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# Pingchuanning decoction attenuates airway inflammation by suppressing autophagy via phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin signaling pathway in rat models of asthma

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## Abstract

**Background:** Pingchuanning decoction is a well-known traditional Chinese medicine for the treatment of airway inflammatory diseases, including asthma. However, the potential mechanism by which Pingchuanning decoction contributes to the amelioration of airway inflammation remains unknown.

**Methods:** A rat model of asthma was well established by inducing ovalbumin. Lipopolysaccharide-stimulated rat tracheal epithelial (RTE) cells were used as cellular model. Lung histopathology and goblet cell hyperplasia were assessed by hematoxylin-eosin (HE) and periodic acid Schiff staining, respectively. Total inflammatory cells count and RTE cell apoptosis were analyzed by flow cytometry. The autophagic activities were evaluated by immunohistochemical and immunofluorescence analysis and Western blot analysis of autophagy-related proteins. We also detected the effects of Pingchuanning decoction on phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) and high-mobility group box 1 (HMGB1)-mediated toll-like receptor 4 (TLR4)/NF- $\kappa$ B pathways-related proteins and inflammatory cytokines using the Western blot analysis and enzyme-linked immunosorbent assay.

**Results:** Pingchuanning decoction effectively attenuated pulmonary pathology and autophagy. Treatment with Pingchuanning decoction activated PI3K/Akt/mTOR pathway and inhibited HMGB1/TLR4/NF- $\kappa$ B pathway, which could be overturned by LY294002, a PI3K antagonist, or rapamycin (Rapa), an autophagy inducer.

**Conclusion:** Pingchuanning decoction exerted a therapeutic effect on asthma by inhibiting autophagy via PI3K/Akt/mTOR signaling pathway.

## KEYWORDS

airway inflammation, asthma, autophagy, Pingchuanning decoction, signaling pathway

## 1 | INTRODUCTION

Asthma is a common chronic airway inflammatory disease characterized by reversible airway obstruction,

abundant mucus production, and infiltration of the airway with inflammatory cells.<sup>1</sup> With the development of global industrialization, the prevalence of asthma has increased in the past decade, which

remains a serious public health problem and causes individual and socio-economic burdens.<sup>2</sup> Therefore, it is urgently required to discover safe and effective therapies.

Autophagy is an evolutionarily and highly conserved catabolic process in eukaryotic cells, referring to the sequestration and degradation of cytoplasmic contents.<sup>3</sup> There is increasing evidence emerging to demonstrate that autophagy is involved in the pathogenesis of various respiratory diseases, such as chronic obstructive pulmonary disease,<sup>4</sup> acute lung injury,<sup>5</sup> pulmonary arterial hypertension,<sup>6</sup> bacterial pneumonia,<sup>7</sup> and so on. It has been widely accepted that autophagy acts as a double-edged sword in inflammatory response. For one thing, autophagy plays a protective role in homeostasis; for the other, it leads to the aggravation of disease.<sup>8</sup> Accumulating studies have existed with regard to the role of autophagy in the pathogenesis of asthma. For instance, Poon et al<sup>9</sup> using TEM suggested that the number of double-membrane autophagosomes in fibroblasts and epithelial cells of patients with asthma were significantly more than that of healthy controls. Interestingly, Zeki et al<sup>10</sup> offer two opposing but plausible hypotheses that autophagy is a regulator of fibrosis, which enhances ECM production in airway mesenchymal cells, leading to airway thickening and rigidity, while autophagy may mitigate fibrosis if it is involved in the degradation of ECM proteins. In addition, higher autophagy levels were observed in sputum granulocytes and peripheral blood eosinophils from patients with severe asthma. Moreover, 3-methyladenine (3-MA), an autophagy inhibitor, down-regulated the expressions of the autophagy-associated protein, microtubule-associated protein 1 light chain 3-II (LC3-II), and eosinophil cationic protein in human eosinophil-like (HL-60) cells.<sup>11</sup> Phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway is a major signal transduction cascade involved in the modulation of autophagy. Malnutrition, hypoxia, or oxidative stress-induced PI3K inhibition could result in decreasing Akt activities and its downstream target mTOR, which contributed to the initiation of autophagy and apoptosis.<sup>12</sup>

Traditional Chinese medicine (TCM) has a long history of treating asthma. Accurate diagnosis combined with appropriate medicinal therapy may achieve satisfactory efficacy. Pingchuanning decoction is a lung-warming, phlegm-resolving, antitussive, and anti-asthmatic TCM, consisting of 11 medicinal herbs. Recently, increasing evidence has strongly implied that Pingchuanning decoction has anti-inflammatory effects on the occurrence and development of asthma. Our previous study has found that Pingchuanning

decoction effectively upregulates Clara cell secretory protein expression in a rat model of asthma, thereby reducing airway inflammation, which in turn inhibits airway remodeling.<sup>13</sup> In addition, Pingchuanning decoction has an inhibitory effect on inflammation biomarkers Raf, extracellular signal-regulated kinases 1, matrix metalloproteinase-9, metalloproteinase tissue inhibitor-1 in lung tissues of asthmatic rats.<sup>14</sup> However, the mechanism by which Pingchuanning decoction exerts a therapeutic effect on asthma remains unclear. Therefore, the objective of our study was to evaluate the molecular mechanism and biological functions of Pingchuanning decoction in the course of treating asthma, providing new mechanistic insight into the role of Pingchuanning decoction against airway inflammation.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental animals and grouping

Male Sprague-Dawley (SD) rats weighing 180–220 g were supplied by the Experimental Animal Center of Anhui University of Traditional Chinese medicine (Hefei, Anhui, China). All rats were housed in temperature-controlled room (20°C–24°C) on 12 hours light-dark cycles with unrestricted access to food and water for 1 week before the following experiments.

Rats were averagely divided into seven groups ( $n = 8$  per group), including normal, asthma, pingchuanning (low dose, middle dose, and high dose), guilong kechuanning, and dexamethasone (Dex) groups. All animal protocols and procedures in this study were ethically reviewed and approved by the Animal Care and Use Committee of Anhui University of Traditional Chinese medicine.

### 2.2 | Establishment of rat allergic asthmatic model

The rat model of allergic asthma was induced by ovalbumin (OVA, Grade V; Sigma-Aldrich, St. Louis, MO) sensitization and challenge in this study. On days 0, 7, and 14, rats were sensitized by intraperitoneal injection of 1 mL antigen sensitization liquid containing 10% OVA diluted in sterile normal saline and 10 mg aluminum hydroxide (Sigma-Aldrich) as an adjuvant. On the 22th day, rats were inhaled with 1% OVA for 20 minutes once daily for consecutive 3 weeks. In the normal group, rats were treated with an equal volume of normal saline instead of OVA.

## 2.3 | Administration of drugs

The preparation of antiasthmatic agents was performed as previously described.<sup>13</sup> Rats were treated with different interventions for 30 minutes before OVA sensitization. From day 7 of normal saline or OVA inhalation, rats in the normal and asthma group were intragastrically administrated with 10 mL/kg normal saline, while rats in the experimental groups were administrated with 3.64, 7.28, 14.6 g/kg Pingchuanning Decoctions (Anhui Weimin Pharmacy, Hefei, Anhui, China), and 10 g/kg Guilong Kechuan-ning capsules (Shanxi Guilong Medicine Co., Ltd., Xinzhou, Shanxi, China) as well as 0.5 mg/kg Dex (Shanghai Xinyi Pharmaceutical Co., Ltd., Shanghai, China) once per day for successive 21 days. All rats were euthanatized by CO<sub>2</sub> asphyxia within 24 hours after the final challenge in each group. Lung tissues and bronchial alveolar lavage fluid were collected for the following procedures.

## 2.4 | Cell culture and treatment

Rat tracheal epithelial (RTE) cells purchased from the Cell Culture Center of the Chinese Academy of Medical Sciences (Beijing, China) were cultured in Minimal Essential Medium (Thermo Fisher Scientific, Inc., Waltham, MA) containing 20% fetal calf serum (HyClone; GE Healthcare Life Sciences, Logan, UT), 100 U/mL penicillin and 100 µg/mL streptomycin in a 5% CO<sub>2</sub> humidified atmosphere at 37°C.

Meanwhile, blood samples were taken from the abdominal aorta of healthy rats and those that received low dose, middle dose, and high-dose of Pingchuanning decoction, respectively. After standing for 30 minutes at room temperature, the supernatant was centrifuged for 20 minutes at 3000g. Serum was incubated in water bath at 56°C for 30 minutes followed by filter sterilization through 0.22 µm.

To further explore the antiasthmatic mechanism of pingchuanning decoction, RTE cells were randomly divided into eight groups, namely, control, lipopolysaccharide (LPS), 3-MA, pingchuanning (low dose), pingchuanning (Middle dose), pingchuanning (High dose), pingchuanning + LY294002 and pingchuanning + Rapa. Briefly, cells were cultured in blank rat serum-containing media or media supplemented with rat serum-containing LPS (Sigma-Aldrich), the autophagy inhibitor 3-MA (Sigma-Aldrich), or pingchuanning (low dose, middle dose, and high dose) alone or together with LY294002 (Sigma-Aldrich), a PI3K antagonist, or rapamycin (Rapa; Sigma-Aldrich), an autophagy inducer. After 24 hours of treatment, cells were harvested for Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-labeled flow cytometry, immunofluorescence staining,

Western blot analysis, and enzyme-linked immunosorbent assay (ELISA), respectively.

## 2.5 | HE staining

Lung tissues were prepared for hematoxylin-eosin (HE) staining to observe the morphological changes of lung tissues and inflammatory cell infiltration. Tissues were fixed in 4% formaldehyde, cut into 5-µm sections, stained with hematoxylin and eosin, and examined under an optical microscope (Leica DM2500M; Leica Microsystems, Wetzlar, Germany).

## 2.6 | Flow cytometry

The number of inflammatory cells (neutrophils, T lymphocytes, and macrophages) in lung tissues was detected by flow cytometry. First, lung tissues were minced, digested with Collagenase IV (Worthington Biochemical, Lakewood, NJ) at 37°C for 1 hour, and then resuspended in ammonium chloride, potassium bicarbonate lysing buffer for 2 minutes at room temperature to remove red blood cells. The dissociated cell suspension was centrifuged at 2000g for 5 minutes, washed twice with phosphate buffered solution (PBS), and then centrifuged at 1000g for 10 minutes. The cell pellets were resuspended in Roswell Park Memorial Institute 1640 medium (Gibco, Carlsbad, CA) and stained with PE-labeled anti-CD11b antibody, APC-labeled anti-GR-1 antibody, FITC-labeled anti-CD3 antibody, PerCP-labeled anti-CD45 antibody, and APC-labeled anti-F4/80 antibody (BioLegend, San Diego, CA) at 4°C for 30 minutes, followed by rinse with PBS twice. After centrifugation at 2000g for 10 minutes, cells were fixed with 4% formaldehyde and examined using FACSCalibur flow cytometer (BD Biosciences, San Diego, CA).

## 2.7 | Periodic acid Schiff staining

After ethanol dehydration and xylene dewaxing, lung tissue slices were soaked in periodic acid solution for 10 minutes, rinsed with distilled water, and then washed by 70% ethanol twice, followed by immersed in Schiff solution for 1–1.5 hours. After dyeing with hematoxylin, gradient elution with ethanol and xylene transparency, the slices were sealed by neutral gum with a cover glass. Double-blind method was applied to observe goblet cell hyperplasia and mucus hypersecretion under the light microscope.

## 2.8 | Immunohistochemical staining for LC3

The localization of autophagosomal LC3 in rat lung tissues was evaluated by immunohistochemistry. In brief,

the glass slides were dried in an incubator at 60°C for 40 minutes, dewaxed in xylene, and dehydrated in an ethanol series. Sections were heated in a microwave oven in 0.3% citrate buffer, treated with 3% H<sub>2</sub>O<sub>2</sub> deionized water, and then incubated with LC3 primary antibody (1:400; Novus Biologicals, Littleton, CO) overnight at 4°C. The slides were subsequently incubated with secondary antibody (Invitrogen, Carlsbad, CA) at 37°C for 30 minutes, washed twice with PBS and incubated with diaminobenzidine (DAB; Invitrogen) for immunolabeling. After hematoxylin counterstain and ethanol dehydration, the sections were sealed by neutral gum with a cover glass and examined under an optical microscope.

## 2.9 | Western blot

Total protein was separated from lung tissues and RTE cells using radio-immunoprecipitation assay buffer

(Santa Cruz Biotechnology, Santa Cruz, CA). Protein was extracted with 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes and blocked with tris-buffered saline tween (TBST) containing 5% skim milk at room temperature for 2 hours. The membranes were probed with primary antibodies against LC3-I, LC3-II, P62, beclin-1, Atg3, Atg5, Atg7, PI3K, phospho-AKT(p-AKT), AKT, p-mTOR, mTOR, high-mobility group box 1 (HMGB1), toll-like receptor 4 (TLR4), phospho-NF- $\kappa$ B p65 (p-P65), and P65 and GAPDH as a loading control (1:1000 dilution; Cell Signaling Technology, Boston, MA) at 4°C overnight, washed with TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) at room temperature for 1 hour. Band intensities were standardized against GAPDH, and the relative density was analyzed on a Molecular Imager

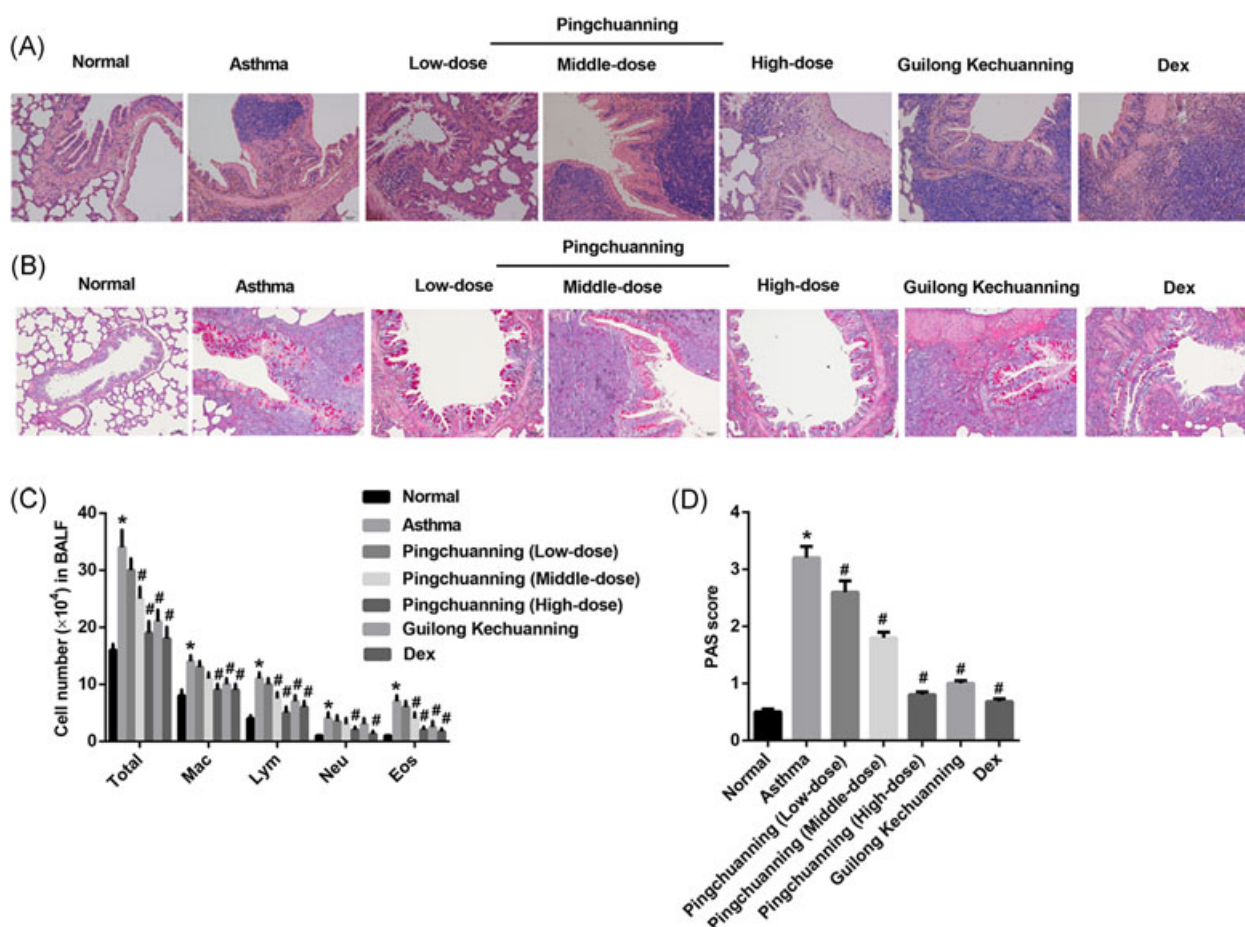


**FIGURE 1** Effect of pingchuanning decoction on pathological pulmonary changes in a rat model of asthma. Representative photomicrographs of morphological changes (A) and inflammatory cell infiltration (B) in HE-stained lung sections, the total number of inflammatory cells (C) determined by flow cytometry and PAS score for assessment of the metaplasia of the mucus-secreting goblet cells using PAS staining (D) in rats from normal, asthma, pingchuanning (low dose, middle dose, and high dose), guilong kechuanning and dexamethasone (Dex) groups. The values are presented as the mean  $\pm$  SD.  $n = 8$  per group. \* $P < 0.05$  compared with the normal group; # $P < 0.05$  compared with the asthma group. HE, hematoxylin-eosin; PAS, periodic acid-Schiff

ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, CA) using enhanced chemiluminescence reagent (Thermo Fisher Scientific, Shanghai, China).

## 2.10 | ELISA

The levels of various proinflammatory cytokines in BALF and lung tissues, including interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  and IL-6 were measured using an ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

## 2.11 | Cell apoptosis assay

The apoptosis of RTE cells was detected by flow cytometry using Annexin V-FITC/PI apoptosis detection kit (Sigma-Aldrich) according to the manufacturer's instructions. RTE cells were harvested, washed twice with PBS, and resuspended in binding buffer. Next, the cells were stained with annexin V-FITC and PI for 15 minutes at 37°C and analyzed by a flow cytometer (Becton-Dickinson, Fullerton, CA).

## 2.12 | Immunofluorescence staining

The autophagic activity was assessed using green fluorescence protein (GFP)-tagged LC3 transfection. RTE cells were transfected with GFP-LC3 plasmid using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. After transfection, cells were washed

twice with PBS and incubated in minimal essential medium. Immunofluorescence was performed to visualize LC3-positive cells using a fluorescence microscope (Leica).

## 2.13 | Statistical analysis

All data were presented as mean  $\pm$  standard deviation (SD) and analyzed by SPSS version 22.0 (SPSS, Chicago, IL). Comparisons between groups were performed using two-sided Student *t* test. *P* value < 0.05 was considered statistically significant.

## 3 | RESULTS

### 3.1 | Pingchuanning decoction alleviated airway inflammation in a rat model of asthma

In the beginning, we investigated the protective effects of Pingchuanning decoction on pathological impairment in a rat model of asthma. HE staining was performed to observe the morphological changes and inflammatory cell infiltration in lung tissues. As illustrated in Figure 1A, there were no abnormal findings in the bronchial and alveolar structure in the normal group; however, rats in the asthma group exhibited observable thickening of the alveolar septum, and visible pulmonary capillary edema, as well as a large number of inflammatory cell infiltrations in the pulmonary capillaries and

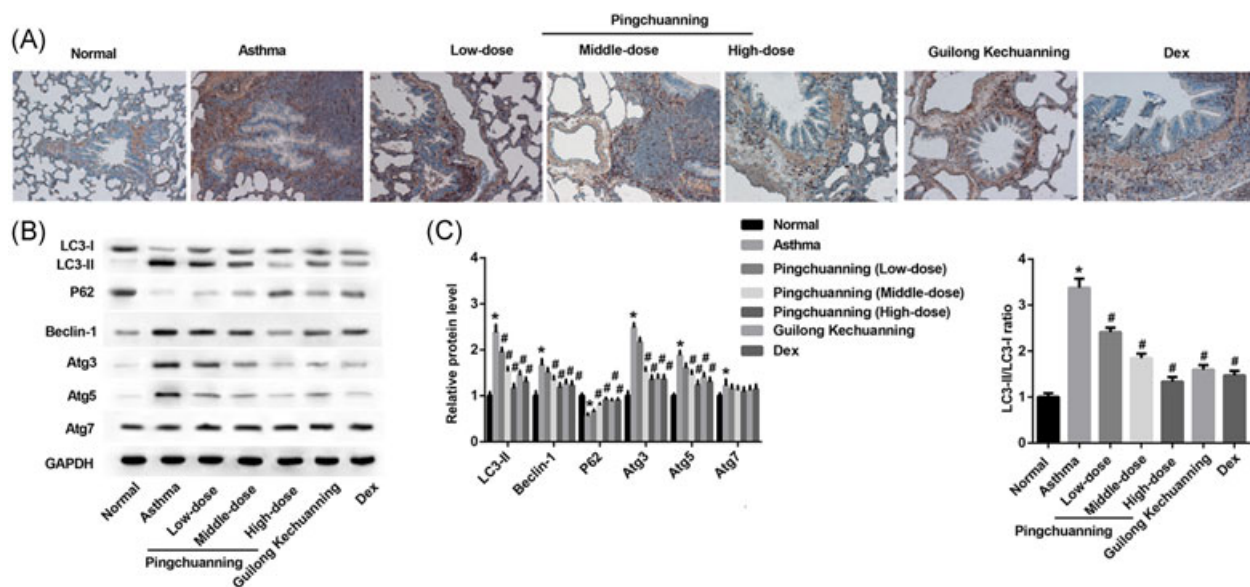


**FIGURE 2** Effect of Pingchuanning decoction on autophagy in a rat model of asthma, LC3 expression visualized by immunohistochemical staining (A), the protein levels (B), and determinations of LC3-I, LC3-II, P62, beclin-1, Atg3, Atg5, Atg7, and LC3-II/LC3-I ratio (C) in lung tissues of rats from normal, asthma, pingchuanning (low dose, middle dose, and high dose), guilong kechuanning and dexamethasone (Dex) groups. Data are presented as the mean  $\pm$  SD. *n* = 3. \**P* < 0.05 compared with the normal group; #*P* < 0.05 compared with the asthma group

alveolar space. Nevertheless, intragastric administration of pingchuanning decoction, guilong kechuanning capsules, or Dex all significantly attenuated the severity of pulmonary lesion compared with those in the normal group.

Flow cytometry showed that the total number of inflammatory cells was significantly increased in asthma group as compared with the normal group, while treatment with pingchuanning decoction, guilong kechuanning capsules, or Dex reduced the total number of inflammatory cells recruited to the lungs (Figure 1C). Lung injury was assessed by the quantification of mucus-secreting goblet cells based on periodic acid Schiff (PAS) staining. No obvious edema on the airway mucosa in the normal group, conversely, in the asthma group, the lung tissue was in irregular structure with obvious edema on the airway mucous membrane. In the pingchuanning, guilong kechuanning, or Dex groups, the goblet cell proliferation was reduced (Figure 1B). Accordingly, PAS score was markedly higher in the asthma group than in the normal group, whereas a lower PAS score was observed in the pingchuanning, guilong kechuanning, or Dex groups (Figure 1D), suggesting that Pingchuanning decoction, guilong kechuanning capsules, or Dex significantly reduced the number of mucus-secreting

goblet cells within the respiratory epithelium. Collectively, all these findings demonstrated that pingchuanning decoction exerted anti-inflammatory effect in asthmatic rats.

### 3.2 | Pingchuanning decoction inhibited autophagy in rat lung tissues

We next estimated the underlying effect of pingchuanning decoction on autophagy in the rat model of asthma. Results of the immunohistochemical analysis showed a significant increase in immunostaining intensity for LC3 in lung tissues in the asthma group as compared with normal controls, and these changes were markedly attenuated by pingchuanning decoction, guilong kechuanning capsules or Dex (Figure 2A). The western blot analysis was used to analyze the protein levels of the autophagy-related proteins LC3-I, LC3-II, P62, beclin-1, Atg3, Atg5, and Atg7 in lung tissues. We observed elevated levels of LC3-II, P62, beclin-1, Atg3, Atg5, and Atg7 as well as the LC3-II/LC3-I ratio in the asthma group as relative to those in the normal group, whereas these levels were decreased in all the treatment groups (Figure 2B and C). These data revealed that Pingchuanning decoction suppressed autophagy in a rat model of asthma.

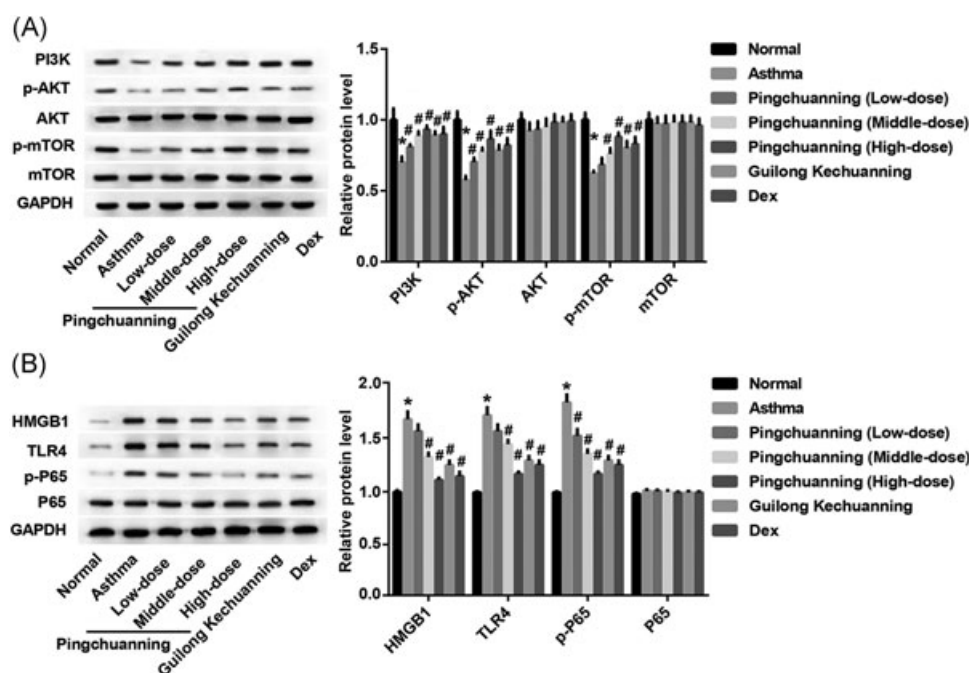


**FIGURE 3** Effect of pingchuanning decoction on PI3K/Akt/mTOR and HMGB1-mediated TLR4/NF- $\kappa$ B pathway-related proteins in lung tissues of asthmatic rats. The protein levels and determinations of PI3K, p-AKT, AKT, p-mTOR, mTOR (A), HMGB1, TLR4, p-P65 and P65 (B) in lung tissues of rats from Normal, asthma, pingchuanning (low dose, middle dose, and high dose), guilong kechuanning and dexamethasone (Dex) groups. Data are presented as the mean  $\pm$  SD.  $n = 3$ . \* $P < 0.05$  compared with the normal group; # $P < 0.05$  compared with the asthma group. PI3K/Akt/mTOR, phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin

### 3.3 | Pingchuanning decoction regulated related proteins of PI3K/Akt/mTOR and HMGB1-mediated TLR4/NF- $\kappa$ B pathway in lung tissues of asthmatic rats

To evaluate the potential mechanism of Pingchuanning decoction in mediating autophagy in asthmatic rats, the protein expressions of the PI3K/Akt/mTOR pathway and HMGB1-mediated TLR4/NF- $\kappa$ B pathway were both examined since PI3K/Akt/mTOR and HMGB1-mediated TLR4/NF- $\kappa$ B pathway were, respectively, antiautophagic, and proautophagic pathways. The PI3K, p-Akt, and p-mTOR proteins were obviously decreased in the asthma group. The administration of pingchuanning decoction, guilong kechuanning capsules, or Dex dramatically enhanced PI3K expression levels and the phosphorylations of Akt and mTOR (Figure 3A). In contrast, the protein levels of HMGB1, TLR4, and p-p65 were elevated in the asthma group. As expected, The administration of pingchuanning decoction, guilong kechuanning capsules, or Dex remarkably inhibited the protein expression of HMGB1, and TLR4 and the phosphorylation of NF- $\kappa$ B p65 (Figure 3B). Taken together, pingchuanning decoction was involved in regulating PI3K/Akt/mTOR and HMGB1-mediated

TLR4/NF- $\kappa$ B pathways in lung tissues of asthmatic rats.

### 3.4 | Pingchuanning decoction mediated the release of proinflammatory cytokines in BALF and lung tissues

The effect of pingchuanning decoction on the proinflammatory cytokines IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in BALF and lung tissues was measured by the ELISA. In BALF, the treatment of pingchuanning decoction, guilong kechuanning capsules, or Dex reversed the decreased IFN- $\gamma$  level and the increased TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels in the asthma group (Figure 4A). The similar effects of pingchuanning decoction, guilong kechuanning capsules, and Dex were found in lung tissues (Figure 4B).

### 3.5 | Pingchuanning decoction ameliorated RTE cell apoptosis via PI3K/Akt/mTOR and HMGB1/TLR4/NF- $\kappa$ B pathway

We continued to explore the antiautophagic mechanism of pingchuanning decoction in vitro. As depicted in

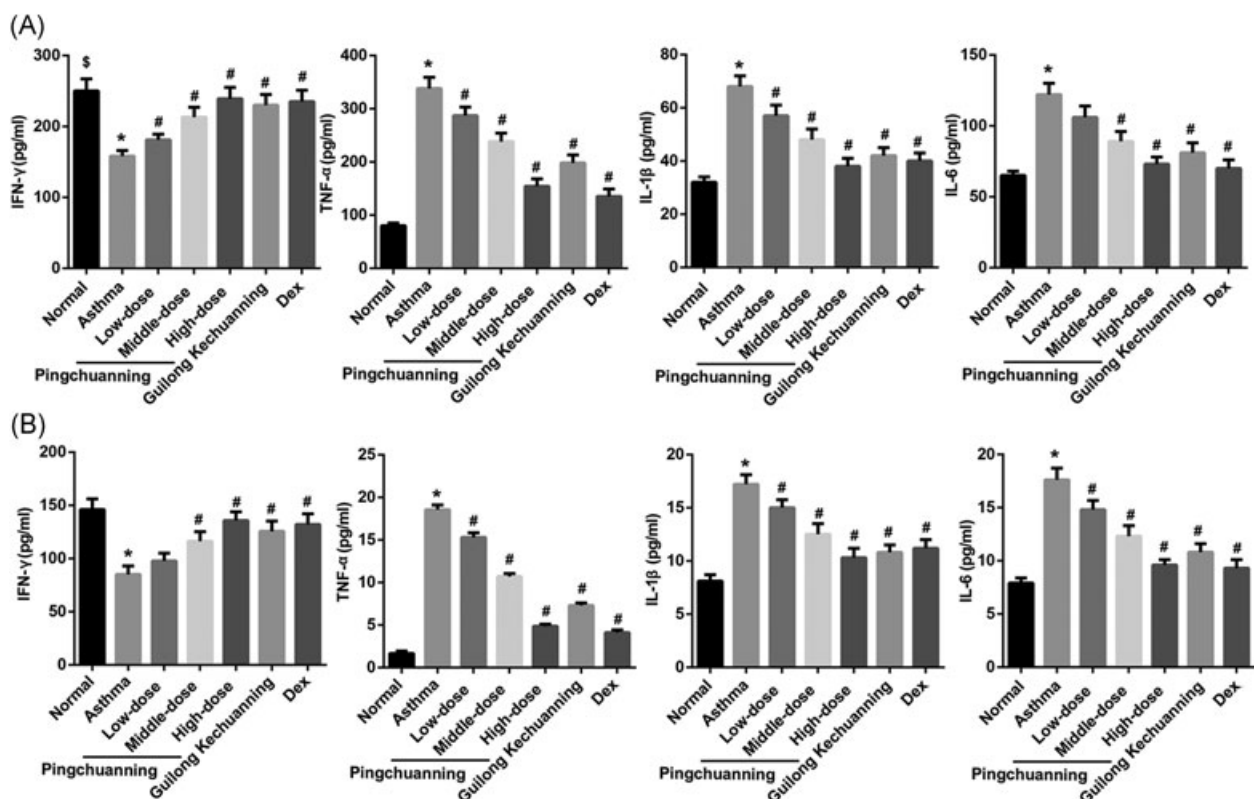


**FIGURE 4** Effect of pingchuanning decoction on the inflammatory contents in BALF and lung tissues. The levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in BALF (A) and lung tissues (B) of rats from normal, asthma, pingchuanning (low dose, middle dose, and high dose), guilong kechuanning, and dexamethasone (Dex) groups determined by ELISA. Data are presented as the mean  $\pm$  SD.  $n = 8$  per group. \* $P < 0.05$  compared with the normal group; # $P < 0.05$  compared with the asthma group. BALF, bronchial alveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; TNF, tumor necrosis factor

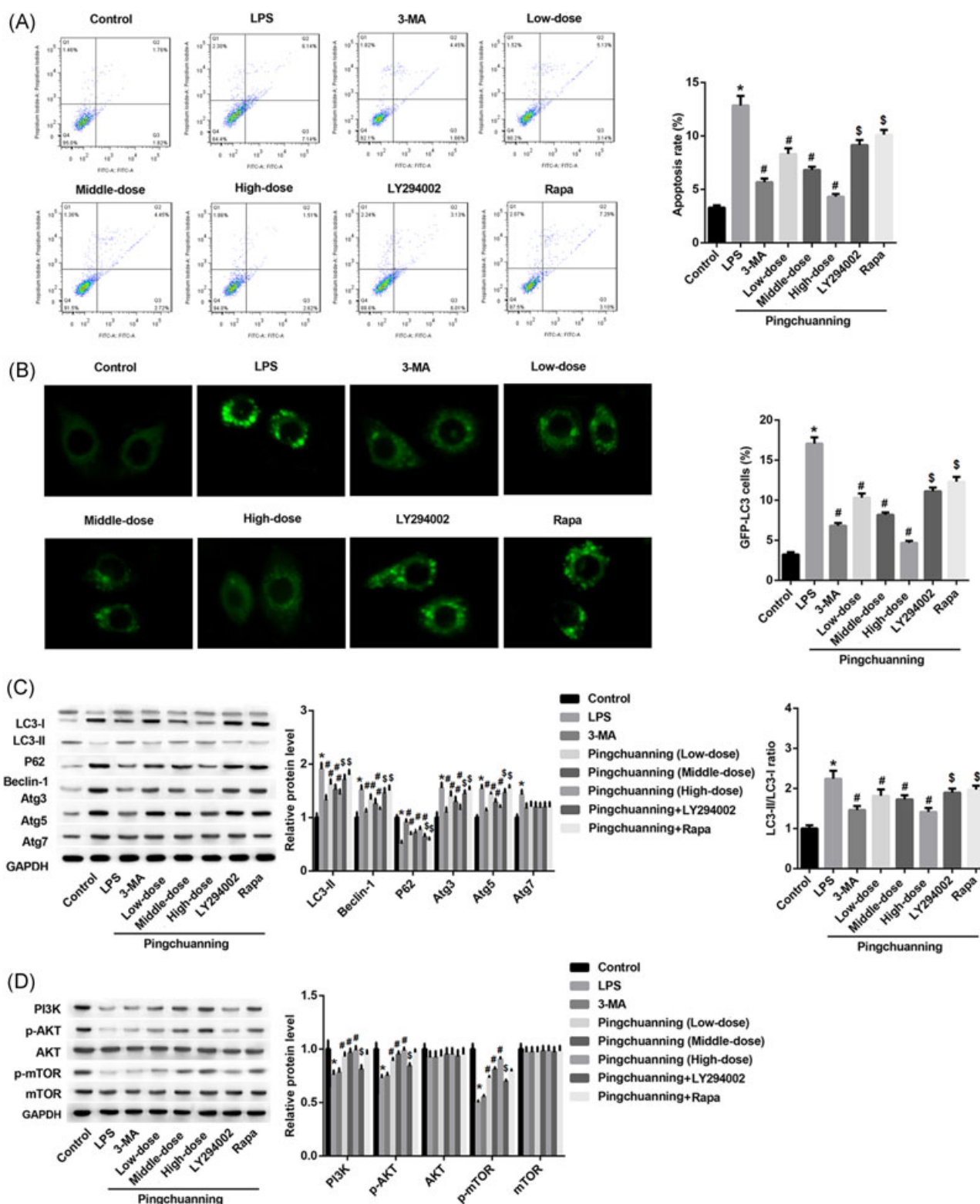


**FIGURE 5** Effect of pingchuaning decoction on apoptosis, autophagy, and PI3K/Akt/mTOR pathway-related proteins apoptosis rate of RTE cells estimated by flow cytometry (A), representative images of GFP-LC3 expression patterns (B), the protein levels and determinations of LC3-I, LC3-II, P62, beclin-1, Atg3, Atg5, Atg7, and LC3-II/LC3-I ratio (C) and the protein levels and determinations of PI3K, p-AKT, AKT, p-mTOR, and mTOR (D) in RTE cells in the groups of control, LPS, 3-MA, pingchuaning (low dose, middle dose, and high dose), pingchuaning + LY294002 and pingchuaning + Rapa. Data are presented as the mean  $\pm$  SD.  $n = 3$ . \* $P < 0.05$  compared with the normal group; # $P < 0.05$  compared with the asthma group; \$ $P < 0.05$  compared with pingchuaning (High dose) group. 3-MA, 3-methyladenine; LPS, lipopolysaccharide; RTE, rat tracheal epithelial

Figure 5A, LPS stimulation promoted the apoptosis of RTE cells, while this proapoptotic effect was mitigated by pingchuanning decoction and the inhibitor of autophagy, 3-MA. Furthermore, pretreatment with the PI3K inhibitor LY294002 or the autophagy activator, Rapa attenuated the inhibitory effect of Pingchuanning decoction.

Next, we detected the LC3 location in RTE cells by employing the immunofluorescence analysis. The formation of GFP-LC3-labeled vacuoles was increased in the LPS group as relative to the control group. Immunoreactivity was weakened after treatment with Pingchuanning decoction or 3-MA, whereas the increased LC3-positive cells were observed in the LY294002 or Rapa groups (Figure 5B).

Furthermore, we detected the protein expressions of autophagy markers and the related proteins of PI3K/Akt/mTOR and HMGB1-mediated TLR4/NF- $\kappa$ B pathways in RTE cells. The expressions of LC3-II, beclin-1, Atg3, Atg5, and Atg7 at protein levels and the LC3-II/LC3-I ratio were upregulated, while p62 protein was downregulated in the LPS group. Incubation with 3-MA or pingchuanning decoction obviously downregulated the protein expressions of above-mentioned autophagy markers and increased p62 expression. The inhibitory effect of Pingchuanning decoction on autophagy was reversed by blocking PI3K/Akt/mTOR pathway or treatment with LY294002 or Rapa, respectively (Figure 5C).

On the other hand, PI3K protein levels and the phosphorylations of Akt and mTOR were decreased in response to LPS stimuli. Treatment with 3-MA or pingchuanning decoction prevented LPS-induced PI3K/Akt/mTOR blockade. The effect of pingchuanning decoction on activation of PI3K/Akt/mTOR pathway was inhibited by addition of LY294002 or Rapa (Figure 5D). Besides, as revealed in Figure 6, Pingchuanning decoction inhibited HMB1 and TLR4 protein expressions and the phosphorylation of p65 induced by

LPS; however, LY294002 or Rapa treatment reversed this effect. Conjointly, our data illustrated that the antiapoptotic and antiautophagic mechanism of Pingchuanning decoction were mediated by HMGB1/TLR4/NF- $\kappa$ B and PI3K/Akt/mTOR pathways.

### 3.6 | Pingchuanning decoction effectively regulated the levels of proinflammatory cytokines in the supernatants of RTE cells

We also verified the effect of pingchuanning decoction on inflammatory contents in vitro. As expected, 3-MA or pingchuanning decoction overturned LPS-induced IFN- $\gamma$  (Figure 7A) reduction and TNF- $\alpha$  (Figure 7B), IL-1 $\beta$  (Figure 7C), and IL-6 (Figure 7D) upregulation in the supernatants of RTE cells. On the contrary, the addition of LY294002 or Rapa led to an opposite effect.

To summarize, these results showed that pingchuanning decoction ameliorated airway inflammation through autophagy inhibition via the activation of PI3K/Akt/mTOR signaling pathway in a rat model of asthma.

## 4 | DISCUSSION

In the current study, we examined the mechanism by which pingchuanning decoction exerted the potential therapeutic effect on asthma. In this experiment, pingchuanning decoction ameliorated histopathological lesions induced by asthma. More importantly, our findings revealed that pingchuanning decoction inhibited LPS-induced-apoptosis of RTE cells, and also suppressed autophagy by regulating the activity of the PI3K/Akt/mTOR signaling pathway.

Asthma is a common inflammatory disease of the airways, affecting millions of people worldwide. Nowadays,

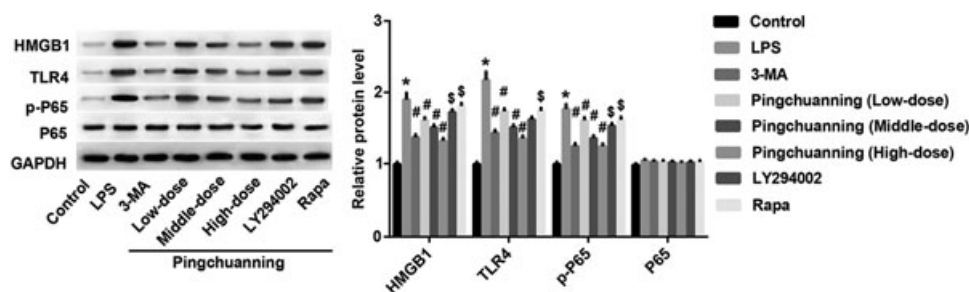


**FIGURE 6** Effect of pingchuanning decoction on HMGB1-mediated TLR4/NF- $\kappa$ B pathway-related proteins. The protein levels and determinations of HMGB1, TLR4, p-P65, and P65 in RTE cells in the groups of control, LPS, 3-MA, Pingchuanning (Low dose, Middle dose, and High dose), Pingchuanning + LY294002 and Pingchuanning + Rapa. Data are presented as the mean  $\pm$  SD.  $n = 3$ . \* $P < 0.05$  compared with the normal group; # $P < 0.05$  compared with the asthma group; \$ $P < 0.05$  compared with Pingchuanning (High dose) group. 3-MA, 3-methyladenine; LPS, lipopolysaccharide; RTE, rat tracheal epithelial

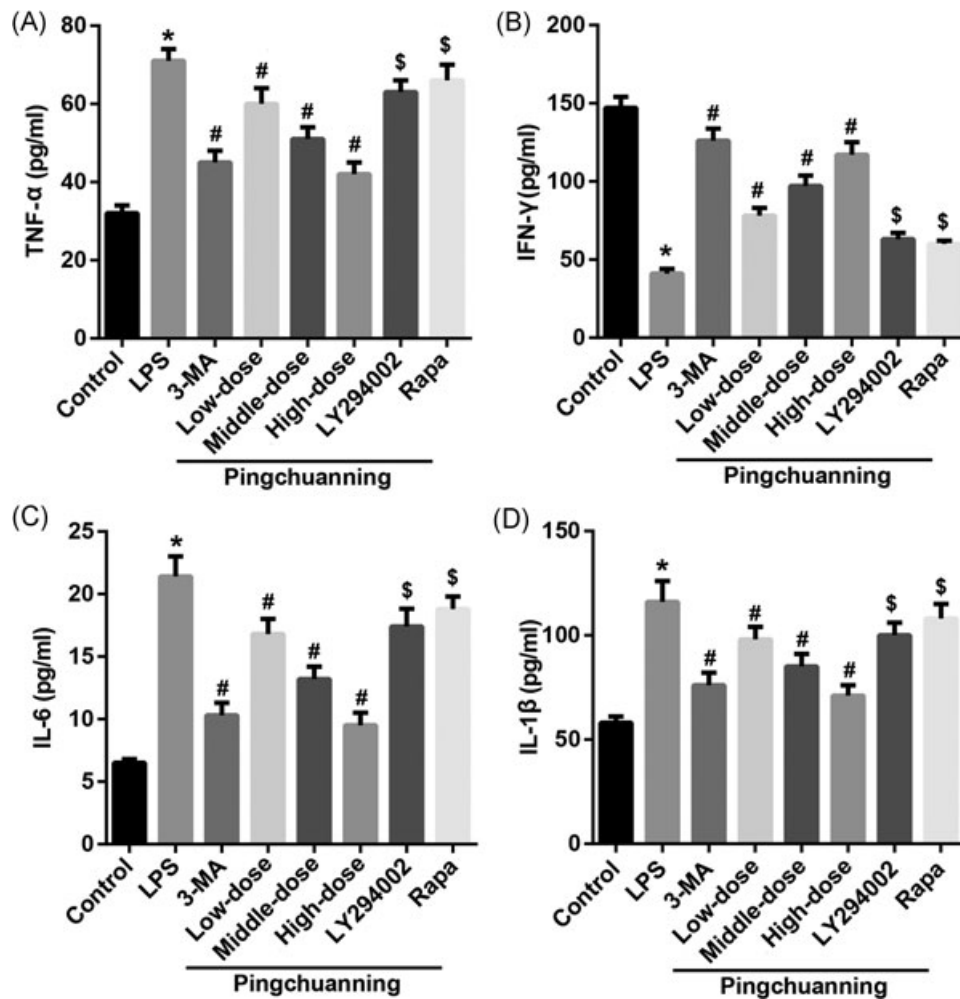


**FIGURE 7** Effect of Pingchuanning decoction on the inflammatory contents in supernatants of RTE cells. The levels of IFN- $\gamma$  (A), TNF- $\alpha$  (B), IL-1 $\beta$  (C) and IL-6 (D) in supernatants of RTE cells in the groups of control, LPS, 3-MA, pingchuanning (low dose, middle dose, and high dose), pingchuanning + LY294002 and pingchuanning + Rapa. Data are presented as the mean  $\pm$  SD.  $n = 3$ . \* $P < 0.05$  compared with the normal group; # $P < 0.05$  compared with the asthma group; \$ $P < 0.05$  compared with pingchuanning (high dose) group. 3-MA, 3-methyladenine; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; RTE, rat tracheal epithelial; TNF, tumor necrosis factor

a variety of effective drugs are available for the treatment of asthma, including TCM. For example, Yan et al<sup>15</sup> reported that Wentong decoction may cure allergic asthma by promoting apoptosis of eosinophils. Wei et al<sup>16</sup> demonstrated that Loki zupa decoction attenuated airway inflammation via regulating Th1/Th2 imbalance in an asthma mouse model. Pingchuanning decoction, composed of Mahuang (Herba Ephedrae), Xi Xin (Radix et Rhizoma Asari), Su Zi (Fructus Perillae), Xing Ren (Semen Armeniacae Amarum), Chen Pi (Pericarpium Citri Reticulatae), Ban Xia (Rhizoma Pinelliae), Huang Qi (Radix Astragali), Tai Zi Shen (Radix Pseudostellariae), Fang Feng (Radix Saposhnikovia), Di Long (Pheretima), and Zhe Bei Mu (Bulbus Fritillariae Thunbergii), is an effective prescription for treating asthma.<sup>17-19</sup> Nevertheless, the exact therapeutic mechanism of this drug has not been elucidated.

Here, we successfully established a rat model of asthma by OVA sensitization. The morphology changes of lung tissues, inflammatory cell infiltration in the bronchiolar walls, airway goblet cell hyperplasia, and mucus hypersecretion were observed in the asthma group. Intragastric administration of pingchuanning decoction effectively attenuated pulmonary inflammation.

Autophagy is an essential catabolic process involving cell metabolism and the renewal of certain organelles.<sup>20</sup> Emerging evidence has demonstrated that autophagy is implicated in certain human diseases, which can contribute to cellular defense but may also induce cell damage. For example, Ahn et al<sup>21</sup> found that autophagy functioned as a tumor suppressor in breast cancer via suppressing Notch1 signaling pathway. On the contrary, autophagy could prevent cell death and protected noninvasive breast cancers against DNA damage.<sup>22</sup> Our

experiments manifested that excessive autophagy was involved in OVA-induced asthma model, which was consistent with previously published research works.<sup>23,24</sup> Oral administration of pingchuanning decoction significantly inhibited autophagy induced by asthma, with decreased formation of autophagosomes as well as the expression of autophagy-related proteins (LC3, P62, beclin-1, Atg3, Atg5, and Atg7).

The PI3K/AKT/mTOR and HMGB1-mediated TLR4/NF- $\kappa$ B signaling pathways are two classical autophagic pathways. It appears that the activation of the PI3K/Akt/mTOR signaling pathway is capable of promoting excessive autophagy and subsequent cell death.<sup>12</sup> Nonetheless, nuclear protein HMGB1 can interact with TLR pathways and activate NF- $\kappa$ B, thereby inducing activating autophagy and leading to the release of proinflammatory cytokines.<sup>25</sup> In the current study, the Western blot analysis was performed to evaluate the expression levels of autophagic proteins. It turned out that pingchuanning decoction treatment evidently activated the PI3K/AKT/mTOR pathway, but inhibited HMGB1/TLR4/NF- $\kappa$ B pathway and the release of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in lung tissues in a rat model of asthma.

We further verified the antiautophagy mechanism of pingchuanning decoction in the pathogenesis of asthma in vitro. It has been shown that LPS promoted apoptosis of RTE cells, autophagy, and HMGB1/TLR4/NF- $\kappa$ B pathway, but blocked PI3K/AKT/mTOR pathway, while inhibition of autophagy or treatment of pingchuanning decoction led to an opposite effect. Interfering with PI3K signaling or activation of autophagy relieved the therapeutical effect of pingchuanning decoction.

## 5 | CONCLUSIONS

In conclusion, pingchuanning decoction significantly relieved airway inflammation after asthma and suppressed the apoptosis of bronchial epithelial cells by inhibiting excessive autophagy. The protective effect of pingchuanning decoction against airway inflammation in asthma was mediated by inhibiting autophagy via the activation of PI3K/Akt /mTOR signaling pathway.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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