Original Article

# 18β-glycyrrhetinic Acid Protects against *Staphylococcus aureus* Infection by Regulating the NF-κB Pathway

**Chunhui Rao[1], Zhuping Hong[2], Yuanyuan Yao[3], Gang Zheng[4], Saisai Wang[3,*]**

[1]Department of Colorectal Surgery, Hangzhou Hospital of Traditional Chinese Medicine, Guangxing Hospital Affiliated to Zhejiang University of Traditional Chinese Medicine, Hangzhou, P. R. CHINA.
[2]Pharmaceutical Informatics Institute, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, P.R. CHINA.
[3]Department of Colorectal Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, P. R. CHINA.
[4]Department of Cardiology, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, P. R. CHINA.

## ABSTRACT

**Background:** 18β-glycyrrhetinic acid (18β-GA) is reported to possess various pharmacological properties of which anti-inflammatory activities has been widely explored. However, the role of 18β-GA in *Staphylococcus aureus* (SA) infection has not been investigated. The aim of the present study was to explore the effects of 18β-GA on the SA infection especially the SA-induced Acute lung injury (ALI) and its related mechanisms. **Material and methods:** We infected the mice or cells with SA and then detected the survival rates of mice, bacterial burden and production of proinflammatory cytokines both *in vitro* and *in vivo*. We then detected the High-mobility group box 1 (HMGB1) expression by RT-qPCR and Western blotting. The effects on NF-κB activation was also determined by Western blotting and luciferase assay. **Results:** 18β-GA could significantly improve the survival rate of SA-infected mice, reduce bacterial burden, suppress infiltration of inflammatory cells and reduce secretion of IL-1β, IL-6 and TNF-α both in lung tissues and cells. 18β-GA treatment decreased high-mobility group box 1 (HMGB1) expression induced by SA infection and neutralizing of HMGB1 could improve the survival rate of mice induced by SA, implying that 18β-GA protected SA infection through down-regulating HMGB1 expression. Finally, we demonstrated that 18β-GA inhibited the NF-κB activation. **Conclusion:** Taken together, our preliminary study suggested that 18β-GA provided protective effects against SA infection via its anti-inflammatory properties possibly through down-regulating the HMGB1/NF-κB activation.

**Key words:** 18β-glycyrrhetinic acid, *Staphylococcus aureus*, Acute lung injury, HMGB1, NF-κB.

## INTRODUCTION

*Staphylococcus aureus* (SA) is *a* gram-positive bacterium that can cause a wide spectrum infection from mild infection of superficial skin to severe infection such as sepsis or septic shock.[1] Lung is a common site of SA infection. Acute lung injury (ALI) is a complex syndrome characterized by hypoxemia, extensive pulmonary edema, tiny atelectasis, intrapulmonary hemorrhage and severely impaired gas exchange.[2] The mechanisms involved in ALI including uncontrolled and excessive production of inflammatory mediators including cytokines, chemokines, adhesion molecules and bioactive lipid products.[3] The primary cause of ALI is bacterial infection, in which SA is one of the most common pathogens. Despite the progress and improvements made in treatment, the mortality of pneumonia with ALI is still high. Strategies such as reducing bacterial load and attenuating exaggerated inflammation are under extensive evaluation.[4]

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**Correspondence:**
***Dr. Saisai Wang,***
Department of Colorectal Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, 79 Qingchun Road, Hangzhou, Zhejiang 310003, P.R. CHINA.
Phone: +86-0571-18767126425
E-mail: saisaiwang@zju.edu.cn

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18β-glycyrrhetinic acid (18β-GA), a major component of *Glycyrrhiza glabra* has been demonstrated to have several pharmacological effects including anti-inflammatory, anti-ulcer, anti-viral, antioxidant and hepatoprotective properties.[5-8] 18β-GA has been found to inhibit the LPS-induced inflammatory response in macrophages and increase the survival rate in LPS-induced ALI.[9-10] Even in Methicillin-resistant *Staphylococcus aureus* (MRSA) infection, 18β-GA could also attenuate the skin lesion and decrease the virulence gene expressions.[11] 18β-GA also exhibits the protective activities in inflammatory liver injury.[12-14] 18β-GA has been showed to attenuate the inflammation in SA-induced ALI.[15] However, the anti-inflammatory effects and molecular mechanisms were unclear.

High-mobility group box 1 (HMGB1) is one of the members of Damage-associated molecular pattern (DAMP) and is associated with delayed and sustained release during infection.[16] HMGB1 is highly released after SA infection and anti-HMGB1 could ameliorate the pulmonary damage,[17] suggesting that HMGB1 might be a target in patients with pneumonia caused by SA.

The main objective of our study is to explore the effects of 18β-GA on the SA infection, especially the induced ALI and the underlying mechanisms. Our study demonstrated that 18β-GA provided protective effects against SA infection *via* its anti-inflammatory properties possibly through down-regulating the HMGB1/NF-κB activation.

## MATERIALS AND METHODS

### Chemicals and Mice

18β-glycyrrhetinic acid (18β-GA) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). Stock solutions were prepared at a concentration of 20 mg/ml and were diluted in medium to the appropriate concentrations indicated in each experiment. C57BL/6 mice (Shanghai Laboratory Animal Company, Shanghai, China) weighing between 16–18 g, age of 6-8 weeks were used. Mice were housed in a specific pathogen-free facility at Zhejiang University School of Medicine. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). This study was approved by the Animal Experimental Ethical Inspection of the First Affiliated Hospital, College of Medicine, Zhejiang University (Permit number is 2017-475).

### Bacterial Strains and Growth Conditions

The SA strain was a kind gift from professor FengXu, the Second Affiliated Hospital, Zhejiang University School of Medicine.[1] The bacteria were allowed to grow at 37°C in Tryptone Soya Broth (TSB) medium. SA stocks were kept at -80$^0$ C in TSB medium supplemented with 50% (vol/vol) of glycerol. Overnight cultures of SA were re-inoculated into fresh TSB and grown to a log phase and then quantified according to a OD600-based bacterial growth curve and Colony forming units (CFU) assay.[18]

### Cell Culture and Treatment

Human macrophage RAW264.7 cells and human liver hepatoma cells (HepG2) were obtained from American Type Culture Collection (ATCC, University Boulevard, Manassas, VA) and were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicilli and 100 mg/mL streptomycin in a 5% $CO_2$ humidified incubator at 37°C. All the cells were pretreated with 18β-GA or DMSO at a final concentration of 20 μM 2 h before infected with SA at a Multiplicity of infection (MOI) of 10 and then continued culture for the indicated times.

### Mice Infection Model

A SA-induced ALI mice model was established as previously reported.[19-21] In brief, mice were randomly divided into three groups: group PBS: mice were intraperitoneally (i.p.) injected with DMSO 2 h prior to mock infection with PBS; group PBS+SA: mice were i.p. injected with DMSO 2 h prior to infection with SA ($1\times10^8$ CFU); group 18β-GA + SA: mice were i.p. injected with 50 mg/kg 18β-GA 2 h prior to SA infection. Mice were anesthesiaded by 1.5% pentobarbital (5 μL/g per mouse) and then 40 μL of SA or phosphate buffered saline (PBS) was inoculated directly intratracheally (i.t.) into the mice.

All the mice were used to perform survival rate, histological and molecular biological examination. The survival rate experiment was performed by examining them every 6 h up to 3 days.

To determine the role of HMGB1 in SA infection, mice were intraperitoneally injected with either 600 μg anti-HMGB1 neutralizing antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or non-immune rabbit IgG in 500 μL PBS 1 h before SA infection.

### Histopathological Changes Examination

Lungs and livers of the mice were fixed in 4% paraformaldehyde for 24 h at 4°C, then embedded in paraffin. 4 μM sections were performed using a rotary microtome

(Leica RM2016, Shanghai Leica Instruments, Shanghai, China) for Hematoxylin and Eosin (H&E) staining. After staining, histopathological changes in the lung and liver tissues were observed under a light microscope.

## SA quantification in the Lung Homogenate (LH) and RAW264.7 Cells

To determine the CFUs of the lung tissues after infection, whole lungs were extracted and homogenized in 1 mL PBS before 100 μL of the homogenates were serially diluted 1:10 in PBS and plated on TSB agar to determine lung CFUs.

For CFUs of the RAW264.7 cells, cell culture medium was collected, and cells were lysed with 0.1% Triton X-100 to release live intracellular bacteria. The total samples were serially diluted 1:10 in PBS and plated on TSB plates to determine the number of CFUs.

## RNA Isolation and RT-PCR

RNA was extracted from lung homogenates and RAW264.7 cells with the Kangwei RNA easy kit (CWBIO, China). After isolation, RNA samples were treated with DNase I for 60 min at room temperature to remove contaminating DNA. Real-time PCR was performed with ABI7500 and a SYBR Primer Script TM RT Reagent Kit (TaKaRa, Japan) following the manufacturer's instructions. The sequences for the primers were listed in Table 1. β-actin RNA was used as an internal control to normalize the data. Quantification was conducted via the comparative cycle threshold method.

## Cytokine Assays

The production of proinflammatory cytokine in the Bronchial Alveolar Lavage Fluid (BALF) and RAW264.7 cells culture medium were detected by ELISA. BAL was performed as previously reported. Briefly, 800 microliters of PBS were instilled into the lung of the mice through the trachea and then carefully removed three times. BAL cells were collected and subjected to a blinded manual cell count. Supernatants of BAL were collected to measure the cytokine production. The concentrations of cytokines in the cell supernatants and BALF were measured using mice ELISA kits for IL-1β, IL-6 and TNF-α (eBioscience, USA).

## ALT and AST Leakage

The production of ALT and AST were determined with commercial kits purchased from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China) according to the manufacturer's instructions.

## Western Blotting

Cells were rinsed with cold PBS and total cellular lysates were prepared using the RIPA buffer (CST, Danvers, MA) supplemented with phenyl methyl sulfonyl fluoride (PMSF, USA) and protease inhibitor cocktail. The protein concentrations were measured with the BCA Protein Assay Kit (CWBIO). The protein samples (20 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk for 1 h at room temperature and probed with primary antibodies against phosphorylated or total proteins for p65, p38, JNK, ERK42/44 and HMGB1 (CST, Danvers, MA). After washing with TBST for three times, membranes were incubated with the HRP-conjugated secondary antibodies (Lianke, Hangzhou, China). ECL reagent were used to detect the immunoreactive bands on a digital image system (FluorChem E; Proteinsimple, Santa Clara, CA, USA).

## Luciferase Assay

RAW264.7 cells were seeded in 96-well plate at a density of $1x10^4$ per well 1 day prior to transfection. Cells were transient transfected with NF-κB luciferase reporter plasmid with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. After 24 h post transfection, cells were pretreated with 18β-GA or DMSO at a final concentration of 20 μM 2 h before infected with SA. After 24 h, cells were collected, and the luciferase activities were determined by using the Bright-Glo luciferase assay system (Promega Corp., Madison, WI).

## Statistical Analysis

All data were analyzed using GraphPad Prism 5 (GraphPad InStatSoftware, San Diego, CA, USA). Results were presented as mean ± SEM with at least three indepen-

**Table 1: Primer Sequences for Real-Time PCR.**

| Targets | Forward primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|
| β-actin | AGAGGGAAATCGTGCGTGAC | CAATAGTGATGACCTGGCCGT |
| IL-1β | CCTCCTTGCCTCTGATGG | AGTGCTGCCTAATGTCCC |
| IL-6 | TTCCAGAAACCGCTATGA | GGTTGTCACCAGCATCAG |
| TNF-α | AATAACGCTGATTTGGTGA | ACC CGTAGGGCGATTACA |
| HMGB1 | GCTGACAAGGCTCGTTATGAA | CCTTTGATTTTGGGGCGGTA |

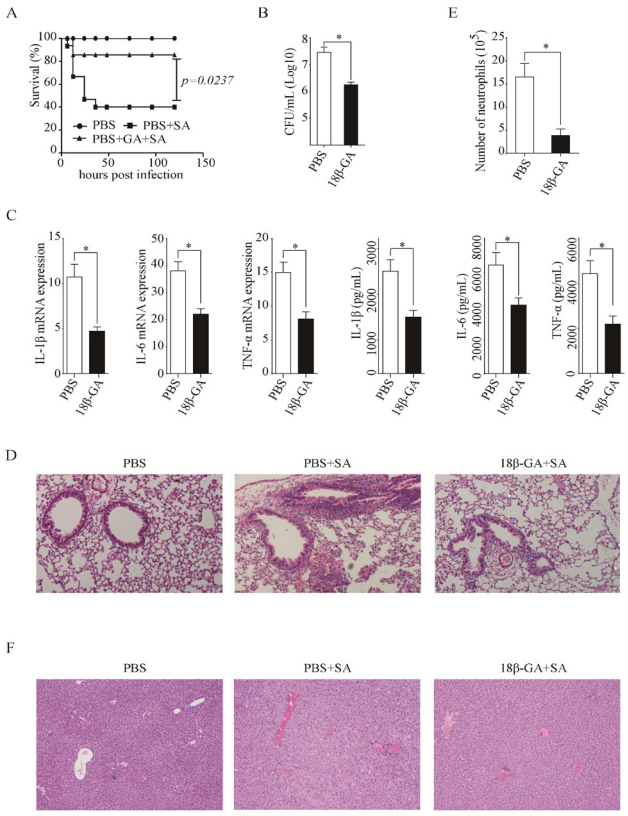


**Figure 1: 18β-GA protects mice against SA-induced ALI and the secondary liver injury. C57BL/6 mice were divided into three groups: PBS, PBS+SA, 18β-GA+SA. Mice were intraperitoneal injected with either 18β-GA (50 mg/kg) or PBS two h before infected with SA ($1\times10^8$ CFU). Mice were kept alive for observation of their viability every 6 h for 120 h (n=15 per group) or sacrificed 12 h post infection (B-F, n=4-8 per group). (A) survival rate was determined (B) CFU counts in LH samples (C) mRNA expressions and protein production of IL-1β, IL-6 and TNF-α in lung tissues and BAL fluid, respectively (D) HE staining of lung tissues (×100) (E) numbers of neutrophils in BAL fluid (F) HE staining of liver tissues in each group (×100). The results represent the mean ± SEM of three independent experiments. *$P$<0.05.**

dent experiments. The Student *t*-test was used to estimate the significant differences between groups. Overall survival rates were calculated using the Kaplan-Meier method. The log-rank test was utilized to compare the survival rates between groups. $P$ values<0.05 are considered to indicate a statistically significant difference.

## RESULTS

### 18β-GA Protects Mice against SA-induced ALI and the Secondary Liver Injury

To evaluate the effect of 18β-GA on SA infection, C57BL/6 mice were pre-treated with 18β-GA 2 h before infected with SA. First, we checked the survival rate of the mice after SA infection. As shown in Figure 1A, 18β-GA treatment significantly improved the survival rate compared with PBS-treated mice ($P$=0.0237). We speculated that this might be resulted from the decreased bacterial load and attenuated inflammation. As shown in Figure 1B, lung tissues from mice treated with 18β-GA showed lower bacterial counts at 12 h post SA infection ($P$=0.043). Furthermore, 18β-GA attenuated the SA-induced increase of TNF-α, IL-1β and IL-6 mRNA expression in lung and protein secretion in BALF (Figure 1C, $P$<0.05). With the challenge of SA, lung tissues were significantly damaged with various histopathologic changes including interstitial edema and hemorrhage, alveolar wall thickenin and notable infiltration of neutrophils in the lung parenchyma and alveolar spaces. While 18β-GA treatment relieved the lung damage (Figure 1D) accompanied with the lower neutrophils infiltration (Figure 1E, $P$=0.0074). We also investigated the liver histopathology. As shown in Figure 1F, SA infected mice showed the infiltration with cells at the peripheral regions of the blood vessels and 18β-GA reduced the cells infiltration.

### 18β-GA Exhibits Antimicrobial activity and Inhibits Proinflammatory Cytokine Production in SA Infected RAW264.7 Cells

To evaluate the effects of 18β-GA on SA infection *in vitro*, RAW264.7 macrophages were implied for SA infection. First, cells were pre-treated with different concentrations of 18β-GA before infected with SA for 6 h and then bacterial numbers were determined.18β-GA significantly inhibited bacterial growth compared with control medium in a dose-dependent manner (Figure 2A). Consistent with the *in vivo* results, pretreatment of 18β-GA significantly decreased the IL-1β, IL-6 and TNF-α expression and production (Figure 2B, $P$<0.05).

### 18β-GA Stabilizes HepG2 Liver Cells During SA Challenge

We also detected the leakage of ALT and AST in culture medium from HepG2 cells exposed to SA infection with or without 18β-GA treatment. As presented in Figure 2C, 18β-GA significantly inhibited the leakage of ALT and AST ($P$=0.028 and 0.018, respectively) compared with the PBS-treated cells after SA challenge.

### HMGB1 Expression was Down-Regulated after 18β-GA Treatment

HMGB1 has been reported to increase sepsis and is positively correlated with the severity of sepsis in animals.[22] Thus, we detected the HMGB1 expression both in lung tissues and in RAW264.7 cells. As shown in Figure 3A-3D, the mRNA and protein levels of HMGB1 in tissues and cells were remarkably increased after SA

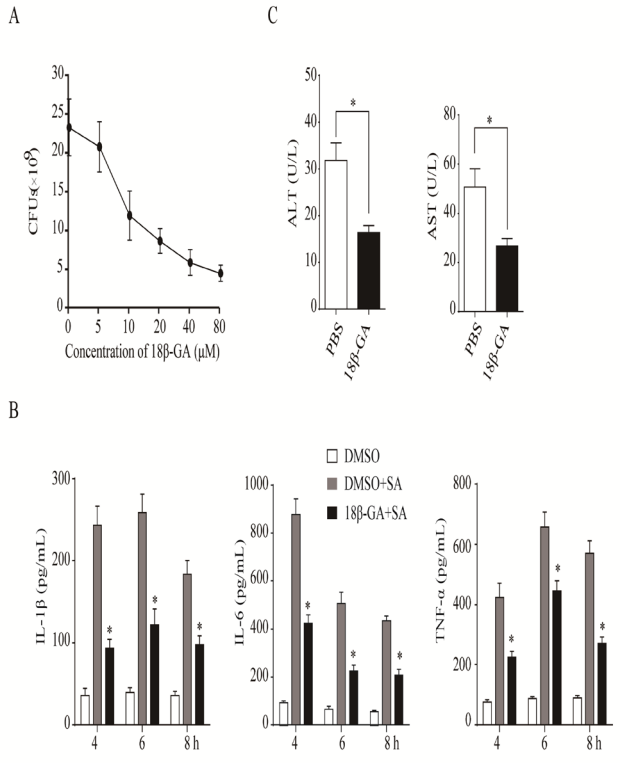


**Figure 2: 18β-GA exhibits antimicrobial activity and inhibits proinflammatory cytokine production in SA infected RAW264.7 cells. (A) RAW264.7 cells were pre-treated with the indicated concentrations of 18β-GA before infected with SA at a moi of 10. At 6 h post infection, bacterial loads were assessed by CFU assay. (B) RAW264.7 cells were pre-treated with 20 μM of 18β-GA 2 h before infected with SA. For the indicated h post infection, supernatants were collected for ELISA to determine the IL-1β, IL-6 and TNF-α production. (C) HepG2 cells were pre-treated with 20 μM of 18β-GA 2 h before infected with SA. 6 h later, the cell culture medium was collected for ELISA to determine the ALT and AST production. The results represent the mean ± SEM of three independent experiments. *$P$<0.05.**

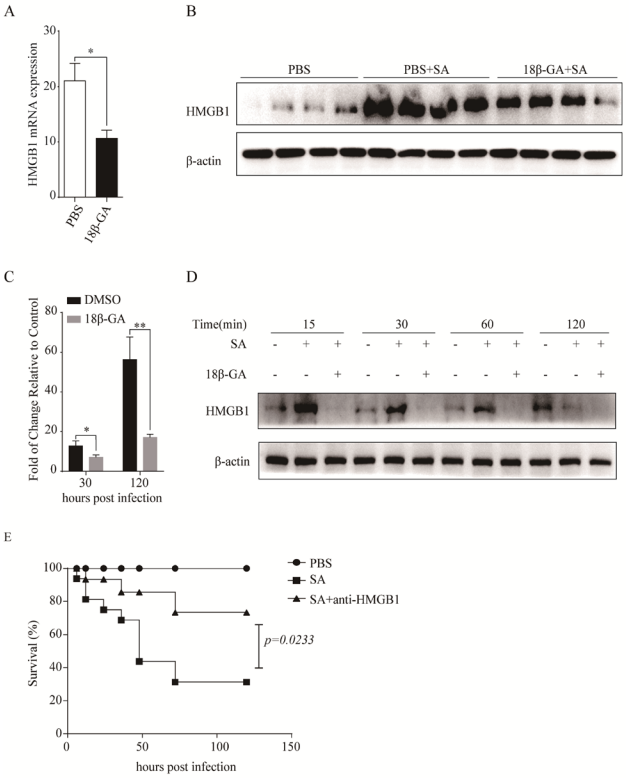


**Figure 3: 18β-GA reduces HMGB1 expression and anti-HMGB1 increases the mice survival rates. Mice were intraperitoneal injected with either 18β-GA (50 mg/kg) or PBS 2 h before infected with SA (1×108 CFU). Mice were sacrificed 12 h post infection, (A) mRNA expression and (B) protein expression of HMGB1 in lung tissues were determined by RT-qPCR and Western blotting, respectively. RAW264.7 cells were pre-treated with 20 μM of 18β-GA 2 h before infected with SA. For the indicated hours post infection, cells were collected to determine the (C) mRNA expression and (D) protein expression of HMGB1. (E) Mice were intraperitoneally injected with either anti-HMGB1 neutralizing antibody or non-immune rabbit IgG before SA infection and the survival rate was determined. The results represent the mean ± SEM of three independent experiments. **$P$<0.01, **$P$<0.01.**

infection and 18β-GA intervention significantly inhibited its expression. Furthermore, mouse HMGB1 antibody pre-treatment could improve the survival rate after SA infection in mice (Figure 3E, $P$=0.0233). These results suggested that the effects of 18β-GA treatment on the reduction of HMGB1 might play a critical role in improving the survival rate of SA-infected mice.

## 18β-GA Inhibited the activation of NF-kB in RAW264.7 Cells

It is generally acknowledged that NF-κB is responsible for the transcription of pro-inflammatory genes and plays critical roles in the pathogenesis of ALI. Many studies have shown that reducing or blocking the activation of NF-κB and MAPK signaling pathway could attenuate the lung injury and inflammation in ALI. Therefore, to clarify the potential mechanism about its anti-inflammatory activity, the effects of 18β-GA on the activation of NF-κB and MAPK signaling pathways were investigated. As shown in Figure 4A, SA infection significantly increased the phosphorylation of p65, p38, JN and ERK42/44 at 15, 30, 60 or 120 min, as compared with control group. However, 18β-GA treatment only markedly decreased the phosphorylation of p65 at 60 or 120 min. We also assessed the NF-κB activation using the luciferase reporter assay. After exposure to 18β-GA, NF-κB activity was significantly decreased (Figure 4B, $P$<0.0001). These results suggested that alleviated effects of 18β-GA on SA-induced inflammatory response were at least partially through regulation of NF-κB activation.

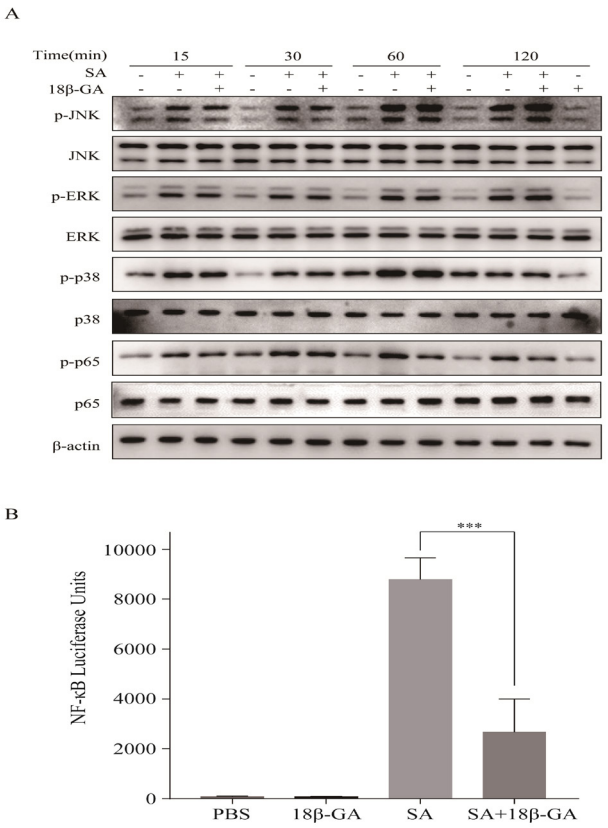


**Figure 4: 18β-GA inhibited the activation of NF-kB in RAW264.7 cells induced by SA. (A) RAW264.7 cells were pre-treated with 18β-GA for 2 h before infected with SA. At the indicated times post infection, proteins were extracted to determine the p65, p-p65, p38, p-p38, JNK, p-JNK, ERK42/44, p-ERK42/44 and β-actin expression by Western blotting. (B) RAW264.7 cells were transfected with NF-κB luciferase reporter plasmid before cells were pre-treated with 18β-GA or DMSO at a final concentration of 20 μM 2 h before infected with SA. After 24 h, cells were collected and the luciferase activities were determined. The results represent the mean ± SEM of three independent experiments. ***$P$<0.001.**

## DISCUSSION

The main findings of the present study were: (a) 18β-GA attenuated SA-induced acute lung injury and improved the survival rate of mice which might through reducing production of inflammatory mediators both *in vivo* and *in vitro*, (b) 18β-GA decreased HMGB1 expression and neutralizing of HMGB1 can improve the survival rate of mice induced by SA, (c) 18β-GA protected mice against secondary liver injury associated with SA-induced ALI and stabilized liver cells, (d) 18β-GA down regulated NF-κB inflammatory signaling pathway. These findings have potentially important implications for the treatment of SA infection or ALI.

*S. aureus* is a gram-positive bacterium which can cause a range of diseases including mild skin soft tissue injury, pneumonia, sepsis, endocarditi and deadly diseases such as toxic shock syndrome.[23] Of these, pneumonia is one of the most prevalent diseases induced by SA infection with high mortality rates up to 8%. SA infection induced AL and more severely, the Acute respiratory distress syndrome (ARDS), is a leading cause of morbidity and mortality in critically ill patients. Current treatments for ALI or ARDS are lacking, so it is important to explore the other possibilities.

18β-GA has been reported to have several pharmacological effects including anti-tumor, anti-inflammation, anti-radiation and so on. Cao *et al.* reported 18β-GA inhibits the initiation and progression of gastric tumors by ameliorating the inflammatory microenvironment through downregulation of COX-2 expression and inhibiting Wnt-1 expression.[24] 18β-GA significantly attenuates UV-induced skin photoaging mainly by virtue of its anti-oxidative and anti-inflammatory properties.[25] The anti-inflammatory effects of 18β-GA have been revealed in recent years. Studies have found 18β-GA exhibits anti-inflammatory activities through inhibition of MIP-1α production in acute propionibacterium acnes-induced inflammatory liver injury and inhibition of LPS/D–galactosamine-induced liver injury and prevention of free fatty acid-induced hepatic lipotoxicity.[12-14] 18β-GA also has been demonstrated to inhibit the LPS-induced inflammatory response in macrophages and increase the survival rate in LPS-induced ALI.[9-10] In accordance with these studies, we revealed that 18β-GA attenuated the inflammation in the SA induced ALI model.

Studies report that HMGB1 is highly expressed in many kinds of inflammatory disorders and contributes to the inflammation process. One study reported that HMGB1 expression is increased in the patients with conjunctivitis or blephariti and 18β-GA impairs antibody recognition of HMGB1 by direct binding to the protein thus prevents HMGB1-dependent $COX_2$ expression and cluster formation.[26] In our study, we also found that 18β-GA decreased the HMGB1 expression induced by SA infection, while the exact mechanisms was not detected further and which was the focus of our future study.

Clinical and experimental studies have shown that ALI is an early and fatal complication of septic shock[27] and the TLR2/4-mediated NF-κB activation could be an early molecular event leading to ALI during septic shock.[28-29] NF-κB is a critical transcription factor in TLR-mediated signaling pathways[30] and plays a critical role in regulation of expressions of a number of genes, including inflammatory cytokines such as HMGB1,TNF-α and IL-1β.[31-32] 18β-GA has been found to attenuate NF-κB activation to alleviate the inflammation.[26] In our study,

we found the same phenomenon that 18β-GA inhibited the activation of NF-κB.

## CONCLUSION

Taken together, we demonstrated that 18β-GA protects mice against lung and liver injury after SA infection. The possible mechanisms are related to the decreased pro-inflammatory factors and HMGB1 expressions which might through the inhibition of NF-κB-regulated inflammation signaling pathways. Thus, 18β-GA may have the potential to serve as a useful therapeutic agent for treating ALI caused by bacterial infection.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**18β-GA:** 18β-glycyrrhetinic acid**; SA:** *Staphylococcus aureus*; **ALI:** Acute lung injury**; HMGB1:** High-mobility group box 1.

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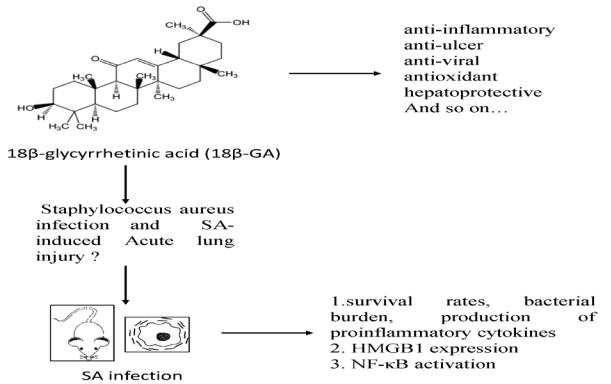
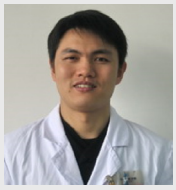
## PICTORIAL ABSTRACT




## SUMMARY

- 18β-glycyrrhetinic acid (18β-GA) is reported to possess various pharmacological properties. In this study, we explored the effects of 18β-GA on the SA infection especially the SA-induced acute lung injury and its related mechanisms. Mice or cells were infected with SA both *in vitro* and *in vivo* and the survival rates of mice, bacterial burden and production of proinflammatory cytokines were detected. The expression of high-mobility group box 1 (HMGB1) and NF-κB activation was further determined. We found that 18β-GA provided protective effects against SA infection *via* its anti-inflammatory properties possibly through down-regulating the HMGB1/NF-κB activation, indicating that 18β-GA may have the potential to serve as a useful therapeutic agent for treating ALI caused by bacterial infection.

## ABOUT AUTHORS



**Mr. Chunhui Rao,** is a surgeon from Department of Colorectal Surgery, Hangzhou Hospital of Traditional Chinese Medicine, Hangzhou, China. He completed his Bachelor's degree in Gannan Medical University in 2004, currently he is pursuing his master degree in Zhejiang University School of Medicine.

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