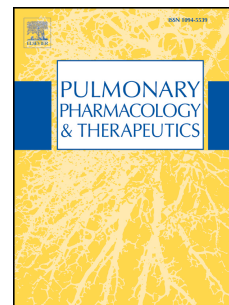


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IL-4R suppresses airway inflammation in bronchial asthma by inhibiting the IL-4/STAT6 pathway

Running title: Roles of IL4R/IL-4/STAT6 in asthma

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ABSTRACT

Objective: This study aims to explore the mechanisms of how IL-4R suppresses airway inflammation in bronchial asthma by inhibiting the IL-4/STAT6 pathway.

Methods: A total of 27 BALB/c male mice were selected and divided into control, asthma and IL-4R groups. Ovalbumin-induced mouse asthma model was established. Maximal pulmonary resistance was recorded. Hematoxylin and eosin (HE) and periodic acid Schiff (PAS) staining were conducted to observe the pathological changes in lung tissue. Optical microscope was used to detect numbers of total cells, mastocytes, eosinophils (EOS), neutrophils, and lymphocytes. Enzyme-linked immunosorbent assay (ELISA) was adopted for the levels of immunoglobulin (IgE), IL-4, IL-5, IL-13 and interferon (IFN)- γ , flow cytometry for the percentages of IL-4⁺ CD4⁺, IFN- γ ⁺ CD4⁺ and IFN- γ ⁺/IL-4⁺ in total thymus-derived (T) cells, qRT-PCR for the mRNA expressions of IL-4, IL-5, IL-13, STAT6, pSTAT6, suppressor of cytokine signaling (SOCS), inducible nitric oxide synthase (iNOS) and vascular cell adhesion molecule (VCAM)-1, and Western blotting for the protein expressions of STAT6 and pSTAT6.

Results: Compared with the control group, the asthma group had irregular tissue structure and severe inflammation, increases in maximal pulmonary resistance, numbers of total cells, EOS, neutrophils, and lymphocytes, levels of IgE, IL-4, IL-5 and IL-13, percentages of IFN- γ ⁺ CD4⁺ and IFN- γ ⁺/IL-4⁺ in total T cells, mRNA expressions of IL-4, IL-5, IL-13, STAT6, pSTAT6, SOCS, iNOS and VCAM-1, and protein expressions of STAT6 and pSTAT6, but decreases in IFN- γ level and percentage of IL-4⁺ CD4⁺ in total T cells. Compared with the asthma group, the IL-4R group had relatively regular tissue structure and light inflammation, declined maximal RL, numbers of total cells, EOS, neutrophils, and lymphocytes, contents of IgE, IL-4, IL-5 and IL-13, percentages of IFN- γ ⁺ CD4⁺ and IFN- γ ⁺/IL-4⁺ in total T cells, mRNA expressions of IL-4, IL-5, IL-13, STAT6, pSTAT6, SOCS, iNOS and VCAM-1, and protein expressions of STAT6 and pSTAT6, but elevated IFN- γ content and percentage of IL-4⁺ CD4⁺ in total T cells.

Conclusion: Our results demonstrate that IL-4R can suppress airway inflammation in bronchial

asthma by inhibited the IL-4/STAT6 pathway, which may provide a new therapeutic approach for the treatment of bronchial asthma.

Keywords: Bronchial asthma; Airway inflammation; IL-4; STAT6; IL-4R

1. Introduction

Bronchial asthma, a world-wide chronic obstructive disease, is characterized by reversible airflow limitation [1]. The prevalence of asthma has steadily increased to the scope from 0.3% to 17.0% in many countries, which is about 1.5% or higher in China [2]. The risk factors for bronchial asthma are divided into host factors including gender, race, obesity and genetic predisposition, as well as environmental factors like air pollution, infection, allergen exposure, diet and smoking [3-6]. To our knowledge, bronchial asthma is characterized by airway inflammation, reversible airway obstruction, and airway hyper-responsiveness [7]. Airway inflammation, a major focus of investigation, referred to a phenomenon that a variety of cells are involved in the process of airway inflammation of asthma, such as eosinophils, T lymphocytes, mastocytes, neutrophils, and dendritic cells (DCs). It is believed that eosinophils play important roles in the pathogenesis of asthma in the way of releasing inflammatory mediators, including radical oxygen species, cysteinyl leukotrienes (CysLTs), major basic protein (MBP) and cytokines [7]. It has been suggested that MBP alters bronchial hyper-reactivity (BHR) through an interaction with epithelium. And evidence also proves that MBP is able to cause BHR through interaction with M2 muscarinic receptors [8].

Interleukin-4 (IL-4) and interleukin-13 (IL-13), two characteristic cytokines secreted by T helper type 2 cells (Th2), basophils and mastocytes with localizations in allergic immunity, are involved in pathological conditions such as asthma and allergy [9]. The main function of Th2 cells is to provide host defense against extracellular parasitic infections while abnormal Th2 response can result in allergic inflammation or asthma, and among Th2 cells, IL-13 sharing the IL-4Ra subunit in their cognate receptors with IL-4, and activate signal transducer and activator of transcription factor-6 (STAT-6) are important for IL-4 release from antigen-stimulated murine splenocytes, which play a predominant role in the immune system [10]. Most interestingly, the activation of the JAK3-STAT6 pathway, as downstream of IL-4, is needed for the acquisition of the expression signature and dendritic-cell-specific demethylation, following STAT6 binding [11]. In addition, one study found that STAT6 played several distinct roles for regulating GATA3 transcription and

demonstrated that the activation of the STAT6 would displace the PcG complex with the TrxG complex, which controlled GATA3 gene expression in Th2 cells [12]. Recently, a study reported that IL-4, IL-13 and STAT6 were crucial components in the progression of airway hyper-responsiveness, airway inflammation, as well as mucus generation in asthma [13]. In this study, we aim to explore the roles of IL-4/STAT6 pathway in airway inflammation in bronchial asthma, which may pave the way for the treatment of bronchial asthma.

2. Materials and methods

2.1. *Experimental Animals and Grouping*

A total of 27 BALB/c male mice (6-8 weeks, 18-24 g) in specific pathogen free (SPF) grade, purchased from the Fourth Military Medical University Laboratory Center. Those mice were separately kept in individually ventilated cages (IVC) at 20 °C with an humidity of 50 %, with free access to standard non-protein pelleted feed and water under 12 h rhythm. The experimental mice were randomly assigned into control, asthma and IL-4R (Recombinant Human IL-4, peProtechEC Ltd, London, UK) groups, with 9 mice in each group. The experiment procedures were in accordance with the ethical standards of the responsible ethics committee on animal experimentation.

2.2. *Establishment of A Mice Model of Chronic Asthma*

Ovalbumin (10 mg) (OVA, Sigma-Aldrich Chemical Company, St Louis MO, USA) was added into 10 g of aluminum hydroxide gel and 100 mL of phosphate buffer saline (PBS) to obtain ovalbumin suspension. On the 1st and 7th days of the rhythm feed, the mice in the asthma and IL-4R groups were undergone intraperitoneal injection of 0.2 mL of OVA suspension for sensitization, and in the control group were injected with PBS suspension instead. From the 14th day, the sensitized mice in the asthma and IL-4R groups were kept in sealed glass container and stimulated by ultrasonic atomization of physiological saline solution containing 5 % OVA with maximum inhalation. This process was carried out once per day lasting 25 min, totally 4 weeks. The mice in

the control group were stimulated by atomizing inhalation of physiological saline for 30 min each time. Mice in IL-4R group were intra-peritoneally injected with 0.5 mL of IL-4 recombinant protein (1 g/h) diluents, while mice in the control and asthma groups were injected with 0.5 mL of physiological saline.

2.3. Measurement of Maximal Pulmonary Resistance

After last stimulation, 24 h later intraperitoneal injection of 1 % pentobarbital sodium (50 mg/kg) (Sigma-Aldrich Chemical Company, St Louis MO, USA) was conducted on mice for anesthesia and then 27 G venous indwelling needle was pierced into the exposed caudal vein. The tube was confirmed successfully-equipped when showed neither resistance nor edema on the skins tested by physiological saline. After anesthesia, the mouse was fixed on the operating table. After the weasand undergone blunt dissection, a 2 mm of plastic pipe was inserted into the weasand through a V-shaped incision. Then, AniRes 2005 animal lung function instrument (Beijing BeiLanbonCo., Ltd., Beijing, China) was connected for mechanical ventilation under the respiratory rate of 90 times/min and tidal volume of 6 mL/kg. When the airway pressure stabilized, acetylcholine (ACH) was injected via vein with various concentrations of 10, 30, 60, 90, 180, and 270 μ g/kg, (Sigma-Aldrich Chemical Company, St Louis MO, USA), and then the maximal pulmonary resistance within 5 s to 1 min were recorded.

2.4. Hematoxylin and eosin (HE) and periodic acid Schiff (PAS) staining

Material collecting and sectioning: after four weeks of atomization of physiological saline solution containing 5 % OVA, all mice were put in sealed glass container with diethyl ether (Sigma-Aldrich Chemical Company, St Louis MO, USA) for anesthesia. One third of mice were fixed on the operating table with chests opened to expose the hearts. Their right atrial appendages were cut after left ventricular intubation, and then 200 mL of 0.9 % physiological saline was rapidly infused. They were washed in a constant speed once physiological saline solution erupted from the right atrial appendages, and until the color of livers and lungs turned from red into white, the infusion was then stopped. Then the lung tissues were clipped and soaked in 10 % methanal for 48

h. And after normal paraffin embedding slicing, the slices were stored at low temperature. After being extracted eyeballs and blood sampling, another one third of mice were fixed on the cricoid cartilage with a transverse incision, and silicone tubes with injectors were inserted into the weasand through incisions. Every 0.4 mL of PBS solution was infused by three times and the bronchoalveolar lavage fluid (BALF) was collected and kept at 4 °C for cells separation within 2 h. For the rest one third mice, their fresh lung tissues were extracted and then reserved at -80 °C.

Hematoxylin-eosin (HE) staining: the fresh lung tissue samples in all groups were put in 10 % formalin (NanJing JianCheng Bioengineering Institute, Jiangsu, China) for 24 h. And after ethanol dehydration, cleaning, dewaxing and embedding, they were made into 4 µm paraffin slices for HE staining. The process of HE staining were present as follows: the slices were put into distilled water after dewaxing, xylene dehydration twice (15 min/time), anhydrous alcohol dehydration was carried out for twice (2-5 min/times), 95 % ethyl alcohol dehydration twice (2-5 min/times), 80 % ethyl alcohol dehydration 2-5 min, and then distilled water was used for washing. Hematoxylin staining was conducted for 5 min followed by rinsing, 1 % hydrochloric-alcohol solution differentiation followed by rinsing and then aqua-ammonia followed by rinsing. Eosin counterstaining was performed for 3 min followed by rinsing, after that dehydration of gradient ethanol including 95 %, 80 % and anhydrous alcohol successively with twice for each (2-3 min/time). Xylene was used two times for transparence around 10 min. After wiping away the xylene on the verge of slices and instilling suitable neutral gum, the slices were sealed by neutral gum with cover glass. Double-blind method was applied to observe the morphological changes of lung tissues under the light microscope. Xylene, ethanol, hematoxylin, eosin and neutral gum were purchased from Sigma-Aldrich Chemical Company (St Louis MO, USA), LM1235 paraffin slicing machine was from Leica Biosystems Co., Ltd. (Shanghai, China), KD-BM paraffin embedding machine was purchased from JinhuaKonor Co., Ltd., (Zhejiang, China), and bio-optical microscope CX-31 was purchased from Olympus Optical Co., Ltd (Tokyo, Japan).

Periodic acid Schiff (PAS) staining: those dewaxed lung tissue slices were rinsed in 3 % acetic

acid for 2 min, and then reacted in 1 % alcanblue acetic solution for 10-20 min, followed by distilled water rinsing twice for 5 min each time. After soaked in 0.5 % periodic acid solution for 5 min, the slices were washed by distilled water twice for 3 min each time and then by 70 % twice for 3 min each time. Those slices were immersed in Schiff solution for 15-30 min and then washed by running water twice for 5 min each time. Lastly, following slight hematoxylon staining of 2-3 min, the dye uncombined was flushed away. And then conventionally dehydrating and neutral gum sealing were carried out. The morphological changes of lung tissues of mice were observed under the microscope. PAS staining kit was purchased from the Sigma-Aldrich Chemical Company (St Louis MO, USA).

2.5. Cell Counts

The mice were undergone intraperitoneal injection of 0.3 mL of 0.3 % nembutal for anesthesia, after which their weasands were cut for tube intubation, infused by PBS twice (0.2 mL each time) and then centrifuged at $1400\sim 1800 \times g$ for 20 min. After sediment of cells was re-suspended and cells were counted, it was centrifuged to remove the supernatant. And then sediment of cells was again re-suspended by 50 μ L of PBS, followed by cell smearing. When being dried out, it was stained by Wright's method. Under optical microscope: 5 fields were selected to classify cells and count the numbers of total cells, mastocytes, eosinophils (EOS), neutrophils and lymphocytes respectively among each group.

2.6. Enzyme-linked Immunosorbent assay (ELISA)

The levels of immunoglobulin (IgE), IL-4, IL-5, IL-13 and interferon (IFN)- γ in the bronchoalveolar lavage fluids (BALF) was tested strictly following the ELISA kit purchased from Shanghai Westang BioTech Co., Ltd. (Shanghai, China). The kit was balanced at room temperature for 20 min, and then cleaning solution was prepared. Ten standard wells on the ELISA plate (2 blank control wells without samples or enzyme-labeled reagent) and the standard samples run on gradient dilution for the standard curve. The diluted samples to be tested were added into the sample wells. Within 15 min taking apoptosis of blank group as reference, we tested the optical

density (OD) value of each well (450 nm). The microplate reader was purchased from Bio-Rad, Inc. (Hercules, CA, USA)

2.7. Flow Cytometry

When the mice were executed, their weasand were exposed under aseptic condition and intubated with the No. 23 tube. After that, 1 mL of PBS solution containing 10 g/L collagenase (4 °C) was infused into the weasand. The lung tissues were cut off, resuspended in Hank's balanced salt solution (HBSS), and then shaken for 30 min. Then the suspension was homogenized, filtrated by 70 µm of nylon net, centrifuged ($1800 \times g$) for 5 min at 4 °C, filtrated by gauze and centrifuged again, and finally resuspended in complete medium to detect thymus-derived (T) cells (over 90 % purity) by flow cytometry. Separated lymphocytes were added with fetal bovine serum (FBS), resuspended and adjusted into $(1\sim2) \times 10^6/\text{mL}$. Then PRMI1640 culture solution with 100 mL/L FBS was added into 24-well plate with 25 ng/mL phorbol ester PMA and 1 µg/mL Ionomycin as irritant and 2.85 µmol/mL Monencin to prevent cytokines from secreting out of the cells. After being well-mixed, they were stored at an incubator with 50 mL /L CO₂ at 37 °C for 5 h. Cytokines on and inside T cells were routinely labeled and detected by FACS Vantage SE flow cytometry. Area was demarcated by CD3⁺, and fluorescence channels (FL1, FL2 and FL3) were used for compensation. A total of 1×10^5 cells of each sample were detected to calculate percentage of Th1/Th2 by Cell QuestTM statistical analysis software.

2.8. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Fresh lung tissues were collected from each group, and Trizol was adopted to extract the total RNA. The OD 260/280 value was tested by ultraviolet spectrophotometer and then used to count the concentration of RNA. Then, we took out 500 ng of RNA to carry out reverse transcription following the specification of Reverse Transcription System A3500 reverse transcription kit (Promega Corporation, Madison, WI, USA). According to the gene sequences of Genbank database, the Primer 5.0 software (Premier Biosoft International, Palo Alto, CA) was used to design the primers (Table 1) that were synthesized by Shanghai Sangon Biotech Co., Ltd (Shanghai, China).

The reaction conditions of the two step method of qRT-PCR (SYBR GREEN method) were as follows: 95 °C for 15 min for once; 95 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min, totally 40 circles. The reaction system: 12.5 µL of Premix ExTaq or SYBR Green Mix, 1 µL of Forward Primer, 1 µL of Reverse Primer, 1-4 µL of DNA template, and 25 µL of ddH₂O. Reduced Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference, the standard of the control group was set as 1 and figured out the Ct value of target genes (amplifying the knee of dynamic curve). Based on the formula of relative quantification $(RQ) = 2^{-\Delta\Delta C_t}$, RQ values of target genes were calculated and used for data analysis. The iQ5 qRT-PCR instrument was purchased from Bio-Rad, Inc. (Hercules, CA, USA).

2.9. Western Blotting

The mice lung tissues proteins were extracted to exam its concentration via (bicinchoninic acid) BCA kit (Beyotime Biotechnology Co., Shanghai, China), added into 5 × loading buffer (Beyotime Biotechnology Co., Shanghai, China), and boiled for 10 min at 95 °C. Each well was added with 30µg of loading buffer and then run on electrophoretic separation in 10 % polyacrylamide gel (Boster Co. Ltd., Hubei, China), polyvinylidene fluoride (PVDF) (Amresco Solon, OH, USA) transmembrane, 5 % bovine serum albumin (BSA) (Sino-American Biotechnology Co. Ltd., Beijing, China) sealing for 1 h at room temperature. Then we added the primary antibody diluted by Tris-buffered saline tween (TBST) (Abcam Co., Ltd., Beijing, China) including GAPDH (ab9485, 1: 2500), STAT6 (ab44718, 1: 200) and pSTAT6 (ab54461, 1: 1000) into the membranes at 4 °C overnight. After the membrane was cleaned by TBST for three times with 5 min for each time, the second antibody was added and reacted at 37 °C for 2 h. Following membrane cleaning again, substrate coloration fluid A and B (Promega Corporation, Madison, WI, USA) were mixed at the rate of 1: 1 and the membrane colored for 1 min at room temperature, wrapped by plastic film and then transferred to dark place. After exposure, X-ray plate was used for developing and photographic fixation. The Gel-Pro analyzer 4. 0 was used for band analysis, and the proportion of the grey value between target protein and GAPDH reflected the expression level of protein. The

primary and second antibodies were purchased from Abcam Co., Ltd. (Beijing, China). Decolorization table (ZD-9500) was purchased from Hualida Company (Jiangsu, China) and vertical transfer electrophoresis tank was purchased from Bio-Rad, Inc. (Hercules, CA, USA)

2.10. Statistical Analysis

All data were analyzed by SPSS 21.0 statistic software and the measurement data were presented as mean $\pm$ standard deviation ($\bar{x} \pm s$). One-way analysis of variance (ANOVA) was used for comparison among three groups, and least significant difference (LSD) test was used for comparison between two groups. $P < 0.05$ means statistically significant difference.

3. Results

3.1. Maximal Pulmonary Resistance of Mice among the Control, Asthma and IL-4R Groups

There was no significant difference in the maximal pulmonary resistance of mice among the control, asthma and IL-4R groups ($P > 0.05$). There was also no significant difference in the maximal pulmonary resistance among the three group when the mice were stimulated by ACH with a concentration of 10 $\mu\text{g/kg}$ ($P > 0.05$); when the concentration of ACH were 30 $\mu\text{g/kg}$, 60 $\mu\text{g/kg}$, 90 $\mu\text{g/kg}$, 180 $\mu\text{g/kg}$ and 270 $\mu\text{g/kg}$, the acetylcholine of mice in the asthma and IL-4R groups was increased as the rise of concentrations ($P < 0.05$). In addition, compared with the control group, the maximal pulmonary resistance in the asthma and IL-4R groups remarkably increased ($P < 0.05$), and the acetylcholine in the IL-4R group evidently declined compared with the asthma group ($P < 0.05$) (Fig. 1).

3.2. Histopathological Changes in Lung Tissues of Mice among the Control, Asthma and IL-4R Groups

The results of HE and PAS staining showed that the lung tissue structure of the mice in the control group had clear structure, with smooth airway epithelium, flat cilium, no obvious edema on the airway mucosa, no obstruction in the smooth bronchial lumen, no evident inflammatory cell infiltration around airway or vascular tissue, very few goblet cell proliferation, and no inflammatory

secretions alveolar cavity (Fig. 2A). Conversely, in the asthma group, the lung tissue was in irregular structure, with incomplete airway epithelium, obvious obscission of cilium, edema on the airway mucous membrane, and narrow or even closed airway lumen. At the same time, in the asthma group, the inflammatory cell infiltration appeared around the submucosa and vascular tissue, mainly including eosinophils and lymphocyte, the goblet cell proliferation increased, and inflammatory secretions appeared in the alveolar cavity. However, in the IL-4R group, slight inflammatory changes occurred to the airway of the mice, but the goblet cell proliferation was reduced. In comparison with the asthma group, the asthma symptoms were reduced significantly (Fig. 2B).

3.3. The Numbers and Classifications of Total Cells, EOS, Neutrophils, mastocytes, and Lymphocytes in BALF and the Levels of IgE, IL-4, IL-5, IL-13 and IFN- γ in Mice among the Control, Asthma and IL-4R Groups

In comparison with the control group, the numbers of total cells, EOS, neutrophils and lymphocytes in the BALF in the asthma and IL-4R groups increased (all $P < 0.05$), while the number of mastocytes had no significant difference (all $P > 0.05$). Besides, the contents of IgE, IL-4, IL-5 and IL-13 were significantly elevated (all $P < 0.05$), while that of IFN- γ declined ($P < 0.05$). When compared with the asthma group, the numbers of total cells, EOS, neutrophils and lymphocytes in the BALF in the IL-4R group, and the levels of IgE, IL-4, IL-5 and IL-13 decreased significantly but that of IFN- γ increased ($P < 0.05$) (all $P < 0.05$), while the number of mastocytes had no significant difference ($P > 0.05$), (Fig. 3).

3.4. Measurements of IL-4⁺ CD4⁺, IFN- γ ⁺ CD4⁺ and IFN- γ ⁺/IL-4⁺ in T cells in Mice among the Control, Asthma and IL-4R Groups

As shown in Table 2, the asthma group exhibited significantly a decreased percentage of IL-4⁺CD4⁺ and an increased percentage of IFN- γ ⁺CD4⁺ and IFN- γ ⁺/IL-4⁺ in total T cells in comparison with the control group (both $P < 0.05$). Besides, no significant difference was observed between IL-4R group and control group in ratios of IL-4⁺CD4⁺, IFN- γ ⁺CD4⁺ and IFN- γ ⁺/IL-4⁺ in

total T cells (all $P > 0.05$). There was a remarkably higher proportion of IL-4⁺CD4⁺ and lower proportion of IFN- γ ⁺CD4⁺ and IFN- γ ⁺/IL-4⁺ in total T cells in the IL-4R group than those in asthma group (all $P < 0.05$).

3.5. The mRNA Expressions of IL-4, IL-5, IL-13, STAT6, pSTAT6, SOCS-1, iNOS and VCAM-1 in Mice among the Control, Asthma and IL-4R Groups

Compared with the control group, the mRNA expressions of IL-4, IL-5, IL-13, STAT6, pSTAT6, SOCS-1, iNOS and sVCAM-1mRNA in the asthma and IL-4R groups were obviously increased (all $P < 0.05$). However, compared with the asthma group, the expression levels of IL-4, IL-5, IL-13, STAT6, pSTAT6, SOCS-1, iNOS and VCAM-1mRNA in the IL-4R group were declined (all $P < 0.05$) (Fig. 4).

3.6. Protein Expressions of STAT6 and pSTAT6 in Mice among the Control, Asthma and IL-4R Groups

In comparison with the control group, increased expressions of STAT6 and pSTAT6 protein were observed in the asthma group and IL-4R group (both $P < 0.05$). Compared with the asthma group, the IL-4R group showed significantly decreased protein expressions of STAT6 and pSTAT6 (all $P < 0.05$) (Fig. 5).

4. Discussion

In this study, our purpose is to explore the mechanism of action of IL-4/STAT6 pathway in bronchial asthma and airway inflammation, as well as the relationship between them, which may be greatly beneficial for the treatment of bronchial asthma and airway inflammation.

With stimulation of ACH, we found that with the increase of ACH concentration, the RL in the asthma group and IL-4R group has increased, while the RL value in IL-4R group has decreased compared to that in the asthma group. ACH was found as a major parasympathetic neurotransmitter in the airways, which participated in airway smooth muscle contraction as well as mucous secretions in the respiratory system [14]. It was produced by nonneuronal cells that were mainly

airway inflammatory cells, and regulated their behaviors, which was known as the nonneuronal cholinergic system [15]. Kang et al. reported that parasympathetic activity was promoted in chronic airway diseases, and over vagal tone seemed to be the only reversible component for airflow limitation [16]. In addition, it has been found in HE and PAS staining that the rats in the asthma group that had an irregular structure of mice lung tissue, incomplete airway epithelium with cilia fallen off and lodged significantly; airway mucosa in absolute edema, narrowed airway lumen and some even completely occluded. Besides, the mucosa layer, submucosa and vascular tissue have been infiltrated around a large number of inflammatory cell, mainly eosinophile cells and lymphocytes; goblet cell has proliferated, and inflammatory secretion was observed in the alveolar cavity. It has been mentioned in the study by Kudo et al. that the lungs are commonly hyperinflated as a result of extensive mucous plugging in peripheral airways, segmental and subsegmental bronchus. Moreover, the composition of mucous includes cellular debris from necrotic airway epithelial cells, inflammatory cells such as eosinophils, lymphocytes, as well as neutrophils, and mucin produced by goblet cells. The airway epithelium presented sloughing of ciliated columnar cells, with squamous cell metaplasia and goblet cell as a sign of airway epithelial repair. There is increased thickness of the subepithelial basement membrane [1].

Compared with those in the control group, the total number of cells, and the numbers of eosinophils, neutrophils and lymphocytes in the asthma group are significantly increased, and the contents of IgE, IL-4, IL-5 and IL-13 also are elevated significantly. One study has suggested that asthma can be more severe in the consequence of the increase of IgE, lymphocytes and neutrophils [17, 18]. Also, another study found in their results that, compared with those in the asthma group, the total number of cells, and the numbers of eosinophils, neutrophils and lymphocytes in the IL-4R group are decreased while the contents of IgE, IL-4, IL-5 and IL-13 also are reduced, suggesting that the inhibition of IL-4/STAT6 pathway can effectively relieve asthma so as to concluded that IL-4 signaling promotes IgE class switching through STAT6 activation and the induction of Ig germ-line transcription [19].

One of the most important results in our study is that IL-4/STAT6 pathway specific inhibitor can reduce the amount of total cells, eosinophils, neutrophils as well as lymphocytes, which have suggested the inhibition of IL-4/STAT6 pathway may alleviate bronchial asthma and airway inflammation. As a key factor responsible for inducing cathepsin activity in macrophages in vitro and in vivo [20], STAT-6 was significant for the progression of mucus generation along with airway inflammation in animal models of allergic airways disease, and both IL-13 and IL-4 activate signal transducer, sharing IL-4R α subunit in the cognate receptors, are activators of STAT-6 [21]. IL-4 stimulation resulted in the activation of multiple signaling pathways, including STAT6 pathway that was required for the production of IL-4 from antigen-stimulated murine splenocytes, while IL-4-triggered increases and expressions of both IL-4 receptor and MHC class II antigens are entirely abrogated, and even lymphocytes from STAT6-deficient animals fail to grow in response to IL-4 [22]. In addition, it was identified that IL-4/STAT-6 pathway participates in the pathogenesis of asthma because STAT-6 activation has been implicated in the differentiation of naïve T-cells into Th2 effector cells, and it also regulated the generation of Th2 chemokine induced by IL-4 and IL-13, including eotaxin from smooth muscle cells, fibroblasts cells, as well as airway epithelial cells. [13]. The up-regulated levels of IL-13 together with its receptor subunits, and some single nucleotide polymorphisms (SNPs) of genes encoding IL-13 was in association with STAT-6 and IL-13R α 1 (signaling molecules) [23]. Also, consistent with our results, in Greiller et al. study, IL-5, iNOS and VCAM-1 promoted vascular permeability and triggered synthesis of acute phase proteins, which were all responsible for the recruitment and activation of cells of the adaptive immune response [24]. To put forward, GATA3 is a key factor for cytotoxic T lymphocyte (CTL) differentiation in response to infection, and its expression was regulated by a distinct, IL-4 independent signaling pathway [25]. T cell factor was shown to activate GATA3 expression so as to promote the development Th2 cells independent of STAT6 signaling produced by IL-4 [26]. Importantly, one study showed that at least partially, the down-regulation of GATA-3 and STAT-6 can attenuate the immunomodulatory actions resulted from Th1/Th2 cytokines in asthma [27].

In conclusion, our study provides evidence that the inhibition of IL-4/STAT6 pathways have great potential to decrease the amount of total cells, eosinophils, neutrophils and lymphocytes to lower the incidence of bronchial asthma and airway inflammation, which may pave the way for therapeutic strategies directed toward IL-4/STAT6 pathways function for the treatment of bronchial asthma and airway inflammation. So far, it was complicated to develop novel and effective anti-inflammatory methods for asthma treatment, but maybe a much better understanding of the inflammatory signaling pathways demonstrated in asthmatic airway cells might result in more effective therapy classes in the future. Nevertheless, we suggest that more prospective studies with larger sample sizes are conducted to provide more precise estimates to confirm the effects of IL-4/STAT6 pathways to get closer to the goal so as to provide new therapeutic strategies for the treatment of bronchial asthma and relieving airway inflammation.

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Disclosure

The authors report no conflicts of interest in this work.

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Legends

Fig. 1. Maximal pulmonary resistance of mice among the control, asthma and IL-4R groups

Note: *, $P < 0.05$, compared with the control group, #, $P < 0.05$, compared with the asthma group; IL, interleukin; ACH, acetylcholine.

Fig. 2. Histopathological changes detected by HE and PAS staining in mice among the control, asthma and IL-4R groups

Note: A, the HE staining images for lung tissues in mice among the control, asthma and IL-4R groups; B, the PAS staining images for lung tissues in mice among the control, asthma and IL-4R groups; HE, hematoxylin and eosin; PAS, periodic acid Schiff; IL, interleukin.

Fig. 3. The numbers and classification of total cells, EOS, neutrophils, mastocytes and lymphocytes in BALF and the levels of IgE, IL-4, IL-5, IL-13 and IFN- γ in mice among the control, asthma and IL-4R groups

Note: A: comparisons of numbers of total cell, EOS and mastocyte in BALF of mice among the control, asthma and IL-4R groups; B: comparisons of numbers of neutrophils and lymphocytes in BALF of mice among the control, asthma and IL-4R groups; C: comparisons of contents of IgE, IL-4, IL-5 and IL-13 and IFN- γ in BALF of mice among the control, asthma and IL-4R groups; *, $P < 0.05$, compared with the control group, #, $P < 0.05$, compared with the asthma group; EOS, eosinophils; IL, interleukin; IgE, immunoglobulin; IFN, interferon; BALF, bronchoalveolar lavage fluid.

Fig. 4. The mRNA expressions of IL-4, IL-5, IL-13, STAT6, pSTAT6, SOCS-1, iNOS and VCAM-1 detected by qRT-PCR in mice among the control, asthma and IL-4R groups

Note: A, the mRNA expressions of IL-4, IL-5 and IL-13 in lung tissues of mice among the control, asthma and IL-4R groups; B, the mRNA expressions of STAT6, pSTAT6, SOCS-1, iNOS and VCAM-1 in lung tissues of mice among the control, asthma and IL-4R groups; *, $P < 0.05$, compared with the control group, #, $P < 0.05$, compared with the asthma group; IL, interleukin; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine signaling;

iNOS, inducible nitric oxide synthase; VCAM, vascular cell adhesion molecule; qRT-PCR, quantitative real-time polymerase chain reaction.

Fig. 5. The protein expressions of STAT6 and pSTAT6 detected by Western blotting in mice among the control, asthma and IL-4R groups

Note: *, $P < 0.05$, compared with the control group, #, $P < 0.05$, compared with the asthma group;

IL, interleukin; STAT, signal transducer and activator of transcription.

TABLE 1. The primer sequences for qRT-PCR

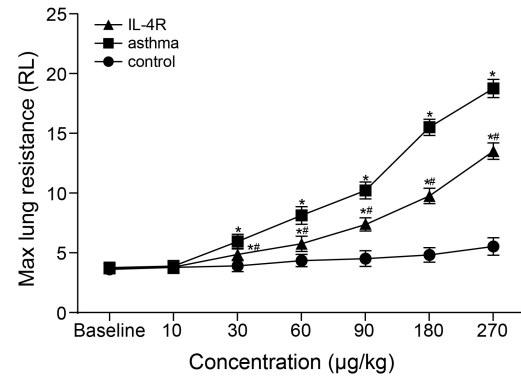
Primer	Sequence
IL-4	F: GGTCTCAACCCCCAGCTAGT
	R: GCCGATGATCTCTCTCAAGTGAT
IL-5	F: CTCTGTTGACAAGCAATGAGACG
	R: TCTTCAGTATATCTAGCCCCTG
IL-13	F: CCTGGCTCTTGCTTGCCTT
	R: GGTCTTGTGTGATGTTGCTCA
IFN- γ	F: ATCAGGAGGGACTCCTTTTCCGCTT
	R: GAAGCCTAGAAAGTCTGAATAACT
STAT6	F: ATGGGACAATGCCTTCTCTGA
	R: AACCACTGCCAAAATGTGAAG
GAPDH	F: CACGGCAAATTCAACGGCACA
	R: TCCAGGCGGCACGTCAGATC

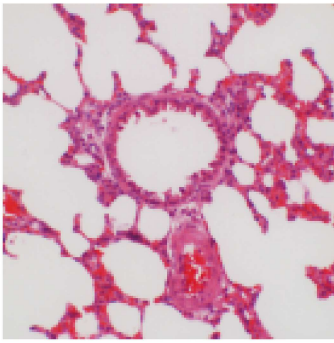
Note: IL, interleukin; IFN, interferon; STAT, Signal transducers and activators of transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction.

TABLE 2. Measurement of IL-4⁺ CD4⁺, IFN- γ ⁺ CD4⁺ and IFN- γ ⁺/IL-4⁺ in T cells detected by flow cytometry in mice among the control, asthma and IL-4R groups

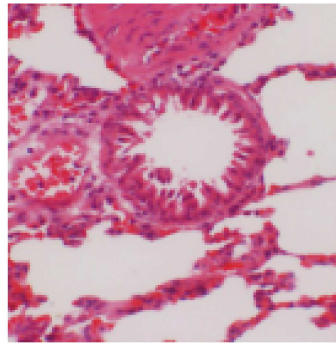
Group	n	IFN- γ ⁺ CD4 ⁺ (%)	IL-4 ⁺ CD4 ⁺ (%)	IFN- γ ⁺ /IL-4 ⁺
Control	9	80.4 $\pm$ 1.9	5.6 $\pm$ 1.1	0.72 $\pm$ 0.21
Asthma	9	91.6 $\pm$ 2.7*	3.1 $\pm$ 0.9*	0.97 $\pm$ 0.21*
IL-4R	9	76.5 $\pm$ 2.2 [#]	6.9 $\pm$ 1.8 [#]	0.66 $\pm$ 0.17 [#]

Note: *, $P < 0.05$, compared with the control group, [#], $P < 0.05$, compared with the asthma group; IL, interleukin; IFN, interferon.

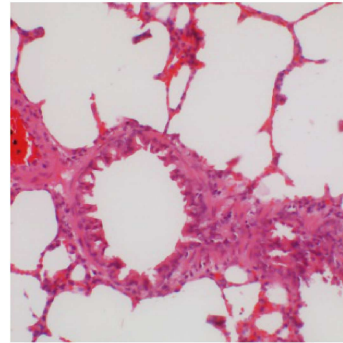


A

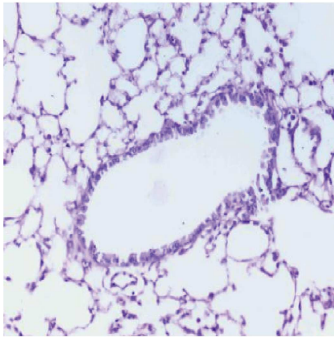
control



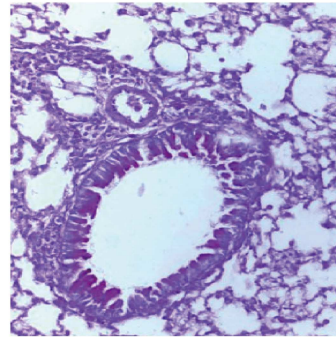
asthma



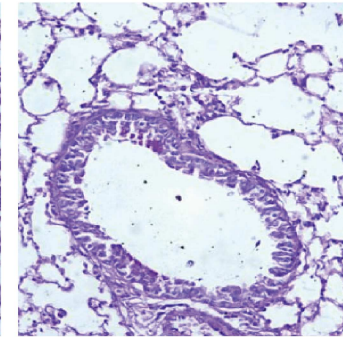
IL-4R

B

control



asthma



IL-4R

