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Glycyrrhizic acid activates chicken macrophages and enhances their *Salmonella*-killing capacity in vitro*#

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Abstract: Objective: *Salmonella enterica* remains a major cause of food-borne disease in humans, and *Salmonella* Typhimurium (ST) contamination of poultry products is a worldwide problem. Since macrophages play an essential role in controlling *Salmonella* infection, the aim of this study was to evaluate the effect of glycyrrhizic acid (GA) on immune function of chicken HD11 macrophages. Methods: Chicken HD11 macrophages were treated with GA (0, 12.5, 25, 50, 100, 200, 400, or 800 μg/ml) and lipopolysaccharide (LPS, 500 ng/ml) for 3, 6, 12, 24, or 48 h. Evaluated responses included phagocytosis, bacteria-killing, gene expression of cell surface molecules (cluster of differentiation 40 (*CD40*), *CD80*, *CD83*, and *CD197*) and antimicrobial effectors (inducible nitric oxide synthase (*iNOS*), NADPH oxidase-1 (*NOX-1*), interferon-γ (*IFN-γ*), LPS-induced tumor necrosis factor (TNF)-α factor (*LITAF*), interleukin-6 (*IL-6*), and *IL-10*), and production of nitric oxide (NO) and hydrogen peroxide (H_2O_2). Results: GA increased the internalization of both fluorescein isothiocyanate (FITC)-dextran and ST by HD11 cells and markedly decreased the intracellular survival of ST. We found that the messenger RNA (mRNA) expression of cell surface molecules (*CD40*, *CD80*, *CD83*, and *CD197*) and cytokines (*IFN-γ*, *IL-6*, and *IL-10*) of HD11 cells was up-regulated following GA exposure. The expression of *iNOS* and *NOX-1* was induced by GA and thereby the productions of NO and H_2O_2 in HD11 cells were enhanced. Notably, it was verified that nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK) pathways were responsible for GA-induced synthesis of NO and *IFN-γ* gene expression. Conclusions: Taken together, these results suggested that GA exhibits a potent immune regulatory effect to activate chicken macrophages and enhances *Salmonella*-killing capacity.

Key words: Glycyrrhizic acid; Chicken macrophage; Macrophage activation; *Salmonella* Typhimurium; Nuclear factor κB (NF-κB); c-Jun N-terminal kinase (JNK)
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1 Introduction

Salmonella is one of the leading causes of food-borne disease worldwide (Scallan et al., 2015). Outbreaks and sporadic cases have indicated that food vehicles such as poultry and poultry by-products are among the most common sources of *Salmonella* infections (Revolledo et al., 2009). In chickens, *Salmonella* Typhimurium (ST) and *Salmonella* Enteritidis

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(SE) are major food-borne *Salmonella* serovars which can colonize or invade the gastrointestinal tract and thus contaminate meat and eggs and cause food poisoning (He et al., 2012). With the restrictions on the use of antibiotics, alternative approaches such as dietary interventions are being evaluated to improve animal health and control *Salmonella* infection. Notable among the interventions is the use of plant extracts in animal feed as they are considered to be "natural" additives and have been shown to be effective immune modulators in response to pathogen infections (Pugh et al., 2005).

Macrophages are key components of the immune system and provide protection against a wide variety of infections. Stimulated macrophages are not only phagocytic cells which detect, phagocytize, and eliminate infectious agents but also serve as antigen-presenting cells for B and T lymphocytes and participate in the stimulation of the adaptive immune system (Setta et al., 2012). However, as a facultative intracellular pathogen, *Salmonella* is able to produce effector proteins which manipulate macrophages to delay the phagolysosomal maturation and thus avoid exposure to lysosomal contents (Haraga et al., 2008). The ability of *Salmonella* to survive and multiply within chicken macrophages is crucial for *Salmonella* virulence and pathogenesis (Barrow et al., 1994). To control intracellular *Salmonella*, macrophages are activated to produce several antimicrobial substances such as nitric oxide (NO) and hydrogen peroxide (H_2O_2) and secrete a group of cytokines and chemokines such as interferon-γ (IFN-γ) and interleukin-12 (IL-12) (Ibuki et al., 2011). Previous studies demonstrated that mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways are involved in macrophage activation and play a critical role in pro-inflammatory effector production and autophagy formation (Mosser and Edwards, 2008; He et al., 2012). These have been shown to be important in controlling intracellular pathogens.

The roots and rhizomes of the *Glycyrrhiza* species (licorice) have been widely used as natural sweeteners and herbal medicines. Glycyrrhizic acid (GA), a major biologically active constituent of licorice root, consists of one molecule of 18-glycyrrhetinic acid and two molecules of glucuronic acid (Matsui et al., 2004). So far, GA has been reported to have anti-viral, anti-cancer, anti-apoptotic, and anti-inflammatory activity (Honda et al., 2012; Wang et al., 2017). Previous studies indicated that GA may act as a potent anti-infectious agent in the process of pathogen invasion by targeting particular immune cells like macrophages and dendritic cells (Bhattacharjee et al., 2012; Hua et al., 2012). In a mouse model, GA treatment caused an enhanced production of NO along with inhibition of intracellular survival of *Leishmania donovani* in macrophages and decreased hepatic and splenic parasite burden in vivo (Bhattacharjee et al., 2012). GA could also increase the productions of IL-12 and IFN-β in macrophages and exhibits a curative effect on several virus infections such as severe acute respiratory syndrome-coronavirus (SARS-CV), human immunodeficiency virus type 1 (HIV-1), and highly pathogenic avian influenza A (H5N1) (Dai et al., 2001; Cinatl et al., 2003; Michaelis et al., 2010). However, most of these studies are focused on mammals, and there is no report on the effect of GA on immune function in chickens. In the present study, we investigated the effects of GA on phagocytic and bacteria-killing activity against ST of the chicken macrophage. Additionally, the expression of cell surface molecules and antimicrobial genes, production of antimicrobial effectors, and its possible mechanisms were analyzed.

2 Materials and methods

2.1 Cells and glycyrrhizic acid treatment

The chicken macrophage cell line (HD11) was generously provided by Dr. Shou-qun JIANG (Guangdong Academy of Agricultural Sciences, Guangzhou, China). Cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA) supplemented with 10% (v/v) chicken serum (Gibco, USA), 100 μg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), nonessential amino acids (1×), sodium pyruvate (1 mmol/L), L-glutamine (2 mmol/L) and 2-mercaptoethanol (5×10^{-5} mol/L) at 41 °C in a 5% (v/v) humidified CO_2 incubator.

GA was purchased from Sigma-Aldrich (purity ≥95.0% (neutralization titration (NT)), St. Louis, MO, USA) and suspended in sterile phosphate-buffered saline (PBS). There was no detectable endotoxin (<0.10 endotoxin units/ml) in the GA samples, as

determined by Endospecy method (Seikagakukougyo, Osaka, Japan). Stocks of GA were frozen in aliquots of 100 μl at 10 mg/ml. The Stock was diluted to the appropriate concentrations in the media indicated by the experiment.

2.2 Cell viability assay

HD11 cells were seeded at 1×10^4 ml^{-1} in 96-well microplates (Corning, USA) and treated with PBS or GA (12.5, 25, 50, 100, 200, 400, and 800 μg/ml) for 48 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA) assay (Mao et al., 2015) was then used to investigate the relative cell viabilities.

2.3 Phagocytosis assay

HD11 cells seeded into 12-well plates (Corning, 1×10^6 cells/ml) were pretreated with PBS or GA (25, 50, and 100 μg/ml) for 12 h, and then incubated with fluorescein isothiocyanate (FITC)-dextran (1 mg/ml, molecular weight 40000; Sigma-Aldrich, St. Louis, MO, USA) at 41 °C for 1 h. After incubation, the cells were washed with PBS to remove excess dextran. The percentage and mean fluorescence intensity (MFI) of intracellular FITC-dextran were determined using FACScalibur flow cytometer (Becton-Dickinson, USA).

2.4 *Salmonella*-killing analysis

The effect of GA on the *Salmonella*-killing capacity of chicken macrophages was measured by a viable count method, as described previously (Ibuki et al., 2011). Briefly, HD11 cells seeded into 24-well plates (Corning, 2×10^5 cells/ml) were preincubated with GA (100 μg/ml) for 12 h. The cells were then washed and incubated with ST (strain CMCC-50115, 2×10^7 colony forming units (CFU)/well) for 1 h at 41 °C to allow bacterial adhesion and colonization. Thereafter, cells were washed twice with PBS and incubated in RPMI-1640 containing gentamicin (25 μg/ml) for 0, 12, and 24 h. Finally, cell lysates from HD11 cells containing intracellular bacteria were serially diluted with PBS and spread onto *Salmonella-Shigella* (SS) agar plates to determine bacterial viability.

2.5 qPCR analysis

HD11 cells were seeded into 12-well plates (1×10^6 cells/ml) and pretreated with PBS, GA (100 μg/ml), or lipopolysaccharide (LPS, 500 ng/ml) for 0, 3, 6, and 12 h, and then washed by PBS three times to collect the cell pellets. The co-cultured cell pellets were re-suspended in RNAiso Plus (TaKaRa, Dalian, China) and then placed in liquid nitrogen. All samples were frozen and kept at −80 °C for no more than one week for further RNA isolation. Total RNA was isolated from the treated cells using RNAiso Plus (TaKaRa, Dalian, China) according the manufacturer's recommendations. Qualitative and quantitative analyses of RNA were determined by the ratio of absorbance readings at 260 and 280 nm (A_{260}/A_{280}) using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and agarose gel electrophoresis (Sangon Biotech, Shanghai, China). One microgram of total RNA from each sample was reverse-transcripted into complementary DNA (cDNA) using PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) following the manufacturer's recommendations. The cDNA samples were then tested for gene expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) performed using SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) on a StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). All samples were denatured for 30 s at 95 °C, followed by 40 cycles of PCR amplification (5 s denaturation at 95 °C, 34 s annealing/extension at 60 °C), and then a final melting curve analysis to monitor purity of the PCR product. Each sample was run in triplicate. Primer sequences for chicken macrophage genes were designed and selected by Primer 6.0 and Oligo 7.0 software and the sequences are listed in Table 1. *β-actin* was used as reference gene and relative quantification was calculated using the $2^{-\Delta\Delta C_q}$ method (Bustin et al., 2009), where C_q is quantification cycle, ΔC_q is $C_{q,\,target}-C_{q,\,reference}$, and $\Delta\Delta C_q$ is $\Delta C_{q,\,treatment}-\Delta C_{q,\,control}$.

2.6 Nitrite generation assay

HD11 cells were seeded into 12-well plates (1×10^6 cells/ml) and pretreated with PBS, GA (25, 50, and 100 μg/ml) or LPS (500 ng/ml) for 48 h. Then NO production was estimated by the Greiss method (Ding et al., 1988). Briefly, equal volumes (100 μl) of cell-free supernatant and Greiss reagent (0.01 g/ml sulfanilamide, 1 g/L naphthylenediamide, and 5% (v/v) phosphoric acid; Sigma-Aldrich, St. Louis, MO, USA) were mixed for 10 min at room temperature. The

Table 1 List of real-time PCR primers

Gene name	Primer (5'→3')	Product (bp)	Accession number
CD40	F: GGCACCTTCTCCAATGTATCTTC	96	NM_204665
	R: GTTCGTCCCTTTCACCTTCAC		
CD80	F: CAGCAAGCCGAACATAGAAAGA	270	NM_001079739
	R: AGCAAACTGGTGGACCTGAGA		
CD83	F: GCTGACTTGCCTCGGGATT	272	XM_418929
	R: TCACTCCGCTATCCGTCTCA		
CD197	F: GACGACTATGACGCCAACAC	211	NM_001198752
	R: CCAGGTTCAGCAAGTAGATGTC		
iNOS	F: CCACCAGGAGATGTTGAACTATG	160	NM_204961
	R: CAGGAGTAATGACGCCAAGAG		
NOX-1	F: CTGGACGGAGCACATCATTG	281	NM_001101830
	R: AGGCAAGCAGGTCATTGAAC		
IFN-γ	F: ACAAGTCAAAGCCGCACATC	83	NM_205149
	R: CACCTTCTTCACGCCATCAG		
LITAF	F: GGACAGCCTATGCCAACAAG	81	NM_204267
	R: GCGGTCATAGAACAGCACTAC		
IL-6	F: CTCCTCGCCAATCTGAAGTC	99	NM_204628
	R: CCTCACGGTCTTCTCCATAAAC		
IL-10	F: ACCAGTCATCAGCAGAGCAT	222	NM_001004414
	R: CCTCCTCATCAGCAGGTACTC		
β-actin	F: ACCCTGAAGTACCCCATTGAAC	107	NM_205518
	R: TGCTCCTCACGGGCTACTCT		

F: forward; R: reverse

absorbency was then read at 550 nm by a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA) and the actual NO concentration was calculated using a standard curve with serial dilutions of sodium nitrite.

2.7 H_2O_2 generation assay

H_2O_2 production was measured using a hydrogen peroxide assay kit (Beyotime Biotech, Shanghai, China). Briefly, cell lysates from cultured cells treated as above for 12 h were mixed with double volumes of test solutions at room temperature for 20 min. The absorbance at 560 nm was then measured using a SpectraMax M5 and the actual concentration was calculated using a standard curve with serial dilutions of H_2O_2.

2.8 Neutralization experiments

HD11 cells (1×10^6 cells/ml) were pretreated with NF-κB inhibitor (BAY 11-7082, 20 μmol/L), c-Jun N-terminal kinase (JNK) inhibitor (SP600125, 20 μmol/L), p38 inhibitor (SB203580, 20 μmol/L), or extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor (U0126, 20 μmol/L) (Beyotime Biotech, Shanghai, China) for 30 min, and subsequently stimulated with GA (100 μg/ml) for 48 or 6 h. Supernatants and cell lysates from cultured cells were analyzed for the production of NO and *IFN-γ* gene expression according to the above methods.

2.9 Statistical analysis

Differences were analyzed by two-tailed Student's *t*-test using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) for Windows and results were expressed as mean± standard deviation (SD) of at least three independent experiments. All statistical analyses were performed using Origin 8.0 (Origin Lab, MA, USA). $P<0.05$ was considered statistically significant.

3 Results

3.1 Cytotoxicity analysis of glycyrrhizic acid on chicken macrophages

No obvious cytotoxicity was observed when chicken macrophage HD11 cells were incubated with GA (12.5, 25, 50, 100, 200, and 400 μg/ml) for 48 h

(P>0.05). In contrast, cells treated with 800 μg/ml GA exhibited a significantly lower survival rate under given experimental conditions (85.22%, P<0.05; Fig. 1).

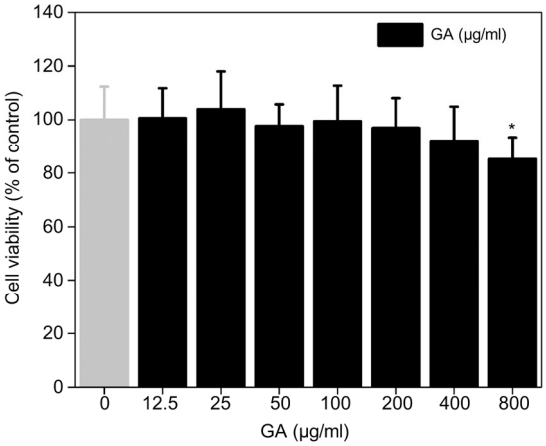

Fig. 1 Cytotoxicity analysis of glycyrrhizic acid (GA) on chicken macrophages

HD11 cells were incubated with PBS or GA (12.5, 25, 50, 100, 200, 400, and 800 μg/ml) for 48 h and cell viability was determined by the MTT method. Results are presented as mean±SD of eight samples. * P<0.05 vs. control (Student's t-test)

3.2 Effects of glycyrrhizic acid on phagocytosis and *Salmonella*-killing activity of chicken macrophages

GA dose-dependently enhanced the uptake of FITC-dextran by HD11 cells and a marked increase of all MFIs and marker 1 (M1) of FITC-dextran was observed in cultured cells pretreated with 100 μg/ml GA when compared to the control group (P<0.05; Fig. 2a). Therefore, 100 μg/ml GA was used for the following experiments.

GA (100 μg/ml) significantly enhanced the uptake of intracellular bacteria ST in cultured cells compared to the control group (P<0.05; Fig. 2b, 0 h). The survival rate of ST in GA-pretreated cells decreased by more than two times at 24 h post infection as compared to uninfected cells (P<0.01; Fig. 2b, 24 h). In addition, we also investigated the antibacterial activity of GA against ST in vitro. However, it was found that GA affected neither the growth and proliferation nor the expression of virulence genes (e.g. *ssrB*, *sipB*, *hilA*, *invA*, and *sopD*) of ST (P>0.05; Figs. S1 and S2, Table S1).

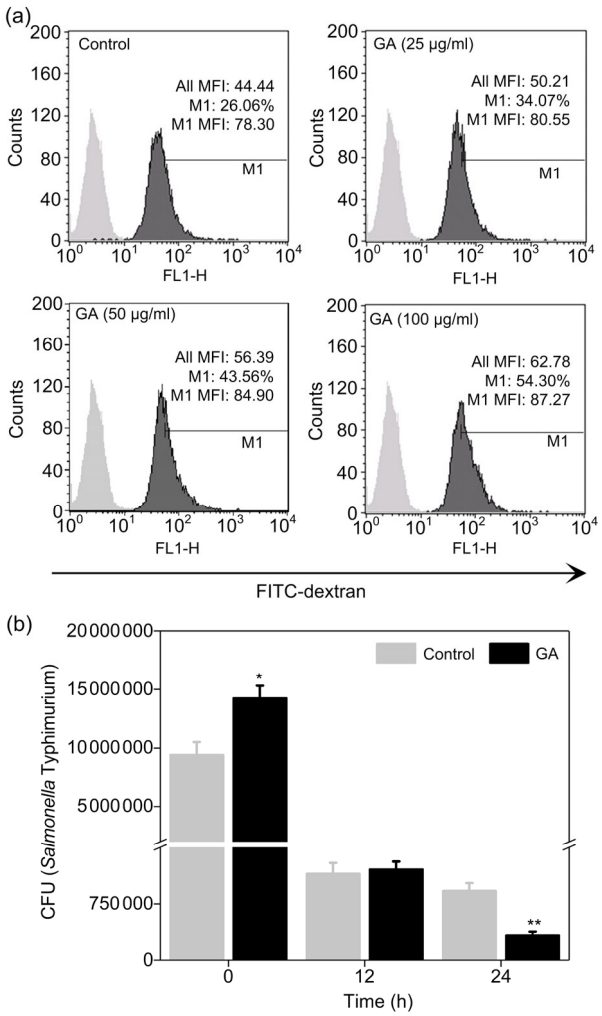

Fig. 2 Effects of glycyrrhizic acid (GA) on the phagocytosis and *Salmonella*-killing capacity of chicken macrophages

(a) HD11 cells were pretreated with GA (25, 50, 100 μg/ml) for 12 h, and then incubated with FITC-dextran (1 mg/ml) at 41 °C for 1 h. The intracellular FITC-dextran was measured by fluorescence-activated cell sorting (FACS). Marker 1 (M1): mean fluorescence intensity (MFI) >50. (b) HD11 cells were pretreated with GA (100 μg/ml) for 12 h, and then infected with ST (2×10^7 CFU/well) for 1 h. The cells were washed and incubated in RPMI-1640 medium with gentamicin (25 μg/ml) for 12 and 24 h, and then these cells were lysed, diluted, and plated on *Salmonella-Shigella* (SS) agar plates for colony enumeration. Data are expressed as mean±SD for three independent experiments. * P<0.05, ** P<0.01, vs. control (Student's t-test)

3.3 Effects of glycyrrhizic acid on activation and antimicrobial factor gene expression of chicken macrophages

As shown in Fig. 3, GA significantly up-regulated the messenger RNA (mRNA) expression of

cluster of differentiation 40 (*CD40*), *CD80*, *CD83* (6 and 12 h), and *CD197* (3 and 6 h) (P<0.05). GA enhanced the expression of both inducible nitric oxide synthase (*iNOS*) and NADPH oxidase-1 (*NOX-1*) (Figs. 4a and 4b) and thereby increased the productions of NO and H_2O_2 (Figs. 4c and 4d) (P<0.05). Furthermore, the mRNA expression of immune-associated cytokines, such as *IFN-γ*, LPS-induced tumor necrosis factor (TNF)-α factor (*LITAF*), *IL-6*, and *IL-10*, was observed to be up-regulated in HD11 cells on GA exposure (Fig. 5).

3.4 Effects of glycyrrhizic acid on the signaling pathways of chicken macrophages

As demonstrated in Fig. 6, BAY 11-7082 (NF-κB inhibitor) and SP600125 (JNK inhibitor), but not SB203580 (p38 MAPK inhibitor), significantly blocked GA-mediated induction of NO and *IFN-γ* (P<0.05), whereas being pre-stimulated with U0126 (ERK1/2 inhibitor) resulted in unanticipated increase in NO level and *IFN-γ* expression (P<0.05).

4 Discussion

Although the mechanisms against *Salmonella* infection are not fully understood, it is known that macrophages play a critical role in the initial recognition and control of *Salmonella* infections (Braukmann et al., 2015). Previous reports have shown that GA could provide protection against several intracellular pathogens in mammals through enhancing macrophage functions (Dai et al., 2001; Bhattacharjee et al., 2012). In the present study, the results found that GA, with a safe range of dose (≤400 μg/ml), could activate chicken macrophages and enhance the cells' phagocytic capacity and ability to clear intracellular ST, which has previously shown to be easily able to survive within chicken macrophages and transcribe high rates of *Salmonella* pathogenicity island 2 (*SPI-2*) genes (Braukmann et al., 2015).

The results verified that GA up-regulated the gene expression of *CD40*, *CD80*, *CD83*, and *CD197*. CD40, CD80, and CD197 are characteristic costimulatory

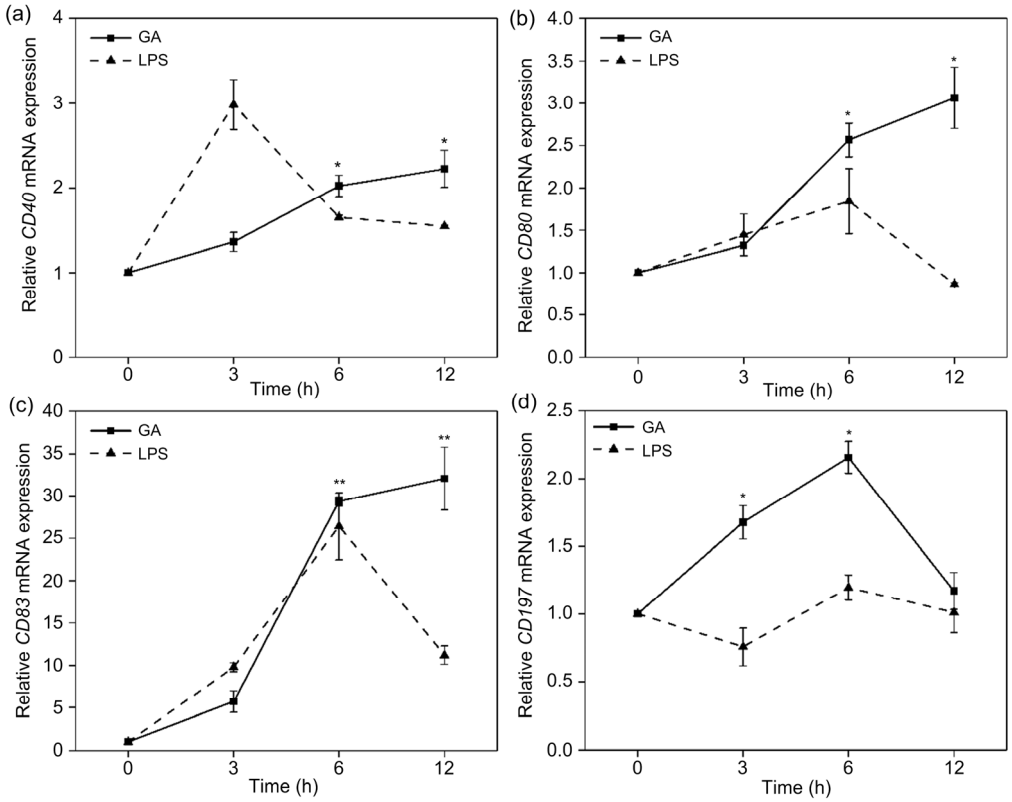

Fig. 3 Effects of glycyrrhizic acid (GA) on the expression of cell surface molecules in chicken macrophages
HD11 cells were incubated with PBS, GA (100 μg/ml), or lipopolysaccharide (LPS, 500 ng/ml) for 3, 6, and 12 h. Total RNA was isolated and the gene expression of *CD40* (a), *CD80* (b), *CD83* (c), and *CD197* (d) was analyzed by real-time PCR. Results are expressed as fold change relative to untreated cells (at time 0 h). Data are expressed as mean±SD for three independent experiments. $^{*}P$<0.05, $^{**}P$<0.01, vs. 0 h (Student's t-test)

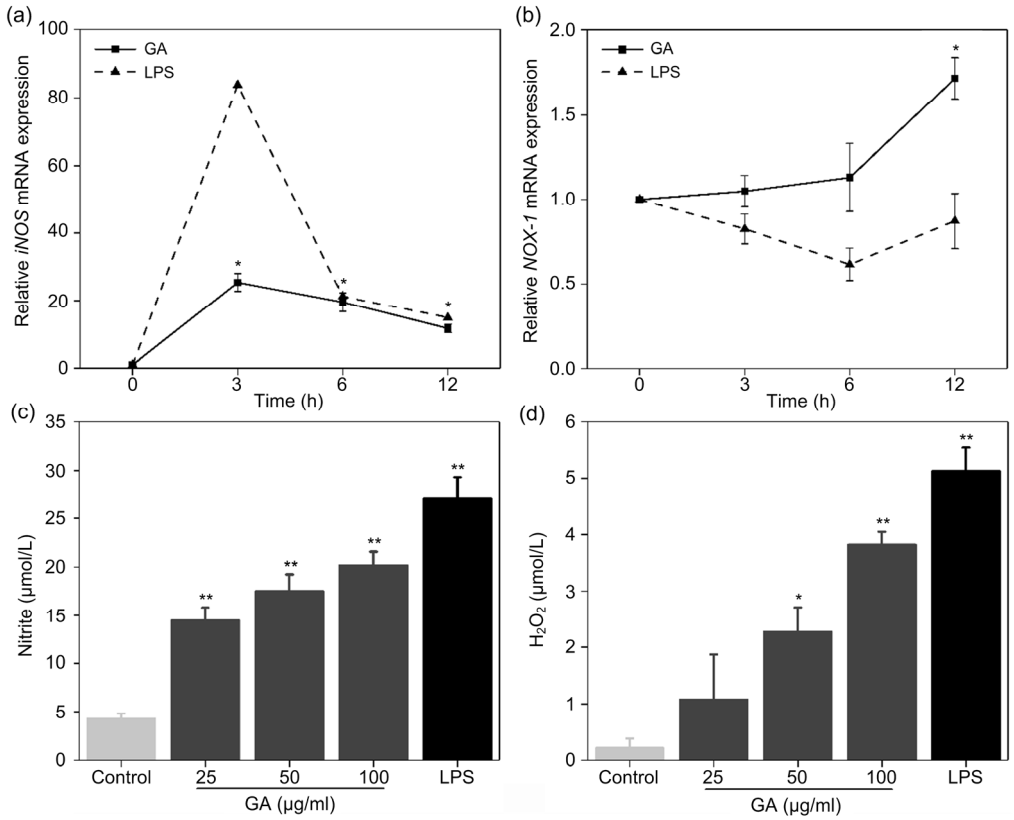

Fig. 4 Effects of glycyrrhizic acid (GA) on the productions of nitrite and hydrogen peroxide in chicken macrophages
(a, b) HD11 cells were incubated with PBS, GA (100 μg/ml), or lipopolysaccharide (LPS, 500 ng/ml) for 3, 6, and 12 h. Then total RNA was isolated and the expression of *iNOS* (a) and *NOX-1* (b) was analyzed by real-time PCR. (c, d) HD11 cells were incubated with PBS, GA (25, 50, and 100 μg/ml), or LPS (500 ng/ml) for 48 h (NO) or 12 h (H_2O_2). Culture supernatants and cell lysates were collected and the productions of NO (c) and H_2O_2 (d) were analyzed. Data are expressed as mean±SD for three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs. 0 h (a, b) or control (c, d) (Student's *t*-test)

molecules or markers for activated macrophages and play an important role in the inflammatory response to pathogen infection (Nolan et al., 2008; Brown et al., 2009). CD83 is essentially expressed on the surface of mature dendritic cells and participates in the interaction between antigen-presenting cells and T lymphocytes (Tang et al., 2005; Rimaniol et al., 2007). This induction of CD83 as well as costimulatory molecules (e.g. CD80) on macrophages suggested that GA could enhance the ability of macrophages to favor T lymphocyte activation. These results not only confirm that GA activates an innate immune response in chicken macrophages to eliminate intracellular ST, but also suggest that GA may contribute to the activation of adaptive immune response in vivo.

iNOS is generally associated with the immune system and can generate high levels of NO, which exhibits potent antivirus and antibacterial effects via several mechanisms such as mutation of DNA and inhibition of protein synthesis (Bogdan, 2001). NOX-1 is a primary NOX in macrophages. It produces superoxide from oxygen and subsequently leads to the generation of other toxic reactive oxygen intermediates, such as H_2O_2 (Rosenberger and Finlay, 2002). Previous reports have shown that both iNOS and NOX activity impaired *Salmonella* replication (Rosenberger and Finlay, 2002), and mice deficient in iNOS and NOX were unable to control ST infection (Mastroeni et al., 2000). In the present study, GA could significantly up-regulate *iNOS* and *NOX-1*

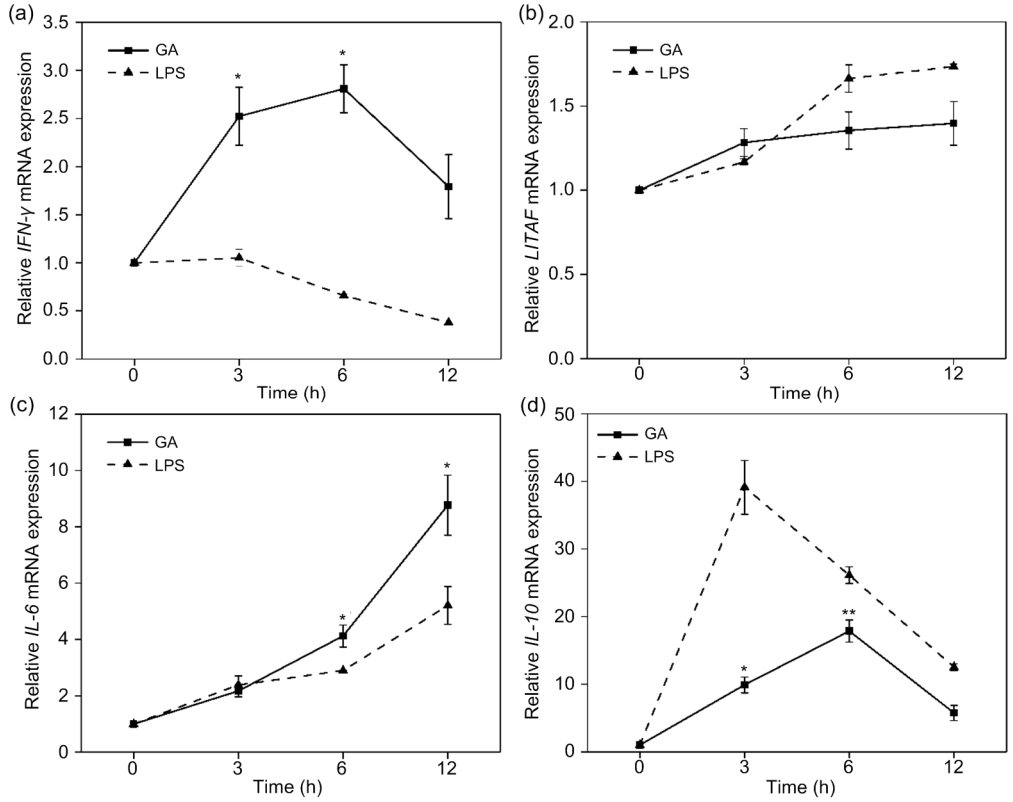

Fig. 5 Effects of glycyrrhizic acid (GA) on the expression of immune-associated cytokines in chicken macrophages
HD11 cells were incubated with PBS, GA (100 μg/ml), or lipopolysaccharide (LPS, 500 ng/ml) for 3, 6, and 12 h. Then total RNA was isolated and the gene expression of *IFN-γ* (a), *LITAF* (b), *IL-6* (c), and *IL-10* (d) was analyzed by real-time PCR. Results are expressed as mean±SD for three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, vs. 0 h (Student's *t*-test)

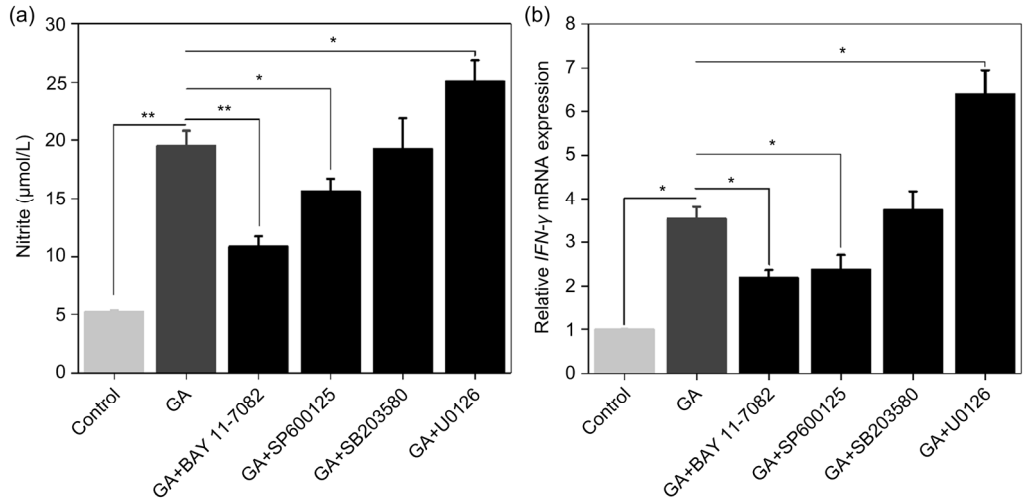

Fig. 6 Effects of glycyrrhizic acid (GA) on the signaling pathways of chicken macrophages
HD11 cells were pretreated with NF-κB inhibitor (BAY 11-7082, 20 μmol/L), JNK inhibitor (SP600125, 20 μmol/L), p38 inhibitor (SB203580, 20 μmol/L), or ERK1/2 inhibitor (U0126, 20 μmol/L) for 30 min followed by the treatment with GA (100 μg/ml) for 48 or 6 h. Then the production of nitrite (a) and expression of *IFN-γ* (b) were measured. Results are expressed as mean±SD for three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ (Student's *t*-test)

mRNA expression and thereby markedly induced the productions of NO and H_2O_2, which led to a marked reduction of ST viability in macrophages. Similar results were obtained from an in vitro study, in which β 1-4 mannobiose enhanced the productions of NO and H_2O_2, and resulted in decreased intracellular survival of SE in chicken MQ-NCSU cells (Ibuki et al., 2011).

IFN-γ, which is involved in the activation of macrophages and T lymphocytes, is another important antimicrobial molecule in host resistance against *Salmonella*. Macrophages primed with IFN-γ appeared to be more sensitive to bacterial components (e.g. LPS) than untreated cells (Sweet et al., 1998; Held et al., 1999), and IFN-γ production was up-regulated in organs after ST infection (Withanage et al., 2005) and thereby conduced to the clearance of ST in chickens (Beal et al., 2004). In our study, GA exposure could up-regulate *IFN-γ*, *LITAF*, *IL-6*, and *IL-10* gene expression, and this may amplify the bacterial signal to macrophages and thus augment the killing of ST.

It is known that macrophage activation requires stimulation of specific transcription factors, among which MAPKs (ERK1/2, JNK, and p38 MAPK) and NF-κB are well characterized in both mammalian and avian cells in response to pathogen infection (He and Kogut, 2003; Han et al., 2009). MAPKs regulate the expression of various inflammatory cytokines (He and Kogut, 2003). NF-κB proteins are detached from its inhibitor inhibitory κB (IκB) after activation, and finally translocate to the nucleus and manipulate the transcription of an array of antimicrobial genes such as *iNOS* (Han et al., 2009). We found that NF-κB and JNK pathway inhibition potently down-regulated the GA-induced NO production and *IFN-γ* expression in stimulated cells, while suppression of ERK1/2 up-regulated NO and *IFN-γ* levels. These results were supported by previous observations, in which NF-κB and JNK inhibitor successfully reduced NO and IFN-γ secretion while ERK1/2 inhibitor markedly increased NO production in murine bone marrow-derived dendritic cells (BMDCs) (Mao et al., 2015; Li et al., 2017). These findings indicated that NF-κB and JNK activation are required for GA-activated chicken macrophages, whereas ERK1/2 signaling may exhibit a regulatory effect to limit excessive inflammation.

In conclusion, our results indicated that GA could activate chicken macrophages and enhance phagocytic and *Salmonella*-killing capacity by enhancing the productions of reactive oxygen and nitrogen species and by increasing the expression of antimicrobial genes. Further in vivo studies are required to validate the efficacy of GA as a dietary intervention to reduce or eliminate *Salmonella* contamination in poultry production.

Contributors

Wei-fen LI, Bai-kui WANG, and Yu-long MAO conceived and designed the experiments; Bai-kui WANG, Yu-long MAO, and Li GONG performed the experiments; Xin XU analyzed the data; Yi-bing WANG made the figures; Bai-kui WANG, Shou-qun JIANG, and Yu-long MAO wrote the paper.

Compliance with ethics guidelines

Bai-kui WANG, Yu-long MAO, Li GONG, Xin XU, Shou-qun JIANG, Yi-bing WANG, and Wei-fen LI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 In vitro antibacterial activity of glycyrrhizic acid against *Salmonella* Typhimurium

Fig. S2 Effect of glycyrrhizic acid on *Salmonella* Typhimurium virulence gene expression in vitro

Table S1 List of real-time PCR primers

中文概要

题　目：甘草酸对体外鸡巨噬细胞免疫和杀菌功能的影响

目　的：探究甘草酸能否激活体外鸡巨噬细胞并增强其免疫和吞噬杀菌功能。

创新点：甘草酸通过核因子 κB（NF-κB）和 c-Jun 氨基端激酶（JNK）信号通路提高一氧化氮（NO）和过氧化氢（H_2O_2）产生量，增强了其吞噬和杀菌的功能。

方　法：以不同浓度的甘草酸（0、12.5、25、50、100、200、400 和 800 μg/ml）处理鸡巨噬细胞系 HD11，采用荧光定量聚合酶链式反应（qPCR）和一氧化氮及过氧化氢测定试剂盒评价甘草酸对鸡巨噬细胞活化和免疫的影响，采用流式细胞技术和涂板计数法测定鸡巨噬细胞吞噬和杀菌能力。

结　论：甘草酸通过 NF-κB 和 JNK 信号通路激活鸡巨噬细胞，提高免疫细胞因子等基因的表达水平和 NO 及 H_2O_2 的产生量，从而增强了鸡巨噬细胞吞噬和清除胞内沙门氏菌的能力。

关键词：甘草酸；鸡巨噬细胞；巨噬细胞活化；鼠伤寒沙门氏菌；核因子κB（NF-κB）；c-Jun氨基端激酶（JNK）