



Luteolin inhibits autophagy in allergic asthma by activating PI3K/Akt/mTOR signaling and inhibiting Beclin-1-PI3KC3 complex

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ABSTRACT

Allergic asthma is a common chronic inflammatory disease characterized by airway inflammation, mucus hypersecretion and airway remodeling. Autophagy is a highly conserved intracellular degradation pathway in eukaryotic cells. There is growing evidence suggesting that dysregulation of autophagy is involved in the pathological process of asthma. Luteolin is a typical flavonoid compound with anti-inflammatory, anti-allergic and immune-enhancing functions. Previous studies have shown that luteolin can attenuate airway inflammation and hypersensitivity in asthma. However, whether luteolin can play a role in treating asthma by regulating autophagy remains unclear. The aim of the present study was to evaluate the therapeutic effect of luteolin on ovalbumin (OVA)-induced asthmatic mice, observe its effect on the level of autophagy in lung tissues, and further elucidate its underlying mechanism. The results showed that OVA-induced mice developed airway hyper-responsiveness, mucus over-production and collagen deposition. The number of inflammatory cells, levels of interleukin (IL)-4, IL-5 and IL-13 in bronchoalveolar lavage fluid (BALF) and OVA-specific IgE in serum were significantly increased. Furthermore, the infiltration of inflammatory cells was observed along with the activation of autophagy in lung tissues. Luteolin treatment significantly inhibited the OVA-induced inflammatory responses and the level of autophagy in lung tissues as well. Moreover, luteolin activated the PI3K/Akt/mTOR pathway and inhibited the Beclin-1-PI3KC3 protein complex in lung tissues of asthmatic mice. In conclusion, this study explored the regulatory mechanism of luteolin on autophagy in allergic asthma, providing biologic evidence for its clinical application.

1. Introduction

Asthma is a common chronic airway disease characterized by airway stenosis, airway wall thickening and increased airway mucus secretion resulting in restricted airflow. Its clinical features include dyspnea, wheezing, chest tightness and cough [1]. According to the 2016 Global Burden of Disease Study, more than 339 million people worldwide suffer from asthma, and in the past 50 years, the incidence and prevalence of asthma have increased significantly [2]. In some countries, the incidence rate even reaches as high as 15–20% [3]. Currently, asthma is considered to be an umbrella diagnosis that includes several different clinical manifestations (phenotypes) and distinct pathophysiological mechanisms (endotypes), among which, allergic asthma is the most common asthma phenotype [4]. The pathological mechanism of allergic

asthma is not yet completely clear. It is generally recognized that it starts with the activation and differentiation of allergen-specific T helper 2 cells (Th2). This is followed by the production of immunoglobulin E (IgE), an allergen-specific immunoglobulin that activates IgE-dependent mast cells, and recruitment of eosinophils into the lungs. Eventually, the persistent airway inflammation and asthma symptoms develop [5].

Autophagy is a highly conserved intracellular degradation pathway in eukaryotic evolution, which can be classified into microautophagy, chaperon-mediated autophagy, and macroautophagy according to their different mechanisms. Among them, macroautophagy (“autophagy” for short) is the most widely studied type [6]. Under normal circumstances, autophagy is conducive to cell survival. It can maintain the homeostasis of cells and organisms by removing damaged and necrotic organelles and proteins in response to environmental pressures ranging from

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nutrient deficiency to growth factor depletion, hypoxia, temperature changes, and infectious pathogens [7]. However, excessive autophagy caused by oxidative stress, inflammation, and other factors can cause adverse effects on cells and even lead to cell death [8,9]. Dysregulation of autophagy is closely related to multiple diseases and has been proved to be involved in the pathological process of allergic asthma [10], such as significant up-regulation of autophagy observed in the lung tissue of ovalbumin (OVA)-induced asthmatic mice [11]. Compared with normal subjects, the lung tissues of asthmatic patients showed inflammation accompanied by low expression of sequestosome 1 (p62) and high expression of autophagy-related proteins coiled-coil myosin-like BCL2-interacting protein (Beclin-1, the first mammalian autophagy protein to be described) and autophagy-related gene 5 (ATG5) [12]. Inhibiting this excessive autophagy can significantly alleviate the symptoms of asthma. For example, activating the autophagy repressor of the kinase mammalian target of rapamycin (mTOR) can reduce airway inflammation in asthmatic mice [13]. Inhibition of autophagy can also significantly reduce the level of eosinophils in the lungs of asthmatic mice [14].

The phosphatidylinositol 3 kinase (PI3K) family is one of the potential targets of anti-asthma treatment [15]. It is mainly divided into three categories: class I, class II and class III. Among them, the class I PI3Ks (PI3KC1) are upstream activators of Protein Kinase B (Akt). mTOR, as a major regulator of autophagy, is a downstream target of PI3K/AKT pathway [16]. Activation of the PI3K/AKT can directly phosphorylate mTOR at Ser2448, which inhibits autophagy and promotes cell survival [17]. Studies have shown that activation of the PI3K/Akt/mTOR signaling pathway effectively attenuates airway inflammation by inhibiting pulmonary autophagy during allergic asthma, while inhibition the PI3K/Akt/mTOR pathway aggravates airway inflammation [18,19]. The Class III PI3Ks (PI3KC3, also known as VPS34) form a protein complex with the autophagic-related protein Beclin-1, which is essential for autophagy [20]. Inhibiting the activity of Beclin1-PI3KC3 core complex represses autophagy [21]. PI3KC3 specific inhibitor 3-methyladenine (3-MA) significantly reduced OVA-induced allergic airway inflammation and oxidative stress in asthmatic mice [14], and decreased the expression of autophagy marker LC3 in lung epithelium [22]. Thus, the class I and the class III PI3Ks may involve in the autophagy regulation process of allergic asthma simultaneously.

Glucocorticoids are mainly used in modern medicine to treat allergic asthma. However, long-term use of glucocorticoids is prone to resistance and has certain side effects. Therefore, it is of great significance to find safe and effective complementary and alternative therapies. Luteolin (3',4',5,7-tetrahydroxyflavone) is a typical flavonoid compound with high medical value for its anti-oxidant, anti-inflammatory, anti-cancer, and enhancement of immunity functions. Previous studies have shown that luteolin significantly reduces the number of eosinophils and the levels of IL-4, IL-5, IL-13, and eotaxin in asthma bronchoalveolar fluid (BALF). It also increases protein levels of IL-10 and IFN- γ , as well as mRNA expressions of IL-10 and TGF- β 1 in the lung tissues and alleviates OVA-induced airway inflammation in asthmatic mice [23]. However, the mechanism of luteolin in treatment of asthma remains unclear. In recent years, studies have shown that luteolin has regulatory effects on PI3K/Akt signaling pathway [24,25] and autophagy [26–28], respectively. Whether it can regulate autophagy through PI3Ks signaling in asthma has not been studied. Therefore, in this study, we observed the effect of luteolin on the autophagy in lung tissues of asthmatic mice and its regulatory effects on PI3K/Akt/mTOR pathway and Beclin1-PI3KC3 core complex.

2. Materials and methods

2.1. Animals

Fifty female balb/c mice (6-week-old, 20–25 g weight range) were purchased from Jiesijie Laboratory Animal Co. Ltd (license number:

SCXK (Hu) 2013–0006), and randomly divided into the control group (Con), asthma group (Ast), low-dose luteolin group (Lut-L), high-dose luteolin group (Lut-H) and dexamethasone group (Dex). The mice were raised under specific pathogen-free (SPF) conditions at a temperature of $25 \pm 2^\circ\text{C}$, 12/12 h light–dark cycle with free access to water and food. All of the animal procedures were approved by the Institutional Animal Care and Use Committee of Fudan University (Authorization number: 2018–10-HSYD-DJC-01) and performed in accordance with National Institute of Health guidelines.

2.2. Experimental model of asthma and treatment

An OVA-induced mice model of allergic asthma was established by intraperitoneally injection of 20 μg OVA (Grade V; Sigma-Aldrich, St. Louis, MO) and 2 mg aluminum hydroxide (Thermo Scientific) at day 0, 7, 14 and 21 (Sensitization stage). From day 22 (Challenge stage), mice were given 2% OVA ultrasonic atomization inhalation for 30 min once a day for consecutive 7 days. Each time of challenge, mice in the Lut-L and Lut-H groups were gavaged with 10 mg/kg and 20 mg/kg [23,29,30] luteolin (Purity 99.50%, Shanghai Winherb Medical Science Co., Ltd) respectively. Mice in the Dex group were administrated with 1 mg/kg dexamethasone, and mice in the Con group were given the same amount of saline. At the same time, mice in the Con group was sensitized and challenged with saline instead of OVA (Fig. 1).

2.3. Measurement of airway hyperreactivity

Mice in each group were anesthetized by intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg) 24 h after modeling. Then, mice were tracheostomized, intubated, and placed in a whole-body plethysmograph connected to the ventilator (Buxco Electronics, Troy, NY) to detect lung function. When mice were exposed to methacholine (Mch, Sigma-Aldrich; St. Louis, MO) at increasing doses of 0, 3.125, 12.5, and 50 mg/mL, lung dynamic compliance (C_{dyn}) and airway resistance (R) were recorded to evaluate airway hyperreactivity (AHR).

2.4. Inflammatory cells count and cytokines detection

Bronchoalveolar lavage fluid (BALF) was collected by intratracheal instillation with 300 μl of cold PBS twice, and centrifuged at 500g for 10 min at 4°C . Mice blood was collected, kept at room temperature for 1 h, centrifuged at 3000 rpm for 30 min, and serum was collected. The supernatants were separated and preserved at -80°C for cytokine detection. The levels of IL-4, IL-5, IL-13 in BALF and serum OVA-specific IgE were determined by ELISA Kits (Cayman Chemical, Michigan, USA) in accordance with the Kit instructions. The cell pellets were resuspended in 100 μl PBS to calculate the number of leukocytes and counts of different populations by using an automatic cell counter (Hemavet 950 instrument; Drew Scientific Group, UK).

2.5. Histological observation and assessment

The left lung of mice in each group were collected and fixed in 4% paraformaldehyde, then embedded in paraffin, sectioned at 4 μm thickness, and stained with Hematoxylin-eosin (HE), Masson's trichrome (Masson) and Periodic Acid Schiff (PAS), respectively. Each section was observed under a light microscope and 3 of fields were randomly selected for photography. For sections stained with HE, the degree of inflammatory cells infiltration was evaluated based on a scoring system describing how many inflammatory cells around the airway [31]: 0, no inflammatory cells; 1, a few inflammatory cells; 2, a ring of inflammatory cells (1–2 cell-layers); 3, a ring of inflammatory cells (3–5 cell-layers); 4, a ring of inflammatory cells (>5 cell-layers). For sections stained with PAS and Masson, area of interest (AOI) was selected and the percentage of positive staining area was calculated by Image J software.

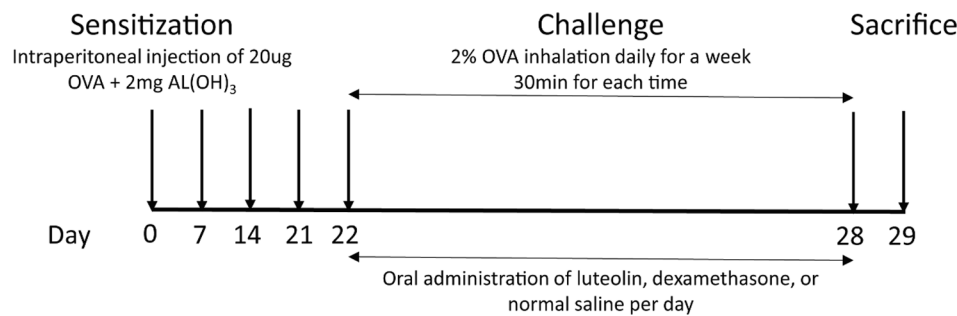


Fig. 1. The modeling process of asthmatic mice. Mice were sensitized, challenged, administered and sacrificed from day 0 to 29. Sensitization was performed on day 0, 7, 14 and 21. Challenged and treatment were performed from day 22 to 28. Lung function of mice in each group was measured 24 h after modeling prior to mice sacrifice. OVA: ovalbumin.

2.6. Immunofluorescence and immunohistochemistry

Tissues were dewaxed to water, and 0.01 M sodium citrate buffer was used for antigen repair. Inactivate endogenous enzymes (only for immunohistochemistry) was inactivated by 3% H₂O₂. After washed with PBS three times (10 min each), tissues were blocked with 5% BSA for 30 min, and incubated with the primary antibody (LC3B, Cell Signaling Technology, Dilution: 1:100; EMBP, Santa Cruz, Dilution: 1:200 for immunofluorescence. LC3B, Proteintech, Dilution: 1:200; Beclin-1, Proteintech, Dilution: 1:400 for immunohistochemistry) overnight at 4 °C. After incubation, samples were washed with PBS for three times (10 min each), then incubated with secondary antibody (Cy3, 1:300; FITC, 1:400 for immunofluorescence. HRP Conjugated Goat Anti-rabbit IgG (1:200) for immunohistochemistry). For immunofluorescence, autofluorescence quencher was added and incubated for 5 min, and then samples were rinsed with running water for 10 min. After washed three times with PBS, samples were incubated with DAPI staining solution for 10 min at room temperature in the dark. After the sections were dried slightly, anti-fluorescence quencher dropwise was added to mount the slides. The slices were observed under a fluorescence microscope (Nikon, Eclipse C1) and collect images. For immunohistochemical assay, sections were stained with DAB and hematoxylin, washed with PBS three times and then mount for microscope observation (CIC, XSP-C204); Area of interest (AOI) was selected and integrated option density (IOD) was calculated by Image-pro plus. The average optical density (AOD, IOD/AOI) was used for analysis.

2.7. Western blotting

Lung tissues were minced and homogenized by RIPA lysate containing protease and phosphatase inhibitor (Sangong Biotech, C500019-0001), followed by 12,000g centrifugation for 10 min at 4 °C, and the supernatants of each group were collected. The concentration of total protein was quantified by Pierce BCA Protein Assay Kit (Thermo Scientific). Electrophoresis separation was performed by 10% SDS-PAGE and transferred with 0.25 μm PVDF membranes. The membranes were blocked with 5% milk at room temperature for 1 h, then incubated with primary antibodies (LC3B, p62, p85, akt, p-akt, mTOR, p-mTOR, beclin-1, pi3kc3, Cell Signaling Technology, Dilution: 1:1000) at 4 °C overnight, followed by HRP-conjugated secondary antibody (Dilution: 1:1000 or 1:10000) incubation at room temperature for 1 h. Images were obtained by ultimate exposure using LAS-4000 mini visualizer (Fujifilm Corporation, Tokyo, Jap).

2.8. Statistical analysis

SPSS 20.0 statistical software was used for data analysis. One-way ANOVA followed by a least significant difference (LSD) post-hoc test was used for data that met the normal distribution. For data that did not meet the normal distribution were examined by Kruskal-Wallis test and

Dunn-Bonferroni post-hoc test for group comparisons. Data were presented as the mean ± standard deviation (SD) or as the median (P25, P75). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Luteolin improves lung function in asthmatic mice

Airway hyperreactivity is one of the characteristics of asthma, evidenced by increased airway resistance (R) and decreased lung dynamic compliance (C_{dyn}) [32]. In the present study, we observed the lung function of mice in each group (Fig. 2). Compared with the control group, an increase in airway resistance accompanied by a decrease in C_{dyn} were observed in the asthma groups by the increase of methacholine concentration, suggesting airways were hyper-responsive. At the concentrations of 12.5 mg/mL and 50 mg/mL methacholine, there was no statistically significant difference in lung function between the low-dose luteolin group and the asthma group. The airway resistance in the high-dose luteolin group and dexamethasone group were significantly reduced ($P_{12.5\text{mg/Lut-H}} < 0.01$, $P_{12.5\text{mg/Dex}} < 0.01$; $P_{50\text{mg/Lut-H}} < 0.01$, $P_{50\text{mg/Dex}} < 0.01$). C_{dyn} was significantly increased in the high-dose luteolin group at both concentrations, and the dexamethasone group increased significantly at 12.5 mg/mL and presented an increasing trend at 50 mg/mL ($P_{12.5\text{mg/Lut-H}} < 0.05$; $P_{12.5\text{mg/Dex}} < 0.01$; $P_{50\text{mg/Lut-H}} < 0.05$; $P_{50\text{mg/Dex}} = 0.053$).

3.2. Luteolin ameliorates lung inflammation in asthmatic mice

The pathological changes of lung tissues in each group were observed (Fig. 3). The results of HE staining showed that the bronchial and lung tissues of mice in the control group had complete structure. In detail, the cilia were neatly arranged, and the bronchial smooth muscle thickness was normal. No inflammatory cell infiltration was found around the bronchial, and the alveoli were normal in size and complete in structure. Mice from OVA-induced asthma showed bronchial stenosis, increased mucus in the airway, swelling or shedding of epithelial cells, irregular cilia arrangement, thickening of bronchial smooth muscle, a large number of inflammatory cells surrounding the airway, and a significant reduction in the number of alveoli. There was no significant improvement in the low-dose luteolin group. In the asthma group and low-dose luteolin group, bronchial lumen stenosis and epithelial cell swelling were seen. Inflammatory cell infiltration was observed around the airway, and the number of alveoli was significantly reduced, accompanied by thickening of alveolar walls and increased spacing. These pathological changes were significantly improved by high-dose luteolin treatment and dexamethasone treatment. The semi-quantitative analysis showed that the airway inflammation score of the asthma group was significantly higher than that of the control ($P_{\text{Ast}} < 0.01$). There was no statistical difference in airway inflammation score between the asthma group and low-dose luteolin group. The scores of the luteolin high-dose

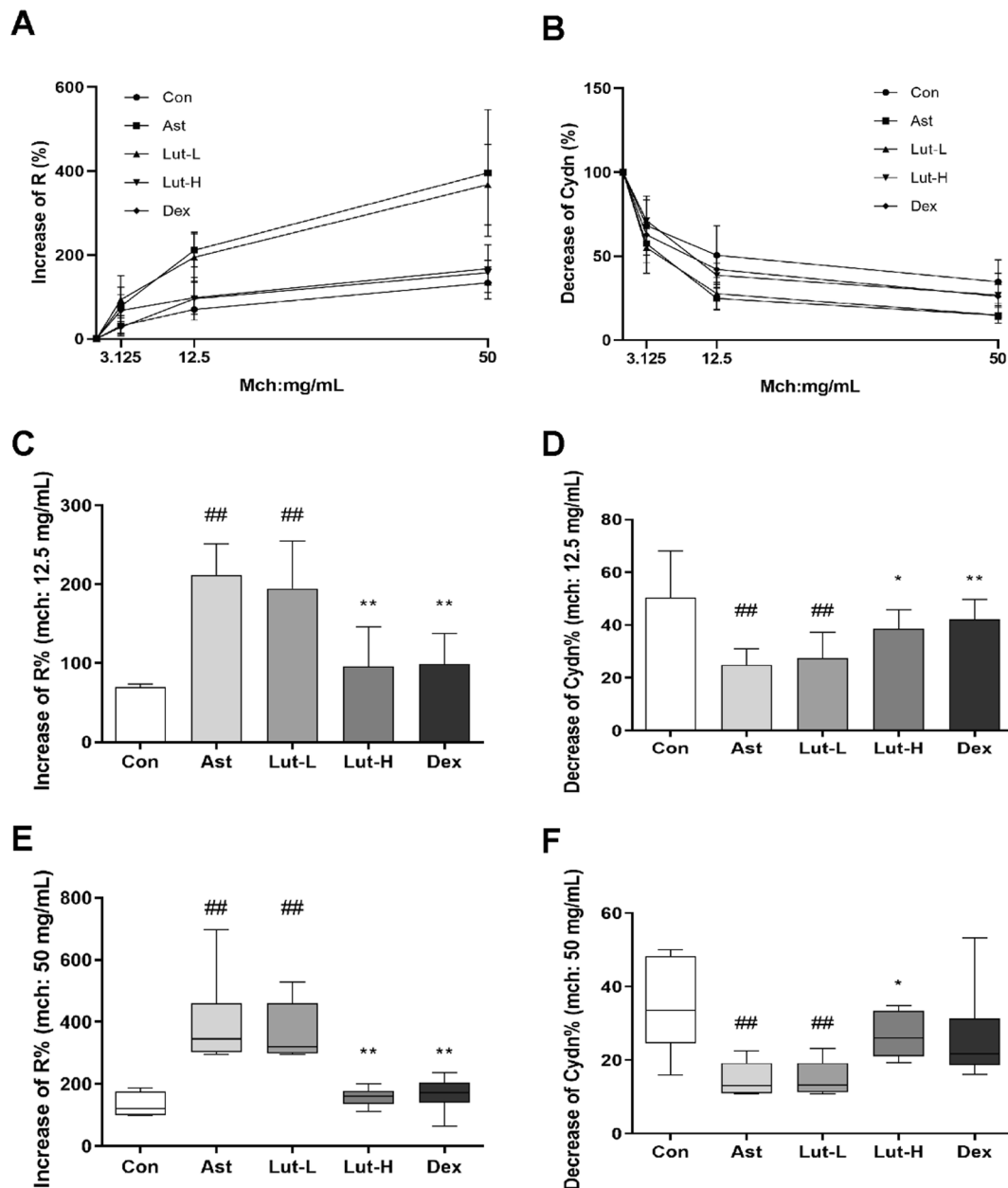


Fig. 2. The effects of luteolin on Lung function of mice in each group. (A–B) Airway resistance and dynamic compliance of mice with different concentrations of methacholine; (C–D) Airway resistance and dynamic compliance of mice in each group at concentrations of 12.5 mg/mL methacholine; Data are presented as the mean $\pm$ standard deviation and one-way ANOVA was applied to detect significant differences; (E–F) Airway resistance and dynamic compliance of mice in each group at concentrations of 50 mg/mL methacholine; Data are presented as the medians (P25, P75) and Kruskal-Wallis ANOVA was used for comparison. N = 6 in each group. [#] $P < 0.05$, ^{##} $P < 0.01$ vs control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs asthma group. R: airway resistance; Cydn: dynamic compliance; Con: control group; Ast: asthma group; Lut-L: low-dose luteolin group; Lut-H: high-dose luteolin group; Dex: dexamethasone group.

group and dexamethasone group were significantly lowered compared with that of the asthma group ($P_{\text{Lut-H, Dex}} < 0.05$).

Next, we classified and counted the inflammatory cells in BALF of mice in each group (Fig. 4). The results showed that compared with the control group, the total number of inflammatory cells in the asthma group was significantly increased ($P_{\text{Ast/Total}} < 0.01$). Among them, lymphocytes, eosinophils, and neutrophils all increased significantly ($P_{\text{Ast/Lym, Eos, Neu}} < 0.01$). Compared with the asthma group, the total number of inflammatory cells in the low-dose luteolin group trended to decrease with no statistical significance. The total number of inflammatory cells in the high-dose luteolin group and dexamethasone group was significantly down-regulated ($P_{\text{Lut-L/Total, Lut-H/Total}} < 0.05$). Among them, lymphocytes, eosinophils and neutrophils showed no

statistically significant difference in the low-dose luteolin group, but lymphocytes and eosinophils showed a downward trend. The number of lymphocytes ($P_{\text{Lut-H/Lym, Dex/Lym}} < 0.05$), eosinophils ($P_{\text{Lut-H/Eos, Dex/Eos}} < 0.01$) and neutrophils ($P_{\text{Lut-H/Neu, Dex/Neu}} < 0.01$) were significantly down-regulated in the high-dose luteolin group and dexamethasone group.

Then we tested the Th2 cytokine levels in the BALF of each group of mice (Fig. 5). The results showed that compared with the control group, the expression levels of Th2 cytokines IL-4, IL-5 and IL-13 of the asthma group were significantly increased ($P_{\text{Ast/IL-4, IL-5, IL-13}} < 0.01$). Low-dose luteolin treatment had few impact on these cytokines, while high-dose luteolin and dexamethasone administration significantly decreased their expression levels ($P_{\text{Lut-H/IL-4, Dex/IL-4}} < 0.05$; $P_{\text{Lut-H/IL-5}} < 0.01$,

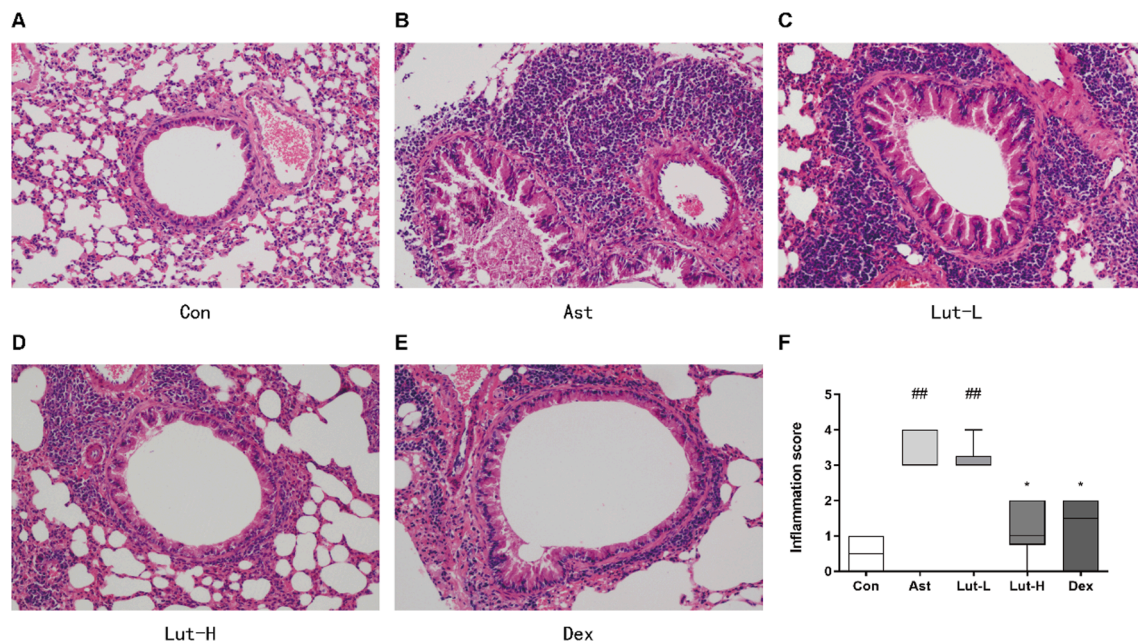


Fig. 3. Hematoxylin-eosin (HE) staining of lung tissues of mice in each group. Panels (A-E) the HE staining of lung tissues of mice in each group; scale bar:10x; Panel (F) the inflammation score for HE staining of each group; n = 6 in each group; Data are presented as the medians (P25, P75) and Kruskal-Wallis ANOVA was used for comparison; #*P* < 0.05, ##*P* < 0.01 vs control group; **P* < 0.05, ***P* < 0.01 vs asthma group. Con: control group; Ast: asthma group; Lut-L: low dose luteolin group; Lut-H: high-dose luteolin group; Dex: dexamethasone group.

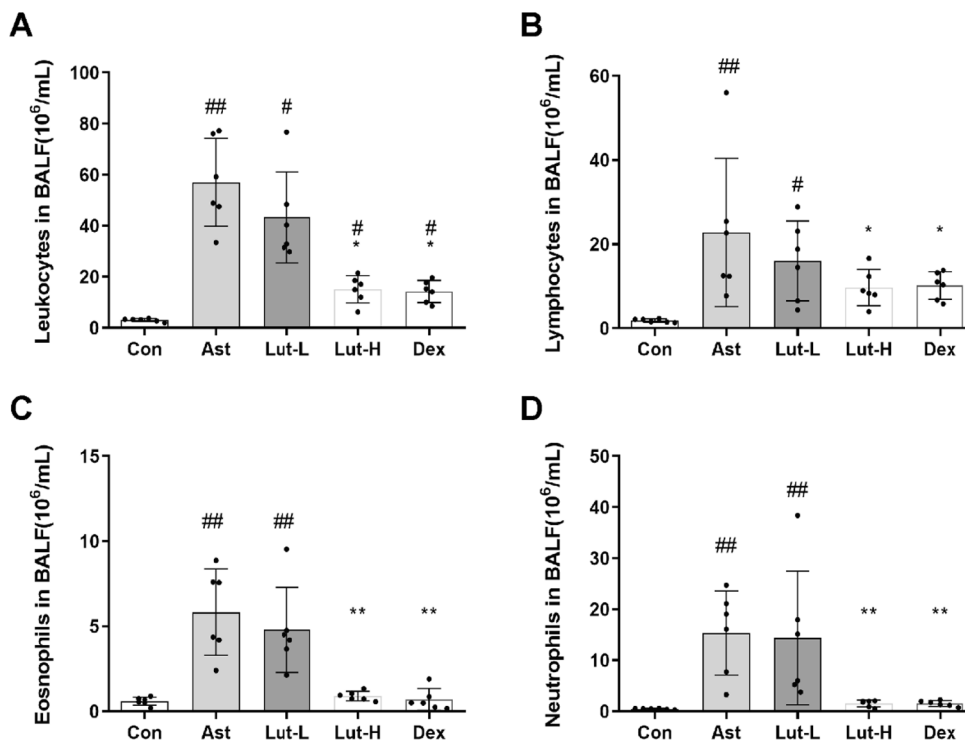


Fig. 4. The effect of luteolin on the classification and count of inflammatory cells in BALF of each group of mice. Panels (A-D) Data are presented as the mean ± standard deviation and one-way ANOVA was applied to detect significant differences. N = 6 in each group; #*P* < 0.05, ##*P* < 0.01 vs control group; **P* < 0.05, ***P* < 0.01 vs asthma group. BALF: bronchoalveolar fluid; Con: control group; Ast: asthma group; Lut-L: low dose luteolin group; Lut-H: high-dose luteolin group; Dex: dexamethasone group.

$P_{\text{Dex/IL-5}} < 0.05$; $P_{\text{Lut-H/IL-13}}$, $P_{\text{Dex/IL-13}} < 0.01$). Since Th2 cytokines are responsible for IgE production [33,34], serum OVA-specific IgE levels in each group were tested (Fig. 5). The results showed that compared with the control group, the level of serum OVA-specific IgE in the asthma group was significantly increased ($P_{\text{Ast/IgE}} < 0.01$). There was no statistically difference in serum OVA-specific IgE levels between the low-dose luteolin group and asthma group, but IgE levels significantly reduced in the high-dose luteolin group and dexamethasone group ($P_{\text{Lut-H/IgE}}$, $P_{\text{Dex/IgE}} < 0.01$).

H/IgE, $P_{\text{Dex/IgE}} < 0.01$).

3.3. Luteolin improves airway mucus secretion and collagen deposition in asthmatic mice

Airway mucus secretion and collagen deposition in each group were observed by using periodic acid-schiff staining (PAS) and Masson staining (Fig. 6). The results showed that the control group could hardly

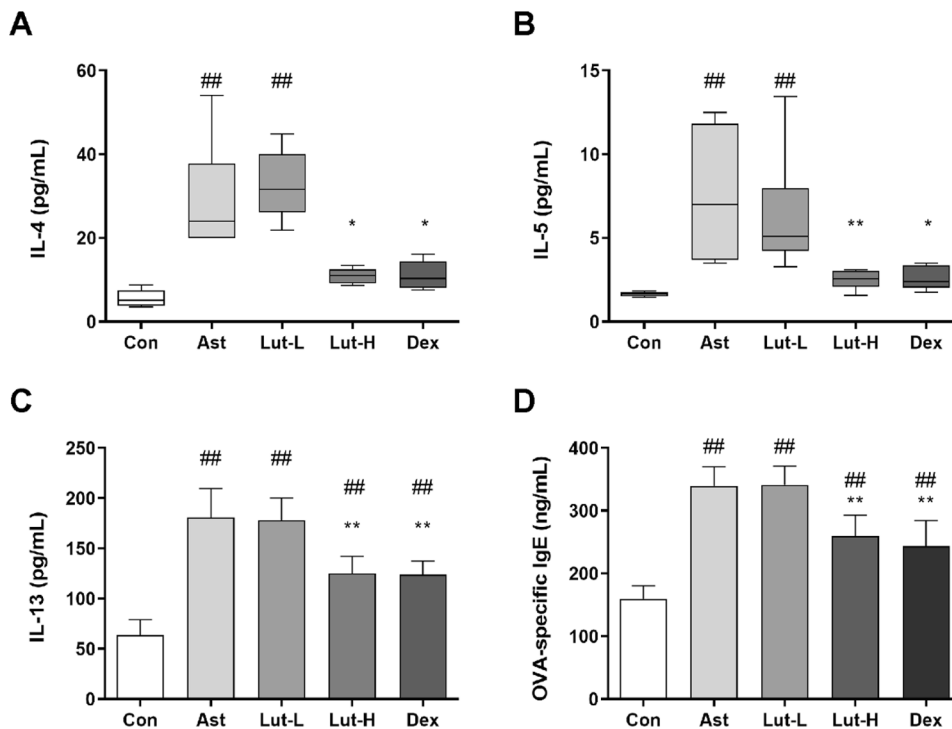


Fig. 5. The effects of luteolin on Th2 cytokines in BALF and serum OVA-specific IgE in each group. (A-B) Data are presented as the medians (P25, P75) and Kruskal-Wallis ANOVA was used for comparison; (C-D) Data are presented as the mean $\pm$ standard deviation and one-way ANOVA was used for comparison. N = 6 in each group; [#] $P < 0.05$, ^{##} $P < 0.01$ vs control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs asthma group. Con: control group; Ast: asthma group; Lut-L: low-dose luteolin group; Lut-H: high-dose luteolin group; Dex: dexamethasone group.

see the PAS staining positive area, and the area of masson positive staining is very small. PAS staining and Masson staining positive area of asthmatic mice were significantly increased (both $P_{\text{Ast/PAS, Masson}} < 0.01$) compared with those of the control group. Compared with the asthma group, the positive staining of PAS and Masson in the low-dose luteolin group was not significantly different, but was significantly reduced ($P_{\text{Lut-H/PAS, Dex/PAS}} < 0.01$, $P_{\text{Lut-H/Masson, Dex/Masson}} < 0.01$) in the high-dose luteolin group and dexamethasone group.

3.4. Luteolin inhibits autophagy in lung tissues of asthmatic mice

LC3B is the main indicator for evaluating autophagy levels [35], and both autophagy and eosinophil are positively correlated with the severity of asthma [36]. Therefore, expressions of LC3B and eosinophils main basic protein (EMBP) in lung tissues of each group were observed. Immunofluorescence results (Fig. 7 A) indicated LC3B was positively correlated with EMBP. In detail, the LC3B and EMBP positive staining were almost invisible in the control group. However, the asthma group and low-dose luteolin group showed positive stainings of LC3B and EMBP in the airway epithelium and tissues around the airway, which were decreased obviously in high-dose luteolin group and dexamethasone group. The results of immunohistochemistry are consistent with immunofluorescence. Semi-quantitative analysis showed that compared with the control group, the AOD (Average optical density) of LC3B in asthma group and low-dose luteolin group was significantly increased ($P_{\text{Ast}} < 0.01$). There was no significant difference between these two groups. Compared with that of the asthma group, the AOD of LC3B significantly decreased in the high-dose luteolin group and the dexamethasone group ($P_{\text{Lut-H, Dex}} < 0.01$) (Fig. 7 B). The western blot showed similar results as those found in immunohistochemical detections ($P_{\text{Ast/LC3B}} < 0.01$ vs Con, $P_{\text{Lut-H/LC3B, Dex/LC3B}} < 0.05$ vs Ast) (Fig. 7 C).

Since p62 is commonly used in combination with LC3B to evaluate autophagy, and LC3B^{high} p62^{low} levels are suggested to indicate activated autophagy [37], the expression level of p62 protein in each group was also observed. The results showed that compared with the control group, the asthma and luteolin low dose groups have no statistical

differences in the expression of p62, which was significantly increased by luteolin high dose and dexamethasone treatments ($P_{\text{Lut-H/p62}} < 0.01$, $P_{\text{Dex/p62}} < 0.05$) (Fig. 7 D).

3.5. Luteolin activates the PI3K/Akt/mTOR signaling pathway in the lung tissues of asthmatic mice

The PI3K/Akt/mTOR plays a key role in regulation of autophagy [38]. Activation of PI3K/Akt/mTOR directly inhibits autophagy [39]. Thus, we observed its expression in the lung tissues of each group. Western Blot results showed that compared with those of the control group, the expressions of PI3K p85 and p-mTOR in the asthma group were significantly reduced (both $P_{\text{Ast/p85, p-mTOR}} < 0.01$). There was no significant difference in PI3K p85 and p-mTOR levels between low-dose luteolin group and asthma group, but their levels increased significantly in the high-dose luteolin group ($P_{\text{Lut-H/p85}} < 0.05$, $P_{\text{Lut-H/p-mTOR}} < 0.01$) and dexamethasone group ($P_{\text{Dex/p85, p-mTOR}} < 0.01$) versus asthma group. Compared with that of the control group, the expression level of p-Akt in the asthma and low-dose luteolin groups showed no statistical differences, but significantly increased in the high-dose luteolin group compared with that of asthma group ($P_{\text{Lut-H/p-Akt}} < 0.05$) (Fig. 8).

3.6. Luteolin inhibits the Beclin1-PI3KC3 protein complex in the lung tissues of asthmatic mice

Beclin1-PI3KC3 protein complex is essential for autophagy [20], and mTOR signaling pathway is involved in Beclin1-PI3KC3 complex regulation process [40,41]. As p-mTOR was up-regulated in high-dose luteolin group (see Fig. 8), we then observed the Beclin1 and PI3KC3 expressions in each group. The immunohistochemistry showed that beclin-1 was mainly expressed in the cytoplasm of tissues that surrounding the airway. The semi-quantitative analysis showed that the AOD of beclin-1 in asthma group was significantly increased compared with that in the control group ($P_{\text{Ast}} < 0.01$). The parameter in low-dose luteolin was not significantly changed, while in high-dose luteolin group and the dexamethasone group it was significantly decreased ($P_{\text{Lut-H, Dex}} < 0.01$) (Fig. 9 A). The western blot results showed that, compared

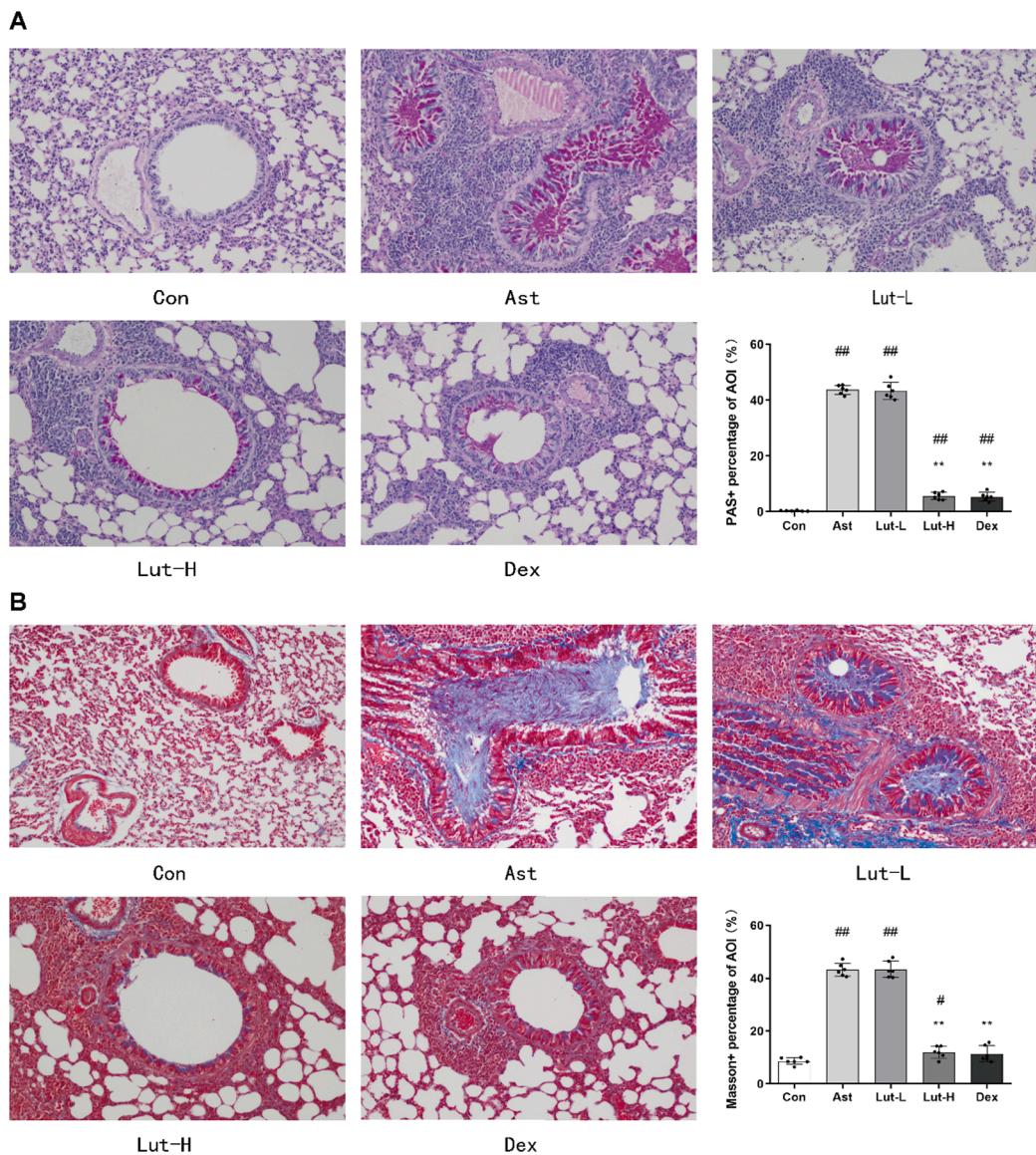


Fig. 6. Airway mucus secretion and collagen deposition in each group of mice. (A) High-dose of luteolin reduced airway mucus secretion; (B) High-doses of luteolin reduced collagen deposition in the airways. Data are presented as the mean $\pm$ standard deviation and one-way ANOVA was applied to detect significant differences. $N = 6$ in each group; $\#P < 0.05$, $\#\#P < 0.01$ vs control group; $*P < 0.05$, $**P < 0.01$ vs asthma group. Con: control group; Ast: asthma group; Lut-L: low-doses luteolin group; Lut-H: high-doses luteolin group; Dex: dexamethasone group; AOI: area of interest. scale bar: $10\times$.

with those in the control group, the expressions of Beclin-1 and PI3KC3 in the asthma group were significantly up-regulated ($P_{\text{Ast/Beclin-1}} < 0.01$, $P_{\text{Ast/PI3KC3}} < 0.05$), suggesting an up-regulation of autophagy level. There was no significant difference between the low-dose luteolin group and the asthma group, while the high-dose luteolin and dexamethasone significantly inhibited their expressions compared with the asthma group ($P_{\text{Lut-H/Beclin-1, PI3KC3}} < 0.01$, $P_{\text{Dex/Beclin-1, PI3KC3}} < 0.01$) (Fig. 9 B, C), indicating that the Beclin-1-PI3KC3 protein complex was inhibited.

4. Discussion

In present study, we used an OVA-induced mouse model of allergic asthma to explore the anti-asthma effect of luteolin on autophagy. Our study showed that the Th2 cytokine levels, the number of inflammatory cells in BALF and the serum OVA-specific IgE of mice in asthma group were significantly increased, accompanied by a significant increase in the level of autophagy marker LC3B in lung tissues, indicating a success in model generation. The low-dose of luteolin had no therapeutic effect on inflammation and over-activated autophagy in lung tissues of asthmatic mice. Administration of high-dose of luteolin not only attenuated the pathological damage and significantly inhibited inflammatory cells/cytokines, but also significantly inhibited the level of autophagy in lung

tissues. One limitation of present study is that no electron microscopy was used to observe the formation of autophagosomes. Since the lung tissues used in this study had been fixed in 4% paraformaldehyde, which is no longer available for electron microscopy detection. However, OVA-induced asthma has been verified by studies to enhance the autophagy level in lung tissues [42], and the inhibitory effect of luteolin on autophagy has also been demonstrated, such as down-regulation of autophagy marker LC3 and beclin-1 proteins [27,28,43]. As for changes in autophagy levels, the mainstream evaluation method is still based on changes in autophagy marker proteins such as LC3, p62, and beclin-1 [35,37]. Our results showed that high-dose of luteolin can significantly reverse these proteins in the lung tissues of asthmatic mice, which is consistent with the above studies.

It is generally accepted that Th2 cytokines are predominant in allergic asthma, although some studies suggest that Th1 cytokines activate autophagy while Th2 cytokines inhibits autophagy. This pattern is mainly derived from the study of immune cells and lacks this characteristic in tissues [44]. In fact, OVA was shown to significantly induce autophagy in lung tissue of asthmatic mice, and the level of autophagy in lung tissues of asthmatic patients was also significantly up-regulated compared with normal subjects [11,12]. Further, immunofluorescence results in our study showed that the expression of LC3B in the lung

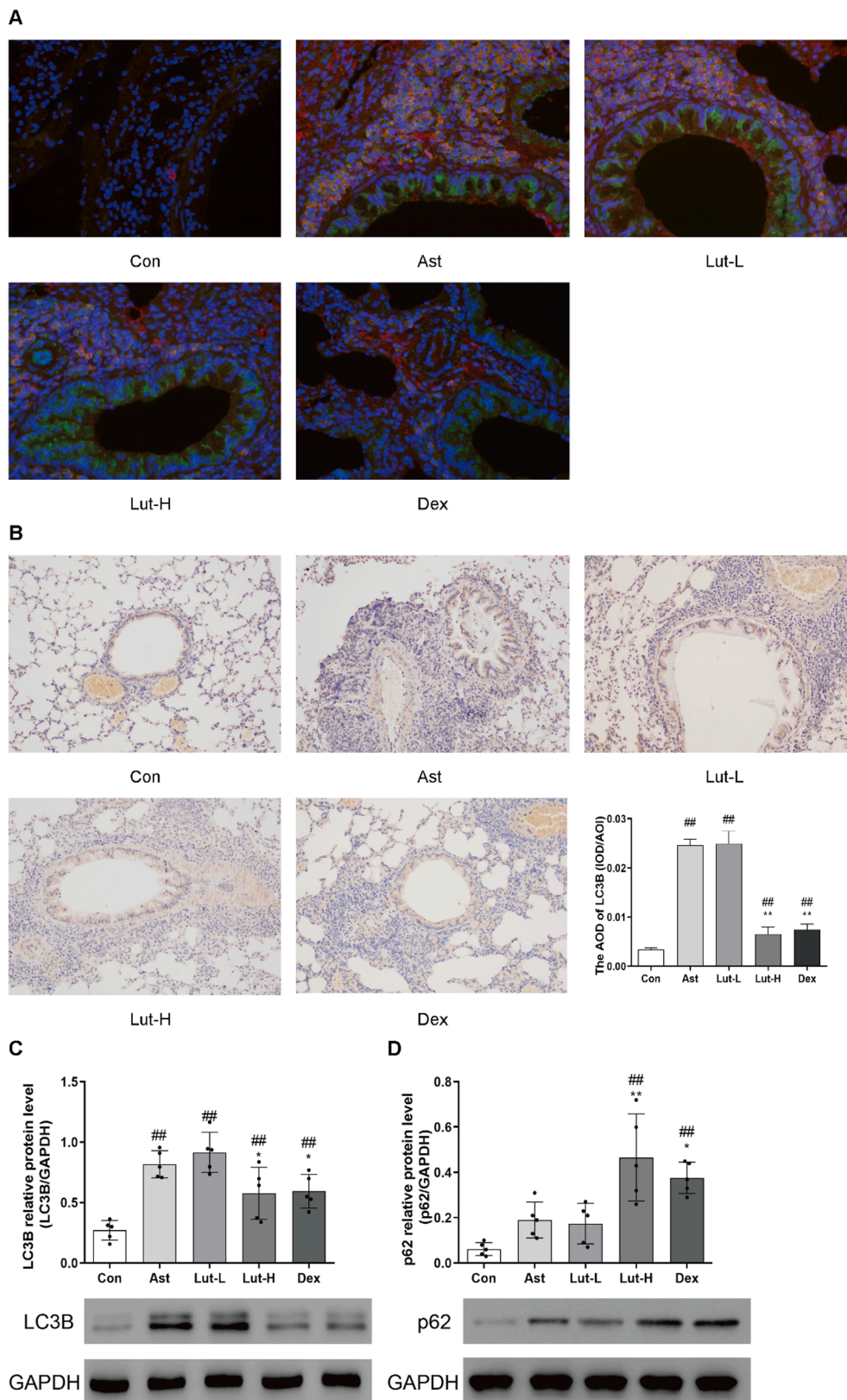


Fig. 7. High-dose of luteolin inhibited the autophagy markers in lung tissues of asthmatic mice. (A) Immunofluorescence of LC3B and EMBP in lung tissues of mice in each group; Green: LC3B; Red: EMBP; scale bar:20 $\times$; (B) Immunohistochemistry of LC3B in each group; scale bar:10 $\times$; (C–D) Expression of autophagy marker proteins LC3B and p62 in each group tested by western blot. Data are presented as the mean $\pm$ standard deviation and one-way ANOVA was applied to detect significant differences. N = 5 in each group; [#] $P < 0.05$, ^{##} $P < 0.01$ vs control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs asthma group. LC3B: microtubule-associated protein 1 light chain 3 beta; EMBP: eosinophils main basic protein; p62: sequestosome 1; Con: control group; Ast: asthma group; Lut-L: Low-does luteolin group; Lut-H: High-does luteolin group; Dex: dexamethasone group; IOD: integrated option density; AOI: area of interesting; AOD: average optical density.

tissues of asthmatic mice was positively correlated with the eosinophils main basic protein, suggesting that autophagy is positively correlated with the severity of eosinophilic inflammation. This may be related to the elevated level of IL-5. Among the Th2 pro-inflammatory cytokines, IL-5 promotes the differentiation, survival, transportation, activation,

and effector functions of eosinophils, thereby participating in eosinophil-mediated inflammation [45]. Eosinophils are a major type of inflammatory cells and play an important role in augmenting AHR, mucus production, airway remodeling and inflammation in allergic asthma by producing IL-13 [46–48]. IL-13 drives airway goblet cells to

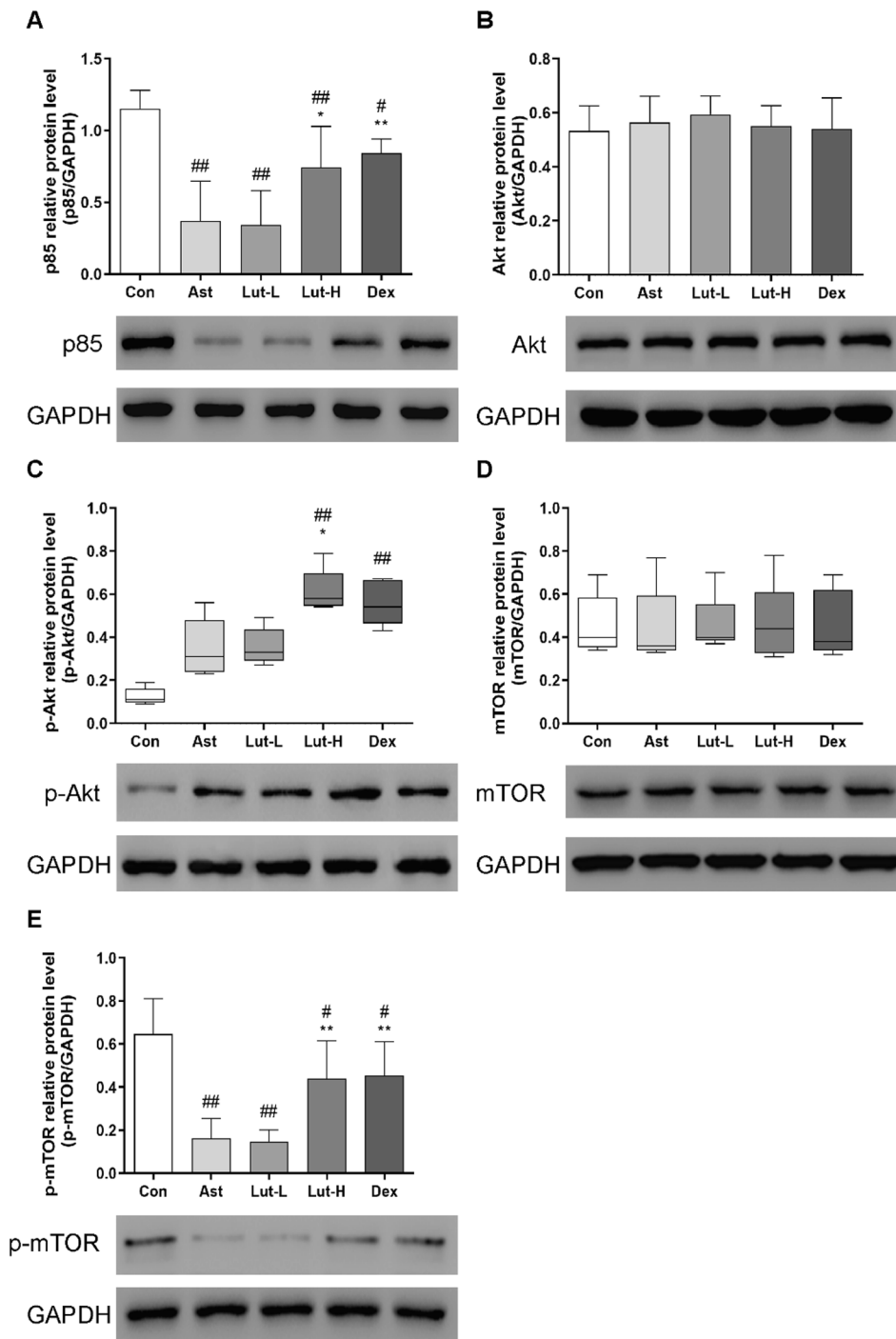


Fig. 8. High-dose of luteolin activated the PI3K/Akt/mTOR signaling in lung tissues of asthmatic mice. Panels (A, B, E) data are presented as the mean $\pm$ standard and one-way ANOVA was applied to detect significant differences; Panels (C, D) data are presented as the deviation or medians (P25, P75) and Kruskal-Wallis ANOVA was used for comparison. N = 5 in each group; [#] $P < 0.05$, ^{##} $P < 0.01$ vs control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs asthma group. p85: class I Phosphatidylinositol 3-kinase p85; (p-) Akt: (phospho-) protein kinase B; (p-) mTOR: (phospho-) mammalian target of rapamycin.

secrete excessive mucus, which is one of the characteristics of airway pathological changes in allergic asthma. During this process, IL-13 activates epithelial autophagy. On the contrary, inhibition of autophagy significantly reduces airway mucus hypersecretion induced by IL-13, and leads to a marked improvement in AHR, reductions in level of IL-5 and in the number of infiltrating eosinophils in bronchoalveolar lavage fluid and histological inflammatory features [36,44]. Consistent with the above studies, our study showed that the high-dose of luteolin significantly alleviated the airway inflammation by down-regulation of the levels of IL-5, IL-13, eosinophils in BALF and OVA-specific IgE in serum. In addition, high-dose of luteolin improved airway hyper-responsiveness by reducing airway mucus secretion and collagen deposition, and inhibiting the expression levels of LC3B and EMBP in

lung tissues of asthmatic mice. However, the low-dose of luteolin seemed to have a slightly activation effect on LC3B and a mildly inhibition effect on p62 with no significant difference. Therefore, the effects of different doses of luteolin on autophagy still need to be further verified by *in vitro* studies.

Interestingly, dexamethasone has been reported to activate autophagy *in vitro*. In present study, we observed that dexamethasone inhibited the level of autophagy in the lung tissue of asthmatic mice. This may be related to dexamethasone suppressed inflammatory cells, especially eosinophils [49]. Previous studies have shown that autophagy is positively correlated with the severity of eosinophilic inflammation in allergic asthma [36], and our study showed that in the lung tissues of asthmatic mice, autophagy marker LC3B is positively correlated with

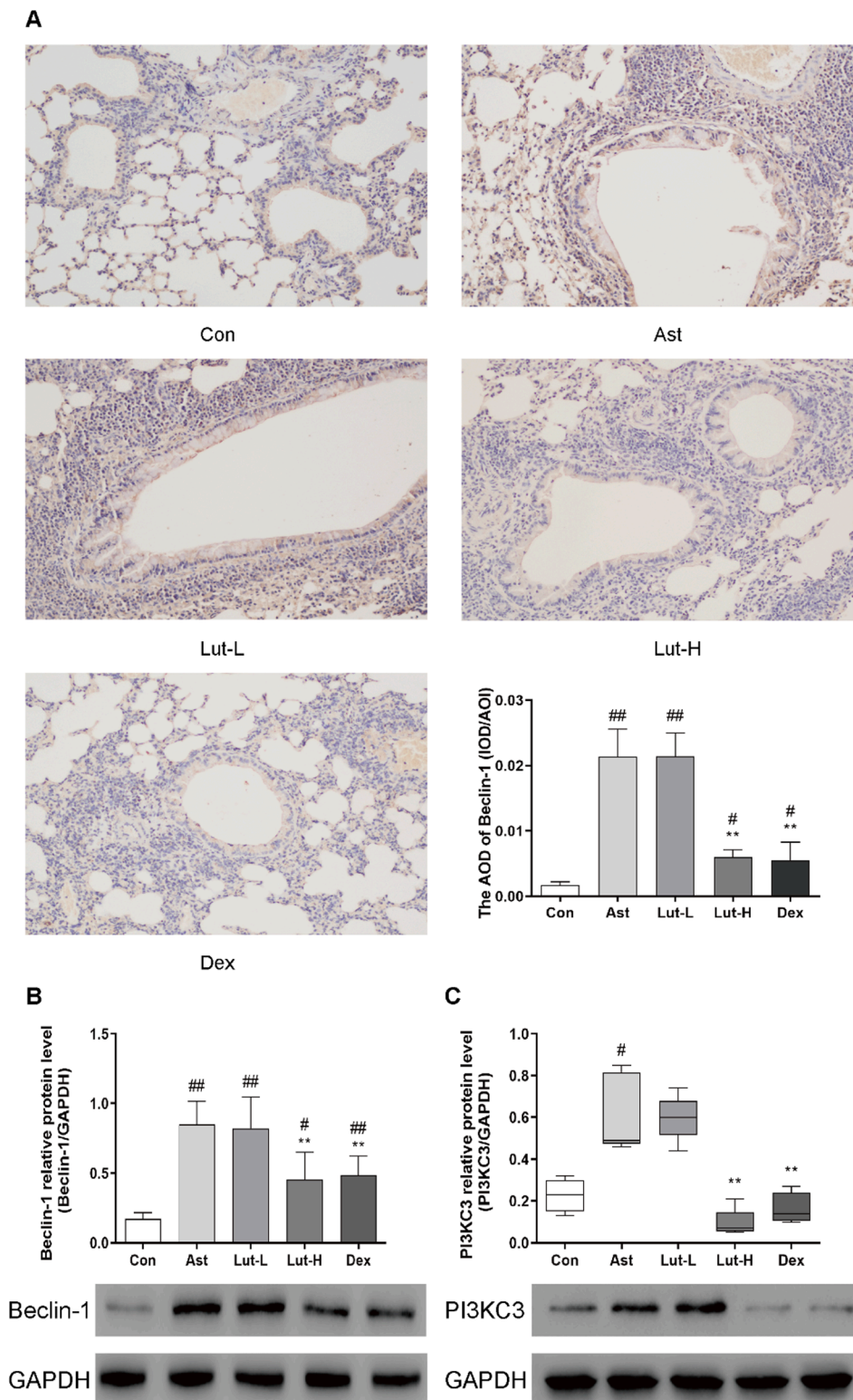


Fig. 9. The high-dose of luteolin inhibited the Beclin-1-PI3KC3 complex proteins in lung tissues of asthmatic mice. (A, B) Immunohistochemistry and western blot of Beclin-1 in each group; scale bar:10 $\times$; Data are presented as the mean $\pm$ standard deviation and one-way ANOVA was applied to detect significant differences; (C) Expression of PI3KC3 proteins in each group; Data are presented as the medians (P25, P75) and Kruskal-Wallis ANOVA was used for comparison. N = 5 in each group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs asthma group. Beclin-1: coiled-coil myosin-like BCL2-interacting protein; PI3KC3: class III phosphatidylinositol 3-kinase; IOD: integrated option density; AOD: area of interesting; AOD: average optical density.

the main basic protein of eosinophils, therefore, dexamethasone may inhibit autophagy in this way. In addition, studies have shown that continuous exposure to glucocorticoids such as dexamethasone can cause a significant decrease in autophagy levels [50]. The effect of dexamethasone on autophagy was also dose-dependent. For example, autophagy markers (LC3-II and Beclin-1) were increased at the low dose of dexamethasone and decreased at the high dose [51]. In fact, dexamethasone has also been shown to inhibit pathologically induced

autophagy [52]. For instance, it inhibits the autophagy in airway epithelium activated by IL-5 in asthmatic patients [18,53], and activates PI3K/Akt/mTOR pathway [54–56]. Additionally, our results showed that both eosinophils and neutrophils were significantly up-regulated in the asthma group, and down-regulated in the dexamethasone group (Fig. 4 C, D). There are evidences suggesting that the effect of glucocorticoids on neutrophils are much weaker than on eosinophils in asthma [57]. However, this situation appears to be mainly present in

some severe persistent asthma patients and long-term use of corticosteroids [57,58]. In fact, increased neutrophils have also been observed under other asthmatic circumstances such as in patients who have a sudden-onset attack [59]. The inhibitory effect of short-term application of glucocorticoids such as dexamethasone on neutrophils has also been proven: the inhibition of neutrophil infiltration by dexamethasone might be due to inhibition of the extravasation of peripheral neutrophils and down-regulation of neutrophil migration [60,61]. Since the treatment period in this study was only 7 days, a relatively short-term, which may explain why the number of neutrophils was reduced.

The PI3K/Akt/mTOR signaling plays a crucial role in the regulation of autophagy [62]. Studies have shown that the inhibition of PI3K/Akt/mTOR signaling pathway can induce autophagy [63], and activation of the PI3K/AKT can directly phosphorylate mTOR at Ser2448, which inhibits autophagy and promotes cell survival [17]. Our study suggested that the low-dose of luteolin had no significant effect on PI3K/Akt/mTOR signaling pathway, while the high-dose of luteolin significantly up-regulated the PI3K p85, p-Akt and p-mTOR protein levels in lung tissues of asthmatic mice, which consistent with other studies [24,64–66]. Additionally, it has been shown that Beclin-1 interacts with a number of proteins to form multiple functional complexes that enable Beclin-1 to respond to different signals [67,68]. Among them, the Beclin-1-PI3KC3 protein complex is essential for the process of autophagy [20]. Studies have confirmed that inhibition of PI3K/Akt/mTOR (such as down-regulation of mTOR phosphorylation level) can directly lead to the activation of autophagy-related protein ULK1, which will phosphorylate Beclin-1, thus, enhancing the activity of Beclin-1-PI3KC3 complex and further promoting the activity of autophagy [69]. In our study, the expression of Beclin-1 and PI3KC3 proteins were significantly up-regulated in the asthma group as expected, while the high-doses of luteolin significantly inhibited their expressions. However, it is still not clear whether the effects of luteolin on PI3K signaling and autophagy are direct effects, or by inhibiting the production of IL-5, IL-13 and eosinophils to reduce the level of autophagy in asthma, which needs to be further verified.

In conclusion, our results suggested that the luteolin can effectively improve lung function, alleviate airway inflammation, reduce airway mucus secretion and collagen deposition in allergic asthmatic mice. Furthermore, luteolin inhibited the level of autophagy in the lung tissues of asthmatic mice, and the mechanism involves activating the PI3K/Akt/mTOR signaling pathway while inhibiting the Beclin-1-PI3KC3 protein complex.

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CRedit authorship contribution statement

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Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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