

ORIGINAL ARTICLE**Effect of Blocking the OX40/OX40L Signaling Pathway by siRNA Interference on Animal Experimental Study of Allergic Rhinitis**

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Background. The identification of new approaches and intervention targets for the treatment of AR is urgently needed. We aimed to investigate the effect of blocking the OX40/OX40L signaling pathway by small interfering RNA (siRNA) on ovalbumin (OVA)-induced AR in a mouse model.

Methods. After establishment of the AR model, the mice were interfered by siRNA-OX40L (experimental group), siRNA-C (negative control group), or PBS (control group). Nose scratching, sneezing and nasal discharge were observed. OX40L mRNA and protein and the IL-5, TNF- α , regulatory T cell (Treg) -specific marker Foxp3, and eosinophil (EOS) levels were analyzed.

Results. The numbers of nose scratching and sneezing were significantly lower in the siRNA-OX40L-treated group ($p < 0.05$). After the intervention of siRNA-OX40L, OX40L mRNA and protein levels were significantly inhibited ($p < 0.05$), but the Foxp3 level was significantly increased in the experimental group ($p < 0.05$). The IL-5 and TNF- α levels were significantly lower in the experimental group ($p < 0.05$), and the reduction was more evident for the Th2-type cytokine IL-5 than for the Th1-type cytokine TNF- α . Few or no EOSs were found in the nasal mucosal epithelium of the experimental group ($p < 0.05$), whereas EOS infiltration was significant in the other two groups.

Conclusions. Blockage of the OX40/OX40L signaling pathway with siRNA-OX40L interference can inhibit allergic reactions and relieve allergic symptoms in AR mice. The underlying mechanism may be related to correcting Th2 immune deviation, inducing immune tolerance, and promoting Treg production. © 2019 IMSS. Published by Elsevier Inc.

Key Words: Small interfering RNA, OX40 ligand, Allergic rhinitis, Mechanism, Dendritic cell.

Introduction

Allergic rhinitis (AR) is a Th2-type immune response and an allergic disease that involves various inflammatory cells and inflammatory mediators (1). Antigen-presenting cells (APCs), including dendritic cells (DCs) and monocytes/macrophages, play important roles in AR. Recently, the prevalence of AR has shown a significantly increasing trend due to increased air pollution, poor lifestyle, and increased

living stress. The high prevalence of AR seriously affects human health and quality of life, increases the social economic burden, and has become a global health problem (2). More than 80% of patients with asthma (AS) are associated with AR symptoms, and 20–50% of AR patients may have concomitant severe AS (3). Although great progress has been made in medication (e.g., antihistamines and glucocorticoids) and immunotherapy for AR, the incidence of AR remains high (4). Therefore, the identification of new approaches and intervention targets for the clinical treatment of AR is urgently needed.

DCs, which are the most powerful professional APCs, are the initiators of the immune response in the body and are thought to be key mediators of immune tolerance and Th1/Th2 differentiation (5). DCs have two different stages

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in the differentiation and development process (immature DCs and mature DCs). Immature DCs are transformed into mature DCs after ingestion of antigens, which is accompanied by upregulated expression of many surface molecules. The most important molecules are the MHC II molecule (signal 1) and the costimulatory molecules CD40, OX40L, CD80, and CD86 (signal 2). DCs contact T cells through their surface molecules, secrete cytokines, and activate T cells, which play a key role in the immunological effect (6,7).

Tumor necrosis factor receptor superfamily 4 (TNFSF4, also known as OX40 ligand, OX40L) is expressed on professional APCs, such as DCs, macrophages and other cells (8). During the immune response, an interaction between OX40L expressed by DCs and its binding partner OX40 provides a costimulatory signal to regulate DC-T cell interaction and plays an important role in the primary and secondary responses of T cells and cytokin productions similar to B7-CTLA4/CD28 and CD40/CD40 ligand (CD40L) (9,10). One study suggested that blockage of B7/CD28 only partially controlled AS and that OX40/OX40L ligation also played an important role (11). In OX40L or OX40 gene knockout AS mice, lung inflammation was significantly attenuated, the accumulation of memory T cells was significantly inhibited, and the characteristic response of AS was significantly reduced, including increases in eosinophils (EOSs), high airway responsiveness, mucus secretion, and Th2 cytokine production (12). Glycyrrhizic acid can regulate the Th1/Th2 balance by inhibiting the OX40/OX40L signaling pathway and thus plays a therapeutic role in the AS mouse model (13). Therefore, the OX40/OX40L signaling pathway may play a major role in the pathogenesis of airway allergic diseases.

Currently, few reports have blocked the OX40/OX40L signaling pathway with small interfering RNA (siRNA) in airway allergic disease models, existing studies have mainly focused on AS. In the present study, we applied siRNA for the targeted inhibition of costimulatory molecule OX40L expression, that is on the surface of bone marrow-derived DCs in AR mice, thereby blocking the OX40/OX40L signaling pathway. The aims of this study were to investigate the targeted therapeutic effect of AR and its mechanism and to provide new approaches and targets for the treatment of AR.

Materials and Methods

Construction of the OX40L Gene siRNA Expression Vector

The OX40L gene sequence was retrieved from GenBank. A pair of specific dimer siRNAs (siRNA-OX40L) targeting OX40L were designed according to the principle proposed by Elbashir et al. siRNA-OX40L and an unrelated siRNA (siRNA-C) negative control were synthesized.

Preparation and Treatment of the AR Mouse Model

The establishment of the AR mouse model was based on previous research report (14). Thirty male BALB/c mice (6–8 weeks of age) were selected. The mice were divided using the random number table method into the experimental group, negative control group, and control group ($n = 10$ mice each). Each mouse was given 10 μ g of ovalbumin (OVA) by intraperitoneal injection with 4 mg of aluminum hydroxide as an immune adjuvant once every other day for a total of eight consecutive injections over 15 d. Then, 60 μ g of siRNA-OX40L (experimental group), 60 μ g of siRNA-C (negative control group), or 60 μ g of PBS (control group) was injected intraperitoneally twice a day at days 27, 30 and 33. From 29–35 d, 5% OVA saline solution was administered as nasal drops on a daily basis (600 μ g per dose, 300 μ g/side) for seven consecutive days. The nose scratching, sneezing, and nasal discharge behaviors of the mice were observed within 20 min after the last nasal sensitization. The mice were sacrificed on day 36 for sample collection. All animal experiments were performed in accordance with a guideline from the Administration of Animal Experiments for Medical Research Purposes issued by the Ministry of Health of China. The protocol was approved by the Animal Experiment Administration Committee of the Affiliated Hospital of Southwest Medical University.

Observation of Animal Behaviors

The scoring criteria for the AR model were as follows. The nose scratching, sneezing, nasal discharge, and feeding behaviors of the mice were observed within 20 min after the last nasal sensitization. We adopted the superposition quantitative scoring method; a total score > 5 points indicates successful model construction. The scoring method was as follows: a) nose scratching: 0 point for no nose scratching, 1 point for slight nose scratching once to twice, and 2 points for intense scratching around the nose; b) sneezing: 0 point for no sneezing, 1 point for sneezing one to three times, 2 points for sneezing four to ten times, and 3 points for sneezing more than 11 times; c) nasal discharge: 0 point for no nasal secretions, 1 point for secretions running to the nostrils, 2 points for secretions beyond the anterior naris, and 3 points for snot running down the face; and d) feeding: 0 point for free movement, 1 point for quiet animals with little movement, 2 points for sluggish movement, and 3 points for lying prostrate without movement.

In Vitro Isolation and Culture of Bone Marrow-Derived DCs

All mice were decapitated within 24 h (d 36) after the last challenge. The femur and tibia of the mice were isolated under aseptic conditions. Bone marrow cells were washed with RPMI 1640 medium. After centrifugation, the

supernatant was discarded, and sterile red blood cell lysis buffer was added to the precipitate to break down the red blood cells. The lysis buffer was removed, and the cells were suspended in the RPMI 1640 medium for culture. The cell surface markers on DCs, including CD11c, CD80, CD86, and MHC II, were evaluated by flow cytometry.

RT-qPCR Analysis of OX40L mRNA Expression

Primers were designed and synthesized according to the corresponding primer sequences retrieved from GenBank. The forward primer was 5'-GCTGAACGATGGTCAAGGA-3', and the reverse primer was 5'-GAGCACAGTATC-CAGGCGTT-3'. After 24 h of transfection, total RNA was extracted from 1×10^6 DCs using the Trizol reagent following the instructions of the RT-qPCR kit. The amplification curves for the samples and the internal reference (GAPDH) were obtained using the corresponding software. The Ct values of the samples were obtained and used to calculate the relative OX40L mRNA expression.

Western Blotting Analysis of OX40L Protein Expression

Total protein was extracted from the cells following the kit instructions. Ten micrograms of total protein was used for SDS-polyacrylamide gel electrophoresis; then, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, followed by blocking with 5% skimmed milk powder at 37°C for 2 h. The membranes were incubated with an OX40L primary antibody at 4°C overnight, followed by a secondary antibody at 37°C for 2 h. After washing, an ECL luminescent reagent was added to the membrane, followed by exposure, development, and fixation.

Isolation of Splenic T Lymphocytes from Normal Mice

Healthy BALB/c mice were decapitated according to the instructions of the mouse lymphocyte isolation solution. The spleens of the mice were removed on a super clean bench under aseptic conditions. A 4.0–5.0 mL aliquot of mouse lymphocyte isolation solution was added to a Petri dish, and the spleen was cut into small pieces and ground on a 400 mesh cell strainer. The isolation solution containing spleen cells was immediately transferred into centrifuge tubes, covered with 500 μ L of RPMI 1640 medium, and centrifuged for 30 min. After centrifugation, the middle gray-white lymphocyte layer was gently aspirated along the tube wall. Then, 10 mL of RPMI 1640 medium was added to the tubes, and the cells were washed by inverting the tubes upside down. The cells were harvested after 10 min of centrifugation.

ELISA Analysis of the IL-5 and TNF- α Levels in the Mixed Lymphocyte Solution Containing DCs and T Cells

Three groups of mouse bone marrow-derived DCs (1×10^4 /well) and healthy mouse T cells (1×10^5 /well) were co-cultured in 96 well plates. OVA was added to each well at a final concentration of 10 mg/L in a final volume of 200 μ L per well. The cells were cultured in a CO₂ incubator for 72 h and then centrifuged. The supernatant was collected, and the absorbance at 450 nm was read on a microplate reader following the instructions of the IL-5 and TNF- α ELISA kits.

Flow Cytometry Analysis of CD4+CD25+ Treg-specific Marker Foxp3 Expression in Lymph Nodes

The groin and axillary lymph nodes were collected from each group of mice and ground in Petri dishes containing sterile PBS until no particles were visible. The cell suspension was transferred to centrifuge tubes for low-temperature, quick centrifugation. The supernatant was discarded, and sterile red blood cell lysis buffer was added to break down the red blood cells, followed by re-suspension in PBS. CD4+ cells were isolated using immunomagnetic bead sorting and considered preliminarily purified CD4+ T cells. The CD4+ T cells were subjected to analysis of CD4, CD25, and Foxp3 expression by flow cytometry.

Pathological Observations

The nasal mucosa of each mouse was collected and immediately fixed in 4% paraformaldehyde solution, followed by paraffin embedding and sectioning. EOS infiltration was observed by hematoxylin and eosin staining. Five random fields in each section were selected for EOS counting under high-power magnification (HPF, $\times 400$) in a blinded manner.

Statistical Analysis

The statistical analysis was performed using SPSS Statistics 19.0. The data are presented as the means $\pm$ standard deviations ($\bar{x} \pm s$). For measurement data, the means among groups were compared using a one-way ANOVA at the level of $\alpha = 0.05$. A p value less than 0.05 was considered significant.

Results

Effect of siRNA-OX40L on Allergic Symptoms in AR Mice

We scored the allergic symptoms of AR mice, including nose scratching, sneezing, nasal discharge and feeding, using the superposition quantitative scoring method. The total score of each mice exceeded 5 points, indicating successful model construction. We also found that the numbers of nose

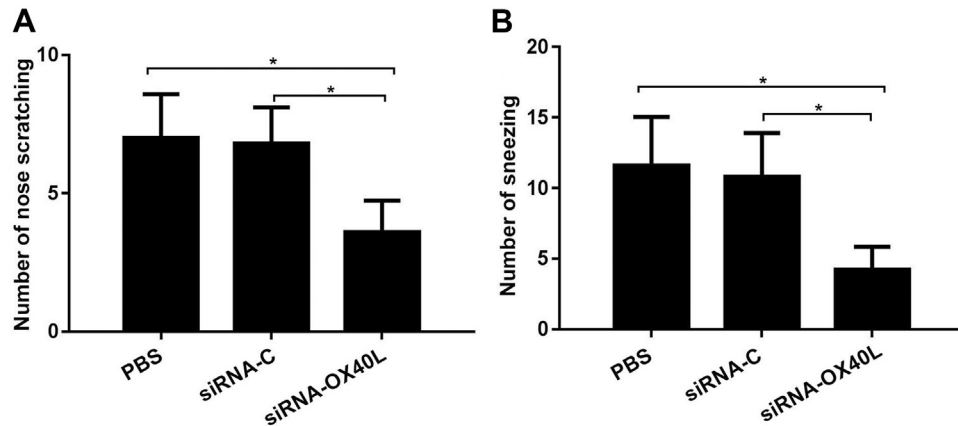


Figure 1. The effect of siRNA-OX40L on allergic symptoms in mice with AR. Mice were treated with PBS, siRNA-C and siRNA-OX40L after immunization with OVA. The numbers of nose scratching (A) and sneezing (B) were counted for 20 min after the last nasal challenge. siRNA, small interfering RNA; OX40L, OX40 ligand; AR, allergic rhinitis; PBS, phosphate buffer saline; siRNA-C, small interfering RNA-control; OVA, ovalbumin. * $p < 0.05$.

scratching and sneezing in mice were significantly lower for the siRNA-OX40L-treated experimental group than for the negative control and control groups ($p < 0.05$; Figure 1A, and 1B). No significant difference was found between the latter two groups ($p > 0.05$; Figure 1A, and 1B).

Morphology and Identification of DCs

The growth of DCs were observed under an inverted microscope. Significant morphological changes of DCs were observed at day 3. Most cells showed suspended growth after day 4, followed by colony-like growth. After 7 d of culture, the suspended cells were further aggregated, and typical dendritic bulges were found on the cell surface (Figure 2A). The CD11c, CD80, CD86, and MHC II surface molecules on mature DCs were detected by flow cytometry. We found that the CD80, CD86, and MHC II levels were significantly lower in the experimental group than in the other groups ($p < 0.05$; Figure 2B), whereas no difference was found between the control and negative control groups ($p > 0.05$; Figure 2B). No significant

difference in CD11c expression was found among the three groups ($p > 0.05$; Figure 2B).

Detection of OX40L mRNA Expression by RT-qPCR

The OX40L mRNA expression level was significantly decreased in the experimental group after siRNA-OX40L treatment ($p < 0.05$; Figure 3A). No significant difference was found between the negative control and control groups ($p > 0.05$; Figure 3A).

Detection of OX40L Protein Expression by Western Blotting

The OX40L protein expression level was detected by Western blotting. We found that the OX40L protein expression level was significantly lower in the experimental group than in the negative control and control groups ($p < 0.05$; Figure 3B, and 3C). No significant difference was found between the latter two groups ($p > 0.05$; Figure 3B, and 3C).

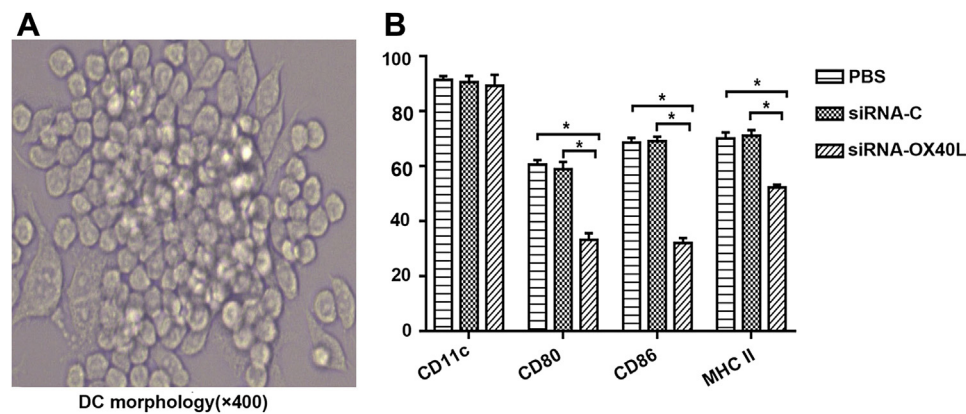


Figure 2. Morphology of DCs and surface marker expression on DCs. The growth of DCs were observed under an inverted microscope. (A) Most cells showed significant morphological changes at d 3 and suspended growth after d 4, followed by colony-like growth. After 7 d of culture, the suspended cells were further aggregated, and typical dendritic bulges were found on the cells surface (magnification $\times 400$). (B) After DCs were mature, surface molecules including CD11c, CD80, CD86, and MHC II levels on that were detected by flow cytometry. DCs, dendritic cells. * $p < 0.05$.

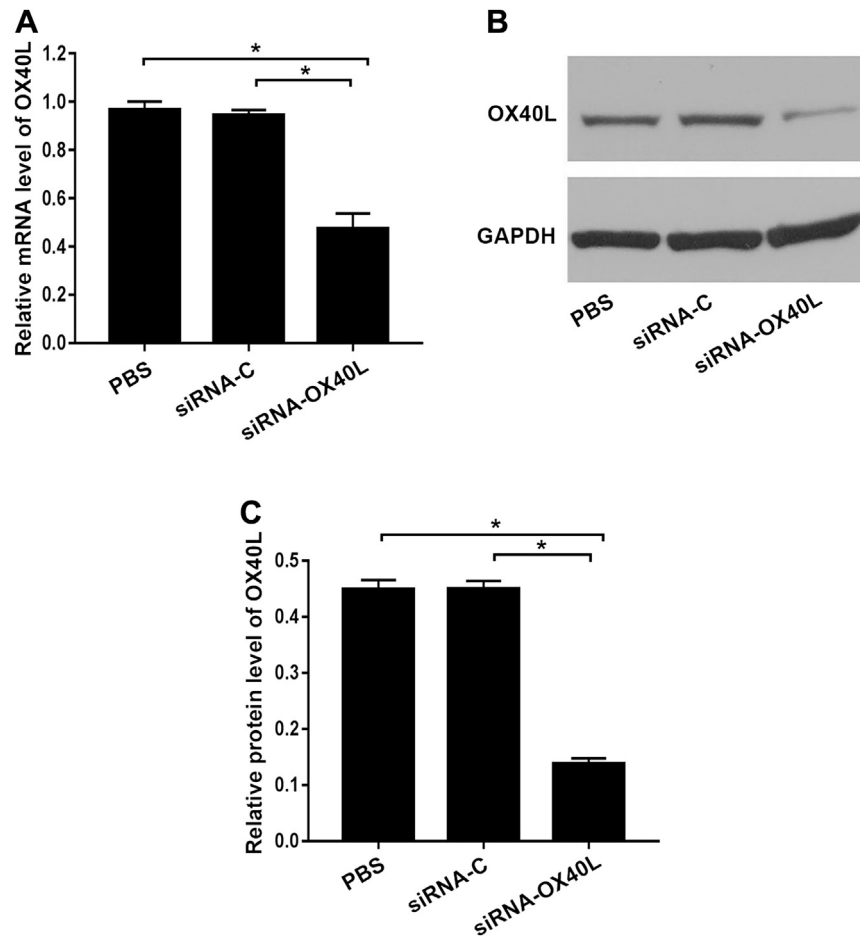


Figure 3. OX40L mRNA and protein expression in AR. (A) The relative expression levels of OX40L mRNA were determined by RT-qPCR in PBS, siRNA-C and siRNA-OX40L groups, respectively. The mRNA levels of OX40L were normalized to GAPDH mRNA. (B) Representative western blot analysis of OX40L in the 3 groups. (C) OX40L band density was normalized to GAPDH band density and represented as blot density. OX40L, OX40 ligand; AR, allergic rhinitis; RT-qPCR, quantitative real-time polymerase chain reaction; PBS, phosphate buffer saline; siRNA, small interfering RNA; siRNA-C, small interfering RNA-control. * $p < 0.05$.

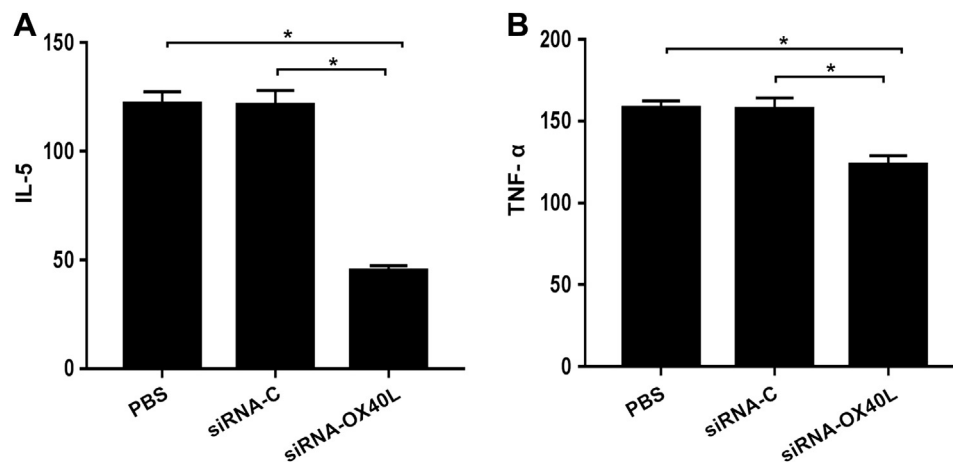


Figure 4. The expression levels of IL-5 and TNF- α . After co-culture of DCs and T cells, supernatants were collected. IL-5 (A) and TNF- α (B) expression levels were measured by ELISA. DCs, dendritic cells; ELISA, enzyme-linked immunosorbent assay. * $p < 0.05$.

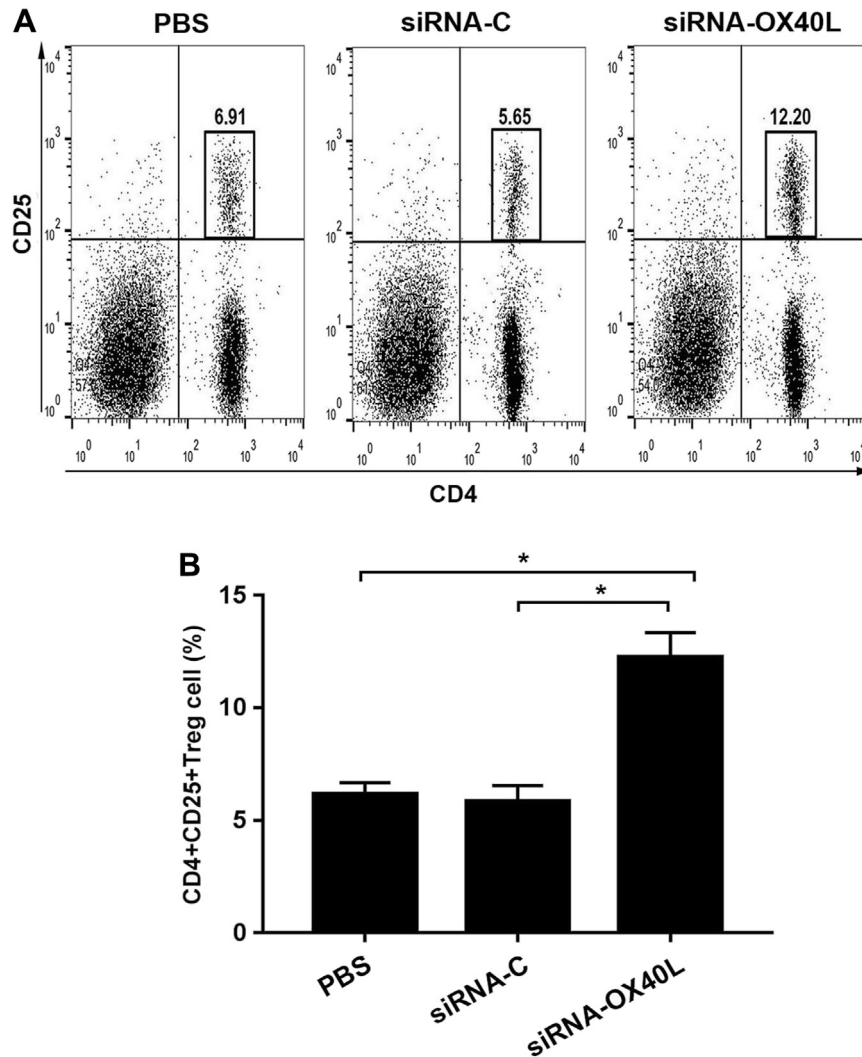


Figure 5. Expression of the CD4+CD25+ Treg-specific marker Foxp3. (A) Expression of the CD4+CD25+ Treg-specific marker Foxp3 was detected. T cell response by Treg induced by siRNA-OX40L. (B) Results of flow cytometry showed that siRNA-OX40L treated animals possessed a population of cells with the Treg phenotype, determined by the significantly increased CD4+CD25+ Treg percentages. Treg, regulatory T cell; Foxp3, forkhead box protein 3; siRNA, small interfering RNA; OX40L, OX40 ligand. * $p < 0.05$.

The Western blotting results were consistent with the RT-qPCR results.

Detection of IL-5 and TNF- α Expression by ELISA

The IL-5 and TNF- α levels in the mixed lymphocyte solution of mature DCs and T cells were significantly decreased in the experimental group. The decrease in the Th2-type cytokine IL-5 was more obvious than the decrease in the Th1-type cytokine TNF- α ($p < 0.05$ for both; Figure 4A, and 4B).

Expression of the CD4+CD25+ Treg-specific Marker Foxp3

The expression of the CD4+CD25+ Treg-specific marker Foxp3 in the three groups of mice was analyzed by flow cytometry. The Foxp3 expression level was significantly

higher in the experimental group than in the other two groups ($p < 0.05$; Figure 5A, and 5B), whereas no significant difference was found between the control and negative control groups ($p > 0.05$; Figure 5A, and 5B).

EOS Infiltration

After treatment with the siRNA-OX40L, no or little EOS infiltration was found in the experimental group, whereas significant EOS infiltration occurred in the nasal mucosal epithelia of the AR mice in the negative control and control groups (Figure 6A–6C). By counting EOS, the numbers of EOS in the PBS (28.96 ± 4.77) and siRNA-C (29.30 ± 3.71) groups were significantly higher than those of siRNA-OX40L (8.12 ± 1.78) group ($p < 0.05$; Figure 6D), but there was no significant difference in the PBS and siRNA-C groups ($p > 0.05$; Figure 6D).

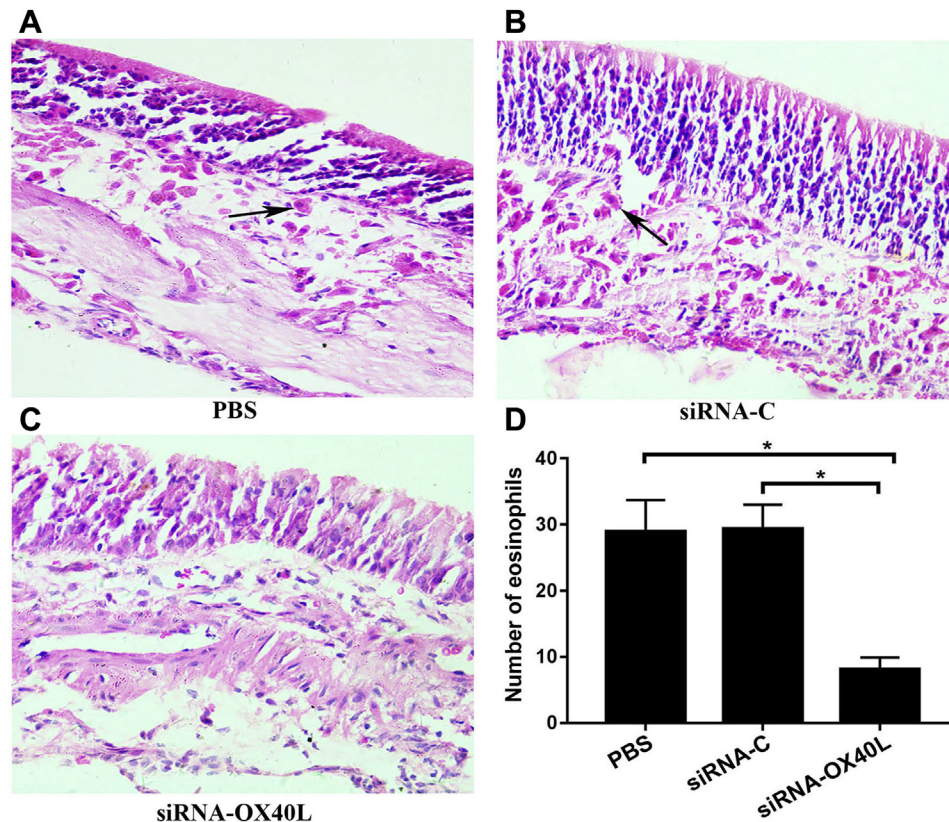


Figure 6. EOS infiltration. The EOS infiltration of nasal mucosal was observed microscopically ($\times 400$). After treatment with the siRNA-OX40L, no or little EOS infiltration was found in the experimental group (C), but significant EOS infiltration occurred in the nasal mucosal epithelia of the AR mice in the other two groups (A, and B), the black arrows showed in A and B indicated EOS. (D) The numbers of EOS of nasal mucosal were counted microscopically. EOS, eosinophils; siRNA, small interfering RNA; OX40L, OX40 ligand; AR, allergic rhinitis. $*p < 0.05$.

Discussion

AR is an IgE-mediated, chronic, allergic inflammatory disease of the airway involving various immune cells (T lymphocytes, DCs, EOS, and mast cells) and inflammatory mediators in atopic individuals after exposure to allergens. DCs are closely associated with allergic diseases and participate in the initiation of allergic reactions. DCs simultaneously provide antigen recognition signals and costimulatory signals during the activation of CD4⁺ T cells and thus are very important for the regulation of immune responses involving T cells. Research into the roles of DCs in allergic diseases has received broad attention (15,16). Our previous study showed that DCs participated in the pathogenesis of AR and AS; in particular, DCs were involved in the pathogenesis of upper and lower airway inflammation. Additionally, we found that the mechanism by which DCs took part in the consistency of upper and lower airway inflammation might be related to the preferential expression of CD4⁺ T cells toward a Th2 cell phenotype that was induced by DCs (17). Moreover, a synergistic effect may exist between DCs and EOSs in nasal inflammatory diseases (18). Based on previous studies, we analyzed the surface costimulatory molecule

OX40L on DCs in the present study. We used siRNA for target inhibition of OX40L expression on DCs, thereby blocking the OX40/OX40L signaling pathway in AR to explore its effect on AR and the underlying mechanism.

The costimulatory molecules OX40 and OX40L, are mainly expressed on the surfaces of activated T cells and APCs, respectively. Binding of the two molecules can enhance the binding ability of T cells to APCs, such as DCs. Additionally, these molecules promote the binding of T cell surface receptors to the MHC-antigen peptide complex and provide necessary auxiliary signals for T cell activation (11,12,19–22). Blockage of the OX40/OX40L costimulatory signaling pathway using an anti-OX40L monoclonal antibody specifically inhibits the function of activated T cells, thereby alleviating symptoms in the acute phase of AS (23,24). Moreover, studies that explored the biological function of OX40/OX40L costimulatory signal molecules in AS found that the Th2 cytokines IL-4 and IL-5, antigen-specific IgE, EOS aggregation, airway responsiveness, and other AS reactions were significantly reduced or not induced in the lungs of AS model mice after blockage of the OX40 signaling pathway or knock out of the OX40 gene; the lesions and clinical symptoms were significantly attenuated in the lungs of these mice; the same

results were obtained in AS mice treated with an anti-OX40L monoclonal antibody (9,25–29). In the present study, we inhibited the expression of the surface costimulatory molecule OX40L on DCs in AR mice through siRNA-OX40L interference, thereby blocking the OX40/OX40L singling pathway. This blockage also significantly alleviated the allergic symptoms of the mice, including nose scratching, sneezing, and nasal discharge, and decreased the expression levels of the Th1- and Th2-type cytokines TNF- α and IL-5, respectively, and EOS infiltration. Our results are similar to the findings of the above studies on AS.

The quantitative imbalance and/or functional imbalance of the Th1/Th2 subsets are the most important immunological abnormalities in allergic diseases (30). OX40/OX40L play important roles in Th1 and Th2 development (11,19,20). OX40 signal defects can deviate T cell response to a Th1-type response (19,31,32). Additionally, OX40L can induce and activate T cells to express IL-4 while inhibiting INF- γ expression. Thus, OX40L is considered a switch for the transformation of CD4+ T cells to the Th2 phenotype (33). A study using inbred mice infected with *Leishmania* spp. as a characteristic animal model found that blockage of OX40/OX40L led to a significant reduction in the production of Th2-type cytokines; meanwhile, the anti-leishmania IgG1 and serum IgE levels decreased, the infection level of the BALB/c mice decreased, and the inflammatory response of the CD4+ Th1 cells was promoted. These findings indicate that the mutual response of OX40/OX40L plays a critical role in the differentiation of CD4+ T cells toward the Th2 response (34). Blockage of the OX40/OX40L interaction using an OX40L immunoglobulin fusion protein increased INF- γ secretion in CD4+ T cells and alleviated EOS infiltration and cryptococcus neoformans infection in the lungs in a *C. neoformans* infection C57BL/6 mouse model (35). By blocking the OX40/OX40L signaling pathway, an anti-OX40 monoclonal antibody effectively inhibited EOSs and Th2 cytokine expression in mice with allergic airway diseases and prevented the progression of inflammation induced by dermatophagoides pteronyssinus (36). In the present study, we found a significantly greater decrease in the Th2-type cytokine IL-5 than in the Th1-type cytokine TNF- α in the experimental group after siRNA-OX40L treatment, which induced deviation of the T cell response to a Th1 type; moreover, the Treg expression level was increased, which was similar to the results Suzuki M, et al. (14) obtained with CD40. Treg cells are a cell population with characteristics of both immune tolerance and immunosuppression. Treg cells can inhibit Th1 and Th2 responses and thus play an important regulatory role in maintaining the stability of the immune system (37). Foxp3, which is a specific marker of CD4+CD25+Tregs (38), is an important switch for Treg cell development (39). One study suggested that blocking one or more DC activation signals resulted in tolerant DCs, which could

inhibit T cell responses through facilitating the induction of anergy or low-energy responses in peripheral T cells and the production of Tregs (40). Thus, we speculate that the mechanism underlying the effect of siRNA-OX40L on AR might be related to correcting Th2 cell immune deviation, inducing immune tolerance, and promoting Treg cell production.

Conclusions

We described suppression of allergic reactions and relief of allergic symptoms in an OVA-induced mouse AR model by blockage of the OX40/X40L signaling pathway with siRNA-OX40L interference. The mechanism may be related to correcting Th2 cell immune deviation, inducing immune tolerance, and promoting Treg cell production. Therefore, the study of OX40/X40L signaling pathway will help us broaden and deepen the understanding of the pathogenesis of AR, and provide a new idea and a therapeutic target for AR.

Acknowledgments

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Conflict of Interest

The authors declare that there is no conflict of interest.

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