

## Original Article

# Glycyrrhizic acid ameliorates LPS-induced acute lung injury by regulating autophagy through the PI3K/AKT/mTOR pathway

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**Abstract:** Acute lung injury (ALI) is a major pathological issue characterized by serious inflammatory response, and a major clinically critical illness with high morbidity and mortality. Glycyrrhizic acid (GA) is a major bioactive constituent isolated from traditional Chinese herb licorice, which has been reported to have positive effects on inflammation. Nevertheless, the effects of GA on lipopolysaccharide (LPS)-treated ALI in mice have not been reported. The purpose of our study is to investigate the inhibitory effects of GA on ALI treated by LPS and to elucidate its possible mechanisms. We found that GA significantly attenuated lung injury and decreased the production of inflammatory factors TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 with LPS treatment. GA induced autophagy which was showed by enhanced number of autophagosomes through upregulating the protein levels of LC3-II/I and Beclin-1 and downregulating SQSTM1/P62. Moreover, pre-treatment of 3-Methyladenine (3-MA), an autophagy inhibitor, reversed the inhibiting effects of GA on the secretion of inflammatory factors in ALI. The PI3K/AKT/mTOR pathway was associated with GA-induced autophagy under ALI induced by LPS. In conclusion, this study indicated that GA inhibited the production of inflammatory factors in LPS-induced ALI by regulating the PI3K/AKT/mTOR pathway related autophagy, which may provide a novel therapeutic perspective of GA in ameliorating ALI.

**Keywords:** Glycyrrhizic acid (GA), ALI, autophagy, inflammation, LPS

## Introduction

Acute lung injury (ALI) is a clinical syndrome characterized by pulmonary inflammation and increased microvascular permeability, which caused by a variety of pathogenic factors, including acute pneumonia, sepsis, severe trauma, and acute pancreatitis, and eventually develops into acute respiratory distress syndrome [1, 2], with a high mortality rate of 35-55% [3, 4]. Currently, there are few effective therapies for ALI [5, 6]. Among all pathogenic factors, bacterial infection is one of the most common causes. Lipopolysaccharide (LPS), the main component of the cell wall of gram-negative bacteria can induce ALI [7, 8]. Once stimulated by LPS, production of inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and in-

terleukin-1 $\beta$  (IL-1 $\beta$ ), is triggered and facilitates in the progression of ALI [7, 8]. Therefore, it is essential to treat inflammation in ALI.

Autophagy is a highly conserved biological process that exists in eukaryotes and maintains cell homeostasis and viability by recycling and reusing energy [9, 10]. The process of autophagy mainly includes five stages: initiation, phagocytic vacuoles, autophagosomes, autophagic lysosome formation and phagosome degradation [9, 11]. The major proteins involved in autophagy process are called autophagy-related proteins, such as LC3-II/Atg8, Beclin-1/Atg6, SQSTM1/P62, and *et al.*, and the key signaling pathways regulating autophagy include PI3K/AKT/mTORC1 and Bcl-2 protein family [12, 13]. 3-Methyladenine (3-MA) is an inhibitor of the

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phosphoinositide 3-kinase (PI3K) that blocks the formation of autophagosomes [14, 15]. Autophagy is generally considered as the protective mechanism of cells, while excessive autophagy leads to autophagic cell death [16, 17]. Studies have shown that autophagy promotes the survival or death of cells depending on factors, such as cell types, environmental conditions, and specific stimuli [17, 18]. Autophagy plays a crucial role in LPS-induced ALI [19], but its specific mechanism remains unknown.

Glycyrrhizic acid (GA), known as glycyrrhizin or qiangling, is one of the most important bioactive ingredients of *Glycyrrhiza uralensis*. GA has many beneficial effects, such as immune regulation, anti-viral, anti-cancer, and anti-inflammatory effects [20, 21]. Studies have found that GA inhibits the inflammatory response in ALI, but the mechanism involved is unknown [22, 23]. In this study, our result indicated that GA could induce autophagy to restrain the production of inflammatory factors, such as TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1, in LPS-induced ALI and the PI3K/AKT/mTOR pathway could be involved in GA induced autophagy.

### Materials and methods

#### *Antibodies and reagents*

Antibodies against LC3 (18725-1-AP, Proteintech), Beclin-1 (11306-1-AP, Proteintech), P62/SQSTM1 (18420-1-AP, Proteintech), PI3K (20584-1-AP, Proteintech), AKT (10176-2-AP, Proteintech), mTOR (20657-1-AP, Proteintech), and HMGB1 (10829-1-AP, Proteintech), TNF- $\alpha$  (11948, Cell Signaling), phospho-PI3K (p-PI3K) (4228, Cell Signaling), phospho-AKT (p-AKT) (4060, Cell Signaling), phospho-mTOR (p-mTOR) (5536, Cell Signaling), IL-1 $\beta$  (bs-0812R, Bioss) were employed. GA (G0150) was obtained from Shanghai TCI Chemical Industry Co.. Dojindo Inc (Japan) provided Cell Counting Kit-8 (CCK-8, CK04), and 3-Methyladenine (3-MA) (S2767) was obtained from Selleck (USA) and LPS (L2880) from Sigma.

#### *Cell culture and treatments*

Mouse macrophage RAW264.7 cells were obtained from the Cell Resources Center of the Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA) supple-

mented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin, and placed in a 37°C 5% CO<sub>2</sub> humidified incubator. The logarithmic growth phase cells were selected for the further experiment. The cells were pretreated with 3-MA (1 mM) or GA (100  $\mu$ g/ml) for 1.5 h or 1 h, respectively. Then the cells were incubated with LPS (1  $\mu$ g/ml) for 24 h and then harvested for further investigation.

#### *CCK8 assay*

RAW264.7 cells were treated as described above and seeded at a density of  $1 \times 10^4$  cells/well in 96-well plates and kept overnight for 24 h at 37°C with 5% CO<sub>2</sub>. Afterwards, 10  $\mu$ l of CCK-8 reagents were added to each well, and the mixture was continuously incubated at 37°C for 2 h. The absorbance values at 450 nm were detected using an EXL-800 Multiscan Spectrum (BioTek, USA).

#### *Confocal laser scanning microscope (CLSM) analysis*

The cells were fixed with 4% paraformaldehyde, washed with PBS, 0.1% Triton X-100 was permeabilized into the cells and then blocked with 5% bovine serum albumin (BSA), and incubated with antibodies of LC3 (1:50) or HMGB1 (1:50) overnight at 4°C. After washing several times with PBS, the cells were incubated with secondary antibodies for 1 h. Then, the cell nucleus was stained with 4'6'-diamino-2-phenylindole (DAPI) and fluorescence images were captured on CLSM (Leica, Germany).

#### *Transmission electron microscopy (TEM)*

RAW264.7 cells were washed 3 times with 0.1 M PBS, and fixed in 2.5% glutaraldehyde at 4°C overnight which was followed by dehydration using an acetone gradient, embedded, sectioned and routinely stained. Visualized autophagosomes were observed under a HT7700 Transmission Electron Microscope from HITACHI.

#### *GA and 3-MA exposure in animal models of ALI*

Male Balb/c mice (7-8 weeks old) were obtained from Experimental Animals Co. Ltd of SJA (Changsha, China), and randomly divided into four groups (12 mice per group), control group, LPS

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**Table 1.** The primers for qRT-PCR

Genes	Forward (5'-3')	Reverse (5'-3')
TNF- $\alpha$	CCCTCACACTCACAACCACC	CTTTGAGATCCATGCCGTTG
IL-1 $\beta$	TCCATCTTCTTCTTTGGGTATTGC	GTGACGTTGACATCCGTAAAGA
$\beta$ -tubulin	GTGACGTTGACATCCGTAAAGA	GTGACGTTGACATCCGTAAAGA

(10 mg/kg) group, GA (200 mg/kg) + LPS group, and 3-MA (15 mg/kg) + GA + LPS group. 3-MA was administered 0.5 h before GA intervention, and GA was administered 1 h before LPS treatment. Control group was given equal amounts of PBS at the same time. Each group of mice were sacrificed after 24 h of LPS treatment. All drugs were administered through intraperitoneal injection. All experiments were approved by the Experimental Animal Management Ethics Committee of the Hunan Normal University School of Medicine. Animal treatment and experimental operation procedures were conducted in line with the ethical requirements of experimental animals.

## Lung weight coefficient

Lung weight coefficient is an important indicator of pulmonary edema. Lung tissues were removed and wiped dry of surface blood and immediately weighed. The lung weight coefficient was evaluated by dividing the lung weight of each mouse by the body weight of the mouse multiplied by 100%.

## Pulmonary histopathology

The lung tissues were 4% formaldehyde-fixed, paraffin-embedded, and cut into 5  $\mu$ m thick sections. Next, the sections were performed hematoxylin-eosin (HE) staining. The changes of pulmonary histopathology were visualized under the microscope and pathological scores were obtained. According to the degree of lung injury, bleeding, edema, exudation, necrosis, congestion, neutrophil infiltration, and atelectasis, they were evaluated on a scale of 0-4 points [24]: no injury = score of 0, lesion field < 25% = score of 1, lesion field 25-50% = score of 2, lesion field 50-70% = score of 3, full field of vision = score of 4. The scores were calculated for statistical analysis.

## Western blot analysis

Proteins were extracted from lung tissue and RAW264.7 cells using a lysis buffer with PMSF,

and electrophoresed on 10-12% SDS-PAGE gels and then transferred into PVDF membranes (Immobilon, IPVH00010). After blocked with 5% skim milk for 1 h, the membranes were incubated with primary antibodies for 12 h

at 4°C. The membranes were washed with TBST and incubated with horseradish peroxidase-linked secondary antibodies for 2 h at room temperature. The immunoreactive bands were analyzed using image analysis software with an ECL system. All values were normalized to the loading control,  $\beta$ -tubulin.

## Immunohistochemistry analysis

The protein level of HMGB1 in lung tissue was evaluated using immunohistochemistry. After conventional dewaxing, lung tissues were repaired by thermal repair, and endogenous peroxidase activity was eliminated using a 3% H<sub>2</sub>O<sub>2</sub> solution. The sections were blocked for 2 h and incubated with the primary antibody for 12 h at 4°C. Subsequently, the secondary antibody and ABC solution were added, and incubated for 2 h at room temperature. Then, the samples were dyed with DAB and haematoxylin, and sealed with a neutral gum. Finally, each sample was observed under a microscope.

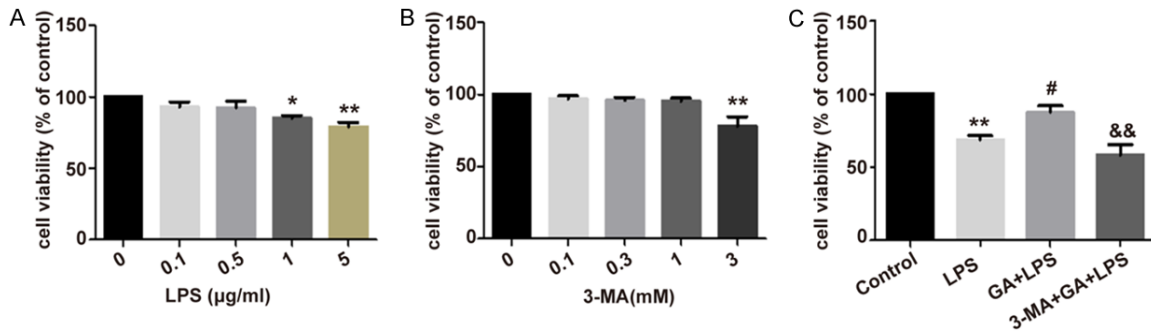
## qRT-PCR

The total RNA was extracted from lung tissues following the instructions of Trizol reagent (Servicebio, China). 2  $\mu$ g of RNA was used for reverse transcription synthesis of cDNA, as a quantitative real-time PCR template. The mRNA levels of TNF- $\alpha$  and IL-1 $\beta$  were measured according to the instructions of SYBR Green Master Mix kit (Roche), and standardized with  $\beta$ -tubulin, as loading control. The 2 <sup>$\Delta\Delta$ Ct</sup> method was used to calculate the expression level of mRNA. The primers for qRT-PCR were shown in **Table 1**.

## Statistical analysis

Data were shown as mean  $\pm$  SEM and obtained from at least four independent samples (n=4). The experimental data was processed through one-way ANOVA analysis using GraphPad 5.0 software (USA).  $P < 0.05$  was considered statistically significant difference.

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**Figure 1.** GA protects cell viability of RAW264.7 cells with LPS treatment. A. After exposure to LPS (0.1, 0.5, 1, 5  $\mu\text{g}/\text{ml}$ ) for 24 h, the viability of RAW264.7 cells were evaluated by CCK8 assay. B. RAW264.7 cells were treated with 3-MA (0.1, 0.3, 1, 3 mM) for 30 minutes and the cell viability was determined using CCK8 assay. C. The viability was assessed for RAW264.7 cells treated with LPS, and with or without GA and/or 3-MA treatment for 24 h. The experiments were performed four independent times ( $n=4$ ) and bars represent as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  in comparison to control group. # $P < 0.05$  in comparison to LPS group. && $P < 0.01$  in comparison to GA + LPS group.

### Results

#### GA protects cell viability of RAW264.7 cells with LPS treatment

To estimate the effect of GA on cell viability in ALI cell model, we first employed LPS to establish RAW264.7 cells model of ALI, and found that the viability of RAW264.7 cells was significantly decreased with LPS treatment (1, 5  $\mu\text{g}/\text{ml}$ ) ( $P < 0.05$ ) (Figure 1A). Then, an autophagy inhibitor, 3-MA, was used to detect whether inhibition of autophagy could decrease viability of RAW264.7 cells, and it was showed that 3 mM of 3-MA obvious reduced viability of the cells ( $P < 0.01$ ) (Figure 1B), but seriously leading to cell death. From the above mention, we employed 1  $\mu\text{g}/\text{ml}$  of LPS and 1 mM of 3-MA as the dose of the reagents for further experiment. Moreover, to detect whether GA could increase the viability of RAW264.7 cells and 3-MA reverse the protective effect of GA, the cells were incubated with 1  $\mu\text{g}/\text{ml}$  of LPS, 100  $\mu\text{g}/\text{ml}$  GA, and/or 1 mM of 3-MA. It was shown that the cell viability was significantly increased with pre-treatment of GA and reversed by 3-MA and GA treatment simultaneously in LPS-induced RAW264.7 cells ( $P < 0.05$ ) (Figure 1C). The above-mentioned results indicated that GA has an inhibitory effect on LPS-induced cell injury.

#### GA inhibits cytokine secretion in RAW264.7 cells with LPS treatment

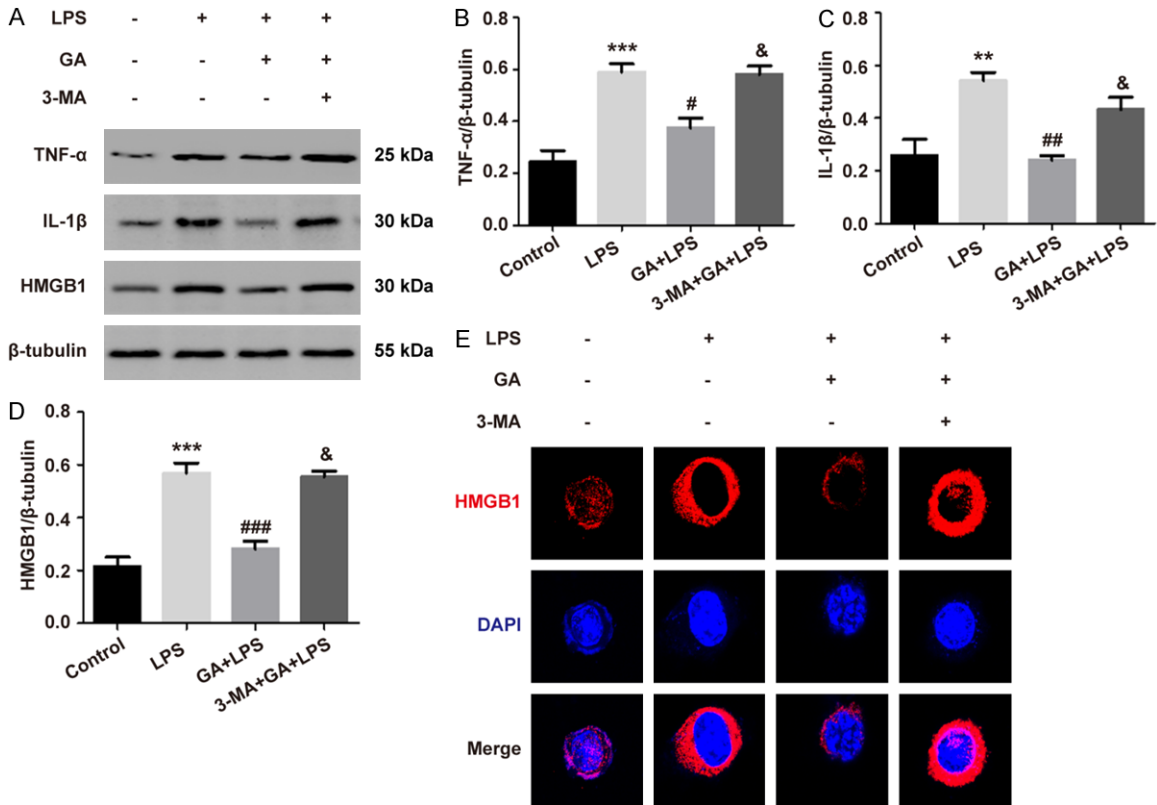
To investigate the effect of GA on inflammatory cytokines in ALI, 100  $\mu\text{g}/\text{ml}$  of GA, 1  $\mu\text{g}/\text{ml}$  of

LPS, and/or 1 mM of 3-MA were incubated with RAW264.7 cells. The results indicated that GA suppressed the levels of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 proteins, but the inhibiting effects of GA were reversed by 3-MA, in RAW264.7 cells treated with LPS ( $P < 0.05$ ) (Figure 2A-D). Subsequently, the HMGB1 expression was analyzed using immunofluorescence staining. The expression of HMGB1 induced by LPS was significantly reduced with pre-treatment of GA, and reversed by 3-MA in RAW264.7 cells ( $P < 0.05$ ) (Figure 2E). These data demonstrated that GA decreased LPS-induced inflammatory responses *in vitro*.

#### GA induces autophagy through the PI3K/AKT/mTOR pathway in RAW264.7 cells with LPS treatment

To explore whether the protective effect of GA on RAW264.7 cells was associated with autophagy activation, we detected the level of several key autophagy-related proteins using Western blotting. As shown in Figure 3A-D, treatment of LPS significantly upregulated the ratio of LC3-II/LC3-I and the level of Beclin-1, while P62 decreased, suggesting autophagy activation ( $P < 0.01$ ). GA could further activate autophagy and 3-MA reversed it, in the LPS-induced RAW264.7 cells ( $P < 0.05$ ). Subsequently, pre-treatment of GA obvious upregulated the fluorescence intensity of LC3 observed under CLSM (Figure 3E), and increased the number of autophagosomes by TEM, which indicating autophagy activation, in the cells with LPS treatment (Figure 3F, 3G). In addition, the fluores-

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**Figure 2.** GA inhibits the production of cytokines in RAW264.7 cells with LPS treatment. A. The levels of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 in RAW264.7 cells detected using Western blotting. B-D. Quantitative analysis of the protein production levels of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1. E. The remarkable images of HMGB1 observed by CLSM in RAW264.7 cells (magnification  $\times 63$ ). The experiments were performed four independent times ( $n=4$ ) and bars represented as mean  $\pm$  SEM. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with control group. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  versus LPS group. & $P < 0.05$  in comparison to GA + LPS group.

cence intensity of LC3 and the number of autophagosomes was significantly decreased in the 3-MA group ( $P < 0.05$ ). The above results indicated that GA could induce autophagy under ALI *in vitro*.

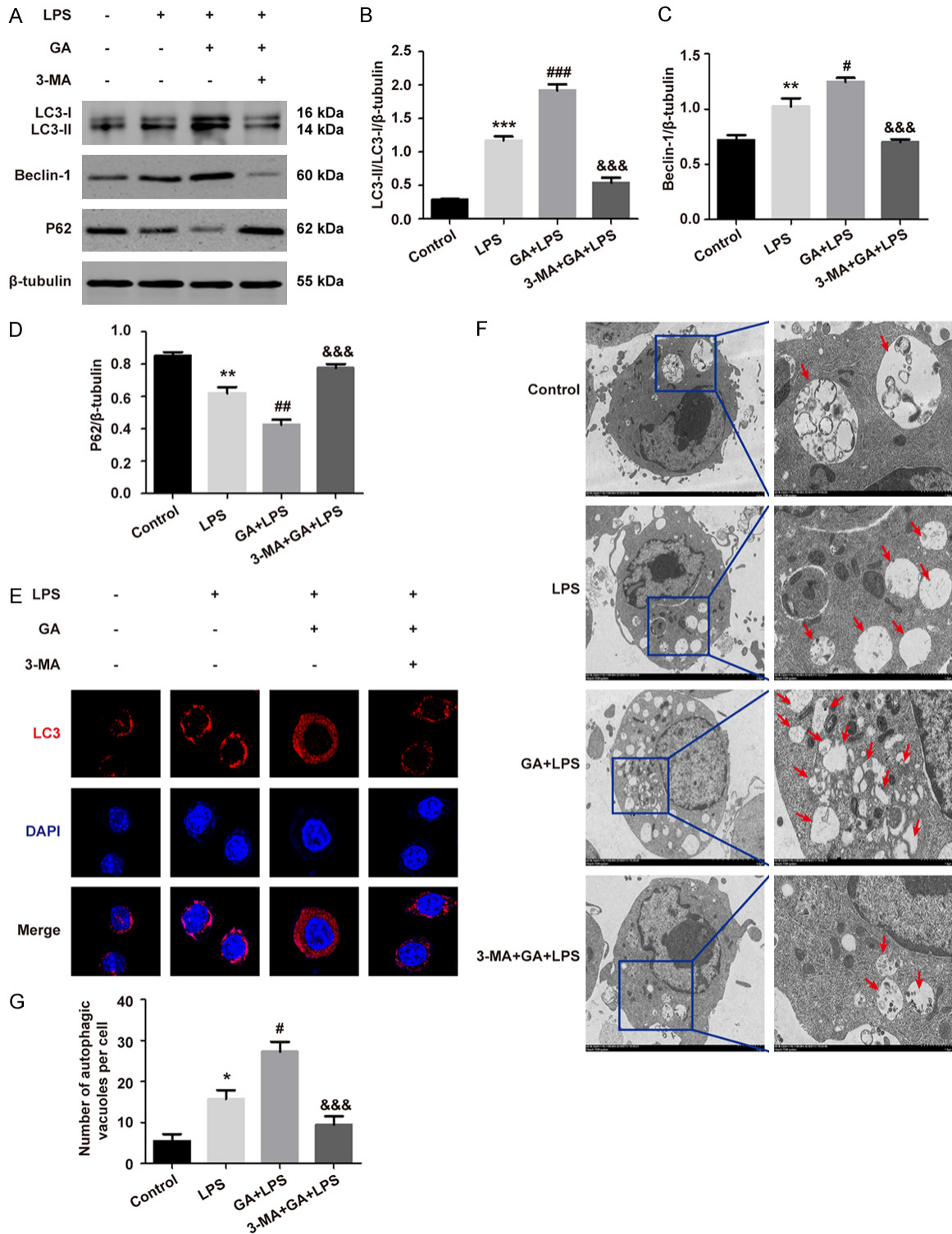
The possible mechanisms of autophagy activated by GA in LPS-induced RAW264.7 cells were being further explored. We investigated the PI3K/AKT/mTOR pathway, the classical autophagy-related pathway. It was found that the levels of p-PI3K, p-AKT, and p-mTOR proteins were decreased with LPS treatment, and further suppressed with the treatment of GA ( $P < 0.05$ ). However, compared with GA group, the levels of p-PI3K, p-AKT, and p-mTOR were downregulated with treatment of 3-MA ( $P < 0.05$ ) (Figure 4). These results suggested that GA induced autophagy at least partly through regulation of the PI3K/AKT/mTOR pathway in RAW264.7 cells with LPS treatment.

### GA inhibits lung inflammatory injury through induction of autophagy in LPS-induced ALI mouse model

Autophagy plays crucial role in regulating inflammatory response by eliminating inflammasome, cytokines, and cellular components, which provides an important method for regulation of inflammation. To explain whether GA decreased inflammation of ALI induced by LPS through autophagy activation *in vivo*, we established the ALI mouse model induced by LPS administration, then the lung weight coefficient and HE staining were to evaluate changes of the pulmonary histopathological features. It was indicated that the lung weight coefficient was significantly increased and the lung tissues accompanied with inflammatory cell infiltration, vascular congestion and bronchial wall thickening in ALI model induced by LPS, compared with control group. Importantly, the histological ch-

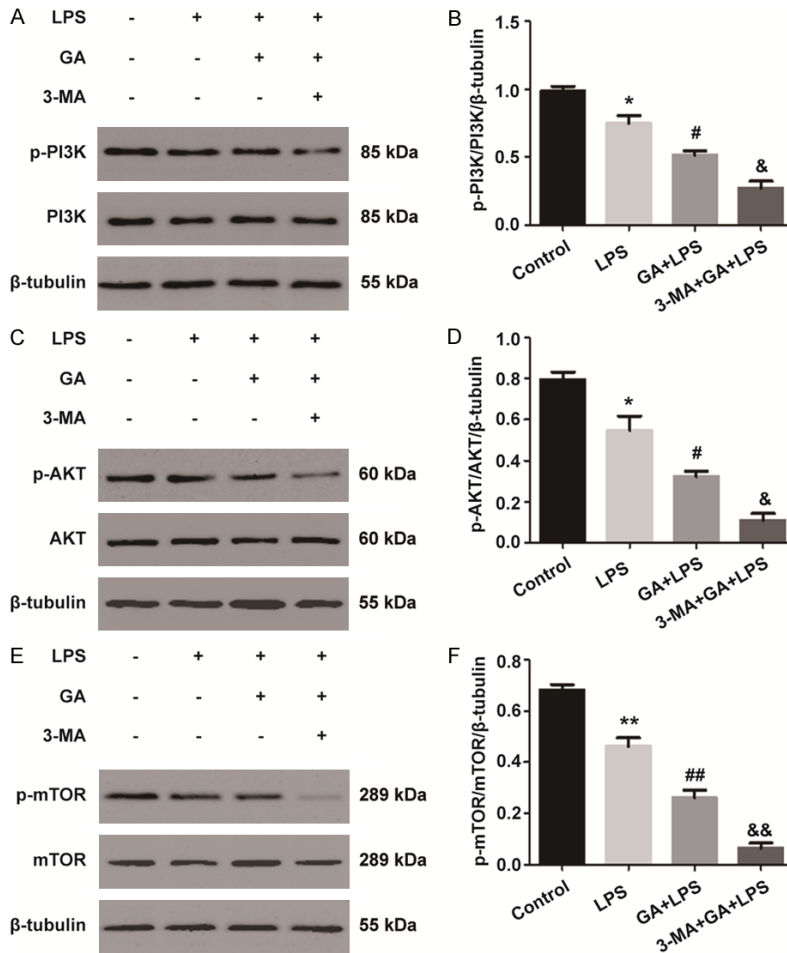


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**Figure 3.** GA induces autophagy in LPS-induced RAW264.7 cells. A-D. The levels of LC3-II/I, Beclin-1, and SQSTM1/P62 were detected by Western blotting in RAW264.7 cells, and the bar graphs showed the quantitative analysis results of the corresponding protein in each group, respectively. E. The representative images of the fluorescence of LC3 in RAW264.7 cells (magnification  $\times 63$ ). F. The remarkable images of autophagosomes ultrastructure by TEM in RAW264.7 cells (magnification  $\times 1500$ ,  $\times 6000$ , respectively). G. Quantitative analysis of autophagosomes numbers in each group, and autophagosomes were indicated shown by the red arrows. The experiments were performed four independent times ( $n=4$ ) and bars represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  in comparison to control group. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  in comparison to LPS group. &&& $P < 0.001$  versus the GA + LPS group.

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**Figure 4.** GA enhances autophagy through the PI3K/AKT/mTOR pathway *in vitro*. Western blotting detected the levels of p-PI3K, PI3K (A, B), p-AKT, AKT (C, D), p-mTOR, and mTOR (E, F). The experiments were performed four independent times ( $n=4$ ) and bars represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  in comparison to control group. # $P < 0.05$ , ## $P < 0.01$  in comparison to LPS group. & $P < 0.05$ , && $P < 0.01$  versus the GA + LPS group.

anges and lung weight coefficients dramatically reduced after GA administration, and these phenomena were reversed with 3-MA treatment ( $P < 0.05$ ) (Figure 5A, 5B). As shown in Figure 5C, the results of the lung injury score according to the changes of pulmonary histopathology of each group by HE staining which was consistent with the lung weight coefficient results.

TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 are representative proinflammatory factors in many infectious diseases. To further explore the effect of GA on inflammation of ALI *in vivo*, we first detected the mRNA levels of TNF- $\alpha$  and IL-1 $\beta$  by qRT-PCR, and the data indicated that LPS increased the production of TNF- $\alpha$  and IL-1 $\beta$  ( $P < 0.01$ ). Nevertheless, pre-treatment of GA significantly re-

strained the production of TNF- $\alpha$  and IL-1 $\beta$  ( $P < 0.05$ ), and the inhibitory effects of GA on inflammatory factors were reversed by 3-MA in LPS-induced ALI mouse model ( $P < 0.05$ ) (Figure 5D, 5E). Moreover, we further evaluated the levels of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 using Western blotting and HMGB1 by immunohistochemical analysis. It was consistent with the mRNA expression of the mentioned inflammatory factors. In briefly, GA obvious downregulated the protein production of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1, while the inhibiting effects of GA on inflammation were reversed by 3-MA administration under ALI *in vivo* ( $P < 0.05$ ) (Figure 5F-J). These above results declared that GA decreased lung inflammation of ALI through activation of autophagy *in vivo*.

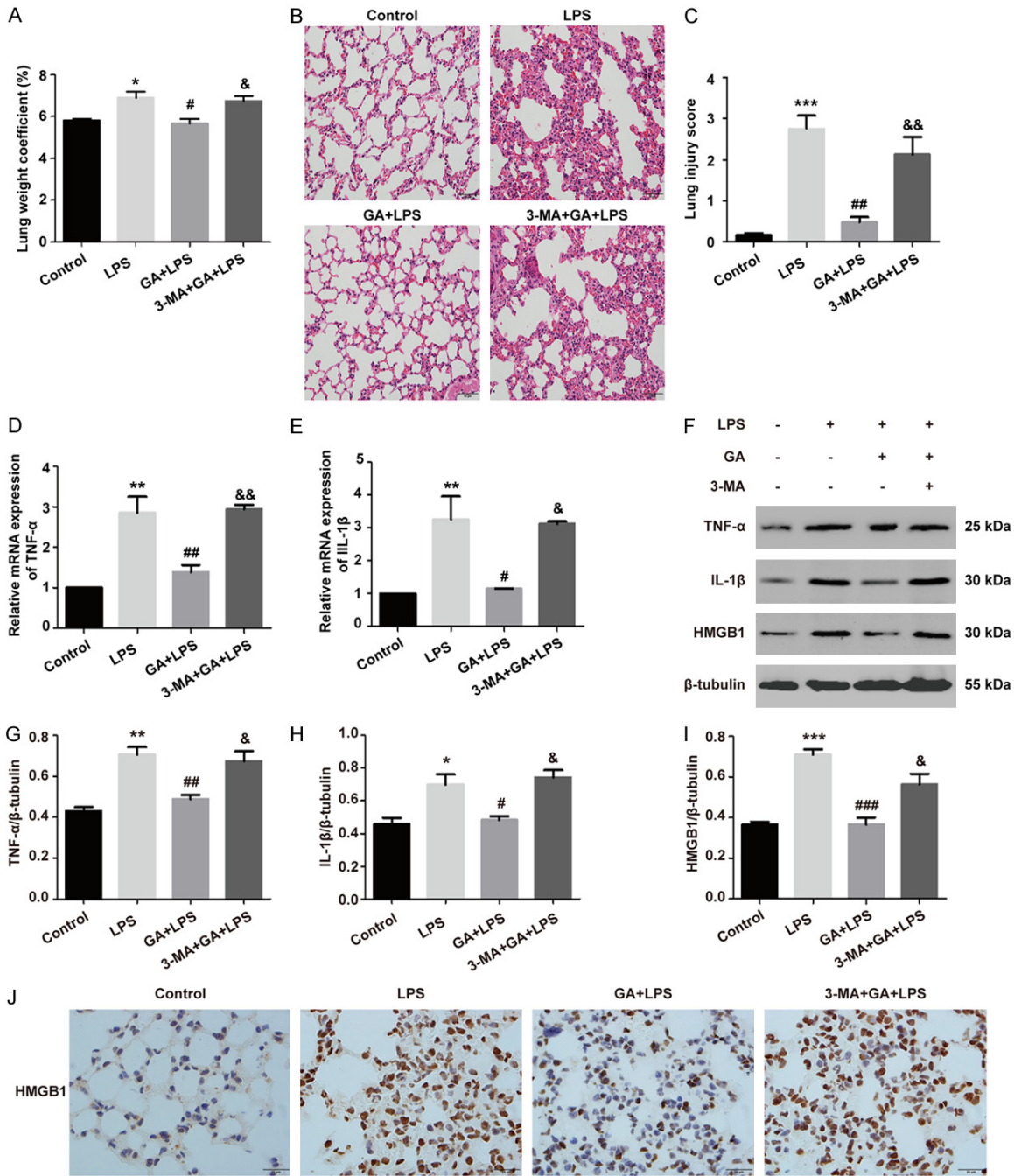
### GA enhances autophagy through the PI3K/AKT/mTOR pathway in LPS-induced ALI mouse model

To elucidate the effect of GA on autophagy in lung injury induced by LPS, we first evaluated the protein levels

of LC3-II/I, Beclin-1, and P62 by Western blotting. As shown in Figure 6, it was shown that the expression of LC3-II/I and Beclin-1 were significantly increased and P62 decreased in the LPS group ( $P < 0.05$ ). Moreover, pre-treatment of GA could further enhance the levels of LC3-II/I and Beclin-1, reduce P62 in LPS-induced ALI mouse model ( $P < 0.05$ ). Importantly, the autophagy activation induced by GA was reversed by 3-MA *in vivo* ( $P < 0.05$ ). Our results indicated that GA enhanced autophagy in LPS-induced ALI mouse model.

In order to further identify the mechanisms of autophagy activated by GA in LPS-induced ALI *in vivo*, we also investigated the PI3K/AKT/mTOR pathway. It was indicated that the levels

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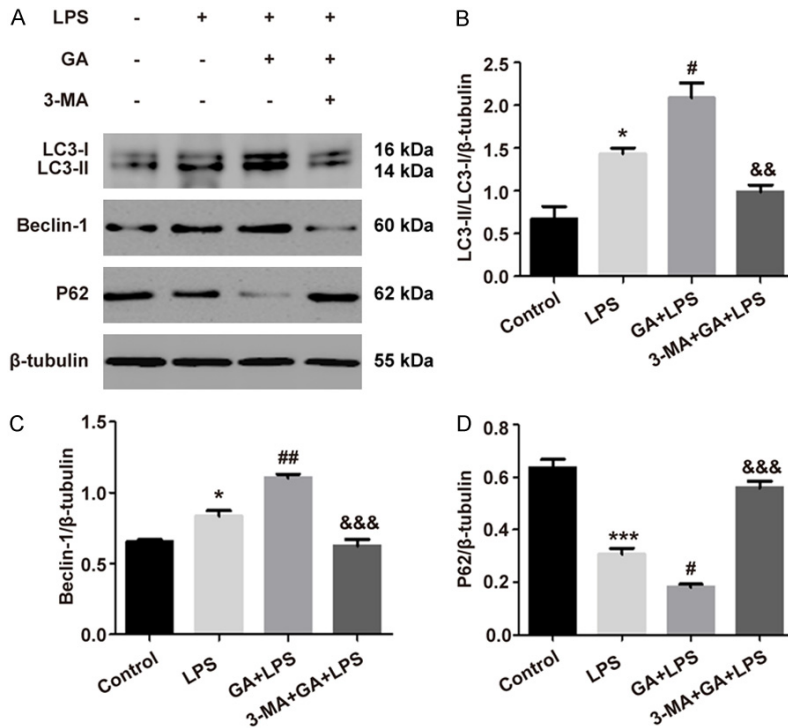
**Figure 5.** GA inhibits lung inflammatory injury through autophagy activation *in vivo*. (A) The pulmonary edema was evaluated by the lung weight coefficient. (B) The histopathological changes of lung tissues were examined using H&E staining (magnification  $\times 400$ ), and (C) morphological damage score for the lung tissues. (D, E) The mRNA levels of TNF- $\alpha$  and IL-1 $\beta$  detected using qRT-PCR. (F) The levels of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 were assessed by Western blotting. (G-I) Quantitative analysis of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 were shown in bar graphs. (J) The representative pictures of immunohistochemical analysis of HMGB1 protein expression. The experiments were performed four independent times ( $n=4$ ) and bars represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with control group. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  in comparison to LPS group. & $P < 0.05$ , && $P < 0.01$  versus the GA + LPS group.

of p-PI3K, p-AKT, and p-mTOR proteins were sharply decrease with GA treatment (200 mg/

kg) ( $P < 0.05$ ), and further decreased after addition of 3-MA ( $P < 0.05$ ), but the total PI3K,



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**Figure 6.** GA induces autophagy *in vivo*. A. Western blotting detected the levels of LC3-II/I, Beclin-1, and SQSTM1/P62 in lung tissues. B-D. Quantitative analysis of LC3-II/I, Beclin-1, and SQSTM1/p62 were shown in bar graphs, respectively. The experiments were performed four independent times ( $n=4$ ) and bars represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\*\* $P < 0.001$  versus control group. # $P < 0.05$ , ## $P < 0.01$  in comparison to LPS group. && $P < 0.01$ , &&& $P < 0.001$  versus sGA + LPS group.

AKT, and mTOR did not change significantly ( $P > 0.05$ ), in the ALI mouse model induced by LPS (Figure 7). These results suggested that the PI3K/AKT/mTOR pathway governed GA enhancement of autophagy under ALI *in vivo*.

### Discussion

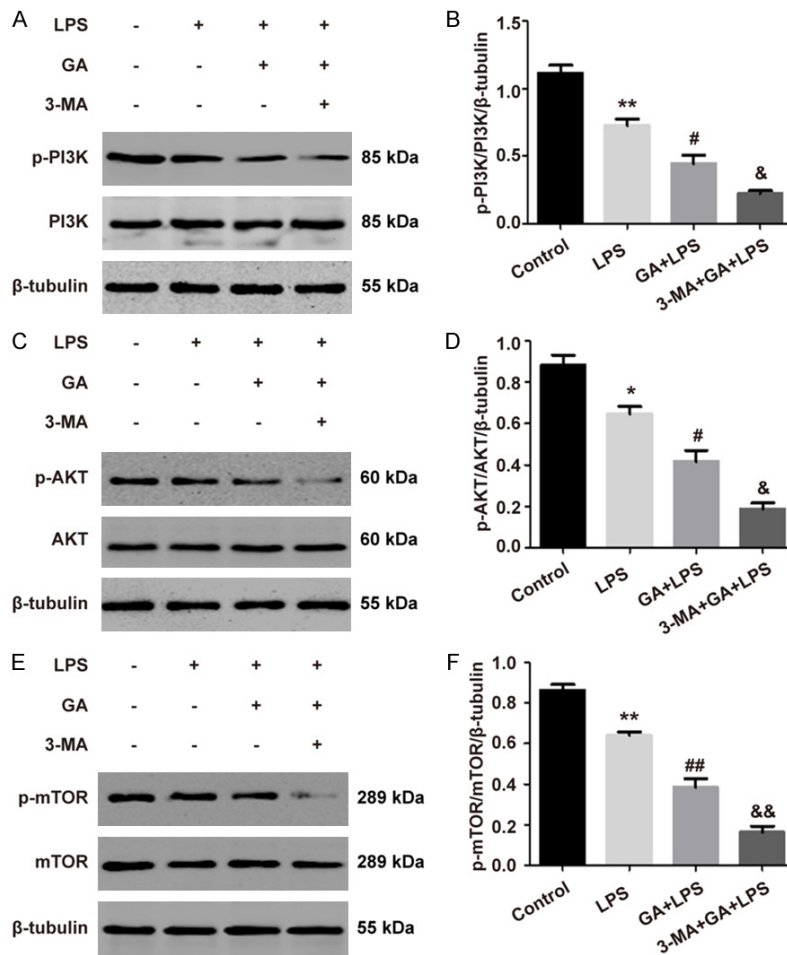
Currently, there is no effective treatment for ALI, therefore, it is urgent to develop the strategy for ALI treatment [5, 25]. The main mechanism of ALI induction by LPS is where multiple intracellular inflammatory signaling pathways cause the excessive release of various inflammatory factors [7, 8, 26]. The imbalance between proinflammatory and anti-inflammatory responses leads the accumulation of large numbers of inflammatory cells and inflammatory cytokines which infiltrating the lung [26, 27]. Nevertheless, the specific mechanism by which inflammation develops into ALI is unknown. Alveolar macrophages are the main resident phagocytes in lung, which participate

in the host's initial defense response through producing inflammatory mediators and chemokines, and regulate the initiation and development of pulmonary inflammation [7, 28]. Meanwhile, excessive activation of macrophages is also the key mechanism of inflammatory damage. TNF- $\alpha$  and IL-1 $\beta$  are the key components of cytokine network and the important inflammatory mediators of ALI initiation, which mainly secreted by mononuclear macrophages [8, 29]. High-mobility group box 1 (HMGB1), a highly conserved protein, is related to the pathogenesis of inflammation and as a proinflammatory mediator when stimulated by LPS [30, 31]. In our study, we examined the levels of the above inflammatory factors and found that LPS induced the production of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 *in vitro* and *in vivo*.

GA has been reported to be widely used in the protection efficacy of liver injuries [32, 33], while the protective effect of GA on lung injury has rarely been reported. Studies have shown that GA has anti-inflammatory and inhibitory effects on HMGB1 [33-35]. In mouse RAW264.7 macrophages induced by LPS, we found that GA increased the cell viability and decreased the production of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1. Moreover, we also showed that GA suppressed the secretion of inflammatory cytokines in ALI mouse model. On this basis, we further investigated the impact of GA on ALI and tried to reveal its potential mechanism.

Autophagy has attracted extensive attention in recent years due to the effects on various physiological and pathological processes. The process of autophagy involves many autophagy related proteins, which can engulf and kill pathogens to protect cells from pathogens, and inhibits inflammasome and the secretion of inflammation factors [19, 36]. It is reported

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**Figure 7.** GA induces autophagy through regulating the PI3K/AKT/mTOR pathway *in vivo*. (A, B) The levels of p-PI3K and PI3K, and (C, D) p-AKT and AKT, and (E, F) p-mTOR and mTOR were evaluated by Western blotting. The experiments were performed four independent times (n=4) and bars represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  in comparison to control group. # $P < 0.05$ , ## $P < 0.01$  in comparison to LPS group. & $P < 0.05$ , && $P < 0.01$  in comparison to GA + LPS group.

that GA significantly enhances autophagy in hepatocellular carcinoma *in vivo* and *in vitro* [37]. Lin *et al.* shows the anti-cancer effect of GA, which is related to the induction of autophagy in breast cancer cells [38]. In this study, we studied GA could activate autophagy in ALI models induced by LPS, and it was indicated that GA significantly increased the protein ratio of LC3-II/LC3-I and the protein levels of Beclin-1 and decreased P62, upregulated the fluorescence intensity of LC3, and enhanced the number of autophagosomes. During the process of autophagy flux, LC3-I converts to LC3-II, and the ratio of LC3-II/LC3-I is used as a quantitative index of autophagy activity [36, 39]. The degradation of P62 protein contributes to the

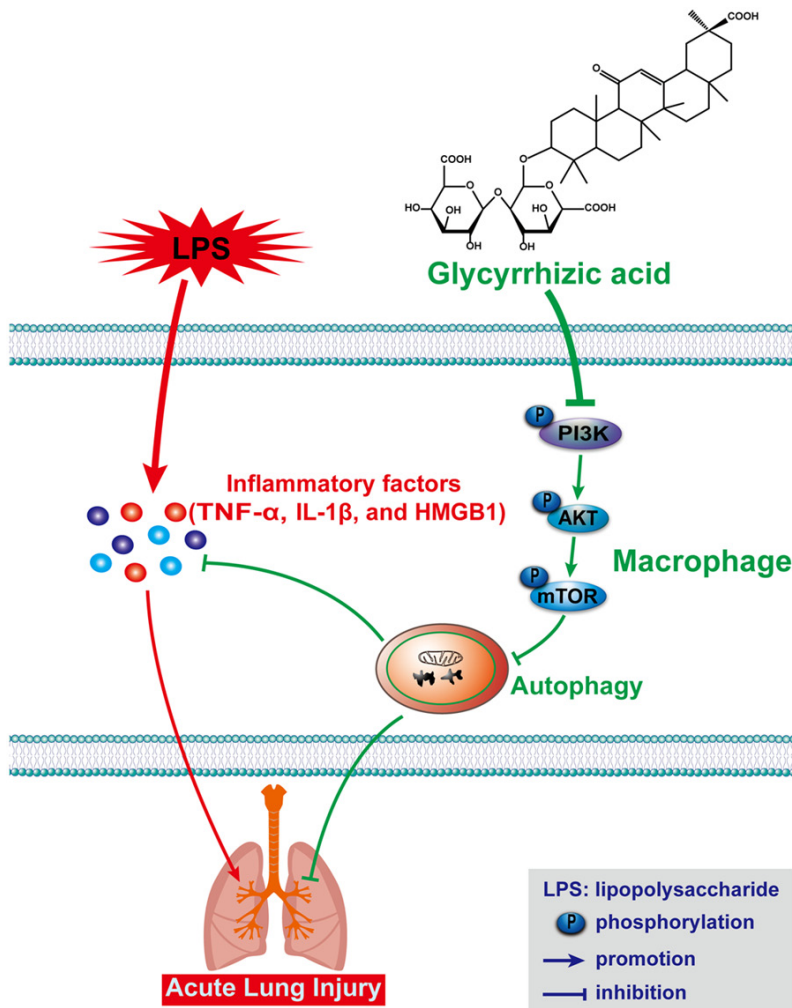
formation of autophagosome, while upregulation of P-62 indicates autophagy inhibition [39, 40]. Thus, we suggested that GA activated autophagy in ALI induced by LPS, and importantly, the activation of autophagy by GA reversed with 3-MA treatment.

Many cytokines are involved in controlling autophagy. Helper T cell cytokines, such as IL-2, TNF- $\alpha$ , and IFN- $\gamma$ , are generally considered as autophagy inducers, while Th2 cell-associated cytokines, such as IL-4, IL-10, and IL-13, can restrain autophagy [41, 42]. In addition, it has shown that autophagy regulates inflammation factors, such as TNF- $\alpha$  and IL-1 $\beta$  [43-46]. Autophagy reduces the secretion of IL-1 $\beta$  by inhibiting the activation of inflammasome [45, 47]. Activation of autophagy inhibits TNF- $\alpha$  secretion [15, 19]. As a classical damage-related model molecule, HMGB1 regulates autophagy mainly by binding to Beclin-1 [48, 49]. Based on these reports and our previous results, we hypothesized that GA regulated the expression of inflammatory

cytokines through regulating autophagy in ALI. The cytokines TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 were suppressed after inhibition of autophagy *in vitro* and *in vivo*. In conclusion, the anti-inflammatory effects of GA might be related to the induction of autophagy.

Zhang *et al.* shows that isoliquiritigenin is a component extracted from licorice, which promotes autophagy and inhibits gastric cancer cells through the PI3K/AKT/mTOR pathway [50]. PI3K regulates autophagy through different mechanisms, as type I PI3K-mTOR is an inhibitory pathway and type III PI3K is an activated pathway [51, 52]. Autophagy plays a protective role in hyperoxia-induced lung injury

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**Figure 8.** A diagram depicting the mechanism of GA ameliorates LPS-induced ALI by activating autophagy through regulating of the PI3K/AKT/mTOR pathway.

[53]. Our results showed that GA reduced the levels of p-PI3K, p-AKT, and p-mTOR proteins, while the total PI3K, AKT, and mTOR had no evident changes, under ALI *in vitro* and *in vivo*, indicating that GA induced autophagy at least partly through the PI3K/AKT/mTOR pathway. Then, 3-MA reduced the levels of p-PI3K, p-AKT, and p-mTOR proteins. Hence, it was indicated that GA ameliorated LPS-induced ALI through autophagy activation by regulating the PI3K/AKT/mTOR pathway.

This study suggested that GA induced autophagy through the PI3K/AKT/mTOR pathway to reduce the secretion of inflammatory factors and ameliorate ALI induced by LPS, as shown in **Figure 8**. The study first discovered that GA activated autophagy through regulating the PI3K/

AKT/mTOR pathway and attenuated LPS-induced ALI, which providing a novel prospect of GA alleviated ALI through inducing autophagy. Nevertheless, there still had some specific molecular mechanisms needed to be explored, such as, how autophagy regulated the changes of cytokines under ALI. Anyhow, this study provided an experimental basis for the application of GA for the clinical treatment of lung injury.

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### Disclosure of conflict of interest

None.

### Abbreviations

ALI, acute lung injury; CLSM, confocal laser scanning microscope; GA, glycyrrhizic acid; HMGB1, high-mobility group box 1; IL-1β, interleukin-1β; LPS, lipopolysaccharide; PI3K, phosphoinositide 3-kinase; TNF-α, tumor necrosis factor-α; TEM, transmission electron microscopy; 3-MA, 3-Methyladenine.

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