High frequency of T helper type 9 cells in Chinese patients with allergic rhinitis

Xiao-Qiang Wang, Guo-Hua Hu, Hou-Yong Kang, Yang Shen, Xia Ke and Su-Ling Hong

Summary

Background: **T helper type 9 cells (Th9) are the most recently discovered subset of Th cells, and are involved in the pathology of several autoimmune and allergic diseases. The significance of Th9 cells in allergic rhinitis (AR) in Chinese patients is unclear.**

Objective: **The aim of this study was to investigate the possible role of Th9 cells in AR in Chinese patients.**

Methods: **Th9 cells and related factors were assessed by measuring levels of interleukin-9 (IL-9), PU.1, interferon-regulatory factor 4 (IRF4), and numbers of Th9 cells. A Th9-polarized milieu was evaluated by determining the levels of IL-4 and transforming growth factor-β1 (TGF-β1). Disease severity was assessed by rhino-conjunctivitis quality of life questionnaires (RQLQ), visual analog scale scores (VAS), and peripheral eosinophils (EOS) count.**

Results: **Levels of IL-4 and TGF-β1 were elevated in AR groups versus healthy controls ($P < 0.05$). Levels of IL-9, PU.1, IRF4, and the numbers of Th9 cells were also significantly higher in the AR groups ($P < 0.05$). Furthermore, positive correlations were identified between IL-9 levels and EOS expression, RQLQ, and VAS scores ($P < 0.01$).**

Conclusions: **Th9 cells and their relative factors were elevated in AR patients. Levels of Th9 polarization-related factors were much higher in AR patients, and the severity of disease was associated with a more severe Th9 response. These results suggest that AR patients present a favorable environment for Th9 differentiation, and that Th9 cells may play a crucial role in the pathology of AR in Chinese patients.** ***(Asian Pac J Allergy Immunol 2015;33:301-7)***

Keywords: *allergic rhinitis, interleukin 9, T helper type 9 cells, Th9 polarization*

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Introduction

Allergic rhinitis (AR), an upper airway inflammatory response induced by allergen exposure, has become a common disease of otorhinolaryngology with a high incidence rate of 20% worldwide.[1] It results in a heavy financial burden and affects quality of life. Although AR has been studied for many years, the exact pathogenic mechanism of this disease remains unclear.

Previous studies have found that T helper (Th) cells play a pivotal role in the development of AR by promoting the development of disease by secreting cytokines; the main participants include Th1, Th2, and Th17 cells.[2-4] With the discovery of new Th cell subsets, studies probing the associations between Th cells and the incidence of AR have been continuously expanding in recent years.

Th9 cells are a recently discovered subset of Th cells, specialized for the secretion of interleukin-9 (IL-9). Th9 cells are generated from the differentiation of naïve $CD4^+$ Th cells in the presence of transforming growth factor-β1 (TGF-β1) and IL-4.[5-7] In addition, studies found that PU.1 and interferon-regulatory factor 4 (IRF4) were essential transcription factors for Th9 differentiation.[8,9] Additional research concluded that Th9 cells have an important regulatory role in the development of various autoimmune inflammatory diseases or allergic airway diseases, and their pathogenic role relies primarily on the secretion of IL-9.[10-12] Since Th9 cells are known to have a pro-inflammatory effect, and particularly, in light of a recent study reporting that serum IL-9 levels were significantly increased in Western AR patients,[13] we hypothesized

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that Chinese AR patients have a severe Th9 cell response, and that these cells contribute to the pathogenesis of AR in Chinese patients.

To test our hypothesis, we investigated the expression of IL-9, PU.1, IRF4, and the levels of Th9 cells in the AR group and controls to assess the status of Th9 response. We then assessed the severity of disease, and measured the levels of IL-4 and TGF-β1 in order to determine the status of a Th9-polarized milieu. To assess the pathogenic effects of Th9 cells in AR, we evaluated the data to identify a potential correlation between disease severity and levels of IL-9 in AR. To the best of our knowledge, this is the first study examining the possible role of Th9 cells in AR, together with revealing a correlation between Th9 response and disease severity in Chinese patients.

Methods

Patients

From January to April 2014, thirty patients who received a diagnosis of AR and were treated in our hospital were enrolled for our study. The AR group consisted of 18 males and 12 females, according to standard validated criteria, the presence of nasal symptoms and positive skin prick tests (SPTs) were used for diagnosis of patients with AR.[14,15] Moreover, SPTs were performed according to the guidelines of the European Academy of Allergy and Clinical Immunology.[16] Clinical data of AR patients, including age, rhino-conjunctivitis quality of life questionnaires (RQLQ), visual analog scale scores (VAS), and peripheral eosinophil (EOS) count, were collected in our study,[14] and the details are presented in Table 1. Patients with non-allergic nasal inflammation, asthma, aspirin intolerance, immunodeficiency disorders, or autoimmune diseases were excluded. Twenty healthy controls from our hospital, including 9 males and 11 females, were simultaneously enrolled in this study. No patients in the control group exhibited symptoms of AR, and all were negative in a skin prick test (SPT). All patients had avoided oral or topical applications of corticosteroids, anti-leukotrienes, or antihistamines for at least 1 month before the specimen collection.[14] This study was approved by the ethical committee of the First Affiliated Hospital of Chongqing Medical University and we obtained informed consent from each participant in the patient and control groups.

Cell preparation and flow cytometric analysis

Anti-coagulated peripheral blood was collected from all subjects. We subsequently isolated peripheral blood mononuclear cells (PBMCs) from samples by standard Ficoll-Hypaque density-gradient centrifugation. For detection of Th9 cells, cell samples were resuspended in RPMI 1640 media to a concentration of 1×10^6 cells/ml.[15]

Table 1. Clinical data of AR patients

	AR group	Controls	*P*-value
Age (years)	34±11	32±10	NS
Sex (male: female)	18: 12	9:11	NS
VAS	39.5±11.22	0	<0.01
RQLQ	3.21±0.84	0	<0.01
EOS (count/mm3)	450.49±119.35	197.7±110.21	<0.01

Significance was accepted at P < 0.05. Not significant=N.S. Data are expressed as mean ± standard deviation.

Since other Th cell subsets together with Th9 cell are the source of IL-9, and producing significantly less IL-9 than Th9 cell, [17, 18] we defined Th9 cells as those with a $CD4^+$ $IL\text{-}9^+$ phenotype, which were from the gated population of $CD4^+$ $IFN\text{-}\gamma^-$ $IL\text{-}4^-$ $IL\text{-}17A^-$ cells.[17] Cells were stimulated with phorbol myristate acetate (PMA 25 ng/ml, Alexis Biochemicals; San Diego, CA, USA) and ionomycin (1 μg/ml, Sigma; St. Louis, MO, USA) in the presence of GolgiStop (2 mmol/ml, BD Biosciences; San Jose, CA, USA) for 4 h at 37°C in an atmosphere of 5% CO_2. Cells were washed with phosphate-buffered saline (PBS), and subsequently incubated with fluorescein isothiocyanate (FITC)-labeled CD4 antibody (eBioscience; San Diego CA, USA). Cells were fixed and then permeabilized by fluorescence activated cell sorter (FACS) Perm Solution (eBioscience), according to the manufacturer's instructions. Cells were incubated with phycoerythrin (PE) anti-human IL-9 antibody, allophycocyanin (APC)-labelled IL-4 antibody, peridinin chlorophyll-cyanin 5 (PerCP-Cy5)-labelled IL-17A anti-body and PE-Cy7-labelled interferon (IFN)-γ antibody (eBioscience) for 30 min at 4°C in the dark. In parallel, we used PE-conjugated PE-Cy5 conjugated rat IgG 2a and mouse IgG1 antibodies as isotype controls. We then analyzed the fluorescence profiles using a FACScan cytometer equipped with Cell Quest software (BD Biosciences).

Real-time polymerase chain reaction (RT-PCR) analysis of IL-9, PU.1, and IRF4

Total RNA was extracted from PBMCs by TRIzol extraction (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. For complementary DNA (cDNA) synthesis, we used a

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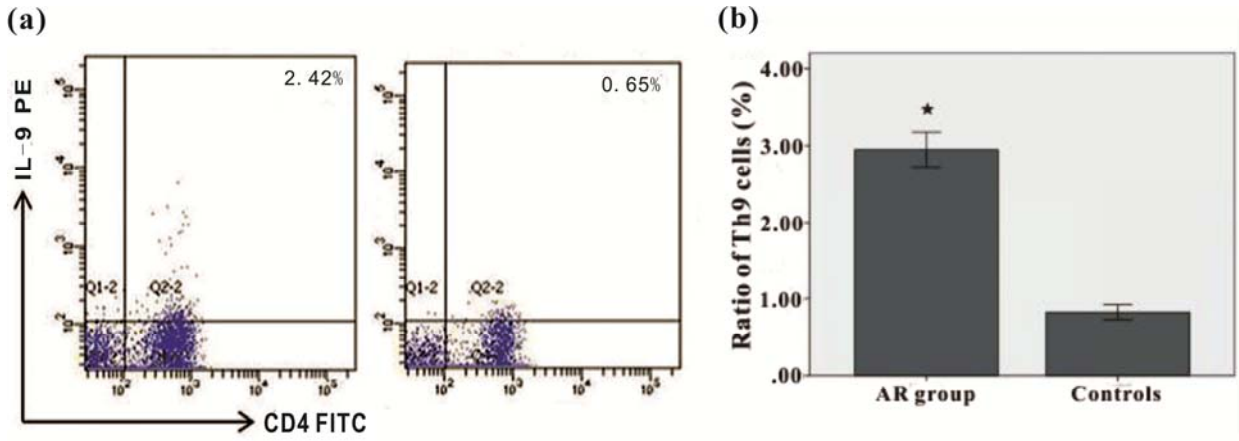

Figure 1. Analysis of T helper type 9 (Th9) cells percentage in allergic rhinitis (AR) and controls by flow cytometry. (a) Representative pictures for ratio of the Th9 cells, the numbers in the upper right quadrant represent the percentage of IL-9 + cells with expression of CD4+cells. (b) The ratio of Th9 cells in PBMCs was significantly higher in AR patients than controls (★: p <0.05, compared with the controls).

Prime Script RT Reagent Kit (TaKaRa; Beijing, China), and performed RT-PCR with a SYBR Real-time PCR Premixture Kit (Bioteke) on an iCycler (Bio-Rad, USA).[14] The following primer sequences were used for RT-PCR: *PU.1* Forward: 5′-AGA AGA AGA TCC GCC TGT ACCA-3′, reverse: 5′-GTG CTT GGA CGA GAA CTG GAA-3′; *IRF4* Forward: 5′-CCC ACC TCG CAC TCT CAG TTTC-3′, reverse: 5′-TATCAGCCTCACACCCCTCCTC-3′; *IL-9* Forward: 5′-GGG ATC CTG GAC ATC AAC TTC-3′, reverse: 5′-GAA GCA TGG TCT GGT GCA GTT-3′; β-actin Forward: 5′-AGTTGCGTTAC ACCCTTTCTTG-3′, Reverse: 5’-TCACCTTCA CCGTTCCAGTTT-3’.[17,19] We performed all PCRs in duplicate. We determined the expression levels of mRNA by using the comparative CT method. For normalization, we used β-actin as a housekeeping gene, and a reaction with no template was run as a negative control.

Enzyme-linked immunosorbent assay (ELISA) for IL-4, IL-9, and TGF-β1

We collected samples of peripheral venous blood and centrifuged the serum within 30 min of blood collection. Sera were stored at −80°C for ELISA.[17]

Because human Th9 cells produce IL-9, but not IL-10,[20] we assayed levels of IL-9 only. We used a cytokine-specific ELISA kit (R&D Systems, USA) to assess the levels of IL-4, IL-9, and TGF-β1 in the sera of patients according to the manufacturer’s instructions. Each sample was assayed in duplicate, and all data are expressed as pg/ml.

Statistical analysis

IBM SPSS ver. 20.0 was used for statistical analysis, and data are presented as mean ± standard deviation. For comparisons between groups, we used the Student’s t-test and nonparametric Mann–Whitney test. The correlations were determined by using the Spearman test. We interpreted $P < 0.05$ as indicating a statistically significant difference.

Results

Clinical data of all patients

All of the clinical data were summarized in Table 1. For the mean age and sex ratio, there was no significant difference between the two groups ($P > 0.05$). By contrast, the peripheral EOS count, VAS, and RQLQ scores in AR patients were significantly higher than controls ($P < 0.01$).

Th9 cell frequency in PBMCs

We used $CD4^+$ $IL\text{-}9^+/CD4^+$ T cells as a marker for Th9 cell frequency in the peripheral blood.[17] We found a significantly higher frequency of Th9 cells in the PBMCs of patients in the AR group (2.95±0.61%) than in controls (0.82±0.21%, $P < 0.05$; Figure 1).

Levels of PU.1, IRF4, and IL-9 mRNA in PBMCs

PU.1 mRNA was significantly higher in samples from the AR group than from controls (1.57±0.34, $P < 0.05$; Figure 2). Compared with the controls (IRF4: 0.86 ± 0.23, IL-9: 1.08 ± 0.35), *IRF4* (4.07 ± 0.68) and *IL-9* (2.72±0.84) mRNA levels in the AR

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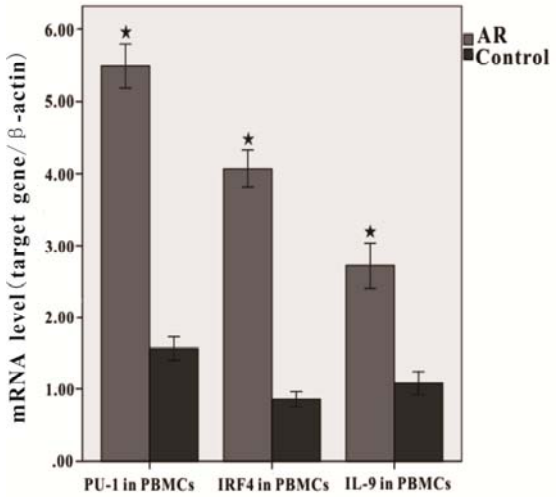

Figure 2. Real time PCR measurement of the expressions of PU.1 IRF4 and IL-9 mRNA in PBMCs from AR groups and controls (★: p <0.05, compared with the controls).

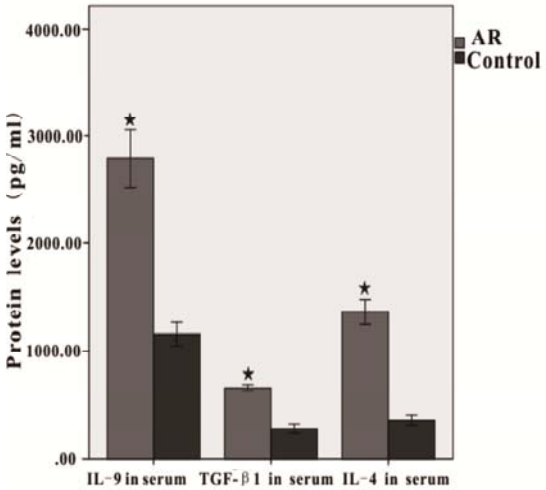

Figure 3. The protein levels of IL-4, IL-9 and TGF-β1 in AR patients and controls were analyzed by ELISA (★: p <0.05, compared with the controls).

group were also significantly higher (P < 0.05; Figure 2).

Serum concentrations of IL-4, IL-9, and TGF-β1

Consistent with the results from RT-PCR analysis, serum concentration of IL-9 was significantly greater in the AR group (2789.27 ± 716.54pg/ml) than controls (1156.35 ± 228.19pg/ml, P < 0.05; Figure 3). The expression of IL-4 was also significantly higher in AR patients (1361.07 ± 312.19 pg/ml) than that in controls (360.9 ± 102.94 pg/ml, P < 0.05; Figure 3), and the control group (281.16 ± 96.26pg/ml) had markedly lower concentrations of TGF-β1 compared to the AR group (661.13 ± 84.49 pg/ml, P < 0.05; Figure 3).

Correlations between the Th9 response and disease severity in AR patients

To further investigate the possible role of the Th9 response in disease severity, we used the Spearman's test for correlation analysis. The result of the correlation analysis demonstrated significant, positive correlations between the expression of IL-9 protein in the serum, *IL-9* mRNA levels, and Th9 cell frequency, with the indexes of disease severity, which included the peripheral EOS count ($r_{IL\text{-}9\ protein}$= 0.685, $r_{IL\text{-}9\ mRNA}$= 0.705, r_{Th9}= 0.584), VAS ($r_{IL\text{-}9\ protein}$= 0.691, $r_{IL\text{-}9\ mRNA}$= 0.676, r_{Th9}= 0.500) and RQLQ ($r_{IL\text{-}9\ protein}$= 0.798, $r_{IL\text{-}9\ mRNA}$= 0.725, r_{Th9}= 0.629) scores in AR patients (P < 0.01; Figure 4).

Discussion

Th9 cells are a newly identified subset of $CD4^+$ Th cells, which are distinguished by utilization of IL-9 as a key cytokine.[6] Recently published articles have demonstrated that Th9 cells may play a crucial role in the pathogenesis of allergic diseases, atopic dermatitis, and asthma.[17, 21] In this study, we observed an increased frequency of Th9 cells in AR patients, suggesting that Th9 cells may have a important role in the development of AR in Chinese patients. Moreover, based on our observation of higher expression levels of IL-4 and TGF-β1 in the AR group, and a previous finding that IL-4 plus TGF-β1 could enhance Th9 cell differentiation[5], we suggest that a micro-environment may exist in AR patients that promotes the differentiation of Th9.

Previous studies have indicated that PU.1 and IRF4 play pivotal roles as key transcription factors for Th9 differentiation.[8, 9] PU.1 can promote Th9 cell and IL-9 production, as well as IRF4, by binding to the *IL-9* promoter. Expressing a short interfering RNA (siRNA) to inhibit PU.1 expression resulted in decreased production of IL-9 and $CD4^+$ $IL\text{-}9^+$ cells, suggesting that PU.1 plays a significant role in IL-9 production in human T cells.[7] Moreover, PU.1 has been found in higher levels in the circulating PBMC samples or biopsy samples of patients with immune inflammatory diseases such as Crohn's disease and ulcerative colitis.[22] Similar

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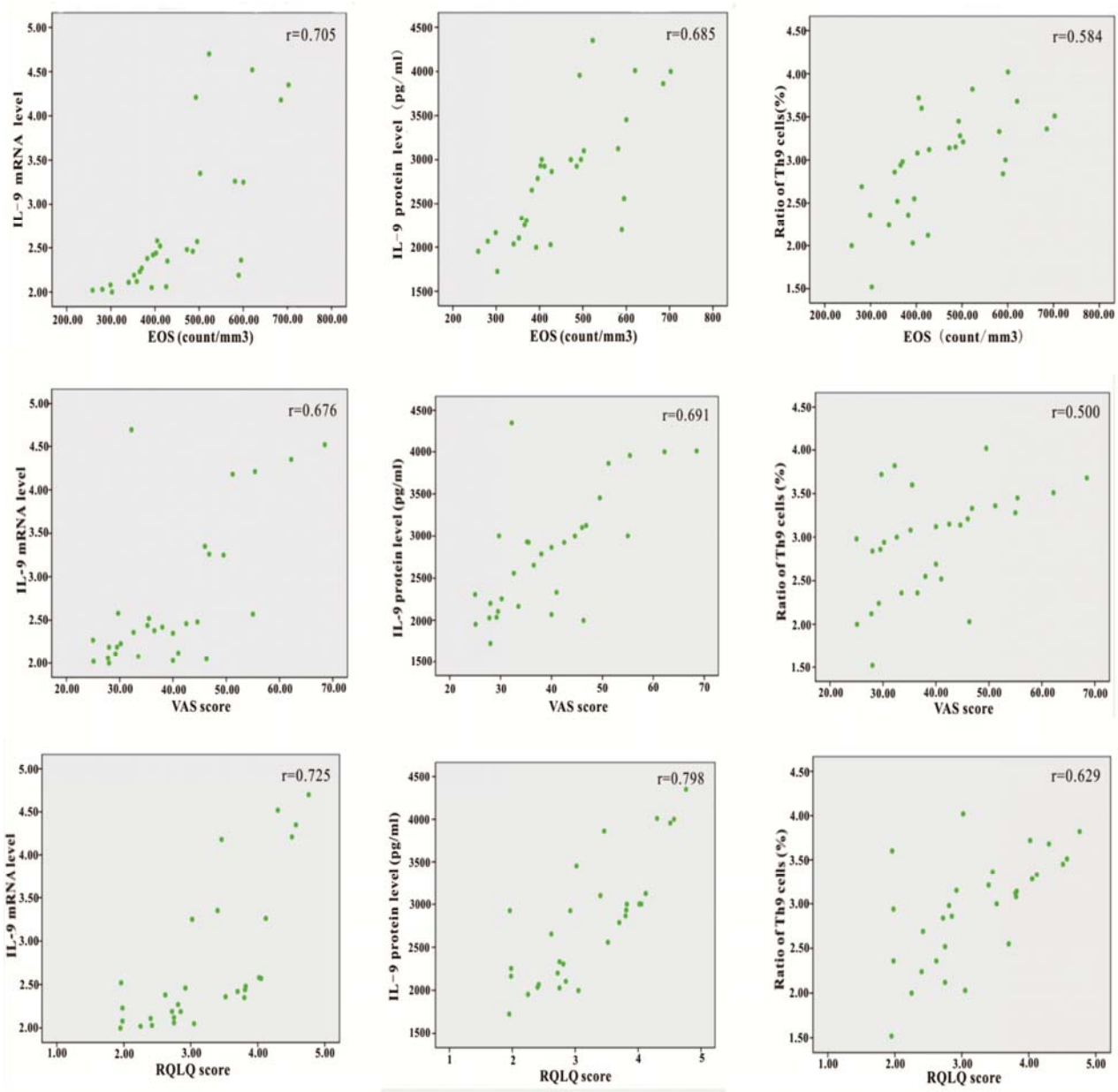

Figure 4. The correlations between Th9 response and index of disease severity in AR patients.

findings were reported from a mouse model of allergic inflammation.[23] Our present findings are consistent with these studies. In addition, consistent with the idea that IRF4 has a synergistic effect with PU.1 to promote Th9 differentiation, we also found a higher level of IRF4 in AR patients than controls. Our results also support the inference of a hyperactive Th9 response in AR. Studies from other groups have found that transduction of PU.1 into Th9 and Th2 cultures leads to both an increased production of IL-9, and a decrease in Th2 cell production, resulting in a PU.1-driven conversion of a subset of Th2 into the Th9 phenotype.[24,25] It is possible that the higher expression of PU.1 observed in AR patients induced Th9 polarization, and also led to the conversion of Th2 cells, causing the ectopic expression of Th9 cells and expediting progression of the disease.

IL-9, which belongs to the family of common γ-chain cytokines, is the main cytokine of Th9 cells. IL-9 has clearly demonstrated effects in the development of allergic inflammation by promoting the growth of mast cells, EOS, and T cells, particularly in asthma.[26, 27] Studies in both an mouse

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asthma model and patients found that blocking IL-9 results in reduced inflammation of the airways, and that the lungs of asthmatic patients expressed high level of IL-9. As an airway inflammatory disease with common features, AR patients have also been observed to express higher levels of IL9 than healthy people.[13] In concurrence with the findings of this previous study, we observed higher levels of PU.1, IRF4, and IL-9 mRNA and protein levels, and an increased frequency of Th9 cells in Chinese AR patients. In addition, we revealed that the frequency of Th9 cells, levels of IL-9 mRNA and expression of IL-9 protein were all correlated positively with the index of disease severity. This novel finding further supports the hypothesis of a crucial role of Th9 cells in the disease progress of AR in Chinese patients. That is, an excessive Th9 immune response in AR patients in the presence of a Th9-polarized milieu may then result in increased production of IL-9, boosting the activation and proliferation of immune cells such as T cells, mast cells, EOS, and so on, particular for EOS.[28] An autocrine positive feedback response in IL-9 secretion has been detected, which amplified the effect of IL-9 and exacerbated the degree of inflammation.[17] All of these pathways may also contribute to the pathogenic mechanisms of Th9 cells in AR.

As a new target for treatment of allergic diseases, studies for agents able to block IL-9 activity have been carried out for several years.[29, 30] However, to date, there still remains much to be learned about the effects of an anti-IL-9 antibody on AR models or patients, study of it may provide an additional therapeutic target and bring about an improved therapeutic outcome.

In conclusion, the results of our study suggest that increased levels of Th9 cells and their components may contribute to the pathogenesis of AR in Chinese patients. Future studies are needed to investigate the more specific mechanism of the Th9 response in the pathogenesis of AR, including *in vitro* experiments. With that consideration, we hope that our study will be helpful for investigators to gain a much deeper insight into the pathogenesis of AR and provide a potential therapeutic target for AR 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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