



Xiao-Qing-Long-Tang attenuates allergic airway inflammation and remodeling in repetitive *Dermatogoides pteronyssinus* challenged chronic asthmatic mice model

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ABSTRACT

Ethnopharmacological relevance: Xiao-Qing-Long-Tang (XQLT) has been used for centuries in Asia to effectively treat patients with bronchial asthma.

Aim of the study: We previously found that single and multiple doses of XQLT administered to sensitized mice before allergen challenge resulted in suppressed airway hyper-responsiveness and airway inflammation. In this study we aimed to investigate whether XQLT has the potential to attenuate the severity of asthma symptoms, and immunomodulatory mechanism of XQLT in a repetitive *Dermatogoides pteronyssinus* (*D. pteronyssinus*)-challenged chronic asthmatic mice model.

Materials and methods: BALB/c mice were intratracheally (i.t.) inoculated with five doses of *D. pteronyssinus* (50 µl, 1 mg/ml) and orally administered of XQLT (1 g/kg) at 1-week intervals. At three days after the last challenge, mice were sacrificed to evaluate airway remodeling, inflammation, lung histological features, and the expression profiles of cytokines and various genes.

Results: XQLT significantly reduced bronchial inflammatory cell infiltration and airway remodeling. It inhibited *D. pteronyssinus*-induced total IgE and *D. pteronyssinus*-specific IgG1 in serum, and changed the “T_H2-bios” in BALF by inhibiting the activation of NF-κB. Collagen assay and Histopathology indicated that XQLT reduced airway remodeling in the lung. Simultaneously, the RT-PCR analysis showed that XQLT downregulated IL-10, IL-13, RANTES, Eotaxin, and MCP-1 mRNA expression in the lung. Moreover, EMSA and immunohistochemistry staining demonstrated that XQLT inhibited NF-κB expression in the nucleus of bronchial epithelial cells.

Conclusions: These results suggest that XQLT exhibits anti-airway inflammatory, anti-airway remodeling, and specific immunoregulatory effects in a chronic asthmatic mice model.

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1. Introduction

Asthma is an inflammatory disease of the airway characterized by airway eosinophilia, goblet cell hyperplasia with mucus hypersecretion, collagen deposition, smooth muscle cell hypertrophy, subepithelial thickening, and hyper-responsiveness

(airway remodeling) in response to inhaled allergens and non-specific stimuli (Hamid et al., 2009). The prevalence of the disease is increasing, and its seriousness has become greater in recent years (Hammad and Lambrecht, 2011).

Patients with chronic asthma who take antiasthmatic drugs (including corticosteroid) for a long and indefinite time may develop serious side effects (Diamant et al., 2007). Xiao-Qing-Long-Tang (XQLT) contains eight herbal complexes and has been commonly used for the clinical treatment of bronchial asthma and allergic rhinitis in Asia (Kung et al., 2006; Nagai et al., 2009). Recently, investigators demonstrated that XQLT improved the quality of life and reduced the symptoms of illness in common cold patients (Byun et al., 2011). In our previous study (Kao et al.,

Abbreviations: HDM, House dust mite; i.t., intratracheal; BALF, bronchoalveolar lavage fluid (s); XQLT, Xiao-Qing-Long-Tang; TCM, Traditional Chinese Medicine

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2000), we found that there was a suppressed airway inflammation and downregulated the allergen-induced intrapulmonary lymphocyte response of sensitized mice that had been treated with XQLT before allergen challenge. These findings indicate that XQLT has immunomodulatory effects.

Activated CD4⁺ T cells, numerous T_H2 cytokines (including TNF α , IL-4, IL-5 and IL-13), IL-6, TGF- β , and chemokines (including EOTAXIN, RANTES and MCP-1) contribute to the pathogenesis of chronic allergic asthma (Barnes, 2008; Akdis et al., 2011; Mukherjee and Zhang, 2011; Katelaris et al., 2011). Moreover, expression of the genes coding for those pro-inflammatory proteins in bronchial epithelial cells is induced as a result of rapid activation of the transcriptional factor NF- κ B and is directly associated with the recruitment of eosinophils (Halwani et al., 2010). The bronchiolar epithelium is an important site of NF- κ B activity as well as expression of NF- κ B-dependent inflammatory mediators. Thus, the induction of NF- κ B by proinflammatory stimuli may be a critical signal in evoking an inflammatory response in the lung during the pathogenesis of chronic asthma (Broide, 2008).

Based on our previous study, we speculate that orally administered multiple doses of XQLT may effectively attenuate the severity of asthma symptoms in a repetitive *D. pteronyssinus*-challenged mice. The purpose of this study is to determine the long-term prophylactic effect of XQLT and to examine whether the attenuate efficacy of XQLT against asthma is associated with reduction of eosinophil infiltration and NF- κ B expression in lung within a mouse model of chronic allergic asthma, in which BALB/c mice are repeatedly challenged by intranasal exposure to house dust mite (HDM) (Yu et al., 1999). Mice used in this model exhibit several symptoms that are similar to those found in patients with chronic asthma, including high levels of immunoglobulin E (IgE) in serum, increased AHR, airway inflammation, and remodeling.

2. Materials and methods

2.1. Mice and reagents

Animal experiments were carried out in agreement with the principles outlined by the Committee of China Medical University (No. 97-93-N). Pathogen-free males, from 6 to 8-week-old BALB/c mice (Laboratory Animal Center, National Cheng Kung University, Tainan, Taiwan, Republic of China) were housed in Microisolator cages (Laboratory Products, Inc., Maywood, NJ, USA) and provided with sterile food and water ad libitum. Care and treatment of all animals followed the guidelines of National Science Council of the Republic of China. Lyophilized house dust mites (*D. pteronyssinus*) were purchased from Allergon (Engelholm, Sweden). Crude mite preparation was extracted with ether. After dialysis with deionized water, the mite extract was lyophilized and stored at -70°C until use. LPS concentration of the *D. pteronyssinus*

preparations was 1.96 EU/mg of *D. pteronyssinus* (Limulus amoebocyte lysate test; E-Toxate; Sigma-Aldrich).

2.2. Preparation of XQLT extracts

XQLT was supplied by Koda Pharmaceuticals Ltd. (Taoyuan, Taiwan). This preparation was a mixture of eight common Chinese herbal medicines, and mixed with amount of 2502.3 g as the ratio in Table 1. Briefly, these were extracted with 17.5 L and 12.5 L of boiled water for 1 h. Poaching liquid was mixed two times and filtered. After filtration, the dregs of a decoction were removed. The filtered liquid was lyophilized, and crushed into a thin powder. The yield of dried extract from starting crude material was 661.8 g (26.4%, w/w). The batch number is 98041021. XQLT was dissolved in distilled water for administration to mice. The dose of administration used in this study was based on our previous study (Kao et al., 2000).

2.3. The HPLC analysis of standard materials to test sample

HPLC method was performed with a system consisting of Hitachi D-7000 Interface, L-7100 pump, L-7420 UV/VIS Detector, L-7200 Autosampler, Mightysil RP-18, GP 250 $\times$ 4.6 mm (5 μm) column was used. The mobile phase composition was Acetonitrile: 0.03% H₃PO₄ (15:85). Detection wavelength was set at 240 nm. 10 μl of sample was injected, and flow rate was 1.0 ml/min. Standard solutions of Paeoniflorin, Methyl paraben and Glycyrrhizin were prepared by dissolving 1.0 mg of each pure substance in 70% methanol to make stock solutions. The dissolved standard solution of Paeoniflorin and Glycyrrhizin were diluted as 0.0033, 0.0132, 0.0396, 0.0528 and 0.1848, 0.1386, 0.0462 and 0.01155 mg/ml, respectively. The 0.04202 mg/ml Methyl paraben was used as internal control. The dried aqueous-extract, 1 g of XQLT was accurately weighed, and dissolved in 10 ml of methanol, and then used an ultra-sonicator for 60 min at room temperature. The 0.5 ml Methyl paraben was added into 1 ml of XQLT filtration and additional methanol was then added to 5 ml. The solutions were filtered through 0.45 μl membrane filter and applied to HPLC. HPLC is offered by Koda Pharmaceuticals Ltd. (Fig. 1A). The quantity of standard materials in XQLT was calculated as follows: the amount (mg/g) of standard materials = measured standard materials (mg/ μl) $\times$ the HPLC injection volume (μl) of XQLT $\times$ dilution factor/the amount (g) of XQLT. From the result of standard calibration curve, R^2 values of two marker substances are 0.9998 and 1.0000. The amounts of standard materials in XQLT are indicated in Fig. 1B.

2.4. Allergen challenge and assessment of airway inflammation and blood eosinophilia

In the *D. pteronyssinus* group of BALB/c mice ($n=6$), allergic airway inflammation and remodeling were provoked by

Table 1
Components of herbal medicines in XQLT.

Botanic family	Botanical nomenclature	Pharmacognostic nomenclature	Amount (g)
(1) Ranunculaceae	<i>Paeonia lactiflora</i> Pall	Paeoniae Radix	3.0
(2) Araceae	<i>Pinellia ternate</i> Breitenbach	Pinelliae Tuber	6.0
(3) Ephedraceae	<i>Ephedra sinica</i> Stapf	Ephedrae Herba	3.0
(4) Lauraceae	<i>Cinnamomum cassia</i> Blume	Cinnamonomi Cortex	3.0
(5) Lamiaceae	<i>Schisandra chinensis</i> Baill	Schisandrae Fructus	3.0
(6) Fabaceae	<i>Glycyrrhiza uralensis</i> Fisch. et DC	Glycyrrhizae Radix	2.0
(7) Zingiberaceae	<i>Zingiber officinale</i> Roscoe	Zingiberis Rhizome	1.0
(8) Aristolochiaceae	<i>Asarum heterotropoides</i> Fr. Schmidt var. <i>mandshuricum</i> Kitag.	Asari herba cum Radice	3.0
Total amount			24.0

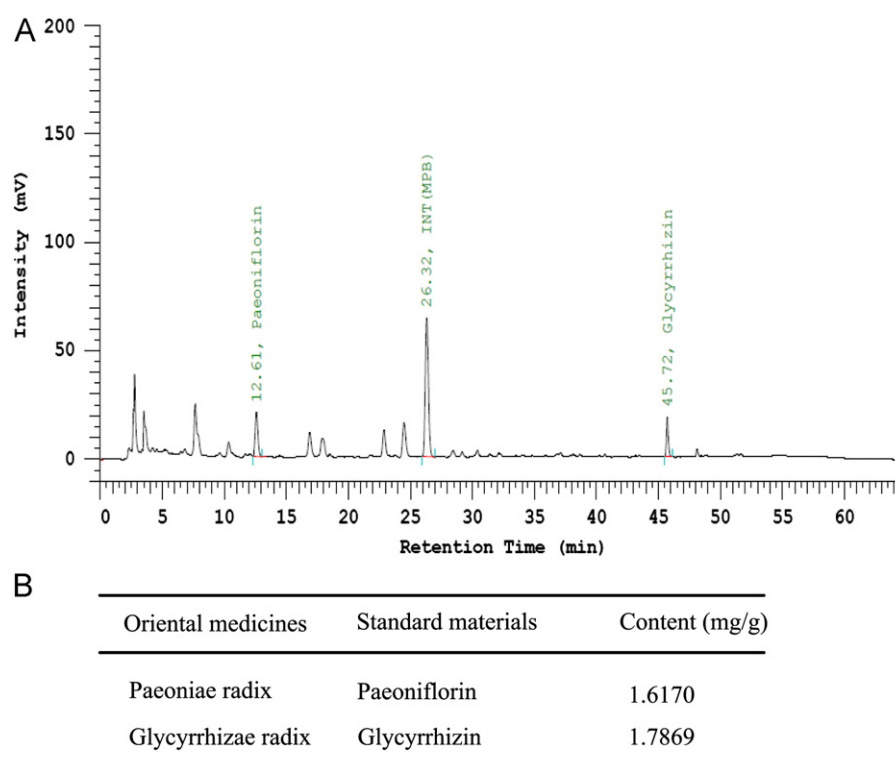


Fig. 1. (A) HPLC chromatogram of XQLT and (B) contents of standard materials in XQLT (unit: mg/XQLT ex. 1 g).

subjecting mice to intratracheally administered *D. pteronyssinus* (1 mg/ml, 50 μ l) in phosphate-buffered saline (PBS) once a week for 4 weeks (total 5 doses). Three days after the last challenge, mice were sacrificed by i.p. injection of xylazine (200 μ g/mice) and ketamine (2 mg/mice). Serum samples were collected and stored at -80°C until assay. The numbers of blood eosinophils were determined using a diagnostic reagent system (Unopette test 5877; BD Biosciences, Rutherford, NJ). BALF were performed (two washes of 1 ml of ice-cold endotoxin-free PBS) according to a previously described procedure (Yu et al., 1999). The BALF were separated (1500 rpm, 10 min, 4°C) and stored at -80°C . After total leukocyte counting, differential counts were performed on cytospin preparations (1×10^5 cells/100 μ l of BALF) stained with Liu stain (Biotech, Taiwan) in a blind manner. For TCM study, mice were orally administered XQLT (1 g/kg) at 1-week intervals with each *D. pteronyssinus* inoculation and naïve mice were also included in the experiment for purposes of comparison. All samples were evaluated in a double-blind manner.

2.5. Measurement of airway hyperresponsiveness

Airway resistance was measured by a single-chamber, whole-body plethysmograph (Buxco Electronics, Inc., Troy, NY) as described in the manual provided. Airway resistance was expressed as Penh (enhanced pause). Increasing doses of methacholine were administered by nebulization for 3 min, and Penh was measured over the subsequent 3 min.

2.6. Measurement of *D. pteronyssinus*-specific IgG1, IgG2a/2b and total IgE

An IgE-specific ELISA was used to measure the total IgE Ab levels in serum samples using matched mAb pairs (BD PharMingen) according to the manufacturer's instructions. A450 readings

of the samples were converted to nanograms per milliliter using a standard curve generated with double dilutions of mouse IgE κ isotype standard (BD PharMingen). For *D. pteronyssinus*-specific antibodies, serum samples were added in duplicate onto ELISA plates coated with *D. pteronyssinus* (2 μ g/ml in 0.1 M NaHCO_3 , pH 8.3). After incubation overnight at 4°C , the plates were washed and incubated with biotinylated rat anti-mouse IgG1 or IgG2a/2b mAb (2 μ g/ml; BD PharMingen) for 1 h, followed by washings with PBS and the addition of streptavidin-HRP conjugate (1:1000 dilution, BD PharMingen). The plates were washed and developed with a tetramethylbenzidine microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and read at OD₄₅₀.

2.7. ELISA of cytokine levels

Concentrations of cytokines were measured by a sandwich ELISA technique. Mouse IL-5, IL-6, IL-12 and TNF- α ELISA Ready-SET-Go! (eBioscience, San Diego, CA), IL-13, INF- γ and TGF- β ELISA DuoSet! (R & D System, Abingdon, UK) were used to detect cytokine concentrations according to the manufacturer's protocol. The reaction was revealed and developed as described above (Kao et al., 2000). Standards were run in parallel with recombinant cytokines.

2.8. Histology and immunohistochemistry of lung specimens

Lungs were inflated with 0.5 ml of formalin (Sigma-Aldrich) and the trachea was tied off. Fixed tissues were embedded in paraffin, and 5- μ m sections were prepared, affixed to glass microscope slides, stained with H&E and periodic acid-Schiff (PAS) stain, and assessed under a light microscope. For immunohistochemistry analyses, cryosections were placed onto slides coated with poly-L-lysine (Sigma-Aldrich), fixed in ice-cold

acetone, and incubated with antibodies against the RelA subunit of NF- κ B (SC-372, Santa Cruz Biotechnology, Santa Cruz, CA). Slides were incubated with an HRP-conjugated goat anti-rabbit polyclonal IgG (ZYMED, San Francisco), washed, and stained using an AEC kit (ZYMED, San Francisco). The slides were then washed and counterstained with hematoxylin.

2.9. Collagen analysis

Collagen content was measured in lung tissue homogenates by a biochemical assay according to the manufacturer's instructions (Sircol collagen assay, Biocolor, Belfast, UK). Lung tissue (100 mg) was homogenized in 2 ml HBSS and centrifuged at 800 g for 10 min. The supernatant was then collected and analyzed.

2.10. Semiquantitative RT-PCR

Total RNA was extracted from total lung (RNeasy Total RNA kit; Qiagen, Hilden, Germany) and converted to cDNA with StrataScript H-reverse transcriptase (Stratagene, La Jolla, CA). PCR amplification (GeneAmp PCR System 2400; PerkinElmer, Branchburg, NJ) was performed on a 1 μ l-sample of cDNA. Amplification cycle numbers and annealing temperatures were optimized for each primer pair. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. Bands were quantified densitometrically (Bio-1D; Vilber Lourmat, Marne La Vallee, France). To control for sample-to-sample variation, the amount of cDNA input in all samples was first adjusted to comparable levels of β -actin transcripts before it was subjected to PCR amplification. The following gene-specific primer pairs (sense and anti-sense) were used: β -actin, 5'-TGGGAATCCTGTGG-CATCCATGAAA C-3' and 5'-TAAAACGAGCTCAGTAACAGTCCG-3'; IL-10, 5'-GGACTTTAAGG GTTACTTGGGTGCCC-3' and 5'-CATTTT-GATCATCATGTA TGCTTCT-3'; IL-13, 5'-CTGCAGTCTGGCTCTCG-3' and 5'-CTTTTCCGCTA TGGCCACTG-3'; RANTES, 5'-GTACATCACCA-TGGCGTATG-3' and 5'-TCTTCTCTGGGTGGCAC ACA-3'; EOTAXIN, 5'-CCATCTGTCTCCCTCC ACCATG-3' and 5'-ATCCCACATCTC CTTT-CATGCC-3'; MCP-1, 5'-ACCAGCCAACCTCT CACTGAAGC-3' and 5'-CAGA ATTGCTTGAGGTGTTGTG-3'.

2.11. Nuclear fractions and EMSAs

Nuclear fractions were prepared from whole lung exposed to *D. pteronyssinus* 72 h after the last challenge according to the manufacturer's instructions (Panomics, Redwood). A LightShift™ Chemiluminescent EMSA Kit SET (PIERCE, Rockford) was used according to the manufacturer's instructions to detect levels of NF- κ B expression.

2.12. Statistical analysis

The cellular accumulation and mediator contents in BALF and serum Ab titer were analyzed by using the Mann–Whitney *U* test. Results are expressed as means $\pm$ SD. A *p* value < 0.05 was considered to represent statistical significance.

3. Results

3.1. The effects of XQLT on *D. pteronyssinus*-induced pulmonary inflammation and airway hyperresponsiveness in a chronic asthmatic mouse model

We first used a repetitive *D. pteronyssinus* challenge experiment to test the effects of XQLT on allergen-induced chronic airway inflammation in a mouse model. BALB/c mice were

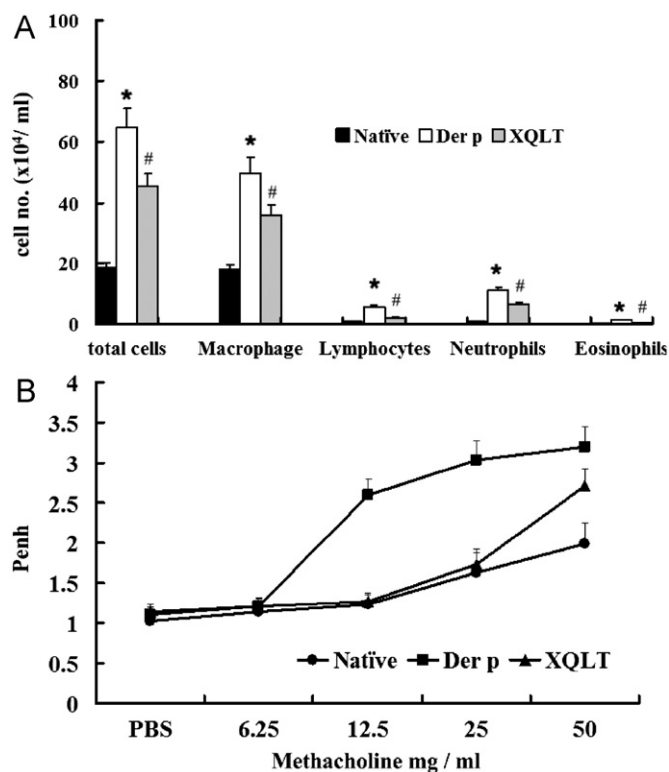


Fig. 2. Effects of XQLT on *D. pteronyssinus*-induced airway inflammatory infiltrates in BALF and airway hyperresponsiveness in repetitive *D. pteronyssinus*-challenged mice. (A) Total and differential counts of BAL cells and (B) airway hyperresponsiveness were determined at day 3 after the last challenge. Values represent the means $\pm$ SD of 6 mice. **p* < 0.05, (vs. control group); # *p* < 0.05 (between non-treated and XQLT-treated group).

intratracheally inoculated with five doses of *D. pteronyssinus* in PBS at 1-week intervals and sacrificed at 72 h after the last challenge. In naïve group mice, the majority of cells in BALF were macrophages. However, the numbers of total cells, macrophages, neutrophils, lymphocytes, and eosinophils were markedly higher in mice that had been exposed to *D. pteronyssinus* (Fig. 2A). In the XQLT group, mice were orally administered XQLT every week after intratracheal *D. pteronyssinus* challenge. The numbers of total cells, macrophages, neutrophils, lymphocytes, and eosinophils in BALF were significantly lower after allergen challenge in XQLT group mice than in untreated challenged mice (the *D. pteronyssinus* group).

To determine whether reduced inflammation was associated with suppression of AHR, we measured methacholine-induced pause (Penh) values by in-liv mice. Penh values in *D. pteronyssinus* group mice were markedly higher than those in naïve mice, indicating the induction of AHR. There was a significant decrease in Penh values at maximal dose of methacholine (50 mg/ml) in XQLT group mice relative to the *D. pteronyssinus* group (Fig. 2B).

3.2. XQLT attenuates *D. pteronyssinus*-induced lung pathology

Inflammation in airway, matrix deposition in subepithelial regions, and excessive mucus secretion from hyperplastic goblet cells are characteristic features of asthmatic airway. To clarify the efficacy of XQLT on lung cells in a chronic allergic asthma murine model, the left lungs were histologically examined 72 h after the final antigen challenge.

Histologic examination showed no evidence of pulmonary inflammation in naïve group mice, (Fig. 3A, top left panel). Conversely, widespread peribronchiolar and perivascular

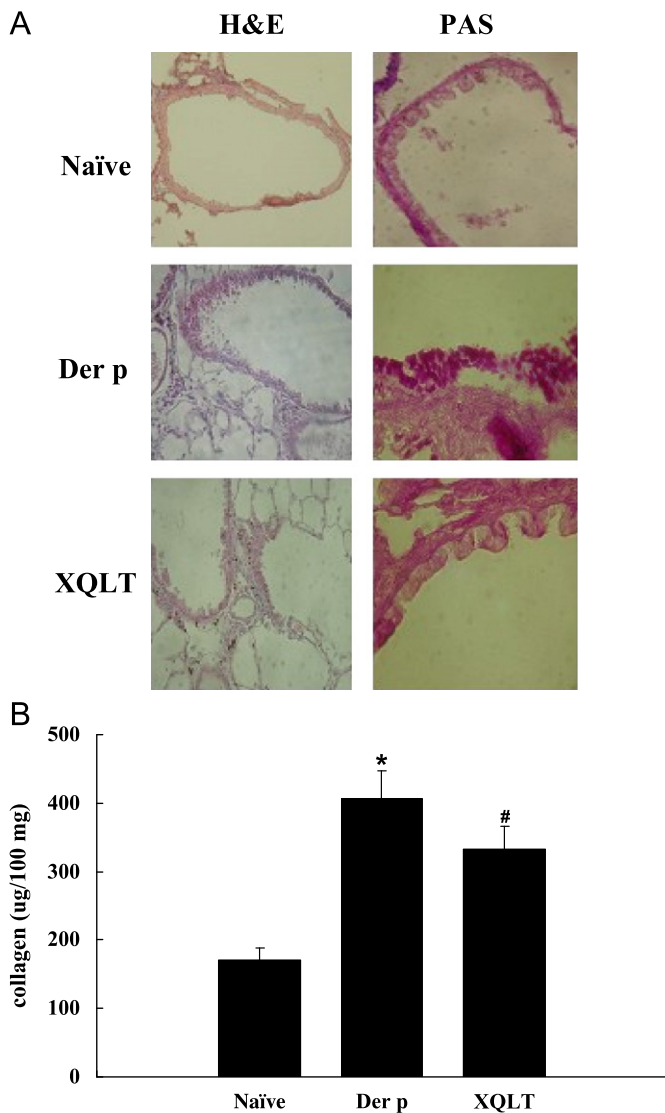


Fig. 3. XQLT suppressed repetitive *D. pteronyssinus* challenge-induced allergic airway inflammation, total collagen, and mucus in mice. (A) Histopathologic examination of the lung tissue revealed that XQLT suppressed infiltration of inflammatory cells around the blood vessels (H & E stain; original magnification, $\times 400$) and activity of goblet cells (PAS; original magnification, $\times 400$) in airway epithelial cells. Data are representative of $n=6$ /group. (B) XQLT suppressed the levels of total collagen in lung homogenate samples in repetitive *D. pteronyssinus*-challenged mice. Values represent the means $\pm$ SD of 6 mice. * $p < 0.05$, (vs. control group); # $p < 0.05$ (between non-treated and XQLT-treated groups).

infiltrates were observed in *D. pteronyssinus* group mice (Fig. 3A, middle left panel). Specimens from XQLT group mice showed significant reduction in peribronchiolar and perivascular infiltrates (Fig. 3A, bottom left panel). Moreover, Bronchial epithelial cells in *D. pteronyssinus* group mice showed extensive PAS-positive staining, indicating the presense of mucus collections (Fig. 3A, middle right panel) relative to PAS staining in naïve group mice (Fig. 3A, top right panel). Treatment of repeatedly *D. pteronyssinus*-challenged mice with XQLT resulted in abrogation of mucus production (Fig. 3A, bottom right panel).

To evaluate the levels of matrix within the lung tissue, total collagen levels were measured quantitatively using a biochemical assay. The levels of total collagen were lower in XQLT group mice than in *D. pteronyssinus* group (Fig. 3B). The above-mentioned findings suggest that XQLT protects against allergenic airway inflammation, the activation of goblet cells, and the deposition of collagen.

3.3. XQLT significantly inhibited serum total IgE and *D. pteronyssinus*-specific IgG1 but slightly enhanced *D. pteronyssinus*-specific IgG2a/2b antibodies

To evaluate the effect of XQLT on the humoral immune response status of repetitive *D. pteronyssinus*-challenged mice, sera were sampled and assayed for serum IgE, IgG1, and IgG2a/2b antibodies. A significant increase in the levels of serum total IgE was observed in the *D. pteronyssinus* group mice relative to the levels in naïve mice. Following the treatments with XQLT in the chronic *D. pteronyssinus*-challenged mice, the serum concentrations of total IgE were significantly lower than in *D. pteronyssinus* group mice. In addition, the concentrations of *D. pteronyssinus*-specific IgG1, which is normally associated with a T_H2 immune response, were markedly lower and those of *D. pteronyssinus*-specific IgG2a/2b were slightly higher in the XQLT group mice than in *D. pteronyssinus* group mice (Fig. 4).

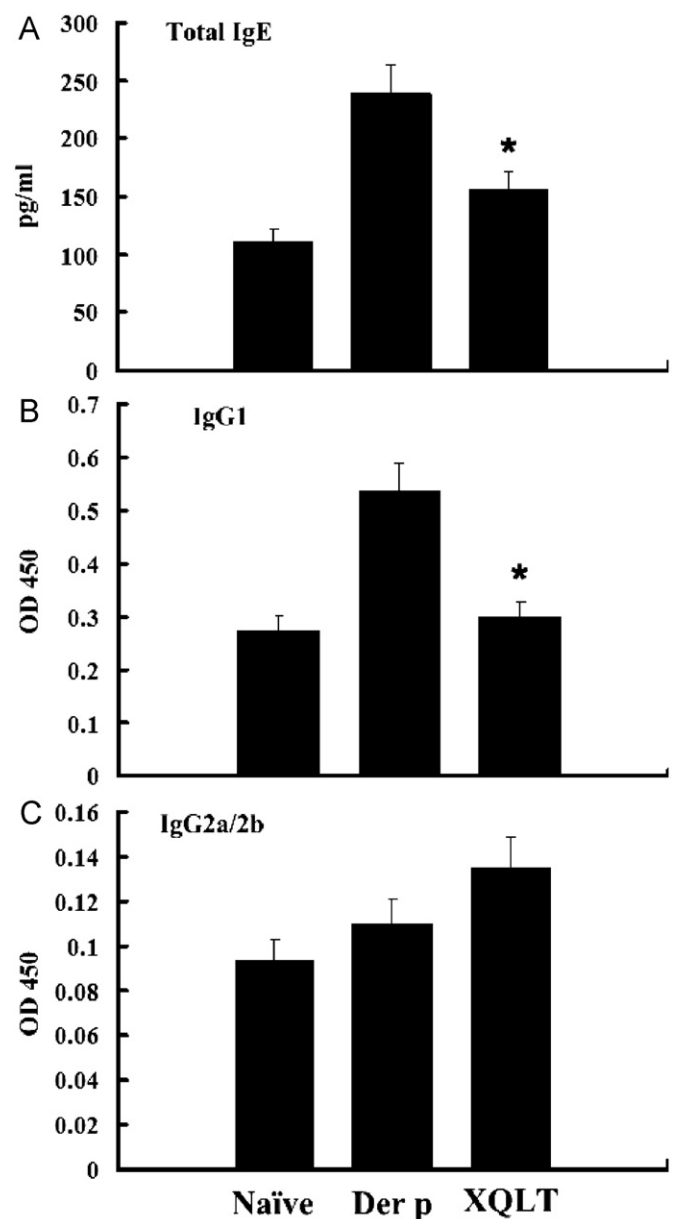


Fig. 4. XQLT inhibited the repetitive *D. pteronyssinus*-induced overexpression of IgE and IgG1 but slightly enhanced IgG2a/b production in serum. Values represent the mean $\pm$ SD of 6 mice. Differences between non-treated and XQLT-treated groups are indicated as follows: * $P < 0.05$.

3.4. XQLT attenuated pro-inflammatory and T_H2 cytokines in BALF and eosinophil in blood

We previously demonstrated that exposure to a crude extract of house dust mites augmented the production of proinflammatory cytokines in the lungs of mice. The animals then developed a T_H2 polarized allergic response with the characteristics of bronchial asthma after repeated challenge (Kao et al., 2000). Fig. 5 shows that in XQLT group mice, the levels of IL-5, IL-6, TNF α , TGF- β and IL-13 were significantly lower, and the level of IL-12 was significantly higher than in *D. pteronyssinus* group mice. In addition, IFN- γ levels were slightly higher in XQLT group mice than in *D. pteronyssinus* group mice, although the difference in production was not significant. Blood eosinophil count was significantly reduced in XQLT group mice than in *D. pteronyssinus* group mice.

3.5. Effects of XQLT on *D. pteronyssinus*-induced proinflammatory cytokine and chemokine expression (mRNA) in total lung

In the *D. pteronyssinus* group mice, the levels of IL-10 and IL-13 mRNA were only slightly higher than those in naïve mice. In the XQLT group, however, the levels of gene expression of IL-10, and IL-13 were markedly lower than those in the *D. pteronyssinus*

group. In addition, XQLT significantly suppressed RANTES, MCP-1 and EOTAXIN. (Fig. 6)

3.6. XQLT suppresses NF- κ B activation in *D. pteronyssinus*-treated lung tissues

The transcription factor Nuclear Factor- κ B (NF- κ B), plays an essential regulatory role in the production of pro-inflammatory cytokines, such as TNF- α , IL-6 and IL-1 β . We hypothesized that the inhibitory effects of XQLT on repeated *D. pteronyssinus*-induced inflammatory cytokine production might be caused by the drug's ability to inactivate NF- κ B. Fig. 7A reveals the results of NF- κ B activation in the whole lungs of XQLT group and *D. pteronyssinus* group mice using electrophoretic mobility shift assay (EMSA). In the present study, NF- κ B activity was not detected in lung cells, endothelium, or bronchial epithelium in naïve mice (Fig. 7B; left panel). However, in the *D. pteronyssinus* group, NF- κ B activity was markedly increased in bronchial epithelium, and partially located in immune cells (Fig. 7B; middle panel). In the XQLT group, XQLT suppressed the activation of NF- κ B in bronchial epithelium (Fig. 7B; right panel). These results indicate that XQLT inhibits proinflammatory and T_H2 cytokines by suppressing NF- κ B activation in bronchial epithelium.

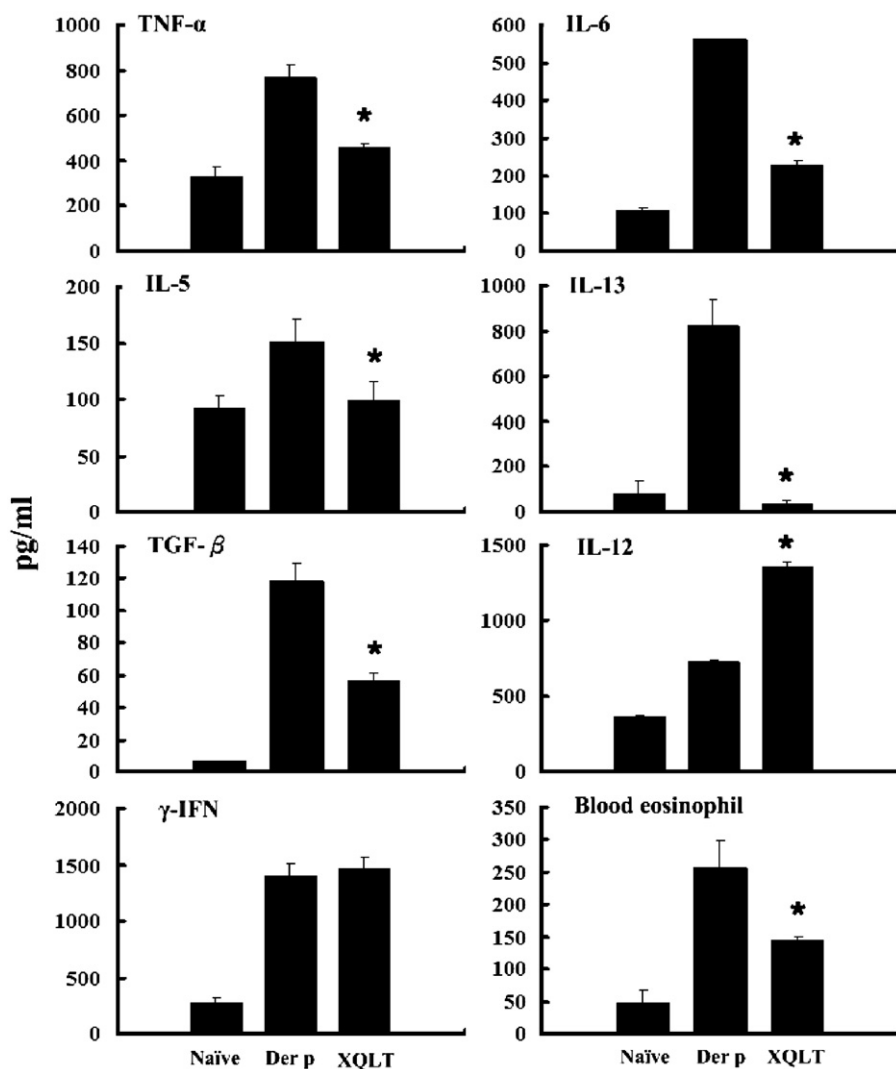


Fig. 5. XQLT reduced TGF- β , TNF α , IL-5, IL-6, IL-13, and blood eosinophil levels, but enhanced IL-12 production. Differences between non-treated and XQLT-treated groups are indicated as follows: * $P < 0.05$.

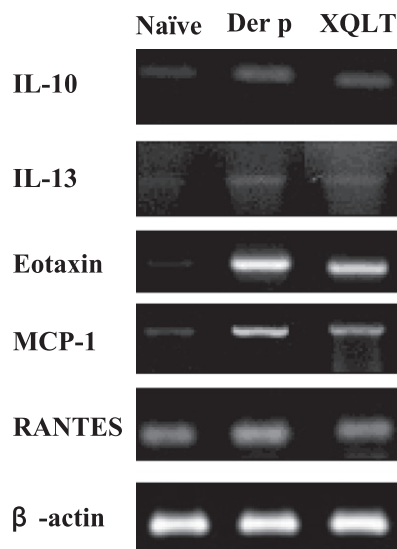


Fig. 6. RT-PCR analysis revealed that XQLT significantly suppressed the expression of T_H2 -related genes and chemokines in total lung cells. Total RNA extracted from total lung was evaluated by RT-PCR. A representative RT-PCR profile from three independent experiments is shown.

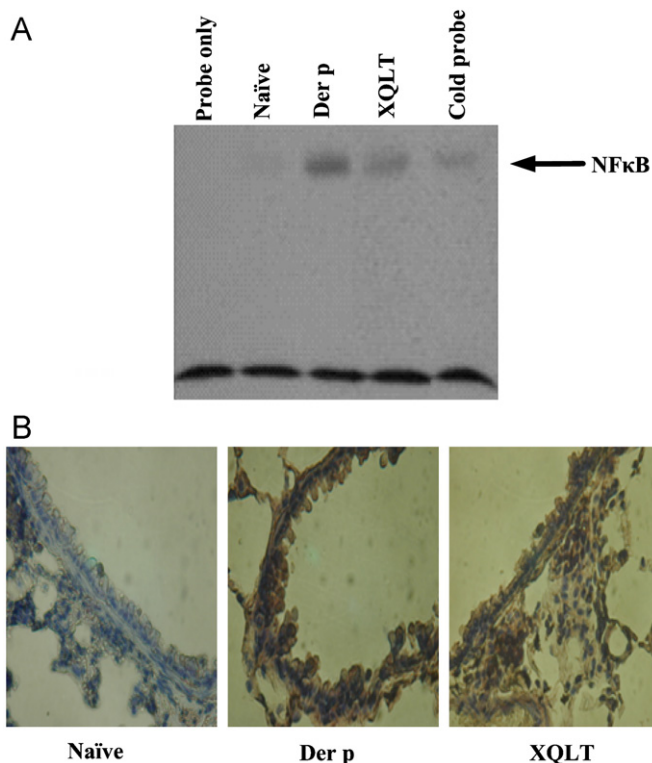


Fig. 7. (A) EMSA revealed that XQLT inhibited NF- κ B activation in whole lung after *D. pteronyssinus* stimulation. Nuclear proteins were extracted from mice 72 h after the last challenge with *D. pteronyssinus*, and activation of NF- κ B was evaluated by EMSA. The arrow indicates the specific DNA-probe complex. (B) Immunohistochemistry to detect the effect of XQLT on the expression of NF- κ B (Rel A; $\times 400$) in bronchiolar epithelial cells of mice after repetitive *D. pteronyssinus* challenge. Paraffin-embedded lung sections were prepared and stained as described in the Section 2. Data are representative of results from three separate experiments.

4. Discussion

We previously showed that repetitive allergen challenge by intranasal exposure to HDM without adjuvant treatment resulted

in pulmonary chronic inflammation. Mice used in this model exhibit several symptoms that are similar to those found in patients with chronic asthma, including high level of IL-5 and IFN- γ in BALF, increased the numbers of goblet cell in the airway, and enhanced costimulatory B7.2 molecular expression on BALF cells (Yu et al., 1999). In this chronic asthmatic murine model, we demonstrated that repetitive *D. pteronyssinus*-challenge induced airway inflammation as well as airway remodeling, including collagen deposition and goblet cell activation in the airway (Fig. 3A and B).

This study assessed the possible protective effects of XQLT that had been administrated during the sensitization period in a murine model of chronic asthma. Although there have been significant advances in our understanding of the efficacy of herbal medicines including XQLT on allergen-induced acute inflammation, very little is known about their in vivo efficacy (Kao et al., 2000, 2001; Ko et al., 2004), especially after prolonged mite exposure. Moreover, classic animal models of allergen-induced airway inflammation fail to mimic all the features of chronic asthma (Ulrich et al., 2008).

In this study, we found that XQLT treatment suppressed AHR and reduced airway inflammation and remodeling. XQLT exhibited anti-inflammatory effects as evidenced by reduction in the total numbers of cells and the percent of macrophages in BALF compared with the *D. pteronyssinus* group. Simultaneously, eosinophils have also been found to be decreased in BALF and serum. XQLT also markedly reduced collagen deposition in the lung. Lung histological features also showed that XQLT reduced inflammation and number of hyperplastic goblet cells. Therefore, the suppressive effect of XQLT on AHR may be associated with reduction of airway inflammation, eosinophilia infiltration and remodeling.

Elevated serum IgE levels may correlate with the incidence or severity of asthma (Broide et al., 2011). Therefore, IgE plays a crucial role in the pathogenesis of atopic diseases, and diminishing IgE synthesis or inhibiting its action are key goals for therapies of asthma and related allergic conditions (Rabe et al., 2011). XQLT treatment significantly decreased serum total IgE in BALF. Furthermore, we also found that IgG1 levels were reduced and IgG2a/2b levels were slightly raised by XQLT treatment. These results indicate that the attenuation of AHR and inflammation by XQLT in this model is associated with specific down-regulation of T_H2 cells but not T_H1 cells in vivo.

T_H2 cytokines play a central role in the pathogenesis of asthma (Zhu and Paul, 2008). We found that XQLT treatment increased the protein level of IL-12 but decreased TNF- α , TGF- β_1 , IL-5, IL-6 and IL-13 levels with inhibiting IL-10, IL-13, EOTAXIN, RANTES, and MCP-1 gene synthesis in lung (Figs. 5 and 6). A previous study showed that IL-5 is predominantly an eosinophil-active cytokine in the late-phase response to antigen challenge, and is important for the recruitment and survival of eosinophils (Akdis et al., 2011; Molino et al., 2011). While both eotaxis and RANTES are potent chemo attractants for eosinophils recruitment (Halwani et al., 2010), RANTES and MCP-1 also involve recruitment of monocytes (Fox et al., 2011). However, animal models and human clinical trials support that eosinophils are active and necessary in the pathogenesis of allergic airway disease (Akuthota et al., 2011). Moreover, accumulation of lymphocyte subsets, metaplasia of mucous cell, remodeling of airway, and production of IL-13 in the lungs were absolutely dependent on eosinophils (Fulkerson et al., 2006; Jacobsen et al., 2008). XQLT treatment can inhibit the secretion of IL-5 in serum and down-regulate mRNA expression of EOTAXIN, RANTES and MCP-1 in lung tissues, which may contribute to the reduction in eosinophils and monocyte recruitment in airway.

However, numerous studies have shown that the development of human bronchial asthma is predominantly associated with T_H2 responds, which promote the eosinophil infiltrate that characterizes asthma (Akdis and Akdis, 2011). XQLT suppression of the

five major T_H2 cytokines and increasing one major T_H1 cytokine may offer some advantage therapeutic. Dexamethasone, a corticosteroids, suppressed antigen-induced AHR and eosinophilic inflammation associate with suppressed T_H1 responses (IFN- γ /IgG2a) as well as T_H2 (IL-4, IL-5, and IL-13/IgE) responses. Nevertheless, they are likely to increase the patient's susceptibility to infection (Lin and Brown, 2009). These findings suggest that XQLT may offer some clinical advantages over corticosteroids.

Several studies have revealed that NF- κ B plays a critical role in T_H2 cell differentiation and regulation of production of pro-inflammatory mediators required for induction of allergic airway inflammation (Pasparakis, 2009; Ogawa et al., 2011). Previous studies have demonstrated that increased NF- κ B activity in bronchial epithelial is observed in patients with asthma and is also induced by inhalation of various infectious (Janssen-Heininger et al., 2009; Ather et al., 2011). Therefore, the NF- κ B signaling pathway has been proposed as a promising target for therapeutic intervention in asthma (Pasparakis, 2009; Ogawa et al., 2011). Indeed, the bronchial inflammatory cells displaying NF- κ B activity secrete high levels TNF- α and IL-1 β to induce of NF- κ B activity in the epithelium of asthmatic airway. The TNF- α and IL-1 β , prototypical proinflammatory cytokines, have important roles in the pathogenesis of chronic inflammatory diseases such as asthma (Lawrence, 2009).

It remains unclear whether the protective activity of XQLT against asthma is associated with a reduction in NF- κ B in the lung. To address these issues, EMSA and immunohistochemistry were performed to detect NF- κ B activation in repeatedly *D. pteronyssinus*-challenged mice (Fig. 7). We found that oral administration of XQLT inhibited NF- κ B activation in the whole lung. Immunohistochemical evaluation revealed a significant reduction in NF- κ B activation, predominantly in bronchial epithelium. The inhibition of NF- κ B activation was associated with attenuation of allergic lung inflammation, eosinophilia infiltration, collagen deposition, mucus production, and IL-5, IL-13, IL-10, EOTAXIN, RANTES, TNF- α , and TGF- β gene expression.

In conclusion, our data provide the first in vivo demonstration that XQLT attenuates allergic airway inflammation, remodeling and antigen-induced AHR through modulating T_H1/T_H2 responses and inhibiting NF- κ B activation within the bronchial epithelium in a murine model of chronic asthma. Therefore, XQLT has the potential to diminish severity of asthma symptoms when patient is challenged by allergy again.

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