



Reciprocal roles of STAT3 and STAT5 in nasal polyposis[☆]

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Abstract

Purpose: Nasal polyposis (NP) is a chronic inflammatory disease that is characterized by increased populations of Th17 cells and impairment of Treg cells function in Chinese patients. Recent studies have shown that signal transducer and activator of transcription 3 (STAT3) and STAT5 are indispensable in the development and maintenance of Th17 and Treg cells. We investigated the roles of STAT3 and STAT5 in the imbalance of Th17 and Treg cells in NP.

Materials and methods: The levels of IL-6, IL-2, pSTAT3, pSTAT5, SOCS3, RORc, Foxp3, IL-17A, and TGF- β 1 were measured in patients with atopic NP, patients with nonatopic NP, and controls. We also evaluated the local distribution of Th17 and Treg cells by double immunofluorescence staining and the correlations between activated STAT3/STAT5 and Th17/Treg cell development were assessed.

Results: Increased levels of IL-6, pSTAT3, SOCS3, RORc, IL-17A, and CD4⁺ RORc⁺ cells, and decreased levels of IL-2, pSTAT5, Foxp3, TGF- β 1, and CD4⁺ Foxp3⁺ cells were detected in both NP groups compared to controls ($P < .05$). The differences in all expression levels (except for IL-6) were significant between atopic and nonatopic patients ($P < .05$). There was a positive correlation between pSTAT3/pSTAT5 levels and Th17/Treg development and a negative correlation between SOCS3 and pSTAT3 in NP ($P < 0.01$).

Conclusions: The results suggest that STAT3 and STAT5 may function through the IL-6 and IL-2 pathways to play a role in the imbalance of Th17/Treg in NP. An even more exaggerated imbalance of Th17/Treg caused by atopy may be correlated to the improper ratio of activated STAT3/STAT5.

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1. Introduction

Nasal polyposis (NP) is characterized by the appearance of hyperplastic polyps in the nasal cavity and paranasal sinuses that can be seen by endoscopy and computed tomography (CT) scanning [1]. The pathophysiology of this disease has been a research focus in the field of otorhinolaryngology owing to its high prevalence and lack of effective treatments [2,3]. Although NP has been widely studied for many years, its pathogenesis is still poorly understood. Previous studies

found that Chinese patients showed a predominantly Th17 response, unlike white patients with the same illness, and that both Chinese and white patients had impaired regulatory T-cell (Treg) function [4,5]. These findings suggested that misregulation of T-effector cells and Treg cells may play a crucial role in the pathogenesis of NP. More recently, Shen et al [6] showed that there is an imbalance of Th17/Treg cells in Chinese patients with NP and that the imbalance was more severe in the atopic group. Although this study provided a deeper understanding of the pathophysiology of NP, the exact mechanism of disease progression remains unknown.

Signal transducers and activators of transcription (STATs) are a family of cytokine- and growth factor-inducible transcription factors that are known to play critical roles in Th cell differentiation [7]. These proteins are phosphorylated at specific tyrosine residues in response to the binding of cytokines to their appropriate receptors. This

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results in their dimerization and translocation to the nucleus, where they modulate gene expression [8]. Among the STAT family of proteins, STAT3 has been implicated in Th17 differentiation via its activation in response to cytokines such as interleukin 6 (IL-6). The IL-6/STAT3 pathway has been illustrated to play a pivotal role in chronic inflammation and autoimmune disorders by promoting Th17 and inhibiting Treg cells [9]. Conversely, STAT5, another member of STAT family, is activated by interleukin 2 (IL-2) and is essential for Treg cell development and has a negative effect on Th17 cell differentiation [10]. Recent studies have focused on the reciprocal effect of STAT3 and STAT5 on T cells, and it appears that STAT3 and STAT5 activities regulate the Th17/Treg balance [11,12]. As an imbalance of Th17/Treg exists in NP, we speculate that the reciprocal effects of STAT3 and STAT5 may contribute to imbalance of Th17/Treg in NP. The aggravated imbalance of Th17/Treg caused by atopy may be correlated to more severe improper ratio of activated STAT3/STAT5.

To test this hypothesis, we measured the expressions of IL-6, IL-2, pSTAT3, pSTAT5, and suppressor of cytokine signaling 3 (SOCS3), a key inhibitor of the STAT3 pathway, in an atopic NP group, a nonatopic NP group, and controls, to determine the level of activation of the STAT pathways. We then evaluated the expression levels of the transcription factors; retinoid acid–related orphan receptor C (RORc) and forehead box P3 (Foxp3); levels of CD4⁺ RORc⁺ cell, CD4⁺ Foxp3⁺ cell, IL-17A, and TGF- β 1 as indicators of Th17; and Treg cell development. Finally, we analyzed the correlations between STAT3/STAT5 activities and development of Th17/Treg. To our knowledge, this is the first study on the relationship between STAT pathways and the imbalance of Th17/Treg in NP.

2. Materials and methods

2.1. Patients

This study was approved by the ethical committee of Chongqing Medical University and informed consent was obtained from all patients and controls. Forty patients with NP were recruited for this study, and the enrollment standard and the diagnosis of atopic status were according to established criteria from previous studies [4–6]. Clinical data about patients included sex, age, duration of disease, history of asthma, and recurrence. Symptom scores were assessed according to a visual analog scale [13]. The preoperative CT scans were graded according to the classification by Lund and Kennedy [14]. The preoperative nasal endoscopy scores were graded according to the classification by Lanza and Kennedy [15]. Patients who had antrochoanal polyps, cystic fibrosis, primary ciliary dyskinesia, fungal sinusitis, or gastroesophageal reflux disease were excluded. Fifteen patients with a deviated septum were recruited as controls. These patients had no history of respiratory disease or atopy, and their skin prick test results were negative. Oral and topical applications

of corticosteroids or antihistamines were withheld for at least 3 months, and all patients received 3 days of antibiotics before surgery [5]. Patients received surgery only when medical treatment had failed. Nasal polypoid samples were obtained from ethmoidal polyp tissue, and the inferior turbinates from patients undergoing septoplasty or rhinoseptoplasty were used as controls.

2.2. Histologic analysis

The paraffin sections of tissue samples (4–5 μ m thick) were stained with hematoxylin-eosin. The overall histopathologic features were observed under low-power field magnification ($\times 100$) and then high-power field (HPF) magnification ($\times 400$). We used an image analyzer to measure the maximal basement membrane (BM) thickness. This included the subepithelial basal part and the thickened fibrosis beneath the basal part in the most severely thickened regions. The absolute number of eosinophils was counted in 10 randomly selected HPFs of 1 section, and these 10 counts were averaged to obtain the mean number of eosinophils per HPF. All analyses were conducted by 2 independent observers who were blinded to the diagnosis or clinical data of the patients from whom the tissue samples had been obtained.

2.3. Immunohistochemistry

Paraffin sections were dewaxed and dehydrated in alcohol, and nonspecific binding was blocked with 2% bovine serum albumin before immunohistochemical staining. The monoclonal antibodies to pSTAT3 (1:100 dilution), pSTAT5 (1:50 dilution), and SOCS3 (1:100 dilution) (Abcam, UK) were used for staining via the streptavidin-biotin complex method. Nonimmune serum IgG and phosphate-buffered saline were used as the negative controls. We analyzed the number of stained cells at a magnification of $\times 400$ as an indicator of protein expression levels. The counting method was the same as that for determining the number of eosinophils.

2.4. Western blotting

We extracted proteins from sample tissue with the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) using the manufacturer's instructions, and supernatants were separated and stored at -80° C for analysis. Samples containing 25 μ g of protein were boiled, separated by acrylamide gel electrophoresis, and transferred electrophoretically to polyvinylidene fluoride membranes (Beyotime). The membranes were blocked with Western blocking buffer (Beyotime) for 1 hour at room temperature and then incubated with rabbit anti-human pSTAT3, pSTAT5, SOCS3, and β -actin polyclonal antibodies (Abcam) at dilutions of 1:1000. After washing, the membranes were incubated with a secondary antibody linked to horseradish peroxidase (mouse anti-rabbit immunoglobulin G, 1:2000 dilution). Proteins were detected with the BeyoECL Plus (Beyotime) kit according to the manufacturer's instructions. β -Actin was used to normalize the results in each lane.

2.5. Quantitative real-time PCR

We analyzed the mRNA levels of Foxp3 and RORc by means of real-time polymerase chain reaction (PCR). Total RNA was isolated via TRIzol extraction (Invitrogen, USA), and all assays were performed in accordance with the manufacturer's instructions. Total RNA (1000 ng) was reverse transcribed to cDNA using random hexamer primers (Invitrogen), and SYBR Premix Taq (TaKaRa, China) was used to perform the real-time PCR. The following primer sequences were used for RORc: 5'-GCT GTG ATC TTG CCC AGA ACC-3' (forward) and 5'-CTG CCC ATC ATT GCT GTT AAT CC-3' (reverse); for Foxp3: 5'-GAG AAG CTG AGT GCC ATG CA-3' (forward) and 5'-AGG AGC CCT TGT CGG ATG AT-3' (reverse). The PCR protocol consisted of 2 cycles at 95 °C for 30 seconds followed by 40 cycles at 95 °C for 5 seconds and 63 °C for 20 seconds. All PCR reactions were performed in duplicate. The comparative CT method was used to calculate the relative gene expression levels [5,6]. Glyceraldehyde-3-phosphate dehydrogenase was used as the housekeeping gene for normalization, and a no-template sample was used as the negative control.

2.6. Enzyme-linked immunosorbent assay

Samples were weighed and homogenized in 1 mL 0.9% sodium chloride solution on ice per 100 mg of tissue. Then, they were centrifuged at 4 °C and 3000 rpm for 10 minutes. After that, the supernatants were collected and stored at –80 °C. We used cytokine-specific enzyme-linked immunosorbent assay kits (R&D Systems, USA) to assay the levels of IL-2, IL-6, IL-1 7A, and TGF- β 1 in the samples according to the manufacturer's instructions. We performed the assay of each sample in duplicate, and all data are expressed in picograms per milliliter.

2.7. Double immunofluorescence staining

We detected CD4 and RORc or CD4 and Foxp3 by double immunofluorescence staining to investigate the

distribution of Th17 and Treg cells in local tissue. Deparaffinized sections were heated at 95 °C for 10 minutes in the presence of 10 mmol/L citric acid sodium for antigen retrieval and then incubated in blocking solution (10% normal goat serum in phosphate-buffered saline) for 10 minutes. The sections were incubated overnight with the primary antibodies anti-CD4 (1:100 dilution) (Santa Cruz, USA), anti-Foxp3 (1:200 dilution), and anti-RORc (1:200 dilution) at 4 °C. After washing, a fluorescein isothiocyanate-labeled antibody against CD4 (1:100 dilution) (Santa Cruz) and then a CY3-labeled antibody against Foxp3 or RORc (1:200) were incubated with the sections away from light. This was followed by nuclear staining with 4',6-diamidino-2-phenylindole (1:1500 dilution) (Santa Cruz) for 1 hour. Negative control sections were obtained by omission of the primary antibody. The method of cell counting was the same as described above.

2.8. Statistical analysis

We used IBM SPSS version 20.0 (IBM SPSS, Chicago, IL) with the data represented by medians and interquartile ranges for statistical analysis. We used 1-way analysis of variance to analyze the statistical significance of the differences between groups. A Kruskal-Wallis *H* test was used when the equal variance test failed, the Mann–Whitney *U* test was used for comparisons between groups, and the Spearman test was used to determine correlations. The significance level was set at $P < .05$.

3. Results

3.1. Clinical data and histologic characteristics of patients

The clinical characteristics of the study subjects are shown in Table 1. Compared with the nonatopic NP and control groups, the CT score and endoscopy score were significantly higher in the atopic NP group ($P < .05$). On the contrary, there were no statistically significant differences in

Table 1
Clinical data and histologic characteristics of patients with NP

	Control	Atopic NP	Nonatopic NP	<i>P</i> value
Age (year), median (range)	28 (21–50)	36 (22–64)	37 (23–67)	NS
Gender	9M/6F	12M/8F	10M/10F	NS
Asthma	0/15	2/20	0/20	NS*
Duration (year)	0	4 (2–7)	3 (1–5)	NS
Recurrence	0	5/20	3/20	NS*
Symptom score	0	10 (9–14)	9 (8–13)	NS
CT score	0	12.5 (11.25–14.75)	10 (9–12)	<.05
Endoscopy score	0	7.5 (5.25–9.75)	4 (3–6)	<.05
Eosinophils (/HPF)	2.9 (2.0–4.3)	39.7 (33.5–48.9)	14.8 (12.1–19.8)	<.05*,†,‡
BM thickness (μ m)	1.33 (0.90–1.43)	21.13 (16.22–27.04)	16.33 (11.87–22.93)	<.05*,†,‡

Student's *t* test or the Fisher exact test (indicated with “★”) was used for determining statistical significance, and significance was accepted at $P < .05$. NS indicates not significant. Data are expressed as medians and interquartile ranges.

* Control vs atopic NP.

† Control vs nonatopic NP.

‡ Atopic NP vs nonatopic NP.

Table 2

Medians and interquartile range for STAT signaling molecules and Th17/Treg-related cytokines and transcription factors

	Control	Atopic NP	Nonatopic NP	P value
IL-6 (pg/mL)	304 (246–349)	2621 (2417.2–2933.5)	2572 (2448.7–2710.7)	<.05* [†] ; 0.74 [‡]
IL-2 (pg/mL)	1973 (1765–2234)	546 (335.7–681.2)	901 (776–1063)	<.05* [†] , [‡]
pSTAT3 (IHC)	12.3 (10.7–13.1)	58 (53.1–60.2)	31.9 (29.1–35.8)	<.05* [†] , [‡]
pSTAT3 (WB)	0.15 (0.13–0.18)	0.72 (0.65–0.81)	0.55 (0.44–0.60)	<.05* [†] , [‡]
pSTAT5 (IHC)	26.7 (20.5–31.2)	4.8 (3.8–5.1)	15.3 (12.5–16.5)	<.05* [†] , [‡]
pSTAT5 (WB)	0.74 (0.68–0.82)	0.09 (0.07–0.14)	0.41 (0.36–0.52)	<.05* [†] , [‡]
SOCS3 (IHC)	6.4 (3.4–7.5)	22.9 (20.5–29.0)	41.4 (38.1–44.3)	<.05* [†] , [‡]
SOCS3 (WB)	0.12 (0.11–0.14)	0.47 (0.38–0.49)	0.61 (0.56–0.67)	<.05* [†] , [‡]
IL-17A (pg/mL)	143 (96–190)	3882 (3734–4136)	1830 (1656–1984)	<.05* [†] , [‡]
TGF- β 1 (pg/mL)	6652 (5891–6832)	1212.5 (989.2–1463.5)	3300.5 (2815.2–3840.2)	<.05* [†] , [‡]
RoRc	1.90 (1.67–2.78)	8.12 (7.32–8.61)	5.83 (5.22–7.15)	<.05* [†] , [‡]
Foxp3	4.13 (3.46–6.04)	0.92 (0.77–1.69)	1.86 (1.42–2.25)	<.05* [†] , [‡]
CD4 ⁺ RORc ⁺ cells	10.9 (10.6–11.7)	77.1 (71.5–79.2)	24.6 (21.4–27.2)	<.05* [†] , [‡]
CD4 ⁺ Foxp3 ⁺ cells	39 (38.1–40.8)	4.8 (3.6–5.9)	14.7 (11.8–16.5)	<.05* [†] , [‡]

IHC indicates immunohistochemistry; WB, Western blotting.

* Control vs atopic NP.

† Control vs nonatopic NP.

‡ Atopic NP vs nonatopic NP.

symptom score, age, sex, duration of disease, or recurrence among the 3 groups ($P > .05$). Subjects with asthma were only found in the atopic NP group. The eosinophil counts and BM thicknesses increased significantly in both NP groups compared to controls, and statistically significant differences were seen between the atopic NP and nonatopic NP groups ($P < .05$).

3.2. Immunohistochemistry findings

There was a significantly higher number of pSTAT3⁺ cells in both NP groups ($P < 0.05$) compared with controls, whereas the number of pSTAT5⁺ cells was significantly downregulated in both NP groups ($P < .05$) compared with controls. Furthermore, compared to the nonatopic group, the atopic group had a markedly upregulated number of pSTAT3⁺ cells ($P < .05$) and a markedly downregulated number of pSTAT5⁺ cells ($P < .05$). The number of SOCS3⁺ cells was found to be significantly upregulated in patients with atopic NP ($P < .05$) and nonatopic NP ($P < .05$) compared to controls, and there were significantly higher SOCS3⁺ cell counts in the nonatopic group ($P < .05$) compared with the atopic group (Table 2, Fig. 1).

3.3. Western blotting findings

Consistent with the immunohistochemistry results, the levels of pSTAT3 and SOCS3 protein were significantly higher in both NP groups ($P < .05$) compared with controls, and the levels of pSTAT5 protein were significantly lower in both NP groups ($P < .05$) compared with the control group. Moreover, the atopic group had markedly downregulated levels of pSTAT5 and SOCS3 protein as well as a markedly upregulated level of pSTAT3 ($P < .05$) compared to the nonatopic group (Table 2, Fig. 2).

3.4. Quantitative real-time PCR

The level of Foxp3 mRNA was significantly lower and the level of RORc mRNA was significantly higher in both NP groups ($P < .05$) compared with the control group. Furthermore, the nonatopic group had markedly upregulated concentrations of Foxp3 mRNA and markedly downregulated levels of RORc mRNA ($P < .05$) compared to the atopic group (Table 2, Fig. 3A).

3.5. Enzyme-linked immunosorbent assay

There were significantly higher levels of IL-6 and IL-17A in both NP groups ($P < .05$) compared to the control group, which is consistent with the expression levels of pSTAT3. Levels of IL-2 and TGF- β 1 were significantly downregulated in both NP groups ($P < .05$) compared to the control group, which is consistent with the expression levels of pSTAT5. Furthermore, the atopic NP group had a significantly higher level of IL-17 and significantly lower levels of IL-2 and TGF- β 1 ($P < .05$) compared to the nonatopic NP group. No statistically significant difference in the level of IL-6 was seen between the 2 groups with NP ($P > .05$) (Table 2, Fig. 4).

3.6. Double immunofluorescence staining

CD4⁺, Foxp3⁺, and RORc⁺ cells were detected in all samples. Moreover, Foxp3 and RORc were seen mostly in cells that were also CD4⁺ cells. Consistent with the PCR results, there were statistically significant differences in the number of CD4⁺ RORc⁺ cells in both NP groups ($P < .05$) compared to controls, and the number of CD4⁺ Foxp3⁺ cells was significantly downregulated in both NP groups ($P < .05$) compared to the control group. The number of CD4⁺ RORc⁺ cells was significantly higher and the number of CD4⁺ Foxp3⁺ cells was significantly lower in the atopic NP group

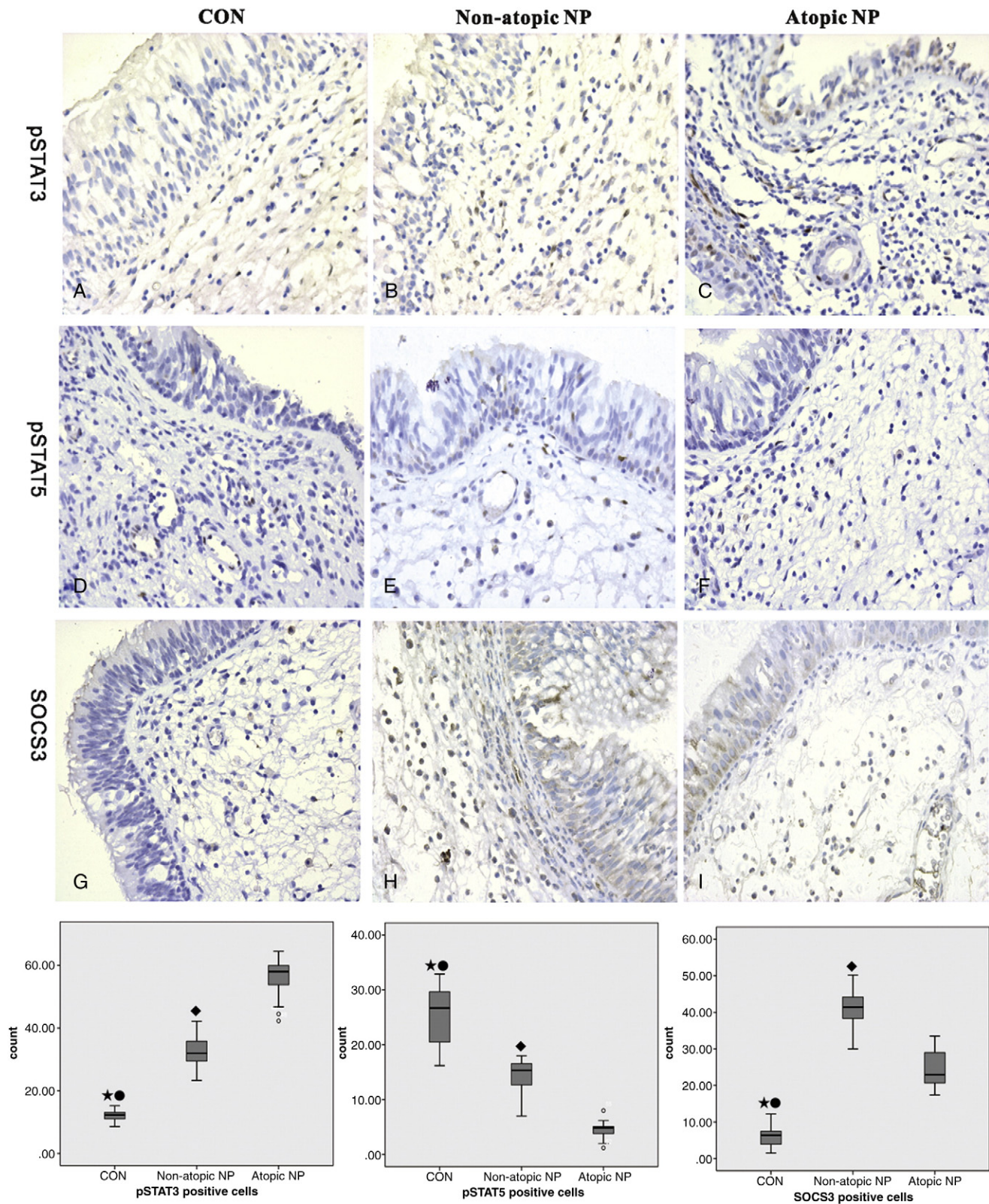


Fig. 1. Expression of pSTAT3⁺, pSTAT5⁺, and SOCS3⁺ cells in nasal mucosa from control and patient samples with atopic NP or nonatopic NP. ★: $P < .05$, controls vs atopic NP; ●: $P < .05$, controls vs nonatopic NP; ◆: $P < .05$, atopic NP vs nonatopic NP. CON indicates controls.

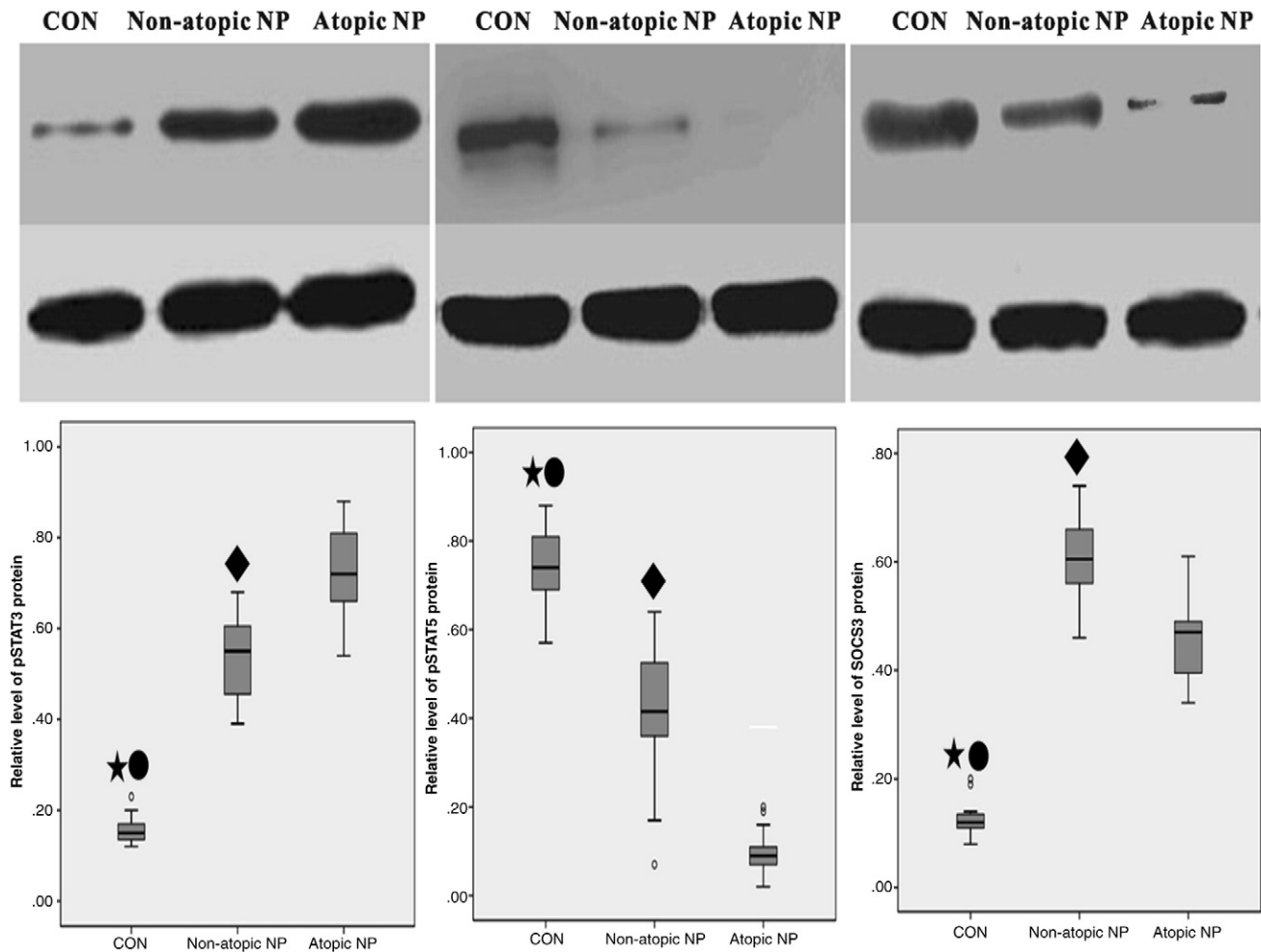


Fig. 2. Western blots of expression of pSTAT3, pSTAT5, and SOCS3 proteins in NP groups and controls. ★: $P < .05$, controls vs atopic NP; ●: $P < .05$, controls vs nonatopic NP; ♦: $P < .05$, atopic NP vs nonatopic NP.

compared to the nonatopic NP group ($P < .05$) (Table 2, Figs. 3B and 5).

3.7. Improper ratio of activated STAT3 and activated STAT5 in patients with NP

We further assayed the improper ratio between activated STAT3 and activated STAT5, which was expressed as the ratio of pSTAT3/pSTAT5. We found that the ratio of pSTAT3/pSTAT5 was highest in the atopic NP group ($P < .05$), lower in the nonatopic NP group ($P < .05$), and lowest in the control group (Table 3, Fig. 3C).

3.8. Correlation between STAT3 and related indicators of Th17 cells

We analyzed the correlations between pSTAT3 expression and RORc and IL-17A expression as well the number of CD4⁺ RORc⁺ cells to assess any correlations between STAT3 and Th17 cells in NP. We observed significantly positive correlations between pSTAT3 and

RORc ($P < .01$), IL-17A ($P < .01$), and CD4⁺ RORc⁺ cells ($P < .01$) (Fig. 6).

3.9. Correlation between STAT5 and related indicators of Treg cells

We also analyzed the correlations between pSTAT5 expression and Foxp3 and TGF- β 1 expression as well as the number of CD4⁺ Foxp3⁺ cells to assess any correlations between STAT5 and Treg cells in NP. We observed significantly positive correlations between pSTAT5 and Foxp3 ($P < .01$), TGF- β 1 ($P < .01$), and CD4⁺ Foxp3⁺ cells ($P < .01$) (Fig. 6).

3.10. Correlation between the IL-6/STAT3 pathway and SOCS3

We assayed the correlation between IL-6, pSTAT3, and SOCS3 expression levels to determine a relationship between the IL-6/STAT3 pathway and SOCS3 in NP. There was a significantly inverse correlation between

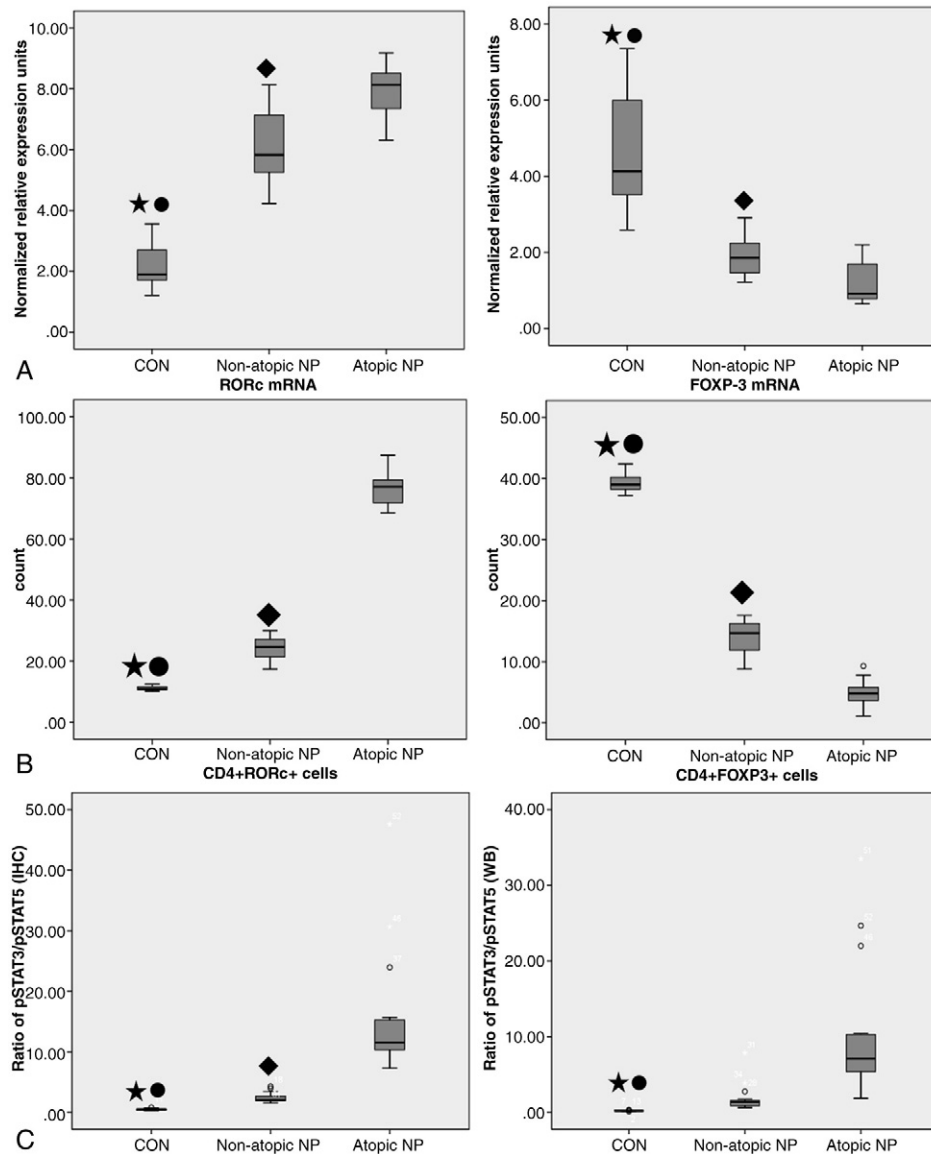


Fig. 3. A, Real-time PCR measurements of the mRNA levels of Foxp3 and RORc in the NP groups and control group. B, Double immunofluorescence stains for CD4⁺ Foxp3⁺ cells and CD4⁺ RORc⁺ cells in the NP groups and controls. C, Ratio of pSTAT3/pSTAT5 by immunohistochemistry (IHC) and Western blotting (WB). ★: $P < .05$, controls vs atopic NP. ●: $P < .05$, controls vs nonatopic NP; ◆: $P < .05$, atopic NP vs nonatopic NP.

pSTAT3 and SOCS3 ($P < .01$) (Fig. 6). However, there was no correlation between IL-6 and SOCS3 ($P > .05$).

3.11. Correlation between pSTAT5 and IL-17A

A significantly negative correlation between pSTAT5 and IL-17A was found in NP ($P < .01$) (Fig. 6).

3.12. Correlations between improper ratio of pSTAT3/pSTAT5 and clinical or histologic features

We tested the relationships between the ratio of STAT3/STAT5 activity and BM and eosinophil count. In accordance with the results of immunohistochemistry (IHC) and Western blotting (WB), the correlation analysis results were expressed as r_{IHC} and r_{WB} . We found that both the

BM ($r_{IHC} = 0.417$, $r_{WB} = 0.405$; $P < .01$) and the eosinophil count ($r_{IHC} = 0.551$, $r_{WB} = 0.484$; $P < .01$) had a significantly positive correlation with the ratio of STAT3/STAT5 activity in NP. Furthermore, the ratio of STAT3/STAT5 activity had a positive relation with CT ($r_{IHC} = 0.451$, $r_{WB} = 0.277$; $P < .01$) and nasal endoscopic check marks ($r_{IHC} = 0.503$, $r_{WB} = 0.490$; $P < .01$).

4. Discussion

Our research expands upon previous studies on the imbalance of Th17/Treg in NP by further investigating the corresponding upstream signaling pathways of Th17 and Treg. Our data show that the IL-6/STAT3 pathway and IL-2/

STAT5 pathway are altered in NP. The increased levels of IL-6 and pSTAT3 proteins and decreased levels of IL-2 and pSTAT5 proteins were consistent with an increase in Th17 cells and a decrease in Treg cells in NP tissues compared to controls. This suggests that an improper ratio of IL-6/STAT3 and IL-2/STAT5 pathways may contribute to the imbalance of Th17/Treg seen in NP and may play a critical role in the development of NP. In the atopic NP group, there was a significantly more improper ratio of pSTAT3/pSTAT5, which represented the activated states of STAT3/STAT5. This is consistent with the larger imbalance of Th17/Treg and the more severe clinical and histologic features seen in the atopic NP group. This suggests that atopy may aggravate the imbalance of Th17/Treg and disease severity by altering the ratio of activated STAT3/STAT5. SOCS3, which is known to have inhibitory effects on IL-6 signaling, was found to be markedly downregulated in the atopic NP group compared to the nonatopic NP group, and there was a significantly negative correlation between pSTAT3 and SOCS3 in patients with NP. This explains why there were significant differences in the expression of pSTAT3 between the 2 groups with NP, but no difference in the level of IL-6 expression.

Treg cells are a critical subset of cells that control inflammatory responses [16]. Foxp3 is believed to be a specific Treg marker that is essential for Treg development and maintenance of the cells in the periphery [17]. Recently, it has been demonstrated that IL-2 can upregulate the expression of Foxp3 through a STAT5-dependent mechanism in human Treg cells, and that is probably due to the fact that STAT5 can directly bind Foxp3's promoter [10,18–20]. Thus, the IL-2/STAT5 signaling pathway should be critical for Treg cell development. In vitro experiments [21] have, indeed, shown that, if STAT5 is deleted in CD4⁺ T cells, the T cells are incapable of differentiating into Treg cells. In the present study, we have demonstrated decreased expression of Foxp3 and TGF- β 1 in patients with NP, which is consistent with the results of previous studies [4–6]. We observed significantly lower IL-2 and pSTAT5 levels in patients with NP and demonstrated a significantly positive correlation between activated STAT5 and Treg cells. These results suggest that the IL-2/STAT5 pathway may have an important effect on the function and development of Treg cells in NP. We suspect that defective IL-2/STAT5 pathway activity may play a role in impaired activity of Treg cells by way of its

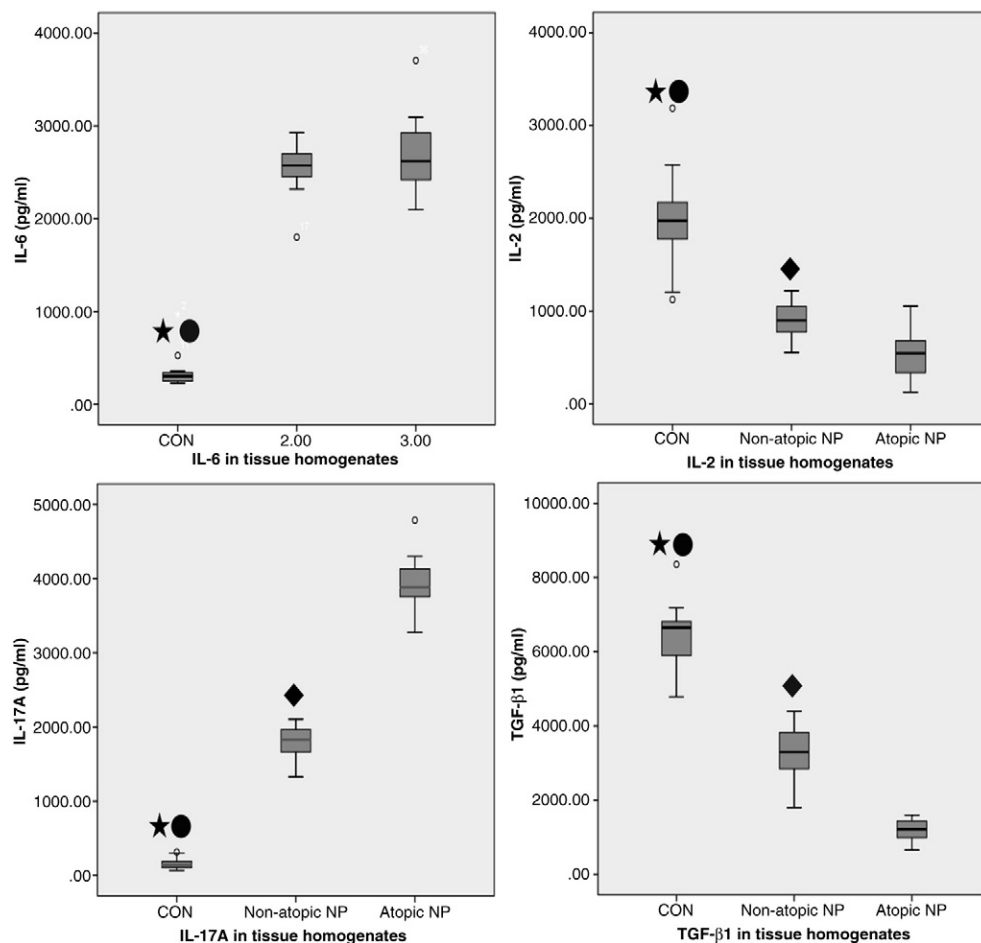


Fig. 4. The levels of IL-2, IL-6, IL-17A, and TGF- β 1 in patients with atopic NP, nonatopic NP, and controls as analyzed by enzyme-linked immunosorbent assay. ★: $P < .05$, controls vs atopic NP; ●: $P < .05$, controls vs nonatopic NP; ◆: $P < .05$, atopic NP vs nonatopic NP.

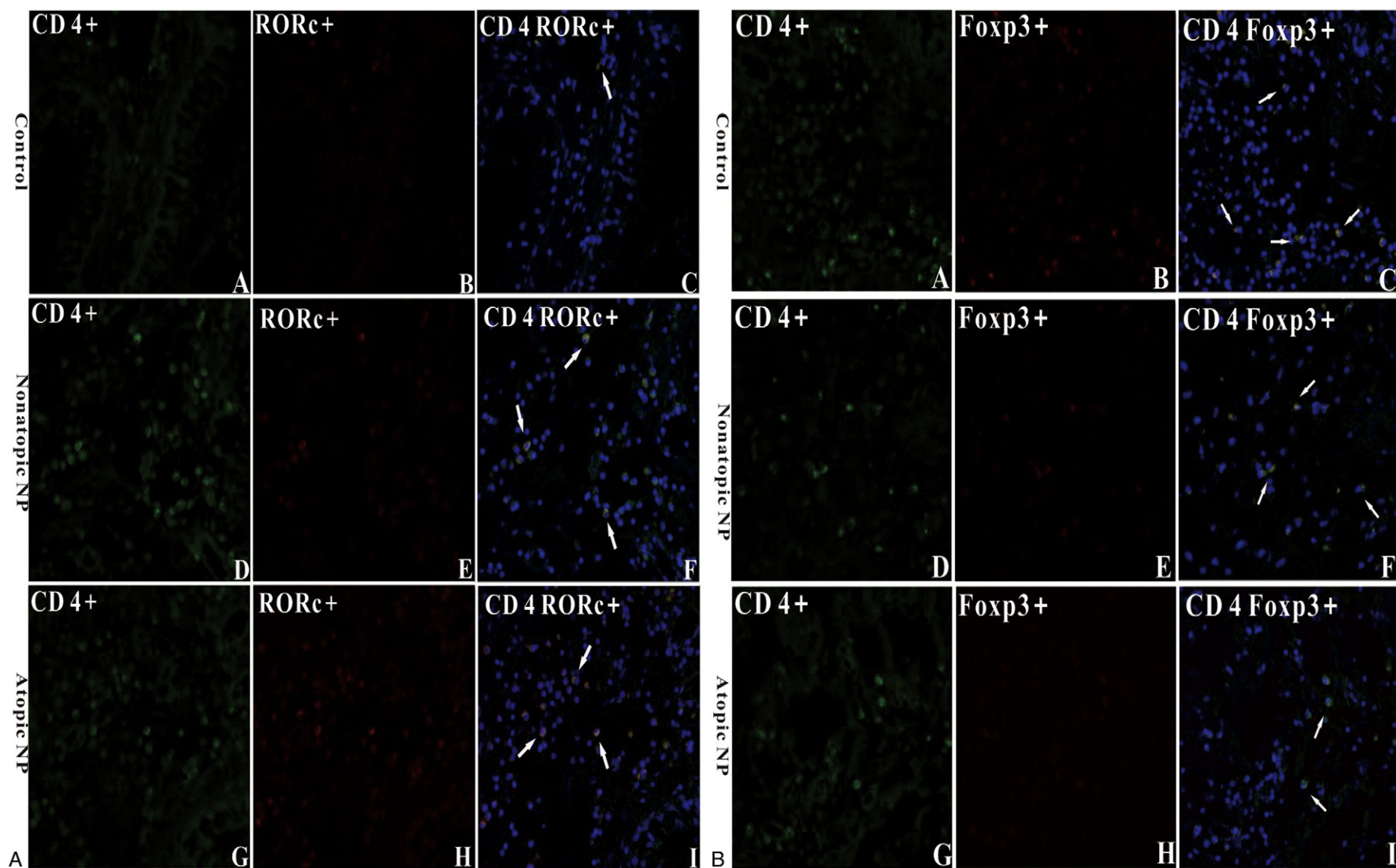


Fig. 5. A, Double immunofluorescence stains for CD4⁺ RORc⁺ cells in the NP groups and controls. CD4 co-localization with RORc in NP: CD4 FITC (green); RORc CY3 (red); double-positive cell (short arrow, orange). B) Double immunofluorescence stains for CD4⁺ Foxp3⁺ cells in the NP groups and controls. CD4 co-localization with Foxp3 in NP: CD4 FITC (green); Foxp3 CY3 (red); double-positive cell (short arrow, orange). FITC indicates fluorescein isothiocyanate.

Table 3

Medians and interquartile ranges for the activated STAT3/ STAT5 ratio

	Control	Atopic NP	Nonatopic NP	P value
pSTAT3/pSTAT5 (IHC)	0.48 (0.40–0.56)	11.56 (10.33–15.30)	2.07 (1.91–2.73)	<.05*,†,‡
pSTAT3/pSTAT5 (WB)	0.21 (0.19–0.24)	7.09 (5.37–10.31)	1.38 (0.87–1.59)	<.05*,†,‡

* Control vs atopic NP.

† Control vs nonatopic NP.

‡ Atopic NP vs nonatopic NP.

effect on Foxp3 expression. This defective immunosuppressive activity may then lead to persistent inflammation in the sinonasal mucosa and the eventual development of NP.

Consistent with the results of Cao et al [5], our findings demonstrate that NP in Chinese patients is Th17-biased with a higher expression rates of RORc and IL-17A, and an upregulated number of Th17 cells when compared with controls. As several studies have illustrated that the IL-6/STAT3 pathway plays an important role in the development of Th17 cells [22,23], we examined the presence of IL-6 and pSTAT3 as a measure of the activated states of the IL-6/STAT3 pathway in NP tissues. In contrast to previous results reported for white subjects [24], our experimental data showed elevations of IL-6 and pSTAT3 in Chinese patients with NP. This suggests that the IL-6/STAT3 pathway may play a pathogenic role in the development of NP in Chinese patients. It is possible that IL-6 induced activation of STAT3, which then binds directly to the IL-17A promoter and regulates RORc to promote Th17 cell differentiation [23]. This hypothesis is supported by the positive correlation we observed in the NP groups between pSTAT3 and IL-17A and RORc expression. Interestingly, similarly to patients with NP, elevations of the IL-6/STAT3 pathway and in levels of Th17 cells have also been seen in patients with ulcerative colitis and Crohn's disease [25,26]. This suggests that increased levels of Th17 cells induced by dysregulation of the STAT3 pathway may play a crucial role in the pathogenesis of inflammatory disease.

In our study, we also observed a higher ratio of pSTAT3/pSTAT5 in patients with NP accompanied by an increased level of Th17 cells and decreased level of Treg cells. In addition, we observed a correlation between pSTAT5 and Foxp3, TGF- β 1, and the levels of Treg cells in polyps. We also observed a significantly negative correlation between pSTAT5 and IL-17A in patients with NP. These results suggest that STAT3 and STAT5 play reciprocal roles in the pathologic progression of NP. This mechanism would entail that STAT3 and STAT5 have overlapping binding sites in the IL-17A genetic locus but have opposite functions [12] and that the IL-2/STAT5 and IL-6/STAT3 pathways induce the Treg and Th17 transcription factors Foxp3 and RORc, which can bind to each other and thus inhibit each other's activity [27]. It seems that STAT3 is indispensable for Th17 cell development and maintenance, and negatively regulates Treg cell differentiation. Conversely, STAT5 appears to be essential for Treg differentiation and to inhibit Th17 cells. Thus, we speculate

that the improper ratio of activated STAT3/STAT5 may be one reason for the imbalance of Th17/Treg in NP and that the impaired distribution of Th17 and Treg cells in polyps may principally result from changes in their upstream signaling pathways.

The role of atopy in polyp formation has been the focus of studies on NP etiology, and it is generally believed to be the root cause of their development [28–30]. Recently, Shen et al [6] found that atopy may aggravate NP by promoting the imbalance of Th17/Treg. Our findings of significantly decreased expressions of Foxp3 and TGF- β 1 and reduced populations of CD4⁺ Foxp3⁺ cells along with increased expressions of RORc, IL-17A, and increased populations of CD4⁺ RORc⁺ cells in patients with atopic NP compared with patients with nonatopic NP agree with this hypothesis. Moreover, our study also demonstrates that there are significant differences in the expressions of the upstream signal pathways of Th17 and Treg differentiation in atopic subjects vs the nonatopic group. It is probable that atopy could cause upregulated levels of pSTAT3 and down-regulated levels of pSTAT5 and result in the larger imbalance of Th17/Treg seen in atopic NP and that the consequences of this are more severe clinical and histologic features including endoscopy scores, CT scores, eosinophil infiltration, and increased BM thickness [6]. We have found positive correlations between imbalance of the ratio of pSTAT3 to pSTAT5 and clinical and histologic features. Thus, we propose that the more severe imbalance of Th17/Treg caused by atopy may be correlated to the expression of activated STAT3 and STAT5, and that this leads to the severity of deterioration and tissue remodeling seen in atopic NP. In such a mechanism, the increased expression of IL-17A in atopic NP may play a pivotal role owing to its involvement in eosinophil infiltration and tissue remodeling [31,32].

To our surprise, we found that there was no significant difference in the level of IL-6 between the 2 groups with NP, although there were significant differences in the level of IL-2 between the 2 groups. These results were inconsistent with the changes in STAT3 and STAT5 in the 2 groups with NP, and it seemed to be that the IL-6/STAT3 pathway was disturbed, particularly in nonatopic NP. To investigate this further, we examined the expression levels of SOCS3, a cytokine-inducible negative regulator of the IL-6/STAT3 pathway that inhibits IL-6/STAT3 signaling by attenuating the phosphorylation of STAT3 [22,33]. We found that the level of SOCS3 was significantly

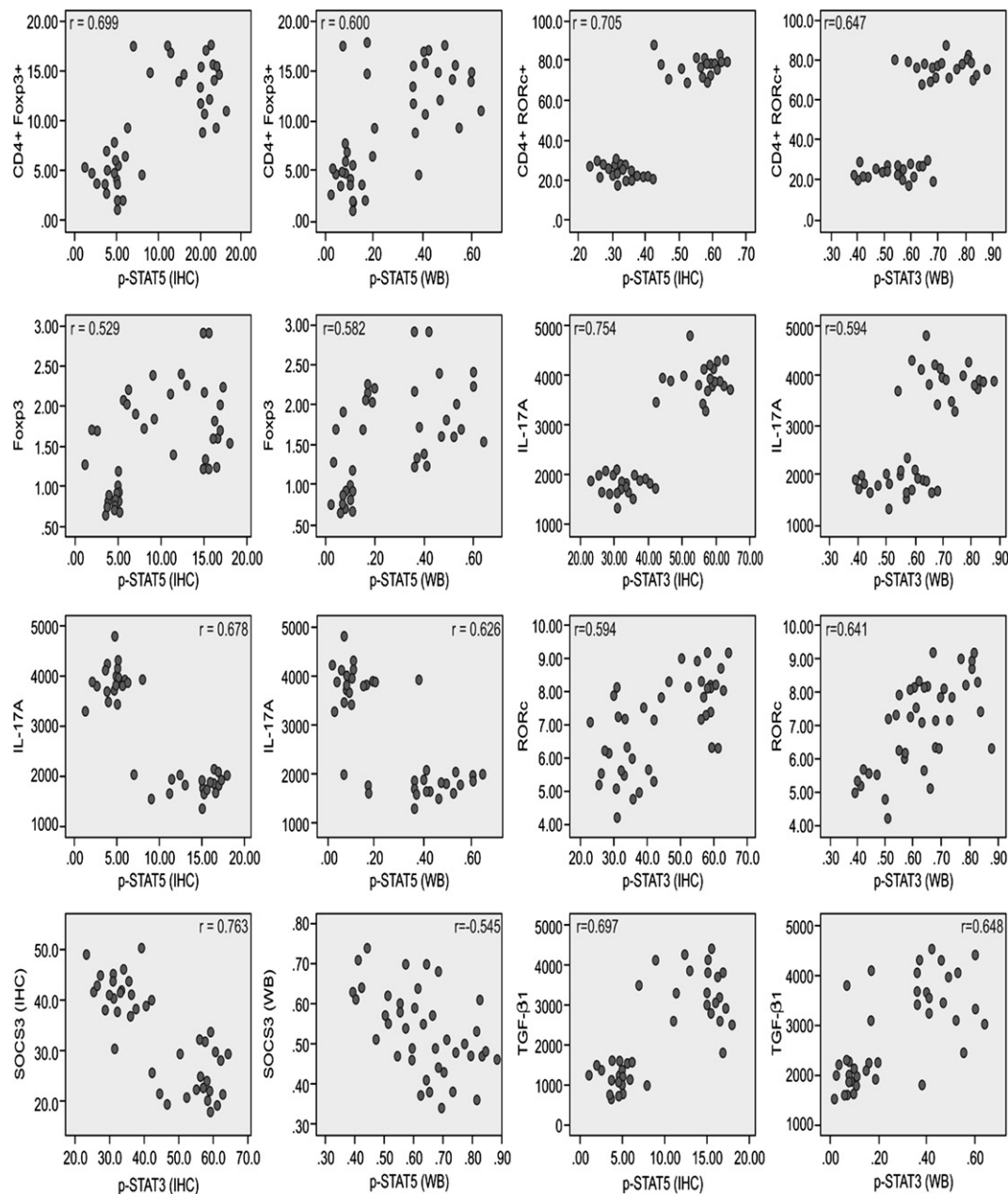


Fig. 6. The correlations between pSTAT3 and RORc, IL-17A, and CD4⁺ RORc⁺ cell levels in polyps, and between pSTAT5 and Foxp3, TGF- β 1, and CD4⁺ Foxp3⁺ cell levels in polyps.

elevated in the nonatopic NP group compared to the atopic NP group, and this result may help explain the reduced level of pSTAT3 in nonatopic NP. Thus we propose that, because atopy restrains the number and function of Treg cells [34,35], atopic NP exhibits a lower activation level of the IL-2/STAT5 pathway, which normally upregulates the expression of SOCS3 [22] and thus leads to a decreased level of SOCS3 that results in higher levels of pSTAT3 and a Th17-dominated response.

In conclusion, our findings demonstrate that there may be important alterations in the IL-6/STAT3 and IL-2/STAT5 pathways in NP. It is possible that these alterations

contribute to the imbalance of Th17/Treg cell populations in NP, and the reciprocal effects of STAT3 and STAT5 may play a pivotal role in this process. We have also shown a relationship between atopy and the ratio of activated STAT3/STAT5 in NP as well as an elevation of the inhibitor SOCS3 in atopic NP. This suggests that the improper ratio of activated STAT3/STAT5, which is regulated by SOCS3, may be correlated to the aggravated imbalance of Th17/Treg caused by atopy. However, our research provides only a preliminary understanding of the pathogenic role of STAT3 and STAT5 in NP. More intensive investigations, including in vitro experiments, will be required to further clarify the

exact mechanism of the IL-6/STAT3 and IL-2/STAT5 signaling pathways in the pathogenesis of NP and how atopy exactly influences the STAT signaling pathways in NP. Overall, our findings may be helpful in gaining deeper insights into the pathogenesis of NP.

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References

- [1] Fokkens W, Lund V, Mullol J. European position paper on rhinosinusitis and nasal polyps 2007. *Rhinol Suppl* 2007;20:1-136.
- [2] Meltzer EO, Hamilos DL, Hadley JA, et al. Rhinosinusitis: establishing definitions for clinical research and patient care. *J Allergy Clin Immunol* 2004;114:155-212.
- [3] Wynn R, Har-El G. Recurrence rates after endoscopic sinus surgery for massive sinus polyposis. *Laryngoscope* 2004;114:811-3.
- [4] Zhang N, Van Zele T, Perez-Novo C, et al. Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus disease. *J Allergy Clin Immunol* 2008;122:961-8.
- [5] Cao PP, Li HB, Wang BF, et al. Distinct immunopathologic characteristics of various types of chronic rhinosinusitis in adult Chinese. *J Allergy Clin Immunol* 2009;124:478-84, 484.e1-2.
- [6] Shen Y, Tang XY, Yang YC, et al. Impaired balance of Th17/Treg in patients with nasal polyposis. *Scand J Immunol* 2011;74:176-85.
- [7] Saleh A, Shan L, Halayko AJ, et al. Critical role for STAT3 in IL-17A-mediated CCL11 expression in human airway smooth muscle cells. *J Immunol* 2009;182:3357-65.
- [8] O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. *Immunity* 2008;28:477-87.
- [9] Atreya R, Mudter J, Finotto S, et al. Blockade of interleukin 6 *trans* signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in Crohn disease and experimental colitis in vivo. *Nat Med* 2000;6:583-8.
- [10] Zom E, Nelson EA, Mohseni M, et al. IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood* 2006;108:1571-9.
- [11] Wei L, Laurence A, O'Shea JJ. New insights into the roles of Stat5a/b and Stat3 in T cell development and differentiation. *Semin Cell Dev Biol* 2008;19:394-400.
- [12] Yang XP, Ghoreschi K, Steward-Tharp SM, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat Immunol* 2011;12:247-54.
- [13] Lund VJ, Holmstrom M, Scadding GK. Functional endoscopic sinus surgery in the management of chronic rhinosinusitis. An objective assessment. *J Laryngol Otol* 1991;105:832-5.
- [14] Lund VJ, Kennedy DW. Staging for rhinosinusitis. *Otolaryngol Head Neck Surg* 1997;117(Suppl):S35-40.
- [15] Lanza DC, Kennedy DW. Adult rhinosinusitis defined. *Otolaryngol Head Neck Surg* 1997;117:S1-7.
- [16] Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 2003;3:253-7.
- [17] Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057-61.
- [18] Murawski MR, Litherland SA, Clare-Salzler MJ, et al. Upregulation of Foxp3 expression in mouse and human Treg is IL-2/STAT5 dependent: implications for the NOD STAT5B mutation in diabetes pathogenesis. *Ann N Y Acad Sci* 2006;1079:198-204.
- [19] Cohen AC, Nadeau KC, Tu W, et al. Cutting edge: decreased accumulation and regulatory function of CD4+ CD25 (high) T cells in human STAT5b deficiency. *J Immunol* 2006;177:2770-4.
- [20] Yao Z, Kanno Y, Kerenyi M, et al. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* 2007;109:4368-75.
- [21] Burchill MA, Yang J, Vogtenhuber C, et al. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol* 2007;178:280-90.
- [22] Chen Z, Laurence A, Kanno Y, et al. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci U S A* 2006;103:8137-42.
- [23] Durant L, Watford WT, Ramos HL, et al. Diverse targets of the transcription factor STAT3 contributes to T cell pathogenicity and homeostasis. *Immunity* 2010;32:605-15.
- [24] Peters AT, Kato A, Zhang N, et al. Evidence for altered activity of the IL-6 pathway in chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2010;125:397-403.e10.
- [25] Mudter J, Neurath MF. IL-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. *Inflamm Bowel Dis* 2007;13:1016-23.
- [26] McLoughlin RM, Jenkins BJ, Grail D, et al. IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. *Proc Natl Acad Sci U S A* 2005;102:9589-94.
- [27] Chen Z, Laurence A, O'Shea JJ. Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation. *Semin Immunol* 2007;19:400-8.
- [28] Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signaling and immune regulation. *Nat Rev Immunol* 2007;7:454-65.
- [29] Zhang N, Gevaert P, van ZT, et al. An update on the impact of *Staphylococcus aureus* enterotoxins in chronic sinusitis with nasal polyposis. *Rhinology* 2005;43:162-8.
- [30] Munoz CF, Jurado-Ramos A, Fernandez-Conde BL, et al. Allergic profile of nasal polyposis. *J Investig Allergol Clin Immunol* 2009;19:110-6.
- [31] Hellings PW, Kasran A, Liu Z, et al. Interleukin-17 orchestrates the g granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am J Respir Cell Mol Biol* 2003;28:42-50.
- [32] Liu Z, Lu X, Wang H, et al. The expression of transforming growth factor-beta 1, interleukin-6, 11 and 17 in nasal mucosa of allergic rhinitis patient. *Lin Chuang Er Bi Yan Hou Ke Za Zhi* 2006;20:625-7.
- [33] Cheng W, Zheng C, Tian J, et al. T helper cell population and eosinophilia in nasal polyps. *J Investig Allergol Clin Immunol* 2007;17:297-301.
- [34] Wu K, Bi Y, Sun K, et al. Suppression of allergic inflammation by allergen-DNA-modified dendritic cells depends on the induction of Foxp3+ regulatory T cells. *Scand J Immunol* 2008;67:140-51.
- [35] Pérez Novo CA, Jedrzejczak-Czechowicz M, Lewandowska-Polak A, et al. T cell inflammatory response, Foxp3 and TNFRS18-L regulation of peripheral blood mononuclear cells from patients with nasal polyps-asthma after staphylococcal superantigen stimulation. *Clin Exp Allergy* 2010;40:1323-32.