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Astragalus polysaccharide modulates ER stress response in an OVA-LPS induced murine model of severe asthma

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ABSTRACT

Endoplasmic reticulum (ER) stress has been recently revealed to play a pivotal role in the pathogenesis of severe asthma. Astragalus polysaccharide (APS), a major bioactive component from *Astragalus membranaceus*, exerts immunomodulatory and anti-inflammatory effects and has been shown to suppress ER stress in chronic diseases such as type-2 diabetes. However, the pharmaceutical application of APS in the treatment of severe asthma is unknown. The results obtained here indicate that APS significantly attenuates eosinophils and neutrophil-dominant airway inflammation by reducing the mRNA levels of *Cxcl5*, *Il8*, and chemokine (C-C motif) ligand 20 (*Ccl20*) and the protein levels of IL13RA and IL17RA. APS also inhibits the activation of unfolded protein response by decreasing the levels of ER stress markers such as C/EBP homologous protein (CHOP), which was associated with a reduction of PERK phosphorylation. Moreover, APS substantially blocks the nuclear translocation of ATF6 and NF- κ B p65. Interestingly, we observed that APS markedly suppresses mucus hypersecretion by decreasing the levels of mucin (MUC) 5AC and MUC5B, which might be due to inhibition of goblet cells differentiation by suppressing the expression of IRE1 β -correlated genes. In summary, APS can have potential pharmaceutical application in treatment of severe asthma.

Keywords: severe asthma, astragalus polysaccharide, endoplasmic reticulum

1. Introduction

Asthma is a common, heterogeneous, respiratory disease characterised by wheezing, shortness of breath, chest tightness and/or coughing, excessive variability in lung function, and expiratory limitation. Although asthma was originally thought to be related to allergies and to involve eosinophils and a dysregulated T helper type 2 (Th2) inflammatory response, there is growing evidence that severe asthma (steroid-insensitive, steroid-resistant, and refractory) does not necessarily exhibit the typical symptoms associated with a classic allergic asthmatic condition [1, 2]. Numerous studies have revolved around understanding steroid resistance in severe asthma pathophysiology, and recent studies have demonstrated that steroid-resistant asthma patients often exhibit neutrophilic airway inflammation [3] due to several inflammatory mediators, including chemokine (C-X-C motif) ligand 5 (CXCL5), interleukin-8 (IL8), and interleukin-17 (IL17) [4-6]. However, our understanding of the underlying mechanisms of the immune response to neutrophil-dominant airway inflammation in severe asthmatics is limited.

The endoplasmic reticulum (ER) acts as a protein-folding factory; it is essential for synthesising functional proteins and transporting secretory and biological membrane proteins in vesicles to the Golgi apparatus [7]. Cells initiate an adaptive feedback mechanism called the unfolded protein response (UPR) to overcome excessive ER stress and restore the functional homeostasis of the

ER. Three protein sensors are localised in the ER membrane and mediate the UPR: double-stranded RNA-activated protein kinase RNA-like ER kinase (PERK); inositol-requiring enzyme 1 (IRE1); and activating transcription factor 6 (ATF6) [8]. Even though there is limited evidence of a direct connection between steroid resistance and airway neutrophilic inflammation, the authors of a recent study proposed that the modulation of ER stress can surmount the refractoriness of neutrophil-dominant asthma to steroids, indicating an emerging molecular mechanism for severe asthma that involves the ER stress response [2]. Therefore, restoration of the functional equilibrium of the ER may be a prospective target for the treatment of severe asthma.

Astragalus polysaccharide (APS) can be extracted from *Astragalus membranaceus*, and has been identified as that herb's major bioactive component. Recent studies indicate that APS may play a role in asthma therapy by regulating immune cell function and cytokine expression [9], as well as by enhancing anti-inflammatory activity [10] and reducing airway remodelling [11, 12]. Furthermore, emerging data have indicated that APS can reduce ER stress and enhance the adaptive capacity of the ER both *in vivo* and *in vitro* [13, 14]. Although APS has attracted much attention in the treatment of some chronic diseases, it remains unclear whether it has similar benefits in severe asthma therapy. Given that severe asthma is correlated with an excessive ER stress response and that APS has modulatory activities on ER function, the aim of the

present study was to determine the impact of APS on ER stress in an ovalbumin–lipopolysaccharide (OVA-LPS)-induced murine model of severe asthma.

2. Materials and methods

2.1 Animals and ethics statement

Four-week-old female specific pathogen-free BALB/c mice (16–18 g) were purchased from Vital River Laboratories Co., Ltd., Beijing, China. The animals were housed in the experimental animal centre 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 sterilised before use. The animals were acclimatised for 7 days prior to initiating the experiments. All experimental procedures were performed in accordance with the National Institutes of Health Guidelines for Laboratory Animals, and were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine [No. SYXK (Su) 2014–0001].

2.2 OVA-LPS sensitisation and challenge

Fig. 1a schematically depicts the protocols used in this study, which were based on a previous report [2]. Sixty specific pathogen-free female BALB/c mice were randomly divided into five groups: a control group; an OVA (Grade V, Sigma-

Aldrich, St Louis, MO, USA) + LPS (Sigma-Aldrich) group; an OVA + LPS + APS (purchased in a hazel-coloured and water-soluble powder form with $\geq 98\%$ purity, Yuanmu Technology Co., Ltd. Shanghai, China) group; an OVA + LPS + 4-phenylbutyric acid (4-PBA, Sigma-Aldrich) group; and an OVA + LPS + dexamethasone (DEX, Sigma-Aldrich) group. There were no significant differences in the weights of the animals among the groups prior to the start of the experiments. For the asthmatic models, mice in the OVA + LPS, OVA + LPS + APS, OVA + LPS + 4-PBA, and OVA + LPS + DEX groups were sensitised on Days 0, 7, and 14 by the intraperitoneal (i.p.) injection of 100 μg OVA emulsified in 1 mg aluminium hydroxide gel (InvivoGen, San Diego, CA, USA) in a total volume of 200 μL . On Days 15, 17, and 19 after the initial sensitisation, the mice were challenged intranasally (i.n.) with OVA_{LPS} (50 μg of OVA combined with 1 μg of LPS in saline) in a total volume of 50 μL . On Days 21, 23, and 25 after initial sensitisation, the mice were challenged for 30 min with an aerosol form of 3% (wt/vol) OVA in saline or with saline as a control.

2.3 APS, chemical chaperone 4-PBA, and DEX administration

A previous *in vivo* study [15] showed that APS supplementation at 100 mg/kg body weight per day elicited a balanced Th1/Th2 response and consequently exerted a beneficial immune regulatory effect, suggesting its potential benefit in the treatment of asthma. Moreover, this treatment did not induce any adverse

effects. Therefore, to examine the therapeutic effects of APS in established asthma, each of the treated mice was administered APS (100 mg/kg body weight per day) and DEX (1 mg/kg body weight per day) (as a positive control) twice by gavage, once 30 min before the first OVA challenge, and again 30 min before the last OVA challenge.

4-PBA (80 mg/kg body weight per day) diluted with phosphate-buffered saline (PBS) at 38 °C was administered twice by i.p. injection to each treated animal, once 1 h before the fourth challenge with OVA_{LPS} and again 6 h after the last challenge in OVA_{LPS} mice, as previously described [2]. The control group was sensitised and challenged with saline solution using the same protocol. The mice were sacrificed 48 h after the last challenge, and bronchoalveolar lavage fluid (BALF) and lung tissues were collected for analysis.

2.4 BALF collection and differential cell counts

Briefly, the mice were anaesthetised by i.p. injection of 400 mg/kg chloral hydrate 48 h after the final challenge; BALF was collected by lavage with 3X 400 µL ice-cold PBS, and 85–90% of the lavage volume was recovered via a tracheal catheter. Thereafter, all lavage samples were centrifuged at 150 *g* for 10 min at 4 °C. The total number of inflammatory cells in the BALF was counted using a chamber slide (Invitrogen, Carlsbad, CA, USA) and the differential cell counts were obtained using Diff-Quick staining for morphology. The number of

cells in the BALF was determined by two independent investigators in a single-blind study, and at least 200 cells were analysed from three different random locations using a microscope. Thereafter, the supernatant was collected, divided into three equal portions, and frozen at -80°C for enzyme-linked immunosorbent assay (ELISA).

2.5 Lung histopathology

Following BALF and serum collection, the lung tissue slices were fixed with 10% neutral-buffered formalin. A series of 3–4- μm microsections 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 = most of the large airways, bronchi, or vessels were surrounded by a thin 1–2-cell layer, a 3–5-cell moderate layer, or a thick >5 -cell layer of inflammatory cells, respectively. Periodic acid–Schiff (PAS) staining was used to quantify airway goblet cells, and Masson’s trichrome staining was used to visualise collagen deposition and fibrosis. Both staining methods were scored as follows: 0 = none, 1 = $<25\%$, 2 = $25\text{--}50\%$, 3 = $50\text{--}75\%$, and 4 = $>75\%$ goblet cells [16]. Histopathology was assessed in a double-blind manner by two independent researchers.

2.6 Measurement of OVA-specific IgE in the serum and BALF

Serum samples were obtained by the centrifugation of peripheral blood at 850 *g* and 4 °C for 10 min, and stored at –80 °C. The levels of OVA-specific IgE in the serum and BALF were measured by ELISA (Xitang Biological Pharmaceutical Co., Shanghai, China) according to the manufacturer's instructions.

2.7 Quantification of MUC5AC and MUC5B in the lung homogenate and BALF

Lung samples from each animal were lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Haimen, China) containing phenylmethanesulphonyl fluoride (PMSF) (Beyotime) and PhosStop (Boster, Wuhan, China). The lysed samples were centrifuged for 30 min at 13,000 *g* to remove debris. MUC5AC and MUC5B were quantified by ELISA according to the manufacturer's instructions.

2.8 Real-time reverse transcription polymerase chain reaction (RT-PCR) assay

Total RNA from the lung tissue was extracted using TRIzol reagent (Takara, Dalian, China), and cDNA was synthesised using the Prime Script® RT reagent kit with gDNA Eraser (Takara, Dalian, China), according to the manufacturer's instructions. Real-time RT-PCR was carried out on the cDNA samples using SYBR® Premix Ex Tap™ II (Takara). The primers used 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 carried out in triplicate and repeated three times. The PCR data were analysed using the QuantStudio 7 Flex detection system (Applied Biosystems Co., Foster City, CA, USA). Cycle threshold (C_T) values were analysed using the comparative C_T ($\Delta\Delta C_T$) method, and the relative amount of target mRNA ($2^{-\Delta\Delta C_T}$) was obtained by normalising to endogenous *Gapdh* levels.

2.9 Nuclear and cytoplasmic protein extraction from lung tissues

Nuclear and cytoplasmic extractions were performed using the Nuclear Extraction Kit (Epigentek, Farmingdale, NY, USA) according to the manufacturer's instructions. Small pieces of lung tissues were lysed with Nuclear Extraction Reagent 1, incubated on ice for 15 min, and centrifuged at 13,000 *g* and 4 °C for 10 min; the supernatant was then collected as a cytosolic extract. The pellet was resuspended in Nuclear Extraction Reagent 2 containing a protease inhibitor cocktail and dithiothreitol. The supernatant was collected as a nuclear extract following centrifugation at 16,000 *g* and 4 °C for 10 min.

2.10 Western blotting

Analysis of binding immunoglobulin protein (BIP), C/EBP homologous protein (CHOP), PERK, p-PERK, IRE1 α , IRE1 β , eIF2 α , ATF4, SAM pointed domain

ETS factor (SPDEF), mouse calcium-activated chloride channel regulator 3 (mCLCA3), anterior gradient 2 (AGR2), IL13RA, and IL17RA proteins was carried out using whole cell lysates, whereas analysis of ATF6 and nuclear factor κ B (NF- κ B) p65 was carried out on both the cytosolic and nuclear extracts. Protein concentrations in the lung samples were measured using a bicinchoninic acid (BCA) protein concentration assay kit (Thermo Fischer Scientific, Waltham, MA, USA). The total protein was mixed with 2X electrophoresis sample buffer (Bio-Rad, Hercules, CA, USA), denatured at 100 °C for 5 min, and then stored at -80 °C. Total proteins were separated by 8–12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. After electrophoresis, the separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (0.2 or 0.45 μ m, Millipore, Billerica, MA, USA), blocked with 5% bovine serum albumin in Tris-buffered saline with 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 anti-IRE1 β (Santa Cruz Biotechnology Inc., Texas, USA), anti-BIP, anti-PERK, anti-IRE1 α , anti-eIF2 α , anti- β -actin (Cell Signaling Technology Inc., Danvers, USA), anti-ATF6, anti-AGR2, anti-mCLCA3, anti-SPDEF, anti-p-PERK (Abcam Ltd., Cambridge, USA), anti-CHOP, anti-ATF4, anti-IL13RA, anti-IL17RA (Ruiying Biology Co., Suzhou, China), and anti-histone H3 (Abbkine Inc., California, USA) overnight at 4 °C. We used goat anti-rabbit horseradish peroxidase-conjugated IgG (Cell

Signaling Technology Inc.) or goat anti-mouse horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology Inc.) to detect antibody binding. The target proteins were visualised using a ChemiDoc™ MP Imaging system (Bio-Rad Co.) and analysed using Image Lab software (Bio-Rad Co.).

2.11 Statistical analysis

The data are expressed as mean $\pm$ standard error of the mean (SEM). All tests were performed using Prism 6.0 (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test to determine the statistical significance. Differences were considered to be significant at $p < 0.05$.

3. Results

3.1 Effects of APS supplementation on the growth of OVA_{LPS}-challenged mice

APS supplementation had no effect on the feed intake of the mice. The body weights of the APS group and control group mice during the aerosol OVA challenge period are given in Fig. 1b. There were no significant differences in mean body weight between the APS and control groups at the same experimental time points.

3.2 APS attenuated airway inflammation and mucus hypersecretion in OVA_{LPS}-sensitised mice

To confirm the establishment of the murine asthma model, we carried out pathological examination of the lung tissues by H&E, PAS, and Masson's trichrome staining, and determined the number of inflammatory cells in the BALF 48 h after the last OVA_{LPS} challenge. Compared with the control group, the model group mice exhibited severe airway inflammatory responses, including extensive infiltration of neutrophils and eosinophils around the large airways, bronchioles, and vessels (Fig. 2a and Fig. 3a) and into the BALF (Fig. 3c). Treatment with APS, 4-PBA, or DEX suppressed the infiltration of inflammatory cells to varying degrees, and induced a remarkable decrease in the total cell counts and in the number of eosinophils, neutrophils, and macrophages compared with the untreated asthmatic mice. These results were confirmed by H&E analysis and inflammation scores. Mice treated with APS and DEX had fewer inflammatory scores compared with the model group mice, whereas 4-PBA did not cause such dramatic decreases (Fig. 2b and Fig. 3b). These findings indicate that the administration of APS during the OVA_{LPS} challenge attenuated the inflammatory responses in the asthmatic airways.

The extent of mucus production and the number of goblet cells were assessed by PAS staining, and the percentages of PAS-positive cells in the large airways and the bronchioles were also evaluated. We observed that the OVA_{LPS}-

challenged mice developed marked goblet cell hyperplasia and mucus hypersecretion in the lumen of the large airways and the bronchioles (Fig. 2a-b and Fig. 3a-b). The APS-treated animals had fewer goblet cells in the airway epithelium, and the mucus scores in this group were significantly reduced. However, no substantial reduction in mucus scores in the large and small airways were observed in the 4-PBA- and DEX-treated mice.

The area of collagen deposition/fibrosis was assessed by Masson's trichrome staining. Collagen deposition was enhanced in the interstitium of the large and small airways in the model group compared with the control group. Airway fibrosis was significantly ameliorated by the administration of APS, as determined by a score of 1.0 ± 0.26 ($p < 0.05$). The 4-PBA-treated mice also showed significantly less fibrosis compared with the untreated asthmatic mice. However, no significant reduction in collagen deposition in the small airways was observed in the DEX-treated mice (Fig. 3a-b).

3.3 APS reduced the levels of OVA-specific IgE in both the BALF and serum

The OVA-specific IgE levels in the BALF and serum were notably elevated by the airway challenge with OVA_{LPS} (Fig. 4). These elevations were markedly reversed following administration of DEX. In comparison with the model group, administration of APS markedly reduced the levels of OVA-specific IgE in the BALF ($p < 0.05$). Additionally, we observed a slight decrease in the levels of

OVA-specific IgE in the serum after APS treatment ($p > 0.05$). These findings indicate that APS can inhibit allergic airway reactions by reducing the OVA-specific IgE levels in the OVA_{LPS}-induced murine asthma model.

3.4 APS suppressed mRNA levels of inflammatory mediators and protein levels of IL13RA and IL17RA in the lung tissue

To evaluate airway inflammation in the lung, we assessed the mRNA levels of a range of inflammatory mediators: *Il4*, *Il5*, *Il6*, *Il8*, *Il10*, *Il13*, *Il17*, *Il33*, chemokine (C-X-C motif) ligand 5 (*Cxcl5*), *Cxcl10*, *Cxcl11*, and chemokine (C-C motif) ligand 20 (*Ccl20*). Consistent with the extensive infiltration of eosinophils and neutrophils observed in the BALF, mRNAs encoding the cytokines and chemokines that mediate allergic inflammation in the lung tissues, including those mediating dendritic cell activation (*Il33*), were significantly increased after the OVA_{LPS} challenge. Likewise, Th2 cytokines (*Il4*, *Il5*, and *Il13*), Th17 cytokines (*Il17*), eosinophilic chemoattractants (*CCL20*), neutrophilic chemoattractants (*Il8* and *CXCL5*), and anti-inflammatory cytokines (*Il6* and *Il10*) were increased (Fig. 5). Administration of APS significantly reduced the mRNA levels of *Cxcl5*, *Il8*, and *Ccl20* in the lung tissues compared with the model group ($p < 0.05$), indicating that APS probably suppressed airway inflammation by inhibiting the recruitment of eosinophils and neutrophils to the respiratory tracts.

As shown in Fig. 5, *Ilg* mRNA levels in the 4-PBA group were substantially lower than those in the model group mice ($p < 0.05$), and treatment with DEX restrained the *Ilg* and *Ccl20* mRNA levels following the OVA_{LPS} challenge. Administration of APS induced the expression of Th2 cytokines (IL4, IL5, IL13, and IL33) at the post-transcriptional level following the OVA_{LPS} challenge, which is partially consistent with a previous report that demonstrated that APS induced the expression of Th2 cytokines (IL4, IL5) at a post-transcriptional level in non-obese diabetic mice [17]. In the pathological state of severe asthma, IL13 is crucial for a mucous metaplastic response, and IL17 is an effector cytokine in neutrophilic inflammation [18, 19]. Because the transmembrane receptor is the major executor and initiator of cellular biological function, we assessed the expression of IL13 receptor A (IL13RA) and IL17 receptor A (IL17RA) at the post-translational level. As shown in Fig. 5d, administration of APS and 4-PBA significantly decreased the expression of IL13RA and IL17RA proteins following the OVA_{LPS} challenge, which was in accordance with the trend observed in the BALF cell counts. These findings indicate that APS inhibits the activation of IL13RA in the mucous metaplastic response and inhibits IL17RA in neutrophilic inflammation.

3.5 APS decreased the ER stress marker CHOP and inhibited activation of the PERK pathway in inflamed lung tissues

ER stress and associated signalling pathways are emerging as significant inflammatory and immune response modulators in the pathogenesis of allergen-induced bronchial asthma, especially in neutrophil-dominant asthma [2]. Accordingly, we hypothesised that ER stress activation was probably involved in OVA_{LPS}-induced airway inflammation. We therefore assessed the roles of two major markers (CHOP and BIP) and three canonical protein sensors (IRE1 α , PERK, and ATF6) that recognise enhanced ER stress responses. Consistent with the previous report [2], the protein expression of the ER stress markers BIP and CHOP was significantly increased following the OVA_{LPS} challenge (Fig. 6b). However, the mRNA levels of the ER stress markers were not significantly increased (Fig. 6a). When assessed at the transcriptional level, the levels of *Perk*, *Atf6*, and *Ire1 α* in the model group mice were nearly twofold lower than those in the control group ($p < 0.05$). Furthermore, oral administration of APS, 4-PBA, and DEX markedly repressed CHOP protein levels ($p < 0.05$) compared with the model group. Upregulation of CHOP expression can be a result of the activation of PERK and its resulting phosphorylation [20]. Therefore, we assessed the phosphorylation of PERK and measured the levels of eukaryotic initiation factor 2 α (eIF2 α) and activating transcription factor 4 (ATF4), which are correlated to the PERK–CHOP pathway

[21]. In contrast to the results for total PERK expression, the expression levels of phosphor-PERK, eIF2 α , and ATF4 were significantly increased in the model group, indicating that the PERK pathway is coupled to increased CHOP expression. As shown in Fig. 6c, activation of the PERK pathway was substantially inhibited by APS and DEX treatment.

3.6 APS blocked the nuclear translocation of ATF6 and NF- κ B p65 in lung tissues

ATF6 consists of an ER luminal domain and a cytoplasmic domain. As the unfolded protein accumulates in the ER, the cytoplasmic domain moves to the nucleus, which is the only location in which ATF6 becomes functional [22]. As shown in Fig. 7a, ATF6 translocated from the cytoplasm to the nucleus following the OVA_{LPS} challenge. Oral treatment with APS, 4-PBA, and DEX significantly inhibited the nuclear translocation of ATF6. Along with the results of the inhibition of the PERK pathway, these findings suggest that APS might attenuate OVA_{LPS}-induced airway inflammation by inhibiting the activation of ER stress response.

Considering the link between excessive ER stress and NF- κ B activation in the pathogenesis of severe asthma [2, 23], we determined the nuclear translocation of NF- κ B p65 to elucidate the link between the expression of inflammatory cytokines. The NF- κ B p65 levels in nuclear and cytoplasmic extracts of the lung

tissue were significantly increased in the OVA_{LPS}-challenged mice compared with those in the control group ($p < 0.05$) (Fig. 7b). In addition, APS, 4-PBA, and DEX blocked NF- κ B p65 nuclear translocation. Interestingly, a marked decline of the NF- κ B p65 level was also observed in the cytoplasmic extracts from the APS- and DEX-treated animals, indicating that APS and DEX inhibited the expression of NF- κ B p65 at the whole cell level. Moreover, several previous studies have demonstrated that the activation of NF- κ B is implicated in IL13 and IL17 receptor signalling [24, 25], which might explain the consistency between the inhibition of NF- κ B p65 nuclear translocation and the reduction of IL13RA and IL17RA protein levels after APS treatment.

*3.7 APS restrained the expression of IRE1 β -correlated genes (*Spdef*, *mClca3*, and *Agr2*) related to mucus production*

IRE1 β , an isoform of IRE1 α , has been reported to play a crucial role in mucin production in an OVA-induced murine model of asthma [26]. In the present study, in contrast to IRE1 α , expression of IRE1 β was substantially higher in the model group compared with the control group ($p < 0.05$) (Fig. 8). Oral administration of APS, but not DEX or 4-PBA, significantly reduced the *Ire1 β* mRNA levels in OVA_{LPS}-challenged asthmatic mice ($p < 0.05$). IRE1 β protein expression in the APS and DEX groups was significantly lower than in the model group ($p < 0.05$). Based on Celsius analysis [27], a community resource

for Affymetrix microarray data, *Ire1 β* gene expression is highly correlated with the expression of genes (*Spdef*, *mClca3*, and *Agr2*) involved in goblet cell differentiation and mucin production [26]. SPDEF, mCLCA3, and AGR2 mRNA and protein levels showed marked upregulation in the lung samples of OVA_{LPS}-challenged mice compared with those of the controls ($p < 0.05$). Oral administration of APS substantially reduced the *Spdef*, *mClca3*, and *Agr2* mRNA expression levels, as well as the protein expression of SPDEF ($p < 0.05$). Furthermore, treatment with either 4-PBA or DEX suppressed the expression of these *Ire1 β* -correlated genes to varying degrees. Based on the Celsius data and our findings, we inferred that APS might reduce airway mucus hypersecretion by restraining the expression of *Ire1 β* -correlated genes.

3.8 APS inhibited the mucins MUC5AC and MUC5B

The mucins MUC5AC and MUC5B dominate the characteristic biophysical properties of the airway mucus [28]. To determine the inhibitory effects of APS on airway mucus hypersecretion, we determined MUC5AC and MUC5B levels in the lung homogenate or the BALF. As shown in Fig. 9, the concentrations of MUC5AC or MUC5B in the BALF and of MUC5B in the lung homogenate of the model group were significantly higher than those in the control group ($p < 0.05$). The MUC5AC and MUC5B levels in the BALF in the APS- and DEX-treated groups were significantly reduced compared with those in the model group (p

< 0.05). Moreover, administration of DEX significantly reduced the MUC5B level in the lung homogenate compared with that in the model group ($p < 0.05$). However, no significant differences in MUC5AC concentration in the lung homogenate were observed between the groups.

4. Discussion

In the present study, we demonstrated that mice challenged with OVA_{LPS} exhibited eosinophil- and neutrophil-mediated airway inflammation, mucus hypersecretion, and substantial collagen deposition, consistent with the phenotype of the murine model of severe asthma described in a previous report [2]. The first finding was that APS inhibited the recruitment of eosinophils and neutrophils to the respiratory tracts by reducing *Cxcl5*, *Il8*, and *Ccl20* mRNA levels and by decreasing the protein expression of IL13RA and IL17RA. The second finding was that the OVA_{LPS} challenge induced ER stress responses consistent with the overexpression of *Ire1 β* -correlated genes and increased mucin concentrations, although these effects were partially inhibited by APS treatment. Therefore, APS might ameliorate OVA_{LPS}-induced severe asthmatic syndrome by alleviating neutrophil-dominant airway inflammation and by suppressing mucus hypersecretion, which is linked to a reduction of the ER stress response and *Ire1 β* -correlated genes.

Severe asthma was defined by the presence of poor asthma control (Juniper

Asthma Control Questionnaire (ACQ) score ≥ 1) or by airflow obstruction (FEV1% predicted < 80 , FEV1/FVC% < 70), despite treatment with high-dose inhaled corticosteroid (ICS) (≥ 1000 μg inhaled beclomethasone equivalent per day) along with long-acting β -agonist [29]. Previous reports have indicated that the underlying biology of the neutrophilic phenotype is linked to asthma severity [30, 31]. To illustrate this, clinical investigations have shown that the pathobiological parameters associated with severe asthma diagnosis are neutrophilic sputum and a rise in peripheral blood neutrophil counts [32]. Patients with severe asthma also appear to have a greater mucus secretory response [33]. In this study, we established a murine asthmatic model to mimic severe asthma in humans through OVA and LPS induction, as previously described [2], and also established that the murine respiratory tracts responded to these challenges by exhibiting neutrophil-dominant airway inflammation and mucus hypersecretion.

Under healthy conditions, mucus is secreted to cover the airway and protect against inhaled material, and is removed by mucociliary clearance. Although acute mucus secretion is an effective airway defence, pathologic mucus hypersecretion is a more sustained response, especially when associated with goblet cell hyperplasia [34]. Goblet cell hyperplasia and the accompanying mucus hypersecretion are pathophysiological hallmarks of asthma [35]. MUC5AC and MUC5B, the major macromolecular components of lung

secretory gel-forming mucin glycoproteins [36], are typically restricted to the goblet and glandular cells in the conducting epithelia of diseased airways [37, 38]. Furthermore, *ex vivo* cultures of fully differentiated bronchial epithelium tissue from severe asthma patients produce abundant mucin [39], indicating that mucus overproduction is highly correlated with asthma severity.

In this study, we observed that the mRNA levels of genes (*Ire1 β* , *Spdef*, *mClca3*, and *Agr2*) directly related to goblet cell hyperplasia were substantially increased by the OVA_{LPS} challenge. This observation strengthens the notion that mucin overproduction requires increased protein folding; such proteins could be processed and synthesised in the ER. A recent study demonstrated that MUC5AC protein production was induced following exposure to the ER stressor tunicamycin *in vitro*, implying that ER stress causes mucus hypersecretion in the airway [40]. In mammals, the ER stress sensor IRE1 has diverged into two paralogues, IRE1 α and IRE1 β [41, 42]. In contrast to the mammalian IRE1 α , the IRE1 β -mediated luminal event involves a direct interaction with unfolded proteins rather than a connection with BIP [43], which might explain the increased *Ire1 β* mRNA levels, as opposed to the *Ire1 α* or *Bip* levels observed in this study. IRE1 β expression, detected in both mouse and human airway goblet cells [26], improves the efficiency of protein folding by affecting the level of mRNA that encodes the major secretory product MUC2 [44]. SPDEF, another transcriptional regulator of the airway goblet cell metaplasia, inhibits LPS-

induced neutrophilic infiltration and bacterial clearance [45]. mCLCA3 is ubiquitously expressed in mucus cells and is linked to inflammatory respiratory diseases associated with mucus overproduction, such as asthma [46]. mCLCA3-deficient mice exhibit decreased numbers of neutrophils infiltrating the BALF during *Staphylococcus aureus* infection [47], suggesting that mCLCA3 imparts a dual effect on neutrophilic airway inflammation and mucus hypersecretion. The ER protein AGR2, a member of the disulphide isomerase protein family, increases with the overproduction of the airway mucins MUC5AC and MUC5B, and a loss of AGR2 is correlated with ER stress in the airway epithelium [48]. Although these goblet cell hyperplasia-related genes were induced in the OVA_{LPS}-sensitised mice, the direct/indirect interactions between IRE1 β and its associated genes (*Spdef*, *mClca3*, and *Agr2*) require further investigation.

ICS is the first line of therapy for the treatment and long-term control of clinical asthma. Moreover, glucocorticoids (GCs) are usually used as the representative standard therapeutic agents of experimental asthma. The authors of a previous study proposed that DEX reduces the expression of MUC5AC, and that this reduction is mediated by GC response elements and receptors [49]. However, the authors of another study found that the treatment of OVA_{LPS}-induced asthmatic mice with DEX had no significant effect on alleviating the neutrophil-dominant airway inflammation or ER stress responses,

suggesting that OVA_{LPS}-induced mice exhibit the steroid-resistant characteristics of either refractory or severe asthma [2]. Moreover, frequent oral corticosteroids use in children with asthma leads to severe decreases in bone mineral accretion [50].

4-PBA, a chemical chaperone with an inhibitory effect on ER stress responses, is currently undergoing clinical trials and shows promise as a candidate for future investigation as a novel therapy for asthma [51]. Furthermore, a recent report showed that PBA alleviates ER stress-mediated epithelial-mesenchymal transition in the pathogenesis of bleomycin-induced pulmonary fibrosis [52], suggesting that PBA could potentially be used in asthma therapy by airway remodelling. In contrast to DEX, 4-PBA significantly attenuated the neutrophil-dominant asthmatic features through inhibition of NF- κ B activation in a mouse model [2], which is consistent with our findings. Nevertheless, the evidence also demonstrated that the anti-mucus overproduction effects of 4-PBA on respiratory diseases is rare and that 4-PBA could bring about gastrointestinal side effects, including epigastric discomfort, nausea, and diarrhoea [53]. In this study, we did not observe inhibition of mucus hypersecretion by 4-PBA in the OVA_{LPS}-challenged mice (Fig. 2b, Fig. 3b, and Fig. 9).

APS possesses several properties that can be exploited for designing asthma treatments. First, APS exhibits a protective effect on LPS-infected Caco-2 cells by repressing inflammatory indicator expression and maintaining monolayer

integrity [10], indicating that APS may have anti-inflammatory and structure-stabilising benefits for the airway epithelium. Second, APS enhances the expression of Th1- and Th2-specific transcription factors and supports a shift towards the Th2 phenotype, suggesting that it exerts a beneficial immune regulatory effect [54]. This finding may explain why the mRNA expression of Th2 cytokines was further induced by APS treatment in our study. Moreover, we observed that APS treatment substantially reduced the protein expression levels of IL13RA and IL17RA. Because transmembrane receptors are the primary executors and initiators of cellular biological function, we infer that APS inhibits Th2 response, although it induced mRNA expression of Th2 cytokines in our study. Third, APS enhances epidermal growth factor-like domain 7 (EGFL7) expression in neonatal rats with bronchopulmonary dysplasia, implying that APS may potentially be used as a therapeutic strategy for airway remodelling [11]. Finally, there has been no study on the adverse effects of APS compared with 4-PBA or DEX. Taken together, we have observed alleviated neutrophilic airway inflammation, decreased levels of mucus secretion, inhibition of the ER stress response, and downregulation of goblet cell hyperplasia-related genes after APS treatment in the OVA_{LPS}-challenged asthmatic mice, which reinforces the potential use of APS as an ancillary therapeutic agent in asthma treatment.

To the best of our knowledge, this is the first report demonstrating the inhibitory

effects of APS on mucus overproduction in an experimental asthma model. Two further questions arose as a result of this study, and they require further elucidation. First, the expression of the genes encoding BIP and CHOP proteins, which increased in abundance by at least twofold, did not exhibit a similar trend at the transcriptional level after the OVA_{LPS} challenge in the lung tissue. Because ER acts as a protein-modification organelle, and based on the theory that mRNA–protein correlation is not strictly linear in nature [55], we hypothesise that the ER stress markers in this severe asthmatic murine model have further intrinsic and complex mechanisms that can differentially affect the amounts of mRNA and proteins. Second, the increased airway mucus hypersecretion was closely associated with the activation of the ER stress transducer IRE1 β [26]. It is not clear whether APS, directly or indirectly, inhibited the activation of IRE1 β , causing the absence of mucus overproduction in the present murine severe asthma model. Further studies with selective overexpression or silencing of IRE1 β in particular APS-intervened cell types will provide insight into the role of IRE1 β in the pathogenesis of airway mucus hypersecretion. In conclusion, the results of this study provide preliminary evidence for an *in vivo* mechanism to link the ER stress response to the anti-neutrophilic airway inflammation and anti-mucin hypersecretion effects of APS in severe asthma.

Conflicts of interest

The authors confirm that there are no conflicts of interest pertaining to the material in this manuscript.

Acknowledgements

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Fig. 1. (a) Experimental protocol of OVA-LPS sensitisation/challenge in mice as described in the Materials and methods section. i.p.: intraperitoneal injection; i.n.: intranasal administration. (b) The body weights of APS group and control group mice during the aerosol OVA-challenge period.

Fig. 2. Treatment with APS reduced inflammatory cells infiltration, goblet cell hyperplasia and collagen deposition in large airways (magnification 200x, Scale bar=100 μ m). (a) Lung sections were stained with H&E to analyze the infiltration of inflammatory cells, PAS to assess goblet cell hyperplasia, Masson's trichrome to evaluate the subepithelial deposition of collagen and fibrosis. (b) The total inflammation scores were calculated. PAS-positive and PAS-negative epithelial cells were counted, and the percentage of PAS-positive cells per large airway was calculated. Masson's trichrome staining analysis of collagen deposition was calculated. Values represented as mean $\pm$ SEM (n= 5 per group). #p < 0.05 compared to the control group, and *p < 0.05 compared to the model group.

Fig. 3. Treatment with APS reduced inflammatory cells infiltration, goblet cell hyperplasia and collagen deposition in bronchioles (magnification 200x, Scale bar=100 μ m). (a) Lung sections were stained with H&E to analyze the infiltration of inflammatory cells, PAS to assess goblet cell hyperplasia, Masson's trichrome to evaluate the subepithelial deposition of collagen and fibrosis. (b) The total inflammation scores were calculated. PAS-positive and PAS-negative epithelial cells were counted, and the percentage of PAS-positive cells per bronchiole was calculated. Masson's trichrome staining analysis of collagen deposition was calculated. (c) Cell numbers and differentiation in BALF were determined by chamber slide, and at least 200 cells were counted. Values represented as mean $\pm$ SEM (n= 5 per group). #p < 0.05 compared to the

control group, and * $p < 0.05$ compared to the model group.

Fig. 4. Treatment with APS inhibited the levels of OVA-specific IgE in BALF or serum. The concentrations of OVA-specific IgE were measured with ELISA. Values represented as mean $\pm$ SEM (n= 6 per group). # $p < 0.05$ compared to the control group, and * $p < 0.05$ compared to the model group.

Fig. 5. Inducement with OVA and LPS increased the mRNA levels of *Il4*, *Il5*, *Il6*, *Il8*, *Il10*, *Il13*, *Il17*, *Il33*, *Cxcl5*, and *Ccl20* in lung tissues. Treatment with APS reduced the *Il8*, *Cxcl5*, and *Ccl20* at mRNA levels and inhibited IL-13RA and IL-17RA at protein levels in lung tissues after OVA_{LPS} challenge. (a-c) The mRNA levels of *Il1 β* , *Il4*, *Il5*, *Il6*, *Il8*, *Il10*, *Il13*, *Il17*, *Il33*, *Cxcl5*, *Cxcl10*, *Cxcl11*, and *Ccl20* were quantified in lung tissues by RT-PCR. (d) The protein levels of IL13RA and IL17RA were determined in lung tissues by Western blotting. The relative density quantification is relative to β -actin. Values represented as mean $\pm$ SEM (n=5 per group). # $p < 0.05$ compared to the control group, and * $p < 0.05$ compared to the model group.

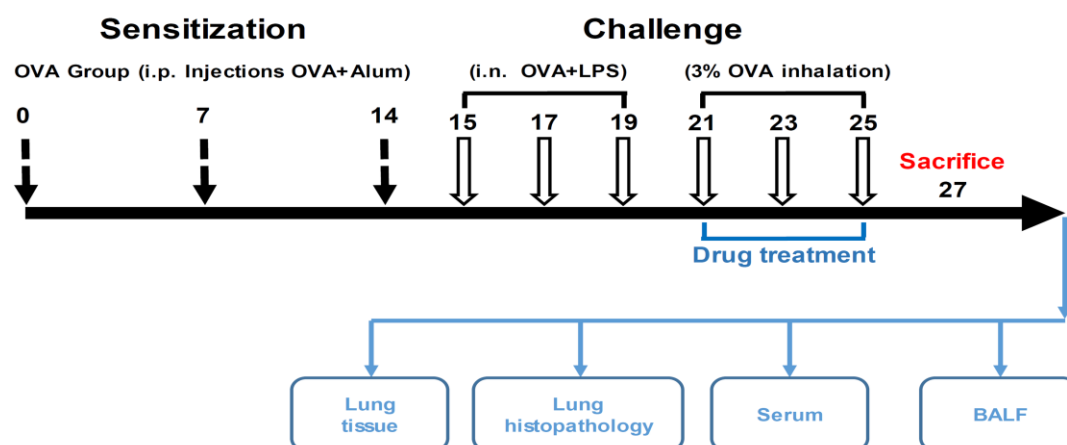
Fig. 6. Treatment with APS decreased the ER stress marker-CHOP by inhibiting the activation of PERK pathway. (a) The mRNA levels of *Chop*, *Bip*, *Perk*, *Atf6*, and *Ire1 α* were analyzed by RT-PCR. (b-c) Western blotting measured the protein levels of CHOP, BIP, PERK, p-PERK, eif2 α and ATF4. The relative density quantification is relative to β -actin. C, control group; M, model group; A, APS group; P, 4-PBA group; D, DEX group. Values represented as mean $\pm$ SEM (n=5 per group). # $p < 0.05$ compared to the control group, and * $p < 0.05$ compared to the model group.

Fig. 7. Treatment with APS blocked the nuclear translocation of ATF6 and NF-

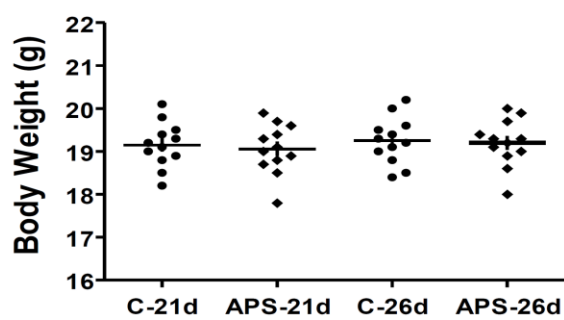
κ B p65 in lung tissues. (a-b) Western blotting measured the cytosolic and nuclear fractions of ATF6 and NF- κ B p65. The cytosolic fraction in which protein levels of ATF6 and NF- κ B p65 were normalized to β -actin. The nuclear fraction in which protein levels of ATF6 and NF- κ B p65 were normalized to Histone H3. C, control group; M, model group; A, APS group; P, 4-PBA group; D, DEX group. Values represented as mean $\pm$ SEM (n=5 per group). #p < 0.05 compared to the control group, and *p < 0.05 compared to the model group.

Fig. 8. Treatment with APS suppressed the expression of *Ire1 β* -correlated genes in the lung tissues. (a) The mRNA levels of *Ire1 β* , *Agr2*, *Spdef*, and *mClna3* were analyzed by RT-PCR. (b) Western blotting measured the protein levels of IRE1 α , IRE1 β , AGR2, SPDEF, and mCLCA3. The relative density quantification is relative to β -actin. C, control group; M, model group; A, APS group; P, 4-PBA group; D, DEX group. Values represented as mean $\pm$ SEM (n=5 per group). #p < 0.05 compared to the control group, and *p < 0.05 compared to the model group.

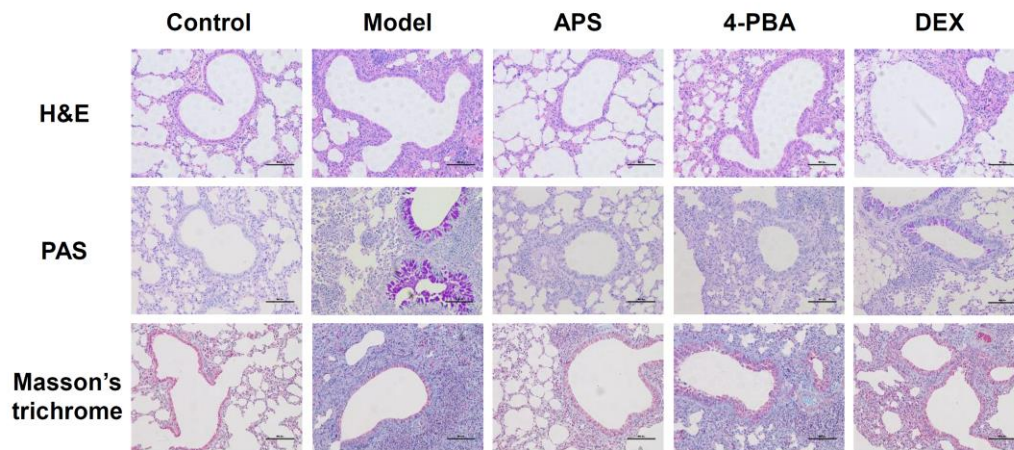
Fig. 9. Effects of APS on MUC5AC and MUC5B levels in lung homogenate and BALF. BALF was collected 48 h after the last OVA challenge. The muc5ac and MUC5B levels were detected by ELISA. Values represented as mean $\pm$ SEM (n=8 per group). #p < 0.05 compared to the control group, and *p < 0.05 compared to the model group.



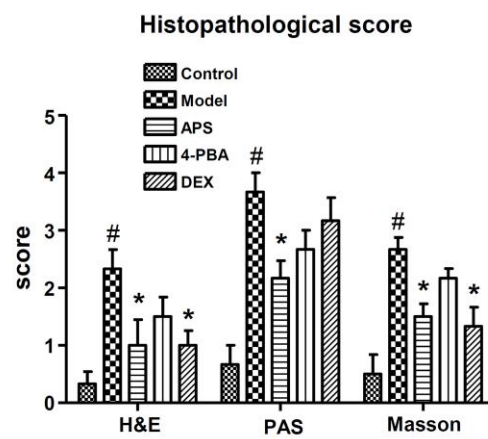
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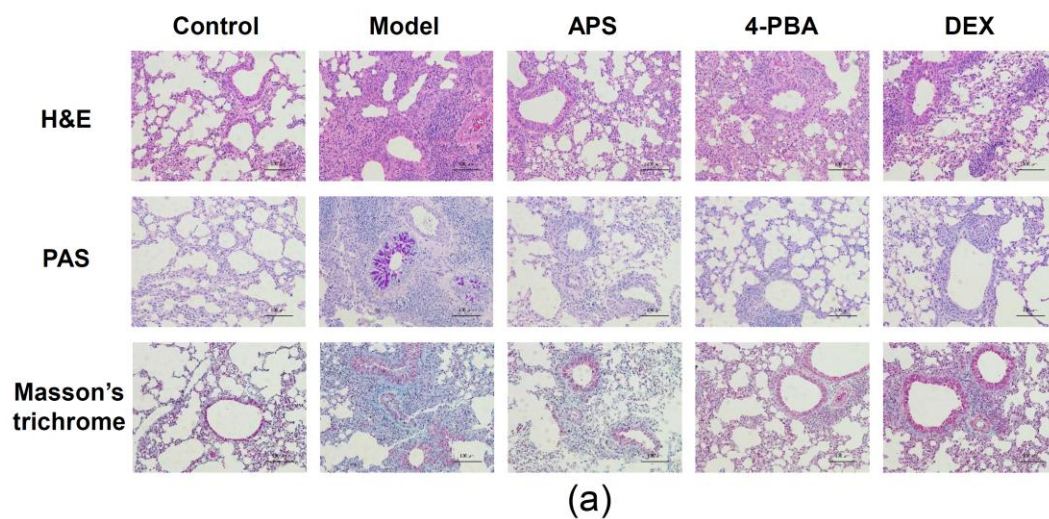
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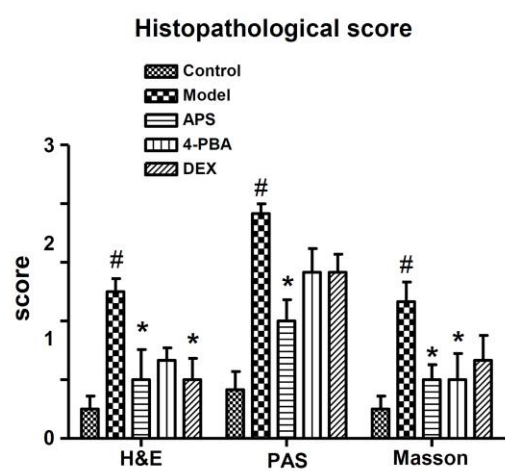
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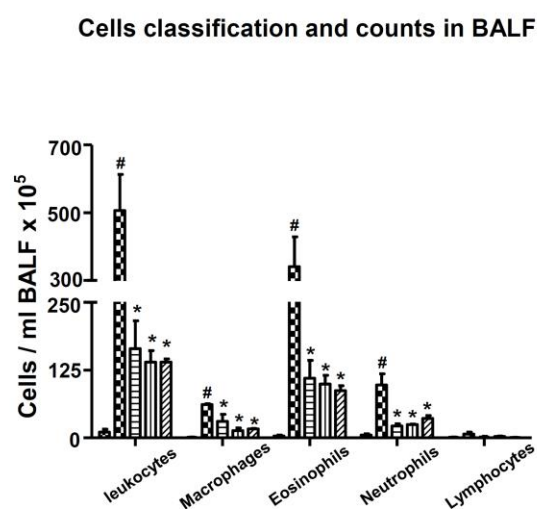
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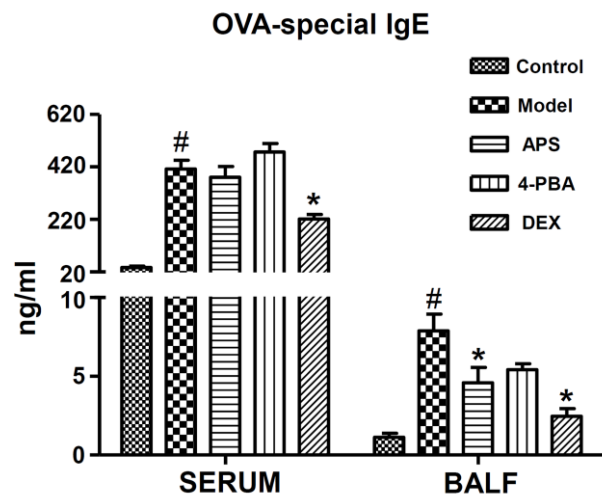
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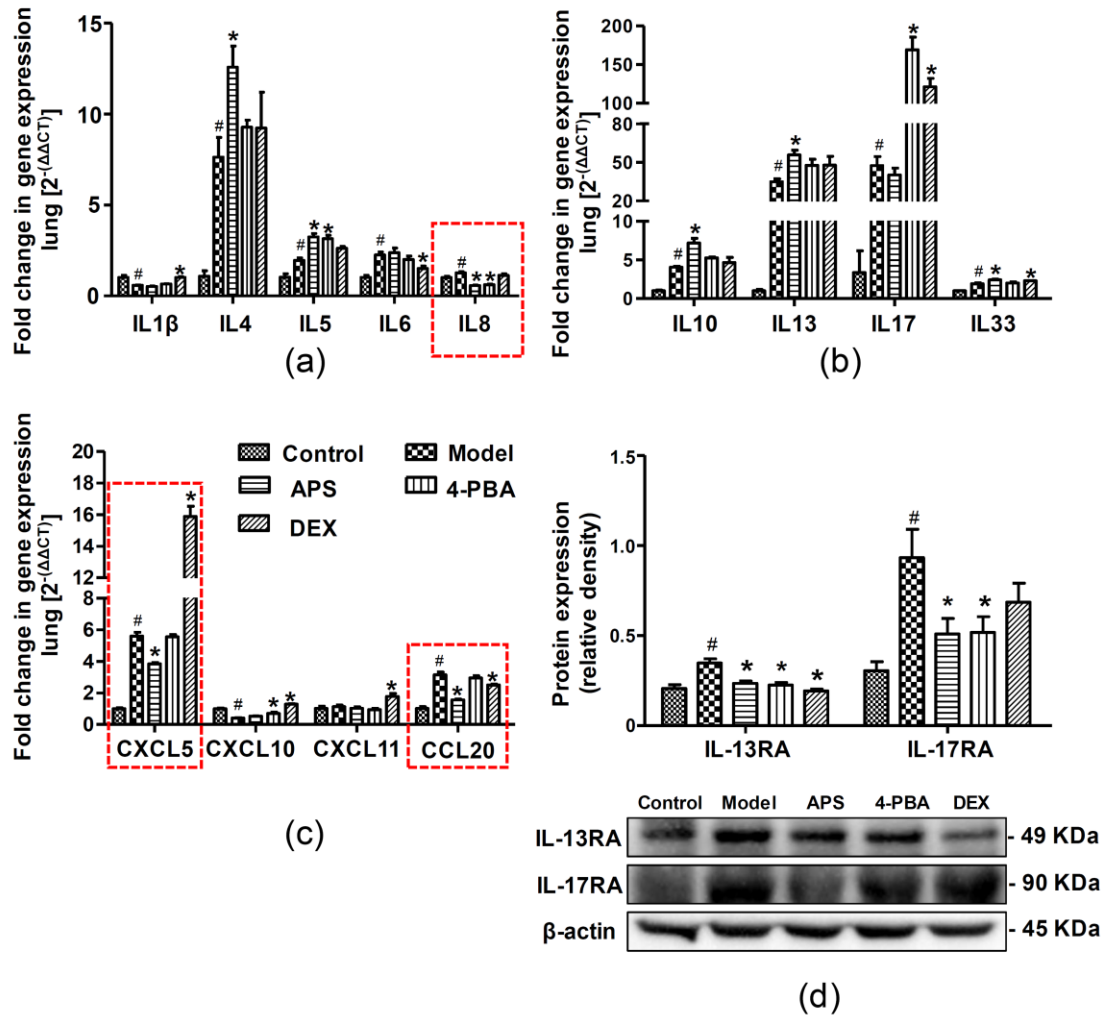


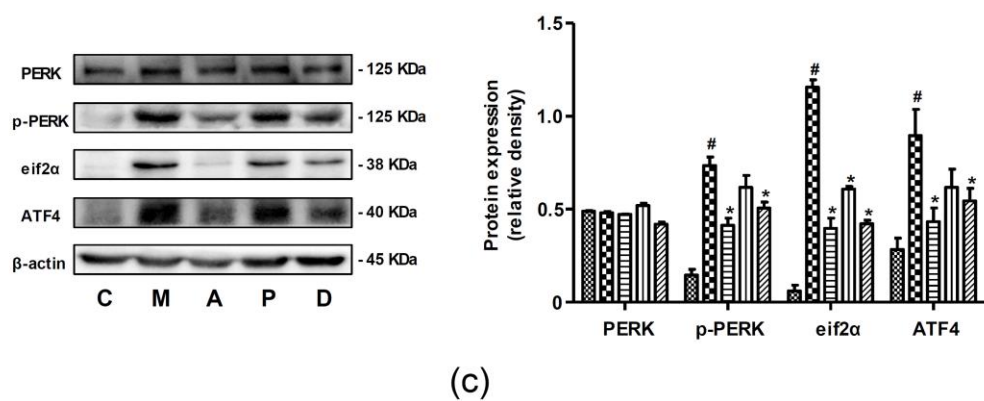
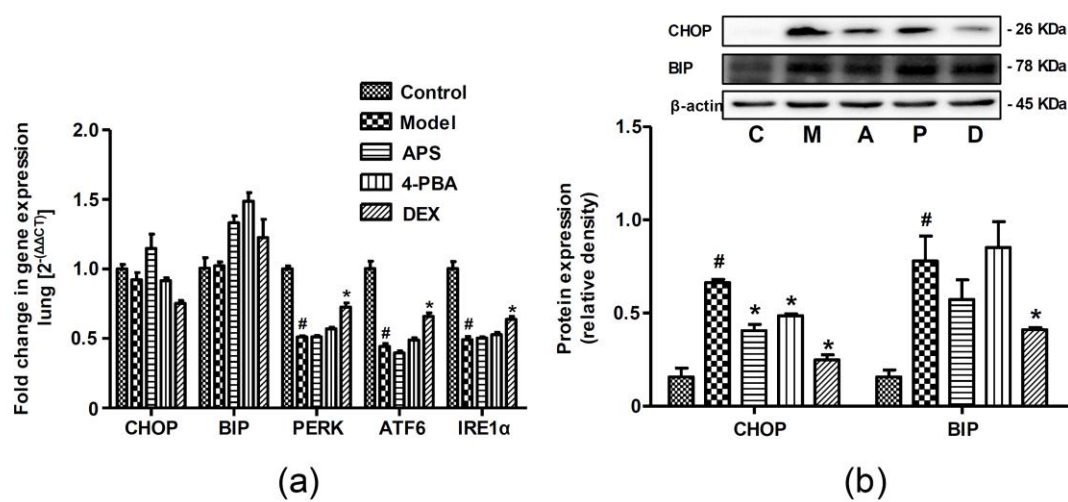
(b)

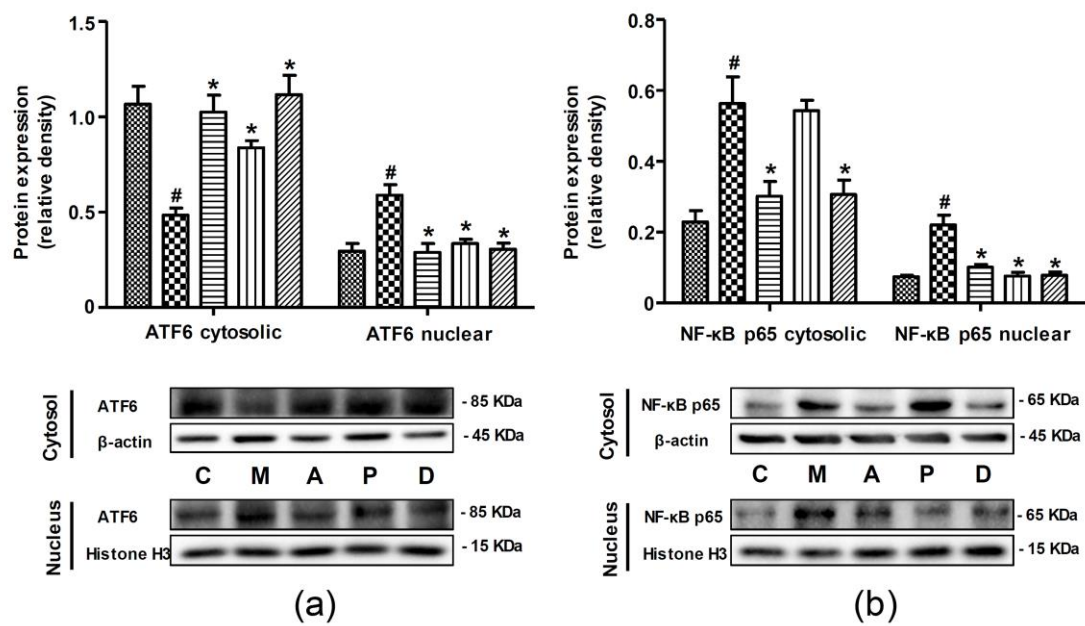


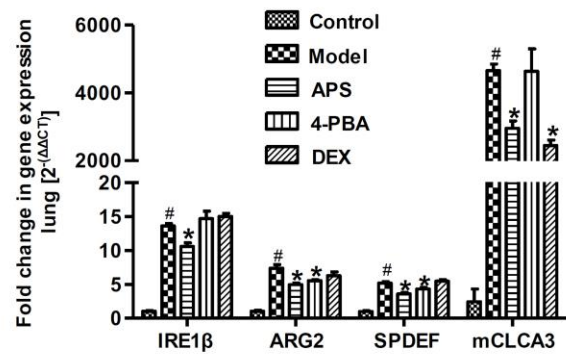
(c)



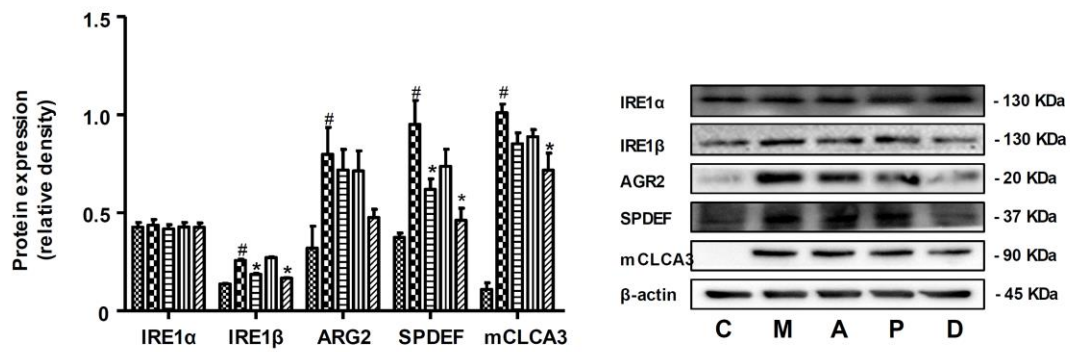








(a)



(b)

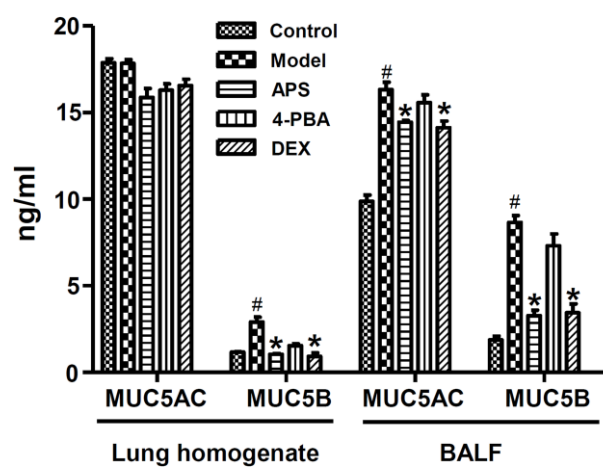


Table 1

The sequence of primes.

Gene	Forward Prime (5'-3')	Reverse Prime (5'-3')	Product (bp)
<i>Bip</i>	TGCGTGTGTGTGAGACCAG	CCACAGTGAACCTTCATCATGCC	126
<i>Chop</i>	TTATCTTGAGCCTAACACGTCGAT	CCAGGTTCTCTCTCCTCAGGT	100
<i>Atf6</i>	ACCTGTTCTTCCTCTGAAATCCAA	AGGACAGAGAAACAAGCTCGG	176
<i>Perk</i>	CCGCAAGAAGGACCCTATCC	GAGTTTCAGACTCCTTCCGCT	143
<i>Ire1α</i>	GCGATGGACTGGTGGTAACT	TGCACCACCTTTCTCAGGAC	121
<i>Ire1β</i>	GCCGCTCATTTTCCAGATCAC	CAGGAAATGCCATCGGTGC	198
<i>Gapdh</i>	CGTGTTCTACCCCCAATGT	TGTCATACTTGGCAGGTTTCT	104
<i>Il4</i>	CATCGGCATTTTGAACGAG	TTGGAAGCCCTACAGACGAG	120
<i>Il5</i>	CTGCCTGCTTCGTCTTGTTA	TCAAATCCAACCTCTTCCACAC	105
<i>Il6</i>	CGGAGAGGAGACTTCACAGAG	ATTTCCACGATTTCCCAGAG	104
<i>Il8</i>	TCACCGATGTCTACCTGCTG	CACAGGGTTGAGCCAAAAGT	112
<i>Il10</i>	TAACTGCACCCACTTCCCAG	AAGGCTTGGCAACCCAAGTA	165
<i>Il13</i>	CTTGCTTGCCCTTGGTGGT	GGGGAGTCTGGTCTTGTGTG	118
<i>Il17</i>	GACTCTCCACCGCAATGA	TCTCAGGCTCCCTCTTCAG	161
<i>Il33</i>	CCGTTCTGGCCTCACCATAA	GACGCAGCAAATGCTTGGAT	165
<i>Cxcl5</i>	GTTCCATCTCGCCATTCATGC	AAGCAAACACAACGCAGCTC	127
<i>Cxcl10</i>	ATCCTGCTGGGTCTGAGTGG	GGATAGGCTCGCAGGGATGA	135
<i>Cxcl11</i>	GGAAGGTCACAGCCATAGCC	CTTTGTGCGCAGCCGTTACTC	171
<i>Ccl20</i>	GCAGAAGCAAGCAACTACGAC	CTTTGGATCAGCGCACACAG	162
<i>Spdef</i>	ACGGATGGTGAGGTGGACT	TGCCTTTCTCCTTGTTGAGC	127
<i>Agr2</i>	GACGAATGCCCACACAGTC	CAGGAGAAAGGTGCTTGTCAG	127
<i>mClca3</i>	TGAACTGGGAGGCAACACTT	CGGGGATGAACACAGACAC	122