ORIGINAL ARTICLE

Characterizing T-Cell Phenotypes in Nasal Polyposis in Chinese Patients

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Abstract

Background: Nasal polyposis has different etiologies in Western and Eastern countries. Furthermore, its pathogenesis is still poorly understood.
Objective: To determine the T-cell phenotypes involved in nasal polyposis in Chinese patients.
Methods: Twenty-four Chinese patients with nasal polyps were studied. CD4, CD8, Foxp3, and interleukin (IL) 17 were analyzed by immunohistochemical staining. Expression of T-bet, GATA-3, Foxp3, and RORγt mRNA was detected by real-time polymerase chain reaction. The levels of T-cell cytokines (IL-4, IL-5, interferon [IFN] γ, IL-10, IL-17, and transforming growth factor [TGF] ß) were determined using enzyme-linked immunosorbent assay, and serum immunoglobulin (Ig) E levels were measured using the UNICAP system.
Results: Increased expression of $CD4^+$ and $CD8^+$ and decreased expression of Foxp3 and IL-17 were detected in nasal polyps compared with control tissue. Furthermore, expression of T-bet and GATA-3 mRNA was upregulated, whereas Foxp3 mRNA expression was markedly downregulated. Furthermore, increased levels of IFN-γ, IL-4 and IL-5 and decreased levels of IL-10 and TGF-ß were found in nasal polyps. There was no association between *Staphylococcus aureus* exotoxin (SAE)-specific IgE and T regulatory cell (Treg) insufficiency in nasal polyps.
Conclusions: Our findings demonstrate that excessive infiltration of $CD4^+$ and $CD8^+$ T cells in nasal polyps may be associated with expression of $Foxp3^+$ by Tregs but not with SAEs in Chinese patients.

Key words: Chronic rhinosinusitis. Nasal polyps. T regulatory cells. Transcription factor. Cytokine.

Resumen

Antecedentes: la poliposis nasal se debe a diferentes etiologías en los países orientales y occidentales. Además, su patogenia es poco conocida.
Objetivo: Determinar los fenotipos de células T implicados en la poliposis nasal en pacientes chinos.
Métodos: Se estudiaron 24 pacientes chinos con pólipos nasales. Se evaluó mediante tinción inmunohistoquímica CD4, CD8, Foxp3, e interleucina (IL) 17. Se detectó la expresión del ARNm de T-bet, GATA-3, Foxp3 y RORγt por reacción en cadena de la polimerasa. Se determinaron los niveles de citocinas (IL-4, IL-5, interferón [IFN] γ, IL-10, IL-17, y TGF-ß) empleando ensayo enzimo-inmunoanálisis adsorbente, y niveles séricos de inmunoglobulina (Ig) E empleando el sistema UNICAP.
Resultados: Se detectó un aumento de la expresión de $CD4^+$ y $CD8^+$ y disminución de la expresión de Foxp3 e IL-17 en pólipos nasales comparados con tejido control. Además, la expresión del ARNm de T-bet y GATA-3 se encontraba sobreexpresada, mientras que la expresión del ARNm de Foxp3 se encontraba marcadamente disminuida. Asimismo, se encontraron niveles elevados de IFN-γ, IL-4 y IL-5 y disminución de los niveles de IL-10 yTGF-ß en los pólipos nasales. No se observó asociación entre la IgE específica frente a la exotoxina del *Staphylococcus aureus* (SAE) y la deficiencia en células T reguladoras (Tregs) en los pólipos nasales.
Conclusiones: Nuestros hallazgos demuestran que un exceso de infiltración de células T $CD4^+$ y $CD8^+$ en los pólipos nasales, podría estar asociado con la expresión de $Foxp3^+$ por las células Tregs, pero no por SAEs en pacientes chinos.

Palabras clave: Rinosinusitis crónica. Pólipos nasales. Células T reguladoras. Factor de transcripción. Citocina.

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a subtype of chronic inflammation of the mucous membrane in the paranasal sinus that can be distinguished from chronic rhinosinusitis without nasal polyps [1]. Histology reveals the selective accumulation of numerous eosinophils, neutrophils, lymphocytes, plasma cells, and mast cells, as well as the formation of nasal polyps [2]. Although CRSwNP has been widely studied, its pathogenesis is still not fully understood [3].

Histologically, nasal polyps can be divided into 4 types: edematous (eosinophilic), fibrotic (noneosinophilic), glandular, and atypical [4]. The types of inflammatory cells that infiltrate nasal polyps differ in Asian and Western patients [5,6]. Bachert et al [5] reported that eosinophilic nasal polyps were found in more than 80% of Western patients with CRSwNP, whereas Hao et al [7] reported that the incidence of fibrotic polyps (mainly accumulation of lymphocytes and neutrophils) was relatively higher (more than 40%) in Asian patients with CRSwNP. More recently, Zhang et al [8] reported a lower incidence of edematous nasal polyps in Chinese patients than in European patients. We also found that fibrotic polyps were present in nearly 60% of Chinese patients with CRSwNP, while edematous polyps were present in less than 40% of those patients (unpublished data). Therefore, the pathogenic mechanisms of nasal polyps vary in Eastern and Western populations; this could be due to different living environments and genetic conditions. However, data on the pathogenesis of nasal polyps in Chinese patients remain scant.

To address this issue, we characterized a group of Chinese patients for whom histology testing revealed typical accumulations of lymphocytes and neutrophils. Based on reports that mixed helper T (T_H) patterns (T_H1/T_H2) are present in CRSwNP and *Staphylococcus aureus* exotoxins (SAEs) are associated with polyp formation in white patients [9,10], we used immunohistochemical staining to investigate the biomarkers CD4, CD8, Foxp3, and interleukin (IL) 17 in nasal polyps. We also detected the expression of T-bet, GATA-3, Foxp3, and RORγt mRNA using reverse transcriptase-polymerase chain reaction (RT-PCR). The levels of T-cell cytokines (IL-4, IL-5, interferon [IFN] γ, IL-10, IL-17, and transforming growth factor [TGF] ß) were determined using an enzyme-linked immunosorbent assay (ELISA) (Bioss, Beijing, China), and serum immunoglobulin (Ig) E levels were measured using the UNICAP system (Phadia, Uppsala, Sweden).

Patients and Methods

Patients

The study population comprised 24 patients with CRSwNP, and all 24 had fibrotic nasal polyps according to histological analysis (Figure 1, A-C). Diagnosis was based on clinical history, anterior rhinoscopy, nasal endoscopy, and paranasal computed tomography (CT) scan. All patients met the American Academy of Otolaryngology-Head and Neck Surgery Chronic Rhinosinusitis Task Force criteria for CRSwNP [11]. CT scans were staged according to Lund and Kennedy [12], and polyps were graded by size and extension in both the left and right nasal fossa on a scale of 0 to 3. Atopic status was evaluated by skin prick tests to common aeroallergens. Asthma was diagnosed by a pneumologist. All patients were refractory to medical treatment (oral antibiotics, topical corticosteroids, decongestants, and mucolytic agents for more than 6 weeks)

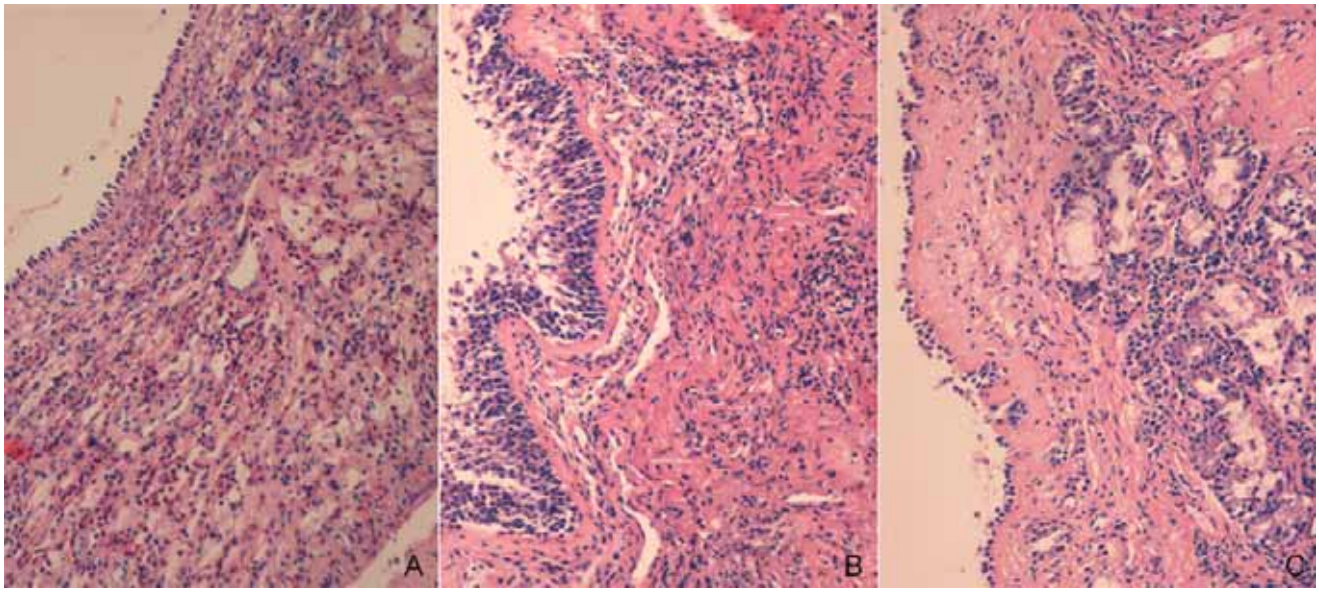

Figure 1. Histological analysis showing the types of nasal polyps in Chinese patients with CRSwNP. A, Edematous (predominantly eosinophilic). B, Fibrotic (predominantly noneosinophilic). C, Glandular (magnification ×200). CRSwNP indicates chronic rhinosinusitis with nasal polyps.

Table. Patient Demographics, and Cell Numbers and Cytokine Levels in Nasal Polyps[a]

	Controls (n=11)	CRSwNP (n=24)	*P* value
Sex (Male: Female)	7:4	14:10	>.05
Age, y	31-55	20-51	>.05
Positive SPT results	0/11	4/24	<.001
Asthma in history and current	0/11	0/24	>.05
Smoking	2/11	5/24	>.05
Duration of CRSwNP, y	0	3.5	
CT score (Lund-McKay)	0	15.8	
Total polyp scores/2 sides	0	4.7	
CD4	887 (314-1678)	1279 (275-1840)	.002
CD8	758 (155-1410)	1050 (187-1329)	.016
Foxp3	83.2 (11-162)	35.4 (7-69)	.036
IL-17	75.3 (12-176)	44.8 (9-106)	.041
IFN–γ	39.2 (15.5-87.4)	114.2 (25.9-174.5)	.013
IL4	8.5 (0-19.1)	18.9 (5.5-39.2)	.008
IL5	7.0 (0-15.2)	14.7 (4.5-29.4)	.004
IL-10	67.2 (19.3-107.2)	32.5 (12.5-55.7)	.019
TGF-ß	115.2 (43.5-158.9)	65.5 (21.3-100.4)	.022
IL-17	37.5 (11.9-90.4)	39.4 (20.4-115.8)	.055

Abbreviations: CRSwNP, chronic rhinosinusitis with nasal polyps; CT, computed tomography; IFN, interferon; IL, interleukin; SPT, skin prick test; TGF, transforming growth factor.
[a] Cell numbers (cells/mm^2) and cytokine concentrations (pg/mL) are expressed as the median (interquartile range).

and had previously undergone endoscopic sinus surgery. Patients with a single polyp (antrochoanal, sphenochoanal) or with other diseases associated with nasal polyps (cystic fibrosis, primary ciliary dyskinesia, and fungal rhinosinusitis) were excluded. The use of local or systemic corticosteroids or other medication was stopped at least 4 weeks before endoscopic sinus surgery. Eleven patients with a deviated septum were recruited as a control group. These patients had no history of respiratory disease or allergy, and their skin prick test results were negative. Patient characteristics are shown in the Table. Our institutional Ethics Committee approved the study, and all patients gave their informed consent.

During surgery, samples of polyp tissue were taken from the patients, and samples were taken from the inferior turbinate in the control group. Each sample obtained was divided into 2 parts: the first was fixed in 4% formalin and embedded in paraffin for histological analysis, while the second was snap-frozen at –80ºC immediately for isolation of mRNA and protein.

Immunohistochemistry

Four-micrometer sections were obtained from the paraffin-embedded specimens and stained using the avidin-biotin complex technique. The slides were dewaxed in xylene and dehydrated in alcohol before the endogenous peroxidase was blocked by incubating the slides in 3% hydrogen peroxide in methanol for 30 minutes. All sections were pretreated with blocking horse serum for 30 minutes before being washed with phosphate-buffered saline (PBS) and incubated overnight at 4ºC with monoclonal antibodies to CD4 (1:200) and CD8 (1:300) (Novacastra, Newcastle, UK), IL-17 (1:200), and Foxp3 (1:100) (Santa Cruz Biotech, Santa Cruz, California, USA), according to the manufacturer's instructions. The samples were then incubated in biotinylated secondary antibody (Zhongshan Co., Beijing, China), followed by avidin-peroxidase complex. After additional washing steps, the slides were stained with 3% diaminobenzidine chromogen, counter-stained with hematoxylin, and affixed with a coverslip. Isotype-matched IgG was used in place of the primary antibody as the negative control.

The sections were coded randomly and counted blind by an observer under a light microscope with an eyepiece reticle at a magnifcation of ×400. The numbers of stained cells in 1 mm^2 of tissue were evaluated using 10 reticles (10×0.1 mm^2) chosen randomly from a single section by 2 independent operators who were blinded as to their origin. A total of 5 sections from each sample were examined.

Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Real-time RT-PCR was performed as in our previous report [13]. Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, Carlifornia, USA) following the manufacturer's instructions. RT was performed and the cDNA was synthesized from 2 μg of total RNA by using an oligo (dT)18 primer and M-MLV reverse transcriptase (TAKARA, Syuzou, Shiga, Japan) for quantitative PCR. Expression of mRNA was determined using the ABI PRISM 7300 Detection System (Applied Biosystems, Foster City, California, USA) and SYBR Premix Taq (TAKARA). The

primer sequences were as follows: T-bet (NM_013351) forward: 5'-GAT GTT TGT GGA CGT GGT CTT G-3'; T-bet reverse: 5'-CTT TCC ACA CTG CAC CCA CTT-3'; GATA-3 (NM_002051) forward: 5'-GCG GGC TCT ATC ACA AAA TGA-3'; GATA-3 reverse: 5'-GCT CTC CTG GCT GCA GAC AGC-3'; Foxp3 (NM_014009) forward: 5'-GAG AAG CTG AGT GCC ATG CA-3'; Foxp3 reverse: 5'-AGG AGC CCT TGT CGG ATG AT-3'; RORγt (NM_001001523) forward: 5'-TGA GAA GGA CAG GGA GCC AA-3'; RORγt reverse 5'-CCACAGATTTTGCAAGGGATCA-3'; ß-actin (NM_001101) forward: 5'-AAG ATG ACC CAG ATC ATG TTT GAG ACC-3'; ß-actin reverse 5'-AGC CAG GTC CAG ACG CAG GAT-3'. PRISM samples contained SYBR Green I Master Mix, 1.5 µL 5 µM primers, and 25 ng of synthesized cDNA in a 25-µL volume. The reaction solutions were heated to 95ºC for 10 minutes, followed by 40 cycles of denaturation at 95ºC for 10 seconds, and an annealing extension at 60ºC for 60 seconds. All PCR reactions were performed in duplicate. Melting curve analysis was used to control for amplification specificity. The mean value of the replicates for each sample was calculated and expressed as a cycle threshold (Ct) value. The relative expression of each target gene was determined as the difference (ΔCt) between the Ct value of the target gene and the Ct value of ß-actin. Fold changes in the target gene mRNA were determined as $2^{-\Delta\Delta Ct}$.

ELISA

The levels of cytokines in nasal tissue were determined using ELISA. Nasal tissue (100 mg) was homogenized in 1 mL 0.9% sodium chloride solution on ice and centrifuged at 4ºC and 3000 rpm for 10 minutes. Their supernatants were collected and stored at –80ºC. The levels of IFN-γ, IL-4, IL-5, IL-10, TGF-ß, and IL-17 in the supernatants were determined using cytokine-specific ELISA kits (Bioss, Beijing, China) according to the manufacturer's instructions. All assays were performed in duplicate. The results are expressed in pg/mL.

IgE Detection

The levels of total IgE and specific IgE (sIgE) to SAEs (determined for a mix of SAEs including SAE A, SAE C, and toxic shock syndrome toxin-1) were measured using the UNICAP system.

Statistical Analysis

Data are presented as the median (interquartile range [IQR]). A nonparametric test (Mann-Whitney U test) was used to compare data. Statistical significance was set at $P<.05$ for all analyses.

Results

Immunohistochemical Analysis of CD4, CD8, Foxp3 and IL-17 Cells in Nasal Tissue

We analyzed expression of CD4 and CD8 using immunohistochemical staining to determine T-cell phenotypes

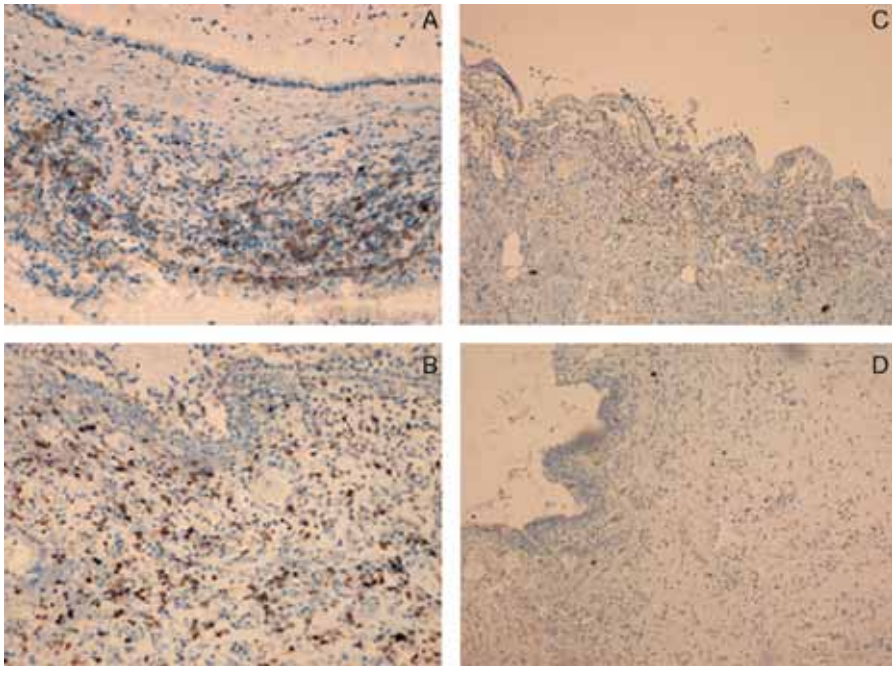

Figure 2. Immunohistochemical staining of the biomarkers CD4, CD8, Foxp3, and IL-17 in nasal tissue. Nasal tissue sections were prepared from patients with CRSwNP and controls. Immunostains of CD4, CD8, Foxp3, and IL-17 were characterized by immunohistochemical techniques using a specific antibody. Data shown are representative of those cells in nasal polyps: A, CD4. B, CD8 (magnification ×200). C, Foxp3. D, IL-17 (magnification ×100).

in nasal tissue, and counted the positive cells. Foxp3 and IL-17 protein were also detected (Figure 2, A-D). As illustrated in the Table, we found that the numbers of $CD4^+$ and $CD8^+$ T cells were significantly greater in nasal polyps than in the mucosa of the control patients ($P<.05$ and $P<.01$, respectively). Interestingly, the numbers of $Foxp3^+$ and $IL\text{-}17^+$ cells in nasal polyps were significantly smaller than in the mucosa of the control patients ($P<.05$ and $P<.01$, respectively).

Relative Levels of T-bet, GATA-3, Foxp3 and RORγt mRNA Expression in Nasal Polyps

We then determined the levels of T-bet, GATA-3, Foxp3, and RORγt mRNA transcripts in nasal polyps and control mucosa using real-time RT-PCR (Figure 3) to detect the expression of T-bet, GATA-3, Foxp3 and RORγt mRNA in all specimens. We found that, when compared with the mucosa of the control patients, expression of mRNA for T-bet and GATA-3 mRNA was significantly upregulated in nasal polyps, whereas expression of mRNA for Foxp3 was significantly downregulated ($P<.05$). However, there was no significant difference between expression of RORγt mRNA in nasal polyps and expression of RORγt mRNA in the mucosa of the control patients ($P>.05$).

Levels of Inflammatory Cytokines and Anti-inflammatory Cytokines in Nasal Polyps

We also examined levels of cytokines expressed in nasal tissue using cytokine-specific ELISA assays. We found that levels of inflammatory T_H1 and T_H2 cytokines (IFN-γ, IL-4, and IL-5) in nasal polyps were significantly higher and that levels of anti-inflammatory cytokines (IL-10 and TGF-ß) were significantly lower than those observed in the mucosa of control participants ($P<.05$). Notably, the mean levels of IL-4, IL-5, and IFN-γ in nasal polyps increased 2.4-, 3-, and 2.4-fold, respectively, over those in the mucosa of control patients, suggesting that mixed T_H1/T_H2 inflammation dominated in these nasal polyps.

Detection of Total IgE and SAE-Specific IgE in Nasal Polyps

SAE-specific IgE has recently been associated with the development of CRSwNP in Western populations [9]. We tested whether a similar IgE response occurred in the nasal polyps of Chinese patients with CRSwNP. The levels of total IgE and SAE-specific IgE in nasal tissue were determined using the UNICAP system. The levels of total IgE in the nasal polyps of patients with CRSwNP were significantly higher than those in the

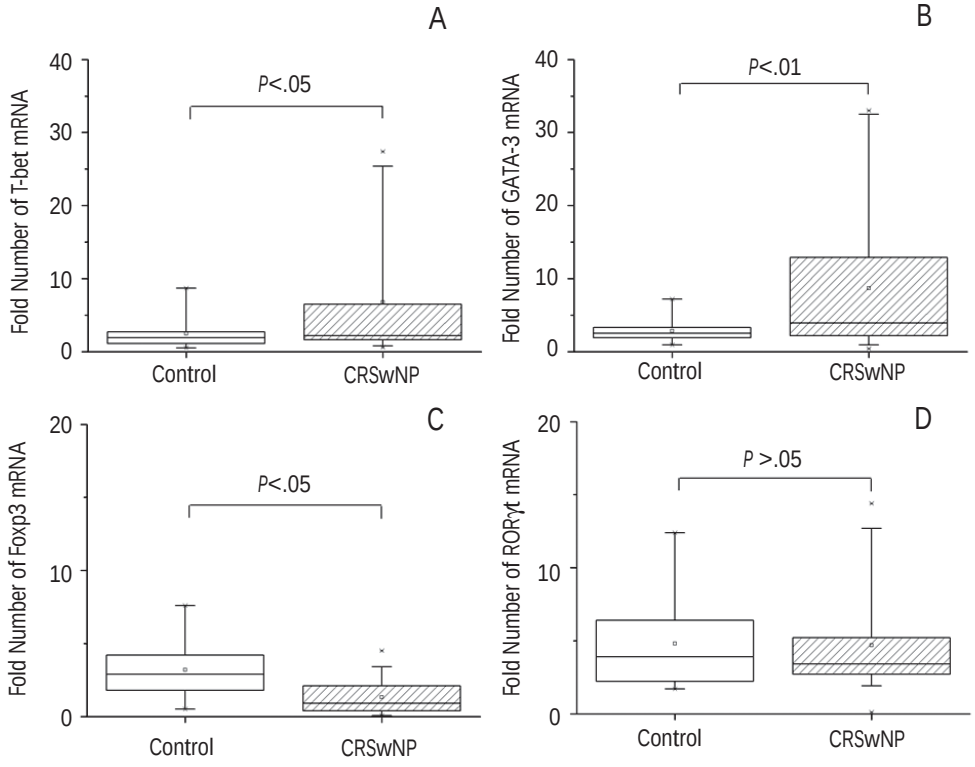

Figure 3. The relative levels of T-bet, GATA-3, Foxp3, and RORγt mRNA in nasal tissue. The relative levels of T-bet, GATA-3, Foxp3 and RORγt mRNA in nasal tissue were determined by RT-PCR. Data are expressed as the median (interquartile range) (n=24 for nasal polyps and n=11 for control mucosa) from 2 independent experiments. RT-PCR indicates reverse transcriptase-polymerase chain reaction.

mucosa of control patients (71.2 kU_A/mL vs. 19.4 kU_A/mL, $P<.05$). However, sIgE was found in only a few patients. Two of the 24 patients with CRSwNP and 1 of the 11 control patients had low levels of sIgE in nasal tissue (10.6 kU_A/mL, 17.4 kU_A/mL, and 16.8 kU_A/mL, respectively). SAE-specific IgE did not seem to be associated with the development of nasal polyps in our patients.

Discussion

Even though a number of guidelines, consensus documents, and position papers have been published during the past few years, data on chronic rhinosinusitis remain limited, and the disease has not been clearly defined [1,2]. Few reports focus on fibrotic nasal polyps in Asian populations [7,14]. Therefore, we designed this study to determine T-cell phenotypes in a Chinese population with CRSwNP. To our knowledge, this is the first study to investigate T-cell phenotypes and evaluate T regulatory cell (Treg) insufficiency in this group.

Using immunohistochemical staining, we show excessive accumulation of $CD4^+$ and $CD8^+$ T cells in Chinese patients with nasal polyps, and a decrease in $Foxp3^+$ and IL-17^+ cell titers. This is consistent with evidence that nasal polyps present mixed T_H1/T_H2 infiltration [15]. We also present the first evidence that $CD8^+$ T cells can contribute to the formation of nasal polyps that have not received timely treatment, thus suggesting the importance of a cytotoxic immune response mediated by $CD8^+$ T cells.

To further understand the mechanisms underlying the formation of nasal polyps, we analyzed the mRNA expression of 4 types of transcription factors. Naive $CD4^+$ T cells are known to develop into at least 4 types of committed T cells, namely T_H1, T_H2, Treg ($Foxp3^+CD4^+CD25^+$), and T_H17 cells (IL-17^+). During differentiation of T cells, each lineage is characterized by its own cytokine profile (IFN-γ for T_H1, IL-4 and IL-5 for T_H2, IL-10 and TGF-ß for Treg, and IL-17 for TH17 cells) and transcription factor (T-bet for T_H1, GATA-3 for T_H2, Foxp3 for Treg, and RORγt for T_H17 cells) [16]. Interestingly, significantly higher levels of T-bet and GATA-3 mRNA and lower levels of Foxp3 mRNA were observed in nasal polyps than in mucosa from control patients. This evidence is consistent with findings in white patients [17]. However, there was little change in levels of RORγt mRNA in nasal polyps, suggesting that T_H17 cells may not be a major player in the pathogenesis of nasal polyps in Chinese patients. We concluded that there was also Treg insufficiency in nasal polyps, based on lower levels of Foxp3 mRNA expression and $Foxp3^+$ cell infiltration. Therefore, the results indicate that the pathogenesis of nasal polyps is based on a common T-cell regulation mechanism in both Eastern and Western populations, despite differences in the accumulation of eosinophils.

Treg insufficiency and dysregulation of the T_H1/T_H2 network are implicated in the development of various autoimmune and allergic diseases [18]. In our previous study, we demonstrated that the expression of transcription factor Foxp3 was downregulated in patients with allergic rhinitis [19]. Similarly, Bruaene et al [17] recently demonstrated in a Western population with CRSwNP that decreased Foxp3 mRNA expression was accompanied by upregulation of T-bet and GATA-3 mRNA and downregulation of TGF-ß1 protein. These results, and those of the present study, enable us to speculate that $Foxp3^+$ Tregs could play an essential role in the regulation of T-cell profiles in nasal inflammation, and that induction of $Foxp3^+$ Tregs may be a potential strategy for the management of nasal polyps and other diseases, as confirmed partially in our recent study about the effect of intranasal corticosteroids on $Foxp3^+$ expression by Treg in nasal polyps [13].

To further prove our hypothesis about the association between excessive numbers of $CD4^+$ and $CD8^+$ T cells and $Foxp3^+$ Tregs in nasal polyps, we measured levels of T-cell cytokines (IL-4, IL-5, IFN-γ, IL-10, IL-17, TGF-ß) and serum IgE. In general, IFN-γ indicates T_H1 cytokine production, IL-4 and IL-5 indicate T_H2 cytokine production, IL-10 and TGF-γ indicate Treg cytokine production, and IL-17 indicates T_H17 cytokine production. The fact that not only Foxp3, but also IL-17, TGF-ß, and IL-10 can originate in cells other than T cells usually leads to confusion, even though Foxp3 was considered the specific marker of Tregs [20]. These markers were still adopted as alternatives, since T cells are considered the major source of cytokines in nasal polyps. Our analysis of T-cell cytokine profiles in nasal tissue revealed that increased levels of IL-4, IL-5, and IFN-γ, as well as decreased IL-10 and TGF-ß production, were observed in nasal polyps. Therefore, the consistency of transcription factors Foxp3 and cytokines IL-10 and TGF-ß confirmed their importance in the pathogenesis of nasal polyps. However, we failed to find increased levels of SAE-specific IgE in the supernatant of homogenized tissue. In fact, we detected specific IgE in only 2 patients with CNSwNP (10.6 kU_A/mL and 17.4 kU_A/mL, respectively) and 1 normal control (16.8 kU_A/mL), while total IgE was significantly higher in nasal polyps than in control tissues. In several cases, we did not ascribe this to atopy, because atopy has been thought not to significantly affect inflammatory mediators in nasal polyps [21,22]. Several authors have recently suggested that SAEs could be associated with inflammatory cell accumulation and polyp formation [9,23]. Seiberling et al [23] detected higher levels of SAEs (14/29) in American patients with CRSwNP, and Bachert et al [9] established a correlation between the presence of IgE and staphylococcal toxins in nasal polyps. Our results on the level of SAE-specific IgE were obviously different from those reported above. Therefore, our findings may provide new insight into the pathogenic mechanism of nasal polyps in Chinese patients. This mechanism is different from that of Western patients with CRSwNP, which mainly consists of edematous nasal polyps. We speculate that the T-cell subtype pattern is associated with infection—possibly fungi or other pathogens—in poor environmental conditions.

In summary, our findings show that Treg insufficiency and excessive accumulation of $CD8^+$ and $CD4^+$ T cells in nasal polyps in Chinese patients are associated with $Foxp3^+$ Treg insufficiency, but not with SAE-specific IgE responses. These new data suggest that the pathogenesis of nasal polyps in Chinese patients is different from that of nasal polyposis in Western patients, a fact which may be helpful in the development of novel therapeutic strategies.

Acknowledgments

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