A non-polar fraction of *Saponaria officinalis* L. acted as a TLR4/MD2 complex antagonist and inhibited TLR4/MyD88 signaling in vitro and in vivo

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**Abstract**

Targeting Toll-like receptor 4/myeloid differentiation factor 2 (TLR4/MD2) signaling is regarded as a potential strategy for treating inflammatory diseases. *Saponaria officinalis* L. is rich in saponin, which include quillaic acid, gypsogenin, saponarin, and hederagenin. We evaluated the pharmacological activity of a *Saponaria officinalis* extract in THP-1 derived macrophages and RAW264.7 macrophages. TLR4/MyD88 complex formation and downstream signals were investigated by co-immunoprecipitation (Co-IP). *In silico* docking simulation was conducted to predict binding scores and perform 3D modeling of saponarin-TRLR4/MD2 complex. A hexane fraction of *Saponaria officinalis* (SH) and fr.1 (a sub-fraction 1 of SH) inhibited mitogen-activated protein kinase (MAPK) signaling, nuclear factor kappa b (NF-κB) activity, cytokine production, and the expressions of marker genes specific for M1 polarization. The inhibitory effects of fr.1 and saponarin on TLR4/MyD88 complex formation were observed by western blotting TLR4 co-immunoprecipitated proteins. Saponarin and fr.1 markedly attenuated LPS-induced inflammatory cytokines, thus reducing mortality and morphological abnormality in zebrafish larvae. Finally, docking simulation revealed that saponarin can directly interact with TLR4/MD2 complex to inhibit downstream signalings. Our findings suggest that saponarin reduces downstream inflammatory response by disrupting TLR4/MD2 complex and blocking MyD88-dependent inflammatory signaling.

**Abbreviations:** CD38, cluster differentiation 38; CD80, cluster differentiation 80; CD86, cluster differentiation 86; COX-2, cyclooxygenase-2; CXCL10, C-X-C motif chemokine ligand 10; IL-1β, interleukin 1 beta; iNOS, inducible nitric oxide synthase; JAK, Janus kinase 2; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa B; NO, nitric oxide; STAT3, signal transducer and activator of transcription 3; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor alpha.

Ga-Ram Yu and Dong-Woo Lim equally contributed to the study.

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1 | INTRODUCTION

Toll-like receptor 4/myeloid differentiation factor 2 complex (TLR4/MD2) complex is a heterodimer that recognizes common patterns in diverse LPS molecules from gram-negative bacteria or endogenous molecules from necrotic cells. TLR4/MD2 is a pattern recognizing receptor (PRR) that initiates immune response to combat pathogens by recognizing lipid components of microbial LPS, that is, pathogen-associated molecular patterns (PAMPs). In response to LPS, two pairs of adaptor proteins, including MyD88 (Myeloid differentiation primary response protein 88) interact with TLR4 to induce signal transduction and MyD88 serves as signaling adaptor to form signaling complexes. Therefore, modulation of TLR4/MyD88 signaling is regarded as a potential means of attenuating inflammation and inflammatory diseases.

Recently, virtual molecular docking has been used to screen and evaluate drug candidates in silico for their antagonistic effects on TLR4/MD2 complexes, and has been demonstrated to be a useful tool for drug discovery and design. Furthermore, this technique can propose the 3D structures of protein-ligand complexes and binding affinities between receptors and their protein ligands, and results obtained to date have resulted in experimental investigations of the antagonistic properties of many synthetic and natural compounds as candidate anti-inflammatory drugs. Drugs such as eritoran (Pubchem CID: 6912404, an LPS mimic) and TAK-242 (Pubchem CID: 11703255) have been shown to bind strongly with TLR4/MD2 complex in virtual docking simulations and to be effective in pre-clinical studies.

Successful blocking of TLR4/MD2 signaling can suppress vulnerability to inflammatory diseases by inhibiting downstream signaling, as has been demonstrated in several TLR4-KO mouse models. TLR4 signaling can be divided into MyD88-dependent and independent (TRIF-dependent) pathways, and inhibition of the MyD88-dependent pathway lead to suppression of the MAPK (Mitogen-activated protein kinase) and NF-κB (Nuclear factor kappa b) pathways which leads to reduced productions of pro-inflammatory cytokines and mediators.

The JAK2/STAT3 (Janus kinase 2/Signal transducer and activator of transcription 3) signaling pathway transduces signals from extracellular cytokines, interleukins, and growth factors, which act through several transmembrane receptors (e.g., interleukin receptors) known to be involved in macrophage polarization, and is a significant pathway in inflammation and cancer. Macrophage polarization refers to the process whereby macrophages produce distinct functional phenotypes in response to specific microenvironmental stimuli and signals. Following activation by lipopolysaccharide (LPS) and Th1 cytokines (such as IFN-γ and TNF-α), macrophages are polarized into M1 macrophages, which are characterized by elevated expressions of surface markers or secretions of CD38, CD80, CD86, HLA-DR, and CXCL10. In particular, M1 macrophages exhibit characteristics of inflammation as they secrete pro-inflammatory cytokines and molecules and recruit immune cells.

Saponins are natural glycosides of steroids or triterpenes and have a range of pharmacological actions, which include anti-inflammatory, antifungal, antibacterial, antitumor, antiviral, and immunomodulatory effects. Certain saponins derived from Quillaja saponaria (The soap bark tree; QS-21) and Panax ginseng (Ginsenosides) have well-established immunomodulatory properties, and other saponins have also been reported to have favorable anti-inflammatory effects.

Saponaria officinalis L. (SO), commonly known as “soapwort”, is a perennial herb belonging to the Caryophyllaceae family native to Europe. Soapwort provides a natural source of saponins, which constitute as much as 21% of its extract, that include quillaic acid, gypso genin, saponarin, and hederagenin. Nevertheless, despite its potential for new drug development, little research has been performed on the topic.

In the present study, considering the preceding data, we used the hexane fraction of a 70% ethanol extract of Saponaria officinalis. We evaluated the effects of this hexane extract of SO (SH) on acute inflammatory responses in monocyte-derived macrophages, and elucidated the molecular mechanism responsible. SH was also subfractionated by silica gel column chromatography, and the impacts of sub-fractions on macrophage polarization were evaluated. The immunoprecipitated proteins were electrophoresed to analyze interactions between TLR4/MyD88 complex in cell membranes, and in vivo study was performed using zebrafish larvae to evaluate the protective efficacy against LPS-induced acute endotoxemia. Finally, in silico molecular docking simulation between saponarin and TLR4/MD2 complex was conducted to predict structures and binding affinities.
2 | MATERIALS AND METHODS

2.1 | Chemicals

Fetal bovine serum (FBS) and penicillin/streptomycin solution were purchased from Invitrogen (Carlsbad, CA, USA), and Dulbecco’s Modified Eagle’s Medium (DMEM) and RPMI 1640 medium were from Hyclone (Logan, UT, USA). Lipopolysaccharide (LPS) and other reagents were obtained from Sigma Chemicals (St. Louis, MO, USA). Human recombinant IFN-γ and IL-4 were supplied by Genscript (Piscataway, NJ, USA). ELISA kits for mouse TNF-α, IL-1β, and IL-6 were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies for iNOS (Inducible nitric oxide synthase), COX-2 (Cyclooxygenase-2), TLR4 (Toll-like receptor 4), MyD88 (Myeloid differentiation primary response 88), lamina B, and β-actin, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz (Dallas, TX, USA). Primary antibodies against p-ERK (Phospho-extracellular signal-regulated kinase), p-JNK (Phospho-c-Jun N-terminal kinase), p-p38 (Phospho-p38 mitogen-activated protein kinase), p-STAT3 (Phospho-signal transducers and activators of transcription 3), p-JAK2 (Phosphor-janus kinase 2), p-IκB-α, and p-NF-κB (p-p65) were obtained from Cell Signaling Technology (Beverly, MA, USA). The oligonucleotide primers used for real-time qPCR were supplied by Macrogen (Seoul, South Korea).

2.2 | Plant materials and subfractionation of extracts

Dried aerial parts of SO were purchased from Herbmaul (Chungcheongbuk-do, South Korea). To prepare the extract, dried aerial parts (200 g) were ground to a powder, extracted with 1.5 L of 70% ethyl alcohol at room temperature for 48 h, and solvent was then removed from the extract under reduced pressure. This extract was then sequentially partitioned using n-hexane, ethyl acetate, and butanol (each for 48 h). The remaining aqueous residue was also treated as a fraction. These four fractions were concentrated and dried to yield a n-hexane fraction (SH, 3.5 g), an ethyl acetate fraction (1.48 g), a butanol fraction (5.7 g), and a water fraction (18.92 g). Silica gel column chromatography was used to sub-fractionate SH. Briefly, 0.063–0.200 mesh silica gel 60 (Millipore) was placed in a column and covered with 300–600 μm sea sand. SH extract (1 g) was added, and the column was step-eluted with n-hexane:ethyl acetate (v/v) mixtures to obtain Sub-fraction 1: 100% hexane-0% ethyl acetate; Sub-fraction 2: 75%–25%; Sub-fraction 3: 50%–50%; Sub-fraction 4: 25%–75%; Sub-fraction 5: 0%–100%; Sub-fraction 6: 0%–100%. Sub-fractions were concentrated and dried to yield fr.1 (Sub-fraction 1, 17%, w/w), fr.2 (Sub-fraction 2, 0.08%, w/w), fr.3 (Sub-fraction 3, 0.11%, w/w), fr.4 (Sub-fraction 4, 5.25%, w/w), fr.5 (Sub-fraction 5, 8.1%, w/w), and fr.6 (Sub-fraction 6, 5.43%, w/w) Supporting Information Table S1. Voucher specimens of Saponaria officinalis L. and the six sub-fractions were deposited at the Institute of Korean Medicine, Dongguk University (DG-DIA018H, DG-DIA018H fr1-fr6).

2.3 | Cell culture, differentiation, and treatment

THP-1 cells (a human monocyte cell line) and RAW264.7 cells (a mouse macrophage cell line) were purchased from the Korea Cell Line Bank (KCLB, Seoul, South Korea). THP-1 monocytes were cultured in RPMI 1640 medium (Welgene, Gyeongsangbuk-do, South Korea) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA), 1% penicillin-streptomycin (Gibco, USA), and 0.1% DMSO at 37℃ in a humidified 5% CO2 atmosphere. RAW264.7 cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% FBS and 1% penicillin-streptomycin at 37℃ in a humidified 5% CO2 atmosphere. To evaluate anti-inflammatory effects, THP-1 monocytes were differentiated into macrophages by treating them with 10 ng/ml of phorbol-12-myristate-13-acetate (PMA; Sigma, USA) for 24 h. After starvation for 24 h, PMA-differentiated THP-1 and RAW264.7 cells were cotreated with LPS (1 μg/ml) and fractionated samples for 6, 12, or 24 h. PMA-differentiated THP-1 cells were subsequently co-stimulated with human IFN-γ (1 μg/ml) and LPS (100 ng/ml) for 12 h to induce the M1 phenotype with or without sub-fractionated samples. Indomethacin (20 μM) was used as a positive control.

2.4 | Cell viability assay

Cell viabilities of THP-1 and RAW264.7 cells were determined using the EZ-Cytox assay kit (Daeil Lab Service, Chungcheongbuk-do, South Korea) according to the manufacturer’s instructions. Optical densities (ODs) of reactants were measured at 450 nm using a microplate reader (Versamax, Molecular Devices, USA).

2.5 | Nitrite assay

Griess reagent was used to investigate the effects of fractionated samples on LPS-induced nitrite levels. Briefly,
RAW264.7 cells were seeded in triplicate on 6-well culture plates at $2 \times 10^5$ cells/ml and incubated at 37°C in a humidified 5% CO$_2$ incubator for 24 h. Cells were then co-treated with LPS (1 µg/ml) and fractionated samples at various concentrations for 24 h. Supernatants were collected, mixed with Griess reagent, and ODs were measured at 570 nm. Nitrite concentrations were calculated using a standard curve.

2.6 | Preparation of nuclear and cytosolic fractions

Nuclear and cytosolic proteins were separated using a Nuclear and Cytoplasmic Extraction Reagents kit from Thermo Fisher Scientific. Briefly, THP-1 or RAW 264.7 macrophages were seeded on 60 mm cell cultures dishes at $2 \times 10^5$ cells/ml and starved for 12 h. Cells were then co-treated with LPS (1 µg/ml) and fractionated samples (10 µg/ml) for 6 h. The nuclear translocation of NF-κB from cytoplasm was assessed by western blot.

2.7 | Western blot analysis and immunoprecipitation

Protein levels of inflammatory markers were determined by Western blot. Briefly, cells were washed with Dulbecco’s phosphate buffered saline (DPBS) and lysed with radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL, USA) containing a protease and phosphatase inhibitor cocktail (Gendepot, Barker, TX, USA). Protein concentrations were measured using the BCA kit (Thermo Fisher Scientific). Protein lysates (20 µg) were loaded into 10% SDS-PAGE gels, electrophoresed, and transferred to PVDF membranes at 100 V for 60 min using an electrophoretic transfer cell (Bio-rad, Hercules, CA, USA). Membranes were blocked with 5% BSA in TBS/T (TBS containing 0.1% Tween 20) for 2 h at room temperature, and blots were incubated with primary antibodies (diluted at 1:1000 in TBS/T containing 3% BSA) overnight at 4°C with gentle shaking. After washing with TBS/T, membranes were incubated with secondary antibodies (diluted at 1:3000 in TBS/T) at room temperature for 2 h. Blots were detected using a western blot imaging system (Fusion Solo, Vilber Lourmat, Collegien, France) and the proteins were visualized using a chemiluminescent ECL buffer (Super Signal West Pico, Thermo Fisher Scientific). For co-immunoprecipitation (Co-IP), differentiated THP-1 cells were cotreated with LPS and fr.1 (Sub-fraction 1 of SH) for 1 h. Co-IP was performed using a Pierce Co-Immunoprecipitation kit (Thermo Fisher Scientific). Briefly, 50 µg of monoclonal TLR4 antibody was first covalently coupled to the resin, incubated with 200 ml of cellular extracts overnight at 4°C, washed, and antibody bound protein complexes were pulled-down and eluted. Eluted proteins were subjected to western blot to investigate the interaction between TLR4 and Myd88.

2.8 | Quantitative real-time polymerase chain reaction

The expression levels of pro-inflammatory mediators and M1 polarization associated genes were determined by qPCR. Total RNA was isolated from THP-1 and RAW264.7 cells using Trizol reagent (Thermo Fisher Scientific), according to the manufacturer’s instructions. Briefly, reverse transcription was performed using AccuPower RT PreMix (Bioneer, Daejeon, South Korea) and oligo deoxynucleotymine (dt) 18 primers (Invitrogen, Carlsbad, CA, USA). Primer-specific binding cDNA was amplified using a Light Cycler 480 PCR system (Roche, Basel, Switzerland) using 10 µl of SYBR green Master mixture (Roche, Switzerland), 8 µl of ultrapure water, 1 pmol/µl of primer, and 1 µl of template cDNA. Amplification was performed using the following schedule: denaturation at 95°C for 10 min, followed by 45 amplification cycles (denaturation at 95°C for 10 s and annealing at 50–60°C for 5 min). Threshold cycle values (Ct value) were used to quantify PCR products. The primers used are described in Supporting Information Table S2. β-actin and GAPDH were used as internal controls, and the results were normalized by dividing gene Ct values by that of β-actin. Data were acquired using Roche LightCycler 480 software (Roche Applied Science, USA).

2.9 | Immunofluorescence microscopy

To follow the nuclear translocalization of NF-κB, cells were grown on Lab-Tek II chamber slides (Nalge Nunc, IL, USA), as previously described with slight modification. Briefly, cells were fixed in 4% formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min at room temperature, blocked with 1% BSA for 1 h at room temperature, and labeled with 2 µg/ml of primary antibody overnight at 4°C. NF-κB in cytoplasm and nuclei was detected by treating cells with PBS containing 2 µg/ml of FITC and 0.2% BSA for 45 min at room temperature. Nuclei were stained using mounting medium containing...
2.10 | ELISA

Concentrations of inflammatory cytokines, that is, TNF-α, IL-1β, and IL-6, in cell culture supernatants were quantified using Quantikine mouse ELISA kits (R&D Systems, Inc. Minneapolis, MN, USA) or human ELISA kits (Invitrogen, Carlsbad, CA, USA). Briefly, cells were seeded at 2 × 10⁵ cells/ml in 24-well plates and cotreated with LPS (1 µg/ml) and different concentrations of fractionated samples for 24 h. Culture media were then collected and TNF-α, IL-1β, and IL-6 concentrations were determined.

2.11 | HPLC-DAD-UV analysis

Chromatographic analyses of SH sub-fractions were performed using a HPLC system (Agilent 1260 infinity HPLC, Agilent, Santa Clara, CA, USA) equipped with a UV-Diode array detector, degasser, and auto-sampler. Saponarin standard (Chemfaces, Hubei, Wuhan, China) and filtered fractionated samples were diluted with pure methanol to 1 mg/ml. The main chemical components of fractionated samples were analyzed using an Agilent Eclipse XDB-C₁₈ chromatographic column (150 × 4.6 mm, 5 µm pore size) at a flow rate of 1 ml/min, a column temperature of 23.5°C, and a detection wavelength of 258 nm by gradient elution (mobile phase A—water and mobile phase B—acetonitrile) as follows: 0–5 min, 95% A; 5–20 min, 95%–0% A; 20–23 min, 0%–95% A; and 23–25 min, 95% A. Sample saponarin content was calculated by using a standard linear regression curve.

2.12 | Zebrafish maintenance

All zebrafish (AB strain) were maintained at 28°C with a 14/10 h light/dark cycle, following the standard guidelines of the Committee of Jeju National University (Jeju, Jeju Province, Republic of Korea; No: 2022-0013). Embryos were harvested from natural mating and cultured in embryo media. Fertilized embryos were incubated in 2 mg/L methylene blue containing E3 embryo media (1.6 g KCl, 34.8 g NaCl, 5.8 g CaCl₂•2H₂O, and 9.78 g MgCl₂•6H₂O in 1 liter of double distilled water, pH 7.2±0.1) at 28.5°C.

2.13 | Toxicity evaluation, morphological observation and heartbeat rate measurement

Zebrafish embryos (n = 20 per groups) were pretreated with 1-phenyl-2-thiourea (PTU, 0.003%) after 1 day post-fertilization (dpf). At 3 dpf, LPS (0.5 mg/ml, 2 nl per each larva) was injected into the yolk sac after anesthesia using a Drummond NANOJECT III injector (Drummond Scientific, Broomall, PA, USA). Same volume of PBS was injected to the untreated group as control. The micro-injected larvae were simultaneously transferred to the embryo medium in the presence of fr.1 or Saponarin (0–100 µg/ml) for 48 h and the morphological abnormality and survival rate were observed. All images were captured using Olympus SZ2-ILST stereo microscope (Tokyo, Japan), and the heart rate of zebrafish was measured to evaluate the cardiotoxicity of fr.1 or Saponarin.

2.14 | Isolation of total zebrafish RNA and RT-PCR

Total RNA was extracted from zebrafish larvae at 18 hpi using an easy-BLUE™ total RNA extraction kit (iNtRON Biotechnology, Seoul, Republic of Korea) according to the manufacturer’s instruction. Two micrograms of the extracted RNA was reverse transcribed using reverse transcription kit (Bioneer). The following PCR conditions were applied for PCR amplification: ziNOS, zIL-1β, and zIL-6, 27 cycles of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, and extended at 72°C for 1 min; zTNF-α and zCOX-2a, 32 cycles of denaturation at 95°C for 45 s, annealing at 57°C for 45 s, and extended at 72°C for 1 min; β-actin, 27 cycles of denaturation at 95°C for 45 s, annealing at 61°C for 45 s, and extended at 72°C for 1 min. The primers used are described in Supporting Information Table S2. zβ-actin was used as internal controls.

2.15 | Molecular docking studies

Ligand preparation: the chemical structures of saponarin and other TLR4 antagonists were downloaded in SDF format from the PubChem (https://pubchem.ncbi.nlm.nih.gov/) and ZINC (https://zinc.docking.org/) databases. Receptor protein preparation: 3D X-ray crystal structures of mouse TLR4/MD2 complex (PDB ID: 2Z64) were obtained from the PDB database (www.rcsb.org/pdb). Molecular docking simulation was used to determine binding energies and to identify the binding site exhibiting best fit between saponarin and TLR4/MD2 complex.
SwissDock (http://www.swissdock.ch) was used for blind docking and Autodock Vina 1.1.2 for local docking simulation (focused view was used for MD2). 3D Structural analysis and ligand-protein interaction analysis were performed using UCSF chimera software 1.15 and PyMOL 2.5.2. Ligplot plus 2.2.4 was used to construct schematic diagrams of the interaction between saponarin and TLR4/MD2 complex.

2.16 Statistical analysis

Experimental data were analyzed using Graph Pad Prism version 5.0 software (Graph Pad, La Jolla, CA, USA). Standard curves were constructed using Excel and PowerPoint (Microsoft, Redmond, WA, USA). Analysis of variance and One-Way ANOVA with Dunnett's multiple comparison tests were used to determine the significances of differences. Results are presented as means ± SDs, and p-values of <.05 were considered statistically significant.

3 RESULTS

3.1 Effects of SH on cell viability

THP-1 or RAW264.7 macrophages were incubated with various concentrations of SH for 24 h. Neither THP-1 nor RAW 264.7 macrophages showed any significant decrease in viability at SH concentrations of <100 μg/ml (107.5% and 95.4% at 100 μg/ml respectively) (Figure 1A and Supporting Information Figure S1A). Subsequent experiments were performed using SH at 10 or 50 μg/ml.

3.2 SH inhibited iNOS and COX-2 expressions in LPS-stimulated macrophages

At a concentration of 50 μg/ml, SH reduced LPS-induced NO production from 23.3 to 15.6 μM in RAW264.7 cells (Supporting Information Figure S1B), whereas treatment with SH alone did not significantly alter NO production. SH significantly and dose-dependently inhibited the LPS-induced production of iNOS and obviously suppressed LPS-induced COX-2 protein levels in THP-1 and RAW 264.7 macrophages (Figure 1B,C and Supporting Information Figure S2A,B). Furthermore, SH co-treatment at 10 or 50 μg/ml significantly reduced LPS induced increases in iNOS and COX-2 mRNA levels in each cell line (Figure 1D,E and Supporting Information Figure S2C,D). No significant difference in protein or gene levels was observed in cells treated with SH alone. Notably, SH significantly inhibited the LPS-induced expressions of iNOS and COX-2 in THP-1 and RAW264.7 macrophages.

3.3 SH regulated MAPK activity in LPS-stimulated macrophages

The inhibitory effects of SH on MAPK signaling in THP-1 or RAW264.7 macrophages were investigated by immunoblotting. LPS markedly elevated the phosphorylation level of ERK1/2, JNK, and p38 MAPKs. However, treatment with SH inhibited LPS-induced activation and dose-dependently reduced the phosphorylations of ERK1/2, JNK, p38, in THP-1 macrophages (Figure 1F,G). Under the same conditions, SH significantly inhibited the LPS-induced phosphorylations of ERK1/2, JNK and p38 in RAW 264.7 macrophages (Supporting Information Figure S3A,B).

3.4 SH suppressed the nuclear translocation of NF-κB in LPS-stimulated macrophages

The impacts of SH on NF-κB family members in THP-1 or RAW264.7 macrophages were investigated by analyzing phosphorylated protein levels. Western blot showed SH significantly inhibited the LPS-induced phosphorylation of IκB-α (Figure 2A), (Supporting Information Figure S4A), and significantly blocked NF-κB translocation to the nucleus (Figure 2B and Supporting Information Figure S4B). Immunofluorescence images indicated SH reduced the LPS-induced nuclear translocation of the p65 subunit of NF-κB (Figure 2C and Supporting Information Figure S4C). However, SH (50 μg/ml) alone had no obvious effect on the translocation of p65.

3.5 SH co-treatment reduced pro-inflammatory cytokine levels in LPS-stimulated macrophages

ELISA and qPCR were conducted to investigate the effects of SH on pro-inflammatory cytokine production. Co-treatment with SH at 10 or 50 μg/ml significantly and dose-dependently inhibited LPS-induced upregulations of TNFα, IL-1β, and IL-6 proteins in THP-1 macrophages (Figure 3A), and qPCR results showed that SH markedly suppressed the gene expressions of these...
pro-inflammatory cytokines (Figure 3B). Similar results were obtained using RAW264.7 cells, SH co-treatment at 50 µg/ml significantly reduced TNFα, IL-1β, and IL-6 protein and mRNA levels (Supporting Information Figure S5A,B).

3.6 | fr.1 inhibited the pro-inflammatory effects of LPS in macrophages

Six sub-fractions of SH were obtained by silica gel chromatography. THP-1 and RAW 264.7 macrophages were
co-treated with LPS and each of these sub-fractions at different concentrations (1–20 μg/ml) for 24 h. No sub-fraction affected cell viability at <10 μg/ml (Figure 4A). Viability results for THP-1 and RAW 264.7 macrophages were similar (Supporting Information Figure S6A). fr.1, fr.2, and fr.3 significantly inhibited the LPS-induced upregulations of iNOS and COX-2 at the protein and gene levels, and fr.1 had the greatest effect, which was comparable with that of indomethacin (Figure 4B). Furthermore, co-treatment with sub-fractions samples decreased LPS-induced MAPK phosphorylation, and fr.1 and fr.6 significantly reduced LPS-induced phosphorylated ERK1/2, JNK, and p38 MAPK levels (Figure 4C). In addition, immunoblot band intensities showed fr.1 markedly inhibited LPS-induced IκB-α phosphorylation and NF-κB nuclear translocation (Figure 4D).
3.7 | fr.1 inhibited the JAK2/STAT3 pathway by reducing LPS-induced pro-inflammatory cytokine production in macrophages

Co-treatment with fr.1 at 10 μg/ml significantly inhibited LPS-induced TNF-α, IL-1β, and IL-6 increases in THP-1 macrophages (Figure 5A). In addition, all six sub-fractions reduced LPS-induced NO production and pro-inflammatory cytokine (TNF-α, IL-1β and IL-6) levels in RAW264.7 macrophages (Supporting Information Figure S6B,C). Furthermore, co-treatments with fr.1 or fr.2 at 10 μg/ml significantly decreased the phosphorylation ratios of JAK2 and STAT3 as compared with LPS treated THP-1 macrophages (Figure 5B).

3.8 | SH sub-fractions inhibited the expressions of genes associated with early M1 polarization induced by LPS plus IFNγ in THP-1 macrophages

To evaluate the effect of SH sub-fractions on genes involved in initial M1 polarization, THP-1 macrophages were treated with sub-fractions (10 μg/ml) in the absence or presence of LPS (100 ng/ml) plus human IFN-γ (1 μg/
ml) for 12 h. Co-treatment with IFN-γ and LPS potently induced M1 markers (a mixture of binding proteins, costimulatory molecules, and chemokines (e.g., CD38, CD80, CD86, HLA-DR, and CXCL10), but co-treatment with fr.1 treatment significantly reduced the mRNA expressions of these M1 markers and its effect was greater than those of other sub-fractions (Figure 5C).

3.9 Profiling and identification of major compounds in sub-fractions by HPLC

Sub-fractions (fr.1-fr.6) were analyzed by HPLC fingerprinting using standards of saponin components in Soapwort reported in the literature. In fr.1, the HPLC major peak had a retention time of 11.595 min, which corresponded to saponarin and a concentration of 23.36 μg/ml (Figure 6A,B). Other fractions contained lower saponarin concentrations, which was in line with the increasing polarity of the extraction solvent used Supporting Information Figure S7.

3.10 fr.1 inhibited the LPS-mediated interaction between TLR4 and MyD88 in macrophages

THP-1 macrophages were co-treated with 10 μg/ml of fr.1 or saponarin in the absence or presence of LPS (1 μg/ml). fr.1 inhibited LPS-mediated TLR4-MyD88 complex formation to the control group level as deduced by western blot of Co-IP samples. However, fr.1 treatment alone did not cause a significant change in treatment-naive cells. In addition, saponarin, which is believed to be the primary bioactive molecule in SH and fr.1, significantly inhibited the LPS-induced interaction between TLR4 and MyD88 in both macrophage types (Figure 7A,B).

3.11 Fr.1 and saponarin prevent LPS microinjection-induced acute endotoxin shock in zebrafish larvae

All zebrafish larvae microinjected LPS had a significant reduction in heartbeat rate by half (Figure 8A,D). About 40%-45% of all zebrafish larvae died and about 40–45% showed severe morphological abnormalities (Figure 8B,E). Morphological abnormalities such as Pericardial edema, head malformation, bent spin, short tail, yolk sac edema, uninflated bladder, and hemorrhagic lesions were observed (Figure 8C,F). In contrast, fr.1 or Saponarin treatment dose-dependently inhibited LPS-mediated mortality and abnormality in the larvae. The highest concentration of sample (100 μg/ml) significantly suppressed mortality by 0% and inhibited morphological abnormalities by approximately 10%.

3.12 Fr.1 and saponarin inhibited LPS microinjection-induced pro-inflammatory cytokines or mediators expression determined by conventional PCR in zebrafish larvae

At 18 hpi, fr.1 treatment significantly reduced LPS-induced overexpression of ziNOS, zCOX-2, ziTNFα, ziIL-6 and ziIL-1β mRNA (Figure 9A). Saponarin also exhibited coincident result, with stronger zCOX-2 inhibitory potential compared to that of fr.1 (Figure 9B).

3.13 Molecular docking studies on saponarin and TLR4/MD2 complex

Docking simulations were conducted to evaluate the binding affinity between saponarin and TLR4/MD2 complex using estimated ΔG values and binding pose. Dexamethasone, which is known to interact with TLR4/MD2, was used for comparison purposes. Blind docking performed using Swissdock revealed that most of the docking clusters of saponarin and dexamethasone are located in a hydrophobic pocket in MD2 (Figure 10A). Moreover, the best model for MD2-ligand docking simulation produced estimated binding energies for saponarin and dexamethasone of −8.2 and −7.0 kcal/mol indicating stronger attraction of saponarin (Figure 10B). Numerous labeled sidechains and residues of protein proximal to ligand showed hydrophobic contact with saponarin in the MD2 pocket (Figure 10C,D). Molecular docking predictions indicated that saponarin interacts with TLR4/MD2 complex and antagonizes the effects of endotoxins like LPS.

F I G U R E 5 fr.1 inhibited the JAK2/STAT3 pathway by reducing pro-inflammatory cytokine production in THP-1 macrophages and inhibited the expression of genes associated with early M1 polarization induced by co-treatment with LPS and IFN-γ. (A) Relative expressions of TNFα, IL-1β, and IL-6 as determined by ELISA, and relative expressions of TNFα, IL-1β, and IL-6 as determined by qPCR. (B) Relative expressions of phosphorylated JAK2/STAT3 as determined by western blot. Results are presented as the means ± SDs of three independent experiments. *p < .01 versus non-treated THP-1 macrophages, and **p < .05, ***p < .01 versus LPS-treated THP-1 macrophages. (C) Relative expressions of M1 markers, that is, CD38, CD80, CD86, HLA-DR, and CXCL10, as determined by qPCR. Results are presented as the means ± SDs of three different experiments. *p < .01 versus LPS-treated THP-1 macrophages, and **p < .05, ***p < .01 versus IFN-γ and LPS co-treated THP-1 macrophages.
**DISCUSSION**

TLR4-MyD88 complex, which acts as a receptor and an adaptor molecule, plays a critical role in the initial steps of innate and acquired immunity. When TLR4 is stimulated by LPS, the TIR (Toll/Interleukin-1 receptor) domain of MyD88 plays a pivotal role in adaptor protein complex assembly with the cytosolic domain of TLR4 and

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**Graphs and Figures:**

- **Graph (A):**
  - TNF-α (pg/ml) and IL-10 (pg/ml) levels in response to various LPS concentrations.
  - TGF-β (pg/ml) levels in response to various LPS concentrations.

- **Graph (B):**
  - JAK2/STAT3 pathway activation in response to LPS treatment.

- **Graph (C):**
  - Gene expression levels of CD38, CD86, and HLA-DR in response to LPS and IFN-γ treatment.
results in TIR-TIR interaction.\textsuperscript{4,49} This assembly of the TLR4-MyD88 signalosome is required for signal transduction from the membrane to downstream signaling protein domains.\textsuperscript{50} At the clinical level, the candidate TLR4 inhibitors eritoran and TAK-242 progressed to Phase III clinical studies after achieving excellent preclinical/phase I and II clinical results.\textsuperscript{51}

However, these drugs failed to reduce mortality significantly in clinical trials on sepsis patients.\textsuperscript{52,53} The authors reported no significant differences in serum interleukins or cytokines in patients administered experimental drugs or placebos. To identify other candidate drugs that modulate TLR4-MyD88 signaling, various molecular types have been investigated as potential ligands of TLR4-MyD88,
**FIGURE 8**  fr.1 and saponarin inhibited LPS microinjection-induced abnormality and mortality in zebrafish larvae.  
(A) Heartbeat rate of zebrafish larvae after LPS injection (0.5 mg/ml) with fr.1 (0–100 μg/ml) treatment.  
(B) Classification of phenotypes of zebrafish after LPS injection with fr.1.  
(C) Critical abnormalities morphologically observed under microscopy after LPS injection in fr.1.  
(D) Heartbeat rate of zebrafish larvae after LPS injection (0.5 mg/ml) with saponarin (0–100 μg/ml) treatment.  
(E) Classification of phenotypes of zebrafish after LPS injection with saponarin.  
(F) Critical abnormalities morphologically observed under microscopy after LPS injection in saponarin.  

### p < .001 versus negative control larvae, and *** p < .001 versus LPS treated larvae. (n = 20 in each group).

**FIGURE 9**  fr.1 and saponarin inhibited LPS microinjection-mediated pro-inflammatory cytokines or mediators expression determined by conventional PCR in zebrafish larvae.  
(A) Relative gene expression of zlNOS, zCOX-2, zTNFa, zIL-6, zIL-1β in LPS-microinjected zebrafish co-treated with fr.1.  
(B) Relative gene expression of zlNOS, zCOX-2, zTNFa, zIL-6, zIL-1β in LPS-microinjected zebrafish co-treated with saponarin.
such as LPS mimetics, synthetic LPS analogs, proteins, and nanotubes.\textsuperscript{9}

In this study, different cell models and ligand-protein virtual docking simulations showed that SH, fr.1, and saponarin all interacted with TLR4/MyD88 complex and suppressed subsequent inflammatory signaling. The LPS-induced assembly of TLR4-MyD88 was significantly hindered by fr.1 or saponarin, and thus, MyD88-dependent downstream signals were not transduced (Figure 6C,D). Furthermore, subordinate pro-inflammatory pathways, and consequently cytokine and interleukins levels, which are subjects of TLR4-MyD88 signaling, were also suppressed by fr.1 or SH. Several studies that have evaluated efficacies of TLR4 antagonists have presented similar results regarding diminished MAPK, NF-κB, and cytokine production.\textsuperscript{54–57}

\textbf{FIGURE 10} Molecular docking studies of the interaction between saponarin and TLR4/MD2 complex. (A) Depiction of ligand clusters of saponarin or dexamethasone binding to TLR4/MD2 complex as deduced by protein-ligand docking simulation in Swissdock. (B) Estimated binding energy and pose of the best MD2-ligand binding simulation model. UCSF chimera depicted MD2 protein as a ribbon and provided a surface image. (C) Sidechains of MD2 protein proximal to ligands (within 5 Å) were labeled and visualized by UCSF chimera. (D) 2D image of non-ligand residues and its corresponding atoms of ligand visualized by Ligplot plus.

Four representative saponins present in soapwort extract were identified by HPLC, which also showed that saponarin contents differed significantly in the six fractions (Figure 6 and Supporting Information Figure S7). However, the unfavorable characteristics of saponarin as a drug regarding molecular (with respect to its molecular weight, Hdon, and Hacc figures) and pharmacological properties (low CaCO-2 permeability and oral bioavailability) have raised questions regarding its mode of action.

In a previous study, we reported that cells co-treated with fr.1 and LPS exhibited significantly lower inflammatory responses than cells pretreated with LPS for 1 h and then treated with fr.1 (data not shown). Therefore, we assumed that the anti-inflammatory properties of fr.1 and saponarin
would manifest during the initial stage of inflammation, and that the mechanism responsible would be directly related to binding between LPS and TLR4-MD2 complex.

Our docking simulation of saponarin with TLR4-MD2 complex revealed that saponarin properly docked into the hydrophobic pocket of MD2, that is, the LPS binding site, with a higher binding score than dexamethasone, a known antagonist of TLR4-MD2 complex (Figure 8A,B). Similarly, ginsenoside Ro (a saponin from Panax ginseng) inhibited TLR4 signaling and antagonized LPS docking with TLR4/MD2 as determined by molecular dynamics simulation. In addition to the interaction between saponarin and TLR4-MD2 complex, we also found that fr.1 had an inhibitory effect on the IL-6/JAK2/STAT3 signaling pathway (another classic pro-apoptotic and inflammatory pathway). The wide spectrum of potential bioactivities offered by saponin glycosides and aglycones suggest that saponarin may have many potential therapeutic uses by the amphipathic nature.

M1 polarization of macrophages is stimulated by microbial components or pro-inflammatory cytokines like LPS, TNF-α, or IFN-γ or other TLR activators, and thus, the inhibition of macrophage M1 polarization is achieved by preventing these external stimuli impacting macrophages. Transition to M1 phenotypes was inhibited by SH as demonstrated by depressed expression of M1 markers of CD38, CD80, CD86, HLA-DR, and CXCL10 and the efficacy was comparable to interleukin-4.

Consistent results acquired from the in vivo study also reinforced our points. As the zebrafish larvae yolk is highly immune-responsive site, infiltration of LPS in the site triggers migration of macrophages and inflammatory cascades. It has been reported that the LPS-mediated excessive inflammation increases mortality and fatal deformity in zebrafish embryo. The results from in vivo experiment demonstrated solid drug potential of our sample (fr.1 and saponarin), highlighting zero mortality in groups with highest concentration (Figure 8B,E).

Taken together, our results suggest saponarin reduces downstream inflammatory response by interfering with binding between LPS and TLR4/MD2 complex, and thus, blocking MyD88 dependent signaling. This report details the pharmacological actions of a soapwort fraction and its major component saponarin and the mechanisms responsible for their inhibition of TLR4/MyD88 signaling in macrophages. We conclude that saponarin is a promising potential treatment for inflammatory diseases.

5 CONCLUSION

In summary, our findings explain the modulating effect of Saponaria officinalis extract and saponarin on innate immunity. Saponarin was found to interact directly with TLR4/MD2 complex, inhibit TLR4/MyD88 signaling, and suppress M1 polarization. Saponarin markedly attenuated LPS-induced mortality and deformity by decreasing inflammatory cytokines in zebrafish larvae. Our results suggest that saponarin is a promising candidate for the treatment of inflammatory diseases.

AUTHOR CONTRIBUTIONS

Ga-Ram Yu performed the cell experiments, quantitative assays, analyzed western blot data, and generated the figures. Dong-Woo Lim wrote the manuscript and designed the experiments. Wisurumuni Arachchilage Hasitha Maduranga Karunarathne performed in vivo experiments. Gi-Young Kim designed and supervised in vivo study. Hyuck Kim conceived and designed the experiments. Won-Hwan Park analyzed the data and revised the manuscript. Jai-Eun Kim supervised the project and contributed to the final draft. All authors read and approved the final manuscript.

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DISCLOSURES

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

All data that support the findings of this study are included.

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