MEDICAL SCIENCE MONITOR

Involvement of T-Helper 9 Activation in a Mouse Model of Allergic Rhinitis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background:
We aimed to investigate the role of T-Helper (TH) 9 cells in the pathogenesis of allergic rhinitis (AR) in mice.

Material/Methods:
An AR model was produced in BALB/c mice, and the viral encoding interleukin (IL)-9 silencing sequence was used to reduce IL-9 expression. The experiment was divided into a control group, an AR group, an IL-9 shRNA+AR group, and a vector+AR group. Hematoxylin and eosin (H&E) staining was used to detect pathological changes. The cytokine expression was detected by ELISA method. Cellular typing was detected by flow cytometry.

Results:
Cells in the control group were regularly arranged, with clear layers and no congestion, edema, or necrosis observable. By contrast, in the AR model group and the vector treatment group, nasal mucosa showed clear hyperemia and edema in upper tissues and infiltration of inflammatory cells, which were ameliorated by IL-9 silencing. Compared with the control group, interferon-γ (IFN-γ) was significantly down-regulated, while IL-4, IL-17, and IL-9 were significantly elevated in the AR model group. TH1 cells in nasal mucosa, lymph, nasal lavage, spleen, and peripheral blood were significantly reduced, while TH2, TH9, TH17, and Treg cells were significantly elevated in the AR group compared with the control group. Importantly, all these changes in AR model were ameliorated by IL-9 silencing.

Conclusions:
AR is related to the changes of cytokines in TH1, TH2, TH9, TH17, and Treg, which are improved by IL-9 silencing. Activation of TH9 cells is involved in the pathogenesis of AR.

MeSH Keywords:
Acalculous Cholecystitis • Receptors, Interleukin-9 • Rhinitis, Allergic, Seasonal

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Background

Allergic rhinitis (AR) is a type I allergic disease caused by contact with specific allergen(s) [1]. AR is a nasal inflammatory disease caused by allergen and mediated by immune globulin E (IgE). Its clinical manifestations mainly include a runny or stuffy nose, sneezing, red, itchy, and watery eyes, and swelling around the eyes [2]. AR is one type of allergy that widely affects a number of people and influences their quality of life. Intranasal corticosteroids are the preferred treatment for the symptoms [3], while understanding the pathogenesis of AR is of extreme importance for the discovery of other effective treatments.

Transforming growth factor-β (TGF-β) and interleukin (IL)-4 can activate CD4 + T and further enhance the secretion of cytokines such as IL-9 and IL-10, but not other Th2 cytokines. Thus, IL-9 is not secreted by T-Helper (Th) 2 cells [4]. Recent studies have also confirmed that the cytokine IL-9 is also not produced by conventional Th1, Th17, or Treg subsets, but rather by a novel helper T cell subset termed "TH9" [5]. Further studies demonstrated that TH9 cells also possess high proliferation ability and can promote the proliferation of other T cells [6]. TH9 cells play an important role in a variety of diseases [7]. The specific mechanism of action is related to the surrounding tissues or inflammatory environment [8]. TH9 cells have proinflammatory effects, especially in some autoimmune disease models [9]. For example, the injection of differentiated TH9 cells can increase body weight and induce related inflammatory changes [10].

The balance of TH1 and TH2 cells is prone to be impaired in AR, i.e., T-helper cells transferring from TH1 into TH2 cells and eliciting the immune responses [11]. Activation of transcription factor PU.1 is found in the differentiated TH9 cells, which can inhibit the differentiation of TH2 cells and reduce the expression of cytokines in TH2 cells, while the expression of IL-9 is significantly increased [12–15]. Therefore, the discovery of this new TH9 group is helpful to understand the pathogenesis of AR. In the present study, an AR model was established in BALB/c mice and viral silencing of IL-9 was applied to investigate the function of TH9 in AR occurrence and development. This study may provide an experimental basis for clinical treatment of AR disease.

Material and Methods

Animal model and groups

Male BALB/c mice (6–8 weeks, 20–28 g) of SPF grade were obtained from the Department of Animal Sciences, Medical School of Nanchang University. The AR model was established as previously described [15]. Mice were sensitized by administration of an intraperitoneal injection of ovalbumin (OVA, 75 μg) in 200 μL of phosphate-buffered solution (PBS) containing 2 mg of aluminum hydroxide (Sigma Aldrich, St. Louis, MO, USA) in a total volume of 200 μL on days 0, 7, 14, and 21. After initial sensitization, mice were challenged with nasal instillation of OVA 500 μg in 20 μL of PBS into bilateral nasal cavities on days 22–30. The control group was challenged with PBS instead of OVA.

The experiments were divided into 4 groups (N=8): control group, AR group, IL-9 shRNA+AR group, and vector+AR group. One the day before AR modeling, IL-9 shRNA or vector (100 μl) was administered 1 time through intravenous injection. After modeling, nasal mucosa, nasal lavage, and lymphatic, spleen, and peripheral blood were collected for subsequent experiments.

Construct of IL-9 shRNA

Based upon the sequence of the IL-9 gene, 3 shRNAs were designed as following: 5-GATCCCGTCCCAACTGATGATTGTACC TTGATATCCGGGTACAATCATCAGTTGGGACTTTTTTCCAAG-3; 5-GATCCCGACTGTTACAGCTGACCAATGTTGATATCCGCAT TGGTCAGCTGTAACAGTCTTTTTTCCAAG-3; 5-GATCCCG CCACACAGAAATCAAGACTCTTGATATCCGGAGTCTTGATTTCT GTGTGGCTTTTTTCCAAG-3. These 3 shRNA sequences were constructed in lentivirus. The silencing effect was detected using real-time PCR in the 293T cells.

Real-time PCR

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and 100 U/mL penicillin-streptomycin (Sigma, Ronkonkoma, NY, USA) in 5% CO_2 at 37°C. After transfection with different viruses for 48 h, mRNA in different groups was extracted using a TRIzol assay kit (Baosheng Science & Technology Innovation Co, Ltd., Shanghai, China). mRNA was transcribed into cDNA according to the reverse transcription kit instructions (639522, Takara Biotechnology Co., Ltd., Dalian, China), and fluorescence quantitative PCR was utilized to detect the expression level of the targeted genes using cDNA as templates. The relevant expression level of IL-9 was normalized to GAPDH. The primers (5'-3') were as follow:
IL-9 (Forward): CTTGTGTCTCTCCGTCCCAAC;
IL-9 (Reverse): ACTATCCTTTTCACCCGATGGA,
GAPDH (Forward): CAATGACCCCTTCATTGACC;
GAPDH (Reverse): GAGAAGCTTCCCGTTCTCAG.

Histologic evaluation of nasal mucosa

After treatment, mice were sacrificed under anesthesia in isoflurane. Their decapitated heads were immersed in 4% PFA
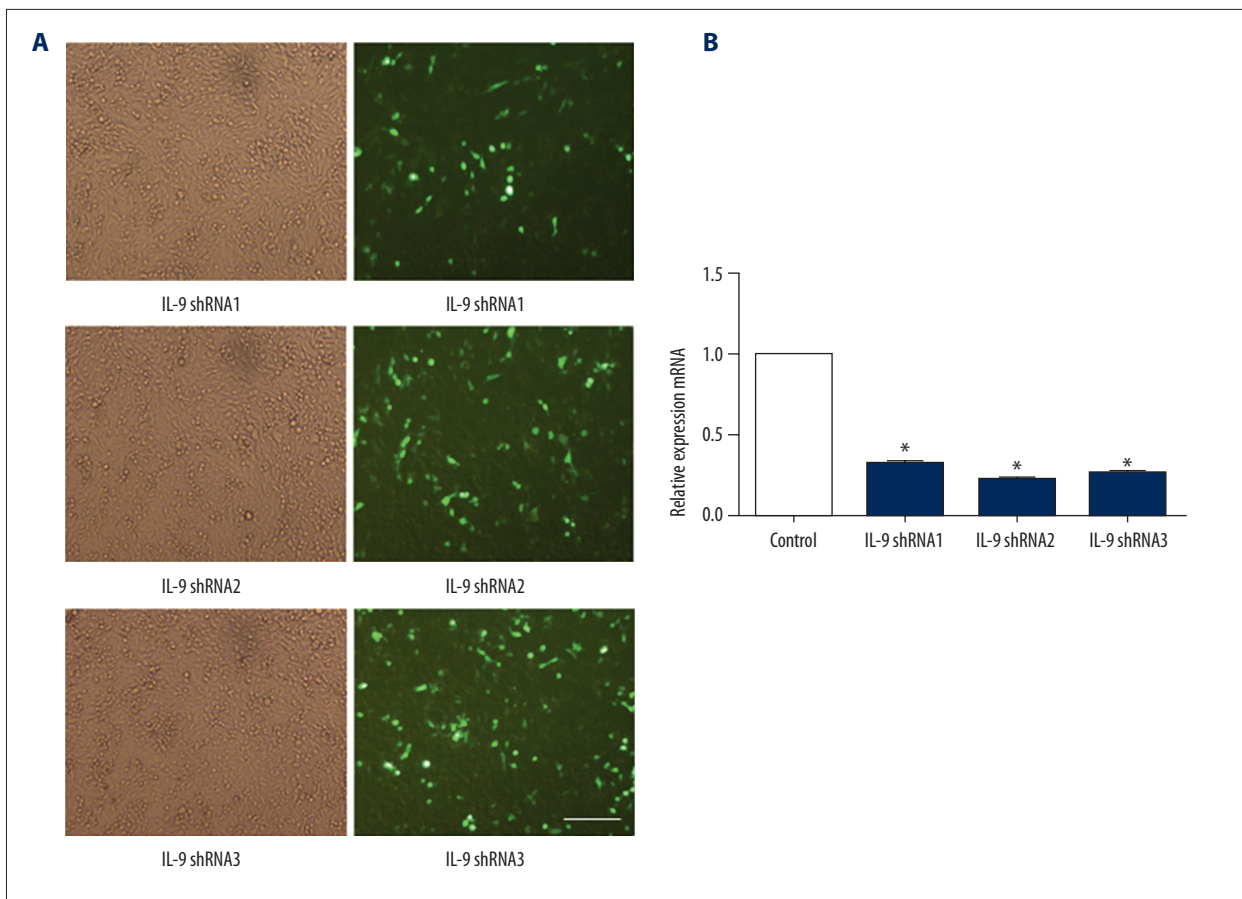
Figure 1. Confirmation of silencing effect by different IL-9 shRNAs. (**A**) Images of cells taken by light microscopy; (**B**) Images of cells taken by fluorescence microscopy after IL-9 silencing; (**C**) IL-9 expression in the cells detected by real-time PCR. * P<0.05 *vs.* control. Scale bar: 100 μm.

overnight. A portion of the nasal sinus was collected for H&E staining. The fixed tissues were embedded in paraffin and sectioned anterior-to-posterior at 5-μm thickness. After that, the slices underwent dewaxing and hydration, and were stained by hematoxylin and eosin. The images were taken under light microscopy.

ELISA

The nasal lavage was kept in –80°C. IFN-γ (SCA049Mu, CLOUD-CLONE, Shanghai, China), IL-4 (SEA077Mu, CLOUD-CLONE, Shanghai, China), IL-17 (SEA077Mu, CLOUD-CLONE, Shanghai, China) and IL-9 (SEA063Mu, CLOUD-CLONE, Shanghai, China) were detected by ELISA method according to the instructions of the kits.

Flow cytometry

Nasal mucosa, nasal lavage, and lymphatic, spleen, and peripheral blood were collected for flow cytometry. Monoclonal antibodies against CD4 (FITC) (Cat. 553047), CD25 (PE) (Cat. 553075, BD), IFN-γ (PE) (Cat. 554552, BD), IL-4 (PE) (Cat. 562044,

BD), IL-17 (PE) (Cat. 560436, BD), FoxP3 (PE-Cy5) (Cat. 563954, BD), and IL-9 (PE) (Cat. 560807, BD) were applied to stain the cells. Flow cytometry method was used to detect the T cell subgroups: TH1 (CD4$^+$ IFN-γ$^+$), TH2 (CD4$^+$ IL-4$^+$), Treg (CD4$^+$ CD25$^+$ Foxp3$^+$), TH17 (CD4$^+$ IL-17$^+$), and TH9 (CD4$^+$ IL-9$^+$).

Statistical analysis

Data are presented as means ± standard deviations and were analyzed by SPSS 19. One-way analysis of variance with post hoc Bonferroni test for multiple comparisons was performed. Differences were considered significant at P<0.05.

Results

Confirmation of the silencing effect

The images taken under light microscope and fluorescence microscope are indicated in Figure 1A. All 3 designed shRNAs displayed good transfection efficiencies and significantly reduced IL-9 expression in 293T cells (Figure 1B). Moreover, IL-9 shRNA-2

Control

AR

IL-9 shRNA+AR

Vector+AR
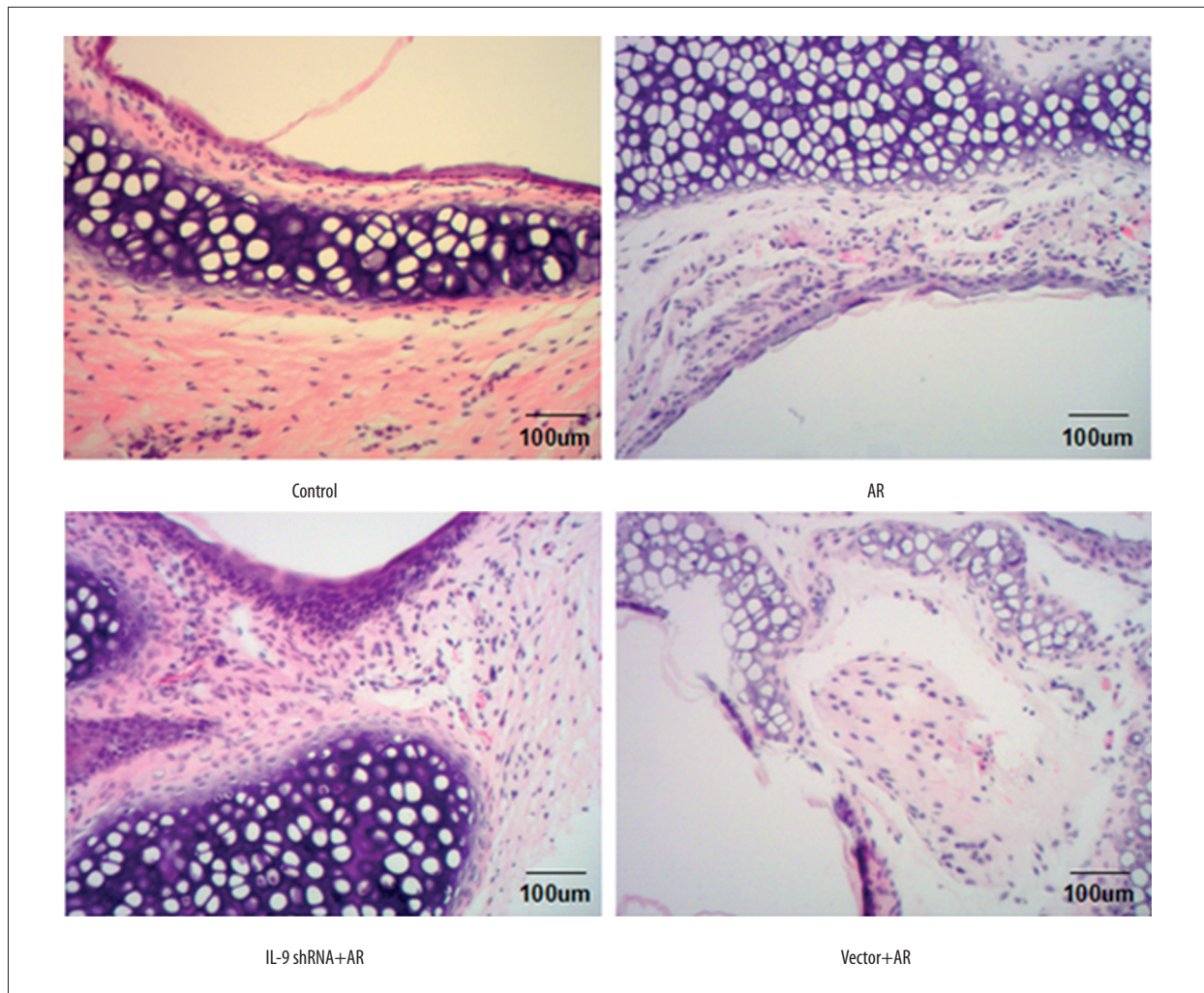
Figure 2. Pathological changes in nasal sinus.

showed the best silencing effect. Therefore, viral encoding IL-9 shRNA-2 was employed in the subsequent experiments.

Pathological changes

As shown in Figure 2, the cells in the control group were regularly arranged integrally with clear layers. There was no congestion, edema, or necrosis in any layers. By contrast, in the AR model group and the vector treatment group, nasal mucosa showed remarkable hyperemia and edema in upper tissues, and infiltration of inflammatory cells. Especially, the cells were irregularly arranged. Although edema and infiltration of inflammatory cells were slight in the IL-9 shRNA+AR group, the cells were regularly arranged, which indicated the protection of IL-9 shRNA.

Changes of IFN-γ, IL-4, IL-17, and IL-9

The levels of IFN-γ, IL-4, IL-17, and IL-9 in nasal lavage are shown in Figure 3. Compared with the control group, IFN-γ
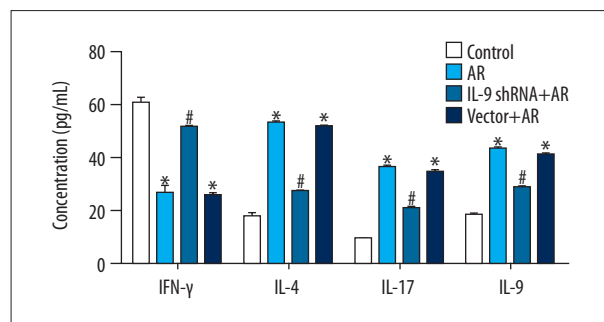
Figure 3. Changes in IFN-γ, IL-4, IL-17, and IL-9. * $P<0.05$ vs control; # $P<0.05$ *vs.* AR.

was significantly reduced, while IL-4, IL-17, and IL-9 were significantly elevated in the AR group ($P<0.05$). Compared with the AR group, IL-9 shRNA ameliorated the changes of IFN-γ, IL-17, and IL-4 caused by AR modeling ($P<0.05$).
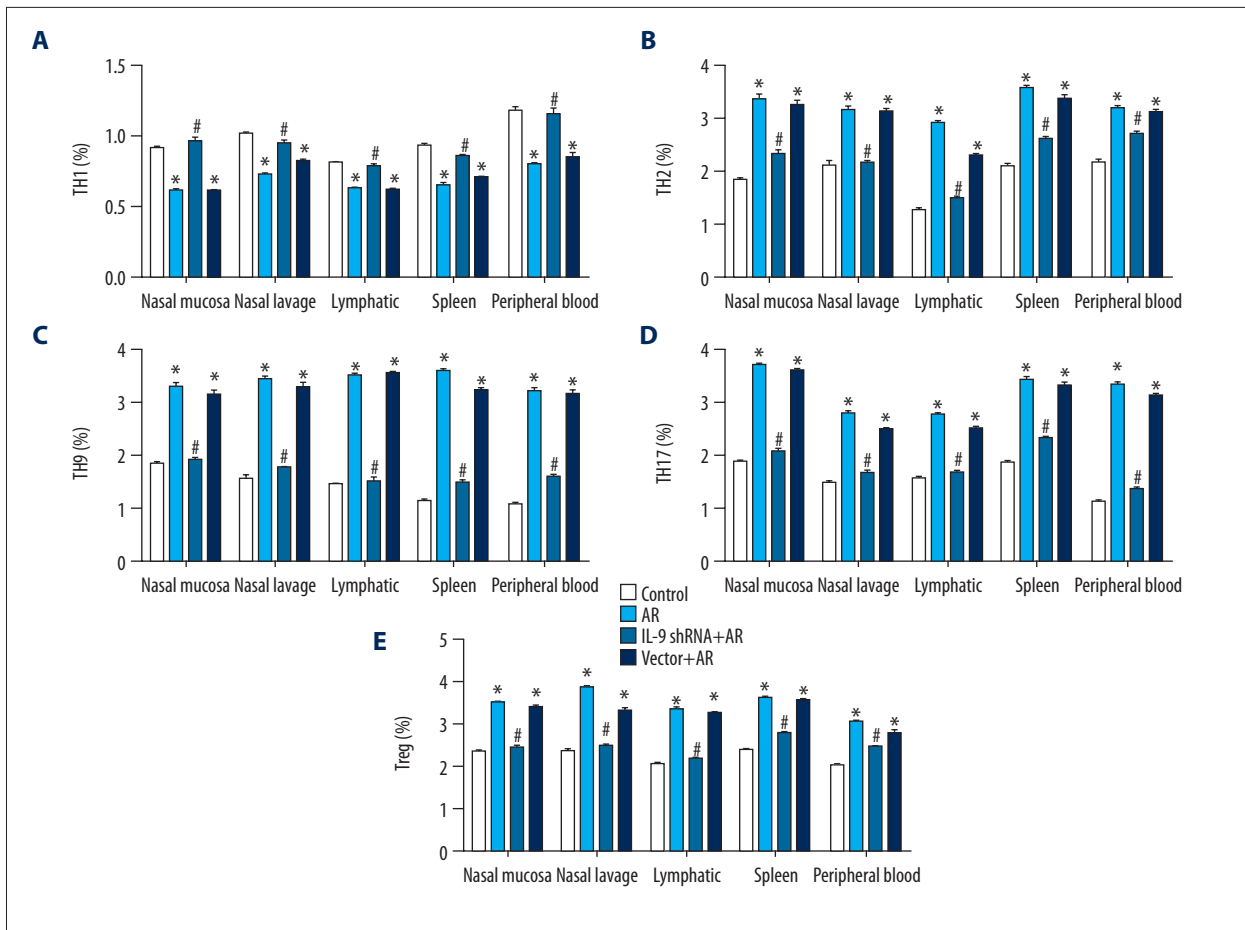
Figure 4. Changes in TH1, TH2, TH9, TH17, and Treg cell subgroups. (**A**) TH1; (**B**) TH2; (**C**) TH9; (**D**) TH17; (**E**) Treg. * P<0.05 *vs.* control; # P<0.05 *vs.* AR.

Changes of TH1, TH2, TH9, TH17, and Treg cell subgroups

The ratio of TH1, TH2, TH9, TH17, and Treg cells in nasal mucosa, nasal lavage, and lymphatic, spleen, and peripheral blood are shown in Figure 4. Compared with the control group, the TH1 cell ratio was significantly reduced in the AR group (*vs.* Control, P<0.05), but it was elevated by IL-9 shRNA silencing (*vs.* AR, P<0.05). Compared with the control group, TH2, TH9, TH17, and Treg cell ratios were significantly elevated in the AR group (*vs.* Control, P<0.05), while they were reduced by IL-9 shRNA silencing (*vs.* AR, P<0.05).

Discussion

In this study, we used ovalbumin as antigen to produce an AR model in mice. Consistent with previous publications [16,17], pathological changes, alterations of cytokines, and cellular typing demonstrated typical AR phenotypes. Importantly, IL-9 silencing, which is a functional inhibition of TH9 cells, reversed or ameliorated the changes. These results reveal that

dysregulation of TH9 cells plays a critical role in the pathogenesis of AR.

AR is an IgE-mediated allergic disease characterized by inflammation of the nasal mucosa and caused by exposure to allergens. The typical features include imbalance of TH1 and TH2, usually caused by excessive differentiation of TH2 cells [18]. The mechanism of rhinitis also involves other inflammatory cells, such as mast cells and eosinophils/basophils. Many cytokines and chemokines also affect the process of inflammation and development of rhinitis [13]. Experimental evidence have also shown that hypersensitive reaction in AR may cause excessive proliferation of TH2, which secrets a many related cytokines (e.g., IL-4, IL-5 and IL-13) [19]. In the present study, ovalbumin was the antigen used to induce the chronic allergic reaction. This model has confirmed utility in investigating the mechanisms of AR or in searching for treatments [20,21]. H&E staining of nasal sinus further indicated the pathological changes after AR modeling. We detected IL-9 level in the established AR model, showing that IL-9 level was significantly elevated in this model. Moreover, IL-9 level was positively

correlated with the symptoms of AR, including sneezing, runny nose, and nasal itching, while it had a slight negative correlation with nasal congestion [19]. The number of IL-9-positive cells in nasal mucosa was correlated with the number of eosinophils in nasal mucosa, and about 1/3 of the cells were T cells. After 2 years of immunotherapy, the levels of IL-9 protein and mRNA were significantly decreased in AR patients [22]. These results suggest that TH9 cells secrete IL-9, which likely contributes to AR pathogenesis.

In this study, we constructed 3 IL-9 lentiviruses, and selected one of the virus with the best silencing effect to treat AR mice. The results of H&E showed that the inflammatory effect of AR model was improved after silencing IL-9. The number of mouse eosinophils in the nasal mucosa increased significantly after the AR model was established, while IL-9 silencing reduced the number of nasal eosinophils. These results indicate that the expression of eosinophils invasion is closely related to IL-9 expression. Our results further confirm that IL-9 is closely related to the AR model, and IL-9 silencing can improve the symptoms of AR [22]. Although TH1 and TH2 are both CD4+ helper cells, their function is based on the secretion of cytokines IFN-γ and IL-4 [22]. TH17 is a newly discovered TH subgroup independent of TH1 and TH2 groups [23]. TH17 is the result of differentiation of CD4+T cells in different environments. TH17 plays an important role in a variety of immune and inflammatory diseases. In those diseases, IL-17 level is generally increased [24]. Treg cells are also a class of immunosuppressive T cells. The function of Treg cells is mainly through immune suppression, to achieve the silencing of antigen. TH9 cells are a newly discovered subset of CD4+T cells, which mainly produce IL-9, an effective cytokine. It has been found that TH9 cells play an important role in the pathogenesis and development of asthma [25]. In our study, we found that TH1 cells decreased, while TH2, TH9, TH17, and Treg cells increased in the AR model in the nasal mucosa, lymph, nasal lavage, and spleen and peripheral blood. By contrast, silencing IL-9 increased TH1 cells but decreased TH2, TH9, TH17, and Treg cells. These results indicated that AR is related to the levels of TH2, TH1, and to TH9, TH17, and Treg cells, whereas knockdown of IL-9 improves the symptoms of AR, which emphasizes the importance of allergen-specific immunotherapy [26].

Conclusions

In conclusion, AR is related to the changes of cytokines in TH1, TH2, TH9, TH17, and Treg, and it can be improved by silencing IL-9. This study may provide an experimental basis for the clinical treatment of AR, especially concerning allergen immunotherapy.

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