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Regulatory Effect of Xiaoqinglong Decoction on Thymic Stromal Lymphopoietin (TSLP) Inflammation Promoter in Mice with Cold Asthma

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ABSTRACT

Allergic asthma is a complex and chronic inflammatory airway disease. The thymic stromal lymphopoietin (TSLP) signaling pathway plays an important role in asthma. Xiaoqinglong Decoction (XQL) is the first choice to treat cold asthma in clinical settings. In this study, the role of the TSLP pathway in the onset of asthma and the protective mechanism of XQL were investigated.

A total of 50 female mice were randomly divided into the following groups: the blank group (A), the model group (B), the XQL group (C), the dexamethasone group (D), and the XQL + dexamethasone group (E). Asthma was induced with ovalbumin, and corresponding drug intervention was carried out for 7 days, after which serum and lung tissue end points were analyzed.

Serum interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α), nuclear factor ϰB (NF-ϰB), and TSLP levels were higher in group B than in group A ($p<0.05$). However these levels were lower in group C and D than in group B ($p<0.05$), and there was no significant difference between groups C and D ($p>0.05$). Interestingly, these end points were significantly lower in group E than in groups C and D ($p<0.05$). Regarding pathologic changes, the inflammatory infiltrate in the lungs of groups C, D, and E was lower than that of group B, especially in group E.

We conclude that the TSLP pathway plays an important role in the course of asthma, and can be used as an important target for asthma treatment; XQL may play a role in reducing inflammation and relieving asthma by regulating the TSLP signaling pathway.

Keywords: Asthma; Inflammation; Mice; Thymic stromal lymphopoietin; Xiaoqinglong decoction

INTRODUCTION

The status of bronchial asthma in juvenile respiratory diseases has increased annually, and the Global Initiative for Asthma (GINA) reports that the prevalence of asthma in children has increased significantly,[1] especially in young children. Recently, the third national pediatric asthma epidemiological survey in China showed that,

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in China, the prevalence of asthma in children is rising. The current 2-year prevalence in urban 0–14-year-old children ranges from 0.42–5.73%, with an average of 2.32%; the accumulative prevalence rate is 0.48–7.57%, with an average of 3.02%.[2] Currently, the GINA recommends inhalation therapy as the preferred treatment protocol for asthma in children, and inhaled corticosteroids and receptor agonists are currently the first-line drugs. However, inhaled corticosteroids cannot reverse airway remodeling, and some patients may be insensitive to hormones due to a polymorphism in the glucocorticoid receptor (GR) gene.[3] Thus, the promotion and application of GINA programs in China are not satisfactory, and the utilization rate of inhaled hormones in Chinese children with asthma is only 58.7%[2]. The advantages of traditional Chinese medicine in treating asthma have received more attention in recent years, and Chinese and foreign scholars have performed in-depth studies about the mechanism of Xiaoqinglong Decoction (XQL) in treating asthma.[4,5] Our previous studies on XQL have investigated its role in the biology of matrix metalloproteinase 9 (MMP-9), IL-13, nitric oxide (NO), and nitric oxide synthase (NOS). Animal experiments and clinical studies have shown that XQL can effectively reduce inflammatory factors involved in the pathogenesis of asthma. However, the targets of XQL in asthma, and whether it can play an anti-inflammatory role in the Thymic stromal lymphopoietin (TSLP) signaling pathway (TNF-α-NF-κB-TSLP-Th2-TNF-α), are unknown.

XQL decoction is the first choice to treat cold asthma, but not hot asthma, and its curative effect is clinically significant. However, the syndrome theory of asthma according to traditional Chinese medicine has always been ignored according to a previous report.[6] In this study, we first established a cold asthma mouse model, and then investigated the effects of XQL. We evaluated the serum levels of interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α), nuclear factor κB (NF-κB), and TSLP, the expression of NF-κB and TSLP in the lung tissues, as well as the pathological changes in lung tissues to investigate the effects of XQL in the pathogenesis of cold asthma.

MATERIALS AND METHODS

Animals and Grouping

A total of 50 specific pathogen free (SPF) grade female BALB/c mice, aged 6–8 weeks and weighing 20–22 g, were obtained from the Henan Provincial Experimental Animal Center, license No. SCXK (Yu) 2010-0002. The 50 mice were divided into groups based on a random-digits table. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Henan University of Traditional Chinese Medicine.

Establishment of Animal Models

As recorded by Justice JP and other studies,[7-9] on the first day of the experiment, the freshly prepared ovalbumin (Sigma Aldrich, St. Louis, MO, USA) (OVA) sensitization suspension was injected into mice in groups B, C, D, and E, whereas group A received 0.9% sodium chloride solution. In each mouse, 10 sites (both posterior foot metatarsi, bilateral groins, bilateral waist sides, bilateral back sides, and bilateral neck sides) were selected for subcutaneous injection of 0.05 mL of the suspension at each site, as well as intraperitoneal (ip) injection of 0.5 mL of the suspension. This was repeated on days 7 and 14. On Day 21, groups B, C, D, and E were treated with 0.4 mg/mL OVA via inhalation for 10 min (0.5 mL/min), once a day for 7 days for excitation (Figure 1).

Successful model establishment was determined by signs of irritability and increased activity, followed by decreased mobility, stooping, shortness of breath, and ruffled fur; chest shrinking, neck craning, abdomen adduction, and urinary and fecal incontinence in mice After mice were sensitized and asthma was stimulated using OVA, the mice in groups B, C, D, and E were placed in an environment with the temperature ranging from -4 to 4°C, with cold wind blowing (AC) twice a day for 40 minutes for 7 consecutive days. Manifestations such as panting-based aversion to cold, clustering, decreased ambulation, cold limbs, hunching, ruffled fur, sneezing, clear nasal discharge, or pale lips indicated success in establishing the model.

Drug Administration

Groups A and B received saline. Group C was orally administered the Xiaoqinglong Decoction (Prescription: Ephedra 9 g; Cassia twig 9 g; Peony root 9 g; Asarum 6 g; Glycyrrhiza 6 g; Dried ginger 6g; Pinellia 9 g; Schisandra chinensis 6 g. Pharmacy of First Affiliated Hospital of Henan University of TCM, Zhengzhou,

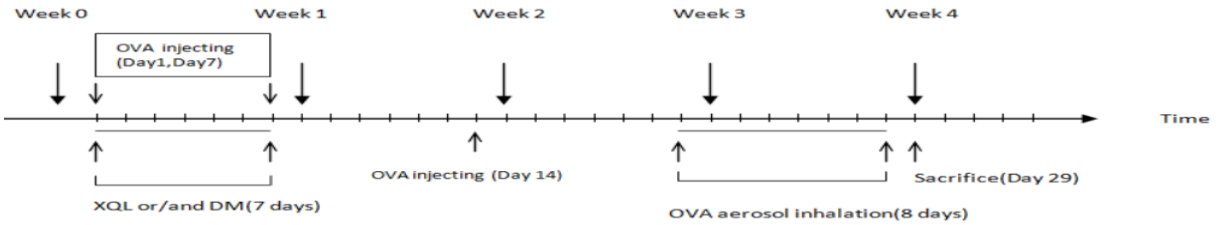

Figure 1. Experimental flow and key time points for the administration. Day 1, Day 7 and Day 14: Groups B, C, D, and E mice were injected with OVA sensitization suspension. From day 1 to day 7, XQL or and dexamethasone were administered to C, D, and E groups for 7 days. From day 21 to day 28, B, C, D, and E mice were provided inhalation of OVA for 10 min (amount 0.5 mL/min), once a day for 8 days for excitation. The mice were sacrificed on day 29.
B: the model group, C: XQL group, D: the Dexamethasone group, E: XQL+dexamethasone group, XQL: Xiaoqinglong, OVA: ovalbumin.

China) from day 1 then once a day for 7 consecutive days (dose conversion as 9.1, according to the normal human dose of 0.9 g/100 g each time). Group D received dexamethasone (Rongsheng Pharmaceutical Co. Ltd, Jiaozuo, China) via ip injection from day 1 of excitation (3 mg/kg/d) for 7 d. Group E was orally administrated the Xiaoqinglong Decoction from day 1 and then once a day for 7 d (dose conversion as 9.1 according to the normal human dose, 0.9 g/100 g each time) together with dexamethasone (ip, 3 mg/kg/d) for 7 consecutive days. The dosages were calculated according to the following formula: "D2 = D1 × K2/K1", in which D2 is the required dose, D1 is the known dose (61 g/d), and K1 and K2 are factors provided in “Experimental animals and animal experimental techniques” by Mingsan Miao).

Specimen Preparation

All mice were killed by using 1% pentobarbital sodium (ip, 30 mg/kg) or cervical dislocation 12 h after the final excitation. Whole blood was collected from eyeballs, centrifuged in a common test tube, and the supernatant was separated and stored at -70°C until use. Lung tissue was collected after whole blood collection. Mice were killed by cervical dislocation, an incision was made in the chest, and lung tissue was removed and divided into two parts: one part was placed in saline to prepare the tissue homogenate, and the other part was fixed in 10% neutral formalin to prepare slides for histopathological analysis.

Tissue homogenates were prepared by rinsing 0.2–1 g of tissue in ice-cold saline followed by addition of a certain amount of pre-cooled homogenate media (pH 7.4; 0.01 mol/L Tris-HCL, 0.0001 MOL/L EDTA-2Na, 0.01 mol/L sucrose, and 0.8% sodium chloride solution) or 0.86% cold physiological saline (ratio 1:9). Tissue samples were homogenized in a tissue pulping machine, centrifuged, and the supernatant was removed and stored at -70°C.

Histopathological Observation

After fixation for 24 h, the mouse lung tissue was processed by gradient dehydration and paraffin embedding. The paraffin section was stored overnight at 4°C, sectioned into 5-μm slices, fully developed, and roasted for 3 h, followed by hematoxylin and eosin (HE) staining, for morphologic observation.

Enzyme Linked Immunosorbent Assay (ELISA)

Serum IL-1β, TNF-α, NF-κB, and TSLP levels were determined using ELISA kits. Briefly, for TNF-α detection, microtiter wells were coated with purified mouse TNF-α antibody to obtain a solid-phase antibody screen. A sample or TNF-α was added to the wells followed by an HRP-labelled antibody against TNF-α to generate an antibody-antigen-enzyme-antibody complex. After washing, the TMB substrate solution was added, leading to the formation of blue color, catalyzed by the HRP enzyme. The reaction was terminated by the addition of sulfuric acid. The color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of porcine TNF-α in the samples was determined by comparing the optical density (OD) of the samples to the standard curve values. Serum IL-1β, NF-κB, and TSLP levels were determined similarly.

Western Blot

TSLP and NF-κB were detected in lung tissue by western blot. Total protein concentrates were extracted from the protein lysate with 1% ethylphenyl polyethylene glycol, 0.5% deoxycholate, and 0.1% sodium dodecylsulfate in PBS, and separated by 10% SDS polyacrylamide gel electrophoresis. The products were transferred onto nitrocellulose membrane and blocked in 5% skim milk at 4°C overnight, followed by the primary antibody for 2 h with gentle shaking. The membrane was washed in TBS-Tween 3 times, incubated with secondary antibody with gentle shaking for 2 h, and then washed in TBS-Tween 3 times. After washing, the membrane was exposed and developed to X-ray films using luminescence agents.

Statistical Analysis

SPSS 19.0 (IBM; Armonk, NY, USA) was used for the data processing, and all the data were used ($\bar{x}$±s). The data were first analyzed by the homogeneity test of variance using one-way ANOVA, followed by pairwise comparison of multiple samples using the SNK method or the Dunnett's T3 method for comparison. The test level was a=0.05, and p<0.05 indicated statistical significance.

RESULTS

Effect on the Histopathology of Lung Tissue

Histopathologic evaluation of paraffin-embedded lung sections was performed by light microscopy (200X). Group A exhibited intact alveolar wall structure, congestion-free blood vessels, and no significant inflammatory cell infiltration; Group B exhibited significantly more inflammatory infiltration in the lung tissue and bronchial sub-membrane, the lung tissue exhibited obvious congestion, proliferation of a large number of goblet cells, and significantly thickened tracheal wall. The vessels exhibited obvious congestion and edema, with shedding of bronchial cilia, accompanied by adhesion, lodging, or necrosis, indicating successful model development. Groups C, D, and E exhibited alleviated inflammatory infiltration, with the most obvious improvement in Group E (Figure 2).

Serum Levels of IL-1β, TNF-α, NF-κB, and TSLP

The serum levels of IL-1β, TNF-α, NF-κB, and TSLP in group B were significantly higher (p<0.05) than those of Group A. Groups C, D, and E showed significantly lower levels of IL-1β, TNF-α, NF-κB, and TSLP (p<0.05) than Group B. There was no significant difference in the levels of IL-1β, TNF-α, NF-κB, and TSLP between Groups C and D (p>0.05), but the levels of these indexes in Group E were significantly lower than those of Groups C and D (p<0.05) (Table 1).

Expression of TSLP and NF-κB in Lung Tissue

The levels of TSLP and NF-κB proteins in Group B were significantly higher (p<0.05) than those of Group A. The expression of TSLP and NF-κB was significantly lower in Group C and E than that in Group B (p<0.05). There was no significant difference in the expression of TSLP and NF-κB between Groups C and D (p>0.05).

These data indicate that XQL and dexamethasone can decrease the expression of TSLP and NF-κB proteins, which are elevated in the lung tissue of mice with cold asthma (Table 2, Figure 3).

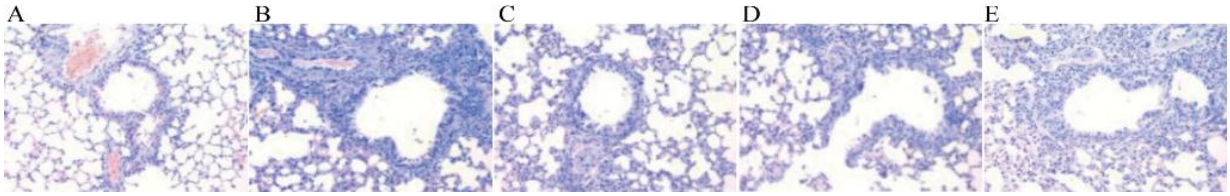

Figure 2. Lung morphology of each group (two hundredfold amplification).

A) Blank group: intact alveolar wall structure, congestion-free blood vessels, and no significant inflammatory cell infiltration. B) Model group: significant inflammatory infiltration in the lung tissue and bronchial submembrane; the lung tissue exhibited obvious congestion, proliferation of a large number of goblet cells, and significantly thickened tracheal wall. The vessels exhibited obvious congestion and edema, and the bronchial cilia shed a lot, accompanied by adhesion, lodging, or necrosis.

C) Xiaoqinglong decoction group, D) dexamethasone group, and E) Xiaoqinglong decoction + dexamethasone group: the pathological changes as shown in group B were alleviated. This result was especially obvious in Group E.

Table 1. The expressions of TSLP, TNF-α, IL-1β and NF-κB in serum of each group ($\bar{x}$±s)

Group	Cases(n)	TSLP(pg/ml)	TNF-α (pg/ml)	IL-1β (pg/ml)	NF-κB (ng/ml)
A	9	41.51±5.97	17.52±6.04	50.65±6.28	8.23±1.93
B	9	171.22±9.58*	70.48±6.31*	182.58±10.91*	30.32±1.98*
C	9	72.46±6.79**	35.91±6.84**	76.13±4.17**	16.36±1.75**
D	9	71.36±6.67***	35.94±6.97***	80.51±5.89***	16.45±1.83***
E	10	42.08±4.79****	18.92±6.41****	51.47±5.42****	8.34±2.04****

Note: Compared with Group A, $^{*}p$<0.05; compared with Group B, $^{**}p$<0.05, $^{***}p$<0.05, $^{****}p$<0.05; compared group C and D p>0.05.

Table 2. The protein expression of TSLP and NF-κB in the lung in all groups (x±s)

Group	Cases(n)	TSLP/β-action	NF-κB/β-action
A	9	0.10±0.05	0.07±0.05
B	9	0.40±0.08*	0.35±0.09*
C	9	0.21±0.08**	0.15±0.05**
D	9	0.22±0.09***	0.18±0.05***
E	10	0.19±0.06****	0.16±0.09****

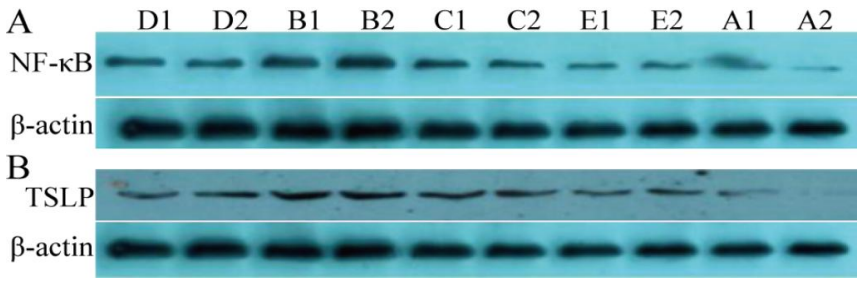

Figure 3. Expressions of NF-κB and thymic stromal lymphopoietin (TSLP) proteins in different groups (western blot).
A: Five groups of mice lung tissue NF-κB western blot protein bands. B: Five groups of mice lung tissue TSLP western blot protein bands.
A1, A2: blank group, B1, B2: model group, C1, C2: Xiaoqinglong decoction group,
D1, D2: dexamethasone group, E1, E2: Xiaoqinglong decoction+dexamethasone group. Compared with Group A, the expressions of TSLP and NF-κB proteins in Group B were significantly increased (p<0.05). Compared with Group B, the expressions of TSLP and NF-κB proteins in Group C and D were significantly reduced (p<0.05). There was no significant difference in the expression of TSLP and NF-κB between Group C and D (p>0.05).

DISCUSSION

Bronchial asthma is a common allergic disease in children. The incidence of asthma has increased in recent years, and therefore it has received significant attention from clinicians and investigators.[9-11] TSLP promotes asthmatic airway remodeling by interacting with various structural cells, such as airway epithelial cells, fibroblasts, or airway smooth muscle cells,[12,13] and has recently become a hot topic of research because it might be an important therapeutic target for the treatment asthma.[14-16] Chen et al[17] found that antagonizing TSLP can significantly reduce airway inflammation and inhibit or even reverse the pathological changes of asthmatic airway structure, suggesting that it is a primary mediator of airway inflammation and airway remodeling. Clinical studies[18] also have confirmed that TSLP is increased in the airways of asthmatic patients, and glucocorticoids can block TSLP receptors, thus reducing allergic

inflammation. The data presented in this study also showed that the expression of TSLP in Group B was higher than that in Group A.

The expression of TSLP in human respiratory epithelial cells is influenced by proinflammatory cytokines such as IL-1β and TNF-α. TSLP then binds to its receptor on the cell surface via the regulation of NF-κB,[19] thus stimulating inflammation and activating dendritic cells (DCs). The TSLP-activated DCs (TSLP-DCs) stimulate the conversion of CD4+ T-cell precursors to Th2 cells; IL-4, IL-5, IL-13, and IL-10 produced by normal Th2 cells do not normally promote the incidence of allergic diseases. However, the Th2 cells stimulated by TSLP-DCs can produce classical Th2 cytokines, including IL-4, IL-5, IL-13, and a large amount of TNF, which can then activate the TSLP gene promoter via the NF-κB pathway.[20,21] This cyclic amplification effect can then form an information-transmission loop, the TSLP signaling loop (TNF-α-NF-κB-TSLP-Th2-TNF-α), resulting in allergic diseases (Figure 4).

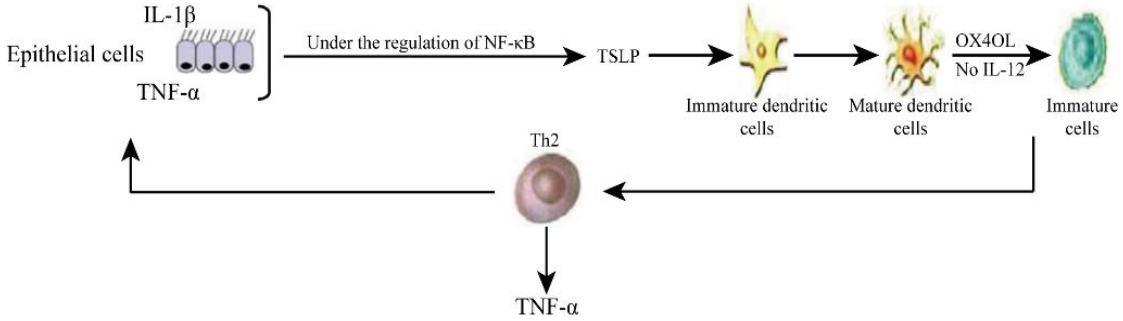

Figure 4. Simplified cartoon to indicate the thymic stromal lymphopoietin (TSLP) signaling loop (TNF-α-NF-κB–TSLP-Th2-TNF-α). The expression of TSLP in human respiratory epithelial cells is influenced by proinflammatory cytokines such as interleukin1β (IL-1β) and tumor necrosis factor-α (TNF-α), and the resulted TSLP then binds to the receptor on the cell surface via the regulation of nuclear factor κB (NF-κB), thus playing its inflammatory effect and activating dendritic cells (DCs). The TSLP-activated DCs (TSLP-DCs) can stimulate the conversion of CD4+ T cell precursors toward Th2. The TSLP-DCs-medicated Th2 cells can produce classical Th2 cytokines, including TNF, and such cytokines as TNF-α can activate the TSLP promoter gene via the NF-κB pathway.

XQL is a classical prescription against cold asthma in Zhang Zhongjing's "Treatise on Cold Pathogenic and Miscellaneous Diseases," exhibiting effects such as warming the lungs, dissipating excessive fluid, and relieving cough and asthma. This prescription is composed of *Herba Ephedrae*, *Ramulus Cinnamomi*, *Radix Paeoniae Alba*, *Asurum uropeum*, *Radix et Rhizoma*, *Rhizoma Zingiberis*, *Pinellia Ternata*, and *Schisandra Chinensis*. These pungent and warm-natured drugs mainly target diffusing and dispersing cold fluid retention, as well as assist in reducing the secretion of gastric acid. Researchers in China and abroad have extensively studied its role in airway inflammation, immunity, allergy, and airway remodeling, but there is currently no research on its effect on the TSLP signaling pathway. This study utilized the mouse model of cold asthma, and showed that the levels of IL-1β, TNF-α, NF-κB, and TSLP in the serum and the expression of TSLP and NF-κB in the lung were elevated in mice with cold asthma (Group B) compared to those of untreated mice in Group A. These data indicate that the cytokines in the TSLP pathway are upregulated in cold asthma and suggest that this loop plays an important role in the onset of asthma. XQL treatment (Group C) significantly reduced the levels of IL-1β, TNF-α, NF-κB, and TSLP in the serum and the expression of TSLP and NF-κB in the lung tissue. Glucocorticoids are also considered classic drugs for the treatment of asthma. This study showed that dexamethasone (Group D) reduced the levels of IL-1β, TNF-α, NF-κB, and TSLP in the serum and the expression of TSLP and NF-κB in the lung tissue of mice with cold asthma, suggesting that XQL and dexamethasone can both regulate the TSLP pathway, and their roles are identical, because they reduced the cytokines to similar levels.

Furthermore, the combination of XQL and dexamethasone on the TSLP transmission loop had an additive effect compared to either treatment alone. Both XQL and dexamethasone attenuated the inflammatory infiltrate in the lungs, and the combined treatment caused the most obvious reduction, suggesting that the combination of these two drugs has the best anti-inflammatory and protective effect against histopathological changes associated with asthma.

In summary, this study confirmed that the TSLP signaling pathway plays an important role in the pathogenesis of asthma, and it can be an important target for asthma therapy. As a representative prescription against cold asthma, XQL can play a role in reducing inflammation and asthma via regulation of the TSLP signaling pathway.

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