Significance of the IL-6 pathway in nasal polyposis in Chinese patients

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Summary

Background: **The interleukin-6 (IL-6) pathway is known to be important in Th17 cell differentiation and in the pathology of many inflammatory disorders. However, the significance of the IL-6 pathway in nasal polyposis (NP) in Chinese patients remains unclear.**

Objective: **The aim of this study was to evaluate the functions of the IL-6 pathway in NP in Chinese patients.**

Methods: **The levels of IL-6 pathway components, including IL-6, soluble IL-6 receptor (sIL-6R), phosphoSTAT3 (pSTAT3), and suppressor of cytokine signalling 3 (SOCS3), were assessed. The Th17 milieu was examined by measuring the levels of retinoid acid-related orphan receptor C (RORc) and IL-17A.**

Results: **Levels of IL-6 pathway components, RORc, and IL-17A were significantly higher in both NP groups than in the control (P <0.05). Furthermore, significantly higher levels of pSTAT3, RORc, and IL-17A, and significantly lower levels of SOCS3 were found in the atopic group than in the non-atopic group (P <0.05). IL-6 and sIL-6R levels were not significantly different between the 2 NP groups (P >0.05). pSTAT3 exhibited significantly positive correlations with RORc and IL-17A (P <0.01).**

Conclusions: **The expression levels of the IL-6 pathway components were significantly higher in NP patients. Moreover, p-STAT3 levels were much higher in the atopic group, and were associated with a more severe Th17 response. These results suggest that the IL-6 pathway may play a crucial role in the pathology of NP in Chinese patients, and atopy may contribute to NP by affecting the IL-6 pathway.** ***(Asian Pac J Allergy Immunol 2012;31:11-9)***

Key words: *Atopy, interleukin-6, IL-6 receptor, nasal polyposis, phosphoSTAT3, Th17 cell*

Introduction

Nasal polyposis (NP), a common inflammatory disease in otorhinolaryngology, is characterised by persistent inflammation of the nasal and paranasalmucosa and accumulation of eosinophils, T cells, neutrophils, and plasma cells in the mucosa. The clinical features include the appearance of hyperplastic polyps in the nasal cavity and paranasal sinuses that can be detected by nasal endoscopy and computed tomographic (CT) scanning.[1,2] The pathophysiology of this disease has become a research priority because of its high prevalence and undesirable outcomes.[2,3] Although NP has been widely studied for many years, its pathophysiology remains unclear. Previous studies have shown that NP in Caucasian patients was associated with a Th2-biased eosinophilic inflammation, whereas Chinese patients showed a predominant Th17 response.[4,5] Moreover, Shen et al. found that Chinese patients with atopic NP exhibit a more severe Th17 response[6]. These findings suggest that Th17 cells and atopy may play important roles in the pathology of NP in Chinese patients. However, the exact mechanism remains unknown.

Interleukin-6 (IL-6), which is now known to be a pro-inflammatory cytokine, binds to a receptor complex comprising soluble IL-6 receptor (sIL-6R) and signal-transducing receptor subunit glycoprotein 130 (gp130) and activates cytoplasmic transcriptional factors, such as signal transducer and activator of transcription 3 (STAT3), to trigger gene expression. This pathway has been named "trans-signalling".[7] Several studies showed that IL-6 plays a crucial role in Th17 differentiation via the "trans-signalling" pathway, and STAT3 is a key transcription factor in this process.[8, 9] It has also been shown that the IL-6

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pathway is associated with the pathogenesis of various inflammatory diseases, such as Crohn's disease, rheumatoid arthritis (RA), and asthma.[9,10,11] Given the importance of the IL-6 pathway in the pathogenesis of inflammatory diseases and Th17 differentiation, studies examining the correlation between the IL-6 pathway and NP pathogenesis are important. More recently, Peters et al. reported that the IL-6 signalling pathway may be blunted in NP in Caucasian patients, and this may be unfavourable to the development of Th17 cells in the local tissue environment in NP in Caucasian patients.[12] However, the exact function of the IL-6 signalling pathway in the pathogenesis of NP in Chinese patients remains unknown. Given that Chinese patients exhibit a predominant Th17 response, we speculate that the IL-6 pathway may play a pivotal role in the pathogenesis of NP in Chinese patients, and atopy may influence the IL-6 pathway to induce a more severe Th17 response.

To test this hypothesis, we evaluated the expression of IL-6, sIL-6R, pSTAT3, and SOCS3, a key inhibitor of the STAT3 pathway, in the atopic NP group, the non-atopic NP group and controls, to determine the status of the IL-6 pathway in NP. We then measured the expression levels of the transcription factor retinoid acid-related orphan receptor C (RORc) and IL-17A as indicators of Th17 cell development. Finally, we investigated the association between the IL-6 pathway components and Th17 cell development in NP. To our best knowledge, this is the first study to evaluate the IL-6 signalling pathway in NP in Chinese patients.

Table 1. Clinical data of patients with NP

	Control	Non-atopic NP	Atopic NP	*P*-value
Age (years)	29 (21–52)	35 (25–66)	34 (20–61)	*N.S.*
Gender	6M/4F	10M/10F	12M/8F	*N.S.*
Asthma	0/10	0/20	3/20	*N.S.* *
Duration (years)	0	2 (1–4)	5 (2–9)	*N.S.*
Recurrence	0	2/20	6/20	*N.S.* *
Symptom score	0	8 (6–12)	11 (9–15)	*N.S.*
CT score	0	9 (8–11)	12 (9–15)	<0.05
Endoscopy score	0	4 (3–6)	7 (5–9)	<0.05

Data are expressed as median (inter-quartile range). Student's *t*-test or the Fisher exact test (marked with *) was used to determine the statistical significance; *P*-value <0.05 was considered significant.

Abbreviations: CT, computed tomography; NP, nasal polyposis; N.S., not significant

Methods

Patients

A total of 40 NP patients were recruited for this study. The enrolment standards and the diagnosis of atopic status were according to established criteria from previous studies.[5,6,13] Clinical data of patients included sex, age, duration of disease, history of asthma, and recurrence. Symptom scores were assessed according to a visual analogue scale (VAS).[14] Preoperative computed tomography (CT) scans were graded according to the classification by Lund and Mackay.[15] Preoperative nasal endoscopy scores were graded according to the classification by Lanza and Kennedy.[16] All of these data are presented in Table 1. Patients who had antrochoanal polyps, cystic fibrosis, primary ciliary dyskinesia, fungal sinusitis, or gastrooesophageal reflux disease were excluded. Ten patients with a deviated septum, no history of respiratory disease or atopy, and a negative skin prick test were recruited as controls. Oral and topical applications of corticosteroids or antihistamines were stopped for at least 3 months, and all patients received 3 days of antibiotics before surgery.[5] Patients received surgery only when medical treatment failed.

This study was approved by the ethical committee of Chongqing Medical University, and informed consent was obtained from all patients and controls.

Tissue preparation

The NP samples were obtained from ethmoidal polyp tissue during surgery, and the mucosa of the inferior turbinates from patients undergoing septoplasty or rhinoseptoplasty were used as control samples. All fresh samples were divided into 2 parts. One part was fixed with 4% paraformaldehyde and embedded in paraffin for immunohistochemical staining and double immunofluorescence; the other part was immediately flash-frozen in liquid nitrogen and stored at -80°C for use in quantitative real-time polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), and western blot analysis.

Immunohistochemistry

Paraffin sections were de-waxed and dehydrated in alcohol and non-specific binding was blocked with 2% BSA before immunohistochemical staining.

Monoclonal antibodies against pSTAT3 (1:100 dilution) and SOCS3 (1:100 dilution) (Abcam, UK) were used with the streptavidin-biotin complex method. Non-immune serum IgG and phosphate-buffered saline (PBS) were used as negative controls. We analysed the number of stained cells at a magnification of 400× as an indicator of protein expression levels. The counting method was the same as that used to enumerate eosinophils.

Western blot analysis

We extracted proteins from sample tissues using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China), according to the manufacturer's instructions. Supernatants were separated and stored at -80°C until analysis. Samples containing 25 μg of protein were boiled, separated by acrylamide gel electrophoresis, and electrophoretically transferred to PVDF membranes (Beyotime). The membranes were blocked with western blocking buffer (Beyotime) for 1 h at room temperature and then incubated with rabbit anti-human pSTAT3, anti-SOCS3, and anti-β-actin polyclonal antibodies (Abcam) at dilutions of 1:1000 each. 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 normalise the results in each lane.

Quantitative real-time PCR

RORc mRNA levels were analysed by real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, California, USA), and all assays were performed in accordance with the manufacturer's instructions. Total RNA (1000 ng) was reverse-transcribed to cDNA by 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: F, 5′-GCT GTG ATC TTG CCC AGA ACC-3′; R, 5′-CTG CCC ATC ATT GCT GTT AAT CC-3′. The PCR protocol consisted of 2 cycles at 95°C for 30 s followed by 40 cycles at 95°C for 5 s and 63°C for 20 s. All PCR reactions were performed in duplicate. The comparative CT method was used to calculate the relative gene expression levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene for normalisation, and a no-template sample was used as the negative control.

Enzyme-linked immunosorbent assay

We used cytokine-specific ELISA kits (R&D Systems, USA) to assay the levels of IL-6, sIL-6R, and IL-17A in the samples, according to the manufacturer's instructions. The assay for each sample was performed in duplicate; all data are expressed in pg/mL.

Double immunofluorescence

The subcellular distribution of the RORc protein was investigated by double immunofluorescence. Deparaffinised sections were heated at 95°C for 10 min for antigen retrieval and then incubated in blocking solution (10% normal goat serum in PBS) for 10 min. The sections were incubated with anti-CD4 (1:100 dilution) (Santa Cruz, USA) and anti-RORc (1:200 dilution) antibodies at 4°C overnight. After washing, the sections were incubated in the dark with a FITC-labelled antibody (1:100 dilution) (Santa Cruz) against the anti-CD4 antibody and a CY3-labeled antibody (1:200) against the anti-RORc antibody. This was followed by nuclear staining with DAPI (1:1500 dilution) (Santa Cruz) for 1 h. Negative control sections were obtained by omitting the primary antibody. Cells were counted as described above.

Statistical analysis

We used IBM SPSS v. 20.0 for statistical analysis. Data are presented as medians and inter-quartile ranges. When comparisons were made between groups, the Kruskal-Wallis test was used to analyse significant inter-group variability. The Mann-Whitney *U* test was used for between-group comparisons, and the Spearman test was used to determine correlations. A P value less than 0.05 was considered significant.

Results

Immunohistochemical analysis

Significantly higher numbers of $pSTAT3^+$ cells and $SOCS3^+$ cells were detected in the NP groups (P <0.05) than in the control group. Moreover, the atopic group exhibited a markedly higher number of $pSTAT3^+$ cells (P <0.05) and lower number of $SOCS3^+$ cells (P <0.05) compared to the non-atopic group (Table 2, Figure 1).

Western blot analysis

Consistent with the immunohistochemistry results, the protein levels of pSTAT3 and SOCS3 were significantly higher in the NP groups (P <0.05) than in the control group. Furthermore, compared to the non-atopic group, the atopic group exhibited a

Table 2. Medians and inter-quartile ranges for IL-6 signalling molecules and Th17-related cytokines and transcription factors

	Control	Versus	Non-atopic NP	Versus	Atopic NP	Versus control
IL-6(pg/mL)	317.5 (237.2–397.2)	P <0.05	2067.5 (1459.2–2642.5)	N.S.	2067.5 (1459.2–2642.5)	P <0.05
sIL-6R (pg/mL)	167 (112.0–267.2)	P <0.05	438.5 (354.2–564.2)	N.S.	506.5 (441.2–639.2)	P <0.05
pSTAT3 (IHC)	11.6 (9.7–12.3)	P <0.05	30 (22.8–32.1)	P <0.05	51.2 (42.8–56.0)	P <0.05
pSTAT3 (WB)	0.14 (0.12–0.20)	P <0.05	0.41 (0.34–0.49)	P <0.05	0.68 (0.60–0.76)	P <0.05
SOCS3 (IHC)	4.1 (3.2–6.4)	P <0.05	31.5 (29.0–38.4)	P <0.05	19.4 (16.9–22.3)	P <0.05
SOCS3 (WB)	0.14 (0.11–0.19)	P <0.05	0.54 (0.47–0.60)	P <0.05	0.37 (0.28–0.43)	P <0.05
IL-17A (pg/mL)	83.5 (61.7–97.5)	P <0.05	355.5 (248.5–488.2)	P <0.05	666.0 (513.7–765.2)	P <0.05
RORc	1.77 (1.52–2.14)	P <0.05	3.72 (3.21–4.17)	P <0.05	5.40 (5.06–6.31)	P <0.05
$CD4^+RORc^+$cells	10.8 (9.2–11.7)	P <0.05	21.4 (19.3–24.3)	P <0.05	47.9 (39.4–54.6)	P <0.05

Data are expressed as median (inter-quartile range).Mann-Whitney U test was used for unpaired comparisons. P-value <0.05 was considered significant.

Abbreviations: IHC, Immunohistochemical analysis; NP, nasal polyposis; N.S., not significant; WB, Western blot analysis.

markedly higher level of pSTAT3 (P <0.05) and a lower level of SOCS3 protein (Table 2, Figure 2).

Quantitative real-time PCR

RORc mRNA levels were significantly higher in the NP groups (P <0.05) than in the control group. Furthermore, the non-atopic group had markedly lower levels of RORc mRNA (P <0.05) compared to the atopic group (Table 2, Figure 3a).

Enzyme-linked immunosorbent assay

Consistent with the expression levels of pSTAT3, the levels of IL-6, sIL-6R, and IL-17A were significantly higher in both NP groups (P <0.05) than in the control group. Moreover, the atopic NP group exhibited a significantly higher level of IL-17A compared to the non-atopic NP group (P < 0.05). However, the levels of IL-6 (P =0.29) and sIL-6R (P =0.07) were not significantly different between the 2 NP groups (Table 2, Figure 3b–3d).

Double immunofluorescence

$CD4^+$ and $RORc^+$ cells were detected in all samples. Moreover, RORc was seen mostly in cells that were also positive for CD4. Consistent with the PCR results, the number of $CD4^+$ $RORc^+$ cells in the NP groups was significantly different from that of the control group (P <0.05). The number of $CD4^+$ $RORc^+$ cells was significantly higher in the atopic NP group than in the non-atopic NP group (P <0.05) (Table 2, Figure 4).

Correlation analysis

We examined the correlations between the IL-6 pathway components and Th17 cell development to determine the possible function of the IL-6 pathway in NP. The correlation analysis results are expressed as r_{IHC} and r_{WB} for immunohistochemistry and western blotting, respectively. pSTAT3 exhibited significantly positive correlations with RORc (r_{IHC} = 0.495, r_{WB} = 0.663, P <0.01), IL-17A (r_{IHC} = 0.476, r_{WB} = 0.559, P <0.01), and $CD4^+$ $RORc^+$ cells (r_{IHC} = 0.557, r_{WB} = 0.704, P <0.01). In addition, we also found a significant inverse correlation between pSTAT3 and SOCS3 (r_{IHC} = -0.509, r_{WB} = -0.508, P <0.01) (Figure 5). However, there was no correlation between IL-6, sIL-6R, and SOCS3 (P >0.05).

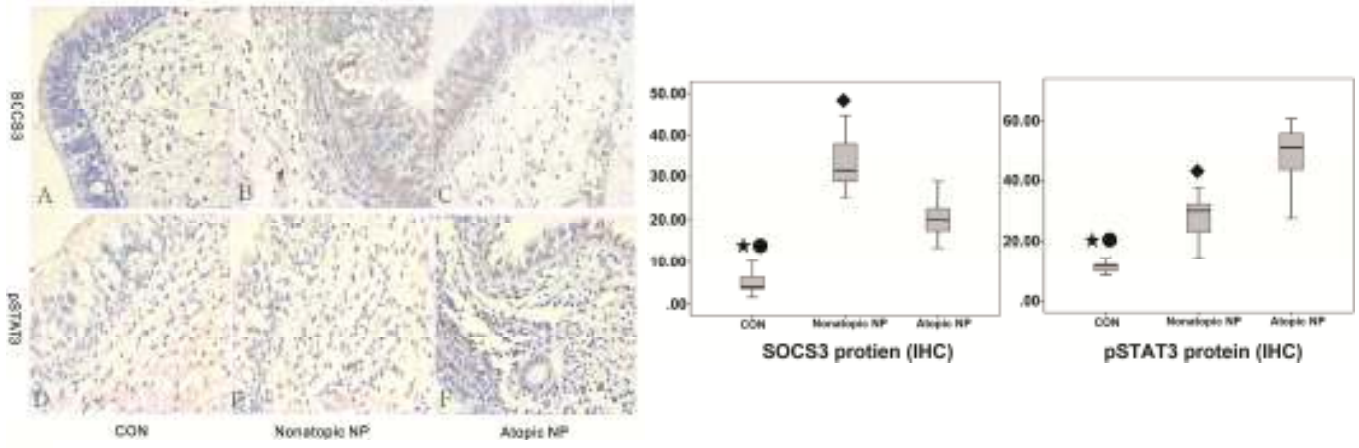

Figure 1. (a)Expression of pSTAT3^{+} and SOCS3^{+} cells in the nasal mucosa of control subjects (CON) and patients with atopic nasal polyposis (NP) or non-atopic NP. ★: $P < 0.05$, CON vs. Atopic NP; ●: $P < 0.05$, CON vs. Non-atopic NP; ◆ $P < 0.05$, Atopic NP vs. Non-atopic NP.

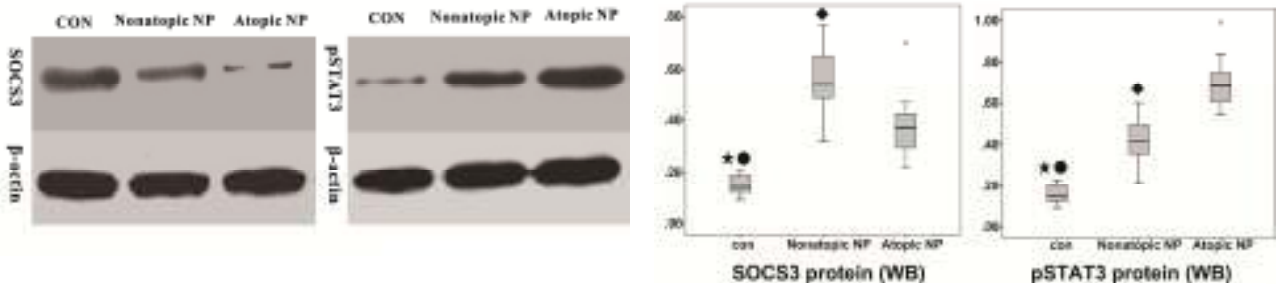

Figure 2. Western blot analysis of pSTAT3 and SOCS3 expression in the control (CON) and NP groups. ★: $P < 0.05$, CON vs. Atopic NP; ●: $P < 0.05$, CON vs. Non-atopic NP; ◆ $P < 0.05$, Atopic NP vs. Non-atopic NP.

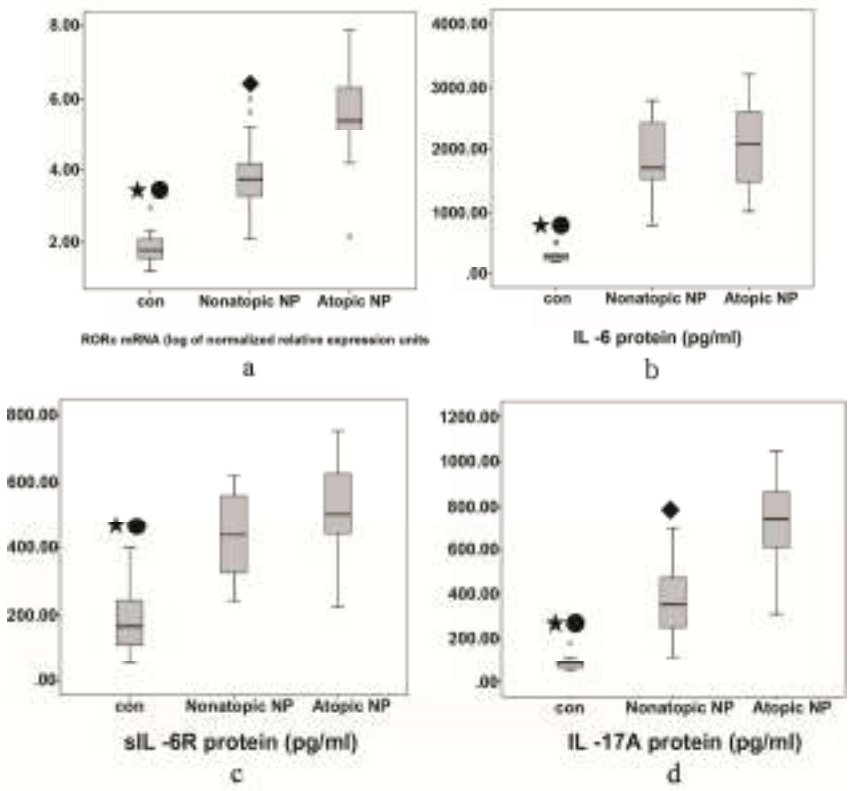

Figure 3. (a) Real-time PCR analysis of RORc mRNA in the control (CON) and NP groups. (b–d) Levels of IL-6, sIL6R, and IL-17A in patients with atopic NP, non-atopic NP, and CON, as analysed by ELISA. ★: $P < 0.05$, CON vs. Atopic NP; ●: $P < 0.05$, CON vs. Non-atopic NP; ◆: $P < 0.05$, Atopic NP vs. Non-atopic NP.

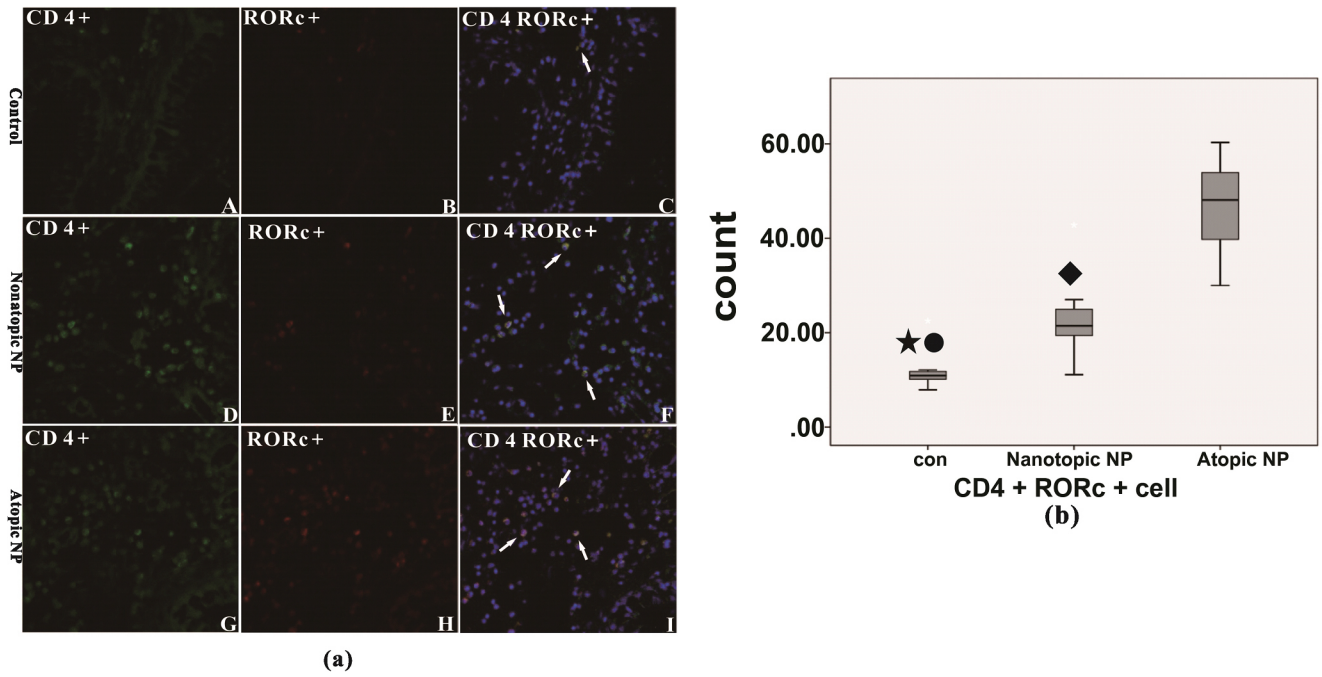

Figure 4. (a) Double immunofluorescence for $CD4^{+}RORc^{+}$ cells in the control (CON) and NP groups. Co-localisation of CD4 with RORc in NP. CD4, FITC (green); RORc, CY3 (red); Double-positive cell (arrowhead, orange). (b) Levels of $CD4^{+}$ $RORc^{+}$ cells in the NP groups and CON. ★: $P < 0.05$, CON vs. Atopic NP; ？: $P < 0.05$, CON vs. Non-atopic NP; ◆: $P < 0.05$, Atopic NP vs. Non-atopic NP.

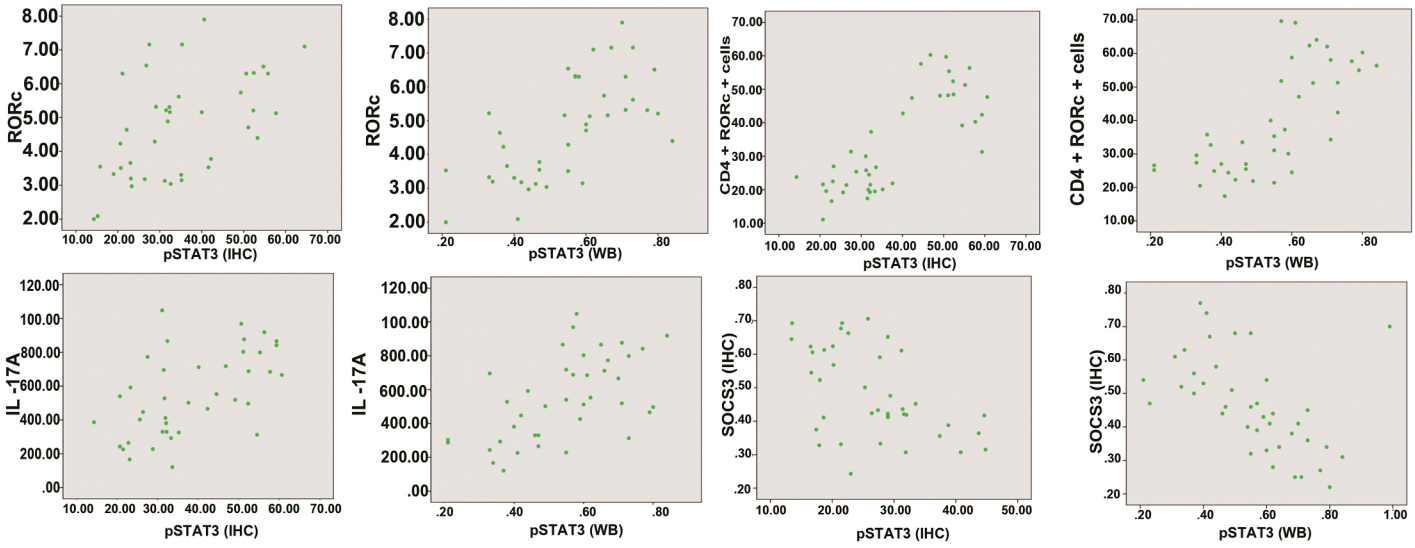

Figure 5. Correlations between pSTAT3 and RORc, IL-17A, $CD4^{+}$ $RORc^{+}$ cells, and SOCS3 levels in polyps.

Discussion

In the present study, we found that the expression levels of the IL-6 pathway components were increased in NP, and the level of pSTAT3, a key transcription factor of the IL-6 pathway, was positively correlated with Th17 cell development in NP, suggesting that the IL-6 pathway may play a crucial role in the development of NP in Chinese patients. Furthermore, the atopic NP group exhibited higher levels of pSTAT3 and a more severe Th17 response. This suggests that atopy may induce a more serious Th17 response by altering the IL-6 pathway in NP in Chinese patients. The levels of SOCS3, a key inhibitor of the IL-6 pathway, were markedly lower in the atopic NP group compared to the non-atopic NP group, and there was a significant negative correlation between pSTAT3 and SOCS3 in NP patients. This explains the significant difference in the expression of pSTAT3 between the 2 NP groups, as well as the lack of a difference in the expression levels of IL-6 and sIL-6R.

Consistent with previous studies,[4,5,6] our findings indicate that NP in Chinese patients is Th17-biased, with higher expression levels of RORc and IL-17A, suggesting that Th17 cells play a pathogenic role in the development of NP in Chinese patients. It has recently been demonstrated that the IL-6 pathway promotes Th17 differentiation via activation of STAT3.[17,18,19] Furthermore, *in vitro* Th17 differentiation is greatly impaired in STAT3-deficient T cells.[20,21] Therefore, we examined the presence of IL-6, sIL-6R, and pSTAT3 as a measure of the activated state of the IL-6 pathway in NP tissues. Unlike the previous results reported for Caucasian patients,[12] elevated levels of IL-6, sIL-6R, and pSTAT3 were observed in Chinese NP patients, suggesting that the IL-6 pathway may play a pathogenic role in the development of NP in Chinese patients. It is possible that IL-6 first binds to the receptor complex containing sIL-6R, then induces the activation of STAT3, which then binds directly to the IL-17A promoter and regulates RORc expression to promote Th17 cell differentiation in NP;[22,23] these mechanisms increase Th17 inflammation, contributing to the persistence of chronic inflammation in NP. The positive correlations of pSTAT3 with RORc and IL-17A observed in our study may support this hypothesis. Thus, the IL-6 pathway may generate a local tissue environment that is favourable for the development of Th17 cells in NP in Chinese patients.

Although the role of atopy in the development of NP has been the focus of studies of NP aetiology for many years, it remains controversial. Some previous studies suggested that atopy did not significantly affect the development of NP, whereas others believe that atopy is an important factor in NP development.[24,25,26,27] Recently, Shen et al. found that atopy may promote a much stronger Th17 response in NP patients.[6] The significantly higher expression of RORc and IL-17A, and the increased population of $CD4^+$ $RORc^+$ cells in patients with atopic NP compared with patients with non-atopic NP in our study are consistent with this hypothesis. Furthermore, we also found significant differences in the expression of the upstream signalling pathway associated with Th17 cell differentiation in atopic NP patients versus the non-atopic group. Based on these results, we propose that atopy could alter the IL-6 pathway and elicit a much higher expression of Th17 cells, thereby aggravating the polyps[6]. Surprisingly, we found no significant differences in the levels of IL-6 and sIL-6R between the 2 NP groups. These results are inconsistent with the change in STAT3 levels in the 2 NP groups, suggesting that the IL-6 pathway is specifically altered in atopic NP. To investigate this further, we examined the expression levels of SOCS3, a cytokine-inducible negative regulator of the IL-6 pathway. SOCS3 inhibits IL-6 signalling by attenuating the phosphorylation of STAT3.[28,29] We found that SOCS3 levels were significantly lower in the atopic NP group compared to the non-atopic NP group, and this result may help to explain the elevated levels of pSTAT3 in atopic NP. Thus, atopy may contribute to the down-regulation of SOCS3, which normally downregulates the expression of pSTAT3, thereby eliciting a much higher level of pSTAT3, which results in a more severe Th17-dominated response in atopic NP.

Currently, corticosteroids are the recommended therapy for nasal polyps and are known to decrease IL-6 production.[2, 30, 31] However, to our knowledge, there are no reports describing the effects of topical corticosteroids on the IL-6 pathway in NP. Previous studies have focused on the treatment of Th2-skewed eosinophil inflammation, the pathological characteristics of Caucasian patients, using topical corticosteroids for NP.[32] The new viewpoint that the IL-6 pathway may play pro-inflammatory roles in Chinese patients with Th17-biased NP should arouse our attention. This pathway may be an interesting

therapeutic target and may result in a distinct therapeutic outcome.

In conclusion, our findings indicate that levels of the IL-6 pathway components were significantly up-regulated in NP in Chinese patients. In addition, our study also revealed a significantly positive correlation between Th17 cell development and pSTAT3 and a relationship between atopy and the IL-6 pathway in NP. Based on these data, we suggest a pivotal role for the IL-6 pathway in NP in Chinese patients and propose that atopy may contribute to NP by altering the IL-6 pathway. However, more thorough studies, including *in vitro* experiments, are required to evaluate the exact mechanism of the IL-6 pathway in the development of NP in Chinese patients. Overall, we expect that our findings will enable us to gain a deeper insight into the pathogenesis of NP and provide a potential therapeutic target for NP in Chinese patients.

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Disclosure Statement

The authors declare that no financial or other conflicts of interest exist in relation to the content of the article.

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