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Glycyrrhizin inhibits lipopolysaccharide-induced inflammatory response by reducing TLR4 recruitment into lipid rafts in RAW264.7 cells

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ABSTRACT

Background: The aim of this study was to investigate the effect of glycyrrhizin on LPS-induced endotoxemia in mice and clarify the possible mechanism.
Methods: An LPS-induced endotoxemia mouse model was used to confirm the anti-inflammatory activity of glycyrrhizin in vivo. In vitro, RAW264.7 cells were stimulated with LPS in the presence or absence of glycyrrhizin. The expression of cytokines was determined by ELISA. Toll-like receptor 4 (TLR4) was determined by Western blot analysis. Nuclear factor-kB (NF-κB) and Interferon regulatory factor 3 (IRF3) activation were detected by Western blotting and luciferase assay. Lipid raft staining was detected by immunocytochemistry.
Results: In vivo, the results showed that glycyrrhizin can improve survival during lethal endotoxemia. In vitro, glycyrrhizin dose-dependently inhibited the expression of TNF-α, IL-6, IL-1β and RANTES in LPS-stimulated RAW264.7 cells. Western blot analysis showed that glycyrrhizin suppressed LPS-induced NF-κB and IRF3 activation. However, glycyrrhizin did not inhibit NF-κB and IRF3 activation induced by MyD88-dependent (MyD88, IKKβ) or TRIF-dependent (TRIF, TBK1) downstream signaling components. Moreover, glycyrrhizin did not affect the expression of TLR4 and CD14 induced by LPS. Significantly, we found that glycyrrhizin decreased the levels of cholesterol of lipid rafts and inhibited translocation of TLR4 to lipid rafts. Moreover, glycyrrhizin activated ABCA1, which could induce cholesterol efflux from lipid rafts.
Conclusion: Glycyrrhizin exerts an anti-inflammatory property by disrupting lipid rafts and inhibiting translocation of TLR4 to lipid rafts, thereby attenuating LPS-mediated inflammatory response.
General significance: Learning the anti-inflammatory mechanism of glycyrrhizin is crucial for the anti-inflammatory drug development.

1. Introduction

Inflammation is a reaction of tissue to irritation, injury or infection, typically caused by various bacterial infections [1]. Bacterial LPS, a potent immune system activator, is an important risk factor for inflammation. Macrophages play an important role in various inflammatory responses [2]. LPS activates macrophages, triggering inflammatory mediators, including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), metalloproteinases cycloxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and prostaglandin E2 (PGE2) [3–5]. These inflammatory mediators lead to inflammation and various other clinical manifestations.

Toll-like receptors (TLRs) are a large family of Type I transmembrane receptors that play an integral role in the innate immune system. A total of 13 Toll-like receptors (TLRs) have been recently identified [6–8]. Located on the cell plasma membrane or within endosomes, TLRs recognize conserved, characteristic molecular structures on infectious agents called "pathogen associated molecular patterns" (PAMPs). LPS, the integral molecules within the outer membrane of Gram-negative bacteria, activates the expression of TLR4 [9–11]. LPS primarily signals via TLR4 receptors. TLR4 associates with CD14 and MD-2 to recognize LPS. First, LPS binds to CD14 to form the CD14–LPS–LBP complex. Then, the complex is presented to the TLR4–MD-2 complex [12]. Finally, the intact plasma membrane microdomains facilitate the formation of the receptor complex to initiate TLR4 signaling.

The activation of TLR4 by LPS induces MyD88-dependent and MyD88-independent signaling pathways. The MyD88-dependent pathway requires signal transduction intermediates such as IL-1RI-associated protein kinases (IRAKs), and transforming growth factor-activated kinase (TAK1), for the activation of NF-κB and the production of pro-inflammatory cytokines [13]. The MyD88-independent pathway requires

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signal transduction intermediates, such as TICAM1 and TICAM2, to ultimately activate the transcription factor IRF3 and to induce the production of IFNβ and IFN-inducible genes [14,15]. The activation of TLR4 by LPS induces NF-κB and IRF3 activation, resulting in the release of cytokines [13]. Therefore, treatments aimed at modulating TLR4 signaling might have potential therapeutic advantages for inflammatory diseases.

Glycyrrhizin, a triterpene glycoside isolated from licorice root (Fig. 1), is responsible for the pharmacological activities of this plant. It has been shown that glycyrrhizin exhibits a broad spectrum of anti-inflammatory effect. Glycyrrhizin inhibits nitric oxide (NO) activity, prostaglandin E_2 (PEG_2) activity and inflammatory cytokine production in LPS-activated macrophages [16] and LPS-induced acute lung injury in mice [17]. Recently, it has been reported that the anti-inflammatory effects of glycyrrhizin are mediated by blocking the activation of NF-κB signaling [18,19]. However, the molecular targets of the anti-inflammatory actions of glycyrrhizin in LPS-stimulated macrophages remain unclear.

The aim of this work was to examine the anti-inflammatory effects of glycyrrhizin in LPS-stimulated macrophage and to identify the molecular targets of glycyrrhizin in the TLR4 signaling pathway. The results showed that glycyrrhizin inhibited LPS-induced NF-κB and IRF3 activation and cytokine production through the inhibition of TLR4 translocation and the disruption of lipid rafts.

2. Materials and methods

2.1. Materials

Glycyrrhizin (99.8%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dimethyl sulfoxide (DMSO), LPS (*Escherichia coli* 055:B5), and 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone. Mouse TNF-α, IL-6 and IL-1β ELISA kits were purchased from BioLegend (CA, USA). Mouse RANTES ELISA kits were purchased from R&D Systems (Minneapolis, MN). Mouse mAb Phospho-NF-κB, mouse mAb NF-κB, mouse mAb Phospho-IRF3 and rabbit mAb IRF3 were purchased from Cell Signaling Technology Inc. (Beverly, MA). HRP-conjugated goat anti-rabbit antibodies were provided by GE Healthcare (Buckinghamshire, UK). All other chemicals were of reagent grade.

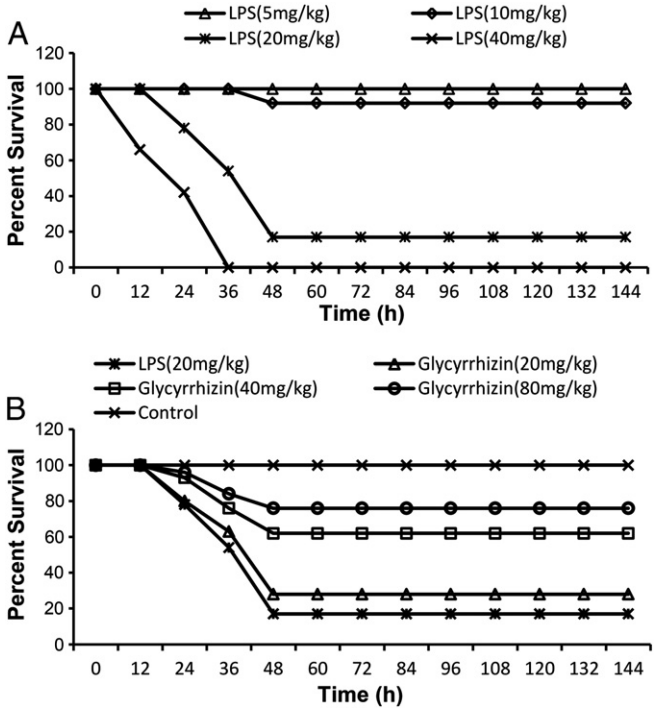

Fig. 1. Effects of glycyrrhizin on LPS-induced lethality in mice. Mice were given an intraperitoneal injection of glycyrrhizin (20, 40 and 80 mg/kg) 1 h prior to LPS challenged. A, the survival rate of mice challenged with LPS of different doses. B, effect of glycyrrhizin (20, 40 and 80 mg/kg) treatment on LPS-induced lethality. The survival was monitored every 12 h for 7 days. $^{\#}p < 0.01$ vs. control group, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. LPS group.

2.2. Cell culture and treatment

The RAW264.7 mouse macrophage cell line was purchased from the China Cell Line Bank (Beijing, China) and cultured in DMEM containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. HEK293 cells, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Media was changed once every 48 h. In all experiments, macrophages were incubated in the presence or absence of various concentrations of glycyrrhizin that was always added 1 h prior to LPS (1 μg/ml) treatment.

2.3. Plasmids

Recombinant vectors encoding TLR4 (NM_021297), MD2 (NM_001159711), MyD88 (NM_010851), TRIF (BC094338), and IKKβ (AF026524; GenBank accession numbers in parentheses) were generated by the PCR-based amplification of RAW264.7 cDNA, followed by subcloning into the pcDNA3.1 eukaryotic expression vector (Invitrogen) as previously described. The TBK1 and IFNβ PRDIII-I luciferase plasmids were obtained from Kate Fitzgerald (University of Massachusetts Medical School) via Addgene. The NF-κB-luciferase reporter plasmid was purchased from Stratagene (La Jolla, CA, USA).

2.4. Animals

Male BALB/c mice, 6–8 weeks, weighing approximately 18 to 20 g, were purchased from the Center of Experimental Animals of Baiqiuen Medical College of Jilin University (Jilin, China). And this study was approved by the Jilin University Animal Care and Use Committee. The protocols were reviewed and approved by the committee. The mice were housed in microisolator cages and received food and water. The laboratory temperature was 24 ± 1 °C, and relative humidity was 40–80%. Mice were housed for 4–6 days to adapt the environment before experimentation. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.5. LPS-induced endotoxemia in mice

The 48 healthy male BALB/c mice were randomly classified into four groups and challenged with LPS (5–40 mg/kg) by i.p. The mortality of mice was observed twice a day for 7 days. In drug testing, the effect of glycyrrhizin (20, 40 and 80 mg/kg) on LPS-induced mortality was assessed by given glycyrrhizin 1 h before LPS challenge. Survival in each group was assessed every 12 h for 7 days.

2.6. Cell transfection and luciferase assay

HEK293 cells were co-transfected with a luciferase plasmid and various expression plasmids or the corresponding empty plasmid vectors using FuGENE HP transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. The β-galactosidase plasmid was co-transfected as an internal control. Luciferase activity was measured using the Luciferase Reporter-Gene Assay (Promega). β-Galactosidase enzyme activity was determined

using the β-galactosidase Enzyme System (Promega) according to the manufacturer's instructions. The luciferase activity was normalized to the β-galactosidase activity.

2.7. MTT assay for cell viability

An MTT assay was used to measure cell viability. Briefly, RAW264.7 cells were plated at a density of 4×10^5 cells/ml in 96-well plates at 37 °C, and 5% CO_2 for 1 h. The cells were subsequently treated with 50 μl of glycyrrhizin at different concentrations (0–300 μg/ml) for 1 h, followed by stimulation with 50 μl LPS. After 18 h of LPS stimulation, 20 μl of MTT (5 mg/ml) was added to each well, and the cells were further incubated for an additional 4 h. The supernatant was removed and the formation of formazan was resolved using 150 μl/well DMSO. The optical density was measured at 570 nm on a microplate reader (TECAN, Austria).

2.8. ELISA assay

RAW264.7 cells were seeded in 24-well plates (4×10^5 cells/well), and incubated in the presence of either 1 μg/ml LPS alone, or LPS plus 50 μg/ml, 100 μg/ml, and 200 μg/ml glycyrrhizin for 18 h. Cell-free supernatants were subsequently employed for the cytokine assays using a mouse enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (BioLegend).

2.9. Total RNA isolation and quantitative real-time polymerase chain reaction

RAW264.7 cells were treated with various concentrations of glycyrrhizin for 1 h followed by incubation with or without 1 μg/ml LPS for 3 h. The total RNA was extracted using TRIzol (Invitrogen) by following the manufacturer's instructions. The RNA was reverse-transcribed into cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Thermo). The relative mRNA concentrations were detected by qRT-PCR using a 7500 Fast Real-Time PCR System (Applied Biosystems) and a SYBR green Plus reagent kit (Roche), as has already been described elsewhere [20]. The primers used for qRT-PCR are listed in Table 1. Each sample was run three times to generate a single product. Melt curves were used to analyze and assess the accuracy of the PCR. $2^{-\triangle\triangle Ct}$ values were chosen to evaluate the expression of candidate genes.

2.10. Western blot analysis

RAW264.7 cells were seeded in 6-well plates and incubated for 24 h, followed by pretreatment with glycyrrhizin for 1 h. After LPS (1 μg/ml) stimulation for 1 h, the cells were collected and washed twice with cold PBS. Total proteins from cells were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo). Nuclear and Cytoplasmic Proteins were extracted from the lungs using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology; China) according to the manufacturer's protocol. Membrane proteins from cells were extracted using Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo). The protein concentration was determined using the BCA method. The proteins were separated by SDS-PAGE using Tris–HCl Precast Gels and subsequently transferred onto PVDF membranes. The resulting membrane was blocked with phosphate buffered saline containing 0.05% Tween-20 (PBS-T), supplemented with 3% skim milk at room temperature for 2 h on a rotary shaker, followed by PBS-T washing. The membranes were washed with a specific primary antibody diluted in PBS-T containing skim milk at 4 °C overnight. Subsequently, the membranes were washed with PBS-T, followed by incubation with the secondary antibody conjugated with horseradish peroxidase at room temperature for 1 h. The blots were again washed with PBS-T and subsequently developed using the ECL Plus Western Blotting Detection System (Amersham Life Science, UK).

Table 1
Sequence of primers used in current investigation in qRT-PCR.

Gene	Primer	Sequence 5′ > 3′	Product size (bp)
TNF-α	Sense	GCCTCCCTCTCATCAGTTCTA	246
	Anti-sense	GGCAGCCTTGTCCCTTG	
IL-1β	Sense	ACCTGTGTCTTTCCCGTGG	162
	Anti-sense	TCATCTCGGAGCCTGTAGTG	
IL-6	Sense	AGTTGTGCAATGGCAATTCTGA	223
	Anti-sense	AGGACTCTGGCTTTGTCTTTCT	
RANTES	Sense	ACCACTCCCTGCTGCTTT	130
	Anti-sense	ACACTTGGCGGTTCCTTC	
β-Actin	Sense	TGCTGTCCCTGTATGCCTCT	224
	Anti-sense	TTTGATGTCACGCACGATTT	

2.11. Immunocytochemistry and lipid raft staining

Cell membrane lipid rafts were labeled with cholera toxin subunit B (CTxB). The cells were fixed in 4% formaldehyde for 30 min at room temperature, followed by incubation with Alexa Fluor 488-conjugated CTxB (5 μg/ml) for 20 min and washing with PBST for 8 min. The cells were stained with Hoechst for 5 min in the dark, followed by washing with PBS three times. The fluorescent images were obtained using Fluoview microscopy (Olympus, Japan).

2.12. Isolation of lipid rafts

Lipid rafts were isolated as previously described [21]. Briefly, RAW264.7 cells were lysed in ice-cold MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl, 1 mM Na_3VO_4, 1% Triton X-100, and protease inhibitors). The lysates were mixed with 4 ml of 40% sucrose, obtained via mixing with 2 ml of 80% sucrose and overlaid with 4 ml of 35% sucrose and 4 ml of 5% sucrose in the MBS buffer. The samples were ultracentrifuged at 100,000 *g* for 18 h and fractionated into 12 subfractions.

2.13. Cholesterol replenishment experiment

RAW264.7 cells were treated with culture medium alone or medium containing glycyrrhizin (50, 100, 200 μg/ml), or MβCD (10 mM) at 37 °C for 60 min. Subsequently the cells were washed with PBS and incubated with medium alone or medium containing water-soluble cholesterol (84 μg/ml) for 30 min. The cells were exposed to LPS. The translocation of TLR4 to lipid rafts was analyzed as mentioned above.

2.14. Statistical analysis

The data are presented as the means ± SD. Comparisons between groups were obtained using ANOVA followed by Dunnett's test. *p*-Values of 0.05 or less were considered statistically significant.

3. Results

3.1. Effects of glycyrrhizin on LPS-mediated mortality

To determine a suitable concentration of LPS for inducing endotoxemia, the mice were challenged with LPS (5–40 mg/kg), the dose response of LPS on mortality was shown in Fig. 2A. Mice were given 5, 10, 20, 40 mg/kg of LPS, the mortality rates were 0%, 8%, 83%, and 100%, respectively. Therefore, 20 mg/kg LPS was chosen as lethal dosage to induce endotoxemia in mice. The effect of glycyrrhizin on LPS-induced mortality was assessed by measuring

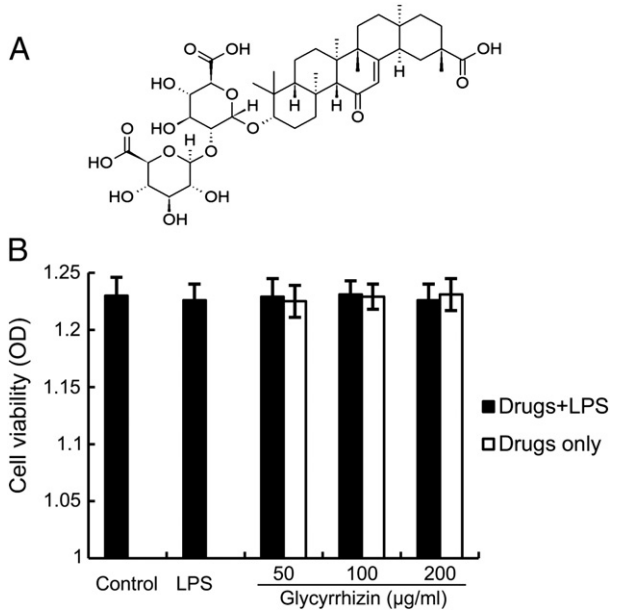

Fig. 2. (A) Chemical structure of glycyrrhizin. (B) Effect of glycyrrhizin on the cell viability of RAW264.7 cells. Cells were cultured with different concentrations of glycyrrhizin (0–300 μg/ml) in the absence or presence of 1 μg/ml LPS for 24 h. The cell viability was determined by MTT assay. The values presented are the means ± SEM of three independent experiments.

survival of mice challenged with 20 mg/kg of LPS. As shown in Fig. 2B, mice receiving 20, 40 or 80 mg/kg carvacrol were 28%, 62% and 76% protective respectively ($p < 0.01$ or $p < 0.05$).

3.2. Effects of glycyrrhizin on cell viability

The potential cytotoxicity of glycyrrhizin was evaluated using the MTT assay after incubating cells for 18 h in the absence or presence of LPS, and the results showed that cell viability was not affected by the glycyrrhizin treatment at the concentrations used (50, 100, and 200 μg/ml) (Fig. 2). Thus, the effects of glycyrrhizin on RAW264.7 cells were not attributable to cytotoxic effects.

3.3. Glycyrrhizin dose-dependently inhibits the expression of cytokines in LPS-stimulated RAW264.7 cells

To analyze the potential anti-inflammatory effects of glycyrrhizin, we determined whether glycyrrhizin affected the expression of cytokines in LPS-stimulated RAW264.7 cells. The expression of TNF-α, IL-6, IL-1β and RANTES was detected by ELISA and qRT-PCR. The results showed that glycyrrhizin suppressed TNF-α, IL-6, IL-1β and RANTES expression in LPS-stimulated RAW264.7 cells in a dose-dependent manner both in protein (Fig. 3) and mRNA levels (Fig. 4).

3.4. Glycyrrhizin inhibits TLR4-mediated IL-8 production in mTLR4 and mMD-2 co-transfected HEK293 cells

To confirm that glycyrrhizin restrains the inflammatory response by targeting TLR4 signal pathways, we detected the effect of glycyrrhizin on the production of IL-8 in LPS-stimulated HEK293-TLR4/MD-2 cells. The cells were treated with various concentrations of glycyrrhizin for 1 h followed by incubation with or without 1.0 μg/ml LPS for 18 h. Cell-free supernatants were subsequently employed for the IL-8 assays. The results showed that glycyrrhizin dose-dependently inhibits IL-8 production in LPS-stimulated HEK293-TLR4/MD-2 cells (Fig. 5).

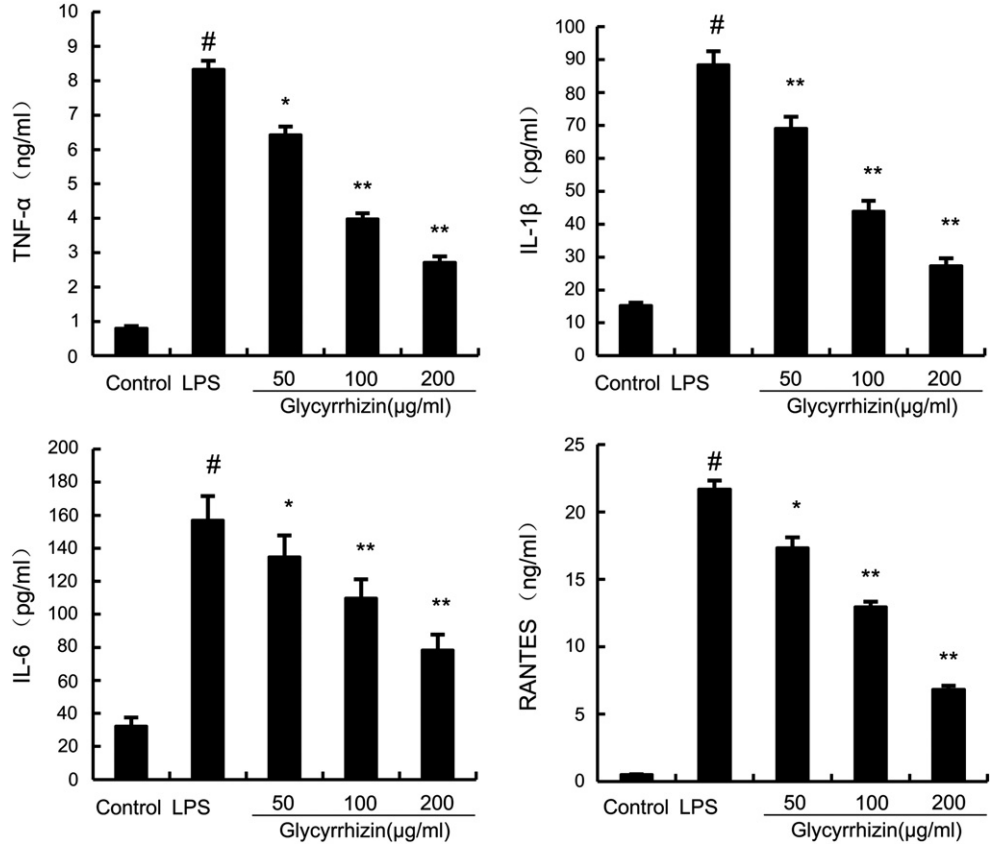

Fig. 3. Glycyrrhizin inhibits lipopolysaccharide (LPS)-induced cytokine production in a dose-dependent manner. Cells were treated with 1 μg/ml LPS in the absence or presence of glycyrrhizin (50, 100, 200 μg/ml) for 18 h. Levels of TNF-α, IL-1β, IL-6 and RANTES in culture supernatants were measured by ELISA. The data presented are the means ± SEM of three independent experiments and differences between mean values were assessed by ANOVA. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group.

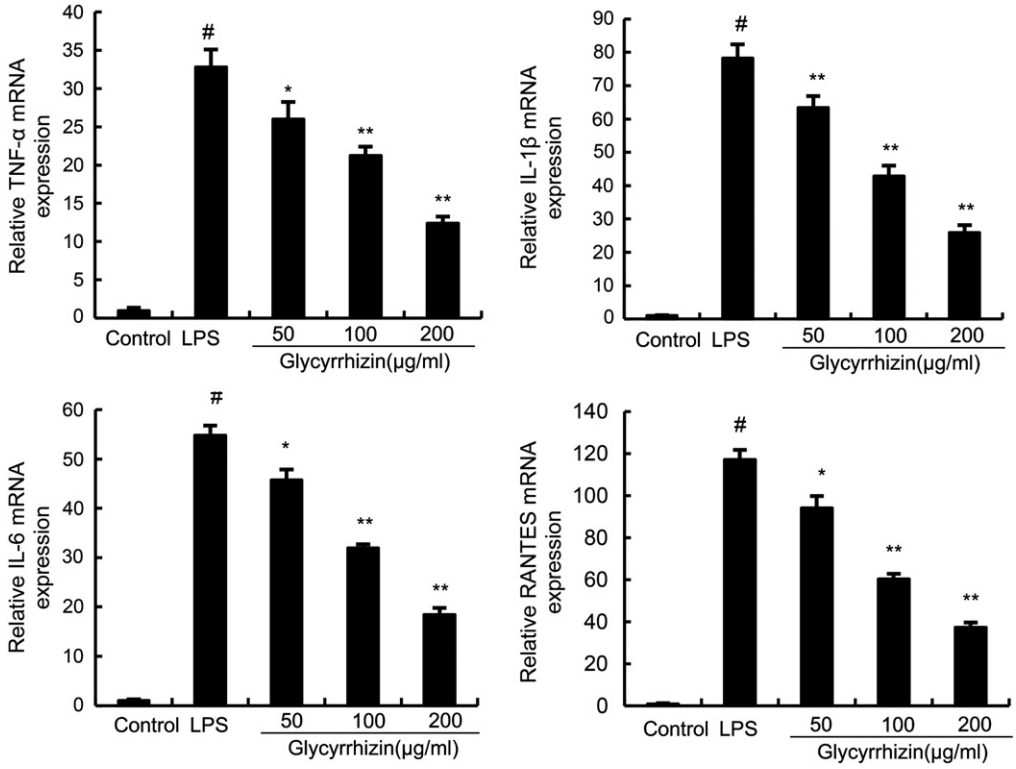

Fig. 4. Glycyrrhizin inhibits lipopolysaccharide (LPS)-induced cytokine production in a dose-dependent manner. Cells were treated with 1 μg/ml LPS in the absence or presence of glycyrrhizin (50, 100, 200 μg/ml) for 18 h. Levels of mRNA levels of TNF-α, IL-1β, IL-6 and RANTES were measured by qRT-PCR. The data presented are the means ± SEM of three independent experiments and differences between mean values were assessed by ANOVA. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group.

3.5. Glycyrrhizin suppresses LPS-induced NF-κB and IRF3 activation

NF-κB and IRF3 are important signaling molecules in the development of inflammatory diseases. The activation of TLR4 induces two signaling pathways: MyD88 and TRIF dependent signaling pathways, which induce NF-κB and IRF3 activation and the eventual release of inflammatory cytokines. To determine whether glycyrrhizin mediates the inhibition of the inflammatory response through the NF-κB and IRF3 pathways, NF-κB and IRF3 protein expression was determined by Western blotting. The results showed that glycyrrhizin significantly inhibited the activation of NF-κB and IRF3 (Fig. 6).

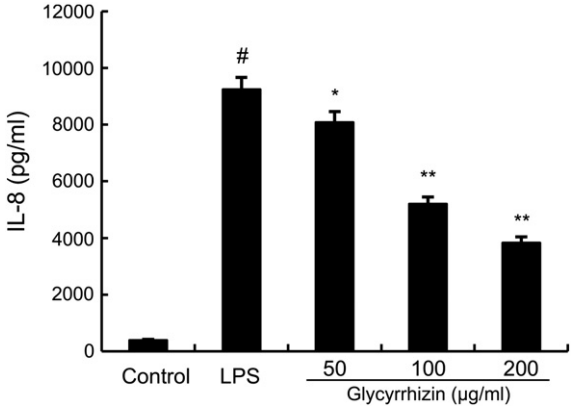

Fig. 5. Glycyrrhizin inhibits IL-8 production in LPS-treated HEK293-mTLR4/MD2 cells in a dose-dependent manner. Cells were treated with 1 μg/ml LPS in the absence or presence of glycyrrhizin (50, 100, 200 μg/ml) for 24 h. Level of IL-8 in culture supernatants was measured by ELISA. The data presented are the means ± SEM of three independent experiments and differences between mean values were assessed by ANOVA. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group.

3.6. Glycyrrhizin does not suppress the induction of NF-κB and IRF3 activation through downstream signaling components of TLR4

The activation of TLR4 induces two signaling pathways: MyD88 and TRIF dependent signaling pathways, which induce NF-κB and IRF3 activation and the eventual release of inflammatory cytokines. In this study, we investigated the inhibitory effects of glycyrrhizin on NF-κB and IRF3 activation induced by the TLR4 downstream signaling components MyD88, IKKβ, TRIF or TBK1. The results showed that glycyrrhizin did not inhibit NF-κB activation induced by MyD88 or IKKβ in HEK293 cells (Fig. 7A, B). Moreover, glycyrrhizin did not inhibit IRF3 activation induced by TRIF or TBK1 in HEK293 cells (Fig. 7C, D). These results demonstrate that the glycyrrhizin-mediated inhibition of LPS-activated TLR4 signaling does not occur through MyD88 or TRIF and their downstream signaling components (the intracellular components of TLR4 signaling pathway). These results indicate that glycyrrhizin exerted anti-inflammatory actions through effects on membrane components, and not through a specific block in the downstream signaling cascades of the TLR4 signaling pathway.

3.7. Glycyrrhizin does not affect CD14 expression and TLR4 expression

The membrane components of LPS signaling involve TLR4, CD14, MD2, lipid rafts and other molecules. In this study, we explored the effects of glycyrrhizin on different components of the membrane complex. TLR4 is the major receptor for LPS. The expression of TLR4 directly affects NF-κB activation in RAW264.7 cells exposed to LPS. To investigate whether glycyrrhizin inhibits LPS-induced NF-κB activation and proinflammatory cytokine production through the suppression of TLR4 expression, we determined TLR4 expression by Western blotting. The results showed that glycyrrhizin did not affect the LPS-induced up-regulation of TLR4 expression (Fig. 7). The cell-membrane CD14 (mCD14) is a glycophosphatidylinositol-linked protein, which is part of the LPS receptor complex. In this study, we detected CD14 expression

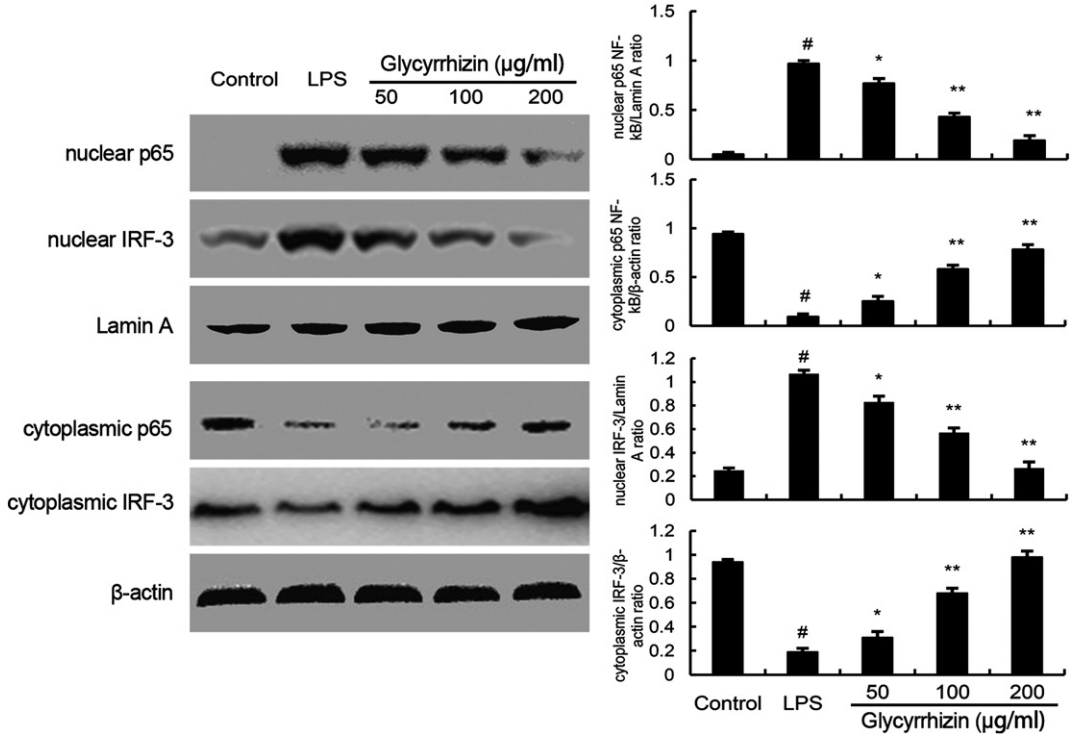

Fig. 6. Glycyrrhizin inhibits lipopolysaccharide (LPS)-induced NF-κB and IRF3 activation. RAW264.7 macrophages were preincubated with glycyrrhizin (50, 100, 200 μg/ml) for 1 h and then treated with 1 μg/ml LPS for 1 h. Protein samples were analyzed by Western blot with specific antibodies. β-Actin was used as a control. The values presented are the means ± SEM of three independent experiments and differences between mean values were assessed by ANOVA. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group.

by Western blotting. The results showed that glycyrrhizin did not affect the LPS-induced up-regulation of CD14 expression (Fig. 8).

3.8. Glycyrrhizin disrupts the formation of lipid rafts in cell membranes by depleting cholesterol

In this study, we examined whether glycyrrhizin exerts an anti-inflammatory property through the disruption of lipid rafts. Using confocal laser microscopy, we detected the accumulation of lipid rafts on the cell surface of macrophages. RAW264.7 cells were pretreated with glycyrrhizin or MβCD and stimulated with LPS for 1 h, followed by staining with Alexa Fluor 488-conjugated CTxB. MβCD was used as a control. The results showed that glycyrrhizin decreased the levels of membrane cholesterol in a dose-dependent manner (Fig. 9).

3.9. Glycyrrhizin inhibits TLR4 translocation to lipid rafts

Lipid rafts are involved in TLR4 signaling. Stimulating cells with LPS recruits TLR4 to lipid rafts. To further address the potential anti-inflammatory effects of glycyrrhizin, we determined the effects of glycyrrhizin on TLR4 translocation to lipid rafts. We isolated raft fractions and examined the translocation of TLR4 through immunoblotting. The results showed that LPS stimulation induced the localization of TLR4 to raft fractions. This effect was inhibited by pretreatment with glycyrrhizin or MβCD (Fig. 10).

3.10. Cholesterol replenishment prevents the effect of glycyrrhizin on translocation of TLR4 to lipid rafts induced by LPS

To further investigate the anti-inflammatory mechanism of glycyrrhizin, cholesterol replenishment experiments were carried out. As shown in Fig. 10, the inhibition effect of glycyrrhizin on LPS-induced TLR4 translocation to lipid rafts was abolished.

3.11. Glycyrrhizin up-regulates the expression of ABCA1 in RAW264.7 cells

ABCA1 is a lipid pump that effluxes cholesterol and phospholipid out of cells [22,23]. Activation of ABCA1 and ABCG1 could induce cholesterol efflux from plasma membrane microdomains known as lipid rafts [24]. To test why cholesterol decreased on the cell surface by glycyrrhizin, the expression of ABCA1 was detected by Western blotting. As shown in Fig. 11, glycyrrhizin up-regulated the expression of ABCA1 in a dose-dependent manner.

4. Discussion

Although several studies have investigated the anti-inflammatory activity of glycyrrhizin, the molecular targets of glycyrrhizin remain unclear. In the present study, we evaluated the anti-inflammatory effects and elucidated the potential molecular mechanism of glycyrrhizin on LPS-stimulated RAW264.7 cells. The results showed that glycyrrhizin suppressed the production of cytokines through the inhibition of lipid raft accumulation and LPS-induced TLR4 signaling.

Cytokines play an important role in inflammatory diseases. TNF-α is the earliest and primary endogenous mediator of inflammatory reactions [25,26]. IL-1β is a major pro-inflammatory cytokine, which mediates the inflammatory response at both the local and systemic levels [27,28]. IL-6 plays an important role in the acute-phase response of inflammation [29,30]. To explore the potential anti-inflammatory effects of glycyrrhizin in vitro, the effects of glycyrrhizin on the production of these cytokines were examined. The results demonstrated that glycyrrhizin inhibited the secretion of TNF-α, IL-1β, IL-6 and RANTES in LPS-stimulated RAW264.7 cells in a dose-dependent manner. The expression of these cytokines is generally modulated through NF-κB and IRF3 pathways. To detect the inhibitory mechanism of TNF-α, IL-1β, IL-6 and RANTES production, we examined the effects of glycyrrhizin on NF-κB and IRF3 activation. The results showed that glycyrrhizin pre-treatment significantly inhibited LPS-induced NF-κB and IRF3 activation in RAW264.7 cells. We further investigated whether the

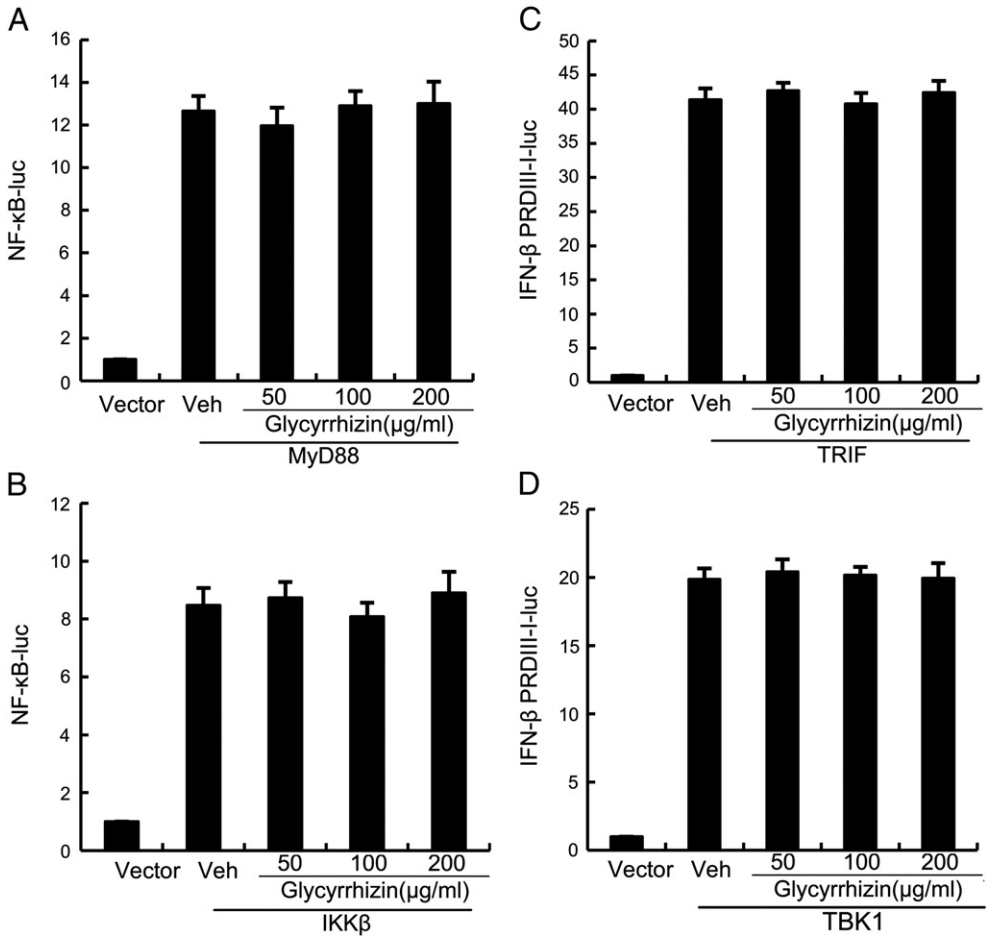

Fig. 7. Glycyrrhizin does not suppress NF-κB activation induced by MyD88 or IKKβ in MyD88-dependent signaling pathways (A and B). HEK293 cells were transfected with NF-κB luciferase reporter plasmid and the expression plasmid of MyD88 or IKKβ. 24 h later, cells were treated with glycyrrhizin (50, 100, 200 μg/ml) for 6 h. Relative luciferase activity was determined by normalization with β-galactosidase activity. The data presented are the means ± SEM (n = 3). Glycyrrhizin does not suppress IRF3 activation induced by TRIF or TBK1 in MyD88-independent signaling pathways (C and D). HEK293 cells were transfected with IFNβ PRDIII-I luciferase plasmid and the expression plasmid of TRIF or TBK1. 24 h later, cells were treated with glycyrrhizin (50, 100, 200 μg/ml) for 6 h. Relative luciferase activity was determined by normalization with β-galactosidase activity. The data presented are the means ± SEM (n = 3).

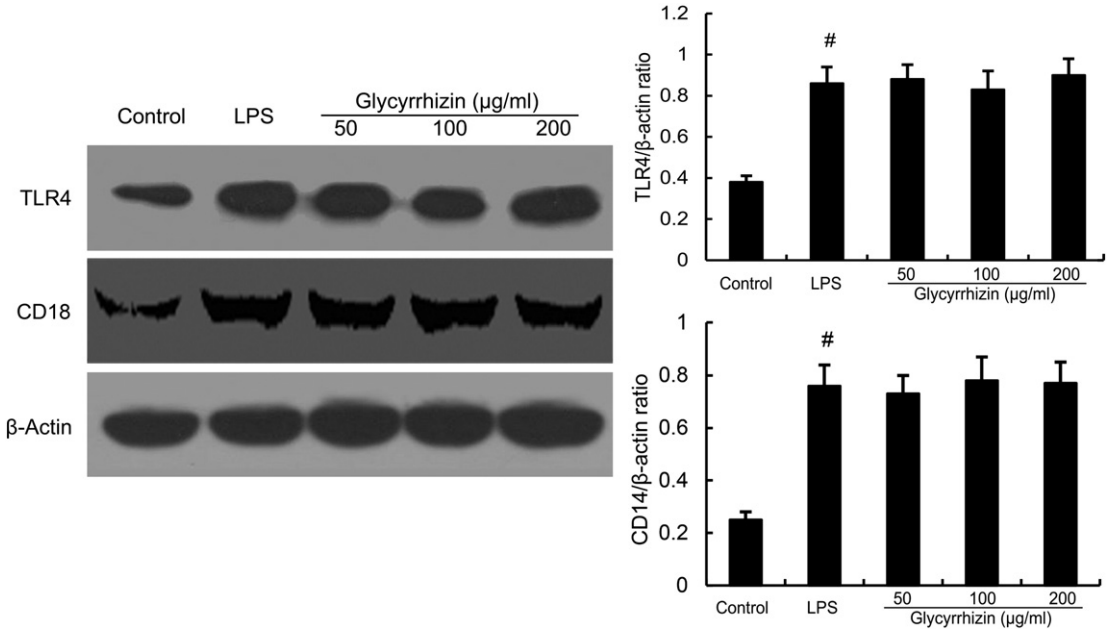

Fig. 8. The inhibition of glycyrrhizin does not affect TLR4 and CD14 expression. Cells were preincubated with glycyrrhizin (50, 100, 200 μg/ml) for 1 h and then treated with 1 μg/ml LPS for 6 h. The protein samples were analyzed using Western blotting with specific antibodies. β-Actin was used as a control. The values are presented as the means ± SEM of three independent experiments, and the differences between the mean values were assessed using ANOVA. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group.

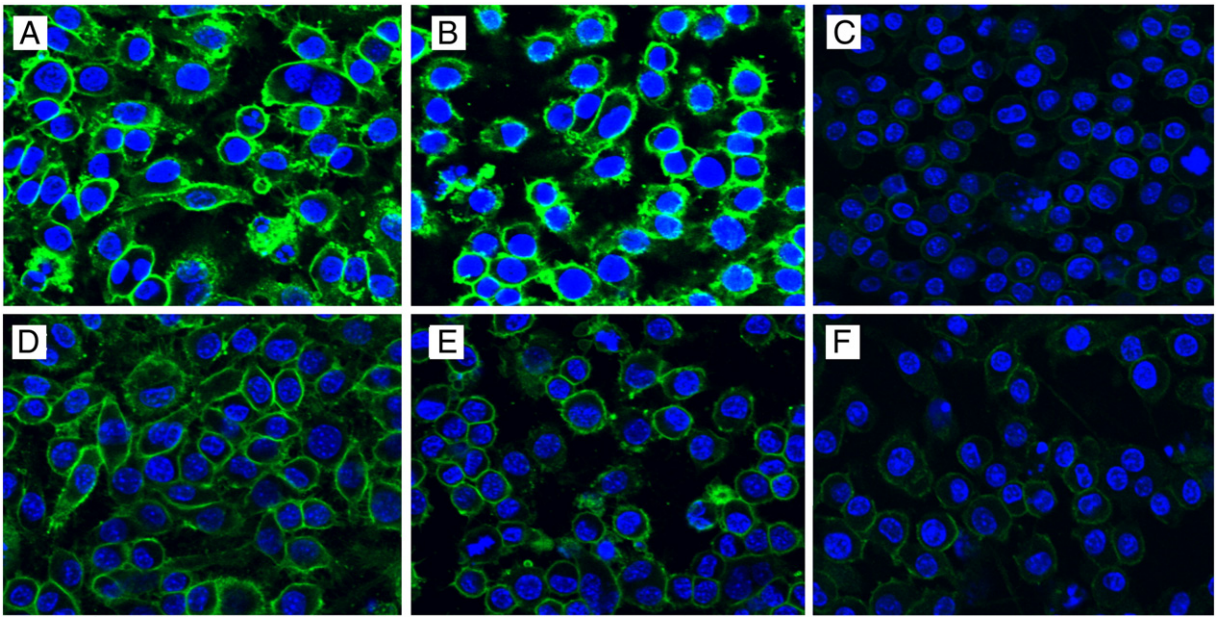

Fig. 9. The inhibition of membrane cholesterol levels by glycyrrhizin. The cells were preincubated with glycyrrhizin (50, 100, 200 μg/ml) for 1 h, followed by treatment with 1 μg/ml LPS for 1 h. The lipid rafts (green) were stained with Alexa Flour 488-conjugated CTxB and the nucleus was stained with Hoechst. (A) control group, (B) LPS group, (C) LPS + MβCD group, (D) LPS + glycyrrhizin 50 μg/ml, (E) LPS + glycyrrhizin 100 μg/ml, (F) LPS + glycyrrhizin 200 μg/ml.

anti-inflammatory activity of glycyrrhizin was exerted through TLR4-mediated signaling. Our results showed that glycyrrhizin inhibited LPS-induced IL-8 production in HEK293-mTLR4/MD-2 cells suggesting that glycyrrhizin exerts anti-inflammatory activity through the inhibition of TLR4 signaling.

LPS primarily signals via TLR4 receptors. LPS activates the TLR4-mediated signaling pathway, and leading to the activation of NF-κB and IRF3 to regulate the release of cytokines [13,31]. To investigate the molecular targets of the anti-inflammatory action of glycyrrhizin, we first detected the effects of glycyrrhizin on the TLR4 intracellular signaling pathway: from MyD88 to NF-κB activation, from TRIF to IRF3 activation. The results showed that glycyrrhizin did not inhibit NF-κB and IRF3 activation induced by MyD88 or TRIF and their downstream signaling components suggesting that the molecular target of glycyrrhizin is upstream of these signaling molecules. In other words, the molecular target of glycyrrhizin might act on the plasma membrane components of the LPS signaling pathway.

TLR4 is the major receptor for LPS. The expression of TLR4 directly affects NF-κB and IRF3 activation in RAW264.7 cells exposed to LPS. To investigate whether glycyrrhizin inhibits LPS-induced NF-κB and IRF3 activation and cytokine production through the suppression of TLR4 expression, we determined TLR4 expression using Western blot analysis. The results showed that glycyrrhizin did not affect the expression of TLR4 up-regulated by LPS. Cell-membrane CD14 (mCD14) is a glycophosphatidylinositol-linked protein, which is part of the LPS receptor complex [32]. Thus, we investigated whether glycyrrhizin

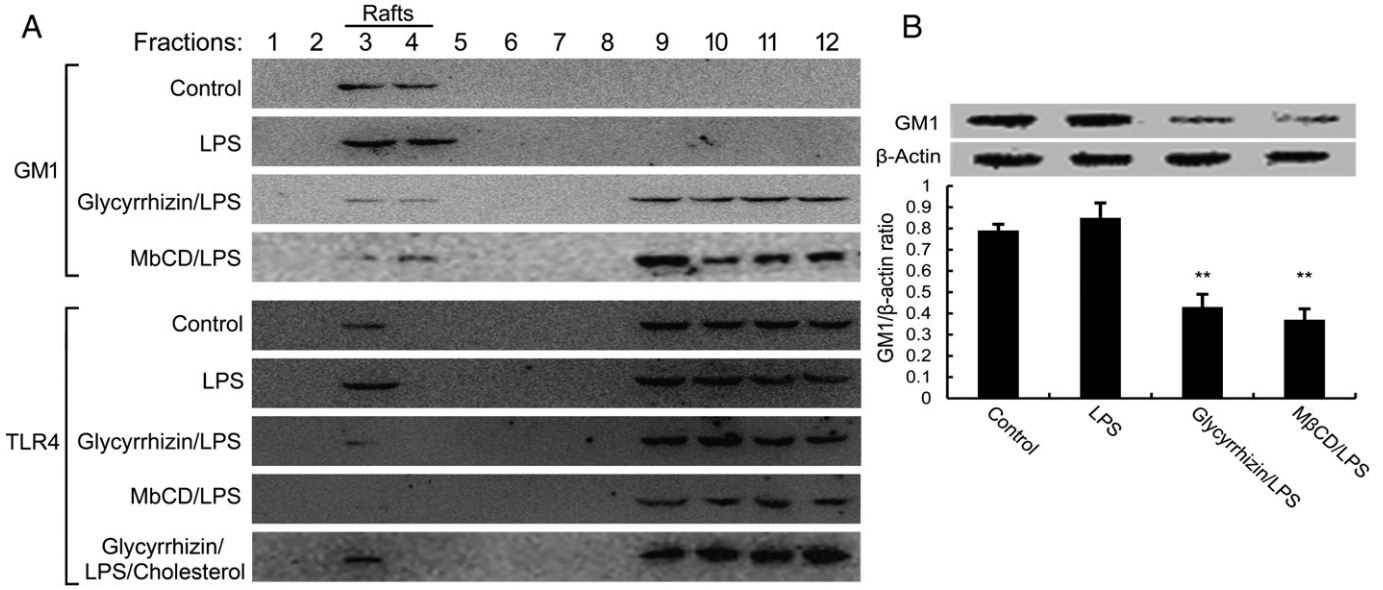

Fig. 10. The recruitment of TLR4 to lipid rafts by glycyrrhizin. RAW264.7 cells were pretreated with glycyrrhizin or MβCD, followed by treatment with 1 μg/ml LPS. The cells were lysed and subjected to discontinuous sucrose density gradient centrifugation as described in the Materials and methods. The fractions were analyzed by Western blotting using CTxB conjugated to horseradish peroxidase (GM1) or anti-TLR4 primary antibody. Fractions 3–4 correspond to lipid rafts. Representative blots of three separate experiments are shown.

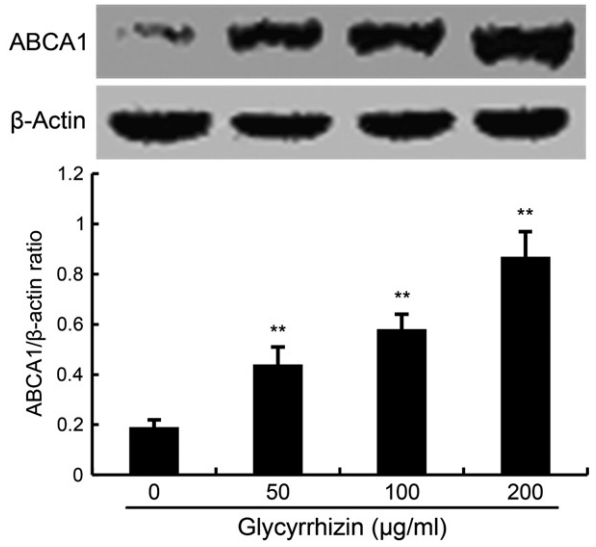

Fig. 11. Effect of glycyrrhizin on ABCA1 expression. Cells were treated with glycyrrhizin (50, 100, 200 μg/ml) for 1 h. Protein samples were analyzed by Western blot with specific antibodies. β-Actin was used as a control. The values presented are the means ± SEM of three independent experiments and differences between mean values were assessed by Student's t-test ($^{*}p < 0.05$, $^{**}p < 0.01$).

exerted anti-inflammatory actions by affecting the expression of CD14. The results showed that glycyrrhizin did not affect the expression of CD14 up-regulated by LPS.

Lipid rafts are plasma membrane microdomains that contain high concentrations of cholesterol and glycosphingolipids [33]. Lipid rafts provide platforms for the formation of receptor complexes and play fundamental roles in signal transduction. Recent, studies have shown that lipid rafts play an important role in LPS-induced signaling in macrophages [34]. Ligand-mediated receptor trafficking to lipid rafts represents an early event in the signal initiation of immune cells. TLR4 was recruited to lipid rafts after the cells were treated with LPS and subsequently induced TNF-α expression [35]. Treatment with raft-disrupting drugs inhibits LPS-induced NF-κB activation and TNF-α production [36,37]. In this study, our results demonstrated that glycyrrhizin inhibited translocation of TLR4 to lipid rafts (Fig. 9). Meanwhile, the results in Figs. 9 and 10 showed that SSa disrupted the formation of lipid rafts by depleting cholesterol. It is suggested that glycyrrhizin disrupts lipid rafts by depleting cholesterol which leads to inhibition of TLR4 translocation to lipid raft and LPS-induced inflammatory responses in RAW264.7 cells.

ATP-binding cassette transporter A1 (ABCA1) is a plasma membrane protein which plays an important role in the movement of cholesterol [38]. Reports have shown that macrophage ABCA1 dampens inflammation by reducing TLR4 trafficking to lipid rafts by reduction of lipid raft cholesterol [24]. To investigate the mechanism of glycyrrhizin reducing lipid raft cholesterol, the effects of glycyrrhizin on ABCA1 were detected. Our results showed that glycyrrhizin increased ABCA1 expression and decreased membrane cholesterol content. These results suggested that glycyrrhizin activated ABCA1 pathway by mediating cholesterol efflux to reduce lipid raft cholesterol content in RAW264.7 cells.

In summary, the results of this study demonstrate that glycyrrhizin inhibits the expression of TNF-α, IL-6, IL-1β and RANTES in LPS-stimulated macrophages. The promising anti-inflammatory effect of glycyrrhizin is associated with up-regulation of the ABCA1 pathway which results in disrupting lipid rafts by depleting cholesterol and reducing translocation of TLR4 to lipid rafts, thereby suppressing TLR4 mediated NF-κB and IRF3 signaling pathways induced by LPS. It has been reported that lipid raft signaling plays an important role in several diseases, such as Alzheimer's, Parkinson's, cardiovascular and prion diseases, systemic lupus erythematosus and HIV [39,40]. Recently, these specific membrane domains have become an interesting target for pharmacological approaches in the cure and prevention of these diseases. Because glycyrrhizin alters lipid rafts, this compound might be a useful drug for the treatment of inflammation-related and other diseases.

Acknowledgements

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