

Role of the TSLP–DC–OX40L pathway in asthma pathogenesis and airway inflammation in mice

Shuang Feng, Li Zhang, Xu-Hua Bian, Ying Luo, Guang-Hui Qin, and Rui-Ming Shi

Abstract: This study aimed to explore the effect of the TSLP–DC–OX40L pathway in asthma pathogenesis and airway inflammation in mice. For this, 65 male BALB/c mice were distributed among the control, asthma, immunoglobulin G (IgG) + asthma (IgG, 500 µg/500 µL, intratracheal injection of 50 µL each time), LY294002 (OX40L inhibitor) + asthma (intratracheal injection of 2 mg/kg LY294002), and anti-TSLP + asthma (intratracheal injection of 500 µg/500 µL TSLP antibody, 50 µL each time) groups. ELISA was applied to measure the serum levels of immunoglobulin E (IgE), ovalbumin (OVA)-sIgE, interleukin-4 (IL-4), IL-5, IL-13, and interferon-γ (IFN-γ); flow cytometry was employed to detect Treg cells and dendritic cell (DC) and lymphopoiesis. RT-qPCR and Western blot assays were used to measure the levels of TSLP, OX40L, T-bet, GATA-3, NF-κB, p38, and ERK. Treatment with LY294002 and anti-TSLP resulted in increases in the numbers of total cells, eosinophils, neutrophils, and lymphocytes in the bronchoalveolar lavage fluid; total serum levels of IgE, OVA-sIgE, IL-4, IL-5, and IL-13; levels of DC cells; lymphopoiesis; and levels of TSLP, OX40L, GATA-3, NF-κB, p38, and ERK, whereas there were decreases in the levels of IFN-γ and CD4⁺CD25⁺Treg cells; CD4⁺Foxp3⁺Treg cells; and T-bet. The TSLP–DC–OX40L pathway may contribute to asthma pathogenesis and airway inflammation by modulating the levels of CD4⁺CD25⁺Treg cells and inflammatory cytokines.

Key words: TSLP–DC–OX40L pathway, asthma, airway inflammation, pathogenesis, mouse model.

Résumé : Cette étude vise à explorer le rôle du sentier TSLP–CD–OX40L dans la pathogenèse de l'asthme et l'inflammation des voies aériennes chez la souris. Soixante cinq souris mâles BALB/c ont été réparties en groupes contrôle, asthme, IgG + asthme (IgG, 500 µg/500 µL, injection intratrachéale de 50 µL à chaque fois), LY294002 (inhibiteur d'OX40L) + asthme (injection intratrachéale de 2 mg/kg de LY294002) et anti-TSLP + asthme (injection intratrachéale de 500 µg/500 µL d'anticorps anti-TSLP, 50 µL à chaque fois). Un dosage par ELISA a été réalisé pour mesurer les niveaux sériques d'IgE, d'ovalbumine (OVA)-sIgE, d'interleukine-4 (IL-4), d'IL-5, d'IL-13 et d'interféron-γ (IFN-γ). La cytométrie en flux a été utilisée pour détecter les cellules Treg, les cellules dendritiques (CD) et la lymphopoïèse. La qRT-PCR et le buvardage Western ont été utilisés pour détecter les niveaux de TSLP, d'OX40L, de T-bet, de GATA-3, de NF-κB, de p38 et de ERK. Le traitement au LY294002 et à l'anti-TSLP résultait en une augmentation du nombre de cellules totales, d'éosinophiles, de neutrophiles et de lymphocytes dans le liquide de LBA, des niveaux sériques d'IgE, d'OVA-sIgE, d'IL-4, d'IL-5 et d'IL-13, du nombre de CD, de la lymphopoïèse, et des niveaux de TSLP, d'OX40L, de GATA-3, de NF-κB, de p38 et de ERK, et en une diminution des niveaux d'IFN-γ, du nombre de Treg CD4⁺CD25⁺, de Treg CD4⁺Foxp3⁺ et de T-bet. Le sentier TSLP–CD–OX40L peut contribuer à la pathogenèse de l'asthme et à l'inflammation des voies aériennes en modulant le nombre de cellules Treg CD4⁺CD25⁺ et le contenu en cytokines pro-inflammatoires. [Traduit par la Rédaction]

Mots-clés : sentier TSLP–CD–OX40L, asthme, inflammation de voies aériennes, pathogenèse, modèle de souris.

Introduction

Asthma is one of the most common chronic diseases around the world (Huerta-Yepez et al. 2011); a disease with chronic inflammation of lower airways and characteristics of recurrent airway obstruction and wheezing (Halim et al. 2012). The airway obstruction can be reversed spontaneously or with treatment, but it also leads to airway remodeling accompanied by structural changes (Detoraki et al. 2010), including mucosal fibrosis, a thickening of the basement membrane, and smooth-muscle hyperplasia (Hamasaki et al. 2014). Asthma has affected ~3%–5% of the total population (Panda and Doddanagali 2011), among which, all of the asthmatic patients have different degrees of allergic airway inflammation, with bronchial allergic inflammation accompanied by airway remodeling being the key factor of allergic/Th2 asthma (Ueno-Iio et al. 2014). Because there are different pathologies in different patients, each

patient has different clinical manifestations at different time periods. Novel therapies for treating different degrees of asthma in patients have been researched (Lee et al. 2010; Taube 2014). However, owing to the uncertainty about the mechanisms of asthma, the results of the research have, regrettably, not been ideal.

Thymic stromal lymphopoietin (TSLP), which is mainly secreted by bronchial and lung epithelial cells, controls inflammatory responses, with mechanisms involving distinct activation at the epithelial–dendritic (DC) cell interface (Wu et al. 2013; Melum et al. 2014). Generally speaking, an altered level of TSLP is often observed in allergic tissues (Ito et al. 2012). Oxford 40 (OX40) is a co-stimulatory receptor that is transiently expressed in activated T cells (Gauvreau et al. 2014a). Further, OX40L, the ligand of OX40, is directly regulated by TSLP, which is generated by airway smooth muscles, epithelial cells, mast cells, and dendritic cells engaged in Th2 responses (Kaur and Brightling 2012). Anti-TSLP antibodies

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in allergen-induced asthmatic responses in humans are effective (Gauvreau et al. 2014b). Although TSLP is an important promoter for allergic inflammation, its underlying mechanism is largely unknown (Li et al. 2011). However, the human TSLP gene has been proven to be one of the most common loci susceptible to asthma (Ober and Yao 2011). Moreover, the TSLP–OX40L signaling pathway mediated by Th2 responses is involved in human inflammation and immune system (Li et al. 2011; Meng et al. 2016). Therefore, the mechanisms of TSLP, DC, and OX40L provided a new direction for us to research, although whether the mechanism of the TSLP–DC–OX40L pathway is connected with asthma and airway inflammation is still a mystery. In addition, activation of the TSLP pathway can contribute to lung inflammation, and is critical for the development of pulmonary pathology and pulmonary human metapneumovirus (hMPV) replication (Lay et al. 2015). Further, the mechanisms for asthma and airway inflammation are related to the index of airway inflammation in asthma (Shimoda et al. 2016). However, there is still little research on the relationship between the TSLP–DC–OX40L pathway and the two kinds of diseases. Thus, in this study, we planned to explore effects of the TSLP–DC–OX40L pathway in asthma pathogenesis and airway inflammation to further the development of treatments for asthma and airway inflammation.

Materials and methods

Ethics statement

This study was carried out in strict accordance along the guidelines for care and use of laboratory animals issued by the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University. All painful procedures were performed under anesthesia, and every effort was made to minimize the suffering of the animals participating in the study.

Experimental animals and grouping

A total of 65 healthy, clean, and specific pathogen free (SPF) male mice aged 6–8 weeks and weighing 13–26 g were selected for this study. All of the mice were bought from the experimental animal center of the Fourth Military Medical University. The mice were kept in separate and ventilated cages in a SPF Animal Laboratory of the experimental animal center under a 12 h light–dark circadian rhythm, and fed with protein feed of standard particle size, with free access to food and water. The room temperature and humidity were maintained at 20 °C and 50%, respectively. The mice were randomly distributed among the control, asthma, immunoglobulin G (IgG; Abcam Inc., Cambridge, Massachusetts, USA; 500 µg/500 µL, intratracheal injection of 50 µL each time) + asthma, LY + asthma (intratracheal injection of 2 mg/kg LY294002; Xitang, China) and anti-TSLP + asthma groups (intratracheal injection of 500 µg/500 µL TSLP antibody, 50 µL each time; Abcam), with $n = 13$ in each group. During the experiment, the animals were treated in accordance with the guidelines for the ethical treatment of laboratory animals.

Model establishment of mice with chronic asthma

A total of 10 mg ovalbumin (OVA; Sigma–Aldrich, St. Louis, Missouri, USA) was added to 10 g aluminum hydroxide gel to make the OVA suspension, with 100 mL phosphate buffered saline (PBS). On the 1st day and 7th day of the 12 h light–dark cycle, mice in the asthma group, IgG + asthma group, LY + asthma group, and anti-TSLP + asthma group were sensitized with 0.2 mL of OVA suspension via intratracheal injections. The mice in the control group were sensitized by intratracheal injections of PBS instead of OVA. After the 14th day, the mice in the asthma group, IgG + asthma group, LY + asthma group, and the anti-TSLP + asthma group were placed in closed glass containers and were allowed to inhale 5% OVA in normal saline atomized ultrasonically at the maximized amount to stimulate mouse's asthma symptoms. The above process was conducted for 25 min once a day, and repeated succes-

sively for 4 weeks. Mice in the control group were stimulated by the inhalation of saline solution. Thirty minutes before the stimulation by inhaling atomized 5% OVA in normal saline, the mice in the IgG + asthma group were given 4 mL/kg body mass of anti-IgG antibody liquid by intratracheal injection; the mice in the LY + asthma group were administered 2 mg/kg body mass LY294002 liquid via intratracheal injection; while the mice in the anti-TSLP + asthma group were injected with 4 mL/kg body mass of anti-TSLP antibody liquid in the same manner.

Airway responsiveness to acetylcholine in mice

Twenty-four hours after the last stimulation of asthma symptoms, the mice were anesthetized with intratracheally injected 1% pentobarbital (50 mg/kg body mass; Sigma–Aldrich). The tail veins of the mice were surgically exposed and a 27G intravenous catheter was inserted. If normal saline was injected through the catheter without resistance and skin edema, the catheterization was regarded as successful. The anesthetized mice were fixed on an operating table, with their tracheas separated by a blunt dissection. The tracheas were cut in a V-shape opening with conjunctival scissors, and a 2 mm plastic pipe was inserted into the opening and then fixed. The other end of the pipe was connected to an animal pulmonary function monitor (type: AniRes 2005, Beijing Bestlab High-Tech Co., Beijing, China). Mechanical ventilation was performed with a tidal volume of 6 mL/kg body mass, with a respiratory frequency of 90 times/min. When the airway pressure became stable, the mice were first injected with normal saline, and then the peak lung resistance (RL) was recorded within 5–60 s for a baseline measurement. Afterwards, the mice were intravenously given increasing concentrations of acetylcholine (10, 30, 60, 90, 180, 270, and 360 µg/kg body mass, respectively (Sigma–Aldrich), administered at a rate of 36 mL/h). When the corresponding doses of acetylcholine were injected into the mice, the RL was recorded within 5–60 s.

Sampling and sectioning

Four weeks later, after the mice were stimulated by inhaling atomized 5% OVA in normal saline, the mice were anesthetized with ether (Sigma–Aldrich), and then fixed on the operating table in a supine position, and their thorax was carefully opened to expose the heart. Pipes were inserted into the left ventricle, then after that, the right atrium was cut open. Then, 200 mL of 0.9% normal saline was used to perfuse the right atrium. When the normal saline welled up from the right atrium, the mice were rinsed at a uniform rate until their kidneys and livers turned white. Afterwards, the perfused lung tissues were clipped with scissors and fixed in 10% neutral polyformaldehyde for 48 h at room temperature, after which the tissues were embedded with paraffin and sectioned to be preserved at a low temperature. Then, 24 h after the last stimulation of asthma symptoms, the eyeballs were removed from the mice to collect blood samples. Then, the collected blood was centrifuged at a rate of 2014g for 20 min at 4 °C to collect the serum, which was then preserved at –70 °C. Then, the mouse tracheas were fixed with vascular clamps on cricoid cartilage, and a transverse incision was made to place silicone tubes that were connected with injectors. PBS was carefully and slowly injected into the tracheas 3 times, 0.4 mL each time. After each perfusion, bronchoalveolar lavage fluid (BALF) was recovered and preserved at 4 °C, and the cells were separated within 2 h. The BALF was centrifuged at 1000g for 10 min at 4 °C to collect the supernatants, which were then preserved at –70 °C. Precipitated cells were re-suspended with 0.5 mL PBS, and 0.1 mL of the cells was taken to count the total cell number under a cytometer, while the rest of the cells were smeared and processed by Wright–Giemsa staining (Sigma–Aldrich). The cells were classified and counted according to cell morphology ($n = 300$). Meanwhile, fresh lung tissues were collected and preserved at –80 °C for subsequent use.

Hematoxylin–eosin (HE) staining

Fresh lung tissues samples from each group were fixed in 10% formalin for 24 h. Then the samples were dehydrated with ethanol, cleared, dewaxed, and embedded to make 4 μ m thick paraffin sections, which were then stained with HE. The HE staining process was carried out as follows: the sections were dewaxed, dehydrated with xylene and a graded series of alcohol, and rinsed with distilled water; then the sections were stained with hematoxylin for 5 min and rinsed with water, counterstained with eosin for 3 min and rinsed with water, and dehydrated once more with a graded series of ethanol. The sections were then cleared with xylene twice for 10 min. After that, the xylene surrounding the sections was wiped out, followed by the immediate addition of neutral gum, and covering of the sections using cover slips. Morphological changes in the mouse lung tissues were observed under an optical microscope (CX-31, Olympus Optical Co., Ltd., Tokyo, Japan) in a double-blind trial. The eosinophils were very obvious and appeared to be colored bright-red under high-power magnification. The cell nuclei were lobed, with the majority of nuclei appearing bilobed, and there were numerous reddish granules in the cytoplasm. The lymphocytes appeared to be small and round, with a relatively high karyoplasmic ratio and royal-purple colored nuclei.

Periodic acid – Schiff (PAS) staining

After dewaxing and rehydrating, the sections of lung tissue were rinsed with 3% acetic acid for 2 min. Then, 1% Alcian blue 8GX and acetic acid solution were added to react with the sections for 10–20 min. Afterwards, the sections were rinsed twice with distilled water (5 min each time), immersed into 0.5% periodic acid solution for 5 min, and rinsed twice with distilled water (3 min each time). Then, the sections were rinsed twice with 70% ethanol (3 min each time). The rinsed sections were immersed in Schiff reagent for 15–30 min, followed by rinsing twice under flowing water (5 min each time). Then, the sections were slightly stained with hematoxylin for 2–3 min, and the unbound dye was rinsed off with flowing water. Finally, the sections were conventionally dehydrated, covered with cover slips, and sealed with neutral gum. The morphology of the lung tissues of the mice was observed under an optical microscope. (The PAS staining kit was from Sigma-Aldrich).

Enzyme-linked immunosorbent assay (ELISA)

The contents of total immunoglobulin E (IgE) and OVA-IgE in the serum, and IgE, interleukin (IL)-4, IL-5, and IL-13 in the BALF were measured according to the specifications of the corresponding ELISA kits (Shanghai Xitang Bio-Tech Co., Shanghai, China). The standards were processed using a gradient dilution, from which the standard curve was drawn. The optical density values for the samples were substituted into the standard curves equation to determine the concentrations of corresponding samples.

Flow cytometry

Fresh lung tissues from each group of mice were sheared and incubated in PBS containing 1 mL 0.2% collagenase (Sigma-Aldrich) for 15 min, and then placed in a stainless steel mesh (300 mesh) and sheared with an eye scissors. Then, the single-cell suspension in the culture medium was collected and centrifuged at the rate of 1398g for 5 min. The supernatant was discarded, and then we added 2 mL of erythrocyte lysate and incubated the cells for 8 min at room temperature, followed by centrifugation with PBS twice, and again the supernatant was discarded. Afterwards, a culture medium containing 10% fetal bovine serum was used to make single-cell suspensions, followed by the addition of 50 ng/mL phorbol, 12-myristate 13-acetate (PMA; Sigma-Aldrich), 500 ng/mL ionomycin (Sigma-Aldrich), and 0.7 μ L/mL GolgiPlug (Becton Dickinson, Franklin Lakes, New Jersey, USA) in 5% CO₂ at 37 °C for 6 min. A total of 10⁶ cells were supplemented with CD4, CD25

(Treg kit was purchased from eBioscience company, San Diego, California, USA), CD11c, MHCII, and their isotype controls, respectively. Then, the cells were cultured for 30 min at 4 °C, rinsed with a flow cytometry staining buffer, centrifuged, and re-suspended to collect the cell pellet. Cells treated with CD4 and CD25 were supplemented with 1 mL of fixative for re-suspension for culturing without light for 60 min at 4 °C. After this, 2 mL of permeabilization buffer was used for rinsing, and the cells were then centrifuged to remove the supernatants. Foxp3-PE was added to further culture the cells for 15 min at 4 °C, then the cells were rinsed with 2 mL of permeabilization buffer and then centrifuged to remove the supernatants. Finally, the cells were re-suspended with an appropriate amount of flowing buffer, and flow cytometry (Becton Dickinson) was used to measure the levels of CD4⁺CD25⁺Treg and DC cells. Meanwhile, a single-cell suspension was made from the mouse spleens, which was then rinsed with PBS. The serum was removed from the suspension, and the suspension was re-suspended to a concentration of 5–10 $\times$ 10⁶ cells/mL. Then, 2 μ L of carboxyfluorescein diacetate, succinimide ester (CFSE) (eBioscience, San Diego, Calif.) was added and blended well with the suspension, which was cultured without light for 10 min at room temperature. Cold complete medium, 4–5-fold the volume of the suspension, was added to terminate the labeling reaction. Then, the suspension was cultured on ice for 5 min, centrifuged, rinsed, cultured for 3 days, rinsed with 2 mL of buffer, centrifuged to remove the supernatant, and re-suspended with the appropriated amount of flow cytometry staining buffer. Flow cytometry was used to determine lymphocyte proliferation. The experiment was repeated 3 times and the mean value was selected for use.

Culture of dendritic cells (DCs)

Fresh lung tissues were digested into a single cell suspension and re-suspended with 20 mL complete medium in a 100 mL culture flask under 5% CO₂ at 37 °C for 2 h. Then, the culture flask was shaken 4 times with 20 mL complete medium to homogenize the suspension. The cells were then incubated with 50 mL/L CO₂ overnight to induce the maturation of DCs. The cultures were replaced on days 3 and 6. On the 6–8 day, the adherent cells, namely, the mature DC cells, were collected using a cell brush. The levels of OX40L in DCs were determined using reverse transcription – quantitative polymerase chain reaction (RT–qPCR).

RT–qPCR

Fresh lung tissues from the mice and freshly prepared DCs were collected and the total RNAs were extracted using Trizol. A Total of 500 ng of RNA was reverse-transcribed according to the specifications of the Transcription System A3500 reverse transcription kit (Promega (Beijing) Biotech, Beijing, China). On the basis of gene sequences issued by GenBank, primers were designed using the software Primer 5.0 (Table 1), and the primers were synthesized by Sangon Bio-Tech (Shanghai). The RT–qPCR reaction conditions (using SYBR Green) were as follows: 95 °C for 15 min, 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, with a total of 40 cycles. The reaction system was as follows: Premix Ex Taq or SYBR Green Mix 12.5 μ L, forward primer 1 μ L, reverse primer 1 μ L, DNA template 1–4 μ L, ddH₂O up to 25 μ L. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference, and the standard of the control group was assigned a value = 1, the C_T values for the targeted genes (peak values in growth curves) were determined, and the relative quantification (RQ) values were obtained based on the equation $RQ = 2^{-\Delta\Delta C_T}$. The RQ values were used for statistical analysis. The RT–qPCR instrument (type iQ5) was purchased from Bio-Rad Laboratories, Inc. (Berkeley, Calif.). This experiment was repeated 3 times and the mean value was selected for use.

Table 1. Primer sequences for reverse transcription – quantitative polymerase chain reaction (RT–qPCR).

PCR primer sequences	Forward (5'–3')	Reverse (5'–3')
<i>TSLP</i>	AATGACCACTGCCAGGCTA	TTGTGAGGTTTGATTGACAGATG
<i>OX40L</i>	GAGCACCAGTTACCGGATGT	TAGGCAATCCTGGGAATGAG
<i>T-bet</i>	GTTCACCAAGCACCAGACAGAG	TGGTCCACCAAGACCACATC
<i>GATA-3</i>	GGATGTAAGTCGAGGCCCAAG	ATTGCAAAGGTAGTGCCCGGA
<i>NF-κB</i>	CGCA TCCA GACCAACAACA	TGCCCCA GAA GGAAACACCA
<i>ERK</i>	TGGTTCTACCGGCGGTTAG	AACGAGGAGGGAGGACAACA
<i>p38</i>	TCGTGTGTTTGGGCATTCTG	TCAGTGTCTCAGGGCTGGAT
<i>GAPDH</i>	AAATGGTGAAGGTCGGTGTG	TGAAGGGGTCGTTGATGG

Note: PCR, polymerase chain reaction; *TSLP*, thymic stromal lymphopoietin; *OX40L*, Oxford 40 ligand; *T-bet*, T-box expressed in T cells; *GATA-3*, GATA binding protein 3; *NF-κB*, nuclear factor-kappa B; *ERK*, extracellular regulated protein kinases; *p38*, p38 mitogen-activated protein kinases; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

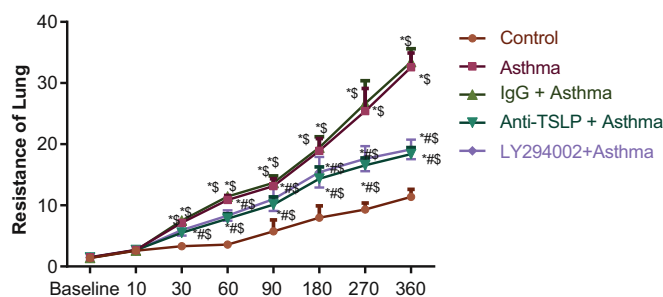
Immunohistochemistry (IHC)

Paraffin-embedded sections of the lung tissues of the mice were heated for 1 h at 60 °C, dewaxed twice with xylene (5 min each time), and hydrated in a graded series of ethanol and distilled water for 5 min. Then, the dewaxed and hydrated sections were boiled in a citrate buffer (pH 6.0) for 15 min to recover the antigen, and then quenched using endogenous peroxidase. The diluted first antibodies (diluted at a ratio of 1:200 for TSLP rabbit anti-mouse and diluted at a ratio of 1:100 for OX40L rabbit anti-mouse) were added to react with the sections overnight at 4 °C. Afterwards, the sections were rinsed 3 times with PBS (5 min each time). In the absence of light, the fluorescent secondary antibody was added (diluted at a ratio of 1:200 for goat anti rabbit fluorescent secondary antibody, from Beijing ZSGB-Bio Co.) to the sections for 2 h at 37 °C. Then, the sections were rinsed 3 times with PBS (5 min each time). The sections were then immersed in anti-quenching mounting medium, and then preserved in the absence of light. A laser scanning confocal microscope was used to observe and photograph the cells under dark conditions. Finally, the levels of TSLP and OX40L in the immunofluorescent photos were calculated and analyzed using Image Pro Plus 6.0 software. The results were represented as integrated optical density (IOD). The experiment was repeated 3 times and the average value was selected for use.

Western blot assay

The protein from the lung tissues of the mice in each group was extracted to determine its concentration, using a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology Co., Ltd, Shanghai). Then, 5× loading buffer (from Beyotime Biotechnology Co., Ltd., Shanghai, China) was added to the extracted protein. Afterwards, the protein was boiled for 10 min at 95 °C with 30 µg of loading buffer in each well. The protein was separated using electrophoresis (Bio-Rad) with 10% polyacrylamide gel (Wuhan Boster Bio-Tech Co., Hubei Province, China), trans-blotted onto a polyvinylidene fluoride (PVDF) membrane (Amresco Inc., Solon, Ohio, USA), and sealed in 5% bovine serum albumin (BSA; Beijing Huamei Biological Engineering Corporation, Beijing, China) for 1 h at room temperature. Then, the protein was supplemented with the first antibody GAPDH [Abcam Inc., Chicago, Illinois, USA; which was diluted with Tris-buffered saline with Tween (TBST; ab8245; diluted at a ratio of 1:1000)], T-box expressed in T cells (T-bet; ab91103, 1 µg/mL), GATA binding protein 3 (GATA-3; ab199428, diluted at a ratio of 1:1000), nuclear factor-kappa B (NF-κB; ab7971, diluted at a ratio of 1:400), TSLP (ab47943, diluted at a ratio of 1:1000), OX40L (EP1168Y, diluted at a ratio of 1:1000), extracellular regulated protein kinases (ERK; ab54230, 1 µg/mL), and p38 mitogen-activated protein kinases (p38; ab31828, diluted at a ratio of 1:1000) for culture overnight. Then, the membrane was rinsed 3 times with TBST (5 min each time). Afterwards, the corresponding secondary antibody (Abcam) was added to react with the protein

Fig. 1. Changes in lung resistance after stimulation by acetylcholine at different concentrations in the control, asthma, IgG + asthma, LY294002 + asthma and anti-TSLP groups of mice (measured in cm H₂O·mL⁻¹·s⁻¹). *, *P* < 0.05 compared with the control group; #, *P* < 0.05 compared with the asthma and the IgG + asthma groups; \$, *P* < 0.05 compared with the corresponding baseline value of each group; there were 13 mice in each group; IgG, immunoglobulin G; TSLP, thymic stromal lymphopoietin. [Colour online.]



for 2 h at 37 °C. Next, the membrane was rinsed and stained for 1 min at room temperature with chromogenic substrate reagents A and B (mixed at a ratio of 1:1 v/v) (Promega Biotech Co., Ltd, Beijing). The membrane, wrapped in film to keep it fresh, was transferred to a darkroom, and then developed and fixed by exposure to X-rays. Gel-Pro analyzer 4.0 software was used for band analysis, in which the protein level was represented by the grey value ratio between the targeted protein and GAPDH. The experiment was repeated 3 times and the mean value was selected for experimental use.

Statistical analysis

Statistical analysis were performed using the SPSS 21.0 statistical software (IBM Corp. Armonk, New York, USA). The measurement data used was the mean ± SD, and comparisons between multiple groups were analyzed using 1-way analysis of variance (ANOVA) and a least significant difference (LSD) test. The significant differences between 2 groups were analyzed using a Student *t* test. Values for *P* < 0.05 were considered to be statistically significant.

Results

General physical condition and airway responsiveness of mice in each group

Asthma models were established and stimulated by OVA in the following groups of mice: asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma. Normal saline was used to stimulate the asthma model in the control group. The mice in the control group were monitored for signs of sneezing, facial pruri-

Fig. 2. Pathological changes to lung tissues in mice among the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups, as measured with HE and PAS staining. (A) HE staining images of lung tissues of mice in the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. (B) PAS staining images of lung tissues of mice in the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. (C) Histogram of inflammation indexes among each group as detected by HE staining. (D) Histogram of inflammation indexes among each group as detected by PAS staining. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the asthma and the IgG + asthma groups; there were 13 mice in each group; IgG, immunoglobulin G; TSLP, thymic stromal lymphopoietin; HE, hematoxylin–eosin; PAS, periodic acid – Schiff. [Colour online.]

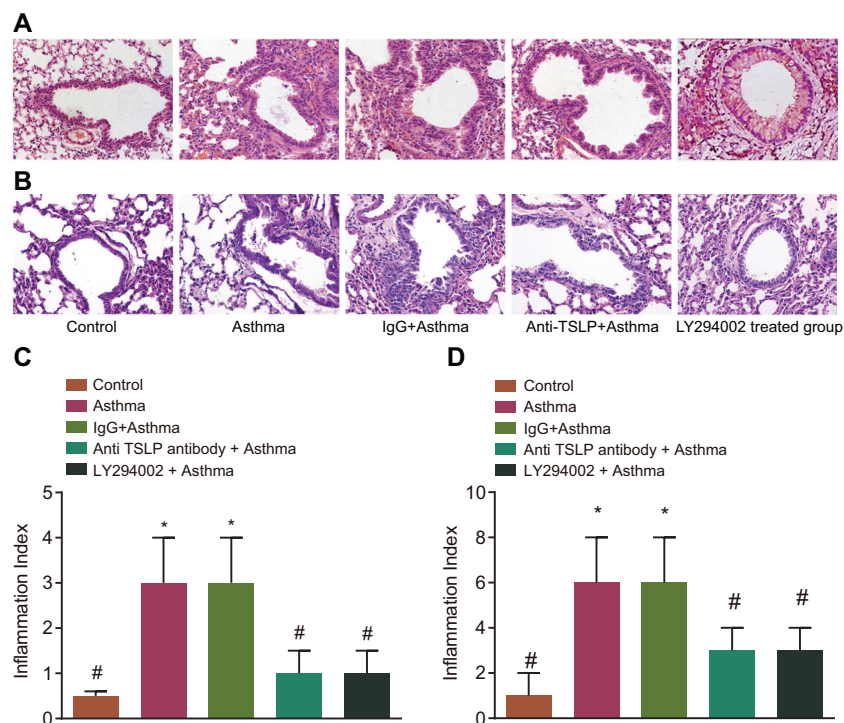


Fig. 3. Numbers of total cells, eosinophils, neutrophils, lymphocytes, and mast cells in mice from the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the asthma and IgG + asthma groups; there were 13 mice in each group; IgG, immunoglobulin G; TSLP, thymic stromal lymphopoietin. [Colour online.]

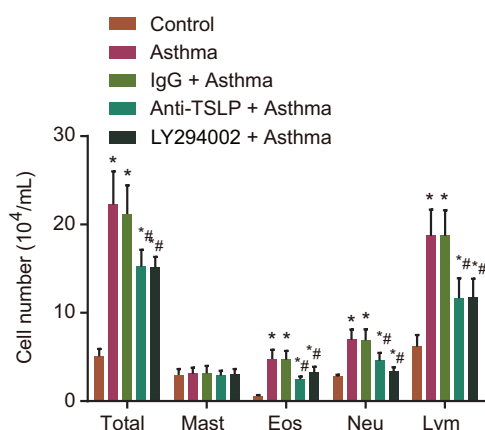
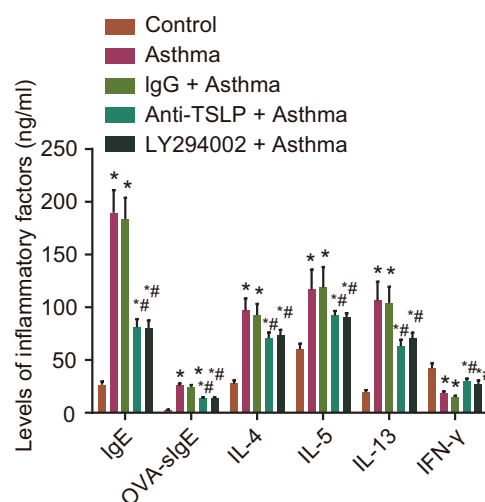


Fig. 4. Levels of total IgE, OVA-sIgE, IL-4, IL-5, IL-13, and IFN- γ detected by ELISA among each group. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the asthma and the IgG + asthma groups; there were 13 mice in each group; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; IgE, immunoglobulin E; OVA, ovalbumin; IL, interleukin; IFN- γ , interferon- γ . [Colour online.]



tus, arching back, or wheezing, and none of these signs were observed in the control mice. However, the mice in the asthma and the IgG + asthma groups exhibited all of the aforementioned signs, as well as rapid breathing, worsening mental states, and dull hair. Interestingly, the mice in the LY294002 + asthma and the anti-TSLP + asthma groups showed significant relief from sneezing, nodding wheezing, and other symptoms of asthma.

These results indicated that the asthma models had been successfully established.

No significant differences in peak RL baseline values were observed among the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups (all $P > 0.05$). No significant

Fig. 5. Levels of CD4⁺CD25⁺Treg, CD4⁺Foxp3⁺Treg, and DC cells in lymphoid tissue in the lung in the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. (A) Flow cytometry charts (left) and histogram (right) of levels of CD4⁺CD25⁺Treg cells in lymphoid tissue from the lungs in the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. (B) Flow cytometry charts (left) and histogram (right) of levels of the CD4⁺Foxp3⁺Treg cells from lymphoid tissue in the lung in the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. (C) Flow cytometry charts (left) and statistical chart (right) of DCs in lymphoid tissue in the lung in the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. *, $P < 0.05$ compared with the control groups; #, $P < 0.05$ compared with the asthma and IgG + asthma groups; there were 13 mice in each group; DC, dendritic cells; IgG, immunoglobulin G; TSLP, thymic stromal lymphopoietin. [Colour online.]

differences in RL values were observed among all of the groups wherein the mice were stimulated using 10 µg/kg body mass acetylcholine ($P > 0.05$), and there were no significant differences between the RL values and the corresponding baseline values for each group ($P > 0.05$). The RL values in the control group did not show any significant changes at different concentrations of acetylcholine (30, 60, 90, 180, 270, or 360 µg/kg body mass; all $P > 0.05$). The mice in the asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups exhibited an increased RL value and concentration of acetylcholine (all $P < 0.05$). Higher RL values were observed in the asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups compared with the control group (all $P < 0.05$). Compared with the asthma group, the RL value in the IgG + asthma group was not significantly different ($P > 0.05$); however, the RL values for the LY294002 + asthma group and the anti-TSLP + asthma group were lower ($P < 0.05$) (Fig. 1).

Pathological changes to lung tissues in mice among each group as detected by HE and PAS staining

The results of HE and PAS staining showed that the mice in the control group exhibited clean lung tissue structure, integrated airway epithelium, full-sized cilia, with no obvious edema in the airway mucosa, smooth bronchi without occlusion, no inflammatory cell infiltration around airway and vessels tissue, little hyperplasia of the goblet cells, and no inflammatory secretion in the alveolar cavity. However, mice in the asthma and the IgG + asthma groups exhibited irregular lung tissue structure, incomplete airway epithelium, loss of cilia in the airway epithelium, obvious edema in the airway mucosa, narrow airway lumen accompanied by absolute occlusion, large numbers of inflammatory cells infiltrating the mucosa, submucosa, and around the vessel tissues, and with the majority of eosinophils and lymphocytes, goblet cell hyperplasia, and inflammatory secretion in the alveolar cavity. Mice in the LY294002 + asthma and anti-TSLP + asthma groups displayed slight inflammation and ameliorated goblet cell hyperplasia compared with the asthma group (Fig. 2).

Total cell number and cell classification in the BALF from the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups

The asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups showed a significant increase in the numbers of total cells, lymphocytes, neutrophils, and eosinophils (all $P < 0.05$), and no significant difference in the number of mast cells compared with the control group (all $P > 0.05$). There were no significant differences in the numbers of total cells, eosinophils, neutrophils, lymphocytes, and mast cells between the asthma and IgG + asthma groups (all $P > 0.05$). Compared with the asthma and IgG + asthma groups, the LY294002 + asthma and anti-TSLP + asthma groups showed a decreased number of total cells, eosinophils, neutrophils, and lymphocytes in the BALF (all $P < 0.05$), and no significant differences in the number of mast cells ($P > 0.05$) (Fig. 3).

Levels of total IgE, OVA-sIgE, IL-4, IL-5, IL-13, and IFN-γ in the mice from each group

In the asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups, the levels of total IgE and OVA-sIgE in the serum as well as IL-4, IL-5, and IL-13 in BALF were increased, and

the levels of IFN-γ in the BALF was decreased compared with the control group (all $P < 0.05$). There were no significant differences in the levels of total IgE, OVA-sIgE, IL-4, IL-5, IL-13, and IFN-γ between the asthma and IgG + asthma groups ($P > 0.05$). When compared with the asthma and IgG + asthma groups, the LY294002 + asthma and anti-TSLP + asthma groups exhibited decreased levels of total IgE and OVA-sIgE in the serum, IL-4, IL-5, and IL-13 in the BALF, and an increased IFN-γ level in the BALF (all $P < 0.05$) (Fig. 4).

Levels of CD4⁺CD25⁺Treg and DC cells in mice from each group

The asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups exhibited lower levels of CD4⁺CD25⁺Treg cells, CD4⁺Foxp3⁺Treg cells, and higher levels of DC cells in the lymphoid tissues of the lung compared with the control group (both $P < 0.05$). No significant differences in the levels of CD4⁺CD25⁺Treg cells, CD4⁺Foxp3⁺Treg cells, and DC cells of lymphoid tissue in the lung were observed between the asthma and IgG + asthma groups ($P > 0.05$). The LY294002 + asthma and anti-TSLP + asthma groups exhibited increased levels of CD4⁺CD25⁺Treg cells and CD4⁺Foxp3⁺Treg cells, and decreased levels of DC cells of lymphoid tissue in the lung compared with the asthma and IgG + asthma groups (all $P < 0.05$) (Fig. 5).

Comparison of lymphopoiesis in mice among the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups

Lymphopoiesis in lung tissue was detected using flow cytometry, and the results indicated that the asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups displayed significantly higher lymphopoiesis compared with the control group ($P < 0.05$). There was no difference in lymphopoiesis between the asthma and IgG + asthma groups ($P > 0.05$). When compared with the asthma and IgG + asthma groups, lymphopoiesis was decreased in the LY294002 + asthma and anti-TSLP + asthma groups (both $P < 0.05$) (Fig. 6).

The mRNA levels of TSLP, GATA-3, OX40L, T-bet, NF-κB, p38, and ERK in the mice from each group

The asthma group, IgG + asthma group, LY294002 + asthma group, and anti-TSLP + asthma group exhibited higher mRNA levels of TSLP, GATA-3, OX40L, NF-κB, p38, and ERK, and lower levels of T-bet in mouse lung tissues compared with the control group (all $P < 0.05$). There was no significant difference in the TSLP, OX40L, T-bet, GATA-3, NF-κB, p38, and ERK mRNA levels between the asthma and the IgG + asthma group (all $P > 0.05$). When compared with the asthma group and the IgG + asthma group, the LY294002 + asthma group and anti-TSLP + asthma group exhibited decreased mRNA levels of TSLP, OX40L, GATA-3, NF-κB, p38, and ERK, and an increased level of T-bet (all $P < 0.05$) (Fig. 7A). In comparison with the control group, the level of OX40L in DCs was increased in the asthma group, IgG + asthma group, LY294002 + asthma group, and the anti-TSLP + asthma group (all $P < 0.05$), with no significant difference between the asthma and the IgG + asthma groups ($P > 0.05$). Compared with the asthma group and IgG + asthma group, the level of OX40L in DCs was decreased in the LY294002 + asthma group and the anti-TSLP + asthma group (all $P < 0.05$) (Fig. 7B).

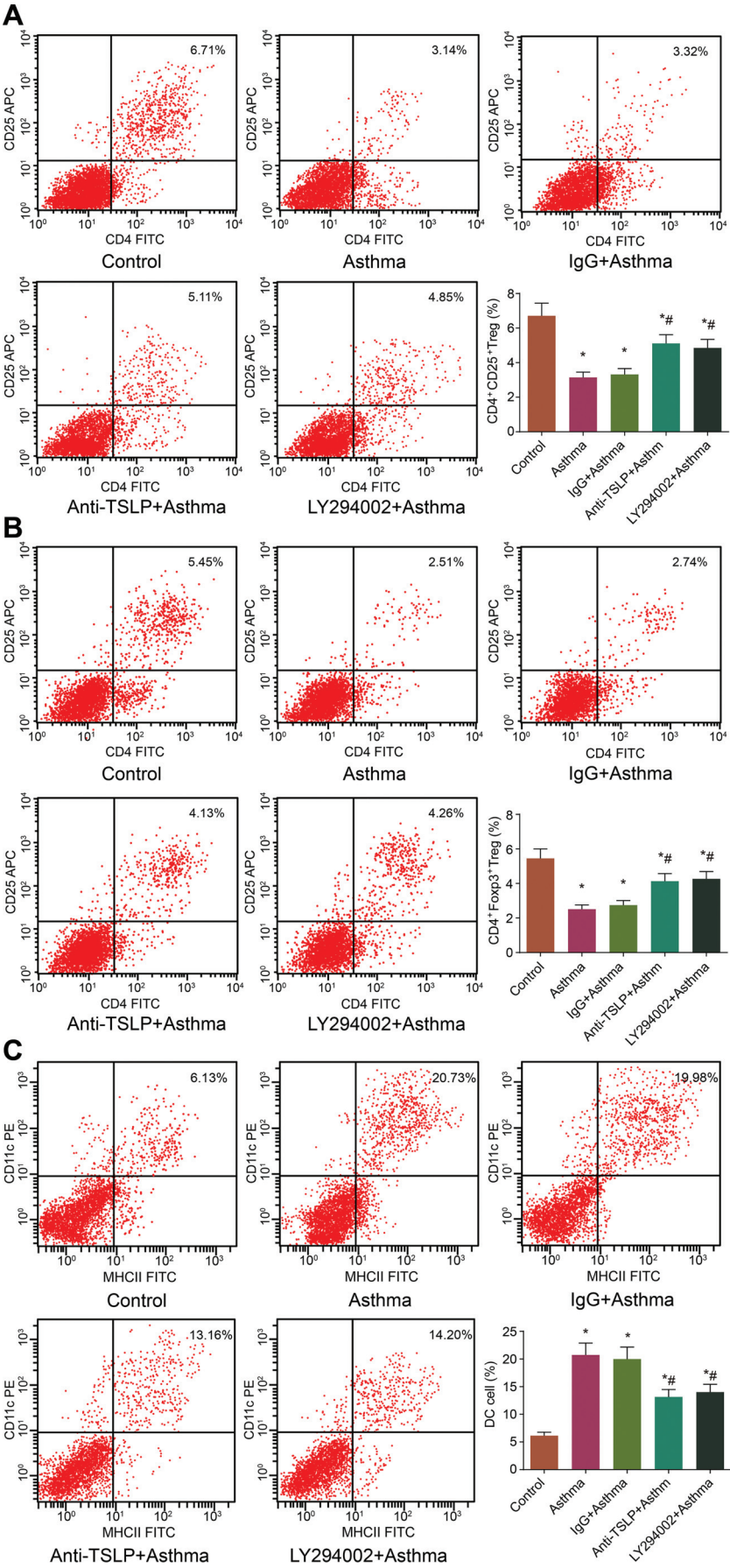


Fig. 6. Lymphopoiesis in the lung of mice among the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups using flow cytometry. (A) Flow cytometry charts of lymphopoiesis among the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. (B) Histogram of number of lymphocytes among the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the asthma and IgG + asthma groups; there were 13 mice in each group; IgG, immunoglobulin G; TSLP, thymic stromal lymphopoietin. [Colour online.]

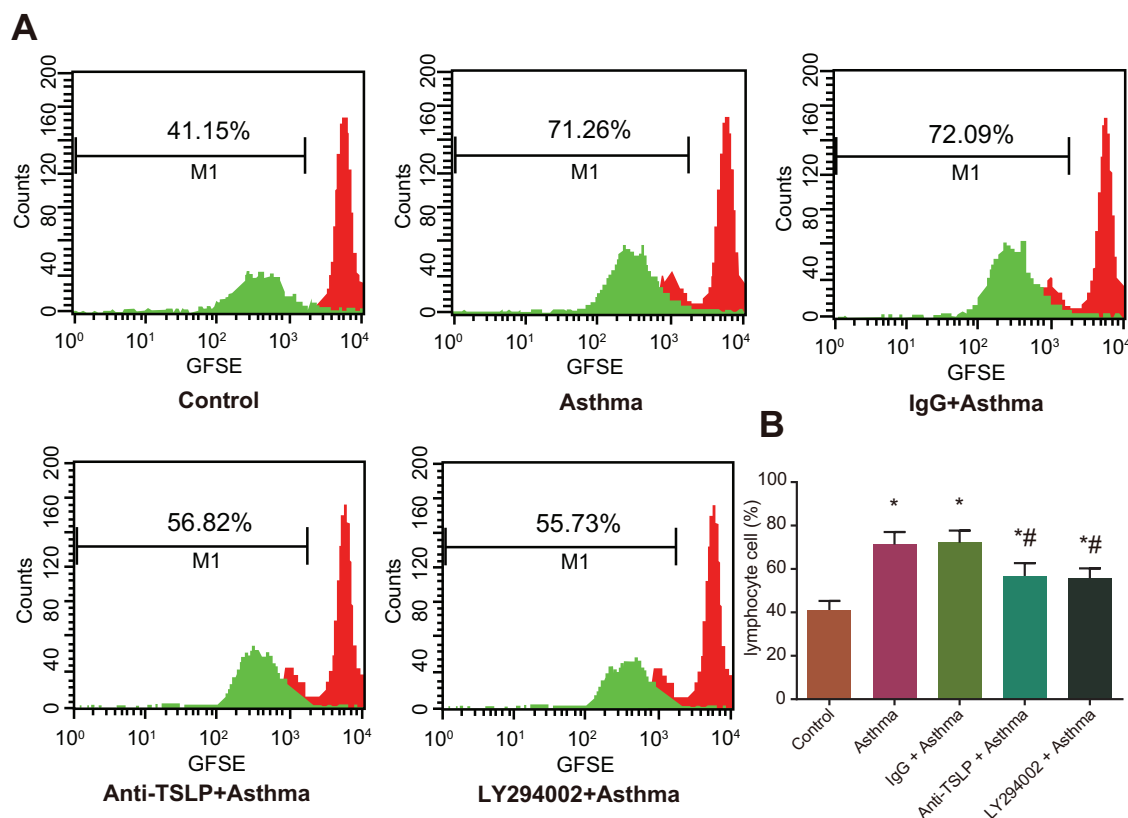


Fig. 7. The mRNA levels of TSLP, GATA-3, OX40L, T-bet, NF- κ B, p38, and ERK (A) and levels of OX40L in DCs (B) in lung tissues among the control, asthma, IgG + asthma, LY294002 + asthma and anti-TSLP + asthma groups. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the asthma and IgG + asthma groups; there were 13 mice in each group; DCs, dendritic cells; IgG, immunoglobulin G; TSLP, thymic stromal lymphopoietin; OX40L, Oxford 40 ligand; T-bet, T-box expressed in T cells; GATA-3, GATA binding protein 3; NF- κ B, nuclear factor-kappa B; ERK, extracellular regulated protein kinases; p38, p38 mitogen-activated protein kinases. [Colour online.]

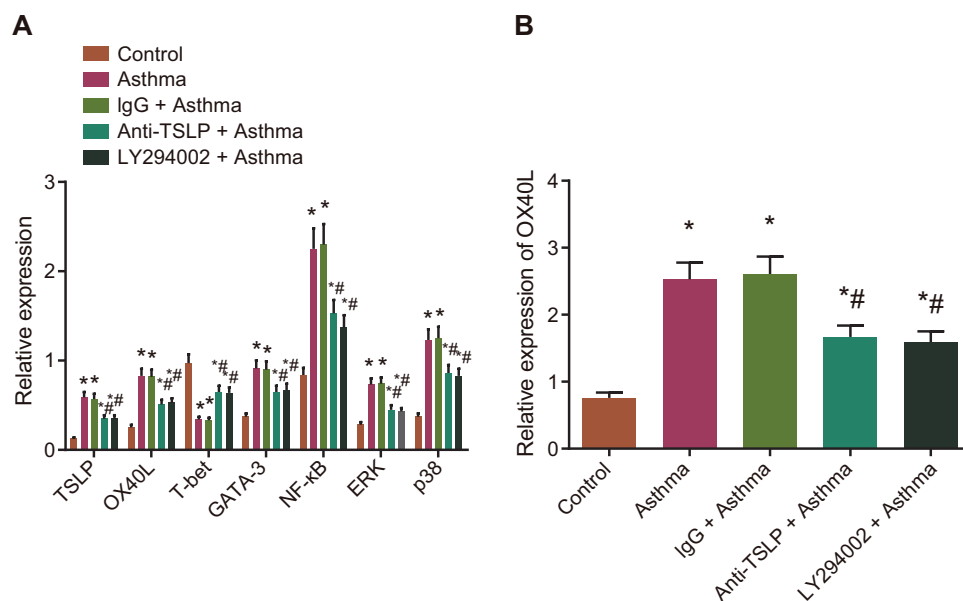
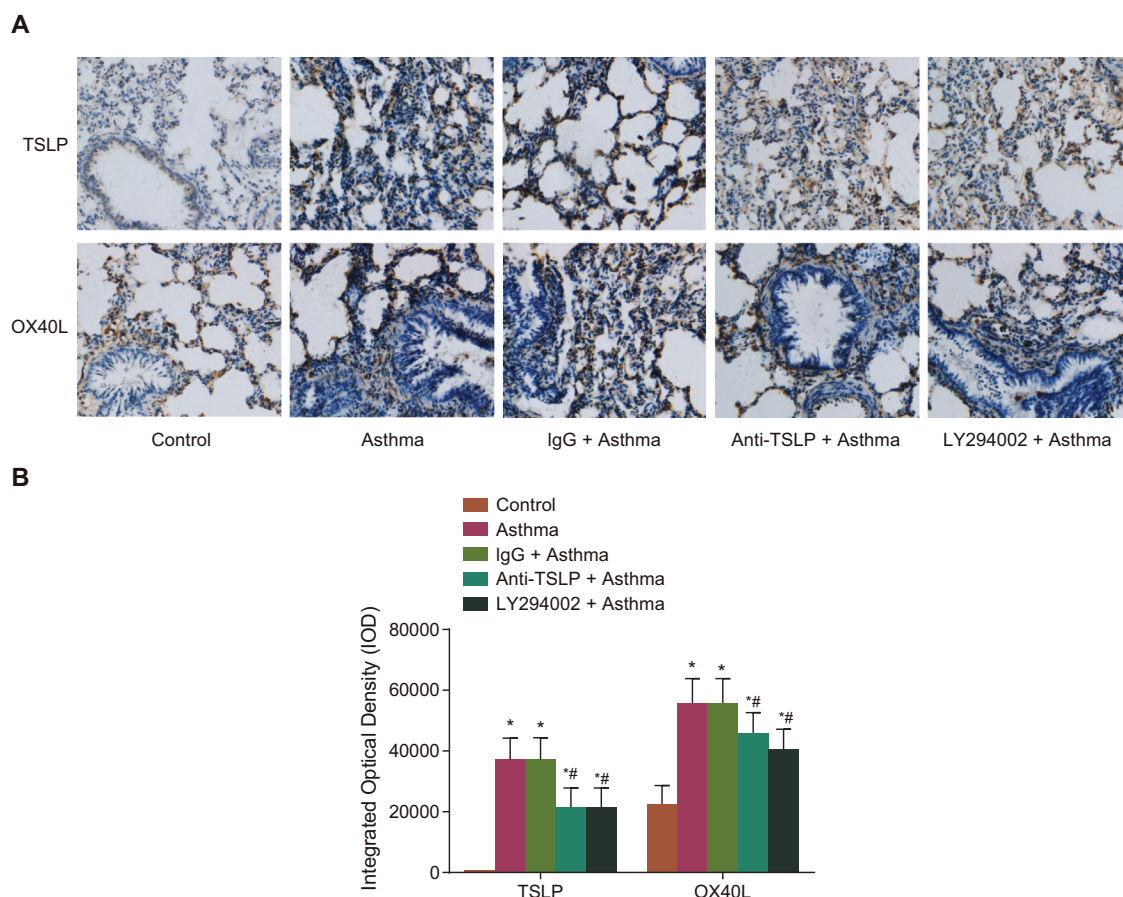


Fig. 8. *TSLP* and *OX40L* levels in mice from the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. (A) IHC images of the levels of *TSLP* and *OX40L* in mice among the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. (B) Levels of *TSLP* and *OX40L* mice in the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the asthma and IgG + asthma groups; there were 13 mice in each group; IgG, immunoglobulin G; IHC, immunohistochemistry; *TSLP*, thymic stromal lymphopoietin; *OX40L*, Oxford 40 ligand. [Colour online.]



Levels of *TSLP* and *OX40L* in mice from each group as detected with IHC

According to the results from IHC, the asthma group, IgG + asthma group LY294002 + asthma group, and the anti-TSLP + asthma group displayed increased *TSLP* and *OX40L* levels compared with the control group (both $P < 0.05$). There was no significant difference in the levels of *TSLP* and *OX40L* between the asthma and the IgG + asthma group ($P > 0.05$). The LY294002 + asthma group and anti-TSLP + asthma group displayed lower levels of *TSLP* and *OX40L* compared with the asthma and the IgG + asthma groups (both $P < 0.05$) (Fig. 8).

Protein levels of *TSLP*, *GATA-3*, *OX40L*, *T-bet*, *NF-κB*, *pp38*, and *pERK* in mice from each group

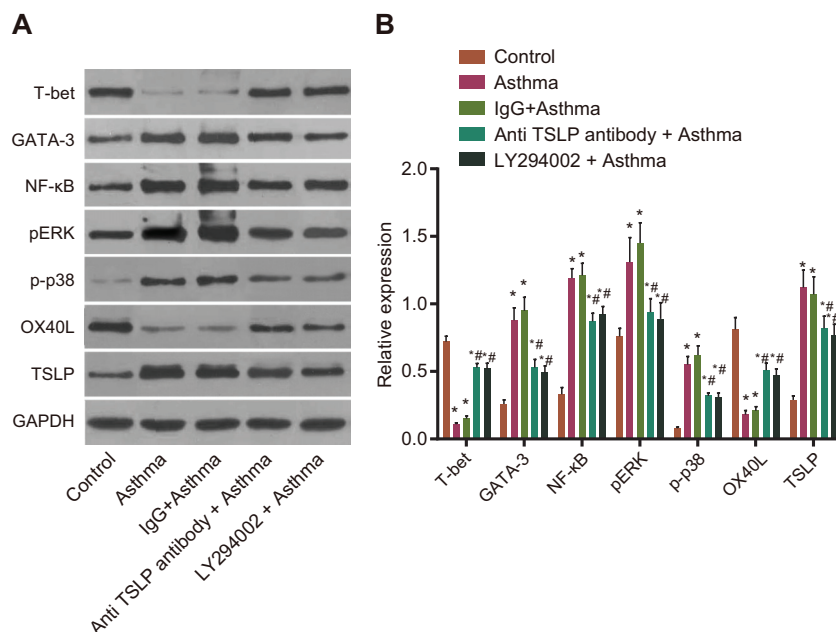
Results from the Western blot assay showed that the asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups had higher protein levels of *TSLP*, *OX40L*, *GATA-3*, *NF-κB*, phosphorylated p38 (*pp38*), and phosphorylated ERK (*pERK*), and lower levels of *T-bet* protein compared with the control group (all $P < 0.05$). There was no significant difference in the protein levels of *T-bet*, *TSLP*, *OX40L*, *GATA-3*, *NF-κB*, *pp38*, and *pERK* between the asthma and the IgG + asthma group (all $P > 0.05$). When compared with the asthma group and the IgG group, the LY294002 + asthma group and the anti-TSLP group had decreased protein levels of *TSLP*, *OX40L*, *GATA-3*, *NF-κB*, *pp38*, and *pERK*, and increased *T-bet* protein levels (all $P < 0.05$) (Fig. 9).

Discussion

Asthma, a highly prevalent chronic inflammatory lung disease, exerts a significant burden and negative influence on the patients' life (Adcock et al. 2008; Sullivan et al. 2011). The pathogenesis of asthma remains unclear (Murphy et al. 2010; Kimura and Ryo 2014), but studies have found that the inflammation in asthma is associated with activated T-cells and T lymphocytes, which provides the theoretical and clinical bases for the treatment of asthma (Shalaby and Martin 2010).

In our study, we inferred that the *TSLP*-DC-*OX40L* pathway, asthma pathogenesis, and airway inflammation may be correlated by means of regulating the level of $CD4^+CD25^+Treg$ cells and inflammatory factors. The major induction factor of airway inflammation and asthma is the imbalance between Th1 and Th2 immune cell responses, resulting in a skew towards the Th2 phenotype (He et al. 2009). DCs can initiate asthma via acquisition and transfer of the inhaled antigens to the pulmonary draining lymph nodes, and can determine the type of immune response by activating naïve T cells. Moreover, the immune response is determined to be a critical initiator of allergic inflammation, which is consistent with the results in our earlier study (Shi et al. 2008). As an interleukin 7 (IL-7)-like cytokine, *TSLP* was originally characterized by its ability to promote the activation of B cells and DCs (Ziegler and Artis 2010). Two forms of *TSLP* splice variants have been reported, among which, the long-form *TSLP* is reported to be markedly induced by poly (I:C)/double stranded RNA stimulation

Fig. 9. Protein levels of *TSLP*, *GATA-3*, *OX40L*, *T-bet*, *NF- κ B*, *pp38*, and *pERK* in mice from each group. (A) Protein levels of *TSLP*, *GATA-3*, *OX40L*, *T-bet*, *NF- κ B*, *pp38*, and *pERK* in mice among the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. (B) Western blot assay images of *TSLP*, *GATA-3*, *OX40L*, *T-bet*, *NF- κ B*, *pp38*, and *pERK* among the control, asthma, IgG + asthma, LY294002 + asthma, and anti-TSLP + asthma groups. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the asthma and IgG + asthma groups; there were 13 mice in each group; *TSLP*, thymic stromal lymphopoietin; *OX40L*, Oxford 40 ligand; *T-bet*, T-box expressed in T cells; *GATA-3*, GATA binding protein 3; *NF- κ B*, nuclear factor-kappa B; *ERK*, extracellular regulated protein kinases; *pp38*, p38 mitogen-activated protein kinases; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; IgG, immunoglobulin G. [Colour online.]



in normal human airway epithelial cells (Harada et al. 2009). Furthermore, it has been proven that *TSLP* plays a significant role in the initiation and maintenance of allergic airway inflammation, and is over-expressed in asthmatic bronchial biopsy specimens (Semlali et al. 2010). In addition, OX40–OX40L is directly mediated by *TSLP*, which is involved in Th2 responses. Thus, animal models have determined that *TSLP* and OX40–OX40L are critical in the development of airway inflammation and hyper-reactivity (Kaur and Brightling 2012). Moreover, The OX40 receptor is preferentially expressed on the surface of activated regulatory CD4⁺CD8⁺T cells, in which the OX40 signal down-regulates T-cell division and survival as well as cytokine release (Croft et al. 2009). In our study, when the OX40–OX40L was blocked by LY294002, inflammation symptoms and levels of T-bet were reduced, while levels of DC cells, lymphopoiesis, and levels of *TSLP*, *OX40L*, *GATA-3*, *NF- κ B*, *p38*, and *ERK* were increased. A previous study showed that the OX40 protein was primarily expressed on the activated CD4⁺ T cells in rats and on activated CD4⁺ and CD8⁺ T cells to promote the proliferation and expansion of CD4⁺ T cells (Ziegler and Artis 2010). In addition, the alleviating effects from blocking *TSLP* receptor (*TSLPR*) are achieved by inhibiting DC maturation and migration and by initiating CD4⁺T cell responses. In other words, blocking *TSLPR* prevents Th2-mediated airway inflammation by regulating DC function, which is of potential significance for novel treatments for asthma (Shi et al. 2008). It is consistent with our results that CD4⁺CD25⁺Treg cells and inflammatory factors are associated with asthma and airway inflammation.

We also concluded that the inhibition of the *TSLP*–DC–OX40L signaling pathway can relieve asthma symptoms and airway resistance in mice, and reduce the serum levels of IgE, IL-4, IL-5, IL-13, as well as the spleen levels of CD4⁺CD25⁺Treg, DCs, *NF- κ B*, *TSLP*, *OX40L*, and *GATA3*. An exaggerated production of IgE antibodies is often the basis for airway inflammation (He et al. 2009). Increased levels of *TSLP* in mice, characterized by an infiltration of CD4⁺ T cells and eosinophils, can positively increase levels of Th2 cyto-

kines and serum IgE (Leyva-Castillo et al. 2013). Further, OX40L is important for the adaptive immune response by promoting the Th2 polarization of T cells, which produce Th2 cytokines such as IL-4, IL-5, and IL-13 (Kaur and Brightling 2012). CD4⁺ Th2 cell infiltration is related to levels of CD4, IL-4, IL-5, and IL-13 immunoreactivity on the ocular surface, accompanied by increased levels of OX40, *STAT6*, and *GATA3* in AC mice (Zheng et al. 2010). Also, CD4⁺ Th2 cells can produce IL-4, IL-5, and IL-13 pro-inflammatory cytokines, which are considered to play the leading role in allergic asthma (Klein Wolterink and Hendriks 2013). Also, it has been proven that the level of OX40–OX40L is positively correlated with the number of IL-4⁺ cells in the lamina propria (Siddiqui et al. 2010), which was in accordance with our results. The ratio of *GATA3* cells is an independent predictive marker of patient survival, owing to the correlation between *GATA-3*⁺ cells and poor prognoses and the clinical outcome of the balance between Th2 and Th1 cells in the tumor microenvironment (De Monte et al. 2011). In addition, expressed *TSLP* can enhance the activation of *STAT5* and level of *GATA3* (Mjösberg et al. 2012), which is consistent with our study result.

In conclusion, our study inferred that the *TSLP*–DC–OX40L pathway is involved in asthma pathogenesis and airway inflammation by means of regulating the levels of CD4⁺CD25⁺Treg and CD4⁺Foxp3⁺Treg cells, as well as inflammatory factors. However, as *TSLP* has a number of effects that can alter antigen-specific immunity, ranging from altered DC recruitment, indication of DC maturation, and altered expression of a number of co-stimulatory molecules, any combination of which could produce the observed phenotypes in our study, more studies are needed to determine how the *TSLP*–DC–OX40L pathway decreases asthma severity.

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