International Immunopharmacology 70 (2019) 504–511

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Glycyrrhizin administration ameliorates *Streptococcus aureus*-induced acute lung injury

Li Yao[a,*,1], Tianle Sun[b,1]

[a] Department of Intensive Care Medicine, Hefei NO.2 People Hospital, Hefei, China
[b] Jiangsu Key Laboratory of Infection and Immunity, Institutes of Biology and Medical Sciences, Soochow University, Suzhou, China

ARTICLE INFO

Keywords:
Glycyrrhizin
Streptococcus aureus
Acute lung injury
Inflammation

ABSTRACT

Streptococcus aureus (*S. aureus*)-induced acute lung injury (ALI) has a high incidence of mortality clinically. Glycyrrhizin (GL) is a traditional Chinese medicine for anti-inflammatory. However, the role of GL in *S. aureus*-induced ALI has not previously been elucidated.

GL (25 mg/kg i.p.) administration exerts potent anti-inflammatory effect in this model. GL administration significantly alleviated inflammation via reduction of multiple cytokines (serum and lung tissue IL-6, TNF-α, IL-8, IL-1β and HMGB1) and immune cells (lung tissue neutrophil and macrophage infiltration). Additionally, we measured the signaling pathways (NF-kB and MARKs) and inflammasome dependent pyroptosis. The results suggest that GL inhibits NF-kB, p38/ERK pathways and pyroptosis. Furthermore, we used different inhibitors to treat infected-A549 cells and found that BMS-582949 (a p38 inhibitor) is the most effective inhibitor for inhibiting pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) production, which suggests that p38 signaling pathway might be the main pathway for *S. aureus*-induced inflammation.

Collectively, the data demonstrates that GL could mitigate inflammation after *S. aureus* infection.

1. Introduction

Gram-positive extracellular growing bacteria, *Streptococcus aureus* (*S. aureus*), has been found that contributes to the severe sepsis in clinical patients [1]. The *S. aureus* infection promotes the production of kinds of pro-inflammatory cytokines and induces multiple organs damage, including acute lung injury (ALI) [2]. Neonatal children and elders are susceptible to *S. aureus* infection, which then leads to alveolar epithelial cells and lung parenchyma destruction known as acute respiratory distress syndrome (ARDS) [3,4]. There are approximately 190,600 patients develop ALI in the United States every year with about 40% mortality [5]. The ALI has serious threat to human life and health, and needs more effective protective strategies to improve its clinical outcome.

The main pathological manifestation of ALI/ARDS is inflammatory infiltration, including accumulation of immune cells (such as neutrophil and macrophage) and pro-inflammatory cytokines [6]. ALI involved *S. aureus* infection disrupts the alveolo-capillar barrier resulting in infiltration of immune cells, which produce pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), IL-8 and IL-1β [7].

Nuclear factor (NF)-kB and mitogen-activated protein kinases (MAPKs) signaling pathways mainly take part in the inflammatory response [8,9]. NF-kB, a principal intracellular transcription factor, usually bounds to its inhibitory protein IkB as a latent cytoplasmic complex, and translocates into the nucleus promote the expression of pro-inflammatory cytokines when IkB degraded [10]. MAPKs signaling pathway consists of c-Jun NH2-terminal protein kinases (JNKs)/stress-activated protein kinases (SAPKs), p38 and extracellular signal-related protein kinases (ERK1/2) [11]. Many inflammatory stimuli have been shown to activate NF-kB and MAPKs signaling pathways, which further promote output of pro-inflammatory cytokines and chemokines [12].

Three components take part in an intracellular multiprotein complex, inflammasome, including the adaptor protein ASC (apoptosis-related speck-like protein containing a caspase recruitment domain) and the cysteine protease procaspase-1 and a cytosolic pattern recognition receptor (such as NLRP3) [13]. First, NLRP3 recruits ASC, which further recruits procaspase-1. Then, procaspase-1 cleaves pro-IL-1β into mature IL-1β. Finally, IL-1β is released into circulation to trigger the inflammatory response. Also, the activated caspase-1 initiates a specific programmed cell death formation named pyroptosis [13]. In addition, the inflammasome plays a significant role in secondary bacterial

* Corresponding author.
E-mail address: ylirn168@163.com (L. Yao).
[1] These authors contributed equally to this study.

https://doi.org/10.1016/j.intimp.2019.02.046
Received 12 November 2018; Received in revised form 10 February 2019; Accepted 25 February 2019
Available online 15 March 2019
1567-5769/

respiratory infection, such as $ASC^{-/-}$ mice are protected from bacterial superinfection [14].

Glycyrrhizin (GL), major active constituent of licorice root consisting of potassium and calcium salts of glycyrrhizic acid, is a triterpene glycoside which has been used as a traditional Chinese medicine for the anti-inflammatory, anti-microbial, anti-protozoal, antiviral, anti-oxidative, anti-tumour and hepatoprotective activities [15]. GL has attracted attention for protecting ALI in mice [16]. One present study was designed to explore the protective role and mechanism of GL in *S. aureus*-induced ALI mice model. In particular, we have also investigated the effects of glycyrrhizin on: (1) the productions of IL-6, TNF-α, IL-8, IL-1β and HMGB1, (2) neutrophil and macrophage infiltration, (3) NF-kB and MAPKs signaling pathways, (4) the different components of multiprotein-complex inflammasome.

2. Methods

2.1. Mice

Female C57BL/6 mice, 6–8 weeks old, were purchased from the Experimental Animal Center of Chinese Academy of Sciences (Shanghai, P. R. China). Before performing any experiment, mice were bred in a pathogen-free room for at least 1 week with a 12 h light and 12 h dark cycle for acclimatization at the Experimental Animal Center of Anhui Medical University, and all animal experiments were performed according to the recommendations in the Guide for the Care and Use of Medical Laboratory Animals.

2.2. Bacteria and infection

S. aureus 834 strain (gift from microbiology lab of the second people's Hospital of Hefei) were cultured on 1% trypticase soy agar (TSA; Oxoid), 0.5% yeast extract (Oxoid) and 1%NaCl plate, inoculated and incubated with tryptone soy broth for 15 h at 37 °C. Then the bacteria were collected, resuspended and adjusted spectrophotometrically concentration at 550 nm.

2.3. Experimental groups

Mice were randomly divided into three groups (5 mice/group): (i) *S. aureus* group. Mice were injected intravenously (i.v.) 0.2 mL of PBS containing 10^7 CFU of viable *S. aureus* at day 0 and intraperitoneally (i.p.) 0.2 mL PBS daily. (ii) Control group. Mice were administered with same volume PBS (i.v.) and 0.2 mL PBS (i.p.) daily. (iii) GL + *S. aureus* group. Same as the *S. aureus* group but mice were injected (i.p.) with glycyrrhizin (GL) (25 mg/kg; Sigma-Aldrich St. Louis, MO) in 0.2 mL PBS daily after *S. aureus* infection.

2.4. Cell experiment

A549 lung cancer cells (ATCC) were cultured in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2 for 12 h, and then stimulated by *S. aureus* (MOI = 25), where some infected-cells were treated with 2 mM GL [17] (Sigma), 50 nM PDTC [18] (Selleck, Shanghai, China), 0.5 μM BMS-582949 [19] (Selleck), 30 μM Ulixertinib [20] (Selleck), 1 μM MCC950 [21] (Sigma) and 50 μM *Z*-VAD-FMK [22] (Selleck) for 6 h, respectively. Supernatant fraction were collected for ELISA.

2.5. Histological examination

The left lung specimens were collected from sacrificed mice, fixed in 10% buffered formalin > 2 days at room temperature, dehydrated by graded ethanol, embedded in paraffin and cut into 5 μm sections. The lung tissues were deparaffinized, stained with hematoxylin/eosin and observed under a light microscope.

Apoptotic cells in lung tissues were detected by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay according to the manufacturer's instructions (Roche Molecular Biochemicals, Inc., Mannheim, Germany).

2.6. Measurement of IL-6, TNF-α, IL-8, IL-1β and HMGB1

Blood and lung tissue homogenates were collected from mice, centrifuged at 4000*g* for 15 min at 4 °C after quantitative same total protein by the bicinchoninic acid assay (BCA; Thermo Scientific, Rockford, IL, USA) and supernatants were measured for IL-6 (eBioscience, San Diego, CA), TNF-α (eBioscience, San Diego, CA), IL-8 (westang, Shanghai, China), IL-1β (eBioscience, San Diego, CA) and HMGB1 (westang, Shanghai, China) by ELISA according to the manufacturer's instructions.

2.7. Lung wet/dry (W/D) weight ratio

The fresh right middle lobe was excised, measured using an electronic scale, blotted of blood and exudate on the surface with filter paper. The lung was measured wet weight using an electronic scale and then dried in an incubator at 80 °C for 48 h to record dry weight. The ratio of wet lung to dry lung (W/D) weight was calculated to assess the lung edema.

2.8. Determination of the number of bacteria in blood and lung

Blood and lung tissue homogenates were diluted in 10-fold steps in sterile water containing 0.5% Triton X-100 (Sigma Chemical Co.). Bacterial CFU was measured by plating each dilution on trypticase soy agar and culturing for 24 h at 37 °C.

2.9. Protein concentration in lung Bronchoalveolar lavage fluid (BALF)

BALF was collected by instillation of 1 mL PBS followed by gentle aspiration for 3 times and concentrated by centrifugation (3000 *g*, 10 min at 4 °C). The BALF supernatant was assessed total protein concentration by BCA assay.

2.10. Flow cytometry

The sediment cells were resuspended in PBS from the BALF, stained with the following monoclonal antibodies: FITC anti-mCD11b, APC anti-mLy6G, APC anti-mF4/80 (Biolegend), and then analyzed on FACS Canto II.

2.11. Western blots

Lung tissues were homogenized in RIPA buffer. Equal quantities of protein samples (approximately 20 μg) were separated by 10% SDS-PAGE, then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat dry milk in PBST for 2 h at room temperature, incubated with antibodies against total and phosphorylated p38, p-p38, ERK1/2, p-ERK1/2, JNK, p-JNK, p65, p-p65, IkBα, p-IkBα, NLRP3, ASC (Cell Signaling Technology, dilution 1:1000), Caspase-1 (Biovision, dilution 1:1000), as well as GAPDH (Sigma, dilution 1:20000) at 4 °C overnight, then washed and incubated with goat anti-rabbit/mouse secondary antibody conjugated with horseradish peroxidase-conjugated anti-rabbit IgG (Southern biotech,1:5000) for 1 h at room temperature. After ECL detection reagent (Thermo Scientific Pierce), photographs were quantified with ImageJ software (NIH).

2.12. Statistical analysis

All data were shown as means ± SEM. Comparison among different

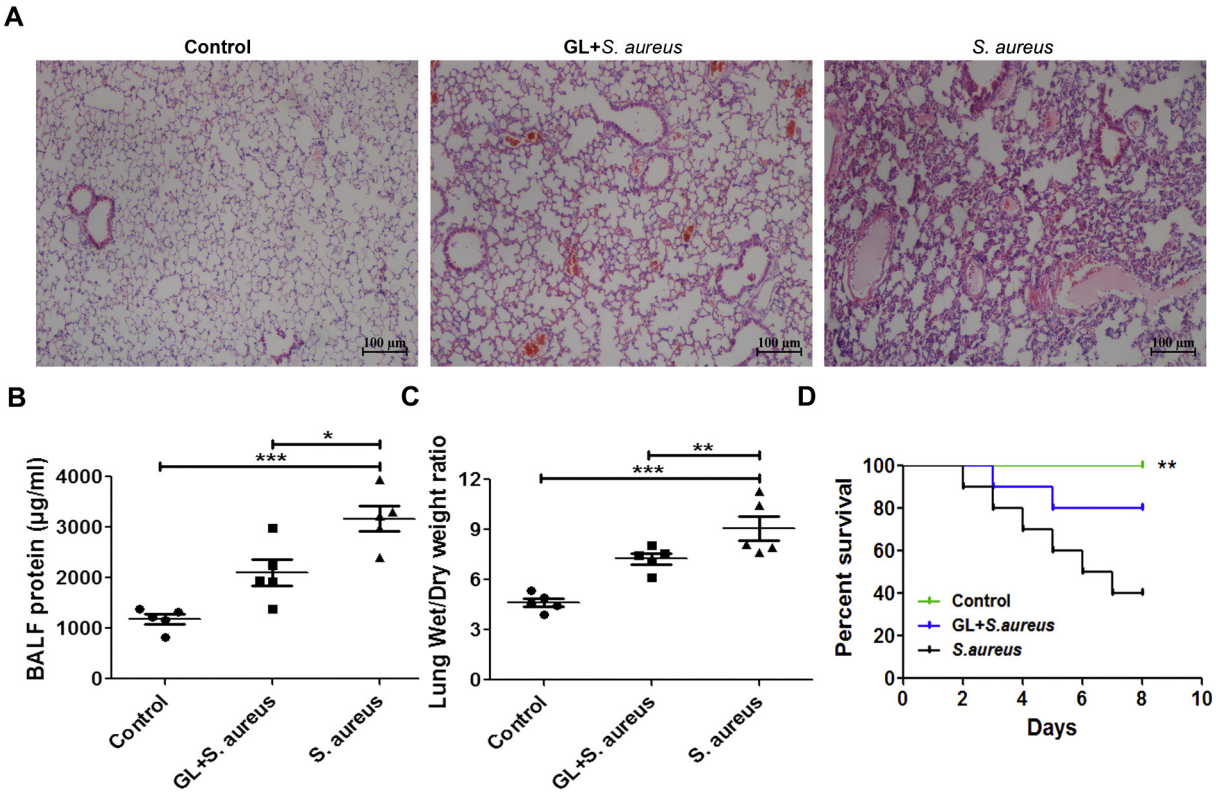

Fig. 1. GL administration attenuates *S. aureus*-induced ALI. (A) Hematoxylin and eosin staining of lung specimens at day 8 after intravenously injecting with *S. aureus*. (B) BALF protein levels. (C) Lung wet-dry weight ratios. (D) Survival rate of mice ($n = 10$). Data are representative of three independent experiments with $n = 5$ per experiment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

groups was analyzed by One-way ANOVA followed by Bonferroni's multiple comparison test using GraphPad Prism 5 software (GraphPad Inc., La Jolla, CA). Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. GL administration reduces lung tissue injury and mortality after S. aureus infection

S. aureus infection caused pulmonary edema, with the characteristics of increased bronchoalveolar permeability and lung wet-dry weight ratio. To test the therapeutic effect of GL, a natural anti-inflammatory triterpene, on *S. aureus*-induced acute lung injury, C57BL/6 mice were challenged with *S. aureus* and then treated with 25 mg/kg GL or equally volume PBS daily. When compared to *S. aureus*-infected lung sections by histological examination, GL treatment could attenuate the lung tissue injury with a fewer number of immune cells infiltration at day 8 (Fig. 1A). The total protein content in BALF was markedly lower in GL + *S. aureus* group compared to *S. aureus* group (Fig. 1B). Similarly, the lung W/D weight ratio was substantially lower in GL treated mice as compared with *S. aureus*-infected mice (Fig. 1C). As show in Fig. 1D, 40% female mice were survival at day 8 after *S. aureus* inoculation, and an increased survival rate (80%) was observed in GL + *S. aureus* group. Taken together, these results indicate that GL treatment could attenuate *S. aureus*-induced acute lung injury. However, there was no increased body weight or better health state with 100 mg/kg GL treatment than *S. aureus* group, which might because of drug toxicity that 200 mg/kg GL treatment would kill mice [23].

3.2. GL suppresses inflammatory cytokines in S. aureus-induced ALI

Although *S. aureus* itself could damage cells directly, there is abundant evidence to support that an excessively activated immune response is the major cause of lung damage by *S. aureus* infection [24,25]. We next analyzed the serum and lung homogenate protein levels of pro-inflammatory cytokines IL-6, TNF-α, IL-8, IL-1β and HMGB1 by ELISA. *S. aureus* infection significantly increased these pro-inflammatory cytokines of both the serum and lung homogenate (Fig. 2A to J). Compared with *S. aureus* group, GL treatment markedly decreased the serum protein levels of IL-6 (approximately 57% decrease), TNF-α (approximately 40% decrease), IL-8 (approximately 27% decrease), IL-1β (approximately 54% decrease) and HMGB1(approximately 34% decrease) (Fig. 2A to E). And GL treatment also significantly decreased the lung homogenate protein levels of IL-6 (approximately 53% decrease), TNF-α (approximately 43% decrease), IL-8 (approximately 49% decrease), IL-1β (approximately 38% decrease) and HMGB1(approximately 18% decrease) (Fig. 2F to J). These data demonstrate that GL attenuates *S. aureus*-induced ALI by decreasing the production of pro-inflammatory cytokines.

3.3. GL treatment inhibits the neutrophil and macrophage infiltration in ALI

Both macrophage and neutrophil are main immune cells that produce inflammatory cytokines and trigger acute lung injury [26,27]. First, the percentage of neutrophil and macrophage both increased from day 0 to 8 (Data not show). Then we measured the percentage of neutrophil and macrophage at day 8, and found that compared with the control group, *S. aureus* significantly increased the percentage of neutrophil ($CD11b^{+}Ly6G^{+}$) and macrophage ($CD11b^{+}F4/80^{+}$) from 3.04% and 3.23% to 34.07% and 13.48%, respectively (Fig. 3A and B). However, GL treatment significant decreased the percentage of macrophage and neutrophil to 12.4% and 8.79%, with ratios reduced to

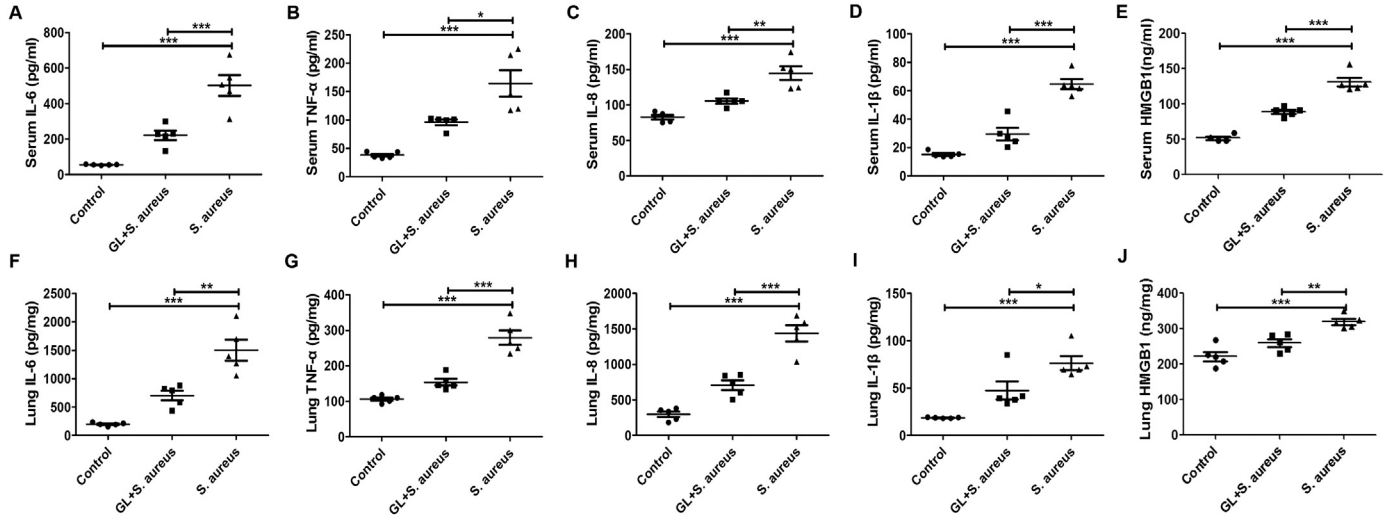

Fig. 2. GL reduces the inflammation in *S. aureus*-induced ALI. Protein levels of pro-inflammatory cytokines IL-6, TNF-α, IL-8, IL-1β and HMGB1 (A-E) in blood and (F-J) lung tissue by ELISA. Independent experiments were performed at least three times with similar results (n = 5). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

0.36 and 0.65 than *S. aureus* group (Fig. 3A and B). Compared to *S. aureus* group, the total numbers of macrophage and neutrophil were also reduced in GL + *S. aureus* group (Fig. 3A and B). These data show that GL robustly reduces neutrophil and macrophage in *S. aureus*-infected lung tissue.

3.4. Effect of GL on S. aureus infection

Next, the number of bacterial cells in the blood and lung homogenate were determined at 8 day after *S. aureus* infection. Bacterial numbers in the blood and lung homogenate were increased in *S. aureus* group compared to control group (Fig. 4A and B). The blood bacterial numbers were decreased whereas lung tissue bacterial numbers has no obvious change in GL + *S. aureus* group compared to *S. aureus* group (Fig. 4B), which suggests that *S. aureus*-induced ALI is independent of bacterial replication in the lung.

3.5. Effect of GL on NF-kB and MAPKs signaling pathways activation

To test the molecular mechanisms of anti-inflammation effect of glycyrrhizin, we measured the changes in activation of NF-kB and MAPKs signaling pathways by western blot. Phosphorylation of IkBα and p65-NF-kB subunit expression, which are indicators of NF-kB signaling activation, substantially increased from infected lung tissues (Fig. 5A). However, GL treatment showed lower levels of the phosphorylation of IkBα and p65 compared to *S. aureus* group (Fig. 5A). Then, the MAP kinase family molecules (JNK, ERK1/2 and p38) were also analyzed. *S. aureus* infection markedly increased the phosphorylation of p38 and ERK1/2; whereas it did not significantly affect the phosphorylation of JNK (Fig. 5B). But the phosphorylation of p38 and ERK1/2 were profoundly reduced in the lung tissues of GL + *S. aureus* group (Fig. 5B). These results indicate that GL might interfere NF-kB and MAPKs signaling pathways after infection with *S. aureus*.

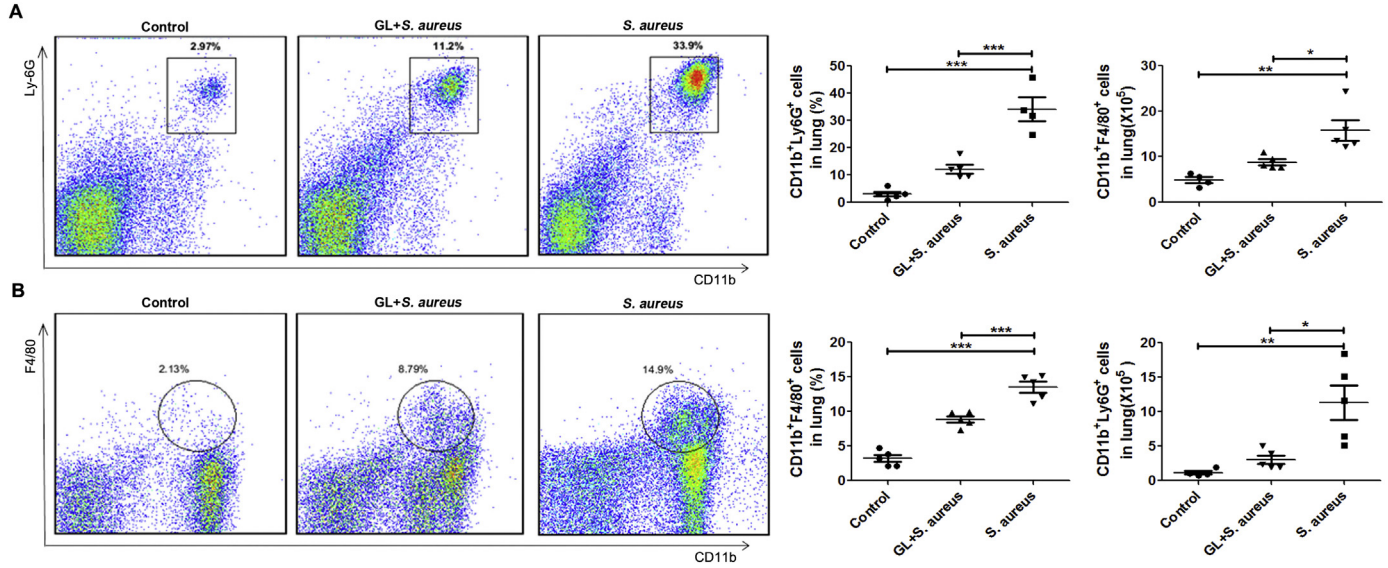

Fig. 3. GL treatment reduces the percent and number of neutrophil and macrophage in ALI. At day 8 post infection, cells from lung tissues were gated by $CD11b^{+}$, then analyzed for expression of (A) $Ly6G^{+}$ and (B) $F4/80^{+}$ subsets, with the numbers representing the frequency of each population as percentages of neutrophil and macrophage. Data are representative of three independent experiments with n = 5 per experiment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

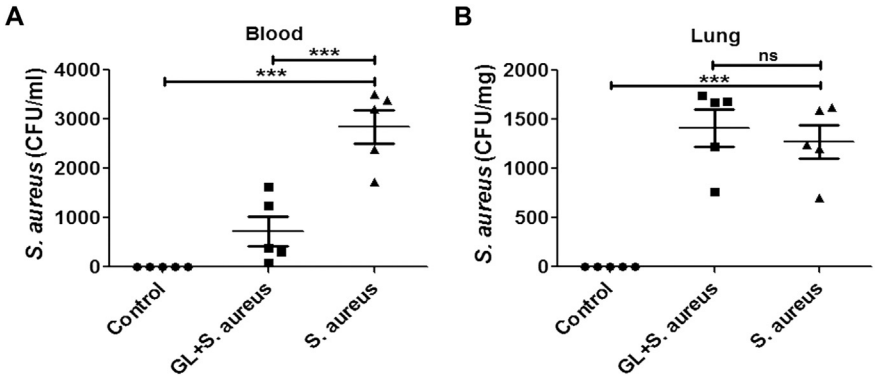

Fig. 4. Numbers of live *S. aureus* in mice after injection of bacteria. (A) The blood and (B) lung tissues were collected at 8 day after injection, and bacterial counts were determined by culture on agar (n = 5). ***$p < 0.001$.

3.6. GL suppresses NLRP3 inflammasome in S. aureus-induced ALI

Recent study has demonstrated that ASC inflammasome contributed to inflammation and mortality during *S. aureus* superinfection, and inhibiting NLRP3 effectively alleviated lung injury [14]. Since GL could mitigate inflammation, we hypothesized GL might ameliorate ALI through the NLRP3 inflammasome activation. The protein levels of NLRP3, ASC, caspase-1 p20 were markedly increased in the *S. aureus* group compared to the control group, and these levels were lower in the GL + *S. aureus* group than the *S. aureus* group (Fig. 6A).

3.7. GL inhibits pyroptosis in ALI

Pyroptosis, a new specific form of programmed cell death, which depends on caspase-1 activation [28]. We found the caspase-1 activity was significantly decreased in the GL + *S. aureus* group than *S. aureus* group (Fig. 6A). In addition, we observed the caspase-1-induced pyroptosis by the TUNEL staining. The GL + *S. aureus* group showed less cell death compared with the *S. aureus* group (Fig. 7). These data suggest that GL might inhibit lung tissue pyrotosis after *S. aureus* infection.

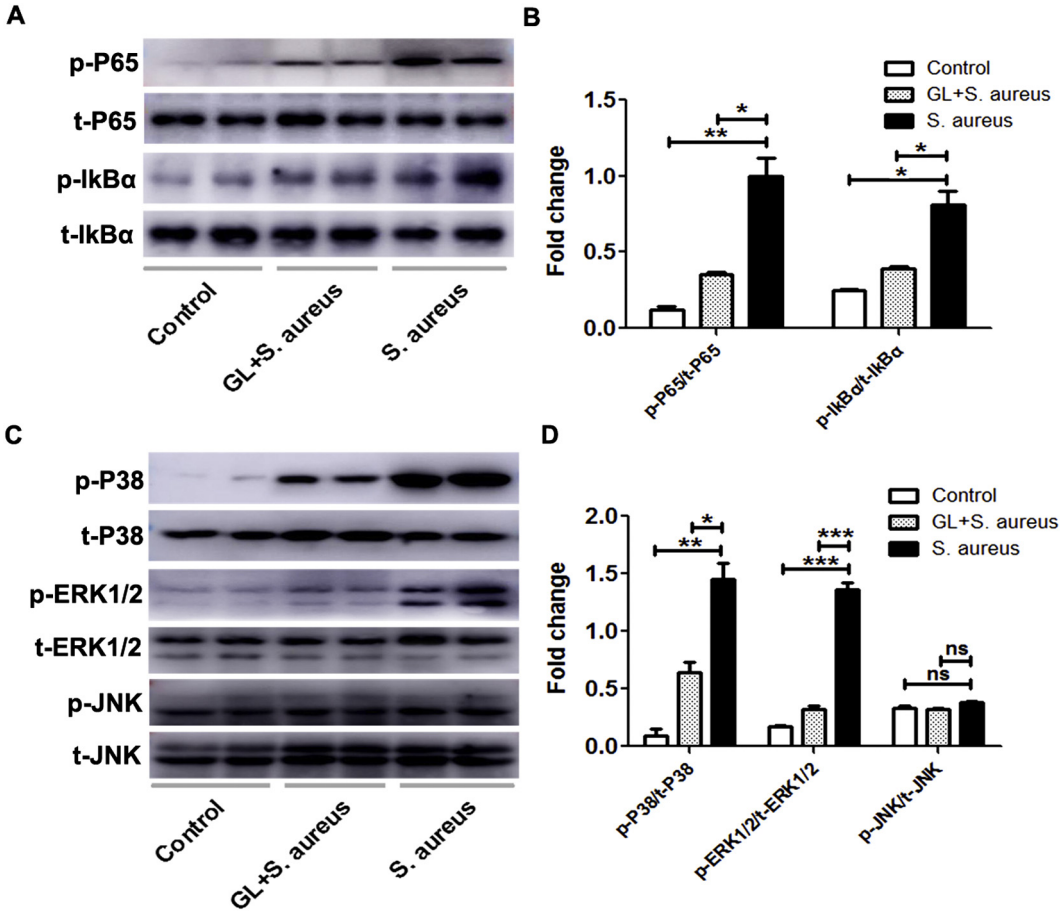

Fig. 5. The effect of GL on *S. aureus*-induced NF-kB and MAPKs signaling in lung tissues. (A-D) Lung lysates were examined for phosphorylation of IkBα, p65, p38, ERK1/2, JNK and protein expression of total IkBα, p65, p38, ERK1/2, JNK. Individual experiments were performed three times with similar results (n = 5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

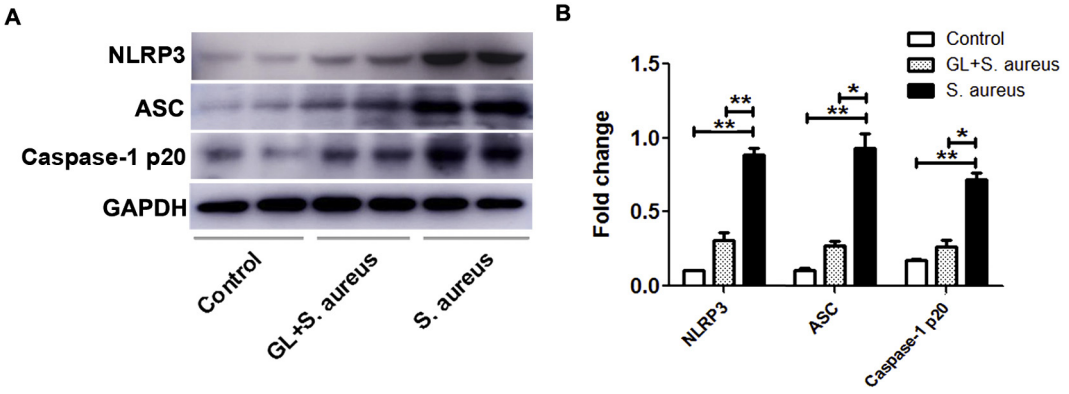

Fig. 6. GL administration decreases the activation of the inflammasome induced by *S. aureus* infection. (A-B) Lung lysates proteins of NLRP3, ASC and caspase-1 p20 were analyzed by western blot. The results are present at least three independent experiments (n = 5). *$p < 0.05$, **$p < 0.01$.

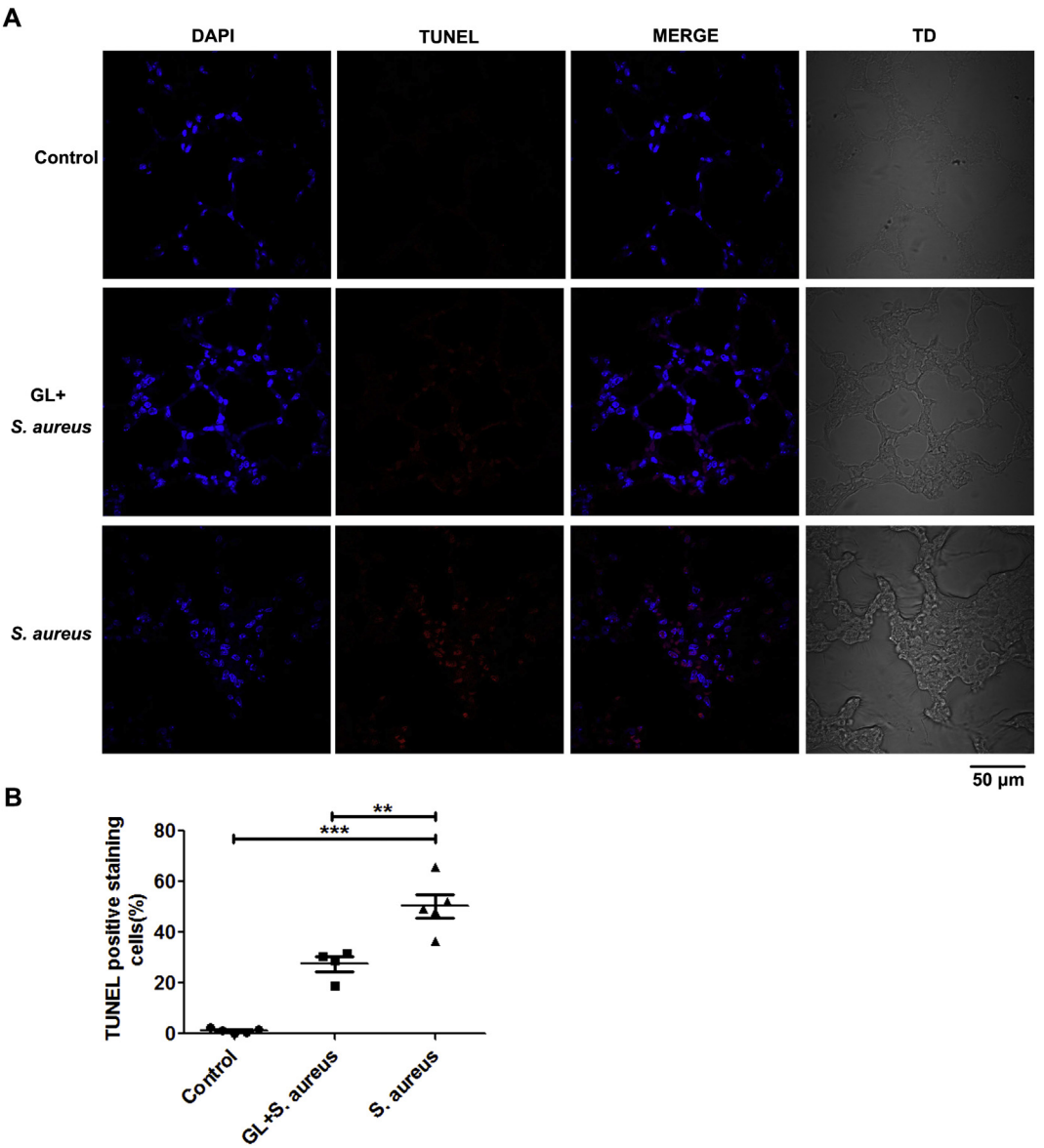

Fig. 7. GL treatment decreases pyroptosis in lung tissues of *S. aureus*-induced ALI. (A) Representative lung TUNEL staining of mice with different treatments. (B) The statistical result showed the TUNEL positive staining cells. Data are representative of three independent experiments with n = 5 per experiment. **$p < 0.01$, ***$p < 0.001$.

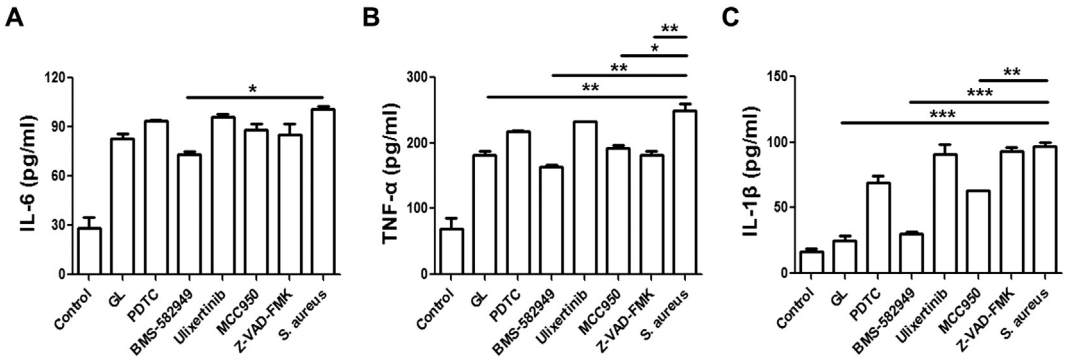

Fig. 8. p38 and pyroptosis pathways get involved the production of pro-inflammatory cytokines in *S. aureus*-infected A549 cells. Protein levels of pro-inflammatory cytokines IL-6, TNF-α and IL-1β (A-C) by ELISA. Independent experiments were performed at least three times with similar results (n = 5). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.8. p38 signaling pathway and pyroptosis get involved in S. aureus-induced inflammation

According to above experiments, signaling pathways (NF-kB, p38, ERK1/2 pathways and pyroptosis) might get involved in *S. aureus*-induced inflammation. Analysis of signaling pathways was performed in the presence of individual inhibitors: (1) PDTC, which blocks NF-kB translocation to the nucleus; (2) BMS-582949, which inhibits p38 phosphorylation; (3) Ulixertinib, which inhibits ERK phosphorylation; (4) MCC950, which inhibits NLRP3 activation; (5) *Z*-VAD-FMK, which inhibits caspase-1 and apoptosis; The supernatant fraction for detecting pro-inflammatory cytokines IL-6, TNF-α and IL-1β. *S. aureus* infection significantly increased these pro-inflammatory cytokines whereas GL treatment markedly decreased them (Fig. 8A to C). BMS-582949 treatment also markedly decreased them (Fig. 8A to 8C). MCC950 treatment could significantly decrease the levels of TNF-α and IL-1β (Fig. 8A and C). In addition, Z-VAD-FMK treatment also significantly decreases the TNF-α level (Fig. 8B). Collectively, these data suggest that pyroptosis get involved in *S. aureus*-induced inflammation and p38 signaling pathway plays the most important role in *S. aureus*-infected A549 cells.

4. Discussion

Acute lung injury induces accumulation of numerous inflammatory mediators and lipid metabolites, and they promote the production of inflammatory cytokines, reactive oxygen species (ROS) and proteases to amplify the inflammatory reaction. A variety of toxins from *S. aureus* induce inflammation with the characteristics of neutrophil and macrophage infiltration, increased vascular permeability and pro-inflammatory cytokines production [29,30]. The useful therapeutic target for *S. aureus*-induced ALI that has not been found. The administration of GL has been proven to effectively inhibit the production of pro-inflammatory cytokines [10]. Our statistics are consistent with the previous researches and indicate that the inhibition of pro-inflammatory cytokines (IL-6, TNF-α and IL-8) production might be a treatment of *S. aureus*-induced ALI. In addition, the percentage and total number of immune cells (neutrophil and macrophage) infiltration were also reduced after the GL administration. Interesting, after GL administration, the number of blood *S. aureus* was reduced whereas the lung *S. aureus* had no obvious change, which might due to the reduction of infiltrating macrophages with phagocytosis [31,32]. The data shows that GL attenuates *S. aureus*-induced acute lung injury, which is independent of bacterial replication in the lung.

GL, as a main active ingredient in licorice, was used as a demulcent and elixir in traditional Chinese medicine and used for the treatment of pulmonary diseases, with pharmacological properties consisting of anti-inflammatory and anti-viral functions [15]. Although most clinical trials by blocking the specific inflammatory mediators were not useful, the blockade of high-mobility group box 1 (HMGB1) has been focused as new strategy in sepsis treatment [33]. GL binds directly to HMGB1 (both Box A and B), and inhibits its activity [34]. HMGB1 presents in almost all eukaryotic cells with only two different aminos between mouse and human, was previously described as a nuclear protein that enhances transcription, and was recently regarded as a crucial late inflammation mediator that exerts pro-inflammatory activity [35,36]. Furthermore, extracellular HMGB1 contributes to the pathogenesis of ALI as a damage associated molecular pattern (DAMP) [37]. The effect of GL decreased the pro-inflammatory cytokines might because of the inhibition to HMGB1. In *S. aureus*-induced ALI, we also found that serum and lung tissue HMGB1 levels were reduced after GL treatment.

The classical view of the NF-kB signaling pathway is the activation of the IkB kinase (IKK) protein complex and the degradation of IkB. The evidence of reduced phosphorylated levels of IkBα and p65 shows that NF-kB signaling pathway was significantly inhibited in infected-lung received GL treatment. To confer MAPKs signaling pathway whether get involved in *S. aureus*-induced ALI, the JNK, ERK1/2, and p38 members were detected. Phosphorylated levels of p38 and ERK1/2 significantly were enhanced rather than JNK after infection, and the GL administration markedly reduced phosphorylated levels of p38 and ERK1/2. Some study suggested that JNK signaling pathway mainly get involved in *S. aureus*-induced cell apoptosis [38,39].

GL may be a great therapeutic drug to prevent *S. aureus*-induced ALI. To date, the inflammasome and IL-1β has been proved to take part in the *S. aureus* infection [14]. But it seems that there is no any other specific strategy to detect pyroptosis except detecting caspase-1 activation [40]. Our results suggest that *S. aureus* activates the inflammasome, enhances caspase-1-induced pyroptosis and induces ALI. GL markedly suppresses the inflammasome activation and pyroptosis. And TUNEL staining also confirms this.

This study demonstrates that GL attenuates the pro-inflammatory cytokines (IL-6, TNF-α, IL-8, IL-1β and HMGB1) production in the serum and lung tissues from *S. aureus* infected mice. Therefore, the inhibition of GL to the production of pro-inflammatory cytokines described in this study is likely due to its effective inhibition toward the activation of NF-kB and MAPKs (Erk1/2 and p38) signaling pathways. Furthermore, the increased IL-1β expression and apoptosis suggest that inflammasome dependent pyroptosis, which is initiated by activated caspase-1, and releases mature IL-1β to promote the inflammatory response. However, infected-A549 cells were treated with different inhibitors (PDTC, BMS-582949, Ulixertinib, MCC950, *Z*-VAD-FMK) for detecting pro-inflammatory cytokines expression. Infected-A549 cells cultured with BMS-582949 produced considerably lower levels of IL-6, TNF-α and IL-1β than other inhibitors. Additionally, MCC950 treatment had lower TNF-α and IL-1β levels, and Z-VAD-FMK treatment had lower TNF-α level. The production of pro-inflammatory cytokines suggests

that p38 signaling pathway and pyroptosis might play a more important role than other signaling pathway in *S. aureus*-induced ALI. So the anti-inflammatory function of GL might be through inhibiting p38 signaling pathway in *S. aureus*-induced ALI. But this still needs more study.

5. Conclusion

In summary, our study indicates that GL attenuates *S. aureus*-induced ALI by decreasing cytokines (IL-6, TNF-α, IL-8, IL-1β and HMGB1) and suppressing NF-kB signaling pathway, p38 and ERK1/2 pathways, the inflammsome activation and pyroptosis. In *S. aureus*-infected A549 cells, GL plays anti-inflammatory activity mainly through inhibiting p38 signaling pathway. In short, GL may be a potential therapeutic drug against *S. aureus*-induced ALI.

Acknowledgement

We thank all the members in the Experimental Animal Center of Anhui Medical University and microbiology lab of the second people's Hospital of Hefei.

Conflict of interest

The authors declare no conflict financial interest.

Author contributions

Y.L and S.T.L. designed the whole study, performed the experiments and wrote the manuscript.

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