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Glycyrrhizin and isoliquiritigenin suppress the LPS sensor Toll-like receptor 4/MD-2 complex signaling in a different manner

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ABSTRACT

Recent evidences suggest that the extracts of plant products are able to modulate innate immune responses. A saponin GL and a chalcone ILG are representative components of *Glycyrrhiza uralensis*, which attenuate inflammatory responses mediated by TLRs. Here, we show that GL and ILG suppress different steps of the LPS sensor TLR4/MD-2 complex signaling at the receptor level. Extract of *G. uralensis* suppressed IL-6 and TNF-α production induced by lipid A moiety of LPS in RAW264.7 cells. Among various *G. uralensis*-related components of saponins and flavanones/chalcones, GL and ILG could suppress IL-6 production induced by lipid A in dose-dependent manners in RAW264.7 cells. Furthermore, elevation of plasma TNF-α in LPS-injected mice was attenuated by passive administration of GL or ILG. GL and ILG inhibited lipid A-induced NF-κB activation in Ba/F3 cells expressing TLR4/MD-2 and CD14 and BMMs. These components also inhibited activation of MAPKs, including JNK, p38, and ERK in BMMs. In addition, GL and ILG inhibited NF-κB activation and IL-6 production induced by paclitaxel, a nonbacterial TLR4 ligand. Interestingly, GL attenuated the formation of the LPS-TLR4/MD-2 complexes, resulting in inhibition of homodimerization of TLR4. Although ILG did not affect LPS binding to TLR4/MD-2, it could inhibit LPS-induced TLR4 homodimerization. These results imply that GL and ILG modulate the TLR4/MD-2 complex at the receptor level, leading to suppress LPS-induced activation of signaling cascades and cytokine production, but their effects are exerted at different steps of TLR4/MD-2 signaling. *J. Leukoc. Biol.* 91: 967–976; 2012.

Introduction

The innate immune system quickly recognizes and responds to pathogenic agents to provide a first line of defense against pathogens [1]. Mediators of these innate responses include TLR family proteins that recognize PAMPs. TLR4 is the first identified TLR and is essential for bacterial LPS responses [2, 3]. To recognize LPS, TLR4 forms a complex with a secreted protein MD-2, which is associated with the extracellular domain of TLR4 [4, 5]. MD-2 reacts with LPS directly at its hydrophobic cavity, and this recognition of LPS induces homodimerization of the TLR4/MD-2 complex, which forms 1:1 of the LPS-TLR4/MD-2 complex [6]. These events at the receptor level are important, initial steps of TLR4/MD-2 signaling in induction of downstream signaling, as the formation of the LPS-TLR4/MD-2 complexes causes the recruitment of an intracellular adaptor protein MyD88 [7]. MyD88 leads to early activation of MAPKs and the transcriptional factor NF-κB to induce inflammatory cytokine genes, such as IL-6 and TNF-α [7]. Interestingly, in addition to the importance of TLRs in sensing pathogen products, a number of nonbacterial TLR4 ligands have been described [8]. These include natural compounds, such as paclitaxel (also known as Taxol) [9], as well as cellular components, such as fatty acids [10], heat shock proteins [11], and hyaluronan [12]. These molecules ligate TLR4/MD-2, leading to activation of inflammatory pathways and cytokine secretion. This is often referred to as “sterile ”

Abbreviations: BMM=bone marrow-derived macrophage, DAMP=danger-associated molecular pattern, DEX=dexamethasone, GL=glycyrrhizin, ILG=isoliquiritigenin, MD-2=myeloid differentiation protein 2, rm=recombinant murine, TLR4F/G=C-terminus of TLR4 was tagged with the FLAG epitope/GFP epitope

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inflammation [13]. Thus, TLR4/MD-2 is a key player of acute infectious and chronic sterile inflammation.

Roots and rhizomes of *Glycyrrhiza* plants (licorice) have been used as herbal medicine worldwide for over 4000 years [14]. Licorice is a well-recognized, natural sweetener and used as a traditional medicine to treat several diseases and symptoms, including diabetes [15], lung diseases [16], and coughs [17]. Previous clinical and experimental studies revealed that licorice has various pharmacological effects, including anti-inflammatory, antiviral, antimicrobial, antioxidative, and anticancer activities and immunomodulatory, hepatoprotective, and cardioprotective effects [18]. Several reports show that Chinese and Japanese herbal traditional medicines or their components regulate TLR signaling. *Panax ginseng* induces production of proinflammatory cytokines via TLR [19]. *Panax notoginseng* reduced the LPS-, CpG-, or polyinosinic:polycytidylic acid-induced production of TNF-α by DC2.4 cells [20]. Chinese herb-derived compound, sparstolonin B, selectively blocks TLR2- or TLR4/MD-2-mediated inflammatory signaling [21]. In addition, roasted licorice extracted by ethanol demonstrates strong anti-inflammatory activity through its ability to reduce NO, PGE2, and proinflammatory cytokines [22].

Among *Glycyrrhiza* plants, *G. uralensis* is one of the mostly used, traditional medicines in Japan. Various components have been isolated from licorice, including triterpene saponins, flavonoids, isoflavonoids, and chalcones. GL, a triterpene saponin, is considered to be a major biological, active component of *G. uralensis*. GL has several pharmacological activities, including anti-inflammatory [23], antiallergic [24], and antiviral activities [25], and has been used for treatment of chronic hepatitis [26]. ILG, another component of *G. uralensis,* is a flavonoid with a chalcone structure and has various biological properties, including antiplatelet aggregation [27], antiallergic [28], and antitumor growth activity [29]. GL and ILG were reported to suppress TLR4/MD-2-mediated NF-κB and MAPK activation, resulting in decreased production of proinflammatory cytokines [30, 31]. Also, GL treatment down-regulated the TLR4 internalization upon LPS stimulation [32], and ILG inhibited LPS-induced homodimerization of TLR4 [33]. Although GL and ILG may modulate TLR4/MD-2-mediated innate immune responses at not only the downstream signaling level but also the receptor level, little is known about whether these components affect the formation of the LPS-TLR4/MD-2 complexes.

In this study, we explored the molecular mechanisms by which the extracts of *G. uralensis* affect TLR4/MD-2-mediated innate immune responses. Among various *G. uralensis*-related components, some saponins and flavanones/chalcones suppressed lipid A-induced cytokine production. Furthermore, GL, a respresentative of saponins, and ILG, a respresentative of chalcones, suppressed LPS-induced homodimerization of TLR4. Accordingly, activation of NF-κB and MAPKs, important molecules for TLR4-downstream signaling, was severely attenuated by both components. Additionally, GL and ILG inhibited NF-κB activation and IL-6 production induced by paclitaxel, a nonbacterial TLR4 ligand. Finally, GL but not ILG inhibited the formation of the LPS-TLR4/MD-2 complexes. Thus, licorice consists of unique components modulating the important initial steps of the LPS sensor TLR4/MD-2 complex signaling in a different manner.

MATERIALS AND METHODS

Mice

C57BL/6 and BALB/C mice were purchased from Japan SLC (Hamamatsu, Japan) and were used at 7–9 weeks of age. They were maintained in under specific pathogen-free conditions with a 12-h light/12-h dark cycle in the animal facility of University of Toyama (Japan) and given free access to food and water. All experiments were performed according to the guidelines for the care and treatment of experimental animals at University of Toyama.

Reagents

The extract from *G. uralensis* was purchased from Tsumura (Tokyo, Japan). LPS from *Escherichia coli* O55:B5 and lipid A purified from *Salmonella minnesota* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycyrrizin was kindly supplied as glycyrrizin-NH_4 from Minophagen Pharmaceutical (Tokyo, Japan). Liquiritin, liquiritigenin, isoliquiritin, ILG, and glycycoumarin were kindly gifted from Maruzen Pharmaceuticals (Onomichi, Japan). Licorice-saponin G2, licorice-saponin H2, liquiritin apioside, and isoliquiritin apioside were isolated from licorice roots. Paclitaxel from *Taxus brevifolia* was purchased from Sigma-Aldrich.

Cell culture

RAW264.7 cells (Riken BioResource Center, Tsukuba, Japan) were cultured in DMEM, containing 10% FCS and antibiotics. Ba/F3 cells, an IL-3-dependent murine pro-B cell line, were cultured in RPMI-1640 medium containing 100 mM 2-ME and 1 ng/ml rmIL-3 (R&D Systems, Minneapolis, MN, USA). The cDNAs encoding murine TLR4 and MD-2 were cloned into the retrovirus vector pMX-puromycin and pBOS, respectively. The pCDNA3 cloned murine CD14 cDNA was kindly gifted from Dr. Yoshiyuki Adachi (Tokyo University of Pharmacy and Life Science, Japan). The pNF-κB-humanized recombinant GFP plasmid was purchased from Agilent Technologies (Santa Clara, CA, USA). Ba/F3 cells expressing murine TLR4, MD-2, CD14, and NF-κB-GFP were established by electroporation. BaF/3 cells expressing TLR4F, TLR4G, MD-2, and CD14 were described previously [34]. BM cells from C57BL/6 mice were plated in 10 cm bacteriological plastic plates with 10% FCS-RPMI 1640, supplemented with 100 ng/ml rmM-CSF (R&D Systems). At Day 7, adherent cells were harvested and used as BMMs. All cells were incubated at 37°C in a humidified 5% CO_2 incubator.

ELISA for TNF-α and IL-6 production

RAW 264.7 cells were incubated with the extract of *G. uralensis* and components from *G. uralensis*. After 1 h, lipid A was added to the culture. After 24 h, culture supernatants were collected, and levels of TNF-α and IL-6 in the culture supernatants were determined by using ELISA kits (R&D Systems).

BALB/c mice (n=5) were administered with GL peritoneally, 24 h and 1 h before LPS administration. Similarly, BALB/c mice (n=5) were administered with ILG orally, 24 h and 1 h before LPS administration. Plasma was collected at 1 h and 4 h after LPS injection to examine TNF-α and IL-6 production, respectively. The levels of TNF-α and IL-6 in the plasma were determined by using ELISA kits (R&D Systems).

Flow cytometry analysis

The following antibodies were purchased from eBioscience (San Diego, CA, USA): PE-conjugated anti-mouse TLR4/MD-2 (clone MTS510) and PE-conjugated rat IgG2a isotype control.

The Ba/F3 transfectants (1×10^5 cells/well) were incubated with purified anti-mouse FcγR (clone 2.4G2) to block binding of the labeled antibodies to FcγR. After 15 min, the cells were stained with predetermined optimal concen-

trations of the respective antibodies. Flow cytometry analyses were conducted on a FACSCanto II (Becton Dickinson, Mountain View, CA, USA), and data were analyzed with Flowjo software (Tree Star, Ashland, OR, USA).

Western blot analysis

Cells were washed and lysed for 60 min in iced lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and phosphatase inhibitor cocktail (Nacalai Tesque). Lysates were subjected to SDS-PAGE and Western blotting analysis. The following antibodies for immunoblotting were purchased from Cell Signaling Technology (Beverly, MA, USA): anti-IκBα, antiphospho-IκB, antiphospho-IKKα/β, anti-IKKα, anti-IKKβ, antiphospho-JNK, anti-JNK, antiphospho-p38, anti-p38, antiphospho-ERK, anti-ERK. Anti-β-actin was purchased from Sigma-Aldrich. Goat anti-rabbit IgG alkaline phosphatase was purchased from Bio-Rad (Hercules, CA, USA). Goat anti-mouse IgG alkaline phosphatase was purchased from American Qualex (San Clemente, CA, USA). The reactive bands were visualized by 5-bromo-4-chloro-3-indolyl-phosphate /NBT color development substrate (Promega, Madison, WI, USA).

Immunoprecipitation and immunoprobing

Ba/F3 cells expressing murine TLR4, MD-2, CD14, and NF-κB-GFP were incubated with GL or ILG. After 30 min, biotinylated LPS (Invivogen, San Diego, CA, USA) was added to the culture. After 30 min, the cells were washed and lysed with lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail (Nacalai Tesque). After 30 min of incubation on ice, nuclei were removed by centrifugation. Beads, coupled with a rat anti-mouse TLR4/MD-2 (clone Sa15-21) [6], were added to cell lysates and rotated for 2 h at 4°C. Beads were washed with lysis buffer, and bound proteins were subjected to SDS-PAGE and Western blotting with streptavidin-conjugated alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA) or goat anti-TLR4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by streptavidin-conjugated anti-goat antibody (American Qualex).

To examine homodimerization of TLR4, Ba/F3 cells expressing TLR4F, TLR4G, MD-2, and CD14 were incubated with GL or ILG for 30 min and then added LPS to the culture. After 30 min, the cells were washed and lysed with lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Nacalai Tesque). After 30 min of incubation on ice, nuclei were removed by centrifugation. Beads coupled with a rabbit anti-GFP (Invitrogen, Carlsbad, CA, USA) were added to cell lysates and rotated for 2 h at 4°C. Beads were washed with lysis buffer, and bound proteins were subjected to SDS-PAGE and Western blotting with mouse anti-FLAG (Sigma-Aldrich) or rabbit anti-GFP (MBL, Nagoya, Japan), followed by HRP-conjugated anti-rabbit antibody (Cell Signaling Technology). The reactive bands were visualized by ECL Plus (GE Healthcare, Uppsala, Sweden).

Cell viability assay

RAW 264.7 cells were plated at a density of 10^5 cells/well in 96-well plates and stimulated with various concentrations of the extract of *G. uralensis,* GL, and ILG. After 24 h, cytotoxicity assay was conducted by using a cell titer (Promega).

Statistical analysis

The data are given as mean ± SD. Statistical significance was evaluated by Student's *t* test.

RESULTS

Extract of *G. uralensis* suppresses TLR4/MD-2-mediated IL-6 and TNF-α production in RAW264.7 cells

To explore effects of components of *G. uralensis* in innate immunity, we first examined if extract from *G. uralensis* could suppress TLR4/MD-2-mediated innate immune responses. We stimulated RAW 264.7 cells, a macrophage cell line, with lipid A moiety of LPS in the presence of extract from the roots of *G. uralensis* and measured the levels of IL-6 and TNF-α in the culture supernatants by ELISA (**Fig. 1**). Cell viability of RAW 264.7 was not affected by the extract at the concentration used (data not shown). Whereas the extract itself did not produce significant amounts of IL-6 and TNF-α, it decreased the levels of IL-6 and TNF-α induced by lipid A stimulation in a dose-dependent manner (Fig. 1). Thus, we confirmed that *G. uralensis* could suppress TLR4/MD-2-mediated innate immune responses.

G. uralensis-related saponins and flavanones/chalcones suppress TLR4/MD-2-mediated IL-6 production in RAW264.7 cells

Our experiments then focused on components of *G. uralensis* responsible for the suppressive effects on TLR4/MD-2-mediated innate immune responses. Among various components of *G. uralensis* [14], we selected saponins, flavanones/chalcones, and coumarins as major components of *G. uralensis* and obtained three saponins (**Fig. 2A**), six flavanones/chalcones (Fig. 2C), and one coumarin (Fig. 2E). All of the tested components themselves did not significantly induce IL-6 production in RAW264.7 cells (Fig. 2B, D, and F). In addition, these components were added to the cultures at the concentrations not affecting the cell viability of RAW 264.7 cells (data not shown). Three saponins similarly suppressed lipid A-induced IL-6 production in dose-dependent manners, but high concentrations were required for these responses (Fig. 2B). Among six flavanones/chalcones, a glycoside isoliquiritin and its aglycone ILG strongly suppressed lipid A-induced IL-6 production (Fig. 2D). Glycycoumarin is a unique coumarin that is included in *G. uralensis* but not other *Glycyrrhiza* plants. The levels of IL-6 production induced by lipid A were not affected by this coumarin (Fig. 2F). These results suggest that some saponins and

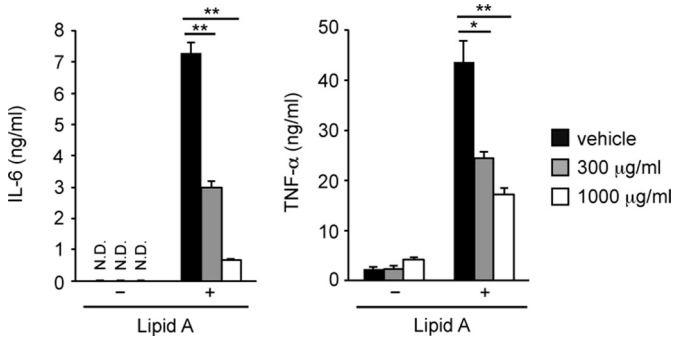

Figure 1. The hot water extract from *G. uralensis* suppresses lipid A-induced TNF-α and IL-6 production in RAW264.7 cells. RAW264.7 cells were stimulated with medium alone or lipid A (1 μg/ml), with or without extract of *G. uralensis*. After 24 h of culture, culture supernatants were collected, and TNF-α and IL-6 production in the culture supernatants was measured by ELISA. Data are shown as the mean ± SD and representative of three independent experiments. *$P < 0.01$; **$P < 0.001$.

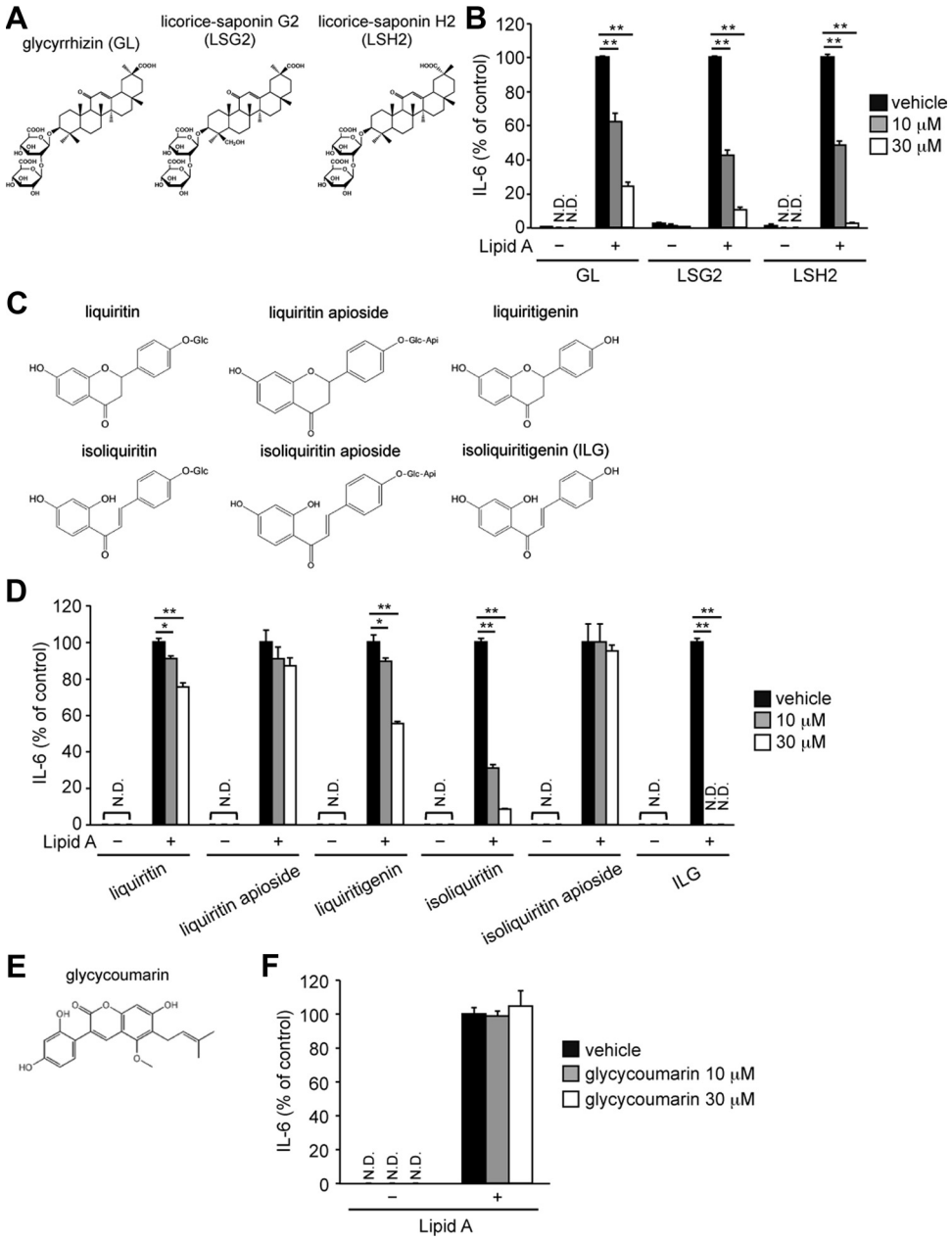

Figure 2. Saponins and flavanones/chalcones of *G. uralensis* suppress lipid A-induced IL-6 production in RAW264.7 cells. (A) Chemical structures of saponins from extracts of *G. uralensis*. (B) RAW264.7 cells were stimulated with medium alone or lipid A (1 μg/ml), with or without the saponins. After 24 h of culture, culture supernatants were collected, and IL-6 production in the culture supernatants was measured by ELISA. (C) Chemical structures of flavanones/chalcones from extracts of *G. uralensis*. (D) RAW264.7 cells were stimulated with medium alone or lipid A (1 μg/ml), with or without the flavanones/chalcones. After 24 h of culture, culture supernatants were collected, and IL-6 production in the culture supernatants was measured by ELISA. (E) Chemical structures of glycycoumarin, a coumarin from extracts of *G. uralensis*. (F) RAW264.7 cells were stimulated with medium alone or lipid A (1 μg/ml), with or without glycycoumarin. After 24 h of culture, culture supernatants were collected, and IL-6 production in the culture supernatants was measured by ELISA. Data are presented relative to the expression in vehicle with lipid A stimulation, set as 100% (B, D, and F). Data were shown as the mean ± SD and representative of three independent experiments. $*P < 0.01$; $**P < 0.001$.

flavanones/chalcones may be responsible components for the *G. uralensis*-mediated immunosuppressive effects on TLR4/MD-2.

GL and ILG suppress LPS-induced TNF-α production in plasma in vivo

Next we examined if these components were effective in an in vivo level. GL and ILG were selected as a representative of saponin and chalcone, respectively. DEX was used as a positive control and suppressed TNF-α and IL-6 production in plasma from LPS-injected mice (**Fig. 3**). ILG significantly decreased the levels of TNF-α and IL-6, but these effects were weaker than those of DEX (Fig. 3A and B). Whereas GL and ILG suppressed LPS-induced TNF-α production, IL-6 production was not affected significantly by GL treatment (Fig. 3A and B). Thus, ILG and GL effectively suppress LPS-induced production of inflammatory cytokines, not only in vitro but also in vivo. However, high doses of ILG and GL were required for their suppressive effects compared with DEX in vivo.

GL and ILG attenuate TLR4/MD-2-mediated activation of NF-κB and MAPKs

Further studies were demonstrated to examine molecular mechanisms by which these components suppressed TLR4/MD-2-mediated production of inflammatory cytokines. As NF-κB is a crucial transcriptional factor for TLR-mediated production, we firstly examined if GL and ILG affected lipid A-induced NF-κB activation. The Ba/F3 cells expressing murine

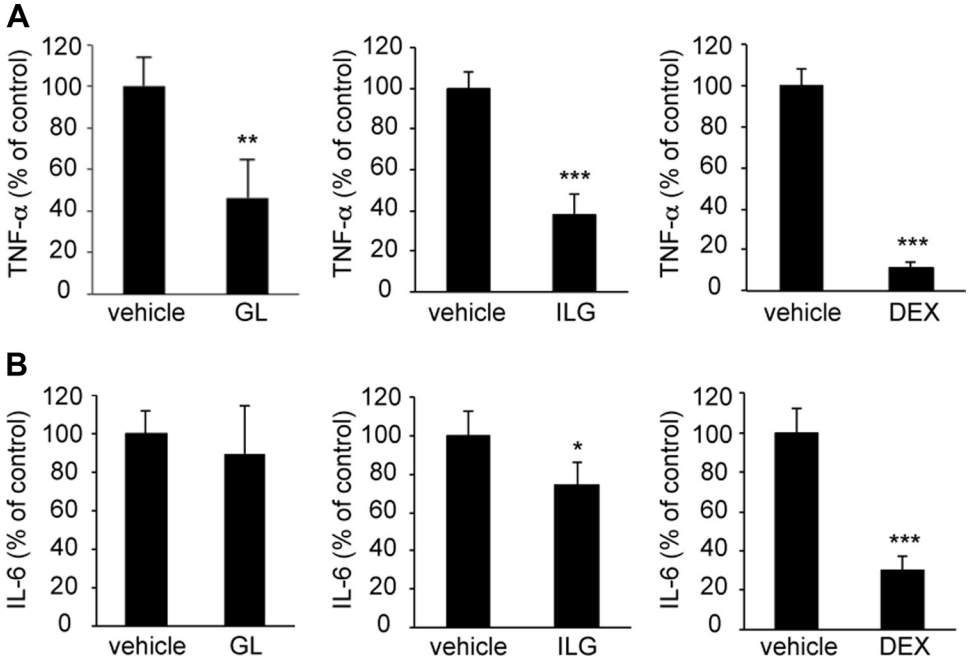

Figure 3. GL and ILG suppress TNF-α production in the endotoxin shock model mice. GL (400 mg/kg), ILG (50 mg/kg), and DEX (5 mg/kg) were administered into female BALB/c mice, 24 h and 1 h prior LPS injection (10 mg/kg). GL was injected i.p. ILG and DEX were administered orally. Plasma was collected at 1 h and 4 h after LPS injection to examine (A) TNF-α and (B) IL-6 production, respectively. The levels of TNF-α and IL-6 in the plasma were measured by ELISA. Data are presented relative to the expression in vehicle, set as 100%. Data are presented as the mean ± SD and representative of three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus vehicle.

TLR4/MD-2 and CD14 were pretreated with GL or ILG and then stimulated with lipid A. NF-κB activation was monitored by measuring GFP expression from a reporter construct using flow cytometry. Neither GL nor ILG alone induced GFP expression (**Fig. 4A**). Lipid A stimulation increased GFP expression within 24 h (Fig. 4B). This lipid A-induced GFP expression was decreased by GL or ILG stimulation, but a high concentration of GL was required for this response compared with ILG.

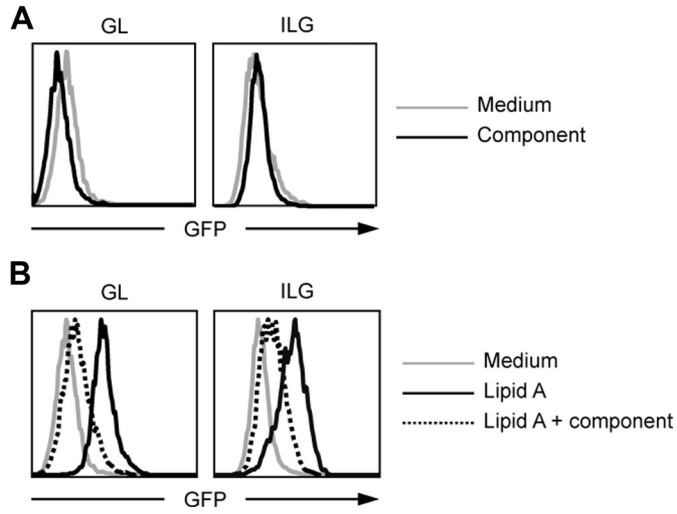

Figure 4. GL and ILG suppress lipid A-induced NF-κB activation in the Ba/F3 cells expressing TLR4/MD-2 and CD14. (A) Ba/F3 cells expressing murine TLR4/MD-2 and CD14 were cultured with medium alone, GL (1 mM), or ILG (30 μM). After 24 h of culture, the cells were harvested, and GFP expression was monitored by flow cytometry. (B) Ba/F3 cells expressing murine TLR4/MD-2 and CD14 were stimulated with medium alone or lipid A (3 ng/ml), with or without GL (1 mM) or ILG (30 μM). After 24 h of culture, the cells were harvested, and GFP expression was monitored by flow cytometry. All data are representative of three independent experiments.

Additionally, we ask whether GL or ILG could suppress innate immune responses induced by a TLR4 ligand other than LPS by using the above protocols. Paclitaxel is an anti-tumor agent from a plant and induces NF-κB activation via TLR4/MD-2 signaling [9]. We detected paclitaxel-induced NF-κB activation by monitoring GFP expression in Ba/F3 cells expressing TLR4/MD-2 and CD14 (Supplemental Fig. 1A). GL and ILG inhibited this response in dose-dependent manners. RAW264.7 cells produced IL-6 in response to paclitaxel (Supplemental Fig. 1B). GL treatment inhibited IL-6 production induced by paclitaxel, as well as lipid A. ILG could also inhibit paclitaxel-induced IL-6 production, but this effect was weaker than that to lipid A. Thus, these components of *G. uralensis* could modulate innate immune responses induced not only by LPS but also by a nonbacterial TLR4 ligand.

Then, we conducted Western blot analyses to extend the above findings. IκBα is usually associated with components of NF-κB and inhibits their translocation into the nucleus [35]. Upon LPS stimulation, IκBα is phosphorylated and degraded to allow NF-κB to be activated [7]. We observed the phosphorylation and degradation of IκBα in lipid A-stimulated BMMs (**Fig. 5A**). Treatment of GL or ILG partially inhibited these responses. Lipid A also caused the phospholylation of IKKα/β (Fig. 5A). Whereas expression of total IKKα or IKKβ was not affected by ILG, this treatment strongly inhibited lipid A-induced IKKα/β phosphorylation. Although GL inhibited lipid A-induced IKKα/β phosphorylation, the response was weaker than by ILG. TLR stimulation also leads to the activation of MAPKs [7]. Lipid A stimulation dramatically induced the phosphorylation of JNK, p38, and ERK1/2 (Fig. 5B). Whereas neither GL nor ILG affected expression of total JNK, p38, and

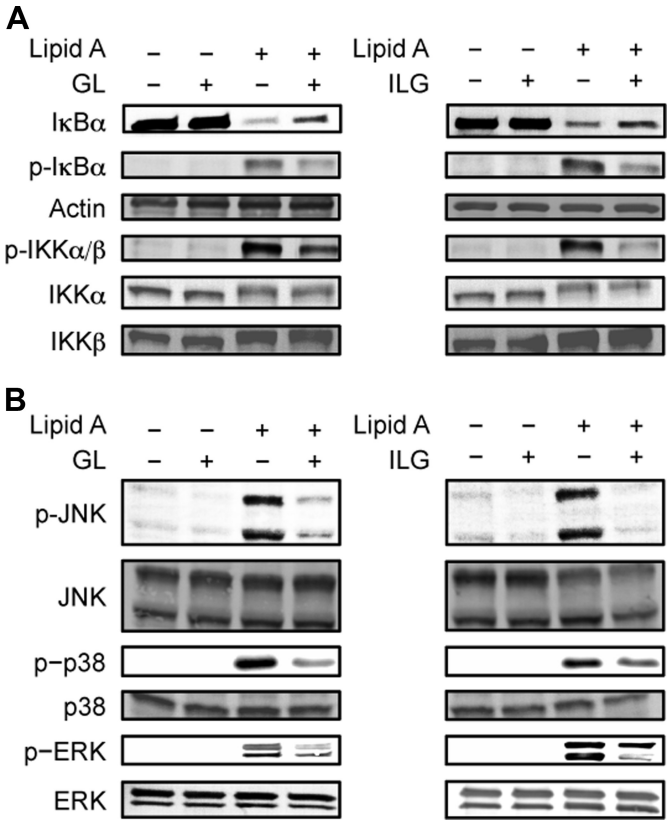

Figure 5. GL and ILG suppress lipid A-induced phosphorylation of IκBα, IKKα/β, and MAPKs in BMMs. BMMs were treated with vehicle alone, GL (1 mM) or ILG (30 μM) for 30 min. Then, the cells were stimulated with medium alone or lipid A (1 μg/ml) for 30 min. Phosphorylation (p) of (A) IκBα, IKKα/β, and (B) MAPKs was examined by Western blotting, as described in Materials and Methods. All data are representative of three independent experiments.

ERK1/2, lipid A-induced phosphorylations were all attenuated by GL or ILG treatment. Thus, GL and ILG inhibited TLR4/MD-2-mediated NF-κB and MAPK activation.

GL but not ILG inhibits LPS-induced down-regulation of TLR4/MD-2 expression

Binding of LPS to MD-2 causes a rapid change in the TLR4/MD-2 complex, resulting in diminished staining with a unique, TLR4/MD-2-specific antibody, MTS510 [36]. This phenomenon may be explained by following three possibilities. First, TLR4/MD-2 may be internalized by LPS stimulation. Second, LPS stimulation may cause the conformational changes of TLR4/MD-2, such as homodimerization, which MTS510 may not recognize. Finally, binding of LPS to MD-2 may physically interfere with recognition of TLR4/MD-2 by MTS510. We exploited this phenomenon to ask if GL and ILG can modulate TLR4/MD-2 signaling at the receptor level. Neither GL nor ILG alone affected TLR4/MD-2 staining on Ba/F3 cells expressing TLR4/MD-2 and CD14 (**Fig. 6A**). LPS stimulation dramatically decreased TLR4/MD-2 staining within 15 min. Interestingly, GL but not ILG treatment partially compensated for the decreased TLR4/MD-2 staining (Fig. 6B). These results indicate that GL affects TLR4/MD-2 signaling at the receptor level.

GL but not ILG inhibits the formation of the LPS-TLR4/MD-2 complexes

The above findings led us to ask whether GL and ILG can affect the formation of the LPS-TLR4/MD-2 complexes on the cell surface. To address this issue, we conducted immunoprecipitation of biotinylated LPS-treated TLR4/MD-2 with another anti-TLR4/MD-2 mAb, Sa15-21, which is able to recognize LPS-treated TLR4/MD-2 [6], and coprecipitation of biotinylated LPS was probed with streptavidin alkaline phosphatase. We detected biotinylated LPS coprecipitated with TLR4/MD-2 by Sa15-21 mAb (**Fig. 7**). Whereas GL stimulation did not affect the levels of precipitated TLR4, amounts of biotinylated LPS bound to TLR4/MD-2 were decreased by GL treatment in a dose-dependent manner (Fig. 7, left panel). In contrast, ILG did not affect the binding of biotinylated LPS to TLR4/MD-2 (Fig. 7, right panel). Thus, GL but not ILG could inhibit the formation of the LPS-TLR4/MD-2 complexes.

GL and ILG inhibit LPS-induced homodimerization of TLR4/MD-2

LPS binding to MD-2 triggers homodimerization of the TLR4/MD-2 complex, resulting in the induction of signal transduction [34]. Therefore, we next examined whether GL and ILG affect LPS-induced TLR4 homodimerization (**Fig. 8**). To investigate the homotypic interaction of TLR4, Ba/F3 cells expressing TLR4F, TLR4G, MD-2, and CD14 were prepared [34]. To detect the homodimerization of TLR4, TLR4G was immunoprecipitated with anti-GFP antibody, and coprecipitation of TLR4F was probed with anti-FLAG antibody. Association between TLR4F and TLRG was observed after LPS stimulation (Fig. 8). This physical association was inhibited by GL or ILG treatment in a dose-dependent manner. Thus, GL and ILG could inhibit LPS-induced TLR4 homodimerization, whereas GL but not ILG inhibited the formation of the LPS-TLR4/MD-2 complexes.

DISCUSSION

GL and ILG have been shown to affect innate immune responses. In this study, we investigated the molecular mechanisms of these components in suppressing TLR4/MD-2-mediated innate immune responses. We demonstrated that GL and ILG suppressed LPS-induced activation of signaling cascades and initiation of receptor signaling. GL and ILG inhibited LPS-induced TLR4/MD-2 homodimerization. Accordingly, lipid A-induced NF-κB and MAPK activation were attenuated by GL or ILG treatment. Interestingly, whereas GL inhibited the formation of the LPS-TLR4/MD-2 complexes, ILG did not affect this formation. Thus, our results clearly suggest that suppressive effects of GL and ILG, two representative components of *G. uralensis*, are exerted at different initial steps of TLR4/MD-2 signaling (**Fig. 9**).

It was reported previously that GL inhibited LPS-triggered TLR4 internalization [32]. Another study reported that ILG

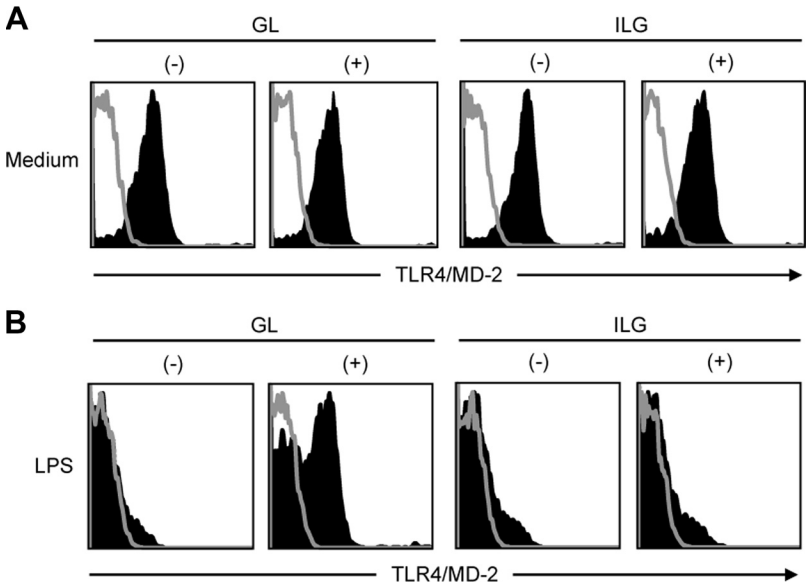

Figure 6. GL but not ILG suppresses LPS-induced down-regulation of the TLR4/MD-2 complex. Ba/F3 cells expressing murine TLR4/MD-2 and CD14 were treated with vehicle alone, GL (1 mM), or ILG (30 μM) for 30 min. Then, the cells were stimulated with medium alone (A) or LPS (25 ng/ml; B) for 15 min. The cultured cells were harvested and stained with anti-mouse TLR4/MD-2 mAb (clone MTS510) or isotype type control antibody (gray lines). FACS analyses were conducted as described in Materials and Methods. (−), Vehicle-treated cells. Data are representative of three independent experiments.

inhibited LPS-induced TLR4 homodimerization [33]. Therefore, it has been suspected that GL and ILG interfere with LPS responses by inhibiting the initiation of TLR4/MD-2 signaling rather than blocking its downstream signaling cascades. However, these studies did not analyze their possible effects on the formation of the LPS-TLR4/MD-2 complexes. Our present results clearly demonstrated that GL but not ILG inhibited LPS binding to the TLR4/MD-2 complex (Fig. 7). This may account for the GL-mediated inhibition of LPS responses; those are inhibition of LPS-induced TLR4 homodimerization, NF-κB, and MAPK activation and IL-6 production. Interestingly, ILG blocked lipid A-induced IL-6 production and activation of NF-κB and MAPKs (Figs. 2, 4, and 5), whereas it did not inhibit LPS binding to the complex (Fig. 7). Thus, inhibition of not only LPS binding but also TLR4 homodimerization may be an important target in suppressing LPS responses. This information could be useful to develop an antiseptic medicine.

The most important question is how GL and ILG interfere with TLR4/MD-2 signaling at the receptor level. As is demonstrated, inhibition of the formation of the LPS-TLR4/MD-2 complexes can be a major inhibitory step for GL. Previous studies suggested that GL might be incorporated into lipid bilayers and suppress the integrity of the plasma membrane [33]. Disruption of the membrane integrity has shown to interfere with the formation of the functional complex of TLR4/MD-2 and CD14, resulting in inhibition of LPS signaling [37]. GL-mediated anti-inflammatory effects were reported not specific to TLR4. GL could inhibit not only TLR4- but also TLR9-mediated inflammatory responses by decreasing the uptake of CpG-DNA [33]. Moreover, it was suggested that CpG-DNA uptake depends on scavenger receptors [38–41]. Thus, GL may not interact directly with TLRs to interfere with their signalings. Altered membrane integrity triggered by GL might be involved in its inhibitory activities of multiple TLRs, which have several cysteine residues in the extracellular and cytoplasmic domain [7, 42]. A previous study reported that an isothiocyanate sulforaphane suppressed oligomerization of TLR4 by forming adducts with cysteine residues in the extracellular domain of TLR4 [43]. It is noticed that ILG has an α,β-unsaturated carbonyl group to react with the thiol group of cysteine [17] Thus, in contrast to GL, ILG could interact with TLR4 directly, and cyteine residues of TLR4 may be a potential target for ILG. Further investigation will be required to reveal the precise mechanisms linking pharmacological activities of GL and ILG to their inhibitory steps of the TLR4/MD-2 receptor.

In our experiments, as others, high concentrations of GL were required to induce anti-inflammatory responses (Figs. 3 and 4) [32, 44]. On the other hand, ILG showed anti-inflammatory responses at much lower concentrations relative to GL

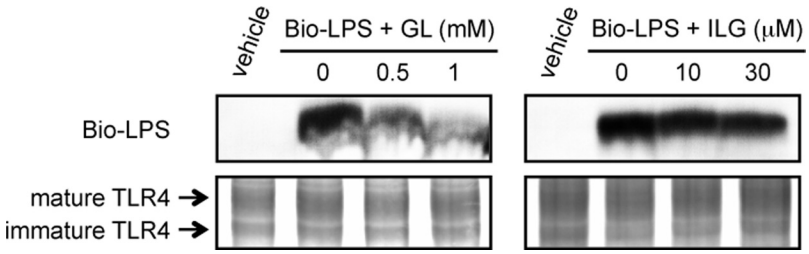

Figure 7. GL but not ILG suppresses the biding of LPS to the TLR4/MD-2 complex. Ba/F3 cells expressing murine TLR4/MD-2 and CD14 were treated with vehicle alone, GL, or ILG for 30 min. Then, the cells were stimulated with medium alone or biotinylated LPS (0.2 μg/ml) for 30 min. The cultured cells were then subjected to immunoprecipitation with an anti-mouse TLR4/MD-2 mAb (clone Sa15-21), as described in Materials and Methods. Binding of biotinylated LPS to the TLR4/MD-2 complex was detected with streptavidin-alkaline phosphatase. TLR4 protein was detected with anti-TLR4 polyclonal antibody. Data are representative of three independent experiments.

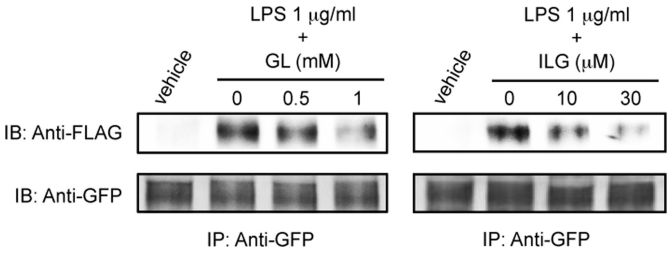

Figure 8. GL and ILG block LPS-induced homodimerization of TLR4. Ba/F3 cells expressing TLR4F, TLR4G, MD-2, and CD14 were treated with vehicle alone, GL, or ILG for 30 min. The cells were then stimulated with medium alone or LPS (1 μg/ml) for 60 min. The cultured cells were then subjected to immunoprecipitation (IP) with anti-GFP and immunoblotting (IB) with anti-FLAG or anti-GFP, as described in Materials and Methods. Data are representative of three independent experiments.

in our in vitro and in vivo experiments (Figs. 3 and 4). We speculated that the hydrophilic character of GL might limit its interaction with the cellular membrane [33], and ILG is more hydrophobic relative to GL. At this moment, a precise reason for their different concentration dependencies suppressing LPS responses is unclear, but ILG rather than GL may be one of the most potent components responsible for *G. uralensis*-mediated anti-inflammatory responses.

As GL has a steroid-like structure, it seems to be incorporated into the nucleus and act as a glucocorticoid receptor agonist [45]. However, another study demonstrated that GL suppressed NF-κB transcriptional activity, independent of glucocorticoid receptors [44]. Although main issues of our study were not to clarify the requirements of glucocorticoid receptors for GL-mediated suppression of TLR4 signaling, we speculated that GL may suppress LPS responses by interfering with cell surface TLR4/MD-2 rather than suppressing activities of transcriptional factors, as GL inhibited LPS-induced activation of multiple signaling cascades, including NF-κB and MAPKs (Figs. 4 and 5). We also demonstrated that ILG could inhibit lipid A-induced activation of multiple signaling cascades (Figs. 4 and 5). As it has been demonstrated that ILG interacts with IKK directly and inhibits its kinase activity [46], ILG may suppress TLR4/MD-2-mediated immune responses in multiple steps, at the receptor level and the downstream signaling level.

The MTS510 mAb specifically recognizes the TLR4/MD-2 complex but not TLR4 alone [36]. Intriguingly, the level of TLR4/MD-2 expression stained with this mAb was rapidly down-regulated after LPS stimulation [6]. A reason for this phenomenon has been speculated as follows. First, TLR4/MD-2 may be internalized upon LPS stimulation. Second, MTS510 may not recognize LPS-induced conformational changes of TLR4/MD-2, such as homodimerization. Finally, LPS binding to MD-2 may physically interfere with recognition of TLR4/MD-2 by MTS510. In this regard, another anti-TLR4/MD-2 mAb Sa15-21 could recognize the complex, even after LPS stimulation [6]. Therefore, the down-regulation of TLR4/MD-2 may not be a result of its internalization. In this study, we demonstrated that GL and ILG treatment inhibited LPS-induced homodimerization of TLR4 (Fig. 8), but LPS-induced down-regulation of TLR4/MD-2 was compensated by GL but not ILG (Fig. 6). Therefore, conformational changes of TLR4/MD-2, such as homodimerization, may not account for the rapid down-regulation. The reason for the down-regulation of TLR4/MD-2 must be LPS-mediated physical interference with recognition of the complex by MTS510. This is consistent with the result that ILG treatment did not inhibit LPS binding to TLR4/MD-2 (Fig. 7). In the presence of ILG, LPS may still bind to MD-2, as MTS510 cannot recognize TLR4/MD-2 in this situation.

In this study, we evaluated anti-inflammatory responses of glycosides (liquiritin, liquiritin apioside, isoliquiritin, and isoliquiritin apioside) and their aglycones (liquiritigenin and ILG), which are all included in extracts of *G. uralensis* [18, 47]. Our in vitro studies revealed that not only an aglycone ILG but also glycosides, such as isoliquiritin, significantly suppressed lipid A-induced IL-6 production in RAW264.7 cells (Fig. 2). In general, it is considered that glycosides of plants are absorbed from the intestine at the form of aglycones, which are hydrolyzed by glycosidase in intestinal flora [48].

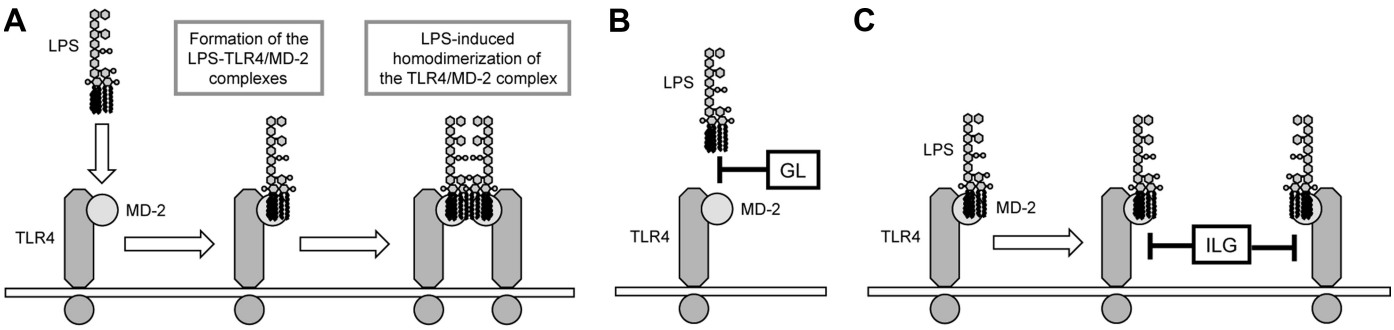

Figure 9. Hypothetical models of GL- and ILG-mediated suppression of the LPS sensor TLR4/MD-2 complex signaling. The suppressive effects of GL and ILG are exerted at different initial steps of TLR4/MD-2 signaling. (A) The models of the formation of the LPS-TLR4/MD-2 complexes and LPS-induced homodimerization of TLR4/MD-2. LPS directly binds to MD-2, which is associated with the extracellular portion of TLR4. This formation of the LPS-TLR4/MD-2 complexes leads to homodimerization of TLR4/MD-2. (B) GL inhibits LPS binding to the TLR4/MD-2 complex (see Fig. 7). Accordingly, the complex cannot homodimerize each other, as shown in Fig. 8. (C) Upon ILG treatment, LPS can bind to TLR4/MD-2 (see Fig. 7). However, LPS-induced homodimerization of TLR4/MD-2 is inhibited by ILG (see Fig. 8).

Therefore, if those glycosides are orally administered, they would be hydrolyzed and absorbed at the form of liquiritigenin or ILG in the intestine. These studies suggested that glycosides by themselves might not affect inflammatory responses without absorbing from the intestine in vivo. However, a previous study demonstrated that a glycoside liquiritin apioside orally administered to guinea pigs was detected in plasma at an early phase after administration, and the concentration of liquiritin apioside in plasma was decreased gradually opposite to the increase of its aglycone liquiritigenin [49]. In addition, liquiritin apioside showed antitutive effects, even when liquiritigenin was not detected in plasma [49]. Thus, not only aglycones but also glycosides may be absorbed from the intestine and show anti-inflammatory effects on immune cells.

TLRs can recognize not only PAMPs but also endogenous ligands, called DAMPs [8]. The interaction between TLR4 and DAMPs leads to activation of proinflammatory pathways and causes chronic, noninfectious inflammation, such as type 2 diabetes [13]. In this study, we demonstrated that GL and ILG suppressed NF-κB activation, and IL-6 production induced by paclitaxel. Thus, GL and ILG might suppress chronic inflammation induced by endogenous TLR4 ligands. This information may provide a good explanation for the suppressive effects of *G. uralensis* on many inflammatory diseases.

In conclusion, we identified critical pharmacological activities of two components of *G. uralensis* in suppressing the initial steps of TLR4/MD-2 signaling. The findings could be useful to understand the molecular mechanisms of anti-infectious agents and develop a novel medicine for infection and sepsis.

AUTHORSHIP

K.T. conceived of the study. H. Honda did the experiments. H. Honda, Y.N., and K.T. wrote the manuscript. S-i.S. contributed to construct the Ba/F3 transfectants. S.A-T. and K.M. provided the anti-TLR4/MD-2 mAb. I.F. and H. Hayashi provided the components of *G. uralensis*. T.M. and A.M. were involved in project planning. K.T. was involved in project planning, financing, and supervision. K.T. and Y.N. are the senior authors.

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KEY WORDS:
innate immunity · *Glycyrrhiza uralensis* · NF-κB · MAPK · IL-6