



Gu-Ben-Fang-Xiao decoction attenuates sustained airway inflammation by suppressing ER stress response in a murine asthma remission model of respiratory syncytial virus infection



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ABSTRACT

Ethnopharmacological relevance: In recent years, asthma has increased dramatically in prevalence with a considerable economic burden all over the world. Long-term remission should be regarded as the promising and meaningful therapeutic goal in asthma management. However, the precise definition criteria and rational therapies for asthma remission have not been well-established. In academia, there is a consensus that even in those who develop asymptomatic remission of asthma, persistent airway inflammation is ubiquitous. Gubenfangxiao decoction (GBFXD) has been widely used in treating asthma remission stage for decades in the Jiangsu Province Hospital of Chinese Medicine, China. We previously demonstrated that GBFXD could downregulate the asthma susceptibility gene *ORMDL3*, a trigger of Endoplasmic reticulum (ER) stress and unfolded protein response (UPR).

Aim this study: To investigate the involvement of ER stress and UPR in the anti-inflammatory effects of GBFXD in Respiratory Syncytial Virus (RSV)-OVA-induced asthma remission mice.

Materials and methods: Mice were orally administered GBFXD at three doses for 30 days after an RSV-OVA challenge. The levels of inflammation mediators in serum were measured using a Luminex assay and the amount of IFN- γ in lung homogenates was detected using ELISA. The splenic CD4⁺ and CD8⁺ T lymphocytes were counted using flow cytometric analysis. The mRNA and protein levels of asthma susceptibility gene *ORMDL3*, ER stress markers (BIP, CHOP), and three canonical UPR branches (PERK-eIF2 α -ATF4, IRE1 α -XBP1/IRE1 α -JNK-AP1 and ATF6-SERCA2b signal pathways) were detected using real-time RT-PCR and western blot.

Results: Histopathological analysis showed that the model group mice still exhibited a sustained airway inflammation even after suspending the OVA-challenge and RSV infections for 30 days. H&E staining results indicated that GBFXD could attenuate sustained airway inflammation. Decreased serum CXCL1 level and increased IFN- γ level in lung homogenate were observed after GBFXD treatment. Reductions in the number of splenic CD4⁺/CD8⁺ T lymphocytes were found after DEX treatment. We further confirmed the previous finding that GBFXD could downregulate the expression of *ORMDL3*. As a result of suppressed UPR, decreased ER stress markers and inhibited UPR branches (PERK and IRE1 α signal pathway) were also observed through the significant reduction of signature mRNA and protein expressions after GBFXD treatment.

Conclusion: GBFXD can significantly attenuate RSV-OVA-induced persistent airway inflammation in

Abbreviations: GBFXD, GuBenFangXiao decoction; RSV, respiratory syncytial virus; OVA, ovalbumin; ER, endoplasmic reticulum; UPR, unfolded protein response; TCM, traditional Chinese medicine; Mont, montelukast sodium; DEX, dexamethasone; HPLC, high performance liquid chromatography; i.p., intraperitoneal injection; H&E, haematoxylin-eosin; PAS, periodic acid-Schiff; Th2-cell, T helper 2 cell; TCID₅₀, 50% tissue culture infectious dose; IL-4, interleukin-4; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL2, chemokine (C-X-C motif) ligand 2; CXCL9, chemokine (C-X-C motif) ligand 9; CXCL10, chemokine (C-X-C motif) ligand 10; CCL2, chemokine (C-C motif) ligand 2; CCL5, chemokine (C-C motif) ligand 5; CCL11, chemokine (C-C motif) ligand 11; IFN- γ , Interferon- γ ; *ORMDL3*, ORM1-like protein 3; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; PERK, protein kinase RNA (PKR)-like ER kinase; eIF2 α , eukaryotic initiation factor 2 α ; ATF4, activated transcription factor 4; IRE1 α , inositol-requiring enzyme 1 α ; XBP-1, X-box binding protein 1; JNK, c-Jun N-terminal kinase; AP1, activator protein 1; ATF6, activated transcription factor 6; SERCA2b, sarcoplasmic/endoplasmic reticulum calcium-ATPase2b; DMEM, Dulbecco's modified eagle medium; BSA, bovine serum albumin; FBS, fetal bovine serum; cDNA, complementary DNA

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murine asthma remission model. These effects may be mediated, at least partially, by inhibiting the activation of ER stress responses.

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1. Introduction

Asthma is a heterogeneous disease characterized by reversible airflow obstruction, bronchial hyperresponsiveness, and non-specific chronic airway inflammation (Boulet et al., 2012). Typical variable and recurring symptoms include wheezing, coughing, chest tightness, and shortness of breath. Among these symptoms, chronic airway inflammation presents a great therapeutic challenge in asthma; it is common even among people who have a symptomatic remission of asthma (Broekema et al., 2010). Although asthma control has improved considerably through the administration of anti-inflammatory therapy such as inhaled glucocorticoids, the merely ameliorative benefits and diversely unexpected side effects of these glucocorticoids make it hard to reach the curative goal (Boulet et al., 2012). Moreover, the treatment goals' focus on asthma control rather than on remission, partly restrict ideas for preventing the relapse of asthma. Thus, there is an urgent need for safe and effective therapeutic options in asthma treatment.

The endoplasmic reticulum (ER) acts as a protein-folding factory that is crucial for synthesizing functional proteins and transporting secretory and biological membrane proteins in vesicles to the Golgi apparatus (Gaut and Hendershot, 1993). Three protein sensors localized in the ER-membrane-double-stranded RNA-activated protein kinase-like ER kinase (PERK), inositol-requiring enzyme (IRE) 1, and activating transcription factor (ATF) 6 mediate the unfolded protein response (UPR) (Hetz, 2012). Under normal conditions, these three protein sensors are bound to a major ER chaperone, Glucose-regulated protein (GRP) 78, which prevents their activation. During special circumstances (Zhang and Kaufman, 2008), GRP78 is released from PERK, IRE1, and ATF6 owing to competitive binding to unfolded and/or misfolded proteins that accumulate in the ER lumen, which leads to the activation of the ER stress response. As a result, ER stress is implicated in the pathogenesis of various inflammatory processes. Particularly, accumulating evidences indicate the involvement of constant ER stress and UPR in the progression of asthma (Osorio et al., 2013). However, no explicit evidence demonstrates the relationship between ER stress and sustained airway inflammation in asthma remission stage.

Respiratory syncytial virus (RSV) infections are a high risk for childhood asthma (Sigurs et al., 2010). Moreover, several long-term prospective studies of children documented with RSV

infections have shown that approximately 40% will continue to have asthma into later childhood (Sly et al., 2010). A meaningful research showed that RSV infections modify miRNA expression, which could maintain a chronic state of airway inflammation (Othumpangat et al., 2012). Nevertheless, the mechanism of RSV-induced airway inflammation in asthma remission is poorly understood. Recent studies demonstrated that RSV infections significantly induced the expression of ER stress-associated genes and were highly related to the accumulation of mutant proproteins, indicating a pronounced activation of the UPR (Bridges et al., 2006; DeDiego et al., 2011). We hypothesize that there is a long-term relationship between RSV infections and sustained airway inflammation in asthma remission stage, caused by the activation of UPR.

Yu Ping Feng San (YPFS) is a well-known traditional Chinese prescription medicine first mentioned in Dan Xi Xin Fa (Du et al., 2013). As the representative formulation of invigorating Qi and consolidating the superficial, YPFS is widely used in influenza and asthma. Gubenfangxiao decoction (GBFXD) (Table 1) is a YPFS formula prescribed by Professor Jiang Yuren, a renowned TCM pediatrician in the modern China. GBFXD contains extra herbs besides YPFS, including *Poria*, *Calcined Concha Ostreae*, *Periostracum Cicadae*, *Pericarpium Citri Reticulatae*, *Radix Saposhnikovia*, *Flos Magnoliae*, *Fructus Schisandrae Chinensis*, and *Radix Glycyrrhiza*. A multi-center randomized controlled trial demonstrated GBFXD is effective in the treatment of asthma remission stage, particularly in reducing the relapse rate (Yuan and Sun, 2010). Our previous studies found that GBFXD could alleviate airway inflammation and downregulate the asthma susceptibility gene *ORMDL3* in an RSV-OVA-induced murine asthma model (Huang et al., 2016). Furthermore, *ORMDL3* could activate UPR and initiate cellular stress in asthma (Cantero-Recasens et al., 2010). Although GBFXD has attracted much attention in TCM treatment of asthma remission, the mechanisms remain unclear. Given that patients with asthma remission still suffer from airway inflammation, the aim of this study was to investigate the anti-inflammatory effects of GBFXD on RSV-induced sustained airway inflammation in a murine asthma remission model. The results obtained here indicate that GBFXD markedly attenuates airway inflammation, which might be due to an inhibition of ER stress signaling.

Table 1
Composition of GBFXD.

Pharmaceutical name	Botanical or zoological name	Family and plant part use	Chinese name	Weight (g)
Radix Astragali Preparata Cum Melle	<i>Astragalus membranaceus</i> (Fisch.) Bge. Var. <i>mongholicus</i> (Bge.) Hsiao	Leguminosae; root	Zhi Huang Qi	15 g
Saposhnikovia Radix	<i>Saposhnikovia divaricata</i> (Turcz.) Schischk.	Umbelliferae; root	Fang Feng	3 g
Atractylodes Macrocephalae Rhizoma	<i>Atractylodes macrocephala</i> Koidz.	Composite; rhizome	Bai Zhu	10 g
Codonopsis Radix	<i>Codonopsis pilosula</i> (Franch.) Nannf.	Campanulaceae; root	Dang Shen	10 g
Poria	<i>Poria cocos</i> (Schw.) Wolf	Polygonaceae; sclerotia	Fu Ling	10 g
Calcined Concha Ostreae	<i>Ostrea gigas</i> Thunberg	Ostreidae; shell	Duan Mu Li	15 g
Cicadae Periostracum	<i>Cryptotympana pustulata</i> Fabricius	Cicadidae; slough	Chan Tui	6 g
Citri Pericarpium Reticulatae	<i>Citrus reticulata</i> Blanco	Rutaceae; peel	Chen Pi	6 g
Magnoliae Flos	<i>Magnolia denudata</i> Desr.	Magnoliaceae; bud	Xin Yi	6 g
Schisandrae Chinensis Fructus	<i>Schisandra chinensis</i> (Turcz.) Baill.	Magnoliaceae; fruit	Wu Wei Zi	6 g
Glycyrrhiza Radix Et Rhizoma	<i>Glycyrrhiza uralensis</i> Fisch.	Leguminosae; rhizome and root	Gan Cao	3 g

The plant name has been checked with www.theplantlist.org mentioning the data of accessing that website.

2. Materials and methods

2.1. Plant materials and reagents

Radix Astragali Preparata Cum Melle (batch number: 140903), Codonopsis Radix (batch number: 140901), Atractylodis Macrocephalae Rhizoma (batch number: 140903), Poria (batch number: 140904), Calcined Concha Ostreae (batch number: 140801), Cicadae Periostracum (batch number: 140406), Citri Pericarpium Reticulatae (batch number: 140910), Saposhnikovia Radix (batch number: 140802), Magnoliae Flos (batch number: 140428), Schisandrae Chinensis Fructus (batch number: 140901) and Glycyrrhiza Radix Et Rhizoma (batch number: 140516) were purchased from Jiangsu Province Hospital of Chinese Medicine, and were authenticated by Dr. Sheng-jin Liu from the College of Pharmacy, Nanjing University of Chinese Medicine, China. Standards of Hesperidin, Prim-o-glucosylcimifugin, Schisandrol A, Magnolin, Lobetyolin, Liquiritin were purchased from Chengdu Push Bio-technology (Chengdu, China), and the purities of all these compounds were above 98% based on HPLC-UV.

2.2. GBFXD preparation

First, the calcined Concha Ostreae was decocted for 30 min while the other herbs were soaked in 8 aliquots of distilled water (v/w). All the herbs were then mixed together and boiled in the 8 aliquots of water for 30 min. After the first decoction was boiled, the residue of the GBFXD (dregs) was boiled in 6 aliquots of distilled water (v/w) for another 30 min. The supernatant obtained from the twice decoction was vacuum evaporated to a final density of 3 g/mL at 65 °C by a rotavapor (Buchi, Switzerland). The extract were stored at 4 °C and dissolved in distilled water at the desired concentrations before use.

2.3. High performance liquid chromatography (HPLC) analysis

HPLC analysis was done by Waters 2695 Alliance (Waters, Milford, USA). Chromatographic separation was performed on Waters-C18 column (250 mm × 4.6 mm, 5 µm) at 30 °C. The mobile phase was a mixture of water containing acetonitrile (A) and 0.5% formic acid (B). A linear gradient elution was conducted as follows: 0–5 min at 10% A, 5–8 min at 18% A, 8–20 min at 18% A, 20–22 min at 25% A, 22–36 min at 25% A, 36–38 min at 30% A, 38–48 min at 40% A, 48–50 min at 45% A, 50–65 min at 45% A, and 65–67 min at 10% A. The flow rate was 1 mL/min, and the total injection volume was 10 µL.

2.4. Animals, cell and virus

Four-week-old female specific-pathogen-free BALB/c mice (16–18 g) were purchased from Suzhou JOINN Laboratories Co., Ltd., Jiangsu, China. The animals were housed in the experimental animal center of the Nanjing University of Chinese Medicine, with a 12 h light/dark cycle at a constant temperature of 22 ± 2 °C. Cages, bedding, food, and water were sterilized before use. The animals were acclimatized for 7 days prior to initiating the experiments. One hundred and five mice were randomly divided into seven groups as follows, A: control group, B: model group, C, D, and E: three GBFXD (36, 24 and 12 g/kg d⁻¹) treatment groups, F: Mont (2.6 mg kg⁻¹ d⁻¹ montelukast sodium, Merck Sharp & Dohme) treatment group, G: DEX (1 mg kg⁻¹ d⁻¹ dexamethasone, Sigma-Aldrich) treatment group. There were no significant differences in the weights of the animals among the groups prior to the start of the experiments. All experimental procedures were performed in accordance with the National Institutes of Health Guidelines for Laboratory Animals and approved by the Animal Ethics Committee

of Nanjing University of Chinese Medicine. [No. SYXK (Su) 2014–0001].

Human RSV (A strain) was purchased from the Type Culture Collection Center, Wuhan University, China. HEp-2 cells were purchased from the Cell Bank Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China. The virus was propagated in HEp-2 cells to prepare a viral stock. HEp-2 cells were cultured in growth medium composed of Dulbecco's Modified Eagle's Medium (DMEM; Gibco Co.) containing Eagle's balanced salt solution and supplemented with 10% FBS (Gibco Co.), 1% penicillin–streptomycin solution (100 ×; Gibco Co.), and 2 mM l-glutamine (Gibco Co.). RSV-infected cells were maintained in DMEM supplemented with 2% FBS (maintenance medium). 3–5 days later cytopathic effect appeared. At maximal cytopathic effect, the cells were harvested as described previously (Chen et al., 2015). Titers were determined by TCID₅₀ method on HEp-2 cells. In this study, the titer of RSV was measured as 1.0 × 10^{4.167} TCID₅₀/mL.

2.5. Sensitisation, challenge and treatment of mice

The mouse model was based on OVA (Ovalbumin, Grade II, Sigma-Aldrich) challenge and RSV infections as described previously (Wang et al., 2014). In brief, on days 1, 8, mice were sensitized with an intraperitoneal injection (i.p.) of 0.2 mL alum-precipitated antigen in NS which contained 100 µg of OVA mixed with 1 mg aluminum potassium sulfate. After 7 days of the sensitisation, the mice were exposed to aerosolized 2.5% OVA for 30 min/day in days 15–28 and 30 min once every three days in days 32–54. Followed OVA-sensitisation condition, mice in B–G groups were intranasally infected with 50 µL RSV on days 29, 42 and 55. From days 56–86, the mice in the treatment groups were administrated GBFXD or Mont or DEX per day, at doses of 36, 24 and 12 g/kg d⁻¹ for GBFXD and 2.6 mg kg⁻¹ d⁻¹ for Mont and 1 mg kg⁻¹ d⁻¹ for DEX. Mice in the control group received the same volume of NS. The protocol for the sensitisation, challenge, virus infections and pharmaceutical intervention was summarized in Fig. 1.

2.6. Lung histopathology

Following serum collection, the lung tissue slices were fixed with 10% neutral-buffered formalin. A series of 3–4 µm micro-sections was stained with haematoxylin and eosin (H&E) to assess inflammatory cell infiltration. The inflammation score was determined as follows: grade 0=no inflammation; grade 1=occasional cuffing with inflammatory cells; and grades 2, 3, and 4=where most bronchi or vessels were surrounded by a thin 1–2 cells layer, a 3–5 cells moderate layer, or a thick > 5 cells layer of inflammatory cells, respectively. Periodic acid-Schiff (PAS) staining was used to quantify airway goblet cells, and the Masson's trichrome staining was used to visualize collagen deposition and fibrosis. Both staining methods were scored as follows: 0: none; 1= < 25%; 2=25–50%; 3=50–75%; and 4= > 75% goblet cells (Ma et al., 2016). Histopathology was assessed in a double-blind manner by two independent researchers.

2.7. Inflammatory mediators

Serum samples were obtained by the centrifugation of peripheral blood (850 g, 4 °C for 10 min) and stored at –80 °C. The levels of inflammatory mediators in the serum, IL-4, CXCL1, CXCL2, CXCL9, CXCL10, CCL2, CCL5 and CCL11 were measured by Luminex kit (Univ, Shanghai, China) according to the manufacturer's instruction.

One lobe of the left lungs from all animals were lysed in a

radio-immunoprecipitation assay (RIPA) buffer (Beyotime, Haimen, China) containing 1% phenylmethanesulfonyl fluoride (PMSF) (Beyotime, Haimen, China) mixture. The supernatant of the lung homogenate was collected by centrifugation with 13,000 g for 30 min at 4 °C. Protein concentrations in the lung samples were measured using a bicinchoninic acid (BCA) protein concentration assay kit (Thermo, USA) and quantified at the concentration of 2 µg/µL. The IFN-γ concentrations from lung homogenate were quantified by ELISA (Multiscience, Hangzhou, China) according to the manufacturer's instructions.

2.8. Flow cytometric analysis of splenic CD4+/CD8+T lymphocytes

The spleens were removed from each sacrificed mice and placed in 1 mL PBS containing 0.5% BSA (Gibco). A single cell suspensions were prepared by disrupting the spleens using Cell disrupter (Beckman), Cells were equally distributed into tubes and washed once in phosphate-buffered saline (PBS). For CD4+/CD8+T lymphocytes analysis, splenocytes were incubated with APC anti-mouse CD3, PE anti-mouse CD4 and FITC anti-mouse CD8a (eBioscience) at the room temperature for 30 min. FACS analysis was performed using the cytomics FC 500 system (Beckman Coulter, USA) with CXP Software.

2.9. Real-time RT-PCR assay

Total RNA from the lung tissue was extracted using the TRIzol reagent (Takara, Dalian, China) and cDNA was synthesized using the Prime Script[®] RT reagent kit with gDNA Eraser (Takara, Dalian, China), according to the manufacturer's instructions. Real-time RT-PCR was performed on the cDNA samples using the SYBR[®] Premix Ex Tap[™] II (Takara, Dalian, China). The used primers are listed in Table 2. The reaction conditions were as follows: 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and extension at 60 °C for 34 s. All amplifications were done in triplicate and repeated three times. The PCR data were analysed using the QuantStudio 7 Flex detection system (Applied Biosystems Co., USA). C_T values were analysed using the comparative C_T (ΔΔC_T) method, and the relative amount of target mRNA (2^{-ΔΔC_T}) was obtained by normalizing to endogenous GAPDH.

2.10. Western blot

Lung samples were lysed in RIPA buffer containing PMSF and PhosStop (Boster, Wuhan, China). The lysed samples were centrifuged for 30 min at 13,000 g to remove debris. Protein concentrations in the lung samples were measured using a BCA

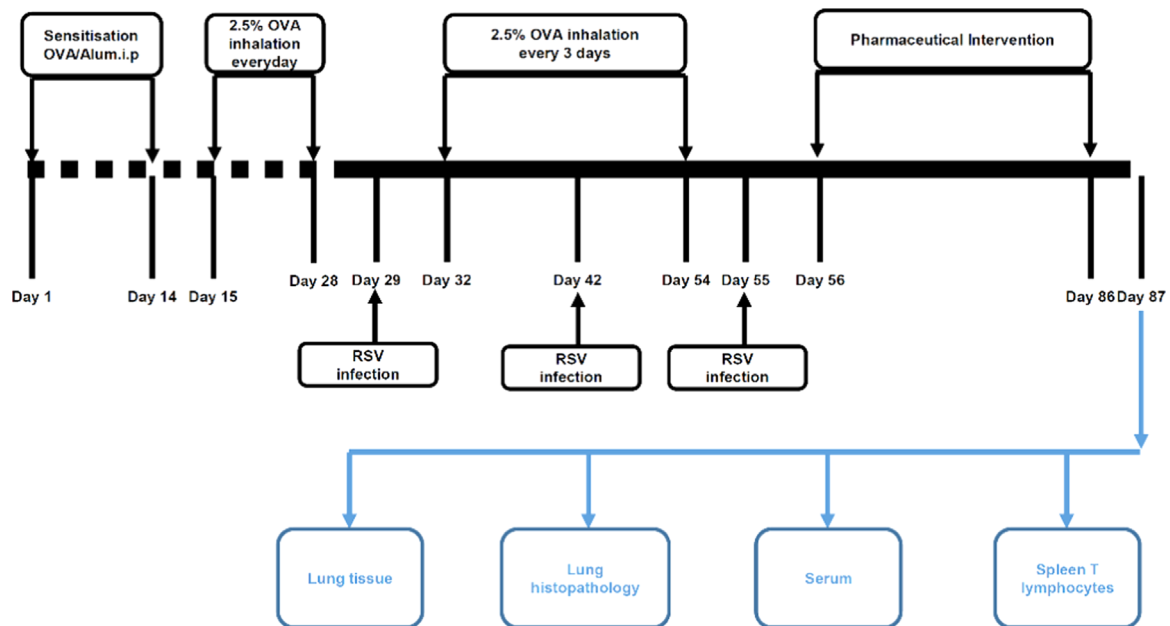


Fig. 1. Experimental protocol of (OVA-RSV) sensitisation and challenge as described in the Section 2.

Table 2
The sequence of primes.

Gene	Forward prime (5'-3')	Reverse prime (5'-3')	Product (bp)
ORMDL3	CCCTACCAACCTTATCCAC	ACCCCGTAGTCCATCTGCT	130
BIP	TGCGTGTGTGTGAGACCAG	CCACAGTGAACTTCATCATGCC	126
ATF6	ACCTGTTCTTCTCTGAAATCCAA	AGGACAGAGAAACAAGCTCGG	176
SERCA2b	GCCGCTCATTTTCCAGATCAC	CAGGAAATGCCATCGGTGC	198
PERK	CCGCAAGAAGGACCCTATCC	GAGTTTCAGACTCCTCCGCT	143
eIF2α	AGCATTTCTCGCCATGTTGC	TAGGCACCGTATCCAGGTCT	122
ATF4	CCTATAAAGGCTTGCGGCCA	TGGATTTCTGTAAGAGCGCC	143
IRE1α	GCGATGGACTGGTGGTAACT	TGCACCACCTTTCTCAGGAC	121
JNK	TTTTGGACTGGCGAGGACTG	TGGGAACAAAACACCACCTTG	172
XPB-1	CTGAGTCCGACGAGGTG	GACCTCTGGGAGTTCCTCCA	188
CHOP	TTATCTTGAGCCTAACACGTCGAT	CCAGGTTCTCTCTCTCAGGT	100
AP1	CTCACACATCTGTGCTCGT	CCTTTCATCCATTGTGCCTC	106
GAPDH	CGTGTTCCTACCCCAATGT	TGTCATACTTGGCAGGTTTCT	104

protein concentration assay kit. The total protein was mixed with 2X electrophoresis sample buffer (Bio-Rad, USA), denatured at 100 °C for 5 min, and then stored at –80 °C. Total proteins were separated by 8–12% SDS-PAGE. After electrophoresis, the separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (0.2 or 0.45 µm, Millipore, USA), blocked with 5% BSA in TBS Tween 20 (TBST, 25 mM Tris [pH 7.5], 150 mM NaCl, and 0.1% Tween 20) for 2 h, and incubated with primary antibodies, including ORMDL3 (Santa Cruz Biotechnology Inc.), BIP/GRP78, SERCA2b, IRE1α, PERK and β-actin (Cell Signaling Technology Inc.), ATF6, XBP-1, eIF2α and p-eIF2α (Abcam Ltd.), CHOP, JNK and AP1 (Ruiying Biology, Suzhou, China), overnight at 4 °C. Goat anti-rabbit horseradish peroxidase-conjugated IgG (Cell Signaling Technology Inc.) was used to detect antibody binding. The target proteins were visualized using a ChemiDoc™ MP Imaging system (Bio-Rad Co., USA) and analysed using the Image Lab software (Bio-Rad Co., USA).

2.11. Statistical analysis

The data are expressed as mean ± standard deviation (SD). All tests were performed using Prism 6.0 (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed with Unpaired Student's *t*-tests (for two experimental groups) or one-way analysis of variance (for three or more experimental groups), followed by Dunnett's post hoc test to determine the differences among multiple comparisons. The significance level was set to $p < 0.05$.

3. Results

3.1. HPLC profile of GBFXD

The major components of GBFXD were analysed using HPLC. As compared with standard reference compounds, six compounds were determined and identified: Liquiritin, prim-o-glucosylcimifugin, hesperidin, lobetyolin, magnolin, and schisandrol. The concentrations of these compounds were 0.195 mg/g, 0.134 mg/g, 0.224 mg/g, 0.087 mg/g, 0.026 mg/g, and 0.027 mg/g respectively (Fig. 2).

3.2. GBFXD attenuated airway inflammation in a RSV-OVA-induced murine asthma model

To confirm the establishment of the RSV-OVA-induced murine asthma model, we conducted lung pathological examinations by H&E, PAS, and Masson's trichrome staining on one day (the 56th day) after the last RSV infection. The inflammation scores, the degree of goblet cell hyperplasia and collagen deposition were significantly increased in the RSV-OVA-induced mice than those of the 56-control group ($p < 0.05$, Fig. 3a and b), indicating a satisfactory establishment of an exacerbated asthma model in this study.

To monitor the symptomatic clinical remission of asthma, we ceased the inducement for 30 days after the last infection. Interestingly, we observed that mice in the model group still exhibited milder/moderate airway inflammatory responses, including a partial infiltration of inflammatory cells around the respiratory tracts and vessels (Fig. 3c). However, we did not observe any goblet cell hyperplasia and collagen deposition in the lung sections of the model group mice (data not shown). The lung pathological results suggested that although the characteristics of respiratory mucin hypersecretion and airway remodeling were absent, persistent airway inflammation still existed.

Treatment with GBFXD or DEX suppressed the infiltration of inflammatory cells to varying degrees. Administration of GBFXD

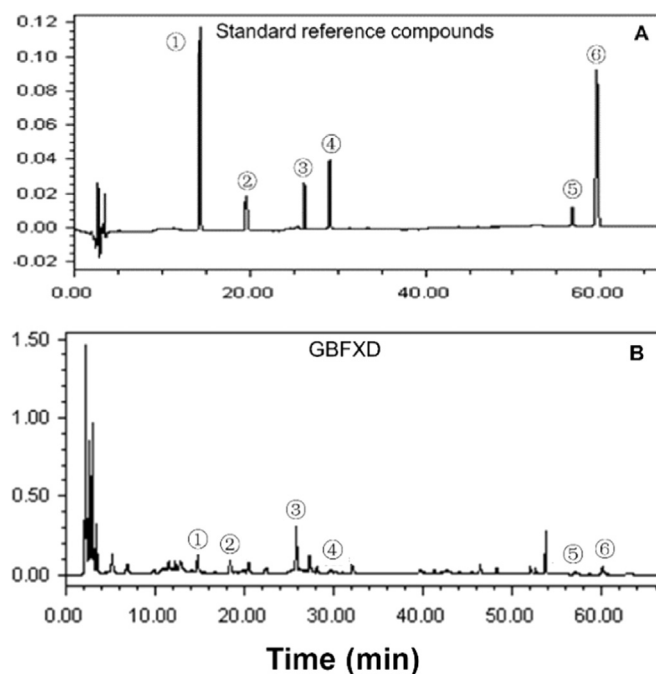


Fig. 2. HPLC-UV chromatograms of GBFXD at 270 nm. Representative chromatograms of standard reference compounds (A) and GBFXD (B) were shown respectively. ①Liquiritin, ②Prim-o-glucosylcimifugin, ③Hesperidin, ④Lobetyolin, ⑤Magnolin, ⑥Schisandrol A.

(12 g/kg and 24 g/kg) induced a remarkable decrease in inflammation scores (1.8 ± 0.45 and 1.6 ± 0.45) compared with that of the model group mice ($p < 0.05$). Mice treated with GBFXD-HD (36 g/kg), Mont (2.6 mg kg^{-1}), and DEX (1 mg kg^{-1}) had fewer inflammatory cells (Fig. 3c), although these three groups did not cause such drastic decreases in the total inflammation scores ($p > 0.05$). These findings indicate that administration of GBFXD further attenuated the inflammatory responses in the murine respiratory tracts 30 days after the RSV-OVA challenge.

3.3. GBFXD reduced CXCL1 level in serum

To determine airway inflammation, we thereafter quantified eight inflammatory mediators (IL-4, CXCL1, CXCL2, CXCL9, CXCL10, CCL2, CCL5, and CCL11) and found that three serum mediators (CXCL1, CXCL2, and CCL11) were substantially upregulated in the model group mice ($p < 0.05$) (Fig. 4a–c). Neutrophils are attracted to the respiratory tracts and are activated mainly by the CXC chemokines CXCL1 and CXCL2 (Rohde et al., 2014). Moreover, CXC chemokines are mainly responsible for neutrophilic airway inflammation in patients with severe and refractory asthma (Kikuchi et al., 2009). In this study, RSV infections induced a neutrophils-dominant chronic inflammation in the airways, which is consistent with the responses of infections with RSV in neonatal mice (Har-ker et al., 2014). Interestingly, we observed that administration of GBFXD (12 g/kg) and DEX significantly reduced the level of CXCL1 in serum than that of the model group ($p < 0.01$). However, there were no differences among the levels of IL-4 and the chemokines CXCL9, CXCL10, CCL2, or CCL5 (data not shown). These findings indicate that GBFXD attenuated neutrophilic airway inflammation by diminishing the level of CXCL1 in serum.

3.4. GBFXD increased the level of IFN-γ in lung homogenate

IFN-γ is the only Type II interferon produced by several cell types and exhibits antiviral and immune regulatory effects (Duerr et al., 2016). Compared with those of the control group, the IFN-γ

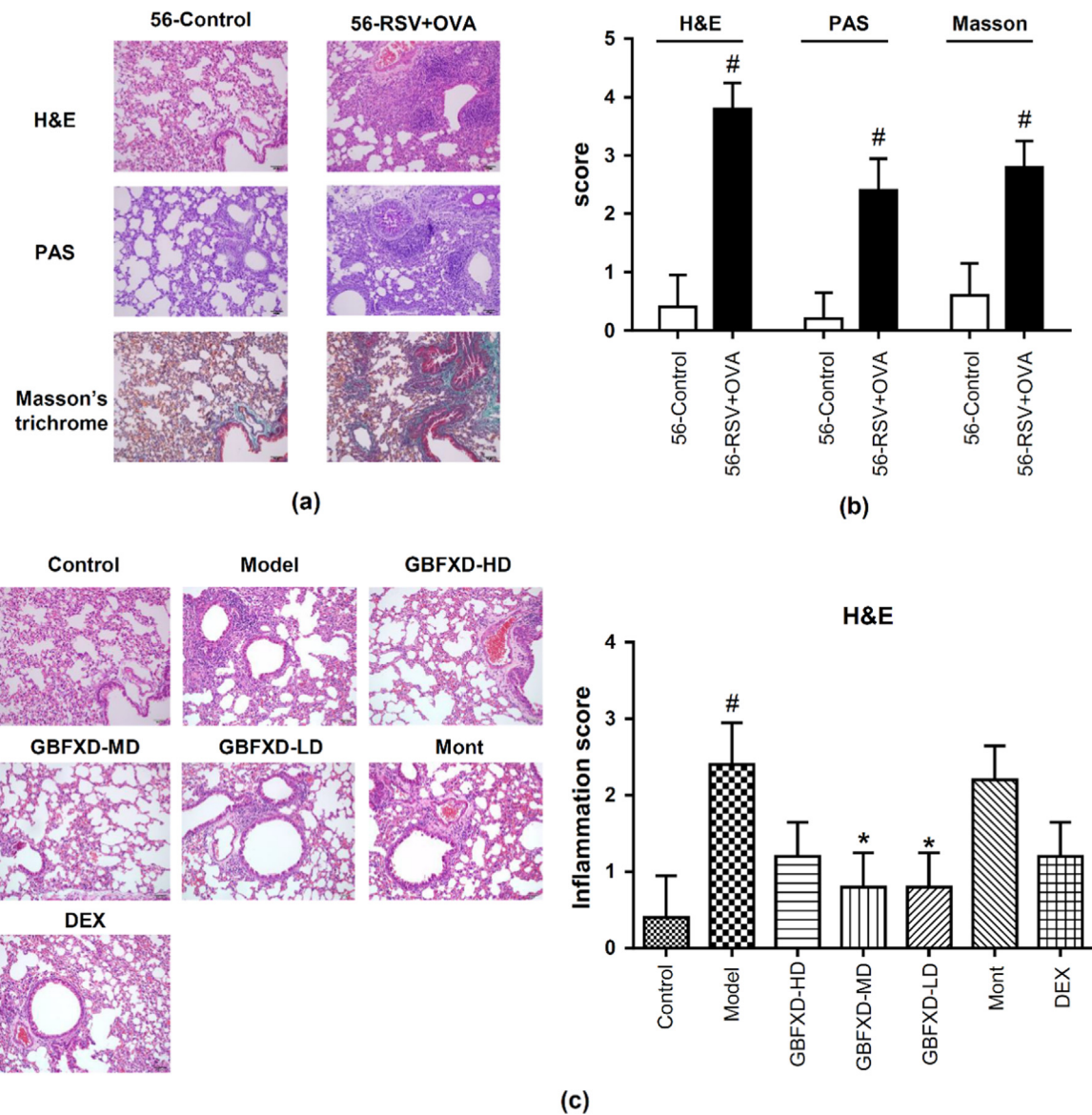


Fig. 3. (a) Histological analysis of lung tissues from control and model group mice sacrificed on one day after last time of RSV infection. Lung sections were stained with H&E to analyse the infiltration of inflammatory cells, PAS to assess goblet cells hyperplasia, Masson's trichrome to evaluate the subepithelial deposition of collagen and fibrosis. Scale bar=50 μ m (b) total inflammation scores in all groups of animals were calculated. The percentage of PAS-positive cells per bronchiole was calculated. Masson's trichrome staining analysis of collagen deposition was calculated. (c) The suppressive effect of GBFXD on RSV-(OVA)-induced airway inflammation in murine model of asthma remission. Scale bar=50 μ m. Total inflammation scores were calculated. Values represented as mean $\pm$ SD ($n=5$ per group). [#] $p < 0.05$ compared with the control group, and ^{*} $p < 0.05$ compared with the model group.

levels in the model group were notably increased (Fig. 4d). Administration of GBFXD (12 g/kg and 24 g/kg) and Mont increased the levels of IFN- γ in lung homogenate than those of the model group mice ($p < 0.05$) (Fig. 4d). These findings indicate that GBFXD could inhibit airway inflammation by enhancing immune activity in the RSV-OVA-induced murine asthma remission model.

3.5. Effect of GBFXD on splenic CD4⁺/CD8⁺ T lymphocytes

CD4⁺ and CD8⁺ T cells are multi-functional effector cells involved in the development of allergic inflammation, with the CD4⁺/CD8⁺ ratio in asthmatic patients being significantly higher than those in healthy controls (Lee et al., 2001). Besides, antigen transfer and antigen-specific CD4⁺ T cell proliferation occur in splenic follicles (Gustavsson et al., 1994). Thus, we detected the splenic CD4⁺/CD8⁺ T lymphocytes after GBFXD treatment. Although RSV infections might impair the CD8⁺ T cells (Erickson

et al., 2012), the ratio of splenic CD4⁺/CD8⁺ T lymphocytes in model group was not influenced. Subsequently, we observed a slightly increased in the number of splenic T lymphocytes after GBFXD treatment ($p > 0.05$) (Fig. 5), implying that GBFXD induced an immune regulatory effect after the RSV-OVA challenge. Besides, oral administration of DEX reduced the number of total splenic T lymphocytes ($p > 0.05$) and markedly decreased the ratio of splenic CD4⁺/CD8⁺ T cells than that of the other groups ($p < 0.05$) (Fig. 5).

3.6. GBFXD restrained the expression of ORMDL3 in lungs

According to our previous study (Huang et al., 2016), we verified again that GBFXD could repress the expression of ORMDL3 in RSV-induced murine asthma models (Fig. 6). The mRNA and protein levels of ORMDL3 in the lung tissues of the model group mice were significantly increased than those in the control group

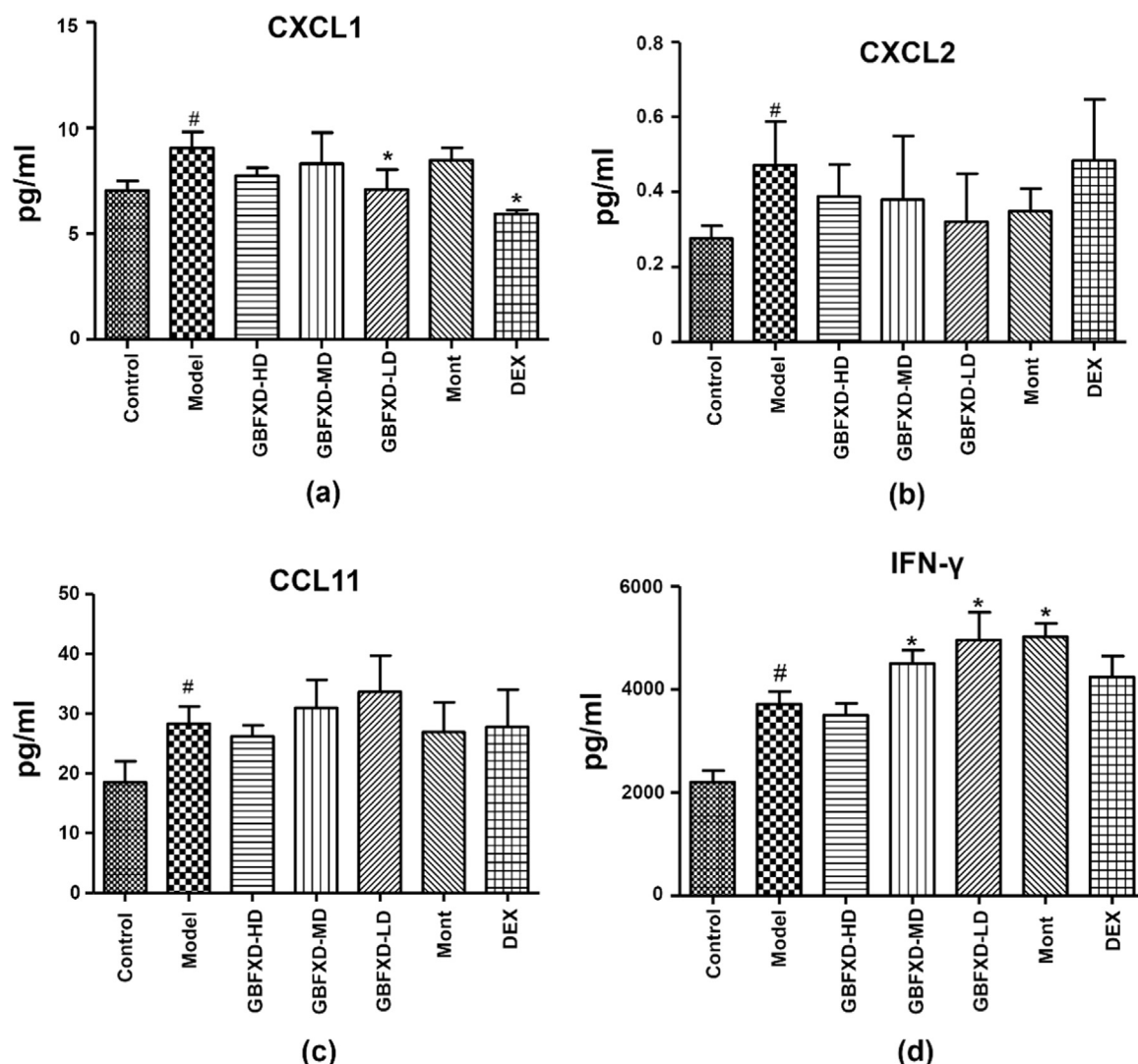


Fig. 4. Inducement with OVA and RSV increased the levels of CXCL1, CXCL2 and CCL11 in serum and IFN- γ in lung homogenate. Treatment with GBFXD inhibited the levels of CXCL1 in serum and further increased the concentrations of IFN- γ in lung homogenate. (a)–(c) The levels of CXCL1, CXCL2 and CCL11 in serum were measured with Luminex assay. (d) The concentrations of IFN- γ in lung homogenate were measured with ELISA. Values represented as mean $\pm$ SD ($n=6$ per group). [#] $p < 0.05$ compared with the control group, and ^{*} $p < 0.05$ compared with the model group.

($p < 0.05$). The mRNA levels of ORMDL3 from the Mont and the GBFXD (24 g/kg and 36 g/kg) groups were dramatically lower than those in the model group ($p < 0.05$). Although Western blot analysis revealed that the differences between the ORMDL3 protein levels of the model group and the medicine intervention groups were insignificant ($p > 0.05$), there was a significant difference in the expression of ORMDL3 mRNA among these groups. A recent study suggested that ORMDL3 may alter ER-mediated Ca^{2+} homeostasis by inhibiting sarco-/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) activity and triggering the unfolded protein response (UPR) (Cantero-Recasens et al., 2010; McGovern et al., 2010). Therefore, we hypothesized that GBFXD could modulate ER stress responses, which might be triggered by ORMDL3.

3.7. GBFXD decreased the expression of ER stress markers in lungs

To help proteins fold correctly, the ER contains a pool of molecular chaperones including GRP78. When protein folding is disturbed inside the ER, BIP synthesis is increased (Walter and Ron, 2011). CHOP is an apoptotic transcriptional factor induced in response to ER stress. Either GRP78 or CHOP, as two ER stress markers, is evaluated to determine the activation of UPR (Kim et al.,

2013). As shown in Fig. 7, the enhancement of the levels of GRP78 and CHOP in the model group indicates the activation of the ER stress involved in airway inflammation. This activation was partially blunted in the GBFXD-, Mont-, and DEX-treated groups. These results suggest that GBFXD may attenuate RSV-OVA-induced airway inflammation in mice by decreasing the levels of ER stress markers. We thereafter investigated the protein sensors that recognized the enhanced ER stress responses.

3.8. GBFXD suppressed activation of the PERK signaling pathway

It has been established that the PERK signaling pathway for ER stress-associated asthma pathogenesis works by modulating the activation of a master inflammation transcription factor NF- κB (Jiang et al., 2003; Kim et al., 2013). ATF4 is crucial in inducing the CHOP apoptosis factor in mammalian cells (Ma et al., 2002). As shown in Fig. 8, we confirmed the effects of RSV-OVA on the activation of PERK, eIF2 α , and ATF4 in the model group mice. Moreover, this activation was partially dampened in the GBFXD-, Mont-, and DEX-treated groups. These results suggest that GBFXD may restore ER homeostasis by suppressing the activation of the PERK signaling pathway.

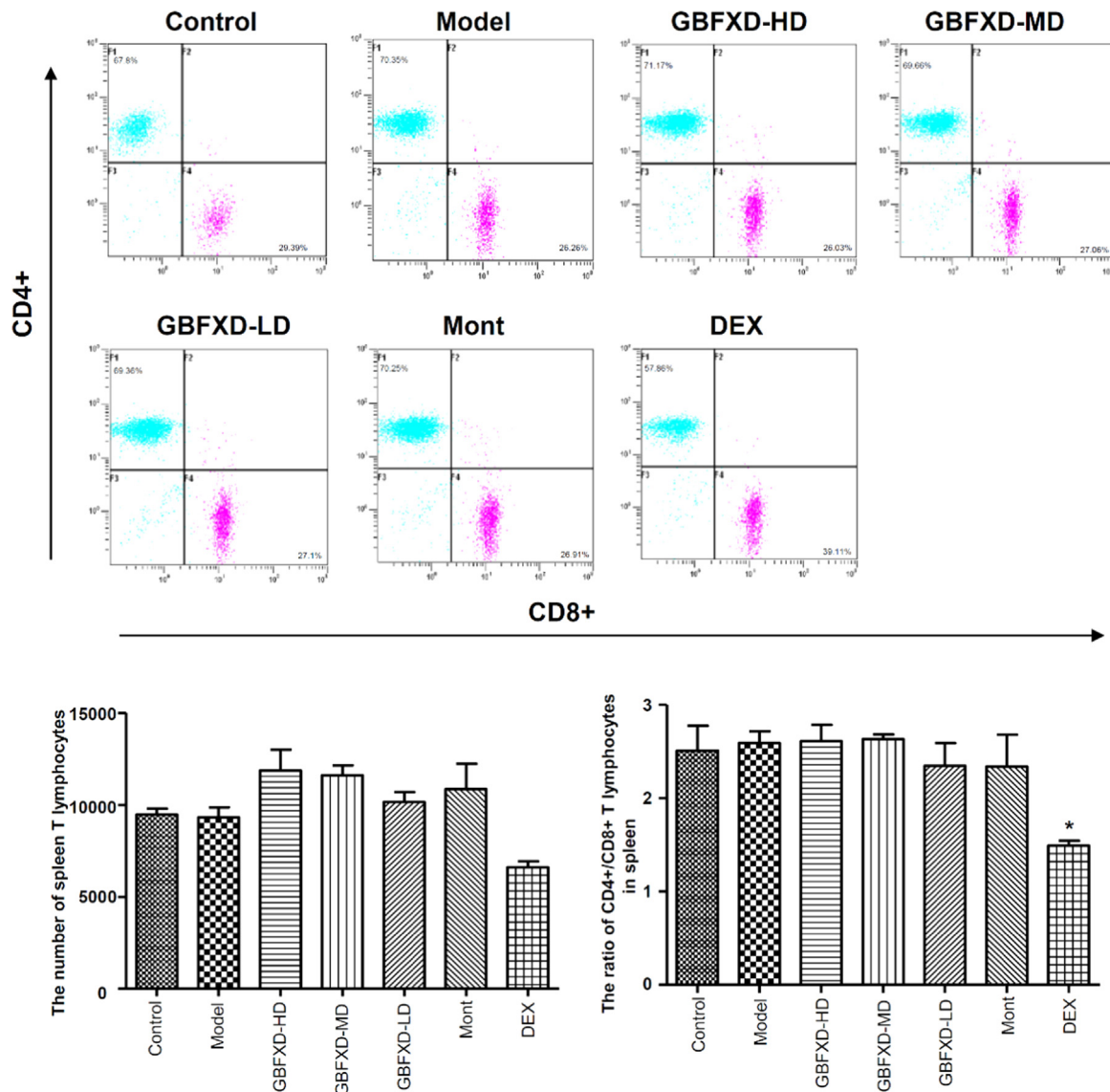


Fig. 5. Effects of GBFXD on splenic CD4⁺/CD8⁺ T lymphocytes. Values represented as mean $\pm$ SD (n=3 per group). #p < 0.05 compared with the control group, and *p < 0.05 compared with the model group.

3.9. GBFXD inhibited the activation of the IRE1 signaling pathway

In mammals, the IRE1 α -JNK signaling pathway is highly associated with inflammatory responses that induce the expression of the transcription factor activator protein 1 (AP1) (Davis, 2000). Moreover, the IRE1 α -XBP-1 branch is essential for the development and survival of many types of cells in the immune system (Brunsing et al., 2008; Iwakoshi et al., 2007; Martinon et al., 2010; Osorio et al., 2014). As shown in Fig. 9, the levels of IRE1 α , JNK, AP1, and XBP-1 in the model group mice were substantially higher than those in the control group. Similar to the observations made in the PERK signaling pathway, the expression of the IRE1 α -JNK-AP1 and the XBP-1 branches were substantially inhibited in the GBFXD-, the Mont-, and the DEX-treated groups.

3.10. GBFXD modulate the expression of ATF6 and upregulate the mRNA of SERCA2b

By activating the third arm of the UPR, ATF6 enhances the transcription of genes encoding ER-associated degradation components (Wu et al., 2007). Moreover, the ATF6 targeted gene,

SERCA2b, was associated with airway remodeling in asthma (Miller et al., 2014). Although there were insignificant differences in ATF6 between the control and the model group (Fig. 10, $p > 0.05$), the levels of ATF6 in the GBFXD (24 g/kg and 36 g/kg), the Mont and the DEX groups were slightly lower than that of the model group. As a broad-range Ca²⁺-dependent pump, SERCA2 is the most highly expressed pump in smooth muscles (Hovnanian, 2007), which could be inhibited by siRNA knockdown of ATF-6 α in lung epithelial cells (Miller et al., 2012). Furthermore, low SERCA expression was observed in airway remodeling (Mahn et al., 2009). In the present study, the expression of SERCA2b in the model group mice was insignificantly lower than those in the control group ($p > 0.05$). Compared with the model group, mice treated with GBFXD, Mont, and DEX displayed increased SERCA2b mRNA expression, albeit a similar effect was not seen at the protein level. These findings may explain the absence of collagen deposition in the lung of the model group mice.

4. Discussion

Five major findings were extracted from this study. 1) Although goblet cell hyperplasia and collagen deposition in the lung tissues

were not apparent, the infiltration of inflammatory cells in the respiratory tracts were still visible. 2) Oral administration of GBFXD alleviated sustained airway inflammation and reduced serum CXCL1 levels in RSV-OVA-induced asthma remission mice.

3) GBFXD may exert some immune regulatory effects by enhancing the level of IFN- γ in lung tissues and increasing the number of splenic T lymphocytes. 4) RSV infections and OVA sensitisation induced ER stress response concomitant with increased UPR

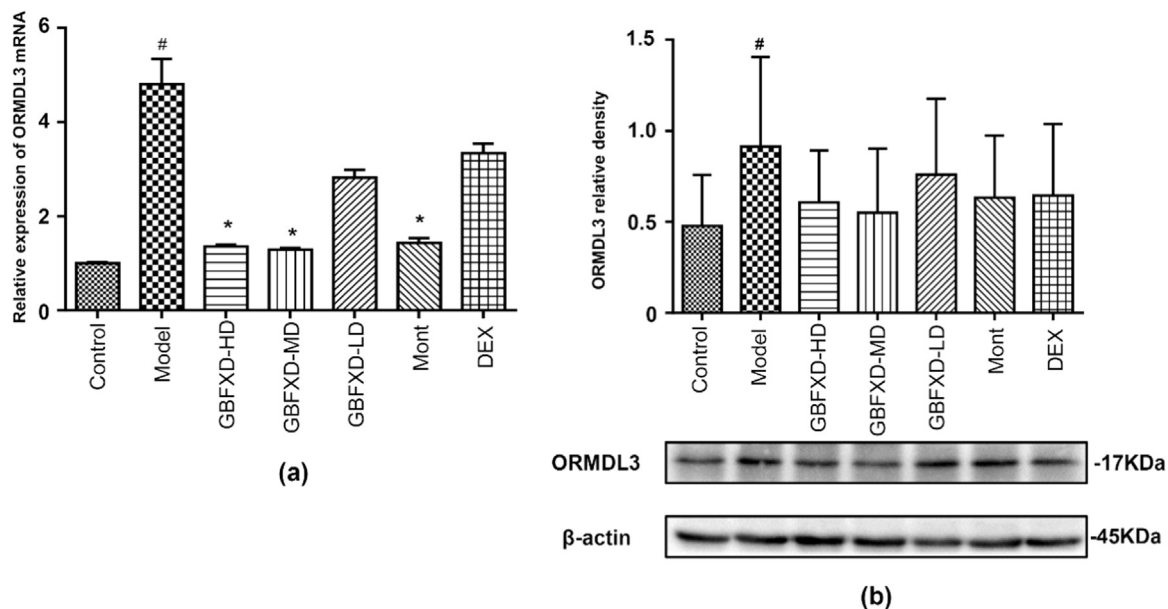


Fig. 6. Treatment with GBFXD downregulated the expression of ORMDL3 in lung tissues. (a) The mRNA expression of ORMDL3 was analysed by RT-PCR. (b)–(c) Western blotting measured the protein expression of ORMDL3. The relative density quantification is relative to β -actin. Values represented as mean $\pm$ SD ($n=3$ per group). [#] $p < 0.05$ compared with the control group, and ^{*} $p < 0.05$ compared with the model group.

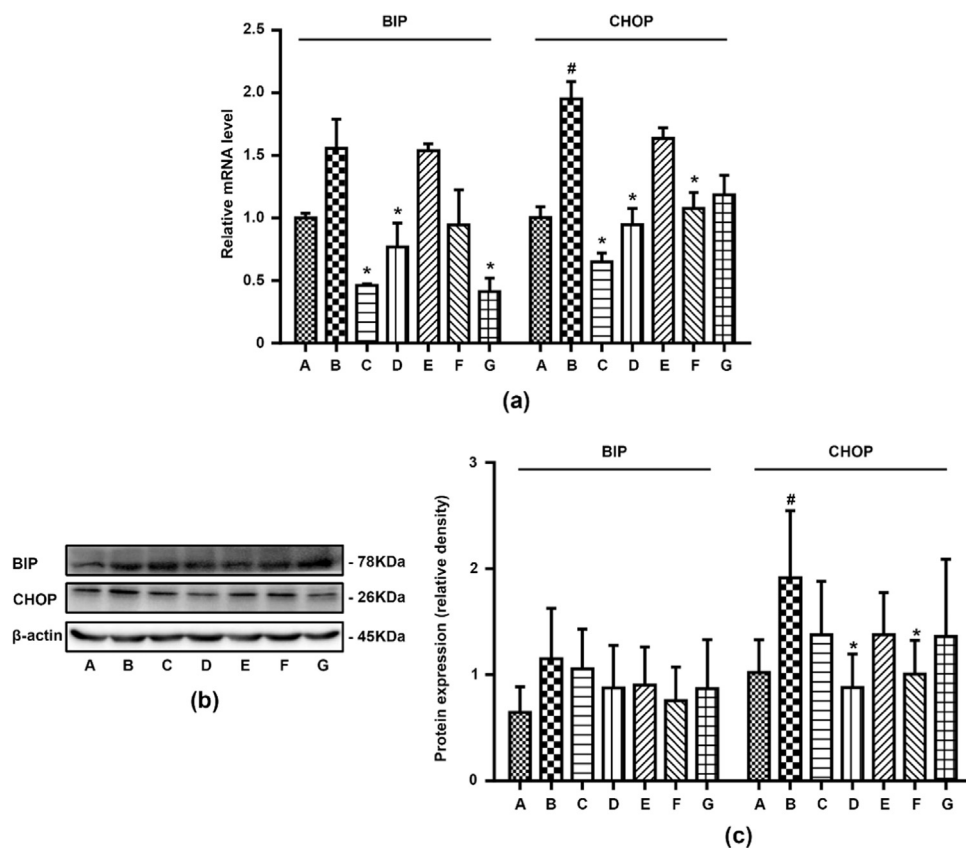


Fig. 7. Treatment with GBFXD decreased the levels of ER stress markers, BIP and CHOP in the lung tissues. (a) The mRNA expression of BIP and CHOP were analysed by RT-PCR. (b)–(c) Western blotting measured the protein expression of BIP and CHOP. The relative density quantification is relative to β -actin. A, control group; B, model group; C, GBFXD-HD group (36 g/kg d^{-1}); D, GBFXD-MD group (24 g/kg d^{-1}); E, GBFXD-LD group (12 g/kg d^{-1}); F, montelukast group (2.6 mg $kg^{-1} d^{-1}$); G, dexamethasone group (1 mg $kg^{-1} d^{-1}$). Values represented as mean $\pm$ SD ($n=3$ per group). [#] $p < 0.05$ compared with the control group, and ^{*} $p < 0.05$ compared with the model group.

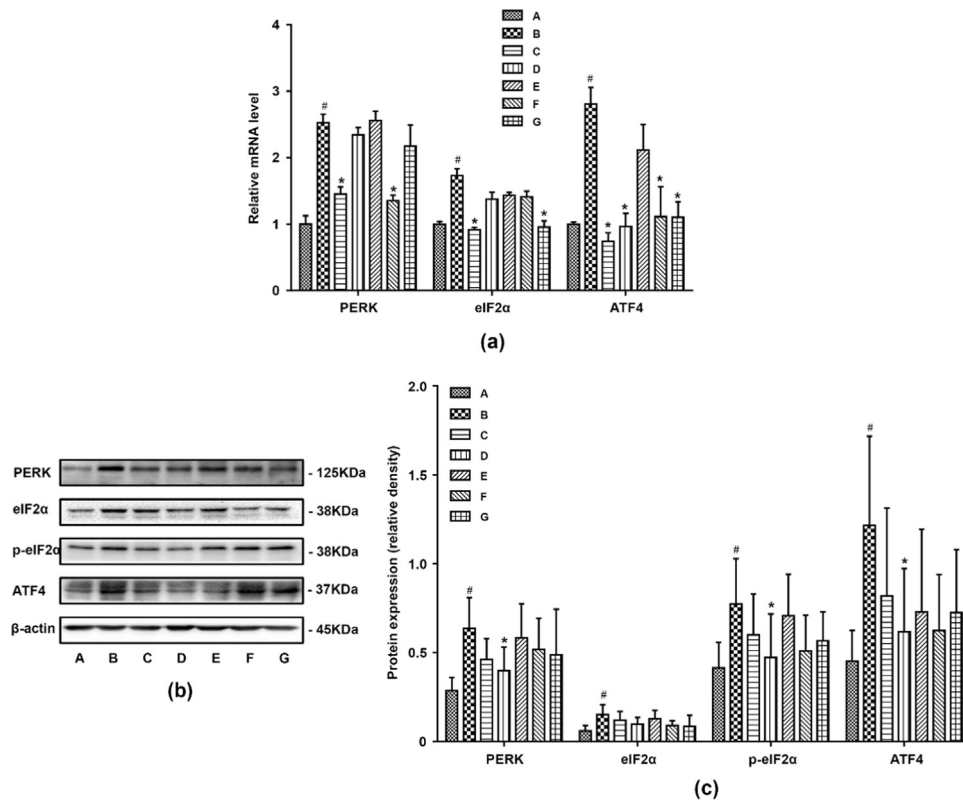


Fig. 8. Treatment with GBFXD suppressed the activation of PERK signal pathway. (a) The mRNA expression of PERK, eIF2α and ATF4 were analysed by RT-PCR. (b)–(c) Western blotting measured the protein expression of PERK, eIF2α, p-eIF2α and ATF4. The relative density quantification is relative to β-actin. A, control group; B, model group; C, GBFXD-HD group (36 g/kg d⁻¹); D, GBFXD-MD group (24 g/kg d⁻¹); E, GBFXD-LD group (12 g/kg d⁻¹); F, montelukast group (2.6 mg kg⁻¹ d⁻¹); G, dexamethasone group (1 mg kg⁻¹ d⁻¹). Values represented as mean ± SD (n=3 per group). #p < 0.05 compared with the control group, and *p < 0.05 compared with the model group.

components and the overexpression of *ORMDL3*, although these effects were inhibited after GBFXD treatment. 5) GBFXD deterred ER stress partially by blunting the activation of the PERK and the IRE1α pathways. Accordingly, GBFXD alleviated RSV-OVA-induced chronic airway inflammation by suppressing lung ER stress response linked to PERK-eIF2α-ATF4 and IRE1α-XBP1/IRE1α-JNK-AP1 signaling.

A remission of asthma is not identical to its cure. Emerging data demonstrate that even during asymptomatic periods, asthmatic patients still suffer from chronic airway inflammation (Locksley, 2010). In this study, we monitored an established RSV-OVA induced murine asthma remission model. Subsequently, we found that even in the absence of the RSV or OVA challenge for 30 days, the model group mice still exhibited a mild airway inflammation. The existence of airway inflammation in this model was demonstrated by the increased lung inflammatory cells and serum chemokines. These findings may give some experimental support to the hypothesis that early infection with RSV would induce a sustained airway inflammation in asthma remission patients (Piedimonte, 2013). However, whether this sustained airway inflammation was induced by the combination of an RSV-OVA challenge or merely by an OVA sensitisation remains to be answered and needs to be further investigated. A recent study demonstrated that RSV infections could induce ER response, leading to an activation of the ATF6 and the IRE1 pathways but not the PERK branch (Hassan et al., 2014). Nevertheless, the selective RSV-induced branches of the ER stress response in their research are not exactly in accordance with our findings. We found that RSV infections induce a non-canonical ER stress response with preferential activation of the PERK and the IRE1 pathways rather than the ATF6 branch. One explanation for these discrepancies could be that they simulated the microenvironment *in vitro*. Hence, the

response of their *in vitro* RSV infection tests may be different from our *in vivo* study. Moreover, the titer and the time points of the RSV infections, the hosts and the intervention methods used in the two studies are not the same. These results suggest that diverse conditions could activate certain UPR pathways leading to very different ER stress responses.

Genome-wide association studies (GWAS) uncovered that the *ORMDL3* locus is associated with asthma pathology (Moffatt et al., 2007). Besides, asthmatic patients exhibit increased *ORMDL3* levels (Toncheva et al., 2015). *ORMDL* proteins are ubiquitously expressed transmembrane proteins anchored in the endoplasmic reticulum (ER). Furthermore, *ORMDL3* has been shown to be critical for endoplasmic reticulum homeostasis by modulating the unfolded protein response and calcium balance. Enhanced *ORMDL3* expression was observed after exposure to human rhinovirus (HRV) or sensitisation with OVA (Caliskan et al., 2013; Miller et al., 2012). Although no evidence has been demonstrated, RSV infections could directly influence the expression of *ORMDL3*; there are two indirect clues that reveal the intricate linkages between them. Firstly, RSV infections were associated with marked changes in the metabolism of sphingolipids (Du et al., 2015). Sphingolipids are essential components of viral and cellular membranes (Spiegel and Milstien, 2003) and act as signaling mediators that regulate *ORMDL3* expression in allergic asthma mice (Oyeniran et al., 2015). Secondly, maternal OVA-tolerant mice infected with RSV exhibited an excessive Th2-type pathology (Castilow et al., 2007). It has been well described that Th2 cytokines could induce the overexpression of *ORMDL3* (Ha et al., 2013). For example, RSV infections induced interleukin (IL)-4 and IL-13 by IL-4 Rα/STAT6-dependent macrophages' differentiation (Shirey et al., 2010). In addition, STAT6 could directly regulate human *ORMDL3* expression (Qiu et al., 2013). These two clues indicate that

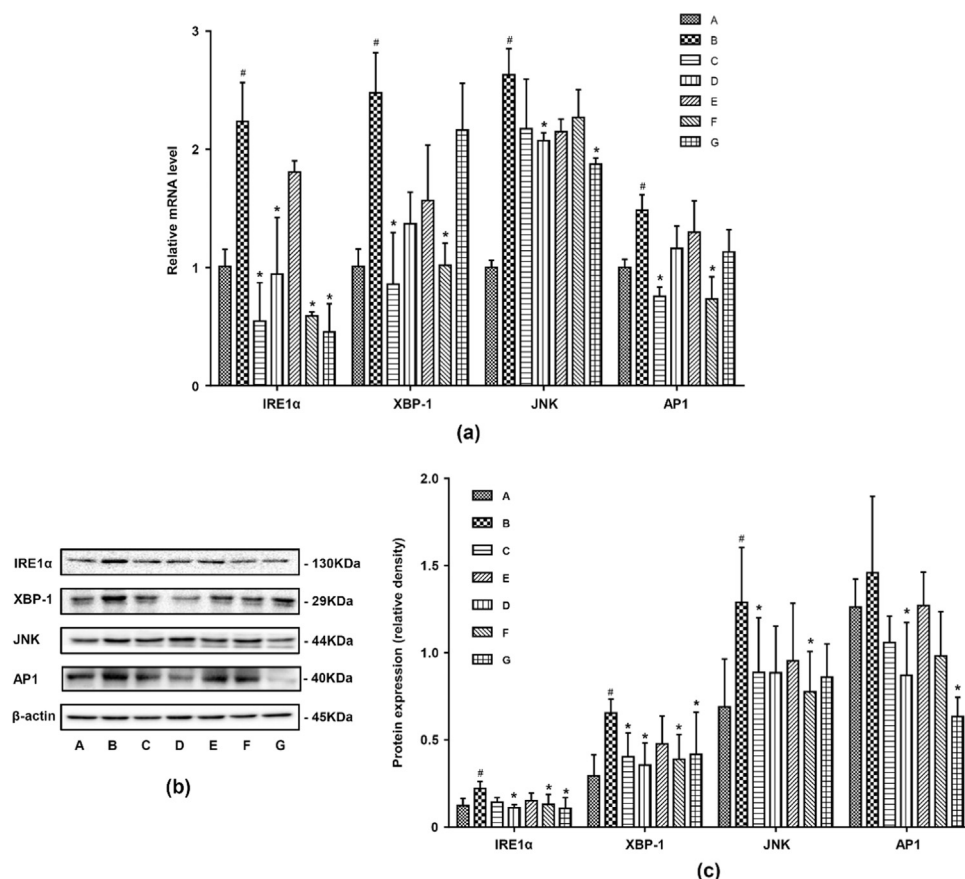


Fig. 9. Treatment with GBFXD restrained the activation of IRE1 α signal pathway. (a) The mRNA expression of IRE1 α , XBP-1, JNK and AP1 were analysed by RT-PCR. (b)–(c) Western blotting measured the protein expression of IRE1 α , XBP-1, JNK and AP1. The relative density quantification is relative to β -actin. A, control group; B, model group; C, GBFXD-HD group (36 g/kg d $^{-1}$); D, GBFXD-MD group (24 g/kg d $^{-1}$); E, GBFXD-LD group (12 g/kg d $^{-1}$); F, montelukast group (2.6 mg kg $^{-1}$ d $^{-1}$); G, dexamethasone group (1 mg kg $^{-1}$ d $^{-1}$). Values represented as mean $\pm$ SD (n=3 per group). #p < 0.05 compared with the control group, and *p < 0.05 compared with the model group.

respiratory infection with RSV may influence *ORMDL3* expression by modulating sphingolipids biosynthesis or inducing STAT6-dependent Th2-cell-deviated responses. Putting our findings together, we observed an increased expression of *ORMDL3* after RSV infections. However, we did not see a Th2-cell mediated airway inflammation at 30 days after RSV infections. Thus, the theory of Th2-mediated *ORMDL3* overexpression could not explain this finding. Moreover, it was difficult to ascertain whether the OVA-challenge is responsible for the *ORMDL3* expression. Therefore, the relationship between RSV infections and *ORMDL3* in this murine asthma remission model need to be further investigated.

Inhaled corticosteroids (ICS) are the first-line therapy for the treatment and long-term control of asthma. The therapeutic role of glucocorticoids (GCs) in asthma is mainly attributed to their anti-inflammatory effects. Furthermore, treatment of OVA-induced asthmatic mice with dexamethasone substantially reduced the increases in GRP78 and CHOP, ER stress markers (Kim et al., 2013). As a kind of leukotriene receptor antagonist, montelukast is used clinically as a maintenance therapy for asthma. Angiotensin II-induced leukotrienes C4 (LTC4) have been extensively studied in the context of asthma and are involved in ER stress-triggered oxidative stress, which can be abrogated by Montelukast (Dvash et al., 2015). Based on these studies and on our findings, DEX and Mont may alleviate airway inflammation in established asthma by reducing ER stress markers. However, asthmatic children who are exposed to long-term administration of ICS exhibit a 1.2 cm-lower mean adult height than normal subjects (Kelly et al., 2012). In this study, administration of DEX exhibited an immune suppressant effect by decreasing the number of splenic T lymphocytes. Moreover, we

observed a dramatic reduction in the average weight of the DEX group mice than in the other groups (data not shown). As an add-on therapy to inhaled corticosteroids, montelukast is commonly prescribed to pediatric asthma patients. However, recent clinical trials indicated that administration of montelukast could induce several adverse drug reactions and its curative effects in asthma were unsatisfactory (Calapai et al., 2014; Nwokoro et al., 2014). Hence, the current asthmatic therapies need to be further improved.

In contrast to the definition of asthma remission stage used in modern medicine, as an “asymptomatic period of more than a month,” practitioners of TCM believe that asthmatic children without clinical symptoms such as coughing, shortness of breath, and wheezing are in the remission stage. Moreover, pathogenesis in the majority of children in the remission stage involves Lung and Spleen Qi deficiency with Phlegm-fluid Retention. Hence, they are treated by invigorating Qi and consolidating the superficial, and strengthening the spleen and reducing phlegm. GBFXD was formulated according to this principle of treatment by supplementing the representative formulation of invigorating Qi and consolidating the superficial, “YPFS” and the representative formulation of strengthening the spleen and reducing phlegm, “Yi Gong Powder” with medicines that dispel the pathogenic wind and consolidate the superficial, and constrict the lung for relieving cough. Radix Astragali Preparata Cum Melle is the primary component that Invigorates the Qi of Lungs and Spleen; Atractylodis Macrocephalae Rhizoma, Codonopsis Radix, Poria, and Citri Pericarpium Reticulatae are ancillary components that strengthen the spleen and reduce phlegm; Saposhnikovia Radix, Magnoliae Flos,

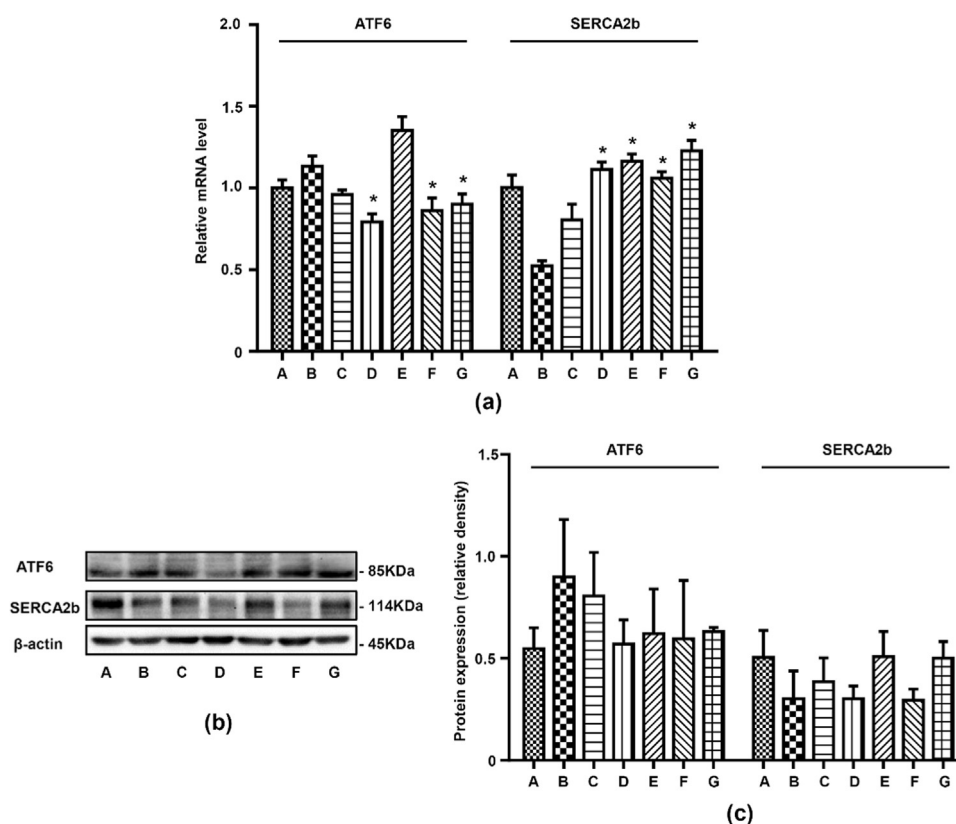


Fig. 10. Treatment with GBFXD modulated the expression of ATF6 and upregulated the level of SERCA2b. (a) The mRNA expression of ATF6 and SERCA2b were analysed by RT-PCR. (b)–(c) Western blotting measured the protein expression of ATF6 and SERCA2b. The relative density quantification is relative to β -actin. A, control group; B, model group; C, GBFXD-HD group (36 g/kg d^{-1}); D, GBFXD-MD group (24 g/kg d^{-1}); E, GBFXD-LD group (12 g/kg d^{-1}); F, montelukast group (2.6 mg $kg^{-1} d^{-1}$); G, dexamethasone group (1 mg $kg^{-1} d^{-1}$). Values represented as mean $\pm$ SD ($n=3$ per group). # $p < 0.05$ compared with the control group, and * $p < 0.05$ compared with the model group.

and Cicadae Periostracum are adjuvant components that promote resuscitation to eliminate pathogenic factors and facilitate the Qi of the lungs, while Schisandrae Chinensis Fructus Constricting Lung for Relieving Cough; Calcined Concha Ostreae is the guiding component that consolidates superficial for arresting sweating, while Glycyrrhiza Radix Et Rhizoma reconciles all components in the formulation. Taken together, all components complement each other to achieve the outcomes of invigorating Qi and consolidating the superficial, and strengthening the spleen and reducing phlegm. The quality control of GBFXD used in this study was undertaken using HPLC, and six reference chemicals were identified as major index components. Hesperidin and Liquiritin exhibited the highest concentrations, followed by Prim-o-glucosylcimifugin, Lobetyolin, Schisandrol A and Magnolin. Hesperidin had a profound inhibitory effect on airway inflammation by suppressing Th2 cytokines in a murine asthma model (Kim et al., 2011). The level of MUC5AC, a subtype of mucins produced during allergic airway inflammation, could be inhibited by Lobetyolin in airway epithelial cells (Yoon et al., 2014). Schisandrol A may have anti-allergic effects by acting as a 5-LOX inhibitory constituent (Lim et al., 2009). A recent study indicated that Magnolin suppressed a process of epithelial-to-mesenchymal transition (Lee et al., 2015), which likely contributes to airway remodeling in asthma (Hackett, 2012). Although the standard reference components from GBFXD contain a sort of anti-asthmatic effects, the explicit mechanisms need to be further elucidated.

In summary, the present study has confirmed that GBFXD suppresses RSV-OVA-induced chronic airway inflammation and downregulates *ORMDL3* expression as well as inhibits the activation of the ER stress-related IRE1 α and PERK signaling pathways. However, there are some questions in the current study that

require further clarification. (I) Although ER stress could be activated by *ORMDL3* and our findings showed that GBFXD significantly decreased the expression of ER stress markers, it is unclear whether the effects of this suppression are mediated by the inhibition of *ORMDL3* expression. (II) We established a murine asthmatic model to mimic human asthma remission by ceasing RSV-OVA sensitisation for 30 days. Even if there are no pulmonary function parameters to characterize this asthma remission murine model, we assumed that the sustained airway inflammation would still exist. This indirectly suggested that although the asthmatic patients exhibited nonsymptomatic or atypical pulmonary function characteristics, the anti-inflammatory treatments were still recommended for use. (III) The increased asthma remodeling was highly associated with the activation of ATF6, the UPR pathway transcription factor (Miller et al., 2014). Does inactivation of the ATF6 pathway cause the absence of airway remodeling in present murine asthma remission model? Further studies with selective overexpression or silencing of ATF6 in particular cell types will provide insight into the role of ATF6 in the pathogenesis of airway remodeling. Overall, our studies provide preliminary evidence for an *in vivo* mechanism to link ER stress response to the anti-inflammatory effects of GBFXD in asthma remission stage.

Acknowledgments

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