Original article

Magnolol inhibits *Streptococcus suis*-induced inflammation and ROS formation via TLR2/MAPK/NF-κB signaling in RAW264.7 cells

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Abstract

Our previous studies have shown that Magnolol (Mag) improves the symptoms and decreases the levels of cytokines during infection induced by *Streptococcus suis* (*S. suis*) in mice. Although some reports show that Mag inhibits lipopolysaccharide-induced inflammatory responses via downregulating mitogen-activated protein kinases (MAPK) and nuclear factor-κB (NF-κB) signaling pathways, the molecular mechanisms underlying Mag-mediated inhibition of *S. suis*-induced inflammatory responses are poorly understood. Here, RAW264.7 cells were stimulated with *S. suis* in the presence or absence of Mag. Cell viability and bactericidal effects were examined, and the concentrations of tumor necrosis factor-a (TNF-α), IL-1β (interleukin-1β), IL-6 (interleukin-6), and IL-8 (interleukin-8) were determined by ELISA. The change in ROS (reactive oxygen species) was determined by fluorescence microscopy and ELISA. The levels of Toll-like receptor 2 (TLR2) and MAPK family proteins and NF-κB signaling were determined by Western blot analysis. *S. suis* induced massive RAW264.7 cell death, a decline in bactericidal activity, the release of inflammatory cytokines, increased oxidative stress, and activation of TLR2/MAPK/NF-κB signaling pathways. Mag treatment significantly suppressed macrophage cell death and caused a decline in bactericidal activity. Furthermore, Mag decreased inflammatory cytokines production and ROS generation. It also prevented p38, extracellular regulated protein kinases (ERK), c-Jun N-terminal kinase (JNK), inhibitor of NF-κB (IκB), and NF-κB phosphorylation induced by *S. suis* in a dose-dependent manner. Our results indicate that Mag exerts anti-inflammatory and cell-protective effects and mediates the activation of MAPK and NF-κB signaling by downregulating the expression of TLR2 upregulated by *S. suis*.

Key words: magnolol, *Streptococcus suis*, Toll-like receptor2, mitogen-activated protein kinase, nuclear factor Kappa B

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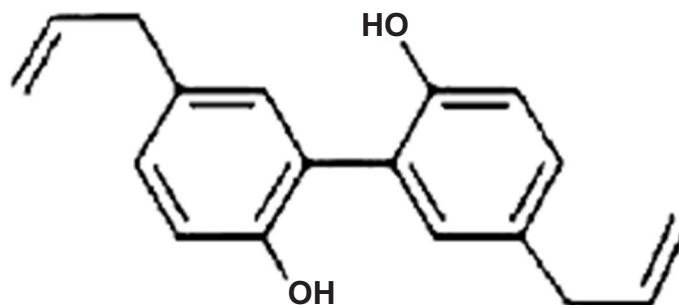
Fig. 1. Chemical structure of magnolol.

Introduction

Streptococcus suis (*S. suis*), a Gram-positive and classical extracellular pathogen, is an important swine pathogen and an agent of zoonosis responsible for considerable economic losses in the swine industry worldwide, particularly over the past 20 years (Gottschalk et al. 2010). Currently, *S. suis capsular type 2* is regarded as the serotype most commonly associated with disease in both humans and pigs (Willemse et al. 2016, Ferrando et al. 2017). To cause disease, *S. suis* must first breach the epithelial barriers, enter and survive in the bloodstream, and then proceed to invade different organs and cause exaggerated inflammation (Fittipaldi et al. 2012). The upregulated expression of several pro-inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and IL-8, has been reported as a consequence of *S. suis* infection (Al-Numani et al. 2003). Furthermore, inflammation is thought to be responsible for most of the clinical signs of meningitis, septicemia, and sudden death (Segura M et al. 2006).

Toll-like receptors (TLRs) are critical sensors that activate the innate immune response (Kawai and Akira 2010). TLR2 has been implicated as the major pattern recognition receptor for ligands derived from Gram-positive bacteria (Kawai and Akira 2005). Because inflammation has been characterized as playing a fundamental role in the pathogenesis of the toxic shock-like syndrome caused by *S. suis* infection, it has been hypothesized that there are differences between the in vivo TLR2 activation patterns arising from different strains of *S. suis* with different potentials for virulence (Lachance et al. 2013).

Magnolol (Mag) is a biologically active compound of *Cortex magnolia officinalis*, a Chinese medicinal herb (Fig. 1). In several studies, Mag has been reported to have many biological effects including anti-oxidant (Lee et al. 2001), antimicrobial (Xia et al. 2014), anti-inflammatory (Wang et al. 2015), anti-

-allergenic (Chen et al. 2006) and anti-tumorigenic properties (Wu et al. 2014). Our previous studies have demonstrated that Mag can improve symptoms and decrease the levels of inflammatory cytokines induced by *S. suis* in mice (Zhang et al. 2016). Recently, it has been reported that the anti-inflammatory effects of Mag are mediated by blocking the activation of NF-κB and MAPKs signaling pathways by interfering with LPS-mediated stimulation of TLR4 (Fu et al. 2013, Wang et al. 2015). In this study, we sought to assess the preventive effects of Mag in *S. suis*-induced infection in RAW264.7 cells and further elucidate the potential mechanisms through which Mag exerts its various effects.

Materials and Methods

Reagents and compounds

Fetal bovine serum (FBS) and Dulbecco's modified Eagle's Medium (DMEM) were purchased from Gibco BRL (Gaithersburg, MD). Mag, which was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (98.5%, Beijing, China), was dissolved in dimethyl sulfoxide (DMSO). 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 2,7-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma-Aldrich. A BCATM protein assay kit was obtained from Wuhan Boster Bio-engineering Limited Co. (Wuhan, Hubei, China). Rabbit antibodies against p38, phospho-p38, ERK (extracellular regulated protein kinases), phospho-ERK, c-Jun N-terminal kinase (JNK), phospho-JNK, inhibitor of NF-κB (IκBα), phospho-IκBα, Nuclear Factor Kappa B (NF-κB) and phospho-NF-κB were purchased from Cell Signaling Technologies, Inc. (Beverly, MA, USA). Rabbit anti-rat TLR2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Mouse macrophage cell line RAW264.7 cells were purchased from the Cell Bank of Type Culture Collection, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were grown in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2.

Bacterial strains and growth conditions

S. suis sero type2 strain SC-19, an isolate obtained from the brain of a pig that died due to the epidemic outbreak of *S. suis* infection in Liaoning province of China in 2016, was selected as the wild-type (WT) strain. Bacteria were grown in tryptic soy broth (TSB) or plated on Tryptic Soy Agar (TSA) plates (Becton Dickinson, MD, USA) supplemented with 5% (v/v) fetal bovine serum at 37°C. Working cultures were obtained by inoculating 100 μL of these primary cultures in 30 mL of Todd-Hewitt broth (THB) and incubating for 16 h at 37°C with agitation. Bacteria in the final suspension were enumerated using the spread plate method on Todd-Hewitt Agar (THA) (Li et al. 2017).

MTT assay for cell viability determination

RAW264.7 cells were seeded in 96-well plates at a density of 0.5×10^5 cells/well. After 24 h, with the exception of control wells, RAW264.7 cells were either incubated with *S. suis* (Multiplicity of infection, MOI=1:100) alone (He et al. 2014) and or incubated with *S. suis* (MOI=1:100) and Mag (25, 50, and 100 μmol/L) at 37°C for 12 h (Auger et al. 2015). After incubation, the plates were washed twice and penicillin G (5 μg/mL) and gentamicin (100 μg/mL) (both from Sigma-Aldrich) were added, and further incubated for 2 h. After treatment, 20 μL MTT working solution (5 mg/mL) was added to the culture medium and the plate was incubated for 2 h at 37°C. The culture supernatant was then discarded and 100 μL DMSO was added to dissolve the formazan crystals. The absorbance of each well was measured at 540 nm with an ELISA reader (Thermo Fisher Scientific, USA) (Zhang et al. 2015).

Effects of Mag on bactericidal effect

RAW 264.7 cells were plated in 24-well tissue culture plates for 24 h and incubated with *S. suis* (MOI=1:100) alone or with *S. suis* (MOI=1:100)

and Mag (25, 50, and 100 μmol/L) at 37°C for 0 or 12 h. Incubation of cells with the agent indicated for the respective period of incubation (0 or 12 hours) is henceforth indicated as either 0 or 12. Subsequent to incubation, the plates were washed twice, and penicillin G and gentamicin were added. The plates were further incubated for 2 h and consequently rinsed with PBS. The cell lysates thus obtained were diluted 10-fold and placed onto THA to calculate the number of viable bacteria. The rate of killing of intracellular bacteria was determined using the formula (1-12 h viable count/0 h viable count) × 100%.

Enzyme-linked immunosorbent assay (ELISA) assay

Cells were seeded in six-well tissue culture plates at a density of 1×10^7 cells and treated for 12 h as described above. The concentrations of TNF-α, IL-1β, IL-6, IL-8 produced *in vitro* by RAW 264.7 cells were measured by ELISA using pair-matched mAbs and cytokine standards obtained from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's recommendations.

Reactive oxygen species (ROS) levels

The intracellular ROS level was determined by DCFH-DA assay. Briefly, the cells were treated for 12 h as described above. They were then incubated with culture medium containing 10 μM DCFH-DA for 30 min at 37°C in the dark. Thereafter, the cells were collected for observation and measured at 540 nm with an ELISA reader (Thermo Fisher Scientific, USA). The ROS-positive cells were visualized and counted using fluorescence microscopy with excitation at 485 nm and emission at 538 nm (Jayaprakash et al. 2015).

Western blotting analysis

After treatment, the cells were washed twice with cold PBS, and total proteins were isolated by the rupturing of the cells by incubation with radio-immunoprecipitation assay (RIPA) lysis buffer on ice. Lysates were then centrifuged at $12,000 \times g$ for 30 min at 4°C. Equal amounts of protein, estimated by using the BCA protein assay kit (Dai et al. 2013), were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA) (Zhang et al. 2015). Blots were blocked

and then immunolabeled with primary antibodies for TLR2 (1:400), p38 (1:500), phosphoryled-p38 (p-p38, 1:1000), ERK (1:500), p-ERK (1:500), JNK (1:500), p-JNK (1:1000), IκBα (1:1000), p-IκBα (1:1000), NF-κB (1:1000), p-NF-κB (1:1000) and β-actin (1:2000) at 20ºC for 1.5 h. The membrane was subsequently incubated with HRP-conjugated secondary antibody (1:2000 dilution) at room temperature for 2 h. An ECL (enhanced chemiluminescence) detection system was used to detect immunoblots, and the protein bands were analyzed by densitometry using Image J (Ver 1.42, National Institutes of Health, USA).

Statistical analysis

Data obtained from five independent experiments are presented as mean ± standard deviation (SD). Statistical analyses were conducted by one-way analysis of variance (ANOVA) using SPSS (version 13.0, SPSS, Chicago, IL, USA) to determine differences among experimental groups, with a p value of < 0.05 being considered as statistically significant.

Results

Effects of Mag on cell viability

To determine the effects of Mag on *S. suis*-induced infection in RAW264.7 cells, the cells were treated with various concentrations of Mag (25 to 100 µmol/L) in the presence of *S. suis*, and the cell viability was determined by MTT assay. As shown in Fig. 2A, the results revealed that the cell viabilities were significantly decreased when incubated with *S. suis* (p<0.01). However, when compared with cells incubated with *S. suis* only, the presence of Mag (25 to 100 µmol/L) resulted in a significant increase in cell viability (p<0.01).

Effects of Mag on bactericidal effect

To investigate the bactericidal effects of Mag in RAW264.7 cells, we performed an experiment to monitor the survival of intracellular bacteria at different Mag concentrations. As shown in Fig. 2B, approximately 17% bacteria were killed after 12 h in the *S. suis* group. However, pretreatment with Mag significantly increased (p<0.01) the bactericidal effect in a dose-dependent manner.

Mag inhibits the secretion of inflammatory cytokines in *S. suis*-stimulated RAW264.7 cells

To analyze the potential anti-inflammatory effects of Mag on *S. suis* infection, we investigated the levels of pro-inflammatory cytokines in *S. suis*-stimulated RAW264.7 cells following Mag treatment. The levels of TNF-α, IL-1β, IL-6 and IL-8 were detected by ELISA. The results showed that *S. suis* stimulated the production of TNF-α, IL-1β, IL-6 and IL-8. In contrast, Mag treatment suppressed the synthesis in a dose-dependent manner (Fig. 2C).

Mag suppressed *S. suis*-induced oxidative stress

During infection, ROS can be generated by the innate immune system (Netzer et al. 2009). To test the generation of ROS induced by *S. suis* in RAW264.7 cells, and the effects of Mag on this induction, ROS production was measured by DCFH-DA assay. As shown in Fig. 3A, *S. suis* treatment induced significant increases in DCF fluorescence (p<0.01); however, ROS induction was inhibited by Mag pretreatment in a dose-dependent manner (p<0.01).

Mag suppressed *S. suis*-induced TLR2 /MAPK/NF-κB pathway

TLR2, MAPKs, and NF-κB play critical roles in the induction of pro-inflammatory gene expression. To determine whether the anti-infective effect of Mag on *S. suis* resulted from the TLR2 mediated activation of the MAPK/NF-κB pathway, the activation of TLR2, p38, p-p38, JNK, p-JNK, ERK, p-ERK, p-IκB, IκB, p-NF-κB, and NF-κB were examined by Western blotting. As shown in Fig. 3B and C, compared with the control group, the levels of TLR2 and phosphorylated proteins were significantly increased in the *S. suis* group (p<0.01). Mag markedly inhibited the *S. suis*-induced upregulation of TLR2 and phosphorylation of these proteins in a dose-dependent manner (p<0.01).

Discussion

Some studies have shown that *S. suis* isolated from pigs had high-level resistance to the antimicrobials of the macrolide, lincosamide and tetracycline families (Aarestrup et al. 1998, Martel et al. 2001), and those isolated from the human body were resistant to norfloxacin (Zalas-Wiecek et al. 2013). In light of such findings, we chose to focus on alternative sources
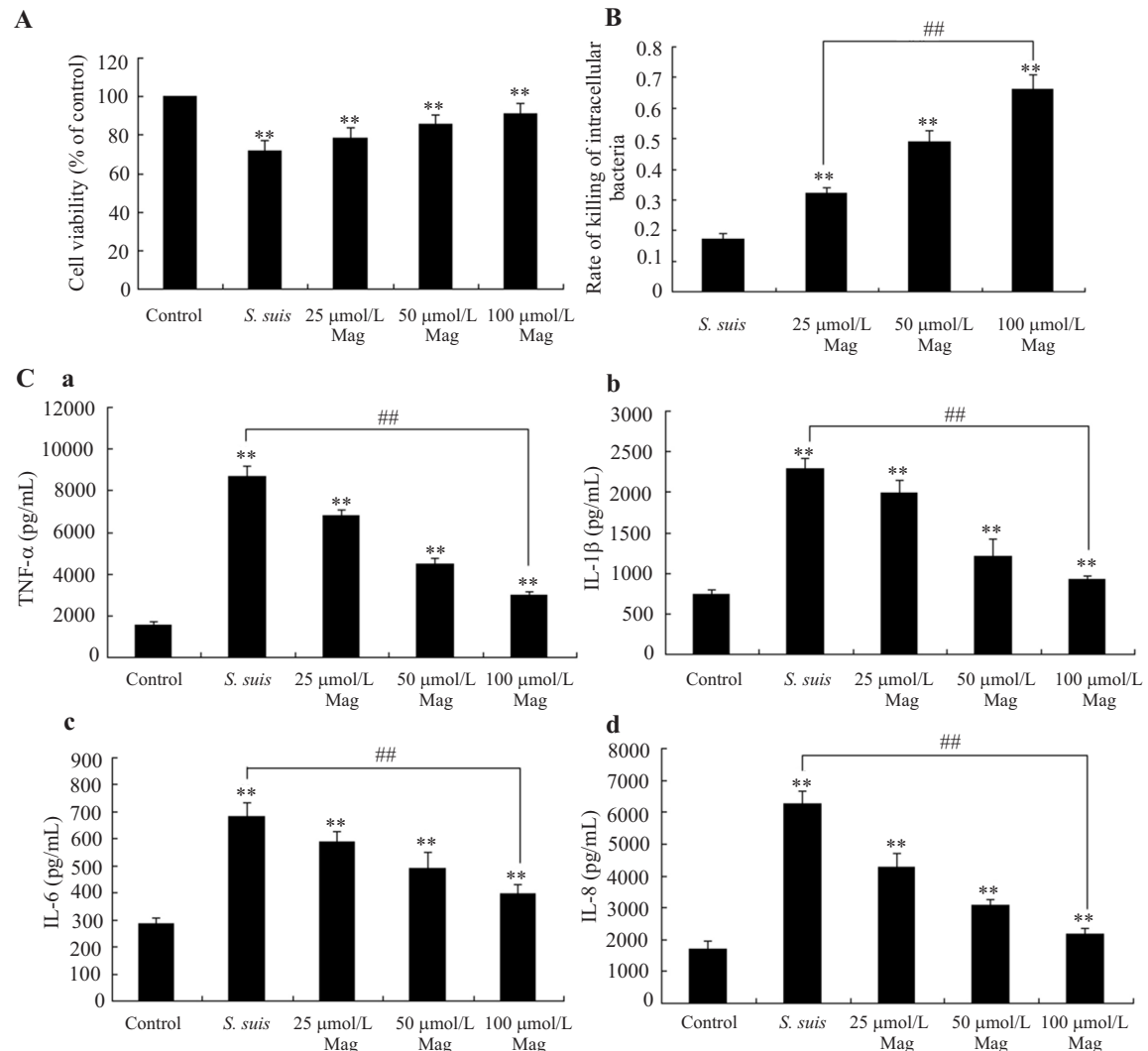
Fig. 2. Effects of magnolol (Mag) on cell viability, bactericidal effect, and cytokines induced by *Streptococcus suis*. **(A)** Effects of exposure to different Mag concentrations (25 to 100 μmol/L) on viability using the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide assay (n = 5). **(B)** Effects of Mag on bactericidal effect. **(C)** Effects of Mag on cytokines using the ELISA. Effects of exposure to different Mag concentrations (25 to 100 μmol/L) on TNF-α (a), IL-1β (b), IL-6 (c) and IL-8 (d) production by RAW 264.7 cells stimulated with *S. suis*. Error bars represent the standard deviation from the mean (SD). Data are expressed as mean pg/mL ± SD from five independent experiments. **p<0.01 versus the control groups; ## p<0.01 for intergroup comparison.

of antimicrobials from natural products to provide alternatives for the prevention and treatment of *S. suis* infection. This research will have a great clinical significance. Mag is extracted from magnolia and is a natural compound with many pharmacological activities. We had previously explored the therapeutic effect of Mag on *S. suis* infection in mice, but the mechanisms through which Mag acts are currently unknown (Zhang et al. 2016). To understand the mechanisms of Mag action we explored the effects of Mag on *S. suis* interactions in RAW264.7 cells *in vitro*.

It has been reported that Mag exerts protective effects on the small intestine in mice infected by *S. suis Serotype 2* (Zhang et al. 2016). This study showed that three different concentrations of Mag had pro-

tective effect on cell viability when infected by *S. suis*. Chen previously reported similar findings that Mag could increase cell viability when cells were heated (Chen et al. 2016). In this study, we also found that Mag could enhance the killing of bacteria by RAW264.7 cells. The results indicate that Mag can help the macrophages to eliminate the bacteria. Mag enhances the protective effect of macrophages against bacteria in a concentration-dependent manner.

The pro-inflammatory cytokines play an important role in inflammatory diseases. Our previous studies demonstrated that Mag could decrease the levels of inflammatory cytokines induced in mice infected with *S. suis* (Zhang et al. 2016). In this study, we examined the effects of Mag on the production of
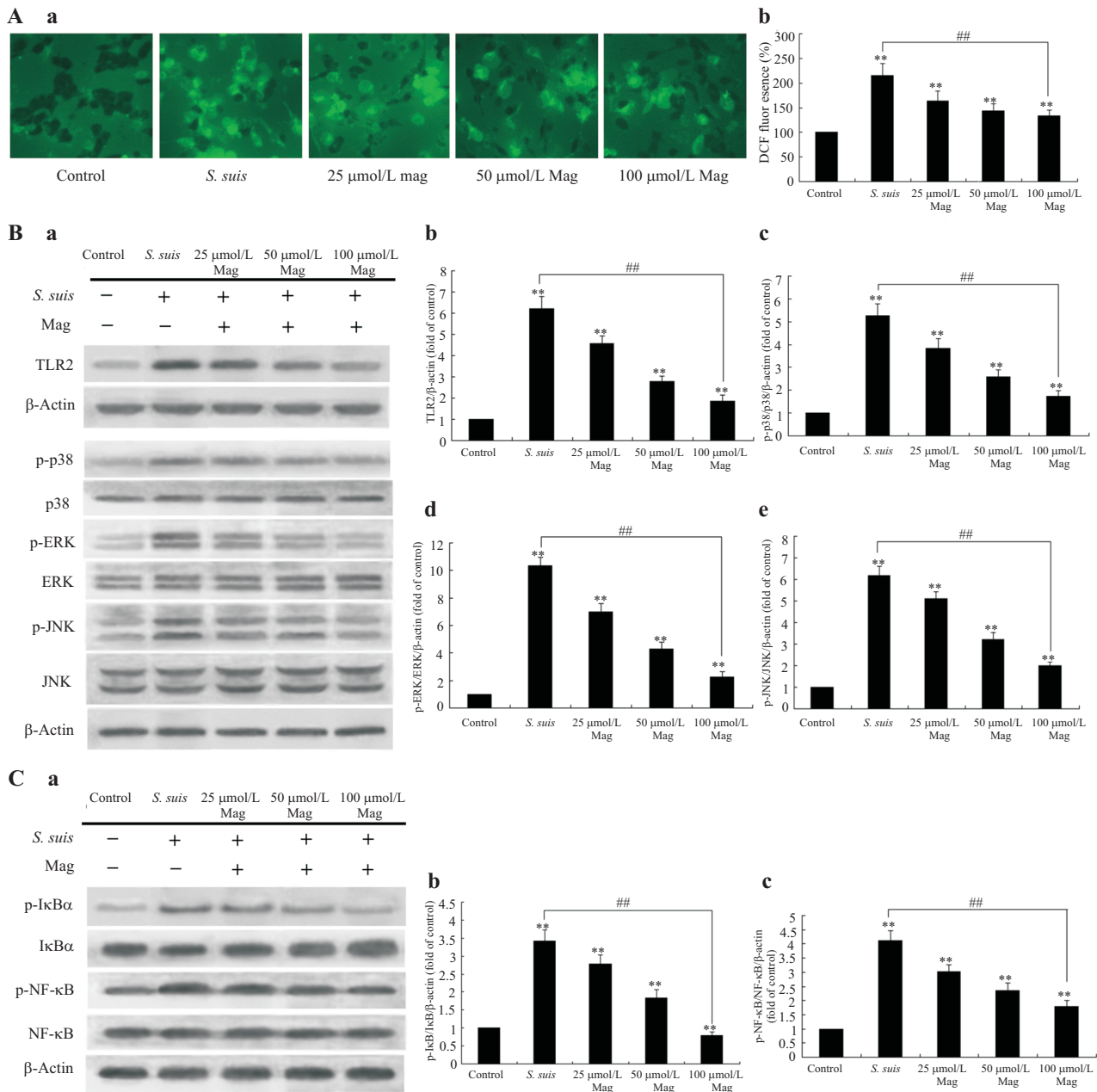
Fig. 3. Effects of Mag on *S. suis*-induced ROS formation and TLR2/MAPK/NF-κB signaling pathway. (**A**) Effects of Mag on *S. suis*-induced ROS formation. Images show the fluorescence of cells treated with *S. suis* alone (MOI=1:100) or when incubated with *S. suis* (MOI=1:100) and Mag in different concentrations (25, 50 and 100 μmol/L) for 12 h after highly fluorescent 2',7'-dichlorofluorescein upon oxidation (DCF fluorescence) staining. The appearance of black staining indicates normal cells, whereas green staining indicates damaged cells in (a). Each bar graph of (b) shows the quantitative results of the DCF fluorescence levels in (a). (**B and C**) Effects of Mag on *S. suis*-induced TLR2 /MAPK/NF-κB pathway. Western blotting analysis of TLR2, p38, p-p38, JNK, p-JNK, ERK p-ERK, p-IκB, IκB, p-NF-κB and NF-κB. β-Actin level was employed as a loading control. Each panel of (B), (b) to (e) shows the quantitative results of the expression levels in (a), respectively. Each panel of (C), (b) to (c) shows the quantitative results of the expression levels in (a), respectively. Bar graph represents mean value ± SD, n = 5. **p<0.01 versus the control groups; ##p<0.01 for intergroup comparison.

pro-inflammatory cytokines *in vitro*. The results demonstrated that Mag inhibited the secretion of TNF-α, IL-1β, IL-6 and IL-8 in *S. suis*-stimulated RAW264.7 cells in a dose-dependent manner. Although some reports indicate that Mag inhibits lipopolysaccharide-induced inflammatory response via downregulating

MAPK and NF-κB signaling pathways, the underlying molecular mechanisms of Mag on inhibiting *S. suis*-induced inflammatory response are not well understood (Lai et al. 2011, Fu et al. 2013, Lu et al. 2015).

Within the family of TLRs, TLR2 has been implicated as the major pattern recognition receptor for

ligands derived from Gram-positive bacteria (Lachance et al. 2013). In the case of *S. suis*, several studies *in vitro* have previously shown that the activation of TLR2 leads to the release of pro-inflammatory cytokines (Takeuchi and Akira 2010, Zheng et al. 2011). It has been found that the expression of these mediators is also modulated by NF-κB and MAPK pathways (Alva-Murillo et al. 2017). To further understand the molecular mechanism of Mag in *S. suis* infection, we explored the TLR2-mediated activation of the MAPK/NF-κB signaling pathway. MAPKs play an important role in inducing cytokine production. *S. suis* stimulates murine macrophages by inducing phosphorylation and activation of p38, ERK1/2 and JNK (Choi et al. 2007). NF-κB is also normally sequestered in the cytoplasm by a family of inhibitory proteins known as inhibitors of NF-κB (IκBs) (Jiang et al. 2017). Once activated, NF-κB subunit p65 dissociates from the inhibitory protein IκBα and translocates from the cytoplasm to the nucleus where it may trigger the transcription of specific target genes such as TNF-a, IL-1β, and IL-6. To detect the inhibitory mechanism of TNF-a, IL-1β, and IL-6 production, we tested the effects of Mag on NF-κB activation and IκBα degradation. We investigated the effects of Mag on *S. suis*-induced TLR2 and phosphorylation of MAPKs and NF-κB in RAW264.7 cells. The results showed that pretreatment with Mag significantly inhibited *S. suis*-induced TLR2 and phosphorylation of ERK1/2, p38, JNK, and NF-κB in RAW 264.7 cells (Fig. 3B and C). Collectively, our results suggest that Mag suppresses *S. suis*-induced proinflammatory cytokine production by preventing TLR2/MAPK/NF-κB pathway activation.

Excessive ROS formation, which arises as a result of unbalanced oxygen consumption, has long been implicated as the major factor, whereby macrophages cause damage (Lee et al. 2014). A growing body of evidence has indicated that ROS is not only the by-product of cellular metabolism, but also contributes to both the activation of cell signaling pathways and transcriptional regulatory factors involved in the cellular functions that are mediated by the activation of MAPK and NF-κB families (Ren et al. 2017). In this study, we have confirmed that Mag decreases the levels of ROS under the experimental conditions and that the mechanism responsible relies on changes in key proteins of the TLR2/MAPK/NF-κB pathway (Fig. 3A).

Conclusions

In general, our data indicate that *S. suis* induces macrophage cell death, with a consequent decline in bactericidal activity, the release of inflammatory cytokines, increased oxidative stress, and the activation of the TLR2/MAPK/NF-κB signaling pathway by infecting RAW264.7 cells. Mag plays a protective role in *S. suis*-induced changes mentioned above. Specifically, we found that Mag (25 to 100 µmol/L) exerts these protective effects through the inhibition of ROS generation and TLR2/MAPK/NF-κB signaling pathway in RAW264.7 cells.

Acknowledgments

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