into the pathogenesis of the subtypes of CRSwNP.

## Patients and methods

### Patients

From September 2013 to December 2013, 24 CRSwNP patients who underwent routine endonasal sinus surgery in the Department of Otolaryngology, Qilu Hospital Shandong University were enrolled in our study. This study was approved by the ethical committee of Qilu Hospital Shandong University and informed consent was obtained from all patients.

All patients underwent a routine medical history inquiry, a physical examination, and a laboratory examination. CRSwNP was diagnosed by medical history, sinus-CT-scan and nasal endoscopy. Visual analogue scales (VAS) [10] were obtained from all patients. The disease extent on the CT-scans was categorized according to Lund-Mackay [11]. The severity of nasal congestion, hypersecretion, nasal irritation, cough, and mid-facial pressure feeling was assessed by verbal rating scales ranging from zero (no complaints) to 10 (maximum symptom severity). The symptoms for diagnosis were: the presence of either one or two major and two minor criteria for a period of 12 weeks [12].

Major criteria include: facial pressure, nasal obstruction or blockage, hyposmia or anosmia, and purulent nasal drainage. Minor criteria include: headache, fever, halitosis, cough, dental pain, fatigue, and ear pain or pressure. Patients with allergic rhinitis, atopy, cystic fibrosis, severe immunodeficiency, autoimmune disease, non-invasive fungal balls and invasive fungal disease, systemic vasculitis, and granulomatous disease were excluded from our study.

### Specimen collection

Oral and topical application of corticosteroids was stopped at least one month before surgery. After general anesthesia and mucosal decongestion, nasal endoscopy surgery was performed; NP and ANP were obtained from the same patients. Adjacent sinus mucosa without obvious swelling was required. Then the specimens were fixed with formalin, stored at a low temperature until homogenization, and then embedded in paraffin.

### Immunohistochemical and hematoxylin-eosin stain

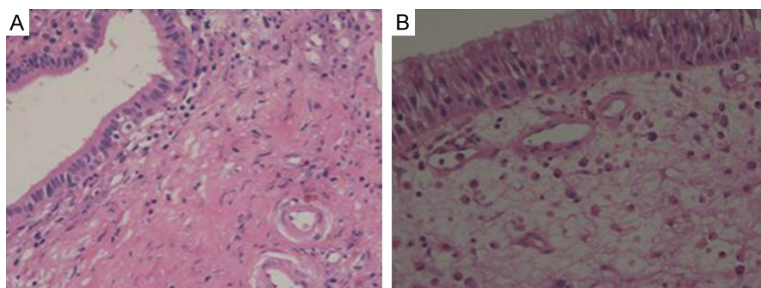
To analyze the histopathological characteristics and inflammatory cell infiltration, sections of 4  $\mu$ m were prepared for hematoxylin-eosin (H&E, Wuhan, China) and immunohistochemical staining. The number of eosinophils and the basal membrane thickness on H&E staining were detected.

Immunohistochemical staining was performed as follows. For dewaxing, the specimens were immersed in 4°C pure acetone for 10 minutes. After washing by PBS several times, the antigen was retrieved by citric acid buffer (PH 6.0) through microwave antigen retrieval (heat to boiling for 4 times, 10 minutes per time). The slides were then soaked with 0.3% hydrogen peroxide solution to close endogenous peroxidase and incubated with 5% goat serum to reduce nonspecific background staining. Monoclonal antibodies: Anti-CD3 (1:100, Abcam), Anti-CD20 (1:50, Abcam), Anti-CD68 (1:200, Abcam), Anti-CD4 (1:200, Abcam), Anti-CD8 (1:200, Abcam), Anti-Foxp3 (1:400, Santa) were added and the sections were incubated at 4°C overnight. PBS was taken as a negative control. The next day, the secondary antibody was added, and the sections were incubated at 37°C for 10-15 min. The liquid labeled with horseradish peroxidase was added, and the sections were incubated at 37°C for 10-15 min. Using 3, 3'-diaminobenzidine (DAB) as a reagent, the sections were counterstained with hematoxylin, dehydrated with a range of different concentrations of ethanol.

### Standard of positive cells

We found that the degree of dying had a great effect on the experiment's results. Different

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**Figure 1.** H&E staining of eosinophils in NP tissues and ANP of the same NP patients.

dehydration rates brought a different count of the positive cells at the same magnification. To reduce experimental error, the number of total inflammatory cells was counted, and the percentage of positive cells was calculated at the same time. Each slice was analyzed by two radiologists blindly. Three individual fields ( $\times 400$  magnification) with infiltration by inflammatory cells were counted. The percentage of each positive cell in the total inflammatory cells was calculated as an average of the positive cell percentage.

### Statistical analysis

Multivariate analysis of Variance (MANOVA) was conducted by SAS (9.4) to determine if the NP and ANP variables were altered by the observer's manipulation of the independent variables. For the analytical method, considering measuring several dependent variables in a single experiment, the MANOVA test gives us a better chance of discovering which factors are important, to protect against Type I errors that can occur, and exclude the affect of the correlation among the dependent variables. Comparisons were made between groups in terms of Eosinophils, CD4+ T cells, CD8+ T cells, CD3+, CD20+, CD68+, regulatory T cells and the thickness of the basal membrane. F-tests were conducted for each independent variable between the two groups. With test criterion  $\alpha = 0.05$ , Wilks' Lambda test was used to detect whether there was a difference between the NP group and the ANP group.

## Results

### Clinical baseline data

Twenty-four patients (12 males and 12 females) with a mean age of 41 years were enrolled in our study. The mean bilateral CT score was 14

(ranging from 2-23). The mean total symptom score according to VAS was 23 (ranging from 9-39). The general clinical data of all patients were listed in **Table 1**.

### Tissue morphology (H&E staining)

**Figure 1** shows the H&E staining results of eosinophils in NP tissues and the ANP of the

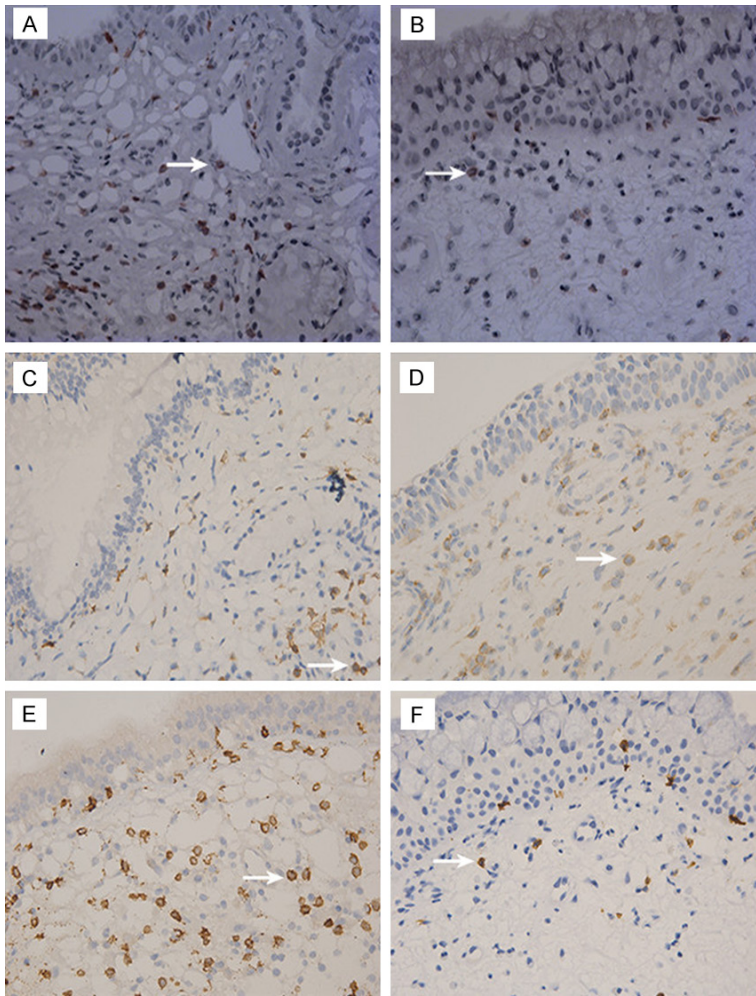
same NP patients, which were observed mainly in lamina propria. Obvious edema and hyperplasia were more frequently judged as pronounced in NP. The percentage of EOS in the total inflammatory cells was calculated. No significant difference was observed in the basal membrane thickness between NP ( $1.6930 \pm 0.6093$ ) and ANP ( $1.3560 \pm 0.2578$ ).

### Immunohistochemical analysis

The immunohistochemical staining results of CD3+, CD4+, CD8+, CD20+, CD68+ and regulatory T cells in NP tissues and ANP of the same NP patients were shown in **Figures 2** and **3**. Predominantly increased cell infiltrations with native macrophages and lymphocyte were found both in NP tissue and the ANP. All cell types were found mainly in the lamina propria. Infiltration of native macrophages and CD8+ and CD4+ T cells could also be found in the epithelium. Numerous CD3+ T cells were observed and scattered evenly in the subepithelial region. The percentage of native macrophages (CD+ 68 cells), regulatory T cells, CD+ 3 cells, CD+ 8 cells, CD+ 4 cells and CD+ 20 cells in the total inflammatory cells was calculated.

MANOVA was operated to determine if the NP and ANP variables are altered by the observer's manipulation of independent variables. F-tests were conducted for each independent variable between the two different groups. With test criterion  $\alpha = 0.05$ , significant differences were found between NP and ANP groups in terms of CD3 (F-Value = 10.47, P-value = 0.0120), CD4 (F-Value = 9.03, P-value = 0.0169), CD8 (F-Value = 17.03, P-value = 0.0033) and regulatory T cells (F-Value = 60.42, P-value < 0.0001). And no highly correlated variables were found which much advantage in including more than one in the test given the resultant loss in degree of freedom.

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**Figure 2.** Immunohistochemical staining of (A, B) CD3+, (C, D) CD4+ and (E, F) CD8+ T cells in NP tissues and ANP of the same NP patients. White arrows represent positive cells.

MANOVA Test Criteria and Exact F Statistics for the Hypothesis of no overall type effect gives us the result of Wilks' Lambda value (F Value = 25.74,  $P$ -value = 0.1513) which showed no significant difference between the NP group and the ANP group (**Table 2**).

The number of regulatory T cells did not decrease noticeably in the NP from the 24 CRSwNP patients, while the percentage of regulatory T cells declined significantly because of the increasing number of total inflammatory cells. So regulatory T cells were more frequently observed in ANP ( $14.6300 \pm 1.8360$ ) than in NP ( $3.7110 \pm 0.2395$ ).

### Discussion

In this study, we used immunohistochemistry and hematoxylin-eosin staining to analyze the

number and distribution of several key inflammatory cells: Eosinophils, T lymphocytes (CD4+ T cells and CD8+ T cells), B lymphocytes, native macrophages, regulatory T cells, and the total of inflammatory cells in the mucosa of each group to help us learn the different patterns of inflammatory infiltration in the different tissues. The MANOVA Test Criteria and Exact F Statistics give us the result of Wilks' Lambda value which showed no significant difference between the NP group and the ANP group in a different type of inflammatory cells.

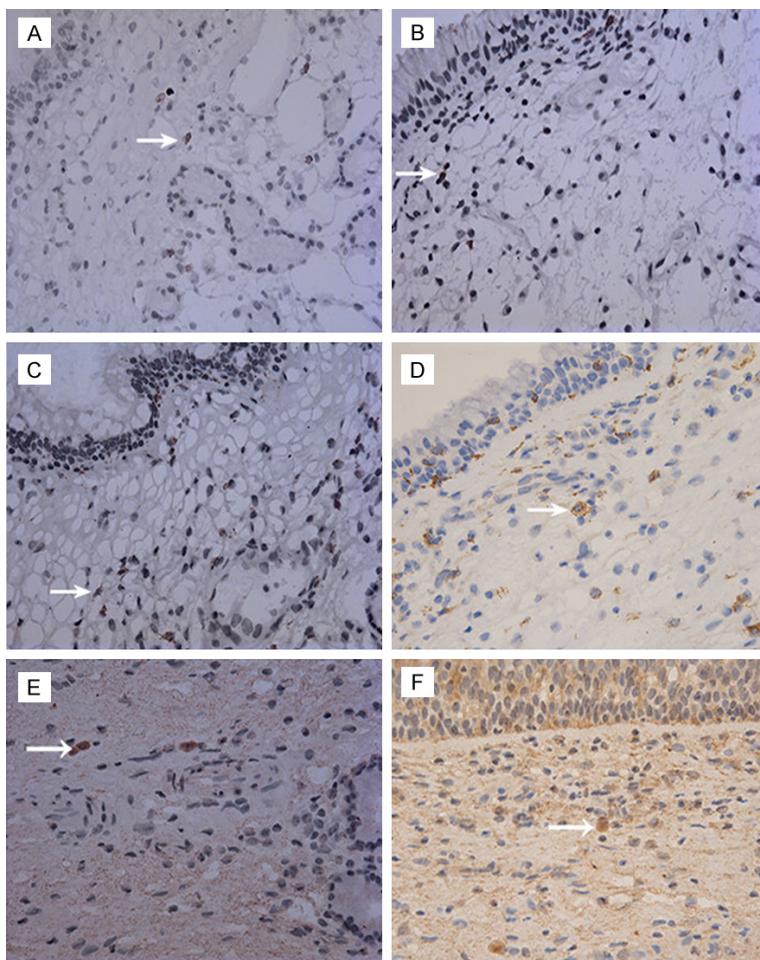
Tissue remodeling is the process in which tissues get repaired and reconstructed, with the production and release of TGF- $\beta$  [13], which then contributes to the regulation of expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), and then finally leads to the change of the extracellular matrix. The thickness of the basal membrane indicates long-lasting inflammation and is considered a marker of airway remodeling [14].

So the change of thickness of the basement membrane can partially reflect the tissue remodeling [15]. In our study, similar changes in the basement membrane thickness were found between NP and ANP.

The results of no obvious difference between NP and ANP suggested similar cellular proliferation activities and tissue remodeling in NP and ANP from CRSwNP patients. Under the assumption of one single disease, tissues in the two groups should have a similar expression of inflammatory cells and tissue remodeling. Thus, we concluded that the adjacent sinus mucosal tissue of nasal polyps and NP are statistically one entity.

However, the inflammatory infiltration was not completely identical between NP and ANP. Furthermore, the inflammatory infiltration in NP

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**Figure 3.** Immunohistochemical staining of (A, B) CD20+ T cells, (C, D) CD68+ T cells and (E, F) regulatory T cells in NP tissues and ANP of the same NP patients. White arrows represent positive cells.

**Table 2.** Analysis of the results in nasal polyps and ANP from 24 CRSwNP patients

Type of Characteristic	F Value	P
CD3	10.47	0.0120
CD4	9.03	0.0169
CD8	17.03	0.0033
CD20	4.42	0.0688
CD68	3.36	0.1043
Treg	60.42	<.0001
EOS	2.40	0.1596
Wilks' Lambda	25.74	0.1513

was stronger than that in ANP. In previous experiments, patients were quantitatively assessed as having granulocyte subsets (eosinophils, neutrophils, basophils) following prednisone administration in nasal polyps, the unaffected adjacent ethmoid sinus mucosa [6]. It

was argued that the immunologic microenvironment within NP lesions was entirely distinct from the surrounding mucosa and it should be studied further. Our results partly confirmed this point of view that the inflammatory infiltration was not completely identical between NP and ANP.

Interestingly, the percentage of regulatory T cells in NP was lower than that in ANP. Owing to its diverse pathways, molecules, and processes, Treg cells take part in the tissue remodeling and have impacts on morphological and functional tissues, such as NP and ANP. So, we argued that Treg cells might be a key in the question of why the typical edema change of mucosa exists in NPs but not in ANP. Several papers have proved that regulatory T cells have a certain inhibiting effect against immunity induced by virus and bacteria [16]. It can be classified into nTreg cells and iTreg cells. It is confirmed that nTreg cells can migrate to sites of inflammation at mucosal surfaces and

inhibit T lymphocytes and B lymphocytes via cell-cell contact, which plays an important part in the pathogenesis of nasal polyps [17]. The iTreg cells suppress immune function in CRSwNP by secreting IL-10 and TGF- $\beta$ , down-regulating generation and expression of IgE, and promoting a phenotype switch of IgG4 [18, 19]. Jaffar et al. used a TCR-transgenic mouse and proved that CD4+ CD25+ T cells play a key role in modulating Eosinophils inflammation by suppressing the development of a Th2 phenotype [20]. These findings may explain why the expression of eosinophils is decreased more in ANP than that in NP. Furthermore, one study reported that up-regulation of Foxp3 by corticosteroids restores Treg cell function to suppress the TH2 response [21]. The increased expression of Treg cells can also suppress persistent mucosal inflammation in nasal polyps, following prednisone administration [22]. The

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result might present a therapeutic approach in nasal polyps, which could benefit the development of safer and more specific drugs to enhance the function and expression of regulatory T cells in CRSwNP.

In conclusion, similar expressions of inflammatory and tissue remodeling were statistically found between NP and ANP from the same CRSwNP patients. Thus NP and ANP can indeed be regarded as one disease entity, supporting the use of the term rhinosinusitis [23-25]. But the inflammatory infiltration was not completely identical between NP and ANP. So, we suggest that NP should be selected as the diseased group, not the ANP. Moreover, without typical edema formation, ANP could not cause nasal congestion and be preserved. With obvious inflammatory infiltration, it could bring great pitfalls for postoperative recurrence. Han and Zhou et al. [26] put forward the treatment of CRSsNP and CRSwNP combined with postoperative follow up and multiple treatments after operation of the nasal polyp with a nasal endoscope. So cooperating with systematized medicine treatment might bring a lower recurrence rate of nasal polyp and might be propitious to the diminished inflammation of ANP [27, 28]. The up-regulation of Treg cells could be a potential target for suppressing persistent mucosal inflammation in nasal polyps and postoperative recurrence.

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### Disclosure of conflict of interest

None.

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