

# Mangiferin Inhibits Apoptosis and Autophagy Induced by *Staphylococcus aureus* in RAW264.7 Cells

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Jun Xu\*  
Hua Yao\*  
Shichen Wang\*  
Huanrong Li  
Xiaolin Hou 

Beijing Key Laboratory of Traditional Chinese Veterinary Medicine, National Demonstration Center for Experimental Animal Education, Department of Veterinary Medicine, Beijing University of Agriculture, Beijing, People's Republic of China

\*These authors contributed equally to this work

**Purpose:** *Staphylococcus aureus* (*S. aureus*) is an important bacterial pathogen, which creates infective inflammation to human being and animals. Mangiferin (MG) is one of the natural flavonoids with anti-inflammatory, anti-bacterial, and anti-oxidative properties. However, the anti-apoptosis and anti-autophagy of MG are unknown. Hence, this study was aimed to research the inhibition of MG on *S. aureus*-induced apoptosis and autophagy in RAW264.7 cells.

**Methods:** The RAW264.7 cells were pretreated with MG, or pretreated with SP600125 or anisomycin synchronously, and then infected with *S. aureus* (MOI=100:1). The viability and proliferation status of RAW264.7 cells were detected by MTT and EdU assay. The relative expression of TNF- $\alpha$ , IL-6 and IL-10 protein was tested with ELISA. The levels of Bax, Bcl-2, caspase-3, c-Jun N-terminal kinase (JNK), extracellular-regulated protein kinase (ERK), p38, LC3, Beclin-1, p62, phosphorylated JNK, phosphorylated p38 and phosphorylated ERK in cells were detected by Western blotting. The apoptosis rate of RAW264.7 cells was analyzed by flow cytometric assay.

**Results:** The study showed that MG significantly attenuated RAW264.7 cells apoptosis and autophagy caused by *S. aureus*. MG alleviated *S. aureus*-induced apoptosis by down-regulating the protein level of active caspase-3 and Bax and up-regulating the level of Bcl-2. MG also inhibited *S. aureus*-induced autophagy via decreasing the protein level of LC3-II/LC3-I and Beclin-1 or increasing the protein expression of p62. This protective role was dependent on the up-regulation of JNK signal pathway, which was confirmed by using JNK agonist and inhibitor.

**Conclusion:** Our results demonstrated that MG might protect RAW264.7 cells from *S. aureus*-induced apoptosis and autophagy via inhibiting JNK/Bax-dependent signal pathway. Therefore, MG may be a potential agent against pathological cell damage induced by *S. aureus* infection.

**Keywords:** mangiferin, *Staphylococcus aureus*, apoptosis, autophagy

## Introduction

*Staphylococcus aureus* (*S. aureus*) is a common gram-positive bacterial pathogen all over the world, which induces a series of infections ranging from skin and folliculated infection to pneumonia, meningitis, osteomyelitis, endocarditis, bacteremia, and septicemia.<sup>1</sup> *S. aureus* has evolved multiple virulence factors such as alpha-hemolysin, panton-valentine leucopenia (PVL), phenol-soluble module (PSM),<sup>2,3</sup> soluble regulatory protein and cysteine protease that are making it one

Correspondence: Xiaolin Hou  
Tel +86 13521095760  
Email hxlx@163.com

of the most infectious and pathogenic bacteria, which seriously threatens the life safety of human and animal. Since antibiotics were widely used to prevent and treat *S. aureus* infectious diseases, it promotes the emergence of antibiotic-resistant bacteria, especially multidrug-resistant (MDR) bacteria and methicillin-resistant *S. aureus* (MASA).<sup>4,5</sup> At present, research had been focused on preventing and treating *S. aureus*.<sup>6</sup>

Toll-like receptors (TLR) are one of the protein molecules involved in non-specific immunity (natural immunity); they are also an important link between non-specific immunity and specific immunity.<sup>7</sup> Previous research demonstrated that TLR2 is closely related to bacterial infection. TLR2 recognises peptidoglycan and phosphatidyl acid on the cell wall of *S. aureus*, thus causing cellular immune response.<sup>8</sup> The level of TLR2 and TLR4 protein was enhanced after macrophages infected with *S. aureus*, thus activating downstream NF- $\kappa$ B and MAPK signalling pathway and promoting the release of cytokines by macrophages.<sup>9–13</sup> *S. aureus* activates TLR2 receptor, and MAPK signalling pathway mediates phagocytosis and autophagy in RAW264.7 macrophage cells by up-regulation of Beclin-1, LC3-II/LC3-I.<sup>14,15</sup> Controlling the pathological changes and protecting the tissue from damage is a usual strategy. ID13 remarkably alleviated pathological status, inhibited the production of pro-inflammatory cytokines,<sup>16</sup> and suppressed the TLR2-NF- $\kappa$ B signal pathway.<sup>17</sup> Daphnetin conferred protection against *S. aureus*-induced pneumonia by anti-inflammation and enhanced mTOR-dependent autophagy.<sup>2</sup> CpG-ODN promotes phagocytosis and autophagy through JNK/P38 signal pathway in *S. aureus*-stimulated macrophage.<sup>2</sup>

Mangiferin (MG) is a flavonoid that originally extracted from the rhizomes of traditional plant Anemarrhena.<sup>18</sup> MG is effective in treating bronchitis<sup>19,20</sup>, and pulmonary through inhibiting TLR4/p65, TGF-beta1/Smad2/3 pathway,<sup>12,19,21</sup> and COX-2.<sup>20</sup> MG ameliorate fat liver through modulation of autophagy and inflammation,<sup>22</sup> also MG boosts autophagy via inhibiting mTORC1 pathway to prevent high glucose-induced cardiomyocyte injury,<sup>23</sup> and protect myocardial insults through inhibition of MAPK/TGF-beta pathways.<sup>24</sup> MG prevents diabetic nephropathy by up-regulation of glyoxalase and autophagy.<sup>24</sup> MG inhibits apoptosis and oxidative stress via BMP2/Smad-1 signalling in dexamethasone-induced MC3T3-E1 cells.<sup>26</sup> Although a large number of research have revealed that MG has a protective role in the inflammatory response, little was known about the role of MG on

*S. aureus*-induced apoptosis and autophagy in RAW264.7 cells model.

In this study, MG treatment protected RAW264.7 cells from *S. aureus*-induced apoptosis and autophagy. These effects mainly involve the inhibition of JNK/Bax-dependent pathways. It may be a potential therapy to control tissue damage caused by *S. aureus*.

## Materials and Methods

### Reagent and Antibodies

MG was purchased from Aladdin Bio-Chem Technology (Shanghai, China). JNK inhibitor SP600125 and JNK activator anisomycin were purchased from Selleck Chemicals (Houston, Texas, USA). The following primary antibodies: rabbit anti-mouse  $\beta$ -actin, caspase-3, Bax, Bcl-2, LC3, p62, Beclin-1, p38, P-p38, ERK, P-ERK, JNK, P-JNK were bought from Cell Signaling Technology (Boston, Mass, USA). The second antibody was Alexa Fluor 488 labelled anti-rabbit from Life Technologies Corporation (Waltham, Mass, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Cells Culture

Mouse mononuclear macrophage leukaemia cells (RAW264.7 cells) (Cat No. TIB-71™) were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, USA) supplemented with 10% FBS (Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin in a constant temperature incubator at 37°C and 5% CO<sub>2</sub>.

### Bacterial Strain

- (A) *S. aureus* (ATCC29213) was cultured in 10 mL of Luria-Bertani (LB; Hopebio, Qinghai, China) at 37°C and harvested at the log phase.<sup>27,28</sup> Then, the *S. aureus* was diluted to achieve a multiplicity of infection (MOI = 100:1). The number of *S. aureus* was determined by serial dilution with the plate counting method.<sup>28</sup>

### Cells Treatment

The study was composed of three parts. The first part involved the blank control group (BC) and *S. aureus* infection (MOI = 100:1) group (SA); on this basis, the second part was set up as three parallel groups, namely BC, SA

(MOI = 100:1 *S. aureus* infection for 4 h) and MG (low, medium and high concentration pretreatment for 1 h) + SA; similarly, the third part was the BC, SA, MG (high concentration pretreatment for 1 h) + SA, SP (JNK inhibitor SP600125 pretreatment for 12 h), SP + SA, SP + SA + MG; finally, the fourth part was the BC, SA, MG (high concentration pretreatment for 1 h) + SA for 4 h, AM (JNK activator Anisomycin pretreatment for 30 min), AM + SA, and AM + SA + MG.

## Cells Viability Assay

The effect of *S. aureus* and MG on RAW264.7 cells viability was assayed using MTT method (Sigma-Aldrich, America). Cells were inoculated into a 96-well plate at  $2 \times 10^4$  CFU/well, after cells growing to monolayer, washing three times with phosphate buffer saline (PBS; Hyclone, China). Then, 200  $\mu$ L of *S. aureus* (MOI = 100:1) was added into each well for different times (0, 0.5, 1, 2, 4, and 6 h) (three wells/each group), or 200  $\mu$ L of MG of different concentrations to each well (three wells each concentration). After washing three times with PBS, the cells were incubated with color-free DMEM (80  $\mu$ L/well) and 5 mg/mL MTT (20  $\mu$ L/well) solution at 37°C for 4 h. After washing three times with PBS, 0.05% DMSO (100  $\mu$ L/well) was added and the tray was shaken gently for 10 min. The cells were observed under a light microscope. The optical density (OD) value of the wells at a wavelength of 490 nm was measured using an enzyme-linked immunosorbent assay reader (Thermo Fisher Scientific, US).<sup>29</sup> The percentage of viable cells was calculated according to the equation: the percentage of viable cells = (OD-value of test team/OD-value of a blank team)  $\times$  100%.<sup>30</sup>

## Measurement of Cells Proliferation by Ethynyl Deoxyuridine (EdU)

RAW264.7 cells were cultured in 6-well plate with  $2 \times 10^4$  CFU/wells under 37°C and 5% CO<sub>2</sub> for 12 h,<sup>31</sup> pretreated with MG (25, 50, 100  $\mu$ M) for 1 h, following by infected with *S. aureus* (MOI=100:1) for 4 h. The supernatant was discarded. Cell Proliferation was assayed using BeyoClick™ EdU-488 Cell Proliferation Assay Kit (Beyotime, Beijing, China) following the user's manual. These results were visualised by a fluorescence microscope (Olympus Corporation, Japan) at a magnification of 400 $\times$ , and the signals were counted in five random visional fields.

## Caspase-3 Activity Assay

The activity of caspase-3 in RAW264.7 cells was detected using Caspase-3 Activity Assay Kit (Beyotime Institute of Biotechnology, China) following the manufacturers' instructions. In short, 100  $\mu$ L lysate was added into the RAW264.7 cells for 15 min on dry ice and centrifuged (20,000 g, 4°C) 15 min, then 50  $\mu$ L supernatant, 40  $\mu$ L detection buffer, and 10  $\mu$ L caspase-3 substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (2 mM)<sup>16</sup> were added into 96-well plates for 60 min. The absorbance of p-nitroanilide was determined at 405 nm using a microtiter plate reader (Bio-Rad, USA). Caspase-3 activity was calculated as a ratio of p-nitroanilide content to total protein amount.<sup>32</sup>

## Detection of IL-6, IL-10 and TNF- $\alpha$ by ELISA

The protein levels of IL-6, IL-10, and TNF- $\alpha$  in a medium of RAW264.7 cells were assayed with ELISA kits (RayBiotech, USA). In brief, RAW264.7 cells were cultured in 6-well plates with  $2 \times 10^4$  CFU/wells under 37°C and 5% CO<sub>2</sub> for 12 h. MG (25, 50, 100  $\mu$ M) treatment 1 h before *S. aureus* infection (MOI=100:1) for 4 h. Then, the cell supernatant was taken for assay following the manufacturers' instructions.

## Flow Cytometry Assay

Flow cytometry assay was used to detected RAW264.7 cells apoptosis and cells cycle progression. We used the Annexin V-FITC/propidium iodide (AV/PI) dual staining commercial kits (Biosea Biotechnology, China), and tested the apoptosis rate of RAW264.7 cells following the manufacturers' instructions. Briefly, RAW264.7 cells were cultured in 6-well plates with  $2 \times 10^4$  CFU/wells for 12 h. After pretreatment with MG and *S. aureus*, the cells were digested with trypsin, collected by centrifugation, and washed with PBS, the cells were stained with Annexin V-FITC and PI, analysis by FCM (Becton Dickson).

## Western Blotting

The whole protein was extracted using an extraction kit (Nanjing Key Gen Biotech, Nanjing, China). The soluble protein supernatant was quantified using Protein Assay Kit (Aid lab Biotechnologies, Beijing, China). Protein samples (20  $\mu$ g/lane) were separated on polyacrylamide gel electrophoresis (Applygen Technologies, Beijing, China).<sup>33</sup> Then, proteins were transferred onto nitrocellulose filter membranes (NC; Pierce Biotechnology, Inc. USA). NC membranes were

incubated with primary antibodies overnight at 4°C and a secondary antibody at room temperature for 40 min. The images of the membranes were displayed by Odyssey dual colour infrared fluorescence imaging system (LICOR, US), and the Image J software was used to analyze these images.

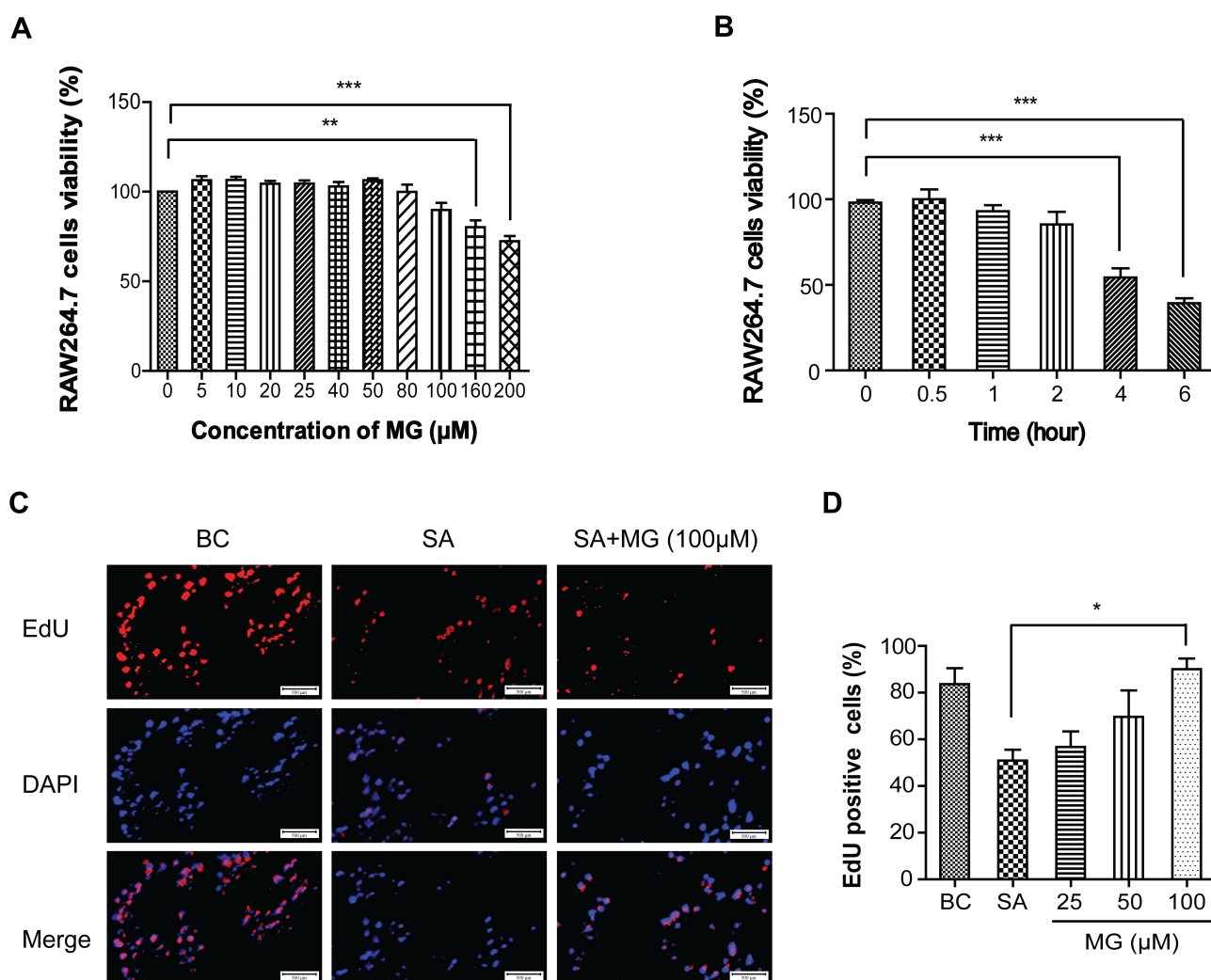
### Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM) from three independent experiments performed in triplicate. Student's *t*-test was used to analyse the comparison between two groups.<sup>34</sup> One-way ANOVA followed by Dunnett's test in GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA).

## Results

### MG Attenuated Cytotoxicity in RAW264.7 Cells Induced by *S. aureus* Infection

The viability of RAW264.7 cells was detected using MTT assay. Cells were pretreated with doses (0, 5, 10, 20, 25, 40, 50, 80, 100, 160 and 200 μM) of MG for 24 hours. As shown in Figure 1A, when the concentration was as high as 100 μM, the viability of RAW264.7 cells were 85.6 ± 0.07% (n=3) no cytotoxicity. However, 200 μM MG induced about 30% cells growth in dose-dependent (Figure 1A). It indicated MG maximum non-cytotoxicity concentration is 100 μM.



**Figure 1** MG attenuated *S. aureus*-induced cytotoxicity in RAW264.7 cells. (A) Cell viability of RAW264.7 cells treated with MG (0, 5, 10, 20, 25, 40, 50, 80, 100, 160 and 200 μM) for 24 hours was determined using MTT assay. Its maximum non-cytotoxicity was 100 μM. \*\**P*<0.01, \*\*\**P*<0.001 compared with 0 μM group. (B) *S. aureus* infection time was determined using MTT assay. The results showed that *S. aureus* induced about 53.4% RAW264.7 cells growth inhibition at 4 hours. \*\*\**P*<0.001 compared with 0 h group. (C) RAW264.7 cells were treated with MG (25, 50, 100 μM) for 1h and infected with *S. aureus* (MOI:100) for 4 h. *S. aureus* infection inhibited RAW264.7 cells proliferation, which was significantly reversed by 100 μM MG pretreatment. Relative fluorescence expression levels were quantified by EdU and DAPI staining. Scale bars: 500 μm. (D) The graph represents a quantitative analysis of the number of EdU positive cells. \**P*<0.05 compared with the SA group.

As showed in Figure 1B, *S. aureus* infection time-dependently inhibited RAW264.7 cells viability and induced to 53.4% cells death at 4 h. Therefore, the infection time of *S. aureus* (MOI=100:1) was set at 4 hours for subsequent research.

In addition, EdU fluorescence staining was used to detect whether RAW264.7 cells were in the proliferation cycle. *S. aureus* reduced positive RAW264.7 cells from  $83.61 \pm 9.66\%$  to  $50.90 \pm 6.53\%$  ( $n=3$ ), 100  $\mu\text{M}$  MG pretreatment increased positive RAW264.7 cells from  $50.90\% \pm 6.53\%$  to  $90\% \pm 6.53\%$  ( $n=3$ ) (Figure 1C and D). These results indicated that MG within 100  $\mu\text{M}$  is non-cytotoxicity to RAW264.7 cells, and 100  $\mu\text{M}$  MG attenuated the cytotoxicity caused by *S. aureus*.

## MG Suppressed Inflammation and Apoptosis in *S. aureus*-Induced RAW264.7 Cells

Proinflammatory cytokines in medium culture were assayed using ELISA kits. Compared with the BC group, the intracellular protein expression of IL-6, IL-10, and TNF- $\alpha$  in RAW264.7 cells of the SA group significantly increased (Figure 2A) ( $n=3$ ). IL-6 increased from  $31 \pm 2.94$  pg/mL to  $192 \pm 6.68$  pg/mL; IL-10 increased from  $18.37 \pm 1.21$  pg/mL to  $133 \pm 2.16$  pg/mL; TNF- $\alpha$  increased from  $52.5 \pm 2.5$  pg/mL to  $165 \pm 15$  pg/mL. On the contrary, compared with the SA group, the level of IL-6, IL-10, and TNF- $\alpha$  protein significantly decreased in the MG group ( $n=3$ ). IL-6 decreased to  $51.7 \pm 3.09$  pg/mL; IL-10 decreased to  $44.3 \pm 1.70$  pg/mL; TNF- $\alpha$  decreased  $65 \pm 5$  pg/mL. The result showed that MG pretreatment significantly ameliorated the inflammation reaction in a dose-dependent manner induced by *S. aureus*.

To characterise the RAW264.7 cells death process, pro-apoptotic proteins (Bax and caspase-3) and anti-apoptotic protein (Bcl-2) were assayed by Western blot. As shown in Figure 2C and D, compared with the BC group ( $n=3$ ), the level of Bax and cleaved caspase-3 protein in the SA group was increased from  $0.22 \pm 0.01$  to  $0.31 \pm 0.01$ , and from  $0.39 \pm 0.06$  to  $0.54 \pm 0.04$ , respectively. While the level of Bcl-2 protein was decreased from  $0.86 \pm 0.003$  to  $0.87 \pm 0.003$ . Compared with the SA group, the relative protein expression level of Bax and caspase-3 in the 100  $\mu\text{M}$  MG groups was significantly decreased from  $0.31 \pm 0.01$  to  $0.16 \pm 0.01$  and from  $0.54 \pm 0.04$  to  $0.22 \pm 0.03$ , respectively. Additionally, the caspase-3 activity in medium culture showed a similar trend with intracellular caspase-3 (Figure 2B). In short, MG pretreatment

significantly prevented the changes induced by *S. aureus* in a dose-dependent manner.

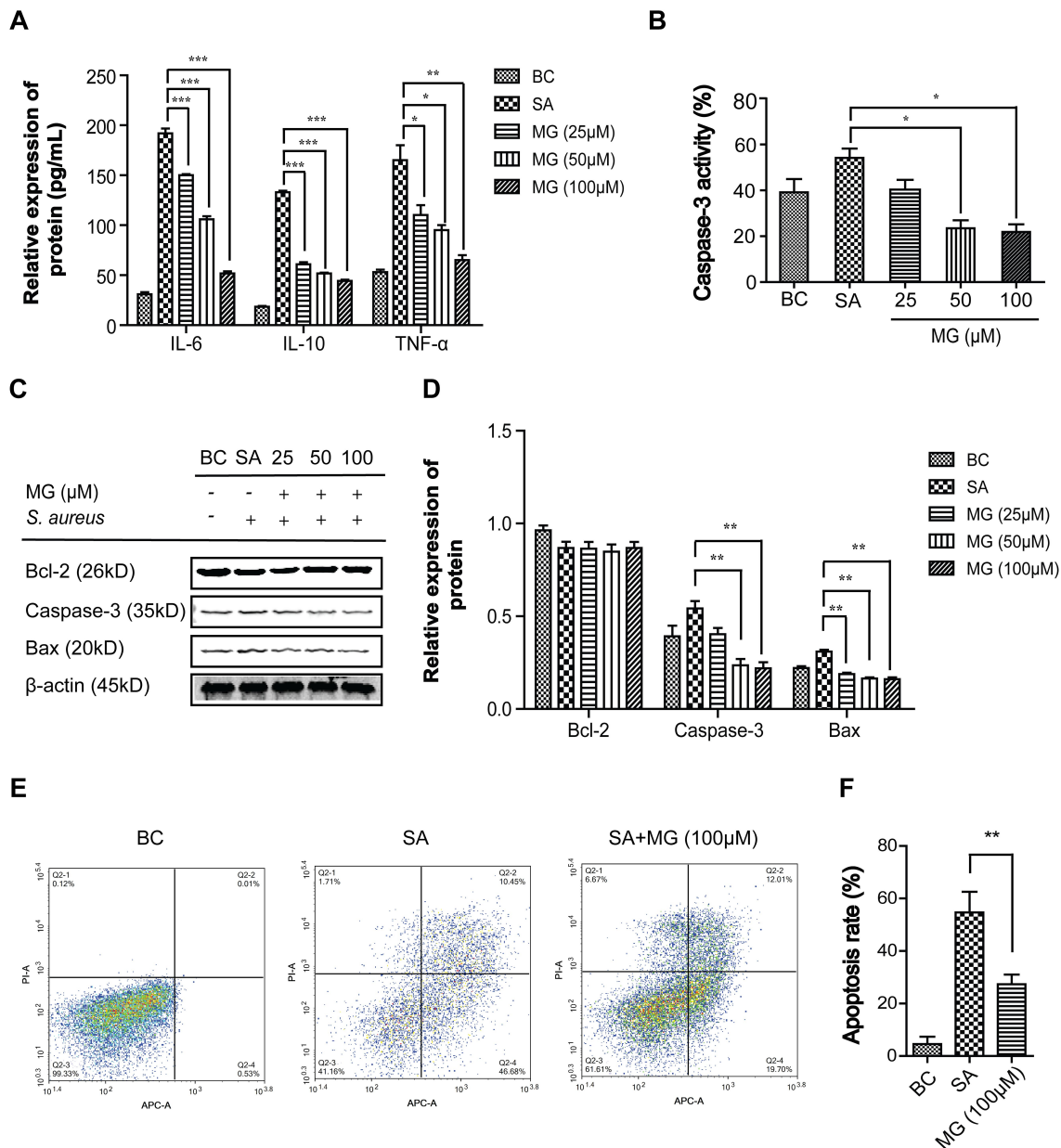
The apoptotic rates of RAW264.7 cells induced by *S. aureus* were measured using flow cytometer. Cells assay (annexin V/PI) including early (annexin V positive and PI-negative) and late apoptotic cells (annexin V positive and PI-positive) were induced by *S. aureus* infection. MG significantly ameliorated the apoptotic death rate from  $57.4 \pm 8.6\%$  to  $27.3 \pm 5.7\%$  ( $n=3$ ) induced by *S. aureus* infection in RAW264.7 cells (Figure 2E and F). All these results illustrated that MG attenuated *S. aureus*-induced inflammatory apoptosis as evidence by decreased expressions of IL-6, IL-10, TNF- $\alpha$ , Bax and caspase-3 and increased expression of Bcl-2 in RAW264.7 cells.

## MG Repressed Autophagy Induced by *S. aureus* in RAW264.7 Cells

The protein levels of p62, Beclin-1 and LC3 associated with autophagy were detected using Western blot (Figure 3). Compared with BC group ( $n=3$ ), the relative expression of Beclin-1 and LC3-II/LC3-I in the SA group showed a significant up-regulation, increased from  $0.29 \pm 0.02$  to  $0.50 \pm 0.02$ , and from  $1.15 \pm 0.05$  to  $2.20 \pm 0.10$ . However, the relative expression of p62 protein in the SA group significantly down-regulated from  $1.50 \pm 0.13$  to  $1.08 \pm 0.06$ . Compared with the SA group, the expression of Beclin-1 and LC3-II/LC3-I in MG group was significantly decreased from  $0.50 \pm 0.02$  to  $0.25 \pm 0.003$  and from  $2.20 \pm 0.10$  to  $0.97 \pm 0.03$ . Besides, the expression of P62 protein significantly increased from  $1.08 \pm 0.06$  to  $1.49 \pm 0.26$ . These results showed that MG attenuated *S. aureus*-induced autophagy in RAW264.7 cells.

## MG Protected RAW264.7 Cells from Apoptosis and Autophagy Induced by *S. aureus* Infection via JNK/Bax Signal Pathway

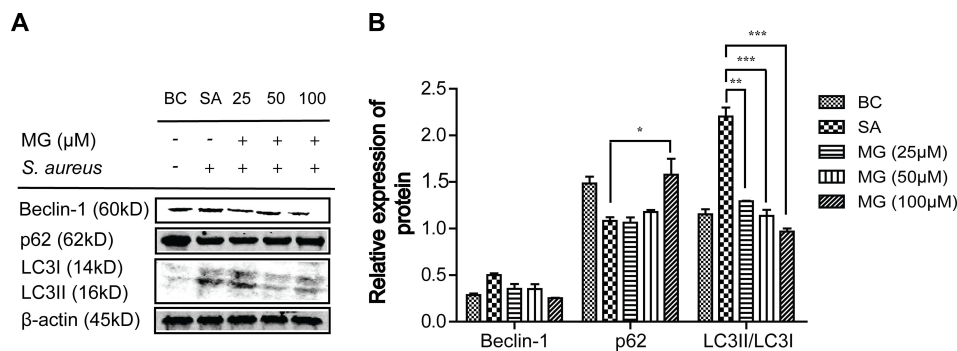
The mitogen-activated protein kinases (MAPK) signalling pathway was shared by four distinct cascades, including the extracellular signal-related kinases (ERK1/2), Jun amino-terminal kinases (JNK1/2/3), p38-MAPK and ERK.<sup>35,36</sup> Activated MAPK transmits extracellular signals to regulate cell growth, proliferation, differentiation, migration, even control the balance of autophagy and apoptosis in response to all kinds of stress.<sup>35,36</sup> To understand whether the protection mediated by MG was dependent on the MAPK signalling pathway, the role of these proteins were determined via



**Figure 2** MG attenuated inhibited apoptosis in RAW264.7 cells infected by *S. aureus*. RAW264.7 cells were infected with *S. aureus* (MOI:100) for 4 hours after pretreated with MG (25, 50, 100 μM) for 1h. **(A)** Detection of pro-inflammatory factor IL-6, IL-10, and TNF-α in medium culture were assayed using ELISA kit. IL-6, IL-10, and TNF-α in the SA group were significantly increased but were significantly decreased in a dose-dependent manner at MG group. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared with SA group. **(B)** Caspase-3 activity was determined using Caspase-3 Activity Assay Kit. Caspase-3 in the SA group were significantly increased but was significantly decreased in a dose-dependent manner at MG group. \*P<0.05 compared with SA group. **(C)** Expressions of Bax, Bcl-2 and active caspase-3 were analysed by Western blotting in RAW264.7 cells. **(D)** Bax, Bcl-2 and active caspase-3 relative expression were quantified by normalising to β-actin. \*\*P<0.01 compared with SA group. **(E)** Apoptosis was determined by a FITC-labeled Annexin V/PI staining and flow cytometry. Representative results from flow cytometry were shown. **(F)** The apoptosis cell rates was calculated. Apoptosis rate in the SA group were significantly increased, but was significantly decreased in the MG group. \*\*P<0.01 compared with SA group.

used Western blot. As **Figure 4** showed that *S. aureus* infection promoted the phosphorylation of JNK, ERK and p38 at different levels, while MG pretreatment only significantly suppressed JNK activation. In a word, *S. aureus* infection suppressed the cells proliferation of by activating MAPK, while MG pretreatment was down-regulated the level of proapoptotic and proinflammatory JNK protein.

MG pretreatment inhibited JNK expression stimulated by *S. aureus*. To study whether the molecular mechanism of JNK phosphorylation and MG inhibition of JNK signalling pathway were related to the dissociation of Beclin1-Bcl-2 and Bax-Bcl-2 complexes, which involved in apoptosis and autophagy. RAW264.7 cells were pretreated with MG for 1 h, pretreated with JNK inhibitor SP600125 for 12 h, or pretreated with



**Figure 3** MG inhibited apoptosis in RAW264.7 cells induced by *S. aureus*. RAW264.7 cells were pretreated with MG (25, 50, 100 μM) for 1h, and infected with *S. aureus* (MOI:100) for 4 h. **(A)** Expressions of Beclin-1, P62 and LC3 protein were analysed by Western blotting in RAW264.7 cells. **(B)** The graph represents a quantitative analysis of the band intensity. \*P<0.05, \*\*P<0.01, \*\*\*p <0.001 compared with SA group.

a JNK agonist anisomycin for 30 min, respectively. The expression level of JNK, P-JNK, Bax, Beclin-1, and LC3-II/LC3-I were measured with Western blot (Figures 5 and 6).

Pretreatment with JNK inhibitor SP600125, the phosphorylation of JNK and Bax were inhibited in *S. aureus* infected cells (Figure 5A and B), which showed similar trends pretreated with MG (Figure 5A and B). In addition, the JNK activator anisomycin further promoted Bax induced by *S. aureus*, indicating that anisomycin aggravated apoptosis induced by *S. aureus*, but MG attenuated the effect (Figure 6A and B).

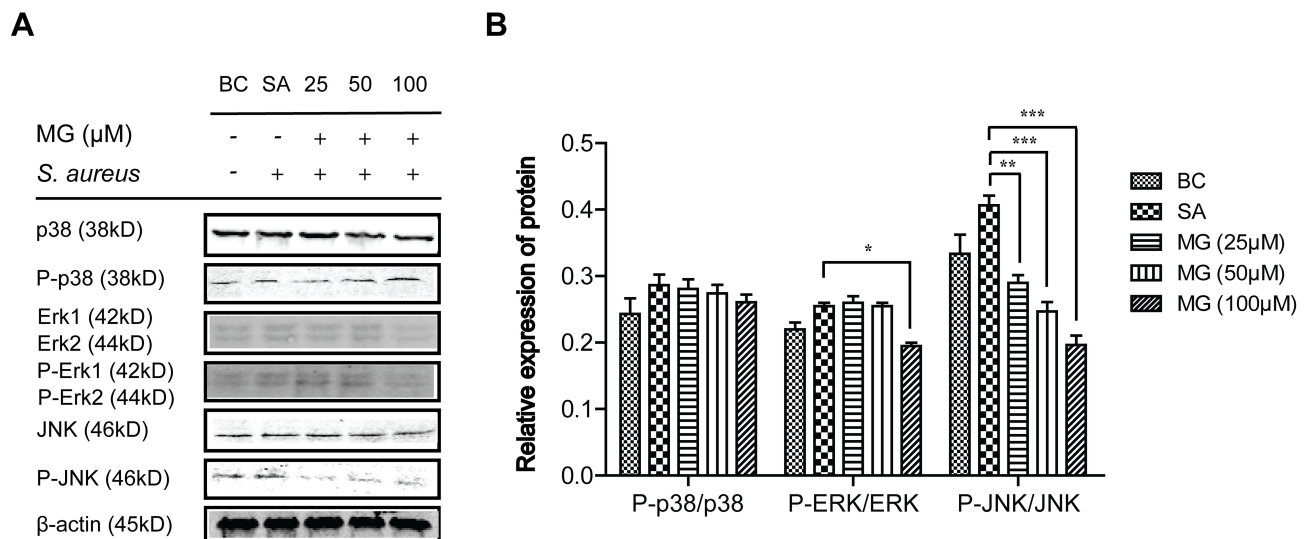
On the other hand, the JNK inhibitor SP600125 decreased the expression of Beclin-1 and inhibited transformation of LC3-I to LC3-II significantly, indicating that SP600125 inhibited autophagy. The effect was similar to

MG (Figure 5A and C). Moreover, the JNK activator anisomycin further promoted the expression of Beclin-1 and the activate of LC3 induced by *S. aureus*. MG pretreatment inhibited Beclin-1 and LC3, indicating that MG and anisomycin exerted an opposite effect on autophagic cell process induced by *S. aureus* infection (Figure 6A and C).

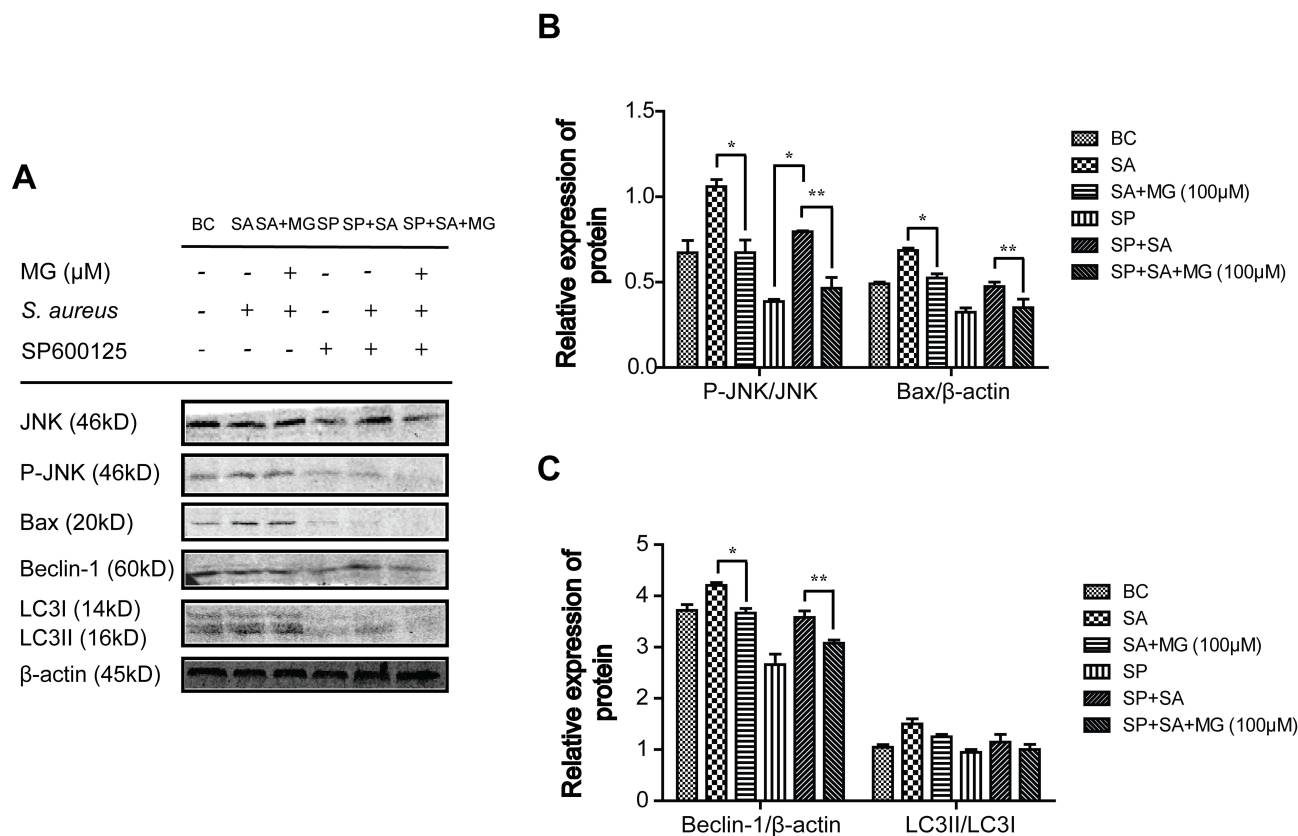
These results indicated that activation of JNK accelerated *S. aureus*-induced autophagy and apoptosis in RAW264.7 cells, and MG inhibited apoptosis and autophagy through inhibiting JNK/Bax signal pathway.

## Discussion

*S. aureus* causes severe systemic infection with high mortality rates for sepsis.<sup>38</sup> Studies have shown that *S. aureus*



**Figure 4** MG inhibited ERK signalling pathway-related proteins expression caused by *S. aureus* infection in RAW264.7 cells. RAW264.7 cells were treated with MG (25, 50, 100 μM) for 1h and infected with *S. aureus* (MOI:100) for 4 h. **(A)** Expressions of p38, P-p38, Erk, P-Erk, JNK and P-JNK protein were analysed by Western blotting in RAW264.7 cells. Gels were representative of three independent experiments. **(B)** The graph represents a quantitative analysis of the band intensity. \*P<0.05, \*\*P<0.01, \*\*\*p <0.001 compared with SA group.



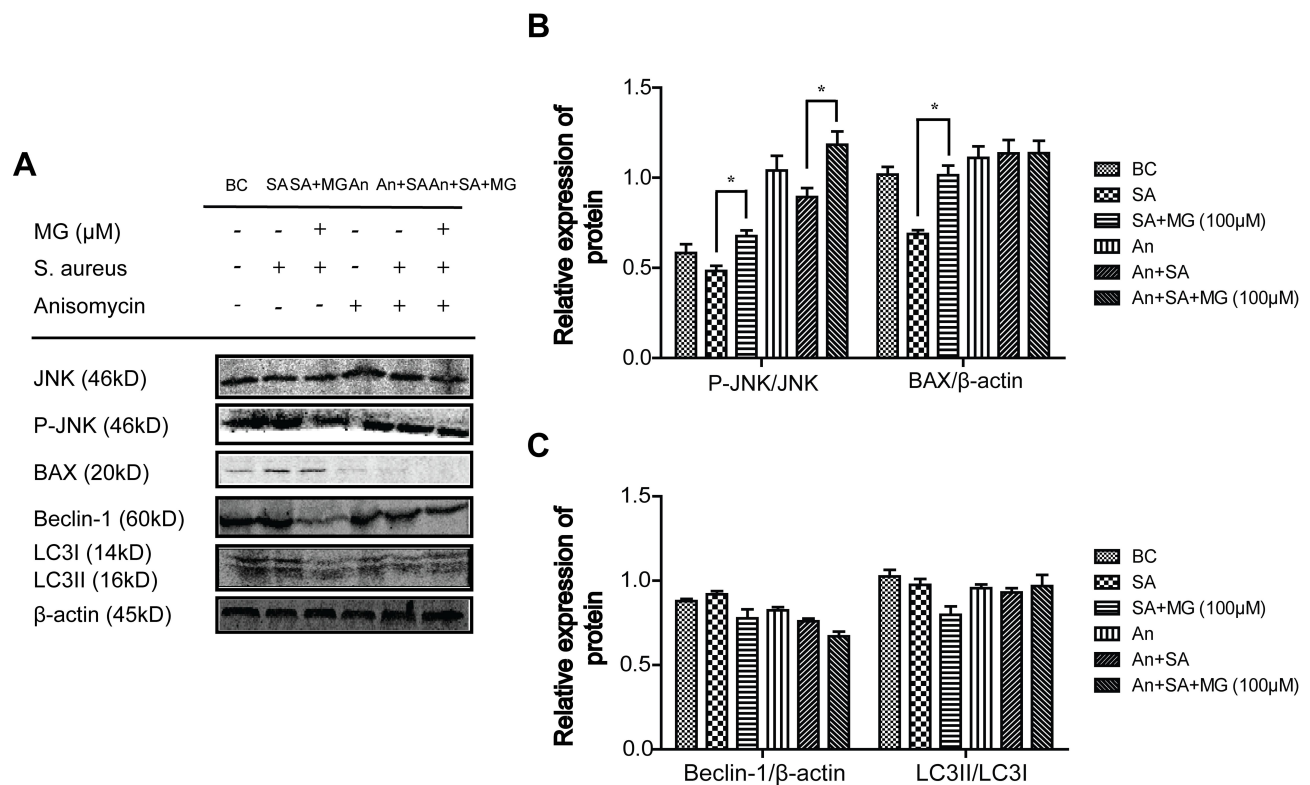
**Figure 5** JNK inhibitor SP600125 obviously affected the related protein of apoptosis and autophagy caused by *S. aureus* infection in RAW264.7 cells. RAW264.7 cells were treated with SP600125 for 12 hour, then treated with MG (25, 50, 100  $\mu$ M) for 1h and infected with *S. aureus* (MOI:100) for 4 h. **(A)** Cell lysates were subjected to Western blot analysis: Representative JNK, P-JNK and Bax band of three independent experiments. Gels were representative of three independent experiments. **(B)** The graph represents quantitative analysis of the band intensity. \* $P < 0.05$ , \*\* $P < 0.01$  compared with SA group. **(C)** The graph represents a quantitative analysis of the band intensity. \* $P < 0.05$ , \*\* $P < 0.01$  compared with SA group.

is recognised by host pattern recognition receptors, and polymorphonuclear cells (PMN) with potent antibacterial activity are recruited.<sup>39,40</sup> However, *S. aureus* resists antimicrobial activities of PMN, requiring additional immune cells to control infection. Macrophages are widely distributed professional phagocytes and kill invading pathogens, facilitate recruitment of immune cells, coordinate adaptive immunity, promote resolution of inflammation and repair damaged tissues.<sup>41</sup> Therefore, *S. aureus* infection is a challenge to the host because the control of the infection requires strong antibacterial immunity while limiting excessive inflammation.

The main mechanism of inflammation is the induction of apoptosis of innate and adaptive immune system cells, and the immunosuppressive effect of these apoptotic cells on surviving immune cells.<sup>42</sup> Apoptosis is a type I programmed cell death, which is closely related to multiple cell systems under both physiological and pathological conditions.<sup>33</sup> The regulation of apoptosis is important in the pathogenesis of the

autoimmune disease, infection and tissue injury. Rectification of the apoptotic-inflammatory imbalance has shown obvious efficacy in animal models, which points out a new path for the clinical treatment of *S. aureus* infection.<sup>42</sup> MG exerts many pharmacological properties, including its antioxidant, antiaging, antiviral, hepatoprotective, analgesic, and immunomodulatory activities.<sup>18,19,24,26,43</sup> MG reduces renal cell apoptosis and enhances the expression of antioxidant molecules HO-1 and SOD2 through the PI3K/AKT pathway.<sup>44</sup> Therefore, MG may be a promising agent for the protection from programmed death and tissues damage. In the study, MG attenuated *S. aureus*-induced inflammation and apoptosis via reducing the expressions of IL-6, IL-10, TNF- $\alpha$ , Bax and caspase-3 and increasing the level of Bcl-2 in RAW264.7 cells.

Autophagy is an endogenous process to remove damaged organelles, maintains essential cellular homeostasis.<sup>45</sup> Physiologic autophagy is involved in the degradation of harmful proteins and damaged organelles to prevent the accumulation of harmful substances and limits the



**Figure 6** JNK activator Anisomycin effected the related protein of apoptosis and autophagy caused by *S. aureus* infection in RAW264.7 cells. RAW264.7 cells treated with a JNK agonist anisomycin for 30 min, then treated with MG (25, 50, 100  $\mu\text{M}$ ) for 1h and infected with *S. aureus* (MOI:100) for 4 h. (A) Expressions of JNK, P-JNK, BAX, Beclin-I and LC3 protein were analysed by Western blotting in RAW264.7 cells. Gels were representative of three independent experiments. (B) The graph represents a quantitative analysis of the band intensity. \* $P < 0.05$  compared with SA group. (C) The graph represents a quantitative analysis of the band intensity.

transmission of harmful signaling.<sup>46</sup> Autophagy is controlled by cytokines and receptors modulating innate and adaptive immunity; but excessive autophagy will cause irreversible injury and transform cells to autophagic cellular death.<sup>47</sup> In the study, MG attenuated *S. aureus*-induced autophagy, the potential mechanisms involved was further investigated, so that it may be exploited as a target for the control of excessive inflammation and tissues damage.

Bacterial recognition receptors trigger a series of cell-activating pathways, including the NF- $\kappa\text{B}$ , MAPK, and type I interferon (IFN I), to induce apoptosis.<sup>48</sup> JNKs belong to the superfamily of MAPK.<sup>49</sup> JNK signalling pathway plays a major role in antibacterial defence against bacterial infection, and inhibition of JNK impairs autophagy upon bacterial infection. *S. aureus* activates Toll-like receptor 2-NF-Kb/MAPK signalling and apoptosis.<sup>50,51</sup> CpG-ODN promotes *S. aureus*-stimulated phagocytosis and autophagy through JNK/P38 signal pathway in macrophage.<sup>12</sup> In our study, MG pretreatment only obviously inhibited JNK signal pathway, which protected RAW264.7 cells from apoptosis and autophagy induced by *S. aureus*. The anti-autophagy efficiency was mediated by inactivation of JNK-dependent

Bcl-2 phosphorylation and its dissociation from the BH3 domain of Beclin. The anti-apoptosis was mediated by inactivation of JNK-dependent Bax. These results provide important insights for controlling apoptosis and autophagy in *S. aureus* infection.

In a word, MG significantly attenuates *S. aureus*-induced apoptosis and autophagy in RAW264.7 cells. Similar effects were shown by treatment with JNK inhibitor SP600125, and opposite effect showed by the treatment with JNK agonist anisomycin. These results indicated that the protective effects of MG against *S. aureus*-induced apoptosis and autophagy were related to JNK inhibition. More should be done in future studies to verify the protective effect of MG on *S. aureus* infection, especially in vivo.

## Conclusion

The present study indicated that pretreatment of MG inhibited apoptosis and autophagy in *S. aureus*-stimulated RAW264.7 cells, which might be mediated by inhibiting JNK/Bax signalling pathway. These findings provided novel insights into the underlying protective mechanisms

for the link between JNK/BAX mediated apoptosis and autophagy upon *S. aureus* infection. This has provides further information regarding clinical treatment strategies using MG.

## Author Contributions

Jun Xu, Hua Yao and Shichen Wang performed the studies. Huanrong Li directed the experiment of bacterial infection. Jun Xu wrote the manuscript. Xiaolin Hou designed the experiment and revised the manuscript. All authors made substantial contributions to conception and design, study design and execution, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. First author with equal contribution: Jun Xu, Hua Yao, and Shichen Wang.

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## Disclosure

The authors report no conflicts of interest in this work.

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