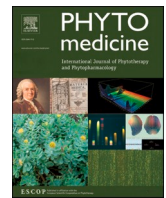




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Paeoniflorin ameliorates airway inflammation and immune response in ovalbumin induced asthmatic mice: From oxidative stress to autophagy

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ABSTRACT

Background: Asthma characterized by airway remodeling is a multiple pulmonary disease, which is associated with various physiological processes including inflammation reaction, immune response, oxidative stress and autophagy.

Purpose: This study aimed to investigate whether these processes are modulated by the total glucosides of *Paeonia lactiflora* Pall (TGP), and its active compound paeoniflorin (PF) with anti-inflammatory and immune-regulatory effects could alleviate ovalbumin (OVA)-induced mouse asthma.

Methods: *In vivo*, models of mouse asthma were established by intraperitoneally with a mixture of OVA and aluminum hydroxide, plus a single nasal injected with OVA to female C57BL/6 mice. The results were observed with PET imaging, TEM, RT-PCR, western blotting. *In vitro*, CD4⁺ T cells were isolated and detected with flow cytometry.

Results: TGP, either in its crude or processed form, and PF effectively ameliorated lung injury in mice induced by OVA, regulated immune/inflammatory response by inhibiting the release of pro-inflammatory cytokines, thereby decreasing Th2 cell proportion, inhibited oxidative stress by recovering mitochondrial membrane potential and regulating metabolic activity in dose-dependent manner. Moreover, PF could inhibit autophagy by regulating mitochondrial function. In addition, the therapeutic effects of TGP and PF on pulmonary injury in asthmatic mice were not affected by processing.

Conclusion: PF may be a valuable agent in ameliorating inflammation and immune response in asthmatic mice, and the possible mechanism involved in this response may range from oxidative stress to autophagy.

Introduction

Asthma is a multiple pulmonary disease characterized by chronic airway disease with wheezing, shortness of breath, chest tightness, coughing, and expiratory airflow limitation. It affects approximately 300 million individuals worldwide (Weronika Barcik, 2020) and has become a major global health concern (Tian et al., 2019). In addition, approximately 250,000 deaths every year are reportedly related to asthma, many of which can be avoidable (Christiansen and Zuraw, 2019). Asthma is mainly caused by many factors, such as airway wall remodeling, chronic eosinophilic, airway inflammation, et al. (Remot et al., 2017). Previous studies indicated that asthma is associated with

various physiological processes, including immune response (Khalaf et al., 2019), inflammation (da Silva et al., 2016), apoptosis (O Potapinska and Demkow, 2009), and autophagy (Lv et al., 2020). Current medicine for asthma mainly focus on anti-inflammatory and anti-symptomatic strategies, such as corticosteroids and anti-cholinergic agents (Alangari, 2014; Gosens and Gross, 2018). Although these treatments can ameliorate airway inflammation and relieve the clinical symptoms of asthma patients, they are ineffective against immune dysfunction and asthma airway remodeling. Therefore, research on the pathogenesis and prevention of asthma is still an urgent task.

Studies showed that pathogenesis of allergic asthma were associated with the increase in the number of type 2 innate lymphoid cells (ILC2s),

Abbreviations: DEX, dexamethasone; IFN- γ , Interferon-gamma; ILC2s, innate lymphoid cells; MMP, Mitochondrial membrane potential; OVA, ovalbumin; PEEP, positive end-expiratory pressure; PET, Positron emission tomography; PF, paeoniflorin; ROS, Reactive oxygen species; TGP, total glucosides of *Paeonia lactiflora* Pall.

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which produce IL-5 and IL-13 in peripheral circulation (Neill et al., 2010). In asthma induced by allergens or pathogens, such as ovalbumin (OVA) (Kearley et al., 2009) and dust mites (Hammad et al., 2009), airway epithelial cells secrete various cytokines, such as interleukin IL-25 and IL-33 (Ealey et al., 2017), which participate in stimulating ILC2s and promoting the production of type 2 inflammatory factors (IL-5, IL-9, interferon (IFN)- γ , and total immunoglobulin (IgE)) (Ealey et al., 2017). ILC2s mainly present in most tissues of the body, including intestines, liver, and brain tissues (Nussbaum et al., 2013), but are relatively rare in the lungs (Monticelli et al., 2011). In an inflammatory environment, ILC2s enhance type 2 immune response by upregulating IL-5 and IL-13 in the lungs of mice (Halim et al., 2012; Monticelli et al., 2011). Indeed, type 2 immune response promotes the differentiation of T cells into Th2 cells, which require a large amount of energy supply that produced in the mitochondria. As a main organ of energy metabolism, mitochondria can regulate glycolysis, fatty acid and amino acid biosynthesis, all of which are closely related to the expressions of HK2, PFK1, PKM1 and other proteins. Besides, mitochondria are also associated with reactive oxygen species (ROS) production and immunity (Shadel and Horvath, 2015). ROS can quickly attack and may molecules nearby cells due to its role in primary immune defense (Gulcin, 2012, 2020; Taslimi and Gulcin, 2018). Moreover, mitochondrial function can be damaged by elevated intracellular levels of ROS, that is, oxidative stress (Schieber and Chandel, 2014), which plays a pivotal role in the pathogenesis of asthma. Increase of ROS lead to the release of Cyt-c, which could activate caspase cascade reaction that participate in apoptosis. Moreover, ROS can induce autophagy and remove oxidized proteins or organelles, and they may present a hopeful pathway for investigation the pathogenesis in asthma (Audrey et al., 2012). Furthermore, autophagy and apoptosis can be triggered by common stimuli, resulting in combined autophagy and apoptosis, which are associated with the expressions of Bcl-2 and Beclin 1 that related to impaired mitochondrial function. Thus, the relationship among immune response, inflammation, oxidative stress, and autophagy may contribute to explore the pathogenesis of asthma and develop effective modalities for preventing or treating for asthma. *Paeonia lactiflora* Pall, a traditional Chinese medicine, has been used as an ingredient in medical formulations for asthma in our previous study by Shou, which showed that the total glucosides extracted from *Paeonia lactiflora* Pall are made up of individual chemical components including benzoyloxypaeoniflorin, paeoniflorin (PF), oxypaeoniflorin, albiflorin, and benzoylpaeoniflorin (Shou et al., 2019). Among these components, PF has particularly exhibits a wide range of pharmacological effects, such as

anti-inflammatory, anti-oxidant (Yuan et al., 2020), and regulation of immune regulation (Tu et al., 2019; Zhang and Wei, 2020; Zheng et al., 2007). PF, a main component of TGP (traditional Chinese medicine with low side effects) at doses of 20 mg/kg and 50 mg/kg has little toxicity on mice as well as on normal tissue, which has been confirmed by previous study (Chen et al., 2015; Yin et al., 2020). With regard to asthma, PF has been shown to regulate the imbalance of the Th1/Th2 ratio due to its anti-inflammatory effects (Zhang et al., 2015). Similarity with inflammatory reaction (Painter et al., 2020), autophagy can also be regulated by PF (Wen et al., 2019). Moreover, Wang et al. found that PF could inhibit oxidative stress and apoptosis that closely associated with immune response (Wang et al., 2020). Therefore, exploring the effects of TGP (or PF) on relationship among inflammation reaction, immune response, oxidative stress and autophagy may provide a new way for treatment of asthma.

Based on above, we hypothesized that PF alleviates asthma by inhibiting the releases of IL-25/IL-33 and promoting metabolic function, all of which were associated with oxidative stress and autophagy. Therefore, our research compared the effect of PF with those of crude or processed TGP in suppressing asthma in an OVA-induced asthma mouse model (refer to Fig. 1B for full experimental timeline) and investigated the mechanisms underlying the activities of PF and TGP. The full experimental timeline is shown in Fig. 1B.

MATERIALS and METHODS

Materials

PF (Fig. 1A) (PubChem CID: 442,534; molecular formula: $C_{23}H_{28}O_{11}$; molecular weight: 480.47 g/mol; purity $\geq 98\%$) was purchased from Chengdu Desite Biotechnology Co., Ltd. (Chengdu, Sichuan, China). Crude and processed *Paeonia lactiflora* Pall with bran stir-baking were purchased from Zhejiang Chinese Medical University Medical Pieces Co. Ltd. (Hangzhou, Zhejiang, China).

Sample preparation

Crude TGP (TGP-C) and processed TGP (TGP-P) were extracted from the crude and processed *Paeonia lactiflora* Pall, respectively, and the quality of the TGP was over 80% as determined by high-performance liquid chromatography. The TGP was processed as follow: 50 g of TGP-C was mixed with 20% Zhenjiang white vinegar. The mixture was kept in an airtight container for 1 h and then placed in an oil bath at

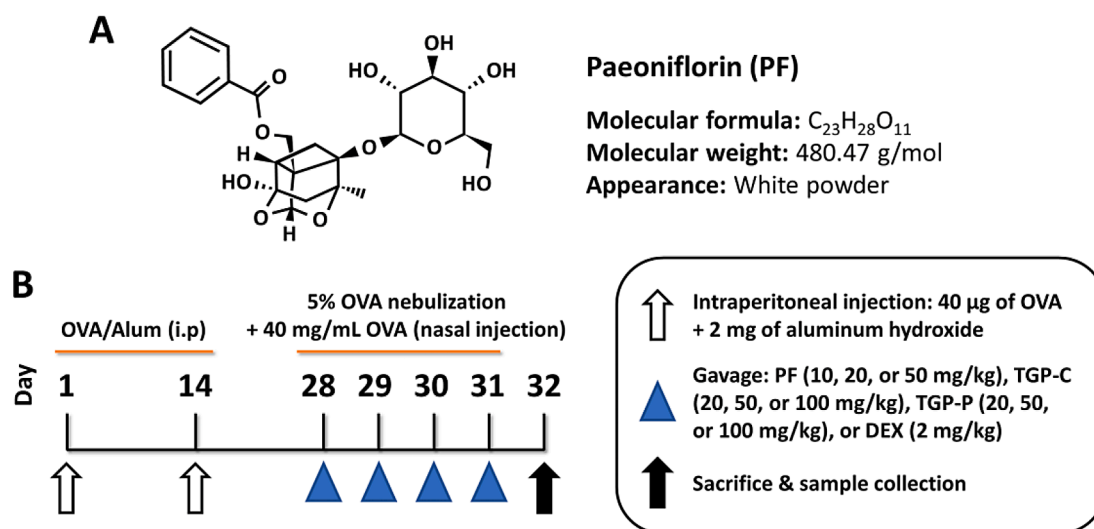


Fig. 1. Overview of animal experiments. (A) Chemical structure of paeoniflorin (PF) and basic properties. (B) Outline of experimental design for establishing the OVA-induced asthma model, illustrating critical points during the experimental timeline.

130 °C with continuously stirred for 10 min. Subsequently, the mixture was removed from the oil bath and then allowed to cool. TGP-C was characterized by high-performance liquid chromatography as described previously (Shou et al., 2019). Finally, both TGP-C and TGP-P were ground into fine powders before use.

Establishment of asthma model

8 weeks old female C57BL/6 mice (20–22 g) were housed in a specific-pathogen-free environment on a 12/12-h light/dark cycle at 22–26 °C with 50–60% relative humidity. After adaption, the mice were randomly divided into 12 groups ($n \geq 5$ for each treatment): control group, OVA group, OVA plus PF groups (10, 25 or 50 mg/kg of body weight), OVA plus TGP-C groups (20, 50 or 100 mg/kg of body weight), OVA plus TGP-P groups (20, 50 or 100 mg/kg of body weight). The dose of PF was based on previous research (Ji et al., 2013; Sun et al., 2015), while the TGP was based on our preliminary study (Shou et al., 2018, 2019). The OVA-induced asthma model was established on the basis of previously published protocols (Fig. 1B) (Hellings et al., 2001; Reddy et al., 2012). For initial sensitization, all mice, except those in control groups, were intraperitoneally injected with a mixture of 40 µg of OVA (A5503–10 g, Sigma-Aldrich) and 2 mg of aluminum hydroxide (AB0023, Sangon Biotech, Shanghai, China) in 200 µl of sterilized phosphate-buffered saline (PBS) on day 1 and 14. From days 28 to 31, mice in OVA plus PF groups, OVA plus TGP-C groups, and OVA plus TGP-P groups were gavaged with PF (10, 25, or 50 mg/kg), TGP-C (20, 50, or 100 mg/kg), TGP-P (20, 50, or 100 mg/kg), and dexamethasone (DEX, 2 mg/kg, D1756–25 g, Sigma-Aldrich) that a positive control drug. After treatment for 1 h, the mice were subjected to 5% OVA nebulization (402b nebulizer, Yuwell) for 30 min daily and then nasally nasal injected with 20 µl of OVA (40 mg/ml). The mice in the control and OVA groups were treated with equal volume of physiological saline. After the last dosing, all mice were sacrificed by overdosing with sodium pentobarbital. Blood and lung tissue were collected for subsequent experiments. All animal procedures were approved by Animal Ethical and Welfare Committee of Zhejiang Chinese Medical University and the permission number was IACUC-20200713–04.

Positron emission tomography (PET) imaging

The pulmonary conditions of the mice induced by OVA were observed and analyzed via positron emission tomography (PET) imaging performed at Wuhan Ruifuning Scientific Co., Ltd. (Wuhan, China). Prior to PET imaging, the mice were fasted for 12 h and then anesthesia with 2% isoflurane (Shandong Keyuan Pharmaceutical Co., Ltd., Jinan, Shandong, China). The tail vein of the mice was injected with ^{18}F -fluorodeoxyglucose (^{18}F -FDG, provided by the Department of Nuclear Medicine at the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) at $200 \pm 10 \mu\text{Ci}$. An hour after the injection, whole-body static scanning was conducted for 14 min by using a TransPET®BioCalibur® LH (Raycan Technology Co., Ltd., Suzhou, China). Reconstruction was performed with iterations, 12 subsets, and no filtering. PET images were acquired from the coronal, sagittal, and transverse planes.

Methacholine challenge test for pulmonary activity

Pulmonary mechanical parameters and airway responsiveness of the mice were evaluated in mice as previously described (Marinas-Pardo et al., 2014). On days 1, 20, and 29, the animals were intraperitoneally anesthesia with sodium pentobarbital (80 mg/kg of body weight) intraperitoneally, after which tracheotomy and intubation were performed. The mice were placed in the supine position and their lungs were connected to a lung function measurement apparatus (AniRes2005, BestLab, Beijing, China) through the tracheal intubation. Mechanical ventilation was performed using a RES3050 ventilator

(BestLab) with 21% oxygen at a respiratory rate of 150 breaths/min, tidal volume of 10 ml/kg, and positive end-expiratory pressure (PEEP) of 2 cm H₂O. Afterward, 50 µl of succinate choline (9 mg/ml, H11021581, China Resources Double Crane Pharmaceutical Co., Ltd., Beijing, China) was intraperitoneally injected to induce muscle relaxation. When breathing was stabilized, the expiratory end was blocked to increase lung pressure to 30 cm H₂O for 5 s to open the partially atelectatic lung tissues. When the PEEP reached 2 cm H₂O after 2 min, respiratory parameters were measured with a single-compartment model (Snapsho-150) and a normal/constant-phase model (Prime-8). Ventilation was performed as described above for 5 min, and methacholine was administered in situ for 10 s by using a FlexiVent nebulizer at 0.025, 0.05, and 0.1 g/ml. In accordance with the constant-phase model, mechanical parameters of lung functions, i.e., inhalation resistance, expiration resistance, and pulmonary compliance, were obtained using the system's built-in software.

Determination of biochemical indices

Releases of inflammatory factors, including IL-5 (MU30011), IL-9 (MU30658), IL-25 (MU30777), IL-33 (MU30762), IFN- γ (MU30038), and IgE (MU30387) in lung tissues were evaluated using enzyme-linked immunosorbent assay kits (Bio-Swamp, Wuhan, China). Lactic acid production in peripheral blood-derived CD4⁺ T cells and lung tissue was examined using an assay kit (A019–2, Nanjing Chengji Institute of Biological Engineering, Nanjing, Jiangsu, China). All assays were performed according to the manufacturer's instructions.

Hematoxylin and eosin (H&E) staining

Upper lobe of left lung tissues was fixed with 2.5% glutaraldehyde and 4% paraformaldehyde prior to histological analysis. Fixed tissues were dehydrated and embedded in paraffin wax. The fixed tissues were dehydrated and embedded in paraffin wax. The paraffin sections were cut into a thickness of 4 µm by using a microtome. For hematoxylin and eosin (H&E) staining, the sections were dewaxed, hydrated, stained with hematoxylin solution (PAB180015, Bio-Swamp), and washed with water, followed by 0.5% eosin solution (PAB180016, Bio-Swamp). Thereafter, the sections were decolorized with gradient ethanol and treated with xylene until they became transparent. Finally, the sections were sealed with neutral gum. All sections were acquired and analyzed by Leica Application Suite.

Isolation of CD4⁺ T cells

Lymphocytes were isolated from the spleen and peripheral blood of the experimental animals. The spleen was cut into small pieces, ground between glass plates, and filtered through a 200-mesh filter. The lymphocytes were separated using a specialized separation solution. Peripheral blood mononuclear cells were obtained via the density gradient centrifugation (Ficoll) method. CD4⁺ T cell subsets were then sorted via flow cytometry.

Flow cytometry

For ILC2s cells, lung tissues were separated into single cells and resuspended in flow cytometry buffer in an Eppendorf tube. Ly-6 G (Gr-1)-PE (12–9668–80, eBioscience), CD25-PE-Cy™5 (15–0251–82, eBioscience), and CD127-FITC (11–1271–82, eBioscience) antibodies were added to each tube. After incubation, the cells were washed with buffer and centrifuged. The supernatant was removed and the cells were resuspended in dyeing buffer and analysis. For Th1, Th2, and Th17 cells, lung tissues were separated into single cells and resuspended in growth medium in an Eppendorf tube. Leukocyte activation cocktail (50 ng/ml, 550,583, BD Bioscience), ionomycin (2 µg/ml, I8800, Solarbio), and BFA (3 µg/ml, M8670, Solarbio) were added to each tube. After

incubation at 37 °C in 5% CO₂, the cells were washed with buffer and centrifuged. The supernatant was removed, and the cells were resuspended in buffer. After the addition of CD4-FITC (85–11–0041–81, eBioscience), the cells were incubated and resuspended in permeabilization buffer (562,574, BD Bioscience). Then, the cells were incubated and centrifuged. The supernatant was discarded, and the previous step was repeated. The cells were resuspended in 100 µl of buffer, and antibodies against IL-17 (12–7177–81, eBioscience), IFN-γ (505,810, BioLegend), and IL-4 (504,104, BioLegend) (2 µl of each) were added. After incubation and washing, the cells were stored at 4 °C before analysis. For regulatory T (Treg) cells, the procedure was similar to the separation of Th1, Th2, and Th17 cells. Flow cytometry was performed using a NovoCyte apparatus (ACEA Biosciences) and the data were analyzed using built-in NovoCyte software.

Mitochondrial membrane potential (MMP) measurement

Mitochondrial membrane potential (MMP) in peripheral blood-derived CD4⁺ T cells and lung tissue was measured with a 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) assay kit (C2006, Beyotime). The cells were suspended in flow cytometry buffer and mixed with JC-1 working solution. The cells were incubated and centrifuged. The supernatant was discarded, and the cells were washed twice with JC-1 dyeing buffer (included in the kit). The cells were resuspended in JC-1 dyeing buffer and centrifuged. Flow cytometry was performed using a NovoCyte apparatus (ACEA Biosciences), and data were collected using built-in NovoCyte software. The JC-1 monomer-to-aggregate ratio is an indication of mitochondrial membrane depolarization.

Reactive oxygen species (ROS) production

Tissue ROS production was evaluated using an ROS assay kit (S0033, Beyotime, Nanjing, Jiangsu) in accordance with the manufacturer's instructions. In brief, lung tissues were separated into single cells and mixed with DCFH-DA probes. Adequate contact between the cells and the probes was ensured by incubating the mixture gentle rotation. Subsequently, the cells were washed with serum-free medium and analyzed via flow cytometric using a NovoCyte apparatus (ACEA Biosciences). Data were collected with built-in NovoCyte software.

Western blot

Lung samples were lysed in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Protein content was measured with BCA protein assay kit (P0010S, Beyotime, Nanjing, Jiangsu) according to manufacturer's instructions. Equivalent proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (GE, Freiburg, Germany) that were pre-soaked in acetone. The membranes were blocked in 5% skim milk at room temperature and then incubated overnight at 4 °C with the following antibodies: anti-hexokinase 2 (HK2, 1:1000, PAB30271, Bio-Swamp), anti-phosphofructokinase 1 (PFK1, 1:1000, 55,028–1-AP, Proteintech), anti-pyruvate kinase isozymes M1 (PKM1, 1:1000, 15,821–1-AP, Proteintech), anti-Bcl-2 (1:1000, PAB30041, Bio-Swamp), anti-Bax (1:1000, PAB30040, Bio-Swamp), anti-cleaved Caspase-3 (1:1000, MAB37300, Bio-Swamp), anti-Cyt-c (1:1000, PAB30480, Bio-Swamp), anti-Becclin 1 (1:1000, Ab62557, Abcam, Cambridge, UK), anti-p62 (1:1000, PAB35470, Bio-Swamp), anti-TOM20 (1:2000, 11,802–1-AP, Proteintech), anti-cytochrome c oxidase 4 (COX IV, 1:1000, PAB34654, Bio-Swamp), and anti-GAPDH (1:1000, PAB36269, Bio-Swamp). Thereafter, the membranes were washed thoroughly with PBS/Tween 20 and incubated further incubated with the appropriate HRP-conjugated secondary antibodies (1:20,000, PAB 160,011, Bio-Swamp). After washing, the membranes were visualized BeyoECL Moon (Beyotime, Nanjing,

Jiangsu, China) and imaged by a Tanon-5200 apparatus (Tanon, Shanghai, China). The bands were quantified by Quantity One software (BioRad, CA, USA).

Real-time RT-PCR

Total RNA was isolated from CD4⁺ T cells using TRIzol reagent (15,596,026, Ambion, Inc., Foster City, CA). Samples of RNA were reverse transcribed into complementary DNA (cDNA). Relative gene expression was assessed by real-time PCR, which was performed on an Agilent Mx3000P QPCR System in a mixture containing Power SYBR® Green PCR Master Mix (KM4101, KAPA Biosystems, Wilmington, MA) and specific primers were as follow: (5'–3'): Bcl2a1b F: CAACCAAGT-GATGGAAAAAG, R: TGCCACAAAACCTGGAAAC; Bcl2a1d F: AATCCA-TAGATACCGCCA, R: GTCACAATCCTTCCCCAG; IL1R2 F: AAGGAACAACACCGGAAC, R: AGCCAACACACACAAT; Ler3 F: ACCATCTCCACACCAT, R: CCTCTTCAGCCATCAA; Syn1 F: GAGGA-GATTCTAACCC, R: CGGACTACTGTAAAGC; Tnfsf18 F: CATCTCC-CAAACCTCACT, R: GTTCATAAACCCCTCTCA; GAPDH F: CCTTCCGTGTTCTCTAC, R: GACAACCTGGTCTCTCA. GAPDH was used as a housekeeping gene to quantify relative fold difference and the method of comparative threshold cycle ($\Delta\Delta Ct$) was also used in this process.

Transmission electron microscopy (TEM)

Upper lobe of right lung tissue was fixed in 2.5% glutaraldehyde and washed with PBS. Following, the samples were then fixed in osmium acid (18,456, TedPella Inc.), washed, dehydrated with gradient ethanol, semi-immersed in a mixture of acetone and epoxy resin 812 (one-to-one ratio), fully immersed in epoxy resin 812, embedded, and heated. Thereafter, the samples were cut into 60-nm sections using an ultramicrotome and subjected to double staining. The sections were first stained with uranium acetate (1005–25 g, Merck) in the dark. The copper mesh is removed and the sections were washed with distilled water. Then sections were stained with lead citrate in the dark. Excess lead solution was removed by distilled water and subjected to transmission electron microscopy (TEM) analysis with a Hitachi HT7700 apparatus (Hitachi, Tokyo, Japan).

Statistical analysis

The results in the experiments presented as the mean $\pm$ standard deviation (SD). The means between more than two groups were analysis by homogeneity of variance with SPSS: 1) if variance was homogeneity, the results were compared by one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests using GraphPad Prism program (Graphpad Software, Inc., San Diego, CA, USA) and statistical significance was defined as $p < 0.05$; 2) if variance was not homogeneity, the results was analyzed with Nonparametric Tests and K independent samples.

Results

PET imaging and methacholine challenge test for pulmonary activity evaluation

The extent of pulmonary inflammation in the mice with OVA-induced asthma was evaluated by detecting pulmonary activity via PET imaging (Fig. 2A). The high Standard uptake value (SUV) is a sign of pulmonary inflammation and confirms the successful establishment of the asthma model in OVA-induced mice (Harris et al., 2011). Results indicated that compared with those in the control group, the mice in OVA-induced group had large amounts of red signal in the lung area with a high standard uptake value (SUV) that represents ¹⁸F-FDG activity (an indication of metabolic activity), PF, TGP-C, and TGP-P reduced the red signals in the lung area increased by OVA, implicating

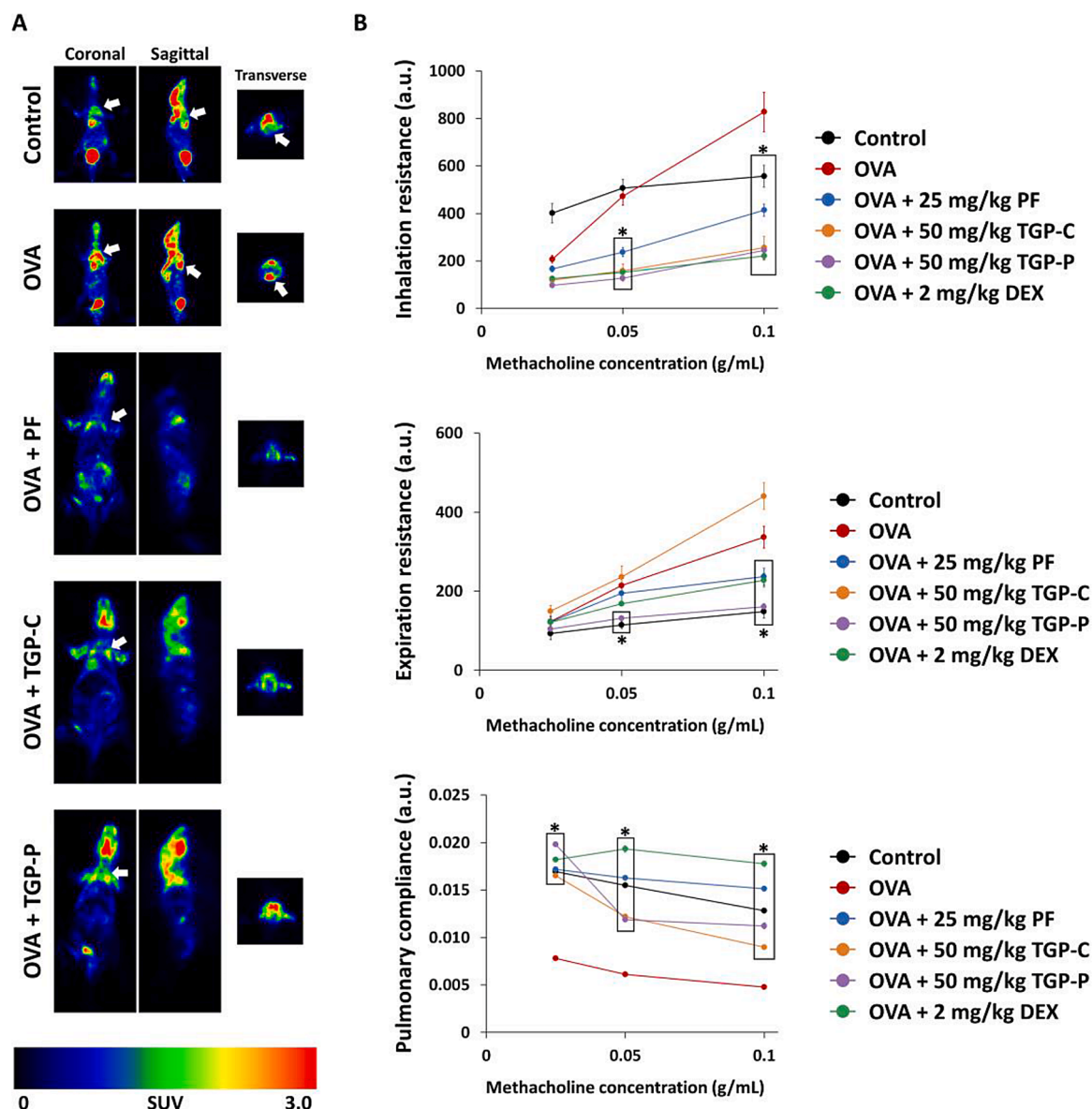


Fig. 2. PET imaging and methacholine challenge test for pulmonary activity evaluation. OVA-induced mice were subjected to various treatments (PF, 25 mg/kg; TGP-C, 50 mg/kg; TGP-P, 50 mg/kg; DEX, 2 mg/kg). (A) PET imaging of the lung region in OVA-induced mice illustrating ^{18}F -FDG activity, which is indicated by standard uptake value (SUV). Images were taken from the coronal, sagittal, and transverse planes. (B) Evaluation of pulmonary activity, performed on day 29 of the animal experiment. Methacholine was administered at 0.025, 0.05, and 0.1 g/ml and the inhalation resistance, expiration resistance, and pulmonary compliance were measured. The data represent the mean $\pm$ SD ($n \geq 5$). * $p < 0.0001$.

that these compounds suppressed ^{18}F -FDG activity. In addition, the high SUV in the OVA-induced mouse lungs was a sign of pulmonary inflammation and confirmed the successful establishment of the asthma model. Results demonstrated that PF, TGP-C, and TGP-P treatments could ameliorate OVA-induced pulmonary inflammation in mice and asthma to a certain degree.

Consistent with reduced SUV levels, the effects of PF, TGP-C, and TGP-P on specific pulmonary mechanical parameters were assessed via a methacholine challenge test (Fig. 2B). As expected, airway resistance (both inhalation and expiration) in the OVA-induced mice were elevated compared with that in the control mice at 0.05 and 0.1 g/ml methacholine, whereas 25 mg/kg PF, 50 mg/kg TGP-P, and 2 mg/kg DEX considerably reduced airway resistance induced by OVA. Interestingly, 50 mg/kg TGP-C exerted the same effect on inhalation resistance but it increased expiration resistance. Furthermore, pulmonary compliance was lower in the OVA-induced mice than in the control mice, and pulmonary compliance was recovered by PF, TGP-C, TGP-P, and DEX. All of

these demonstrated that PF and TGP (C and P) could possibly regulate pulmonary activity by reducing inflammation in asthma.

PF reduced the established airway inflammation

Inflammation response plays a critical role in the development of asthma. The effects of PF, TGP-C and TGP-P on inflammatory response in the lungs induced by OVA were detected by detecting the secretions of inflammatory cytokines were detected. As shown in Fig. 3A, the expressions of IL-5, IL-9, IL-25, IL-33, IFN- γ , and IgE in OVA-induced mice were increased compared with those in the control mice, while these were reversed by DEX (a positive control drug) treatment. Similar to DEX, PF (10, 25, and 50 mg/kg), TGP-C (20, 50, and 100 mg/kg), and TGP-P (20, 50, and 100 mg/kg) also reduced the releases of these inflammatory cytokines (including IL-5, IL-9, IL-25, IL-33, IFN- γ , and IgE) induced by OVA in a dose-dependent manner. Furthermore, the histopathology of mice lungs sections was examined by H&E staining (Fig. 3B

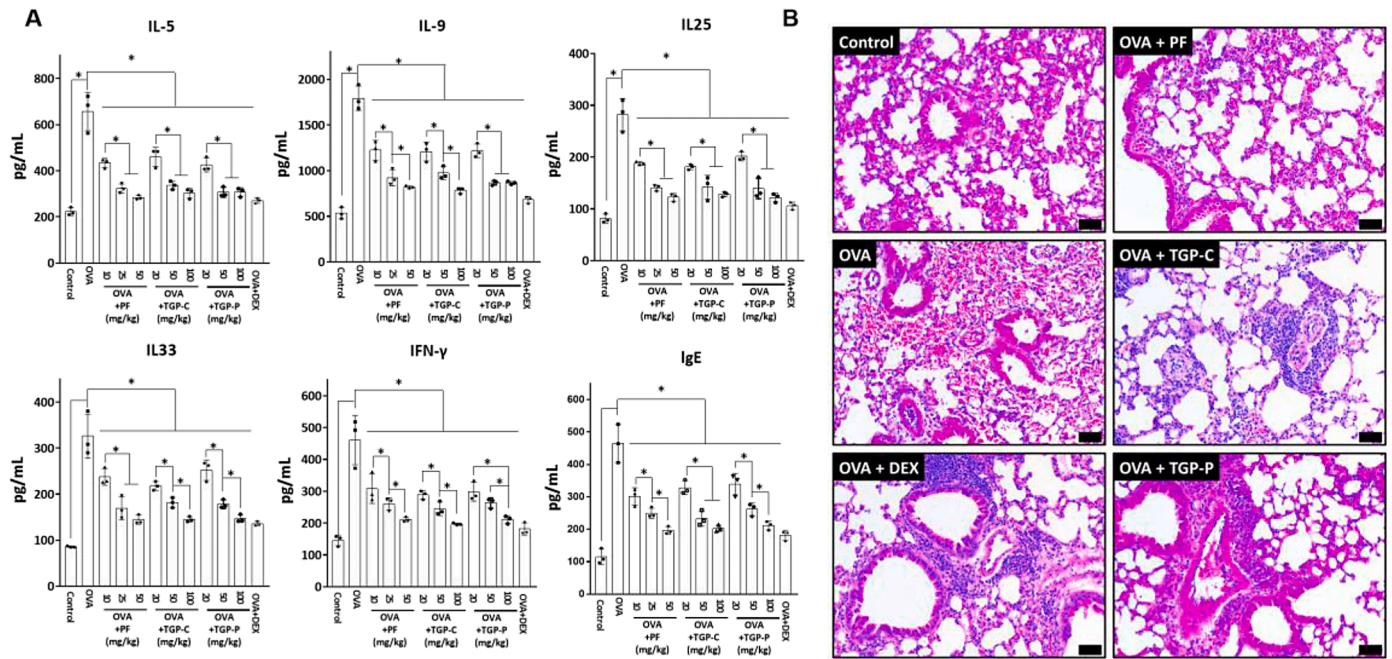


Fig. 3. Secretion of inflammatory cytokines in OVA-challenged lung tissues. ELISA of the secretion of IL-5, IL-9, IL-25, IL-33, IFN- γ , and IgE in lung tissues of OVA-induced mice subjected to various treatments (PF, TGP-C, TGP-P, or DEX). DEX was administered at 2 mg/kg. The data represent the mean $\pm$ SD ($n \geq 3$). * $p < 0.0001$. (B) H&E staining of lung tissues collected on day 32 after mice were sacrificed. Administered doses: PF, 25 mg/kg; TGP-C, 50 mg/kg; TGP-P, 50 mg/kg; DEX, 2 mg/kg.

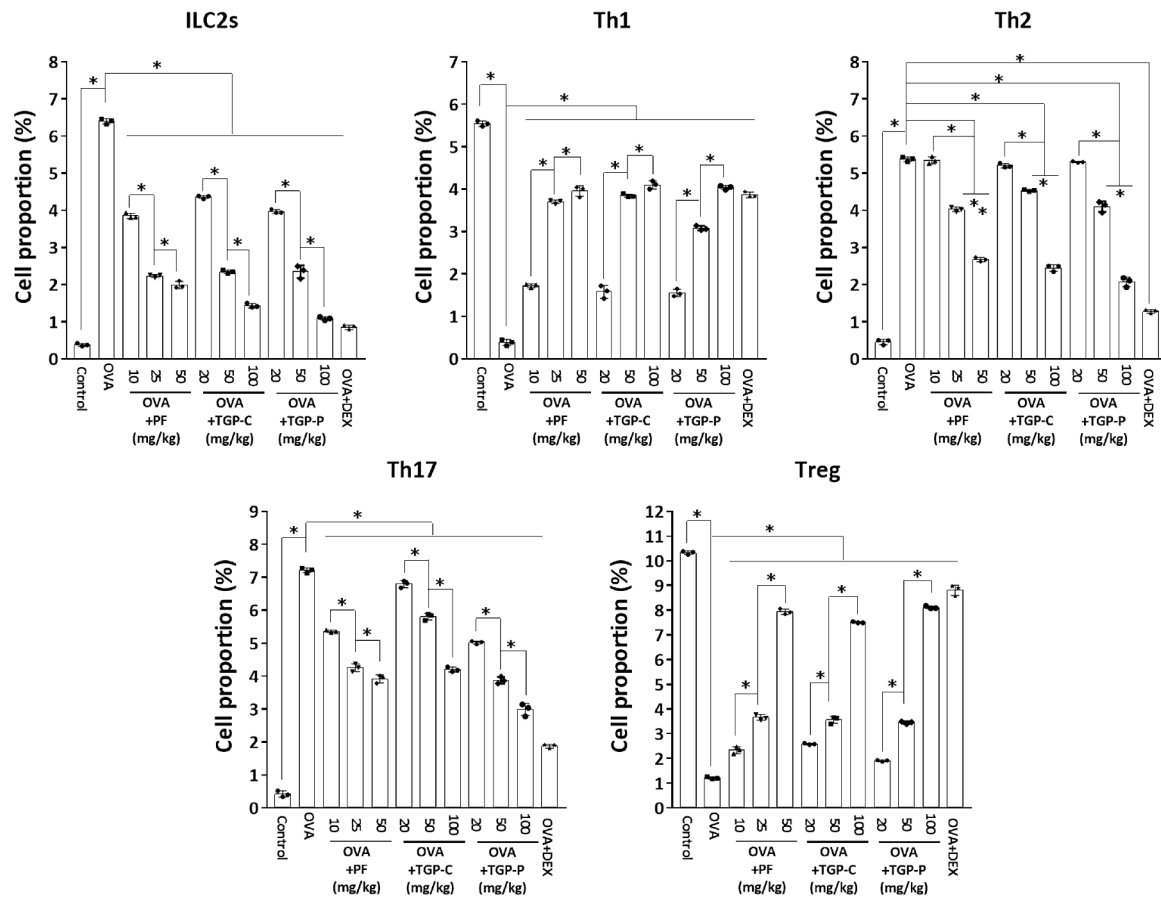


Fig. 4. ILC2s and Th cell subtypes in OVA-challenged lung tissues. The proportion of ILC2s, Th1, Th2, Th17, and Treg cells (%) in lung tissues of OVA-induced mice subjected to various treatments (PF, TGP-C, TGP-P, or DEX) was evaluated by flow cytometry. DEX was administered at 2 mg/kg. The data represent the mean $\pm$ SD ($n \geq 3$). * $p < 0.0001$.

and Supplementary Figure S6). Results indicated that airway epithelium and alveolar epithelial cells in the lung and lung structure in the normal group did not have evident abnormal changes. By contrast, the lung tissues in the OVA group showed had large amounts of inflammatory infiltration and destruction of cell structure. After PF, TGP-C, TGP-P, or DEX treatments, the lung cell structure and inflammatory cell infiltration were obviously ameliorated. These results demonstrated that PF could inhibit inflammatory reaction in OVA-induced asthma.

PF regulated immune response in asthma

The production of IgE and other inflammatory cytokines in allergic asthma were associated with Th2 immune response (Ramakrishna et al., 2012; Ying et al., 2016). The suppressive effects of PF and TGP (C and P) on immune function via T cell differentiation were investigated by measuring the proportion of T cell subsets in lung tissues (Fig. 4 and Supplementary Figure S1). Results showed that the proportions of ILC2s, Th2, and Th17 cells were substantially increased in the OVA-induced group compared those in the control group. By contrast, proportions of Th1 and Treg cells decreased. These results were reversed by PF, TGP-C, and TGP-P treatments in a dose-dependent manner. Therefore, inflammatory and immune response may be important direction for PF in therapy of OVA-induced asthma.

PF regulated the metabolic activity induced by OVA in CD4⁺ T cells

In asthma, the upregulation of glycolysis, (a main pathway of metabolism,) is required for proportions of Th2 (Chen et al., 2019). Thus, the factors (including HK2, PFK1, and PKM1) involved in glycolysis were detected in this study. As shown in Fig. 5D, in the OVA group, the expressions of HK2 (a rate-limiting enzyme), PFK1 (phosphofructokinase 1), and PKM1 (pyruvate kinase) in CD4⁺ T cells were

decreased compared with those in the control group, while these were reversed by PF, TGP (C and P) treatments. As the end-product of glycolysis, pyruvate can stimulate mitochondrial function. This assertion was clarified by measuring the MMP of CD4⁺ T cells isolated from the spleen and peripheral blood from experimental mice (Fig. 5A and Supplementary Figure S2/3). A dramatic increase in the JC-1 monomer proportion was observed in OVA group, indicating a drop in MMP. However, these increases in JC-1 monomer proportion were increased by PF and TGP (C and P) treatments, demonstrating that PF recovered lost MMP in a dose-dependent manner in both the spleen and peripheral blood-derived CD4⁺ T cells. Similar to MMP, the increases of JC-1 monomer proportion in spleen CD4⁺ T cells and peripheral blood were reduced by PF. Except for pyruvate, glycolysis produced lactic acid in an anaerobic environment, lactic acid production in CD4⁺ T cells was determined (Fig. 5C). As expect, lactic acid production increased in the OVA group, but this increase was reduced by PF, TGP (C and P) treatments. These results showed that TGP reduced glycolysis by regulating mitochondrial function in CD4⁺.

PF reduced the immune response by inhibiting autophagy in CD4⁺ T cells

Autophagy plays an important role in asthma (Kielan et al., 2019). In this study, autophagy-related factors (*Bcl2a1b*, *Bcl2a1d*, and *Syne1*) in CD4⁺ T cells were detected via real-time PCR (Fig. 6). The results showed that the expressions of these factors were increased by OVA, which were reduced by PF, TGP (C and P) treatments. Autophagy is also a cell degradation and recycling system that can interact with immunity response (Gerada and Ryan, 2020). Similar to the expressions of autophagy-related factors, those of genes related to immune reaction related genes (*Ler3* and *Tnfrsf18*) were also increased in OVA-induced group compared with the control. Moreover, the expression of *IL1R2* (an inflammatory factor) in OVA group was higher than it in the control

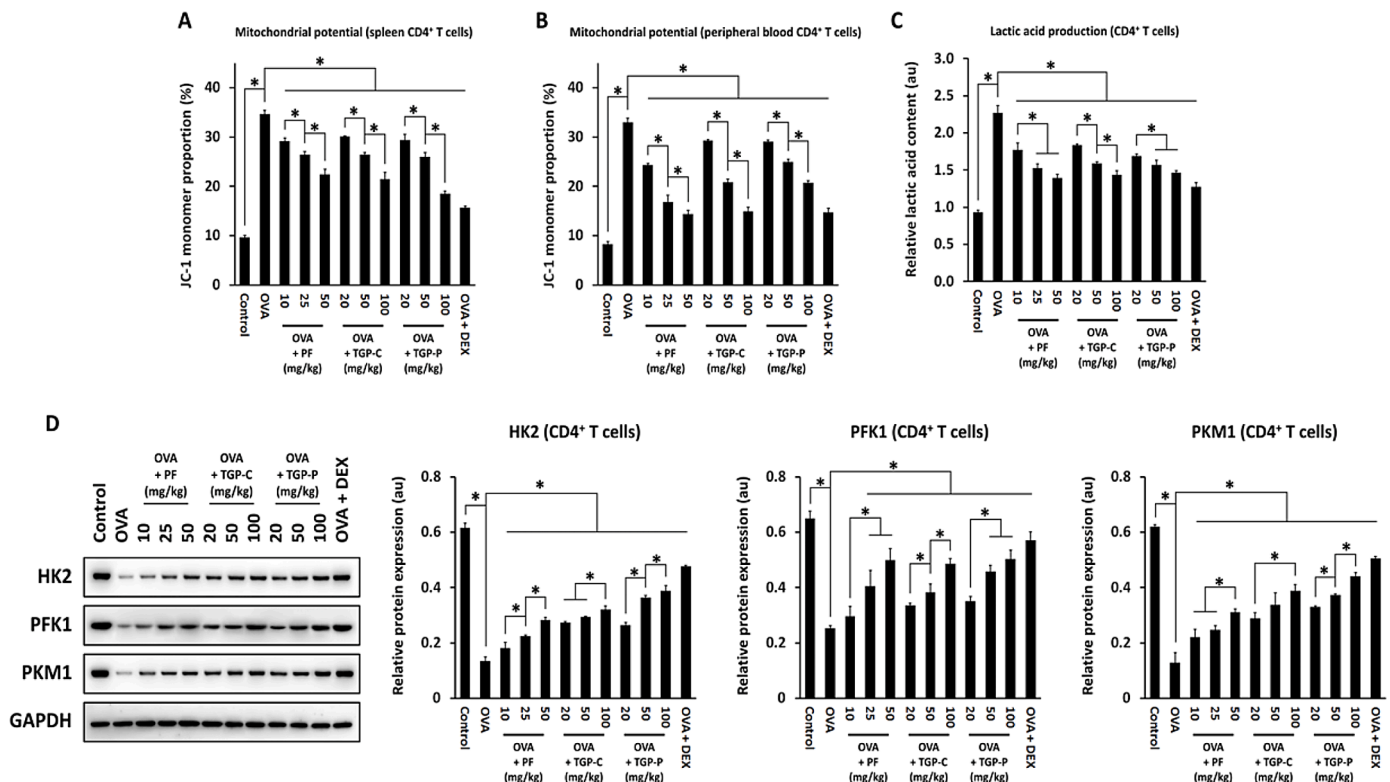


Fig. 5. MMP and metabolic activity in CD4⁺ T cells. OVA-induced mice were subjected to various treatments (PF, TGP-C, TGP-P, or DEX) and CD4⁺ T cells were isolated. DEX was administered at 2 mg/kg. MMP of CD4⁺ T cells isolated from the (A) spleen and (B) peripheral blood was examined by flow cytometry using the JC-1 assay. The data represent the mean $\pm$ SD ($n \geq 5$). * $p < 0.0001$. (C) ELISA of lactic acid production in CD4⁺ T cells. The data represent the mean $\pm$ SD ($n \geq 5$). * $p < 0.0001$. (D) Western blot of enzymes associated with metabolic activity (HK2, PFK1, and PKM1) in CD4⁺ T cells.

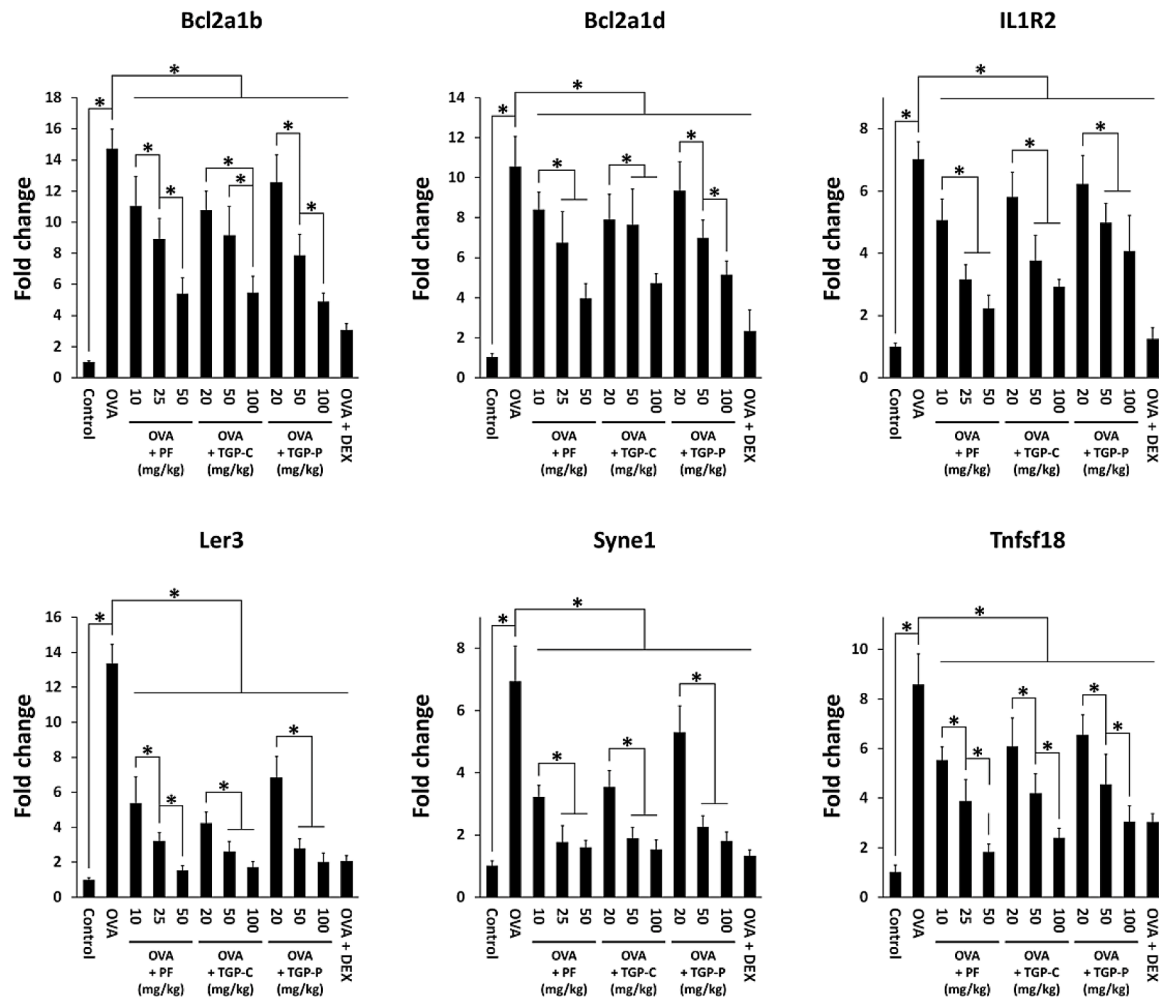


Fig. 6. Immunological memory evaluation in CD4⁺ T cells. OVA-induced mice were subjected to various treatments (PF, TGP-C, TGP-P, or DEX) and CD4⁺ T cells were isolated. DEX was administered at 2 mg/kg. qRT-PCR evaluation of memory-associated gene signatures (Bcl2a1b, Bcl2a1d, IL1R2, Ler3, Syne1, and Tnfsf18). The data represent the mean $\pm$ SD ($n \geq 5$). * $p < 0.0001$.

group (Fig. 6). In turn, these genes were downregulated by PF and TGP (C and P) treatments in a dose-dependent manner. These results indicated that PF regulated immune factors may associated with the inhibition of autophagy.

PF regulated autophagy and apoptosis in OVA-challenged lung tissues

The role of autophagy in immune response and inflammation in asthma was further examined by observing lung characteristics via TEM (Fig. 7A). The ultrastructure of lung epithelial cells in the control group did not have evident abnormal changes, whereas the number of autophagic vacuoles (thick black arrows) in the OVA group was greater than in the control group (Fig. 7A). Furthermore, compared with the control group, OVA treatment also caused a clear increase in number of dense euchromatin (thin black arrows labeled "Eu") in the nucleus. However, these increases were notably reduced by DEX (2 mg/kg), PF (25 mg/kg), TGP-C (50 mg/kg), or TGP-P (50 mg/kg).

Bcl-2 and Beclin 1 can not only regulated autophagy but also mediated apoptosis. Thus, these protein expressions of Bcl-2 and Beclin 1 were detected via Western blot (Fig. 7B). In terms of autophagy, the expressions of Beclin 1 and p62 in OVA-challenged lung tissues were increased, the expressions of TOM20 and COX IV were decreased, all of which were associated with impaired mitochondrial function. In terms of apoptosis, the anti-apoptotic protein Bcl-2 was downregulated, the proapoptotic markers Bax was upregulated, the cleaved form of

caspase3 was increased, and the level of cytochrome-c (cyt-c) were elevated in OVA-induced group compared with the control group, all of which could be reversed by PF, TGP-C, and TGP-P treatments in a dose-dependent manner. These observations suggested that autophagy plays a critical role in therapy of PF by regulating mitochondrial function in asthma.

PF regulated oxidative stress by affecting glycolysis in asthma

Consistent with the results observed in CD4⁺ T cells, PF and TGP (C and P) reduced glycolysis and ameliorated damaged mitochondrial function in mice induced by OVA (Fig. 8A and Supplementary Figure S4). Moreover, OVA also damaged mitochondrial function, which mainly manifested as the reduction of MMP in lung tissue and increase of JC-1 monomer proportion. Mitochondrial dysfunction is associated with oxidative stress, which was also aggravated by OVA, as demonstrated by the increase in ROS production (Fig. 8B and Supplementary Figure S5). Given that oxidative stress could activate glycolysis to meet their energy metabolism requirements, the expressions of HK2, PFK1 and PKM1 in lung were detected by Western Blot (Fig. 8D). OVA led to a drastic increase in lactic acid production in lung tissues (Fig. 8C), which was decreased by PF, TGP-C, and TGP-P treatments. These results demonstrated that PF can protect mitochondria metabolism from oxidative stress.

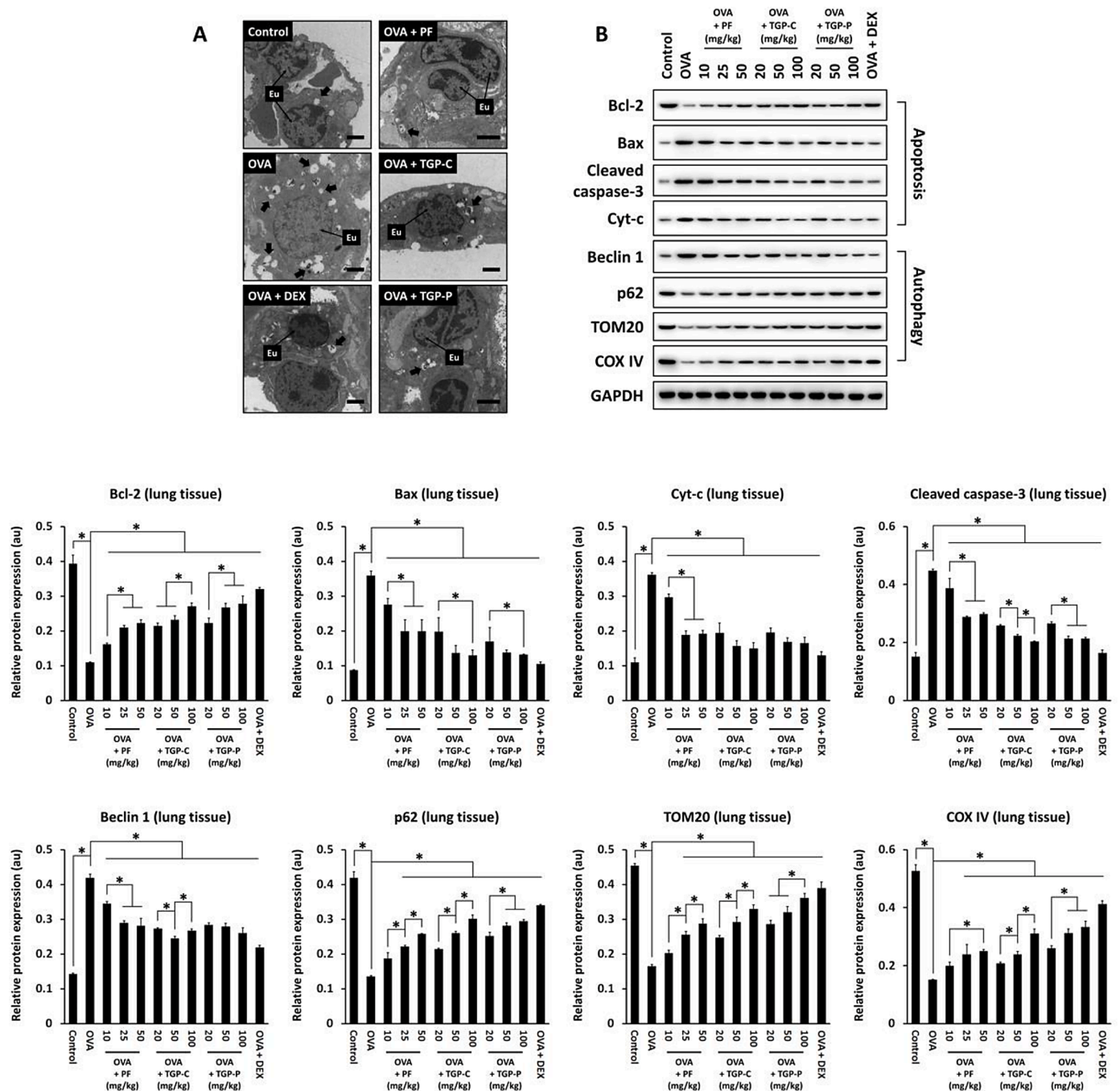


Fig. 7. Apoptosis and autophagy in OVA-challenged lung tissues. OVA-induced mice were subjected to various treatments (PF, TGP-C, TGP-P, or DEX) and lung tissues were collected. DEX was administered at 2 mg/kg. (A) TEM of lung tissues. Thick black arrows point to autophagic vacuoles and narrow black arrows labeled “Eu” point to euchromatin in the nucleus. Scale bar = 2 μ m. (B) Western blot of enzymes associated with apoptosis (Bcl-2, Bax, cleaved caspase-3, and cyt-c) and autophagy (beclin 1, p62, TOM20, and COX IV) in lung tissues.

Discussion

In China and other oriental countries, traditional Chinese medicine has been used for thousands of years to treat various diseases, such as allergic asthma (Li, 2007). The therapeutic basis and molecular mechanisms underlying the effect of individual compounds in herbal and natural formulations have recently attracted the attention of researchers in various fields. Many herbal drugs are processed before administration (Chen et al., 2018; Wu et al., 2018). For example, TGP used in this study was processed with vinegar. PF, the main active ingredient of the TGP, has shown anti-asthma effects (Sun et al., 2015; Zhou et al., 2018), but

the precise immune and inflammation regulation underlying its mechanism of action is unclear. In this study, we revealed that TGP (either in its crude or processed form) and PF regulated pulmonary inflammation and immune response by mediating oxidative stress and autophagy in an OVA-induced mouse model of asthma. We demonstrated that this process is mainly associated with changes in the release of inflammatory factors, T cell subset distribution (differentiation), MMP, glycolysis, ROS production and autophagosome formation.

Inflammation is the key feature of asthma that is manifested by airway hyperresponsiveness, eosinophilia, mucus production, and tissue remodeling (Murdoch and Lloyd, 2010). In present research, specific

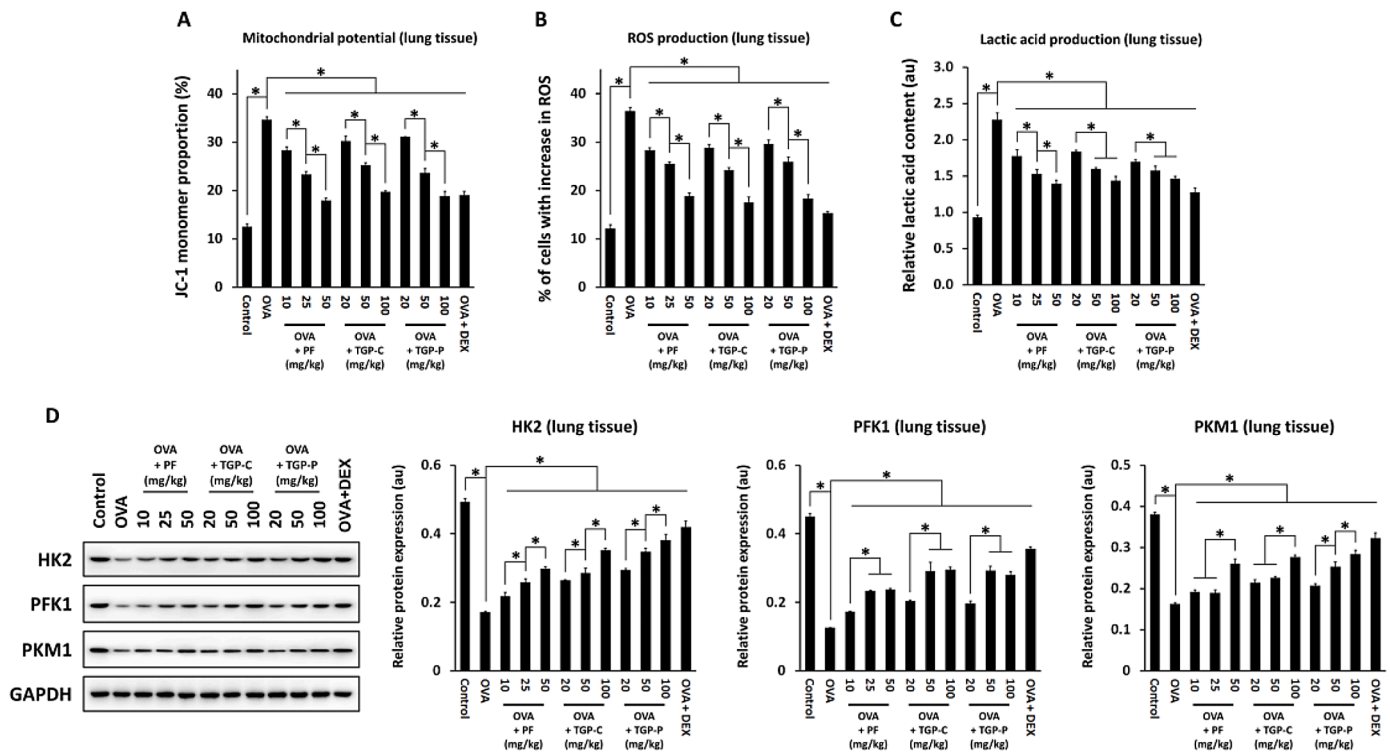


Fig. 8. MMP, ROS production, and metabolic activity in OVA-challenged lung tissues. OVA-induced mice were subjected to various treatments (PF, TGP-C, TGP-P, or DEX) and lung tissues were collected. DEX was administered at 2 mg/kg. (A) MMP of lung tissue was examined by flow cytometry using the JC-1 assay. The data represent the mean $\pm$ SD ($n \geq 5$). * $p < 0.0001$. (B) The percentage of cells in lung with an increase in ROS content was examined by flow cytometry using the DCFH-DA assay. The data represent the mean $\pm$ SD ($n \geq 5$). * $p < 0.0001$. (ANOVA). (C) ELISA of lactic acid production in lung tissues. The data represent the mean $\pm$ SD ($n \geq 5$). * $p < 0.0001$. (D) Western blot of enzymes associated with metabolic activity (HK2, PFK1, and PKM1) in lung tissues.

pulmonary mechanical parameters in present study were changed by OVA, and reversed by TGP and PF treatments. Moreover, airway inflammation is also associated with immune and metabolic dysregulation (Asosingh et al., 2020). The present studies explored the relationship between immune/inflammation response and metabolism in asthma. In our research, activated ILC2s triggered a type 2 inflammatory response in the lungs, resulting in eosinophilia, and excessive production of IL-5, which was consistent with the findings of a previous research (McKenzie, 2014). Given that IL-5 plays critical functions in immune response, it triggers eosinophil differentiation, recruitment, and activation (Hamelmann and Gelfand, 2001). The other cytokines secreted by ILC2s, such as IL-4 and IL-9, can further activate ILC2s, all of which play a critical role in the regulation of immune response in the lungs conversely (Cavagnero and Doherty, 2017; Mindt et al., 2018). In addition, IL-4 promoted the production of IgE by B cells, which induced activation of mast cells and basophils as well as the release of inflammatory mediators (Steinke and Borish, 2001). The proportions of Th2 and Th17 increased, Th1 and Treg decreased along with the up-regulation of ILCs in OVA-induced asthma could trigger immune response. However, the increases of ILC2s and various inflammatory cytokines were reduced by TGP and PF, which manifested that TGP and PF could improve asthma by inhibiting inflammation and immune reaction.

The differentiation of T cells into Th2 cells was in immune response require high levels of energy metabolism (Michalek and Rathmell, 2010), which was closely associated with mitochondrial function that provides energy supply. In OVA-induced allergic asthma, MMP, JC-1 and other indicators related to mitochondrial membrane potential were significantly changed, the release of ROS was increased, all of which indicated that OVA could induce impaired mitochondrial function. Moreover, damaged mitochondria lead to the accumulation of lactic acid along with inhibition of glycolysis-related proteins

expression, such as HK2, PFK1 and PKM1, which also aggravated oxidative stress and inflammation. (Dietl et al., 2010; Pucino et al., 2019). It is worth noting that these changed by OVA were reversed by TGP and PF. Inflammation and irregular immune response may also lead mitochondrial damage that indicated by up-regulation of JC-1 monomer proportion because of disruptions to the proper maintenance of MMP, resulting in oxidative stress (ROS production), depolarization of the mitochondrial membrane, and ultimately cell apoptosis or autophagy/mitophagy (Prakash et al., 2017; Wang et al., 2019), such as the regulation on the expressions of Bcl-2, Bax, Beclin1 in OVA-induced asthma. Furthermore, the elevation of Cyt-c release and cleaved Caspase3 expression indicated that OVA could induced apoptosis, which was ameliorated by TGP and PF. Duo the relationship between autophagy-related factors and mitochondrial damage as well as ROS release, our data showed that TGP and PF exerted obvious effects against asthma by regulating mitochondrial metabolism from oxidative stress to autophagy in lung after OVA induction.

In conclusion, we demonstrated that TGP in its crude or processed form, as well as its active compound PF, effectively suppressed asthmatic features induced by OVA in mice. Improvement in pulmonary parameters was associated with the changes in inflammatory cytokine secretion as well as immune reaction regulated by TGP, all of which was connected with mitochondrial metabolic that form oxidative stress to autophagy. It will be interesting to initiate clinical research to test the efficacy of TGP in treating asthma in the future.

CRediT authorship contribution statement

Gang Cao designed the study. Xin Han, Shaoqi Hu, Qiao Yang and Dongxin Tang performed the experiments. Xin Han, Shaoqi Hu, Xianan Sang and Qiao Yang participated in data analysis. Gang Cao and XH wrote the manuscript. All data were generated in-house, and no paper

mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.phymed.2021.153835](https://doi.org/10.1016/j.phymed.2021.153835).

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Supplementary Figure S1: the percentages of Th1, Th2, Th17, ILC2s and Treg cells detected by flow cytometry. Representative FACS dot plots of Th1 (CD4-H IFN-r-H), Th2 (CD4-H IL-4-H), Th17 (CD4-H IL-17A-H), ILC2s (Lin-H SSC-H, CD25-H, and CD127-H) cells in lung tissues in OVA-induced mice (and treatment with PF, TGP-C, TGP-P, or DEX) and controls.

Supplementary Figure S2: The expression of MMP in CD4⁺ T cells isolated from the spleen. JC-1 monomer proportion was observed in OVA group by flow cytometry, indicating a drop in MMP in CD4⁺ T cells isolated from the spleen.

Supplementary Figure S3: The expression of MMP in CD4⁺ T cells isolated from peripheral blood. JC-1 monomer proportion was observed in OVA group by flow cytometry, indicating a drop in MMP in CD4⁺ T cells isolated from peripheral blood.

Supplementary Figure S4: The expression of MMP in lung tissue. JC-1 monomer proportion was observed in OVA group by flow cytometry, indicating a drop in MMP in lung tissue.

Supplementary Figure S5: ROS production in lung tissue. The increase in ROS production was detected by flow cytometry with FITC.

Supplementary Figure S6: H&E staining of lung tissues.

Supplementary Figure S7: UPLC chromatogram of samples extracted and separated from raw Radix Paeoniae Alba and fried Paeoniae Radix Alba with bran. A UPLC analysis mixed standards, including oxypaeoniflorin, albiflorin, benzoylalbiflorin, paeoniflorin, galloylpaeoniflorin. B UPLC analysis of raw Paeoniae Radix Alba samples. C UPLC was used to analyze the processed samples of wheat bran fried Paeoniae Radix Alba.

Figure S1

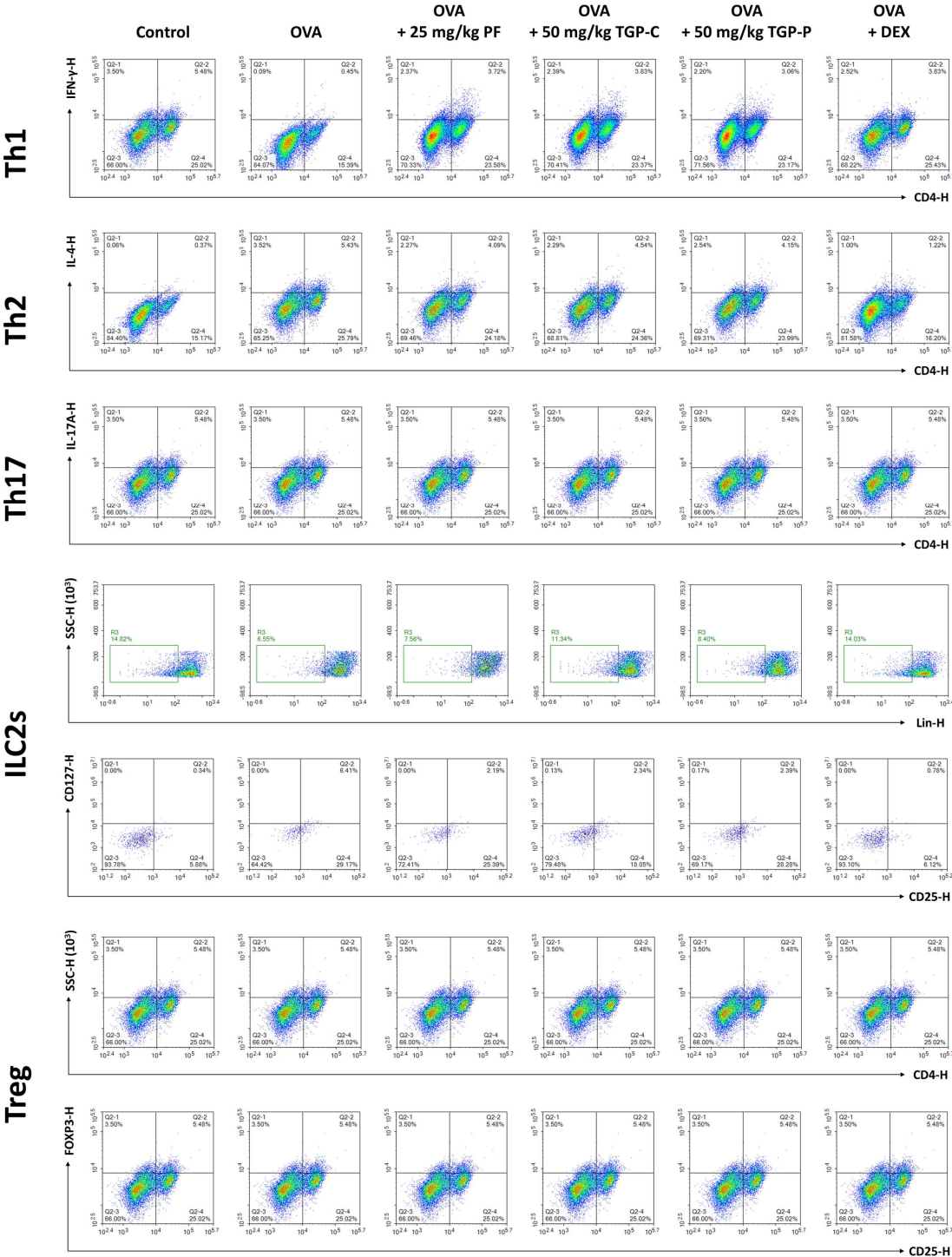


Figure S2

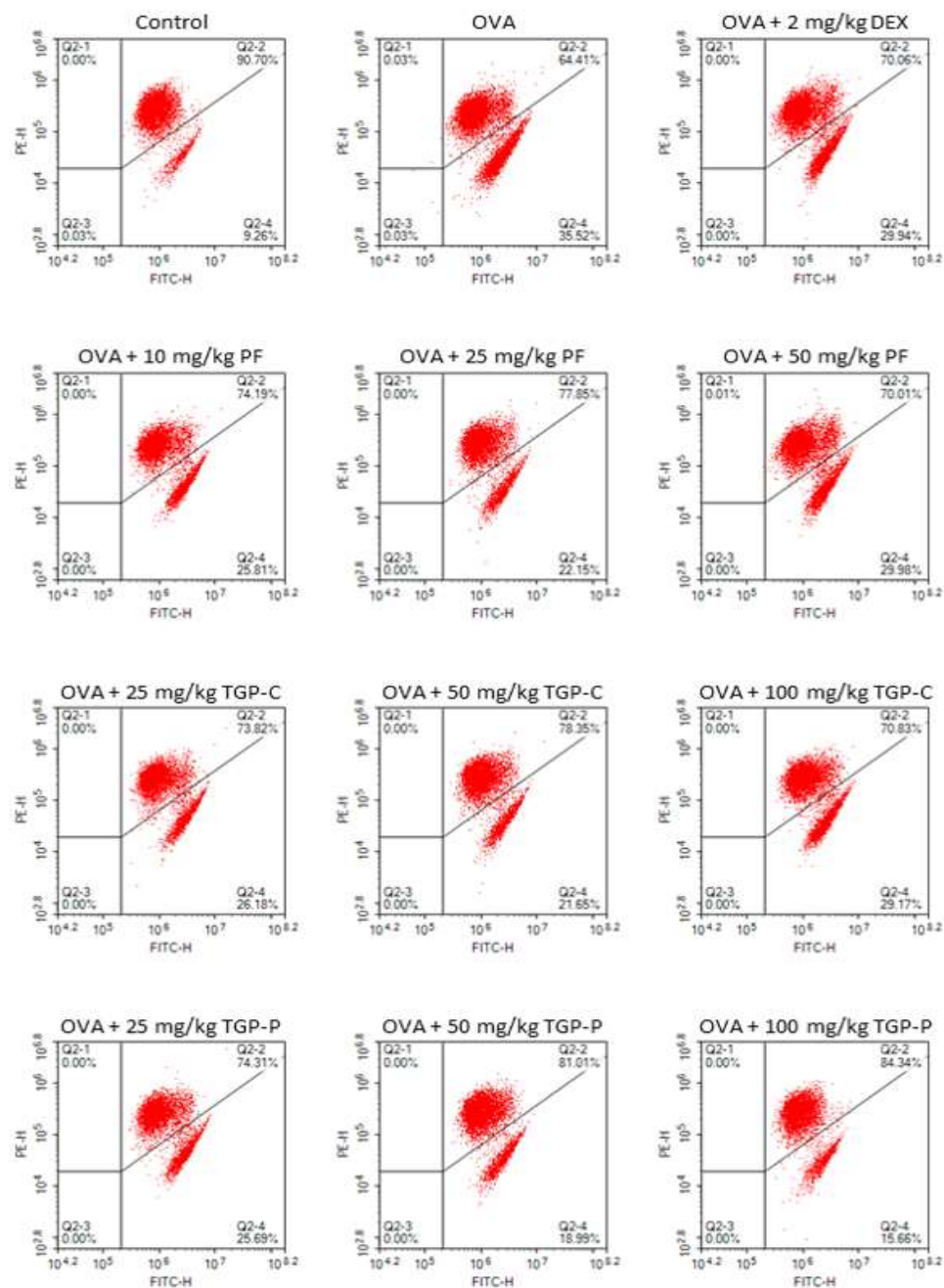


Figure S3

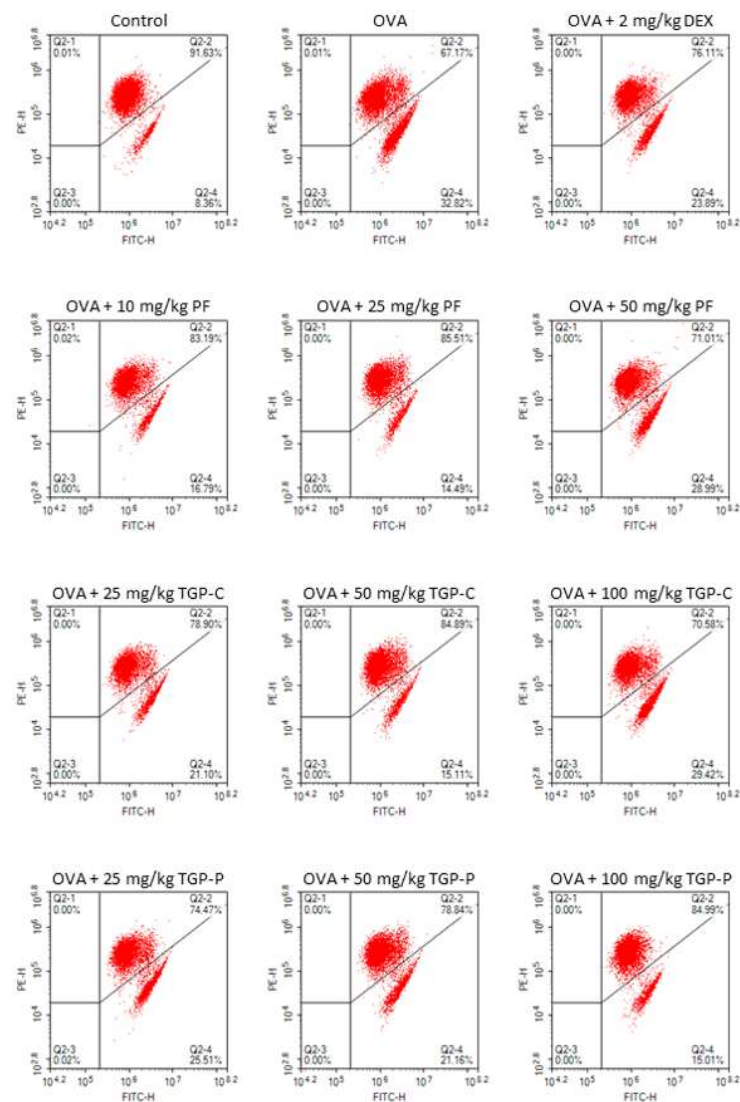


Figure S4

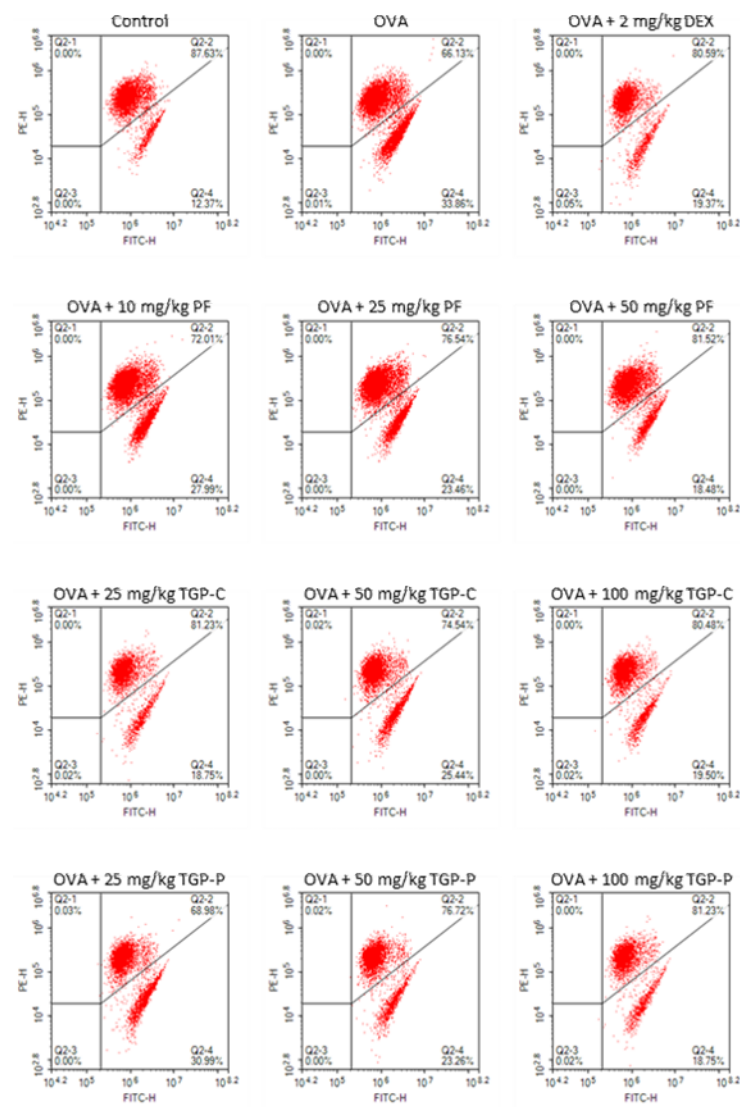


Figure S5

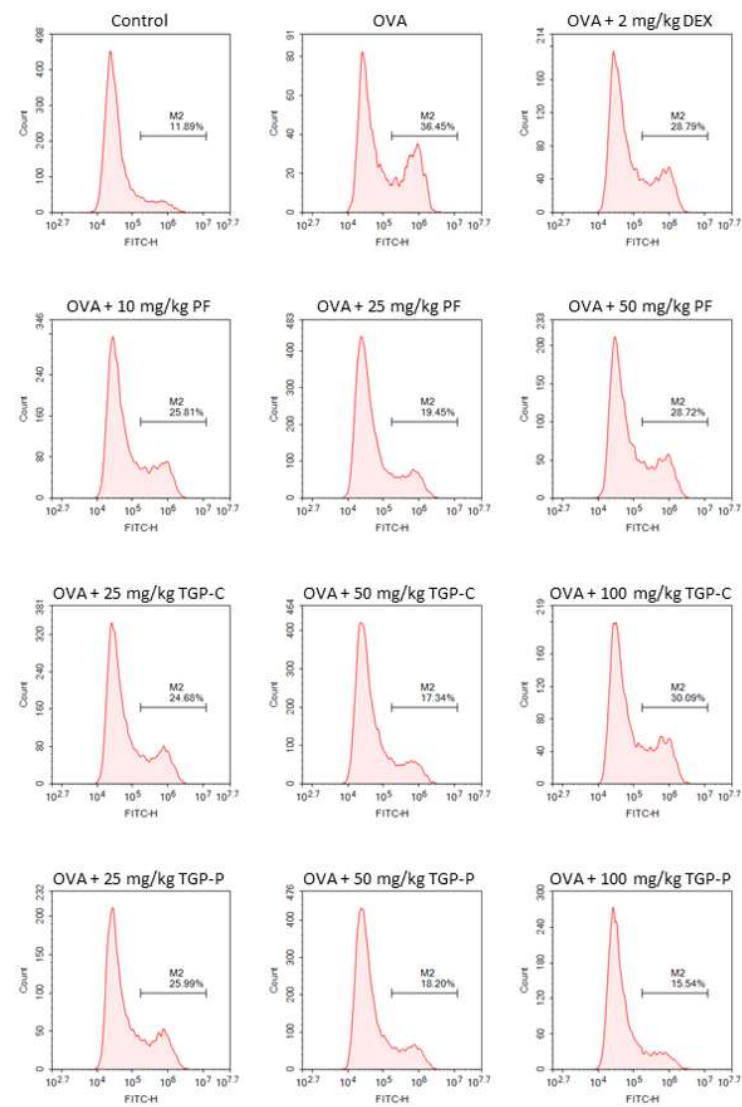


Figure S6

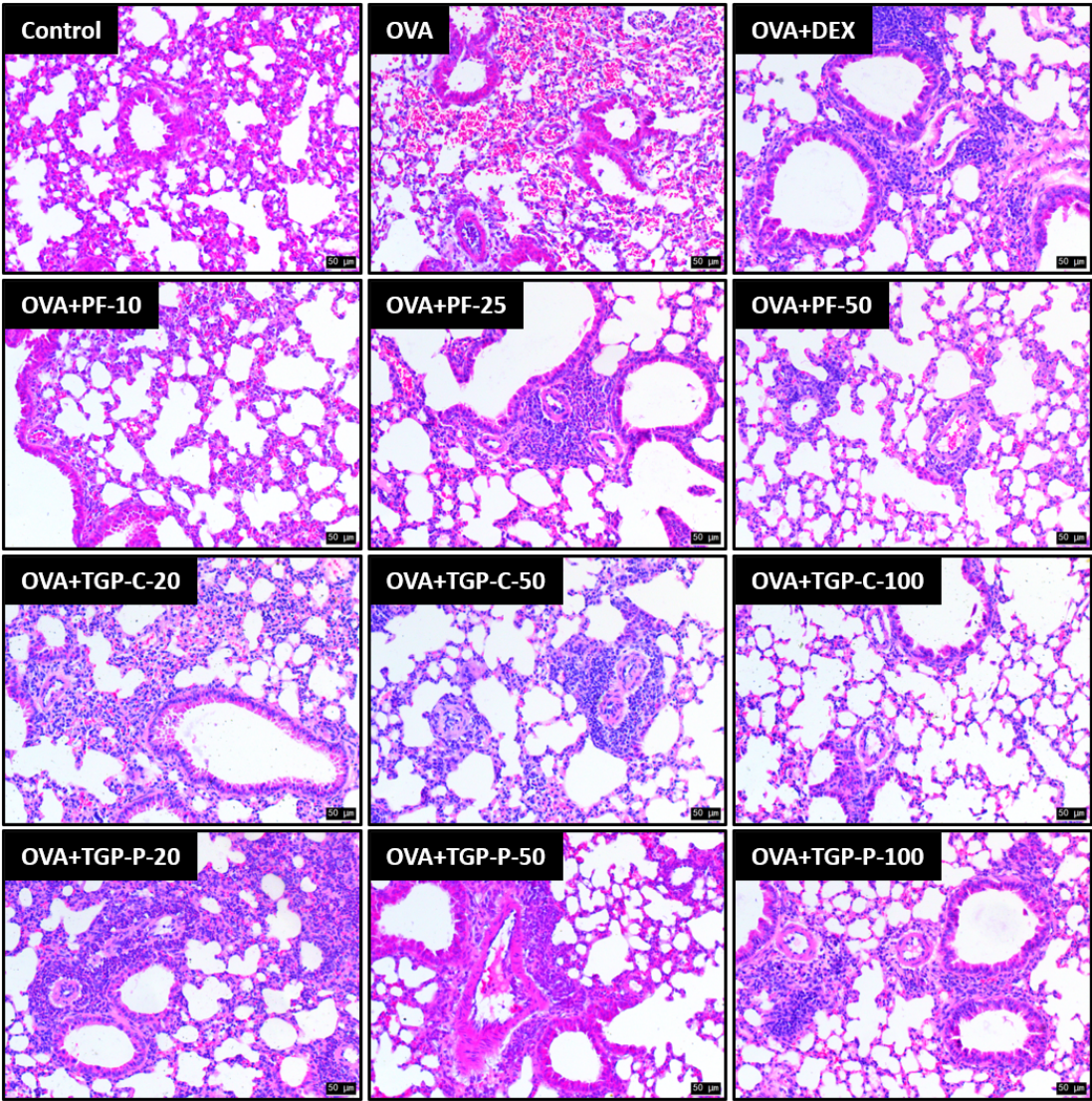


Figure S7

