



The significance of the levels of IL-4, IL-31 and TLSP in patients with asthma and/or rhinitis

Aim: To investigate the clinical significance of the levels of IL-4, IL-33 and thymic stromal lymphopoietin (TSLP) in patients with asthma and/or rhinitis, then do the simple verification in animals. **Methods:** Levels of IL-4, IL-31, IL-33 and TSLP were detected by ELISA and real-time PCR in 64 asthma patients (sIgE[+]: 32 cases, sIgE[-]: 32 cases), 64 rhinitis patients (sIgE[+]: 32 cases, sIgE[-]: 32 cases), 64 asthma complicated with allergic rhinitis patients (sIgE[+]: 32 cases, sIgE[-]: 32 cases) and 32 healthy controls. Then we detected the IL-4, IL-31, IL-33 and TSLP in the sensitized mice. **Results:** Results showed that levels of IL-4, IL-31, IL-33 and TSLP in asthma and rhinitis patients, and those complicated with allergic rhinitis, had significant differences compared with the control group ($p < 0.05$). It was found that the indicators of mugwort and dust mite allergic patients were significantly higher than that of other allergic patients ($p < 0.05$). We got the same tendency in *in vivo* experiments. **Conclusion:** IL-4, IL-31, IL-33 and TSLP may be involved in the pathogenesis of asthma and rhinitis; dust mite and mugwort allergy could increase them significantly.

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At present, allergic diseases include asthma, rhinitis and dermatitis. In industrialized countries, the incidence of allergic diseases is high; for example, the incidence of asthma in many countries is more than 10%. The incidence rate of allergic rhinitis varies from 15 to 30% [1,2]. With the improvement of China's modernization and industrialization level, the incidence of allergic diseases is increasing year by year.

Allergic rhinitis is due to the allergen of type I allergic nasal disease in the nasal mucosa; its primary symptoms are nasal itching, continuous sneezing, water sex mucous secretion volume and nasal resistance. The common features of allergic inflammation include leukocyte infiltration, MDC, increased chemokine CCL17 and CCL22, and increased Th2 type cytokines and IgE levels [3,4].

Bronchial asthma (asthma) is a chronic airway allergic inflammation with genetic susceptibility to multiple genes, which is mainly caused by mast cell reaction and eosinophilic infiltration. A variety of inflammatory cells have been found to be involved in its pathogenesis; inflammatory mediators, complex cytokine network, the increase of serum IgE, eosinophilic infiltration in lung tissue and bronchial hyper-responsiveness all are significant clinical features in asthma. With successful cloning and sequencing of TSLP and TSLP receptor, TSLP activates dendritic cells and starts the molecular mechanisms of the immune response of Th2; the function of TSLP in asthmatic inflammation upstream stage has been identified; and the knowledge of people on asthma immunological pathogenesis got updated [3,4]. In clinical trials it was found that the application of IL-5

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blocking antibody did not produce a treatment effect in Th2 response, also other studies confirmed the three different types of asthma, and different types of asthma driven by different pathological mechanisms [5]. IL-33, as a new member of the IL-1 family, was found to be closely related to asthma. It can promote the selective activation of macrophages and the aggregation, maturation and survival of eosinophils. More importantly, IL-33 was able to induce airway responsiveness to allergen by mast cells and IL-13 [6]. In addition, TSLP causes many respiratory allergic diseases (asthma and dermatitis). The expression of TSLP was positively correlated with the expression of Th2 [7,8].

However, whether the levels of IL-4, IL-31, IL-33 and TSLP are related to the pathogenesis of asthma or rhinitis, or which allergies are more likely to induce the expression of these three cytokines, has not yet been reported. This paper analyzes the role of these cytokines in the pathogenesis of asthma and rhinitis by testing their levels in the serum of patients with asthma, rhinitis and patients with both asthma and rhinitis, and healthy people. Furthermore, through animal experiments it is confirmed that dust mites and mugwort allergies are more likely to induce these cytokines; we provide new pharmacological strategies for therapies in asthma and/or rhinitis.

Materials & methods

Patients

All patients were being treated from October 2011 to May 2012 in our hospital allergy clinic.

In 64 cases of patients with asthma, 32 patients (sIgE[+]), 18 cases of male and 14 cases of female, age ranged from 11 to 59 years, mean age (31.9 ± 15) years, and course from 3 months to 5 years, average (15.2 ± 3.4) months. The allergen in single allergy in seven cases (three cases of mites, one case of mugwort, one case of freshwater fish, one case of *Penicillium* and one case of soybean). The rest are various types of allergy: 21 cases of mite allergy, 14 cases of food allergy and nine cases of mugwort allergy. In 32 cases (sIgE[-]), 16 cases of male and 16 cases of female, age ranged from 14 to 52 years, mean age (32.3 ± 11) years, and course of disease from 3 months to 5 years, average (16.5 ± 5.2) months.

In 64 cases of patients with rhinitis, 32 patients (sIgE[+]), 15 cases of male and 17 female patients, age ranged from 13 to 50 years, mean age (30.1 ± 12.6) years, and course from 3 months to 5 years, average (16.2 ± 4.6) months. The allergen in single allergy in nine cases (five cases of mugwort allergy, three cases of mite allergy and one case of cockroach allergy); the rest are various types of allergy: 23 cases of mugwort allergy, 18 cases of mite allergy and 10 cases of food

allergy. In 32 cases (sIgE[-]), 14 cases of male and 18 cases of female, age ranged from 11 to 51 years, mean age (31.7 ± 11.6) years, course of disease from 3 months to 5 years, average (17.5 ± 5.2) months.

In 64 cases of patients with asthma complicated with allergic rhinitis, 32 cases (sIgE[+]), 16 cases of male and 16 cases of female, age ranged from 10 to 48 years, mean age (30.1 ± 8.8) years, course from 3 months to 5 years, average (17.5 ± 5.1) months. The allergen in single allergy in 11 cases (four cases of mugwort allergy, three cases of mite allergy, three cases of freshwater fish allergy, one case of cockroach allergy and one case of *Penicillium* allergy); the rest are various type of allergy, 20 cases of mugwort allergy, 13 cases of mite allergy, and ten cases of food allergy. In 32 cases (sIgE[-]), 13 cases of male and 19 cases of female, age ranged from 10 to 54 years, mean age (31.4 ± 12) years, and course of disease from 3 months to 5 years, average (18.5 ± 7.2) months.

Above patients who have autoimmune disease, tumor, other serious systemic diseases, other allergic diseases and genetic family history of allergy, and for 3 days prior to the visit had not taken antihistamine agents or for 1 month had not used glucocorticoid were excluded.

Controls were selected from 32 normal subjects at the sports health center in our hospital physical examination. Controls have no systemic disease, endocrine system and heart, lung, liver or kidney disease. Controls have no recent local or systemic infection, nor non atopic dermatitis or other allergic diseases. The study protocol was approved by Medical Ethics and Human Clinical Trial Committee.

Specimen collection

5 ml of venous blood was taken from all patients in the early morning. After centrifugation ($2000 \times g$, 10 min), the upper serum was moved into the Eppendorf tubes, then set in a -20°C refrigerator.

ELISA detection method

Using human IL-4, IL-31, IL-33 and TSLP detection kit, the ELISA method was used to determine the concentration of IL-4, IL-31, IL-33 and TSLP in serum samples. The operation method is strictly in accordance with the specification. sIgE was determined using the German European Union Series (EUROALLER) detection system. Measurement unit is in pg/ml.

Reverse transcription & quantitative real-time PCR

Total RNA was extracted using TRIzol® (Thermo Fisher Scientific) according to the manufacturer's protocol. About 1 µg of total RNA was reverse transcribed

to cDNA using an RT Reaction Kit (Promega). Real-time PCR was performed using an Mx 3000P real-time PCR system (Applied Biosystems) according to the manufacturer's instruction and SYBR Premix Ex Taq (TaKaRa) as a DNA-specific fluorescent dye. PCR was carried out for 50 cycles at 95°C for 10 s and 60°C for 30 s, respectively. Primer sequences for detection of mRNA expression were synthesized as shown in Table 1.

All the reactions were repeated at least three times. Gene expression levels were calculated relative to the housekeeping β -actin by using Stratagene Mx 3000P software.

Western blot analyses

To determine the expression of protein, whole cell extracts (lysate) were prepared from 1×10^6 cells in lysis buffer (20 mM Tris pH 7.4, 250 mM sodium chloride, 0.1% Triton-X-100, 2 mM EDTA, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 4 mM sodium orthovanadate and 1 mM DTT), and 60 μ g of the protein was resolved on 10% SDS–polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, the membrane (Amersham) was blocked with 5% nonfat dry milk in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween-20 Tris Buffered Saline, with Tween-20) for 1 h at room temperature, and the proteins were probed with specific antibodies bought from California, USA: IL-4 (sc-32242) 1:600; IL-31 (sc-515415) 1:300; IL-33 (sc-130625) 1:200; thymic stromal lymphopoietin protein (TSLP) (sc-83871) 1:600. To ensure equal loading, gels were stripped and reprobed with antibodies against β -actin (Kangchen Bio-tech Inc., Shanghai, China). All polyvinylidene fluoride (PVDF) membranes were detected by chemiluminescence (ECL, Pierce Technology).

Animal model

C57BL/6 mice were randomly divided into control group, mite group and mugwort group. Mice in the mite and mugwort groups were sensitized by mite and mugwort extracts through intraperitoneal injection at day point 0, 7 and 14, phosphate-buffered saline instead for the control group. On day 28, the mice of these two groups were challenged by mite and mugwort extracts for 7 consecutive days and control group mice were administrated phosphate-buffered saline. We observed and recorded the symptoms of asthma in each group. Finally, the serum and lung tissues were extracted for the experiment.

Statistical analysis

All data were analyzed with PRISM 6.0 (GraphPad, Inc.). Group comparison and χ^2 test were performed to analyze the results. Statistical significance was defined as $p < 0.05$.

Results

The cytokine of asthma, rhinitis, & asthma combined with rhinitis & normal person

As everyone knows, many diseases will lead to changes in the levels of IL-4, IL-31, IL-33 and TSLP content; we wonder whether there is any difference between these cytokines in asthma patients and normal person. ELISA and real-time PCR were performed after we got peripheral blood and serum from patients with asthma and normal persons (Figure 1A & B). The results showed that for positive or negative sIgE patients, IL-4, IL-31, IL-33 and TSLP were higher than those of normal human. However, there were almost no difference between patients with sIgE(+) and sIgE(-). This means that these indicators can be used to determine whether the patient has asthma, but it cannot determine whether the patient is combined with rhinitis. In patients with rhinitis we also got the same results

Table 1. The primers of the mRNAs.

Name	Forward primer(5'→3')	Reverse primer(5'→3')
IL-4 (human)	GCACCGAGTTGACCGTAACA	CCAACGTACTCTGGTTGGCT
IL-31 (human)	CACTTGCGGAAGCTGTGTC	AGGTTGGGCCAGTGATTAGC
IL-33 (human)	GACTCCTCCGAACACAGAGC	CCCAGCTTGAAACACAAGGC
TSLP (human)	CTCTCGACTTGTGTTCCCCG	GGCGGTGGGATTGAAGGTTA
β -actin (human)	TCGTGCGTGACATTAAGGAG	ATGCCAGGGTACATGGTGGT
IL-4 (mice)	CCATATCCACGGATGCGACA	AAGCCCGAAAGAGTCTCTGC
IL-31 (mice)	GGACCGGGAAGCATTAAACCA	TCCAGTCCCTACAGGGTAGC
IL-33 (mice)	GGCCTTCTTCGTCTTCACA	CTCCAGTTGGAGGGCTTCAG
TSLP (mice)	CCCTGCACACACCTTACTC	TGACTGCCCGAACTGTCATT
β -actin (mice)	GTTGGAGCAAACATCCCCCA	CGCGACCATCCTCCTTAG

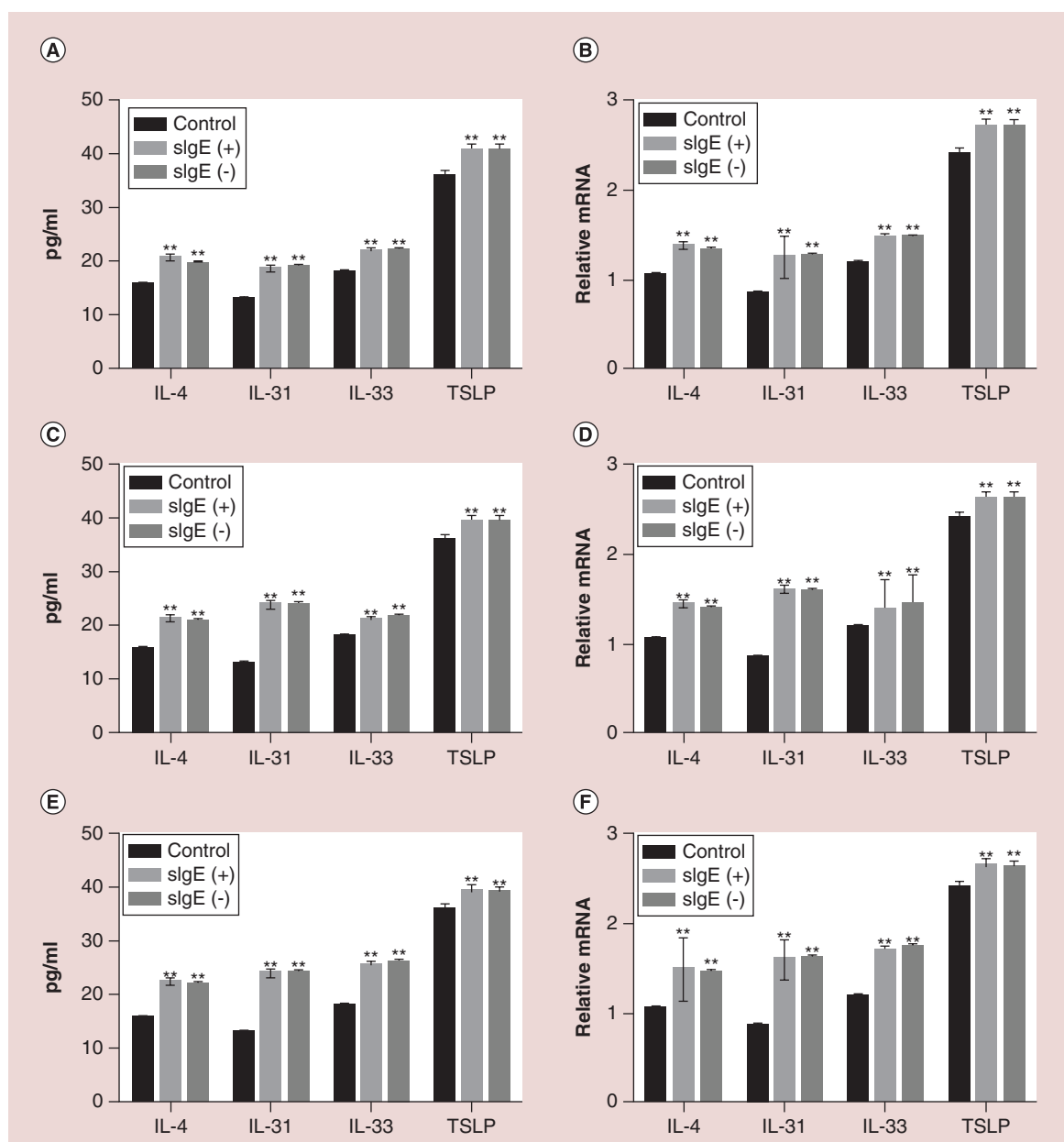


Figure 1. The cytokines of asthma, rhinitis, and asthma combined with rhinitis, and normal person. (A) IL-4, IL-31, IL-33 and TSLP in the serum of asthma patients and normal person were detected by ELISA. (B) IL-4, IL-31, IL-33 and TSLP in asthma patients and normal person were detected by real-time PCR. (C) IL-4, IL-31, IL-33 and TSLP in the serum of rhinitis patients and normal person were detected by ELISA. (D) IL-4, IL-31, IL-33 and TSLP in rhinitis patients and normal person were detected by real-time PCR. (E) IL-4, IL-31, IL-33 and TSLP in the serum of asthma combined with rhinitis patients and normal person were detected by ELISA. (F) IL-4, IL-31, IL-33 and TSLP in asthma combined with rhinitis patients and normal person were detected by real-time PCR. TSLP: Thymic stromal lymphopoietin.

through the same experiments (Figure 1C & D). For patients with asthma combined with rhinitis, we also found the same phenomenon (Figure 1E & F).

Mite & mugwort may more likely to arise IL-4, IL-31, IL-33 & TSLP in patients with asthma

Asthma is usually due to allergies, so the detection of sIgE is a very important examination for patients

with asthma. When sIgE is positive, it means that the patient is allergic to some phenomenon. At present we were interested in which allergens will be more likely to lead to the patient's disease. We analyzed the levels of cytokines in serum of patients with different allergens (Figure 2A & B). The dust mites and mugwort could cause allergy easily and promoted IL-4, IL-31, IL-33 and TSLP expressions.

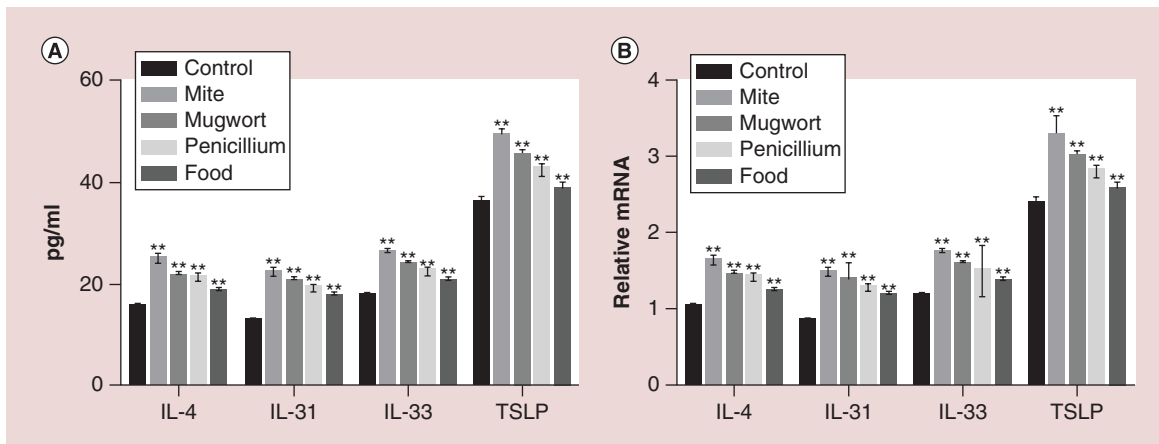


Figure 2. Mite and mugwort may be more likely to cause rises in IL-4, IL-31, IL-33 and TSLP in patients with asthma. (A) IL-4, IL-31, IL-33 and TSLP in the serum of rhinitis patients (slgE is positive) and normal person were detected by ELISA. (B) IL-4, IL-31, IL-33 and TSLP in rhinitis patients and normal person were detected by real-time PCR. TSLP: Thymic stromal lymphopoietin.

Mite & mugwort may more likely to arise IL-4, IL-31, IL-33 & TSLP in patients with rhinitis

Like asthma, allergies are a common cause of rhinitis. We analyzed the levels of cytokines in serum of patients with different allergens (Figure 3A & B). The dust mites and mugwort could cause allergy easily and promoted IL-4, IL-31, IL-33 and TSLP expressions.

In vivo experiments confirmed that dust mites & mugwort can result in elevated cytokines

To study whether mites and mugwort are responsible for allergy in patients, a mice model was constructed. After the successful construction of the mice allergic model, we took the serum and lung tissue of mice as the research object. Results showed that IL-4, IL-31, IL-33 and TSLP significantly increased in the serum of allergic mice (Figure 4A & B). Meanwhile, western blot and real-time PCR were showed that IL-4, IL-31, IL-33 and

TSLP significantly increased in the lung of allergic mice (Figure 4C & D).

Discussion

IL-31 is a newly discovered cytokine, which is mainly composed of activated Th2 cells. The receptors are composed of two elements – IL-31R and IL-31R beta recombinant (OSMR). The study showed that the expression of IL-31 receptor was increased in the allergic animal model [8]. IL-33 as a new member of the IL-1 family has a variety of immune effects, mainly involved in Th2 cell-mediated immune response and regulates mast cell function and the balance between Th1/Th2 responses. IL-33 has been found to be involved in the occurrence of a variety of chronic inflammations and autoimmune diseases. It can promote the innate immune response; it can also promote the adaptive immune response. Nowadays,

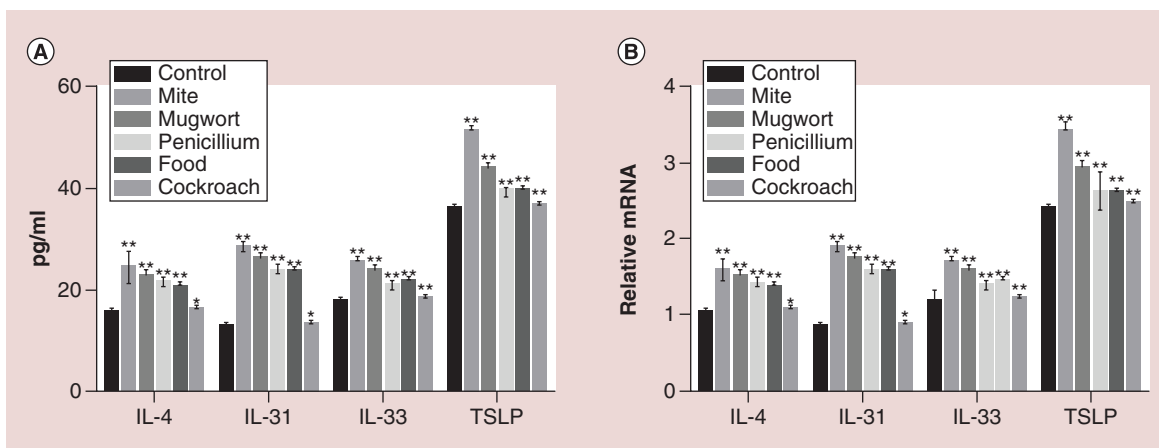


Figure 3. Mite and mugwort may be more likely to cause rises in IL-4, IL-31, IL-33 and TSLP in patients with rhinitis. (A) IL-4, IL-31, IL-33 and TSLP in the serum of rhinitis patients (slgE is positive) and normal person were detected by ELISA. (B) IL-4, IL-31, IL-33 and TSLP in rhinitis patients and normal person were detected by real-time PCR. TSLP: Thymic stromal lymphopoietin.

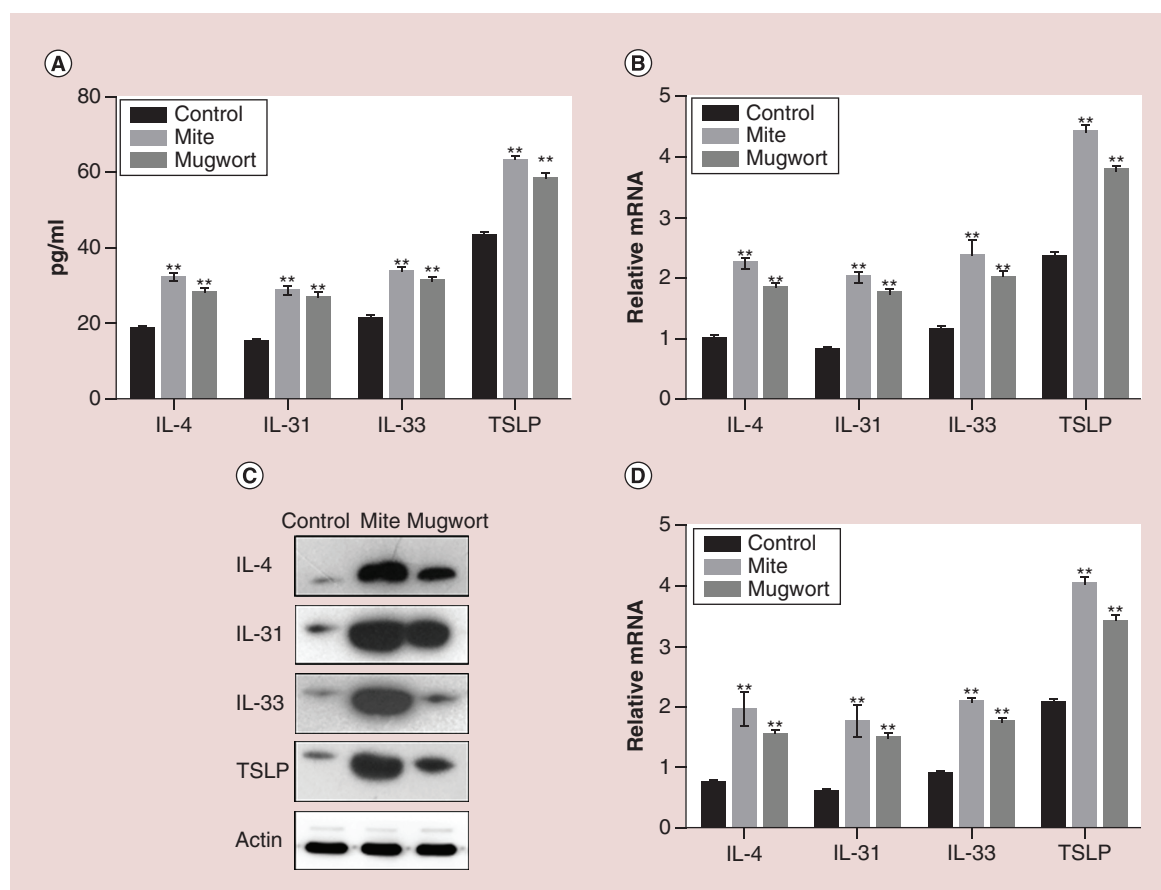


Figure 4. In vivo experiments confirmed that dust mites and mugwort can result in elevated cytokines. (A) After the successful construction of the mice allergic model for mites and mugwort, the serum of the mice was taken to perform ELISA. **(B)** After the successful construction of the mice allergic model for mites and mugwort, the peripheral blood of the mice was taken to do the real-time PCR. **(C)** After the successful construction of mice allergic model for mites and mugwort the lung tissue of the mice was taken to perform the ELISA. **(D)** After the successful construction of mice allergic model for mites and mugwort, the lung tissue of the mice was taken to do the real-time PCR.

TSLP: Thymic stromal lymphopoietin.

mainly the study of IL-33 focused on its function in inflammatory and autoimmune diseases [9]. IL-33 is associated with a typical Th2 type inflammatory disease. Increased expressions of IL-33, ST2 and sST2 in lung tissue were found in asthma and allergic airway inflammation induced by chicken egg albumin of mouse models. IL-33 might induce a series of autoimmune diseases, such as bronchial asthma, systemic lupus erythematosus and allergic shock, so as to accelerate the production of inflammatory cytokines in Th2 cells. In mast cells and natural killer cells, IL-33 can promote the production of inflammatory cytokines and Th2-related factors and chemokine [10]. IL-33 has been found to be widely expressed in epithelial cells of mice and human in recent years. Studies showed that IL-33 and its ligand binding can promote mast cells and IL-4, IL-5, IL-13 and other cytokine levels of cell secretion, and then promote the

inflammatory reaction [11]. IL-33 and ST2 receptor binding in the signal transduction pathway induced an immune response in the body; they played an important role in various diseases of mucosal immunity and allergic immune response to a type of disease [11–13]. However, the roles of IL-31 and IL-33 in asthma and rhinitis were not shown clearly yet.

TSLP was a signal molecule secreted by the damaged skin. As IL-4, TSLP could promote the differentiation and proliferation of B and T cells [14]. Recent studies have found that TSLP might be an initial factor in allergic disease, due to its impact on early allergic inflammation, induced Th2-type allergic inflammation, and then promote the expression of proinflammatory cytokines IL-4, IL-5, IL-13 and TNF- α [15].

In our study, we first found that the levels of IL-4, IL-31, IL-33 and TSLP in patients with asthma, rhinitis or asthma combined with rhinitis were

generally higher than those in normal subjects. It is believed that asthma or rhinitis are often due to allergies, so we were curious as to whether allergic reaction caused by different substances would lead to a difference in the content of these cytokines. We analyzed the levels of these cytokines in the serum of allergic patients. Results showed that the levels of IL-4, IL-31, IL-33 and TSLP in the serum of patients with mites or mugwort allergy were much higher than in those with allergies due to other factors. We then used mice to establish the mites and mugwort allergic models; through observation of the content of cytokines in serum and lung tissue we confirmed our conjectures.

In a summary, we found that there were high levels of IL-4, IL-31, IL-33 and TSLP in asthma or rhinitis patients, and mites and mugwort were more likely to lead to the high expression of IL-4, IL-31, IL-33 and

TSLP. This finding may bring a new theoretical foundation to clinical diagnosis and treatment of asthma, rhinitis or allergic patients.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Animal study received China Medical University Animal Care and Use Committee approval.

Executive summary

- This paper analyzes the role of IL-4, IL-31, IL-33 and thymic stromal lymphopoietin (TSLP) in the pathogenesis of asthma and rhinitis by testing their levels in the patients with asthma, rhinitis, asthma and rhinitis and healthy people.
- Furthermore, through animal experiments, it is confirmed that dust mites and mugwort allergies are more likely to induce these cytokines.
- We found that IL-4, IL-31, IL-33 and TSLP may be involved in the pathogenesis of asthma and rhinitis.
- Dust mite and mugwort allergy could increase IL-4, IL-31, IL-33 and TSLP significantly.
- We provide new pharmacological strategies for the therapies in asthma and/or rhinitis.

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