

The influence of house dust mite sublingual immunotherapy on the TSLP-OX40L signaling pathway in patients with allergic rhinitis

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Background: This study aimed to investigate the clinical efficacy of sublingual immunotherapy (SLIT) with house dust mite (HDM) extract and to examine T helper 2 (Th2)-type immune responses mediated by the thymic stromal lymphopoietin (TSLP-OX40L) signaling pathway in patients with moderate to severe allergic rhinitis (AR) after 12-month HDM SLIT.

Methods: Forty-six cases of HDM-sensitized patients with persistent AR in southern China were enrolled in this study. Clinical efficacy of SLIT was assessed by determining the individual nasal symptom score (INSS) and total nasal symptom score (TNSS) after 12-month HDM SLIT. Moreover, the TSLP-OX40L signaling pathway was investigated through measurements of TSLP by enzyme-labeled immunosorbent assay (ELISA) and OX40L by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and flow cytometry.

Results: After 12 months of HDM SLIT, TNSS and INSS were significantly decreased overall compared with baseline values ($p < 0.001$). By the end of the 12-month HDM SLIT, TNSS had declined by ~50% compared with baseline, and the corresponding level of TSLP in nasal lavage decreased

significantly ($p < 0.05$). The level of OX40L messenger RNA (mRNA) in blood was markedly decreased significantly after 12-month HDM SLIT compared with baseline ($t = 12.300$, $p < 0.05$). Furthermore, significant decreases in OX40L expression on the surface of peripheral blood mononuclear cells (PBMCs) ($t = 13.100$, $p < 0.05$) and OX40L expression on the surface of CD11c+CD86+ cells in PBMCs ($t = 9.946$, $p < 0.05$) after 12-month HDM SLIT were observed.

Conclusion: HDM SLIT downregulated Th2-type immune responses mediated by the TSLP-OX40L signaling pathway in patients with persistent moderate to severe AR. ©2016 ARSAAOA, LLC © 2016 ARS-AAOA, LLC.

Key Words:

allergic rhinitis; sublingual immunotherapy; TSLP; OX40L; T lymphocytes

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Allergic rhinitis (AR) is becoming 1 of the greatest public health challenges in China and is considered to be a

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risk factor for subsequent development of asthma through natural processes.¹ According to epidemiological studies, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are the most commonly inhaled allergens that can cause allergic diseases such as AR and asthma.²

AR is characterized by so-called T helper 2 (Th2)-type immune responses.³ Systemic immunity and mucosal immunity are both involved in Th2-type responses in AR by independent but interacting ways.⁴ These 2 types of immunity do not function synchronously.⁴ Therefore, evaluating Th2-type immune responses in AR with criteria of mucosal immunity or systemic immunity separately would not be comprehensive. Although much progress has been achieved toward understanding Th2-type immune responses in recent years, many aspects remain unknown. Recently, an emerging concept regarding the role of the airway epithelium in the pathogenesis of Th2-type responses has received increasing attention.⁵ Thymic stromal

lymphopoietin (TSLP), an epithelium-generated cytokine, is produced mainly by damaged epithelial cells and is considered to have the ability to directly induce robust Th2 responses in AR.⁶ TSLP represents a master switch at the interface between environmental allergens and has been demonstrated to be a necessary and sufficient factor for the initiation of allergic airway inflammation.⁷ Downstream of the TSLP signaling pathway are OX40 and its ligand OX40L. OX40L is a type II transmembrane glycoprotein expressed on the surface of mature dendritic cells (DCs), macrophages, and activated T and B cells.⁸ OX40L interacts with OX40 in a range of memory T cell activities and in maintaining the survival and proliferation of effector T cells.⁹ Therefore, TSLP-OX40L has been considered an important signaling pathway in the initiation and maintenance of Th2-type immune responses in AR.

Allergen-specific immunotherapy has been used more than 100 years as a desensitizing treatment for allergic diseases. This is a potentially curative and specific method of treatment and is believed to be the only treatment with the capacity to change the natural course of allergic diseases.¹⁰ Since sublingual immunotherapy (SLIT) was first applied in clinical practice in 1986,¹¹ many randomized double-blind placebo-controlled trials with a large number of samples have confirmed the clinical efficacy of this treatment in patients with AR.¹² However, the mechanism of SLIT remains unclear. Until now, studies on the use of SLIT in the treatment of allergic airway disease have mainly been conducted in patients from European countries who suffered seasonal AR with sensitivity to grass or pollen allergens.¹² Whether house dust mite (HDM) SLIT would have the same clinical efficacy for persistent moderate to severe AR as that achieved by pollen allergens and the potential mechanisms remain unclear.

SLIT regulates Th2-type immune responses in AR through the mucosal route. The Th2-type immune responses mediated by the TSLP-OX40L signaling pathway are also initiated in mucosa, thereby triggering a cascade of effects in the systemic immunity. Whether an interaction exists between HDM SLIT and Th2-type immune responses mediated by TSLP-OX40L signal requires investigation. Therefore, the main purpose of this study was to assess the efficacy of HDM SLIT in patients with AR and to investigate the effect of this treatment on Th2-type immune responses mediated by the TSLP-OX40L signaling pathway.

Patients and methods

Patients

A total of 46 AR patients were enrolled in our study. The diagnosis of moderate to severe persistent AR was made based on clinical criteria, including sneezing, itching, rhinorrhea, and nasal congestion. According to the Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines,¹³

inclusion criteria were as follows: (1) clinical criteria of moderate to severe persistent AR for at least 2 years; (2) sensitization to HDM including *D. pteronyssinus* and/or *D. farinae* with a positive skin-prick test (wheal diameter >6 mm) and/or CAP-Pharmacia score > class 2 (Phadia, Uppsala, Sweden). Exclusion criteria were as follows: (1) having a systemic immunological disorder; (2) received intercurrent oral corticosteroid treatment and/or β -blockers in the previous 6 months. The study protocol was approved by the Ethics Committees of our hospitals. Written consent was obtained from each patient or guardians for patients less than 18 years old before the trial.

HDM allergen extract

The HDM allergen extract (CHANLLERNGEN, *D. farinae* Drops; Zhejiang Wolwo Bio-Pharmaceutical Co., Ltd., Huzhou, Zhejiang, China) was used in this study. Briefly, according to the manufacturer's instructions, all patients were asked to take a daily dose of HDM allergen extract by dropping it in the floor of the mouth and keeping it under the tongue for 2 minutes before swallowing. No. 4 solution was taken daily during the maintenance phase by patients less than 14 years old. No. 5 solution was taken daily during the maintenance phase by patients older than 14 years old after No. 4 solution was used up. The step-up dosage protocol was standardized as described.¹⁴

Study protocol

Each patient was observed for a 12-month period. Changes of clinical symptoms and laboratory assessments of molecules in the TSLP-OX40L signaling pathway in all patients were dynamic observed and analyzed before and after the 12-month HDM SLIT. For ethical reasons, a control group could not be used in our studies.

Sample collection

Nasal lavage fluid samples and blood samples were collected before and after the 12-month HDM SLIT in all patients. Nasal lavage fluid was obtained from subjects using previously described methods.¹⁵ Briefly, 5 mL of saline solution (prewarmed to 37°C) was applied with spray atomization into each nasal cavity of subjects with their heads bent down. The fluid was collected by asking the subjects to lean forward and expel the nasal fluid into a container. After 20 seconds, nasal lavage fluid samples were filtered to remove the nasal mucus and centrifuged immediately at 4000 rpm (1800 g) for 5 minutes. The collected samples were aliquotted into graded test tubes and frozen at $\pm 80^{\circ}\text{C}$ until assayed. Venous blood samples (10 mL) were also collected from fasting patients. Ethylenediamine tetraacetic acid (EDTA) anticoagulant was added to 2 mL of the blood sample for RNA extraction, while the remaining 8 mL of blood was processed with heparin anticoagulant for

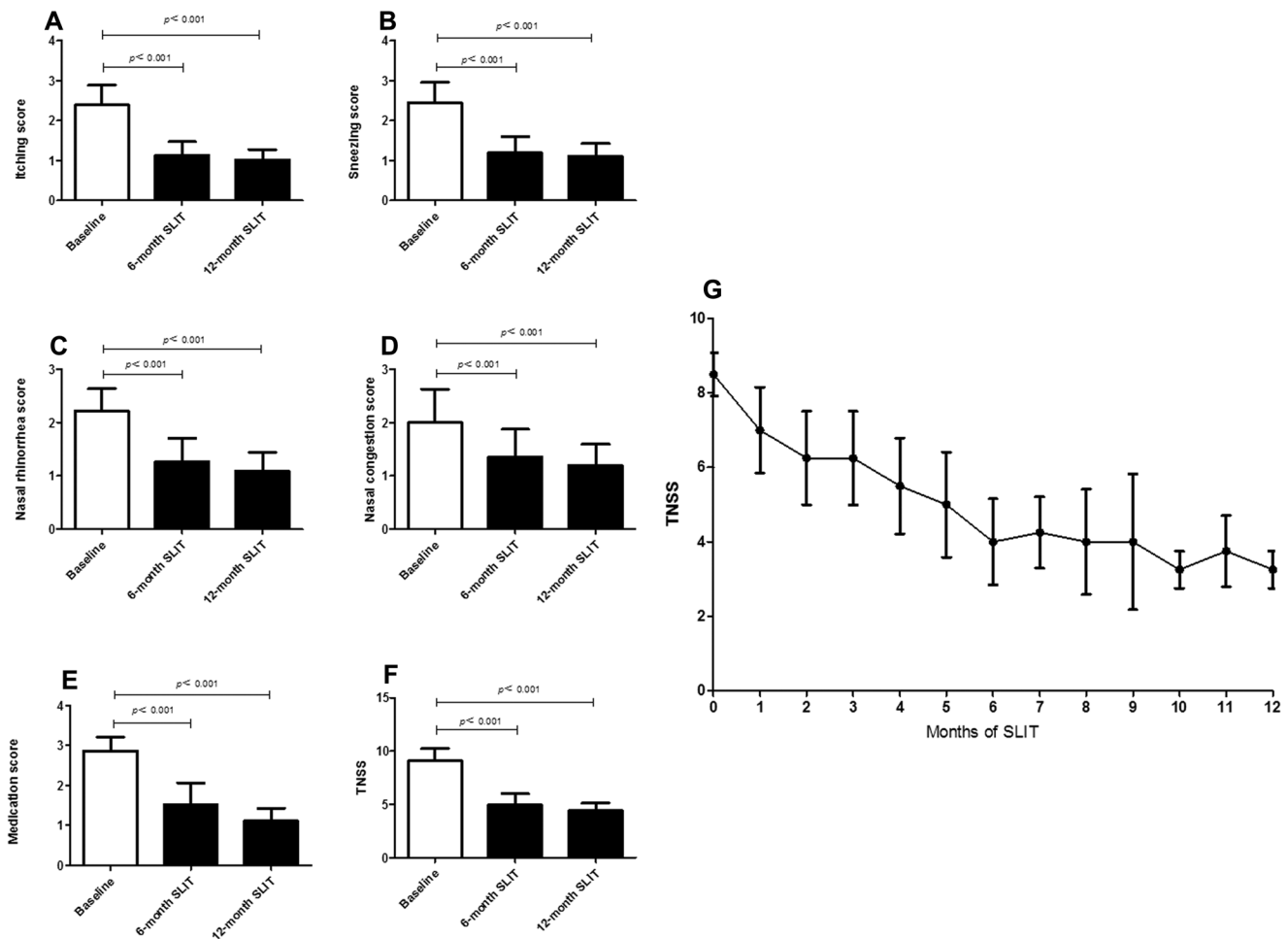


FIGURE 1. Change of symptom scores and medication scores after 12-month SLIT treatment. (A-D) INSS. (E) Medical score. (F) TNSS. (G) TNSS score trends within 12-month after SLIT treatment. All symptom scores and medication scores (A-F) decreased significantly compared with baseline value ($p < 0.001$). TNSS had declined by about 50% compared with baseline value after 12-month SLIT treatment ($p < 0.001$). Data are mean $\pm$ SEM. INSS = individual nasal symptom score; SEM = standard error of the mean; SLIT = sublingual immunotherapy; TNSS = total nasal symptom score.

separation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation with Ficoll.

Clinical evaluation

During the 12-month SLIT treatment, patients were required to record their daily symptom score and medicine score to evaluate clinical efficacy of HDM SLIT. Symptom scores were assessed as individual nasal symptom scores (INSSs) and a total nasal symptom score (TNSS) as described.¹⁶ The INSS included evaluations of nasal rhinorrhea, sneezing, itching, and congestion on a scale of 0 to 3 (0 = no symptom, 1 = mild, 2 = moderate, 3 = severe). The TNSS was defined as the sum of the 4 individual symptom scores.

Laboratory assays

ELISA

In this study, commercial ELISA kits were used to measure concentrations of TSLP (R&D Systems, Minneapolis, MN) interleukin (IL)-4, IL-13, and interferon

(IFN)- γ (eBiosciences, San Diego, CA) in nasal lavage according to the manufacturer's instructions. Briefly, nasal lavage samples were added in duplicate to 96-well plates with 100 μ L per well. The appropriate biotin-conjugated antibodies were added to each well for incubation with the samples at room temperature for 2 hours. The liquid in the wells was then aspirated, and each well was washed 5 times. The substrate solution was added to each well for incubation at room temperature in the dark for 30 minutes. Finally, amounts of TSLP, IL-4, IL-13, and INF- γ in the nasal lavage were determined by detecting the optical density.

Quantitative real-time PCR

Heparinized blood samples were collected from all patients before and after the 12-month SLIT to isolate PBMCs by Ficoll-Hypaque density gradient centrifugation (TBD, Tianjin, China). The total RNA was extracted from PBMCs using TRIzol reagent (Life Technologies, Carlsbad, CA). The procedure for quantitative real-time PCR (qRT-PCR) was performed as described.¹⁶ Briefly, the

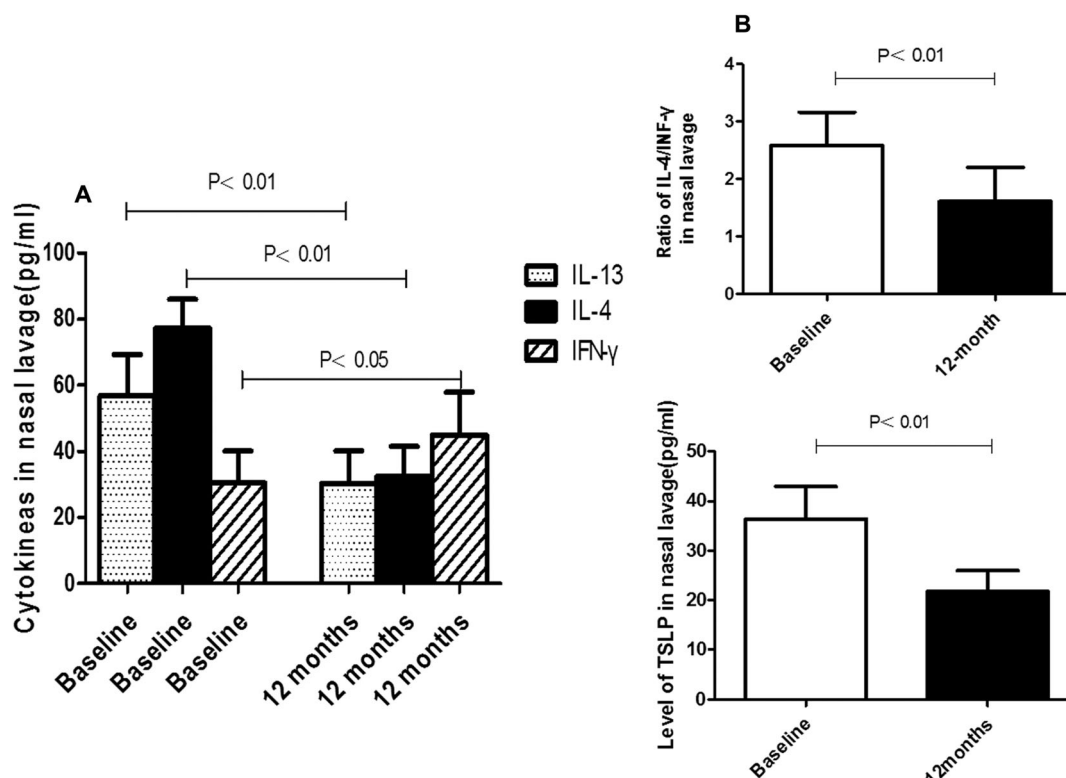


FIGURE 2. (A) The expressions of cytokine in nasal lavage fluid before and after 12-month SLIT treatment. Levels of Th2 cytokine IL-4 and IL-13 in nasal lavage samples decreased significantly ($p < 0.01$) and Th1 cytokine INF- γ increased significantly ($p < 0.05$) after 12-month HDM SLIT. (B) The ratio of IL-4/INF- γ in nasal lavage fluid before and after 12-month SLIT treatment. The ratio of IL-4/INF- γ decreased was statistically significant ($p < 0.01$) after 12-month SLIT treatment. (C) The expressions of TSLP in nasal lavage fluid after 12-month SLIT treatment decreased significantly ($p < 0.01$). Data are mean $\pm$ SEM. HDM = house dust mite; IFN = interferon; IL = interleukin; SEM = standard error of the mean; SLIT = sublingual immunotherapy; Th1 = T helper 1; Th2 = T helper 2; TSLP = thymic stromal lymphopoietin.

messenger RNA (mRNA) expression was measured using an ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA) and SYBR Premix TaqTM (Takara, Japan). Primer sequences were as follows: OX40L forward, 5'-CCCAGATTGTGAAGATGGAA-3'; OX40L reverse, 5'-GCCTGGTTTTAGATATTGCC-3'; GATA-3 forward, 5'-ACCGGCTTCGGATGCAA-3'; GATA-3 reverse, 5'-TGCTCTCCTGGCTGCAGAC-3'; T-bet forward, 5'-AACACAGGAGCGCACTGGAT-3'; T-bet reverse, 5'-AACACAGGAGCGCACTGGAT-3'; β -actin forward, 5'-GGCCAACCGCGAGAAGAT-3'; and β -actin reverse, 5'-CGTCACCGGAGTCCATCAC-3'. The PRISM samples contained 1 $\times$ SYBR Green Master Mix, 1.5 μ L of 5- μ M primers, and 25 ng of synthesized complementary DNA (cDNA) in a 25- μ L reaction volume. The mean value of the replicates for each sample was calculated and expressed as a threshold cycle (Ct) value. The relative expression of the target gene in 1 sample was determined as $2^{-\Delta C_t}$ (ΔC_t , difference between the Ct value of the target gene and the Ct value of β -actin). Fold changes in the target gene mRNA in different samples before and after SLIT treatment were determined as $2^{-\Delta\Delta C_t}$.

Flow cytometry

PBMC samples were centrifuged at 2000 rpm (900 g) for 10 minutes at 4°C, and the cell pellets were resuspended in phosphate-buffered saline (PBS) to obtain single-cell suspensions. After counting, the single-cell suspensions were stained with combinations of fluorescein isothiocyanate (FITC)-A-conjugated anti-human CD11c, phycoerythrin (PE)-A-conjugated anti-human OX40L, and allophycocyanin (APC)-A-conjugated anti-human CD86 antibodies, followed by staining with the appropriate fluorochrome-conjugated isotype control immunoglobulin (Ig). The antibodies were purchased from eBiosciences. The samples were incubated with the antibody cocktail for 20 minutes at room temperature, followed by centrifugation at 1500 rpm (700 g) for 5 minutes at 4°C. The cells were washed twice with PBS. After staining, the cells were resuspended in PBS and analyzed using a FACS LSRII (BD Biosciences, San Jose, CA). The dead cells and debris were excluded based on light-scatter properties. DCs were sorted based on expression of CD11c on forward scatter (FSC)/side scatter (SSC) plots. The sorted CD11c+ populations were analyzed for the expression of the surface molecules OX40L and CD86. After acquisition, flow cytometry data were analyzed using BD FACSDiva software (BD Biosciences).

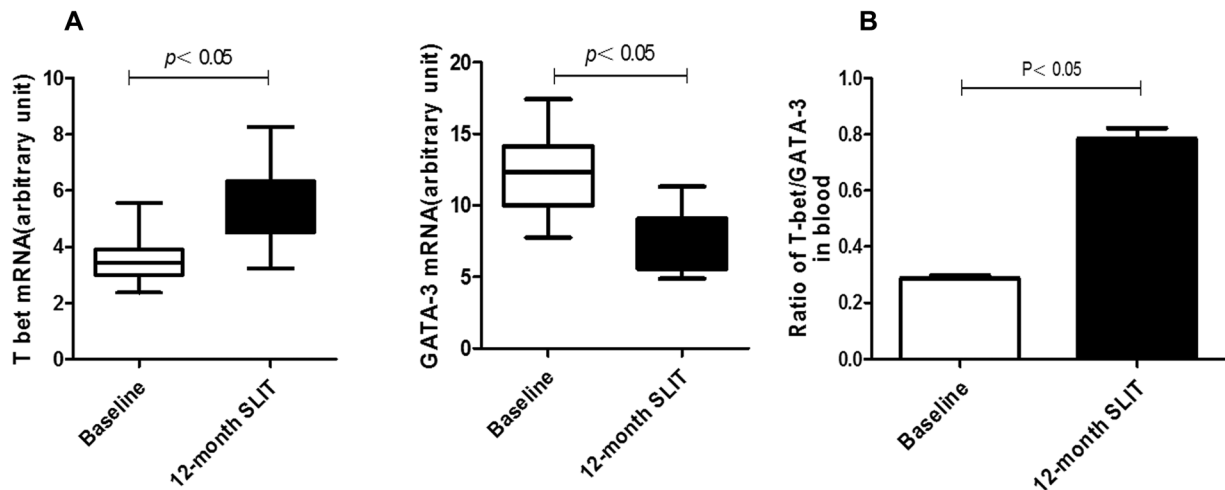


FIGURE 3. (A) The arbitrary units of T-bet mRNA, GATA-3 mRNA in blood before and after 12-month SLIT. The relative quantitative T-bet mRNA and GATA-3 mRNA in the blood was detected using RT-PCR. After 12-month SLIT treatment, T-bet mRNA (A) level was significantly increased compared with the baseline value ($p < 0.05$) and GATA-3 mRNA (A) level was significantly reduced compared with the baseline value ($p < 0.05$). (B) The ratio of T-bet mRNA/GATA-3 mRNA in blood before and after 12-month SLIT. The ratio of T-bet mRNA/GATA-3 mRNA in blood (B) was significantly increased after 12-month SLIT treatment ($p < 0.05$). Data are mean $\pm$ SEM. mRNA = messenger RNA; RT-PCR = reverse transcription polymerase chain reaction; SEM = standard error of the mean; SLIT = sublingual immunotherapy.

Data analysis

The statistical significance of differences between baseline and treatment values was determined using the Student *t* test, nonparametric Mann-Whitney *U* test, or Wilcoxon signed-rank test. The Spearman rank correlation test was used to analyze the correlation between different biomarkers. A *p* value < 0.05 was considered significant. All of the results are presented as the means $\pm$ standard error of the mean (SEM).

Results

Clinical efficacy of 12-month SLIT

After the 12-month SLIT, significant decreases were observed in TNSS, medication score, and all INSS (nasal rhinorrhea, itching, sneezing, and nasal congestion) compared with baseline values ($p < 0.001$) (Fig. 1). TNSS significantly decreased after 12-month SLIT to 4.413 ± 0.717 compared with the baseline value of 9.065 ± 1.162 ($X^2 = 82.667$, $p < 0.001$), while the medication score decreased from 2.870 ± 0.341 to 1.109 ± 0.315 ($X^2 = 79.590$, $p < 0.001$). All INSS values showed significant changes after the 12-month SLIT: the itching score decreased from 2.391 ± 0.493 to 1.022 ± 0.257 ($X^2 = 86.681$, $p < 0.001$); nasal congestion score decreased from 2.000 ± 0.633 to 1.196 ± 0.401 ($X^2 = 60.057$, $p < 0.001$); sneezing score decreased from 2.457 ± 0.504 to 1.109 ± 0.315 ($X^2 = 74.146$, $p < 0.001$); and nasal rhinorrhea score decreased from 2.217 ± 0.417 to 1.087 ± 0.354 ($X^2 = 83.532$, $p < 0.001$). The results showed a significant improvement of clinical symptoms for patients with persistent moderate to severe AR after the 12-month HDM SLIT. The dependence on drugs was reduced significantly for all patients. No serious adverse events and no cases of aggravated symptoms were

reported during the 12-month HDM SLIT, and by the end of the treatment period, TNSS had declined by about 50% compared with baseline (Fig. 1). Changes of INSS during the 12-month HDM SLIT were similar to those of TNSS. Altogether, these results demonstrated the safety and efficacy of HDM SLIT.

Determination of cytokine levels after 12-month SLIT by ELISA

Levels of Th2 cytokine (IL-4, IL-13) and TSLP in nasal lavage samples decreased significantly ($p < 0.05$) and Th1 cytokine (INF- γ) increased significantly ($p < 0.05$) after 12-month HDM SLIT (Fig. 2). The level of IL-4 decreased from 76.010 ± 9.394 pg/mL to 32.910 ± 8.752 pg/mL ($t = 27.010$, $p < 0.05$); IL-13 decreased from 55.68 ± 12.86 pg/mL to 30.000 ± 9.453 pg/mL ($t = 14.050$, $p < 0.05$); INF- γ increased from 30.600 ± 6.755 pg/mL to 43.890 ± 2.528 pg/mL ($t = 4.890$, $p < 0.05$); and TSLP decreased from 36.390 ± 6.610 pg/mL to 21.780 ± 4.231 pg/mL ($t = 11.110$, $p < 0.05$). Meanwhile, the ratio of IL-4/INF- γ decreased from 2.584 ± 0.581 to 1.833 ± 0.223 , and the difference was statistically significant ($t = 3.331$, $p < 0.01$) (Fig. 2).

Transcriptional analysis of T-bet, GATA-3, and OX40L by qRT-PCR

Expression of T-bet mRNA in the blood significantly increased from 2.9-fold to 4.1-fold to 4.2-fold to 6.6-fold after the 12-month SLIT compared with the baseline ($t = 12.230$, $p < 0.05$). Expression of GATA-3 mRNA in the blood significantly decreased from 9.9-fold to 14.9-fold to 5.3-fold to 9.3-fold after the 12-month SLIT compared with baseline ($t = 11.920$, $p < 0.05$) (Fig. 3). The ratio of T-bet mRNA/GATA-3 mRNA increased significantly from

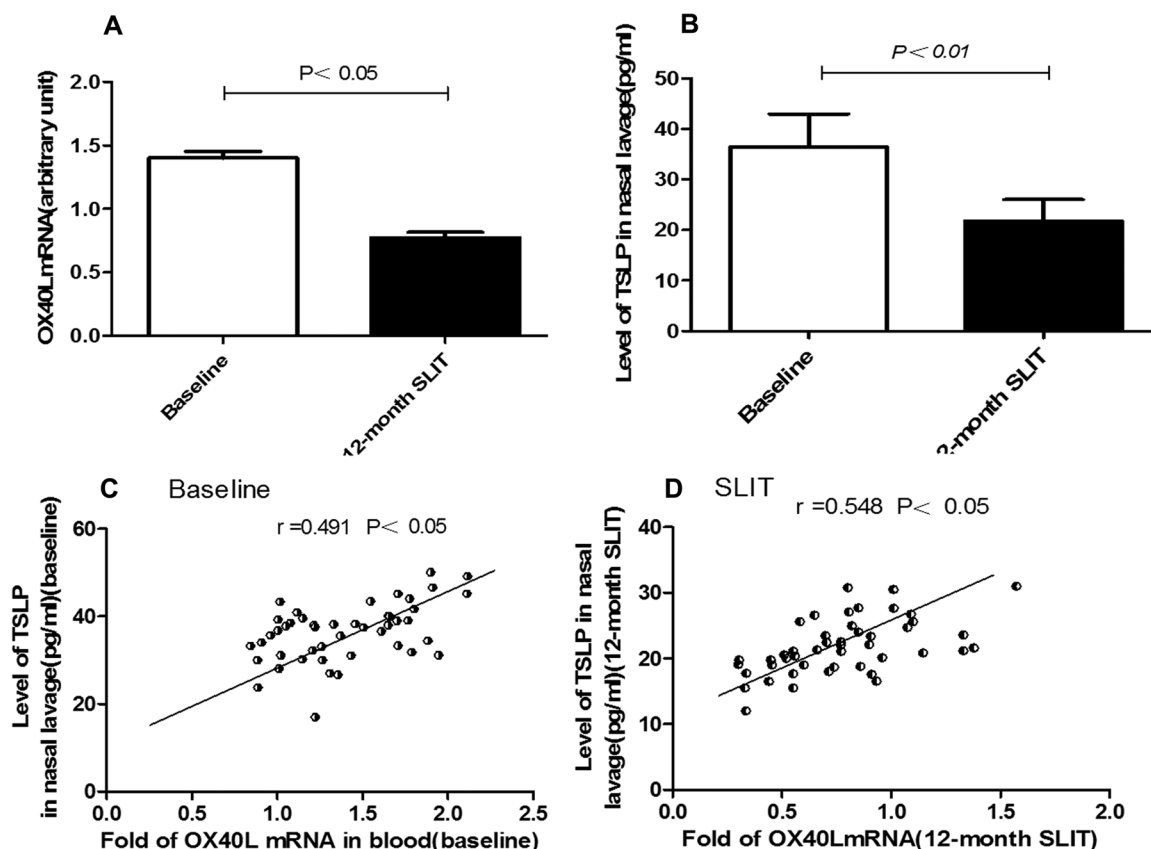


FIGURE 4. (A) The arbitrary units of *OX40L* mRNA in blood before and after 12-month SLIT were detected using RT-PCR. The level of *OX40L* mRNA in blood decreased significantly after the 12-month SLIT compared with baseline value ($p < 0.05$). (B) The TSLP protein concentration in nasal lavage before and after 12-month SLIT was detected using an ELISA kit. The level of TSLP in nasal lavage decreased significantly after the 12-month SLIT compared with baseline value ($p < 0.01$). (C, D) The positive association between *OX40L* mRNA in blood and TSLP in nasal lavage before and after 12-month SLIT, respectively, was observed ($p < 0.05$). Data are mean $\pm$ SEM. ELISA = enzyme-linked immunosorbent assay; mRNA = messenger RNA; RT-PCR = reverse transcription polymerase chain reaction; SEM = standard error of the mean; SLIT = sublingual immunotherapy; TSLP = thymic stromal lymphopoietin.

0.288 ± 0.071 to 0.785 ± 0.251 ($t = 13.890$, $p < 0.05$) (Fig. 3). The level of *OX40L* mRNA in blood decreased significantly after the 12-month SLIT from 1.1-fold to 1.8-fold to 0.5-fold to 1.0-fold compared with baseline ($t = 12.300$, $p < 0.05$) (Fig. 3). Statistically significant negative associations of levels of *OX40L* mRNA and the ratios of *T-bet* mRNA/*GATA-3* mRNA in the blood were observed before and after the 12-month SLIT (before SLIT: $r = -0.440$, $p < 0.05$; after 12-month SLIT: $r = -0.565$, $p < 0.05$) (Fig. 3). Furthermore, significant positive associations of levels of *OX40L* mRNA in the blood and levels of TSLP in nasal lavage fluid also were observed before and after the 12-month SLIT (before SLIT: $r = 0.491$, $p < 0.05$; after 12-month SLIT: $r = 0.548$, $p < 0.05$) (Fig. 4).

Measurement of *OX40L* expression on PBMCs by flow cytometry

OX40L expression on the surface of PBMCs decreased significantly ($t = 13.100$, $p < 0.05$) from $2.712\% \pm 0.511\%$ before treatment down to $1.630\% \pm 0.471\%$ after the 12-month SLIT (Fig. 5). *OX40L* expression on the surface of CD11c+CD86+ cells in PBMCs also significantly de-

creased after the 12-month SLIT from $5.839\% \pm 2.841\%$, to $2.098\% \pm 0.870\%$ ($t = 9.946$, $p < 0.05$) (Fig. 6).

Discussion

The importance of respiratory tract mucosa and TSLP-*OX40L* signaling pathway in the pathophysiology of allergic diseases have received increased attention recently.^{5,7} The current view regarding achieving immunity in the upper respiratory tract is that mucosal immunization would be the most effective way to induce local long-term immune memory.¹⁷ TSLP-*OX40L* signaling pathway appears to be a critical pathway in the context of allergic inflammation. In order to better understand the influence of HDM SLIT on Th2-type immune responses in AR, we evaluated it from the 2 aspects, mucosal and systemic immunity in our studies.

From our clinical observations, we demonstrated that after the 12-month HDM SLIT, all of the AR symptoms including TNSS and INSS had improved significantly compared with baseline values. The dependence on drugs for all patients also was reduced significantly. Wang et al.¹⁴ had achieved similar results with the same dust mite allergens

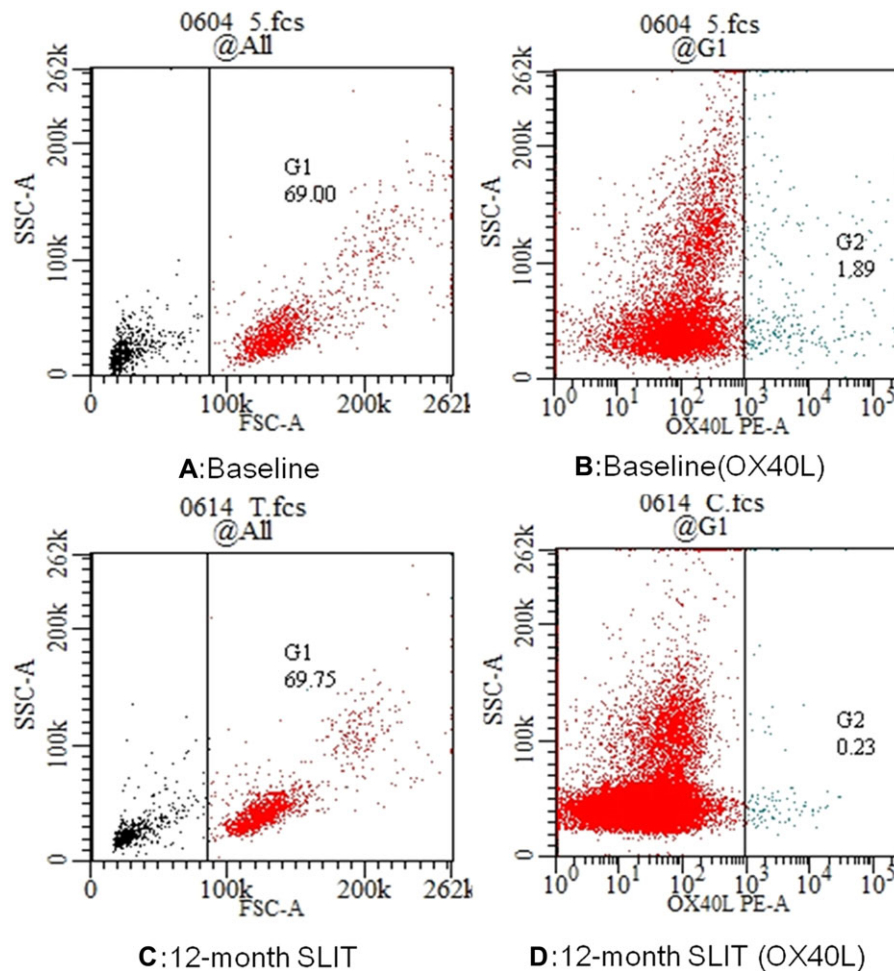


FIGURE 5. The expressions of OX40L on the surface of PBMC before and after 12-month SLIT. PBMCs (red color) were stained with PE-A-conjugated anti-human OX40L antibody. The dead cells (black color) were excluded based on FSC/SSC plots. OX40L (blue color) expression on the surface of PBMCs decreased significantly ($p < 0.05$) after 12-month SLIT treatment. Data are mean $\pm$ SEM. FSC = forward scatter; PBMC = peripheral blood mononuclear cell; PE = phycoerythrin; SEM = standard error of the mean; SLIT = sublingual immunotherapy; SSC = side scatter.

in 120 cases for 6 months. Because of the climatic characteristics of the subtropical zone in our region, the seasonal alteration is not obvious, so the influence of seasonal alteration for AR is neglected accordingly in our studies.

Nasal lavage fluid has been used widely to determine mediators involved in AR, and can directly better reflect the local pathophysiology than systemic mediators,¹⁵ so we selected it to evaluate the status of Th2-type immune responses in nasal mucosa through the expression of Th2-associated cytokines IL-4 and IL-13, as well as Th1-associated cytokine INF- γ . As we expected, the expressions of Th2-associated cytokines (IL-4 and IL-13) were all significantly decreased and Th1-associated cytokine (INF- γ) increased significantly in nasal lavage fluid after 12-month HDM SLIT. Moreover, the ratios of IL-4/INF- γ also decreased. The relative changes of representative cytokines had been used as an index to reflect the balance between Th1-type and Th2-type immune responses. Therefore, the balance of Th2 and Th1 responses in nasal mucosal immunity shifted to the direction of a Th1-type immune response.

To further observe the effects of 12-month HDM SLIT on systemic immunity, the expressions of T-bet mRNA and GATA-3 mRNA in PBMCs were detected in our studies. T-bet is responsible for the induction of Th1 cell differentiation, whereas GATA-3 is mainly responsible for the induction of Th2 differentiation. Therefore, the equilibrium between T-bet and GATA-3 determines the state of balance between the Th1 and Th2 cells. Consistent with changes of Th2-type responses in nasal mucosa, *T-bet* mRNA expression increased significantly and *GATA-3* mRNA expression decreased significantly in PBMCs after 12-month HDM SLIT. Thus, the T-bet/GATA-3 homeostasis was altered and shifted toward the T-bet direction. This result suggested that the balance of the Th1/Th2 responses in systemic immunity had shifted to the Th1 direction.

Although the efficacy of HDM SLIT has been reported by many studies, the mechanisms underlying the success of this treatment are not completely understood. The TSLP-OX40L signaling pathway was demonstrated to be a critical pathway for the initiation and maintenance of

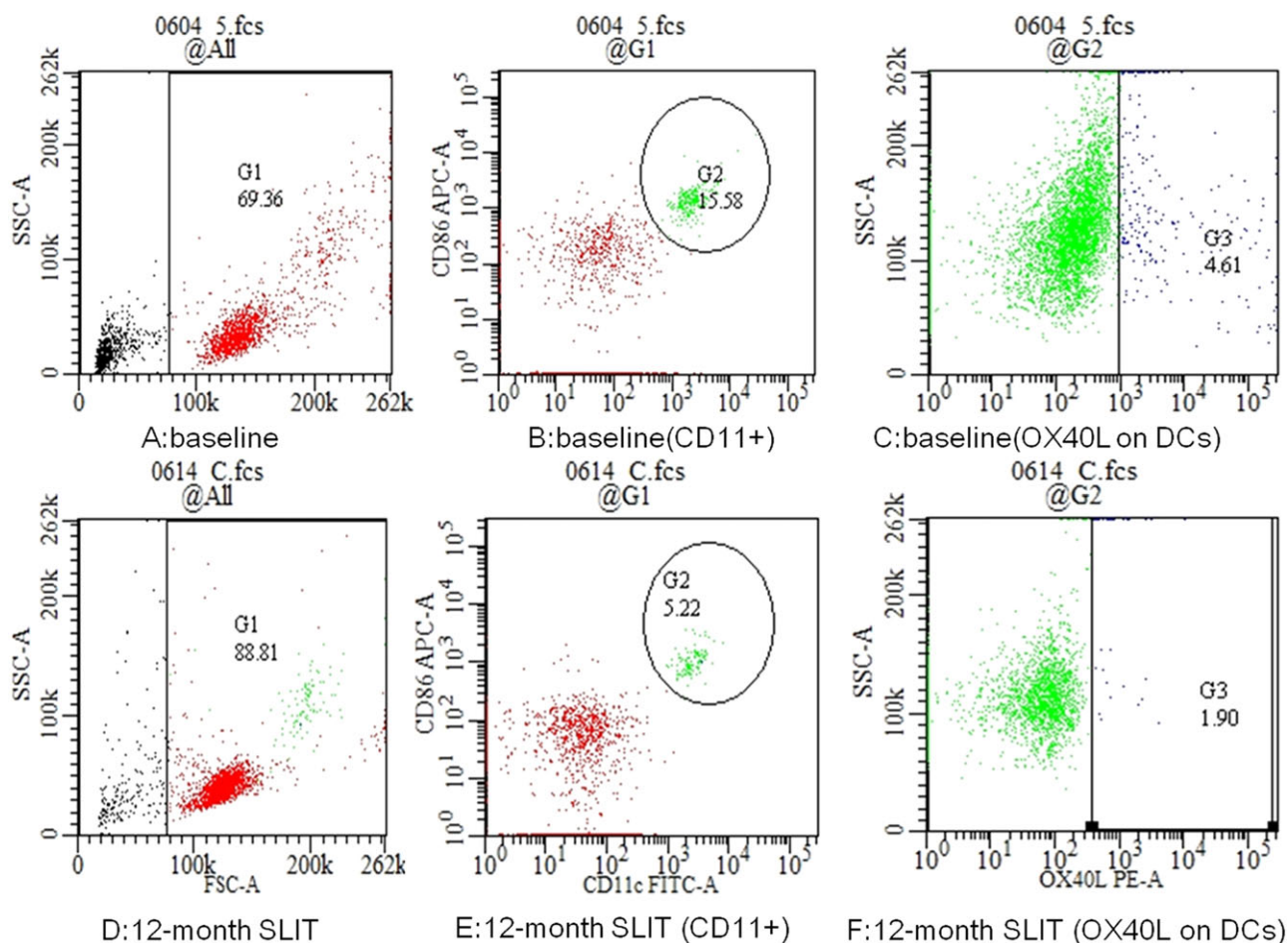


FIGURE 6. The expressions of OX40L (blue color) on the surface of CD11c+CD86+ cells (green color) in PBMCs (red color) before and after 12-month SLIT. PBMCs were stained with combinations of FITC-A-conjugated anti-human CD11c, APC-A-conjugated anti-human CD86, and PE-A-conjugated anti-human OX40L antibodies, followed by staining with the appropriate fluorochrome-conjugated isotype control Ig. Gating strategy: G1 (A, D): Based on FSC/SSC plots to show PBMC; G2 (B, E): CD11c+ was designed as gating strategy in PBMCs to show the expression of CD86; G3 (C, F): The expression of OX40L on the surface of CD11c+ CD86+ cells in PBMCs. DCs were sorted based on expression of CD11c on FSC/SSC plots. The sorted CD11c+ populations were analyzed for the expression of the surface molecules CD86. The expressions of OX40L on the surface of CD11c+CD86+ cells in PBMCs were decreased significantly ($p < 0.05$) after 12-month SLIT treatment. Data are mean $\pm$ SEM. APC = allophycocyanin; DC = dendrite cell; FITC = fluorescein isothiocyanate; FSC = forward scatter; Ig = immunoglobulin; PBMC = peripheral blood mononuclear cell; PE = phycoerythrin; SEM = standard error of the mean; SLIT = sublingual immunotherapy; SSC = side scatter.

allergic immune responses. Moreover, the significance of TSLP-OX40L signaling pathway in Th2-type immune responses is that it can link mucosal and systemic immunity and form a unified whole through this pathway. In this signaling pathway, TSLP is responsible for the initiation of Th2-type immune responses in mucosal immunity; OX40L is responsible for the maintenance of immune memory in systemic immunity. Mou et al.¹⁸ reported that the expression of TSLP in the nasal mucosa was increased significantly in AR, and its expression was tightly correlated with IL-4 and severity of AR. However, little is known about the influence of HDM SLIT on the TSLP-OX40L signaling pathway in AR. TSLP is produced mainly by damaged epithelial cells and OX40L plays critical roles on the maintenance and reactivation of TH2 memory responses during TSLP-mediated allergic responses.¹¹ So we selected nasal lavage fluid to detect TSLP level and PBMCs to detect OX40L level. Our

data showed that the expressions of TSLP in nasal lavage fluid and OX40L in PBMCs both declined significantly after 12-month HDM SLIT. These results were consistent with our clinical observation and laboratory assessments of Th2-type responses in mucosal and systemic immunity. The positive association of OX40L mRNA in PBMCs and TSLP in nasal lavage fluid was observed (Fig. 4). Combining all of these data, we proposed that Th2-type immune responses mediated by TSLP-OX40L pathway was downregulated by HDM SLIT. To further confirm the speculation, we detected the expression of OX40L on myeloid DCs (mDCs). TSLP was revealed to initiate Th2-type immune responses through its activation of CD11c+ DCs and to upregulate OX40L in the TSLP-OX40L signaling pathway.¹⁹ CD11c was identified as a marker of DCs¹⁹ and CD86 was identified as a surface marker that was highly expressed on mature DCs.²⁰ In our trials, the expression of OX40L on mDCs was detected by using flow cytometry.

Not unexpectedly, the expression of OX40L on mDCs decreased significantly compared with baseline values. Therefore, the results implied that the TSLP-OX40L signaling pathway in our trials was downregulated, which was accompanied by weakening of Th2-type immune responses from the mucosal and systemic immunity. The mitigation of the TSLP-OX40L signaling pathway might be an underlying mechanism for the deficiency of Th2-type immune activities.

However, it should be noted that the data provided in this study are merely indicative of the TSLP-OX40L axis as a signaling mechanisms for HDM SLIT of AR. It is possible that the observed downregulation of TSLP (an indication of a loss of the immune initiation) in nasal mucosa was not due to a direct effect of SLIT, rather a consequence of decreased TSLP production in epithelial cells after a prolonged period of stimulation with the standardized allergens. Next, following the downregulation of TSLP in nasal mucosa, the fact that OX40L expression was robustly downregulated in PBMCs and mDCs implies that Th2-type immune memory also waned. The balance of Th1/Th2 responses shifted toward Th1. Therefore, we propose that through the TSLP-OX40L signaling pathway, HDM SLIT extended the downregulation of Th2-type immune responses from mucosal immunity to systemic immunity. Clearly, both of

the immune initiation and immune memory of Th2-type immune responses were alleviated by HDM SLIT. Further studies are needed to acquire more conclusive evidence for the role of the TSLP-OX40L signaling pathway in HDM SLIT. Among them, we believed that the relationship between the productions of TSLP by nasal epithelial cells with HDM SLIT would be a key point. So further experiments focused on the characters of TSLP that might help establish a better connection between HDM SLIT and TSLP-OX40L pathway.

Conclusion

Collectively, the present study demonstrated that alleviation of the Th2-type responses from both of the mucosal and systemic immunity accounted for the observed efficacy of HDM SLIT in patients with moderate to severe persistent AR. Our data further indicated that expression downregulation of the TSLP and OX40L likely underlay the mitigation of Th2-type immune responses, thereby the benefit of HDM SLIT. These findings prompted us to propose that TSLP-OX40L signaling pathway might be a prospective mechanism of HDM SLIT, and that targeting the TSLP-OX40L signaling pathway using HDM SLIT and the like might be a vivid approach for the treatment of persistent AR. 

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Tab.1. HDM SLIT Treatment Protocol and Daily Dose of D.f. and D.p. Extract.

	Week 1(ml)	Week 2(ml)	Week 3(ml)	Week4-5(ml)	Week6+(ml)
Day1	0.05	0.05	0.05		
Day2	0.10	0.10	0.10		
Day3	0.15	0.15	0.15		
Day4	0.20	0.20	0.20	0.15/day	0.10/day
Day5	0.30	0.30	0.30		
Day6	0.40	0.40	0.40		
Day7	0.50	0.50	0.50		

Children younger than 14 years old use 250lg/ml as maintenance dose; subjects older

than 14 years old use 750lg/ml as maintenance dose