Research Article

Nonsaponin fraction of Korean Red Ginseng attenuates cytokine production via inhibition of TLR4 expression

Huijeong Ahn 1,*, Byung-Cheol Han 1,4,*, Jeongeun Kim 1,*, Seung Goo Kang 2, Pyeung-Hyeun Kim 1, Kyoung Hwa Jang 4, Seung Ho So 4, Seung-Ho Lee 4, Geun-Shik Lee 1,*

1 College of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chuncheon, Republic of Korea
2 Division of Biomedical Convergence, College of Biomedical Science, Kangwon National University, Chuncheon, Republic of Korea
3 Department of Molecular Bioscience, School of Biomedical Science, Kangwon National University, Chuncheon, Republic of Korea
4 Korea Ginseng Research Institute, Korea Ginseng Corporation, Daejeon, Republic of Korea

1. Introduction

Ginseng is a root of Panax ginseng Meyer of the family Araliaceae and is cultivated in northeastern Asian countries such as Korea and parts of China. Although the various pharmacological properties of ginseng have been verified, its ability to modulate immune systems is continuously studied [1]. Ginsenoside, a saponin of ginseng, is mostly noted as an immune modulator. Korean Red Ginseng is made by repetitive steaming and drying cycles of ginseng root and contains more ginsenosides than fresh ginseng [2,3]. Ginsenosides attenuate production of cytokines of immune cells through inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling and maturation of cytokines through attenuation of inflammasome activation [4,5]. In addition to saponin components of ginseng acting as immune modulators, nonsaponin (NS) ingredients such as polysaccharides possess...
immunomodulatory properties [6–9]. Ginsan and red ginseng acidic polysaccharide have been shown to induce inflammatory cytokines through toll-like receptor (TLR)/NF-κB signaling [6–9].

The effects of ginsenosides and NSs isolated from Korean Red Ginseng extracts (RGEs) on inflammasome activation have been progressively studied [4,10]. Inflammasome, an intracellular surveillance factor that detects dangerous molecules derived from pathogens and endogenous metabolites, mediates maturation of interleukin (IL)-1β and IL-18 through cleavage of caspase-1 and induces pyroptosis by forming membrane pores of gadermin D [11,12]. RGE and its fractions, saponin fraction (SF) and nonsaponin fraction (NS), present contrasting effects on inflammasome activation [4,10]. SF containing ginsenosides attenuates cytokine secretion by inhibiting the priming and activation steps of inflammasome activation, whereas NS induces upregulation of IL-1β precursor and nucleotide-binding and oligomerization domain (NOD)-2–like receptor protein 3 (NLPR3), which are key inflammasome components, via interaction with TLRs [4,10]. In addition, a high dose of RGE presents a more SF predominant cytokine profile [4,10].

In this study, we elucidated the roles of SF and NS of RGE on the effects of ginsenosides and NSs isolated from Korean Red Ginseng (Panax ginseng) provided by Korea Ginseng Corporation (Daejeon, Korea) on peritonitis by observing expression of TLRs using in vitro and in vivo systems.

2. Materials and methods

2.1. Preparation of SF and NS of RGE

RGE was manufactured from roots of 6-year-old fresh Panax ginseng provided by Korea Ginseng Corporation (Daejeon, Korea). Subfractions of RGE, SF and NS, were prepared according to previous studies [10,13]. Briefly, RGE (2.0 Kg) was subjected sequentially to adsorption chromatography using H2O, 20% ethyl alcohol (EtOH), and absolute EtOH (Daejung Chemicals and Materials Co., Siheung-si, Gyeonggi-do, Korea) as eluents. No ginsenosides were detected on H2O or 20% EtOH elution, which was combined and evaporated to dryness in vacuo (NS, 1.1 kg). Absolute EtOH yield was 135.4 g of SF. According to the results of the component analysis (Supplementary data), SF contained higher saponin content (223.4 mg/g) than NS (5.5 mg/g). NS showed fourfold higher acidic polysaccharide content and sixfold higher arginine–fructose–glucose content than SF. Generally, the ginsenoside content of Korean Red Ginseng powder was 18.5 mg/g. The contents of SF, NS, and RGE are summarized in Table 1.

2.2. Animals study

Male C57BL/6 mice (8 weeks old) obtained from Narabio Co. (Seoul, Korea) were maintained under a 12-h light/dark cycle at 24 ℃. Mice were supplied standard sterile food and water ad libitum, after which they were allowed to adjust to the environment for 1 week. Dosage of RGE to mice was elevated threefold [2 mg/mouse (20 g)/day] than the recommended daily intake of Cheongkwanjang [2 g/human (60 kg)/day], and the concentrations of NS and SF were based on the ratio of elution (RGE: NS: SF = 2: 1.1: 0.1354). Mice were orally administered 200 μL of filtered water as vehicle (Non; nontreatment), RGE (2 mg/mouse/day), NS (11 mg/mouse/day), or SF (135.4 μg/mouse/day) for 7 days and then intraperitoneally (ip) injected with monosodium urate crystal (MSU, 10 mg/mouse; U2875; Sigma-Aldrich Co., St. Louis, MO, USA), aluminum potassium sulfate (Alum, 5 mg/mouse; 039-4404; Daejung Chemicals & Materials Co.), or lipopolysaccharide (LPS, 10 μg/mouse; L4130; Sigma-Aldrich Co.). After 6 h, mice were anesthetized with ether inhalation and sacrificed by decapitation. Peritoneal cavities were washed with 5 mL of phosphate-buffered saline, and peritoneal exudate cells (PECs) were collected for further analysis. To eliminate intestinal bacteria, mice were daily fed 200 μL of antibiotic cocktail containing vancomycin (50 μg/mL), ampicillin (100 μg/mL), kanamycin (100 μg/mL), neomycin (100 μg/mL), streptomycin (50 μg/mL), penicillin (100 U/mL), gentamycin (150 μg/mL), cefazolin (100 μg/mL), and cefradine (50 μg/mL) for 7 days. For assessment of bacterial burden, peritoneal lavage (10 μL) was dropped onto Luria-Bertani (LB; Laboratories Conda, Madrid, Spain) plates and incubated at 37 ℃ overnight. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Kangwon National University (IACUC; approval no. KW-170110-1).

2.3. Cell culture and treatment

Unless otherwise indicated, all materials for cell culture were purchased from GenDEPOT Inc. (Barker, TX, USA). Bone marrow–derived macrophages (BMDMs) were obtained by differentiation of bone marrow progenitors from tibia and femur bones of C57BL/6 mice (8–15-weeks old; Narabio Co.) in L929 cell-conditioned medium as a source of macrophage colony-stimulating factor [14]. The progenitors were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum, 50% L929 cell-conditioned medium, 100 μU/mL of penicillin, and 100 μg/mL of streptomycin. Cells were seeded in nontissue culture–treated Petri dishes (SPL life science Co., Pocheon-si, Gyeonggi-do, Korea) and incubated at 37 ℃ in 5% CO2 atmosphere for 7 days. For inflammasome activation, BMDMs (1.0 × 106 cells per well) were primed with 1 μg/mL of LPS (Sigma-Aldrich Co.) and treated with MSU (0.8 mg/mL) with/without RGE, NS or SF, for 3 h [15]. Cellular supernatant (Sup) and lysate (Lys) were collected for further analysis.

For example, gene expression (2.0 × 105 cells per well for RNA extraction or 1.0 × 106 cells per well for protein analysis) were plated on 6- or 12-well plates (SPL Life Science Co.) and treated with NS (1 mg/mL). In addition, BMDMs were treated with MSU (0.5 mg/mL) or NS (1 mg/mL) with/without CU-CPT22 (5 mM, 4884; Tocris Bioscience, Minneapolis, MN, USA), Anti-mTLR2-IgG

### Table 1

| Components | RGE (mg/g ± SD) | Saponin fraction (SF, mg/g ± SD) | Nonsaponin fraction (NS, mg/g ± SD) |
|------------|----------------|-------------------------------|-----------------------------------|
| Ginsenoside |                 |                               |                                   |
| Rg1        | 1.06 ± 0.02     | 13.33 ± 0.70                  | 0.35 ± 0.61                       |
| Re         | 1.21 ± 0.03     | 15.68 ± 0.77                  | 0.40 ± 0.70                       |
| Rf         | 1.04 ± 0.02     | 13.39 ± 0.89                  | 0.35 ± 0.6                       |
| Rh1        | 0.96 ± 0.01     | 11.54 ± 1.05                  | 0.32 ± 0.56                       |
| Rg2s       | 1.43 ± 0.08     | 15.75 ± 0.96                  | 0.48 ± 0.82                       |
| Rb1        | 5.19 ± 0.03     | 64.20 ± 5.02                  | 1.73 ± 3.00                       |
| Rc         | 2.02 ± 0.05     | 25.98 ± 2.54                  | 0.67 ± 1.17                       |
| Rb2        | 1.88 ± 0.02     | 22.96 ± 1.63                  | 0.63 ± 1.08                       |
| Rd         | 0.67 ± 0.00     | 8.29 ± 0.75                   | 0.22 ± 0.39                       |
| Rg3s       | 2.10 ± 0.03     | 21.69 ± 2.95                  | 0.70 ± 1.21                       |
| Rg3r       | 0.96 ± 0.01     | 10.60 ± 1.30                  | 0.32 ± 0.55                       |
| Arginine–fructose–glucose | 28.18 ± 1.06 | 6.75 ± 2.97 | 39.78 ± 10.06 |
| Acidic polysaccharides | 63.10 ± 0.53 | 21.40 ± 2.53 | 95.43 ± 28.05 |
| Water      | 4.42 ± 1.22     | 3.38 ± 1.30                   | 5.64 ± 1.24                       |

RGE, Red ginseng extract; SD, standard deviation.
(100 ng/mL; InvivoGen, San Diego, CA, USA), TAK-242 (5 μM, CLI-095; InvivoGen), polymyxin B (100 μg/mL; thr-lpm, InvivoGen), MyD88 inhibitor peptide (50 μM, thr-lpmyd, InvivoGen), and Bay 11-7082 (5 μM; 1744, Torcis Bioscience) for 3 h or 9 h. BMDMs were pretreated with NS (1 mg/mL) for 3 h and then replaced with fresh media. After 30 h, cells were treated with LPS (5 ng/mL) or heat-killed Salmonella typhimurium (HKST, 0.001%) for 3 h. Total RNA and cellular lysates were prepared for further analysis.

2.4. Reverse transcription polymerase chain reaction and quantitative real-time polymerase chain reaction

Total RNA was extracted using NucleoZOL (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and reverse transcribed to first-strand complementary DNA (cDNA) using an M-MLV cDNA Synthesis kit (Enzymomics, Daejeon, Korea) [16]. For reverse transcription polymerase chain reaction (RT-PCR), transcription was amplified by a SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc. Grand Island, NY, USA) and nqαf polymerase (Enzymomics). PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. For quantitative real-time PCR (qPCR), gene expression was quantified using an Eco Real-Time PCR system (Bio-Rad, CA, USA) and TOPReal qPCR Premix from Bio Vision (Mountain View, CA, USA). The qPCR reactions contained SYBR Green (Enzymics). Quantitation was normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Gene-specific primers are listed below. Pro-IL-1β (GeneBank ID: NM_008361) primers 5′-CCG ACC CAA TAC CCA AAG AA-3′ and 5′-GCT TGT GCT CTG CTT GA-3′; TNFα (NM_013693) 5′-ACG GCA TGG ATC TCA AAG AC-3′ and 5′-GTG GGT TCC AGC AAG AT-3′; IL-6 (NM_031168) 5′-CCG ACC TTA TTT TCT TCT GG-3′ and 5′-CTG GGT GAG GAG CAC GTA CTG-3′; IL-1α (NM_010554) 5′-CCG ACC TTC TGT TCT TCT GG-3′ and 5′-CTG GGT GAG GAG CAC GTA CTG-3′; IL-1β (NM_010548) 5′-TCA TTT CCG ATA AGG CTT GG-3′ and 5′-TGG ATC TCA AAG AC-3′; IL-18 (NM_010553) 5′-CGG AAT TGG GGT AGG AAG GA-3′ and 5′-GGA AAT TGG GGT AGG AAG GA-3′; TLR1 (NM_003263) 5′-AGG GCA TGG GGT GAG GAG CAC GTA CTG-3′ and 5′-GGA AAT TGG GGT AGG AAG GA-3′; TLR2 (NM_004415) 5′-CTG CTT GGT GAG GAG CAC GTA CTG-3′ and 5′-GGA AAT TGG GGT AGG AAG GA-3′; TLR3 (NM_003265) 5′-TCA TTT CCG ATA AGG CTT GG-3′ and 5′-TGG ATC TCA AAG AC-3′; TLR4 (NM_001289726) 5′-CCG ACC TCA TTT TCT TCT GG-3′ and 5′-CTG GGT GAG GAG CAC GTA CTG-3′; TLR7 (NM_0003268) 5′-GGG CGA TTA AGG CTT TAT CG-3′ and 5′-AGG GCA TAT AAT ACC TCT CG-3′; TLR8 (NM_006066) 5′-GGT CTT GGG TGA AAA GTG AAA-3′ and 5′-GAA AAT GGT GTT GGG TGA AA-3′; TLR9 (NM_017442) 5′-GGA AAA CCT TCT TCT TCT CA-3′ and 5′-TGG ATC TCA AAG AC-3′.

2.5. Western blot analysis

Sup and lys samples were separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (10% or 16%), transferred onto a polyvinylidene fluoride (PVDF) membrane (Pall Corporation, Port Washington, NY, USA), and blocked with 3% skim milk. The membrane was probed with primary antibodies against antimouse IL-1β antibody (AF-401-NA; R&D Systems, MN, USA); anticaspase-1 antibody (AG-208-0042; AdipoGen Co., San Diego, CA, USA); anti-TLR4 antibody (sc-293072; Santa Cruz Biotechnology, Santa Cruz, CA, USA); or antiactin antibody (sc-1615; Santa Cruz Biotechnology) overnight at 4°C. The membranes were further probed with Horseradish Peroxidase (HRP)-conjugated second antiserum (Santa Cruz Biotechnology) and visualized using Power-Opti ECL solution (BioNote Co., Gyeonggi-do, Korea) and a cooled CCD camera System (AE-9150, EZ-Capture II; ATTO Technology, Tokyo, Japan). Band intensity was measured by CS Analyzer, version 3.00 (ATTO Technology).

2.6. Cytokine assay

To quantify secretion of IL-1β, IL-6, and IL-18, peritoneal lavage fluids or cell culture supernatants were measured by a mouse IL-1β/IL-1F2 Salmonella typhimurium ELISA Kit (MTA00B; R&D Systems), mouse IL-6 Quantikine ELISA Kit (M6000B; R&D Systems), and mouse IL-18 platinum ELISA (eBioscience, San Diego, CA, USA). The plates were read out using a Synergy H1 microplate reader (BioTek, Winooski, VT, USA).

2.7. Statistical analyses

Statistical analyses were performed using a t test (Mann–Whitney test) for the two groups or one-way analysis of variance (Tukey’s multiple comparisons test) for multiple groups using GraphPad Prism 6 (GraphPad Software, San Diego CA).

3. Results

3.1. Oral administration of NS inhibits peritoneal IL-1β and IL-6 secretion in MSU-induced peritonitis

To elucidate the in vivo properties of NS of RGE in an inflammammasome-mediated disease model of MSU-induced peritonitis [17,18], mice were fed Korean RGE, SF, or NS for 7 days and then ip injected with MSU. Peritoneal IL-1β secretion was measured as a readout of inflammammasome activation. As seen in Fig. 1A, MSU-induced peritoneal IL-1β secretion, whereas secretion was significantly attenuated in RGE- or SF-fed mice. This result implies that NS has antiinflammammasome properties. Then, we treated RGE, SF, or NS on LPS-primed BMDMs to confirm their antiinflammammasome properties in an in vitro system (Fig. 1B). RGE or SF treatments blocked MSU-mediated caspase-1 and IL-1β secretions, whereas NS did not. This result suggests that NS does not have antiinflammammasome properties. We then observed peritoneal IL-6 secretion in MSU-injected mice fed RGE, NS, or SF (Fig. 1C). Interestingly, IL-6 secretion levels in RGE- or SF-fed mice were significantly lower than those in control and SF-fed mice, similar to the results for IL-1β secretion. Based on these data, NS ingestion might inhibit inflammatory cytokine secretion resulting from MSU-induced peritonitis independent of inflammammasome activation.

3.2. NS intake inhibits cytokine production resulting from peritonitis

We further confirmed the inhibitory effect of NS intake on peritoneal IL-1β secretion in the context of inflammammasome-mediating peritonitis. Mice ingested NS for 7 days and were then ip injected with LPS, a TLR4 ligand and noncanonical inflammammasome trigger [16,19,20], or alum, an inflammammasome trigger [21]. In our results, NS ingestion attenuated peritoneal IL-1β and peritoneal IL-6 secretion in both LPS- and alum-injected mice (Fig. 2A and B). Then, we tested peritoneal IL-18 production, another readout of inflammammasome activation [12,22]. As seen in Fig. 2C, IL-18 secretion in response to MSU or alum was not altered by NS feeding. This result implies that NS attenuated peritoneal production of IL-1β and IL-6 independent of inflammammasome activation. In addition, we elucidated the mRNA expression levels of pro-IL-1β, IL-6, TNFα, and IL-10 in PECs isolated from MSU-injected mice fed with/without NS (Fig. 2D). Similar to peritoneal IL-1β and IL-6 secretions, gene expression
Fig. 1. Effects of RGE, NS, and SF on peritoneal cytokine secretion and inflammasome activation in response to MSU injection. Mice (total n = 57) were fed Non (nontreatment, 200 mL of water, n = 12), Korean Red Ginseng extracts (RGE, 2 mg/mouse/day, n = 15), nonsaponin fraction (NS, 1.1 mg/mouse/day, n = 15), or saponin fraction (SF, 135.4 mg/mouse/day, n = 15) for 7 days and then intraperitoneally (ip) injected with PBS (3 mice for each group) or monosodium urate crystal (MSU, 10 mg/mouse) to induce peritonitis. After 6 h, peritoneal (A) IL-1β and (C) IL-6 secretions were measured. (B) LPS-primed BMDMs were treated with MSU (0.8 mg/mL) in the presence of RGE, NS, or SF as indicated. Secretions of caspase-1 (Casp1) and IL-1β were assayed by immunoblotting and ELISA. Bar graph presents the mean ± SD. All data shown are representative of at least two independent experiments. BMDM, bone marrow–derived macrophages; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; Lys, lysate; PBS, phosphate-buffered saline; SD, standard deviation; Sup, supernatent.

Fig. 2. Effects of NS intake on peritoneal cytokine secretion in peritonitis. Mice (n = 6 per group) were fed Non (200 mL of water) or NS (1.1 mg/mouse/day) for 7 days and then ip injected with (A) LPS (100 μg/mouse) or (B) alum (5 mg/mouse). (C) After 6 h, peritoneal IL-1β, IL-6, and IL-18 secretions were measured. (D) PECs were collected, and mRNA expression levels of pro-IL-1β, IL-6, TNFα, and IL-10 were analyzed. Bar graph presents the mean ± SD. IL, interleukin; ip, intraperitoneally; LPS, lipopolysaccharide; MSU, monosodium urate; NS, nonsaponin fraction; PEC, peritoneal exudate cell; SD, standard deviation.
levels were downregulated by NS ingestion. Based on these data, we can conclude that NS intake inhibits expression of inflammatory cytokines in peritonitis.

3.3. NS ingestion inhibits TLR4 expression via TLR4-MyD88-NF-κB signaling

Although MSU crystals are known to induce production of IL-1β via inflammasome activation [17], they also induce production of inflammatory cytokines by interacting with TLR2 and TLR4 [23]. Therefore, we focused on cytokine production by TLR signaling and tested which TLR is involved in pro-IL-1β expression in response to MSU. BMDMs were treated with MSU in the presence of CU-CPT22 (CU, TLR1/2 antagonist), anti-mTLR2-IgG (αTLR2, TLR2 neutralizing antibody), TAK-242 (TAK, TLR4 inhibitor), polymyxin B (PMB, TLR4 inhibitor), and Bay 11–7082 (Bay, NF-κB inhibitor). As seen in Fig. 3A, upregulation of pro-IL-1β expression by MSU treatment was not altered by the TLR2 blockers, whereas expression was

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**Fig. 3.** Effects of MSU on TLR signaling and NS on TLR4 expression. (A) BMDMs were treated with MSU (0.5 mg/mL) in the presence of TLR inhibitors (CU for TLR1/2, αTLR2 for TLR2, and TAK and PMB for TLR4) or NF-κB inhibitor (Bay) for 3 h. The protein levels of pro-IL-1β were measured as a readout of MSU–TLR interaction. The below bar graph indicates band intensity. Mice (n = 6 per group) fed Non (200 μl of water) or NS (1.1 mg/mouse/day) for 7 days and then ip injected with MSU (10 mg/mouse) to induce peritonitis. PECs were isolated from mice, and (B) TLR4 mRNA and (C) protein expression was analyzed by qPCR and immunoblotting. BMDMs were treated with NS for 3 h, and (D) mRNA and (E) protein expression levels of TLR4 were measured using RT-PCR/qPCR and immunoblotting. (F) BMDMs were treated with NS in the presence of TLR2, TLR4, NF-κB, or MyD88 (MyDi) inhibitor, and TLR4 mRNA expression was assayed by RT-PCR/qPCR. Bar graph presents the mean ± SD. All data shown are representative of at least two independent experiments. MW, molecular weight (100 bp ladder). BMDM, bone marrow–derived macrophages; IL, interleukin; ip, intraperitoneally; MSU, monosodium urate; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NS, nonsaponin fraction; PEC, peritoneal exudate cell; qPCR, quantitative real-time polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation; TLR, toll-like receptor.
Interestingly, the expression of TLR4 was significantly reduced by inhibition of TLR4 and NF-κB signaling. This result implies that MSU induces cytokine expression via TLR4. Then, we confirmed the transcriptional and translational levels of TLR4 in PECs isolated from MSU-injected mice (Fig. 3B and C). Interestingly, the expression of TLR4 was significantly attenuated in MSU-fed mice. In sequence, we assessed whether or not NS directly inhibits TLR4 expression in an in vitro system. BMDMs were treated with NS, and expression of TLR4 mRNA and protein was measured (Fig. 3D and E). NS treatment significantly downregulated TLR4 expression in BMDMs, whereas increased pro-IL-1β expression in macrophages. Furthermore, we tested which downstream pathway transmits NS signaling for downregulation of TLR4 expression. BMDMs were treated with NS in the presence of inhibitors for TLR2 (αTLR2), TLR4 (TAK), NF-κB (Bay), or MyD88 (MyDi, MyD88 inhibitor peptide). As shown in Fig. 3F, decreased TLR4 mRNA expression by NS treatment was recovered by TLR4 and NF-κB inhibitors but not by TLR2 blocker. In addition, MyD88 inhibitor interrupted NS-mediated TLR4 downregulation. Taken together, NS attenuates the expression of TLR4 via the TLR4-MyD88-NF-κB signal pathway, in which the receptor mediates cytokine production in response to MSU injection.

3.4. Intestinal bacteria are a key factor for peritoneal IL-1β secretion in response to MSU injection

Then, we assessed the effects of intestinal bacteria, which might modify the chemical components of NS [24] and/or directly stimulate TLR4 signaling. To eliminate intestinal bacteria, mice were supplied an antibiotic cocktail with/without NS, and peritonitis was induced by MSU injection (Fig. 4A). In the results, antibiotic administration significantly reduced peritoneal IL-1β secretion in both mice fed with/without NS. Although NS ingestion significantly reduced IL-1β production, antibiotic administration showed much more potent effects than NS intake alone, implying that intestinal bacteria directly stimulates peritoneal IL-1β secretion rather than chemical modification of NS. We further tested whether or not bacteria invade the peritoneal cavity during peritonitis. We collected peritoneal lavage from MSU- or LPS-injected mice and cultured the lavage to assess bacterial burden (Fig. 4B). Interestingly, bacterial burden was observed in the peritoneal cavity of MSU- or LPS-injected mice, implying that intestinal bacteria invade into the peritoneal cavity during peritonitis and stimulate TLR4 signaling of peritoneal immune cells to produce cytokines.

3.5. NS attenuates cytokine production via inhibition of TLR4 expression

We then elucidated whether or not NS pretreatment attenuates TLR4-mediated cytokine expression. Macrophages were treated with NS 1 day before treatment with LPS, a TLR4 ligand, or HKST, a TLR4 and TLR5 ligand. In the results, both TLR ligands induced pro-IL-1β expression in BMDMs, whereas increased pro-IL-1β expression in BMDMs pretreated with NS was significantly lower than that in intact BMDMs (Fig. 5A). In addition, NS pretreatment interrupted the upregulation of LPS-mediated cytokine transcription (Fig. 5B). This implies that the efficacy of LPS- and HKST-mediated cytokine production was abrogated because NS

![Fig. 4. Effects of intestinal bacteria on IL-1β secretion in MSU-induced peritonitis. (A) Mice (n = 6 per group) were fed Non (200 μL of water) or NS (1.1 mg/mouse/day) with/without antibiotic cocktail for 7 days and then ip injected with MSU (10 mg/mouse) to induce peritonitis. Peritoneal IL-1β secretion was measured. (B) Mice (n = 4 per group) were ip injected with PBS (200 μL), MSU (10 mg/mouse), or LPS (100 μg/mouse). After 6 h, peritoneal lavage was collected and cultured onto an LB plate to observe bacterial burden. Bar graph presents the mean ± SD. IL, interleukin; ip, intraperitoneally; LB, Luria-Bertani; LPS, lipopolysaccharide; MSU, monosodium urate; NS, nonsaponin fraction; PBS, phosphate-buffered saline; SD, standard deviation.](image)

![Fig. 5. Effects of NS pretreatment on LPS- or HKST-mediated cytokine expression. BMDMs were pretreated with NS (1 mg/mL) for 3 h and then further administered LPS (TLR4 ligand) or HKST (TLR4/5 ligand) for 3 h as indicated in the below schematic graph. (A) Protein levels of pro-IL-1β were measured by immunoblotting. (B) Gene expression levels of pro-IL-1β, IL-1α, TNFα, IL-6, and IL-10 mRNAs were analyzed by RT-PCR. Bar graph presents the mean ± SD. All data shown are representative of at least two independent experiments. BMDM, bone marrow-derived macrophages; HKST, heat-killed Salmonella typhimurium; IL, interleukin; LPS, lipopolysaccharide; MW, molecular weight; NS, nonsaponin fraction; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation.](image)
pretreatment downregulated TLR4 expression in BMDMs. In addition, we determined the expression levels of other TLRs in PECs (Fig. 3B and C) and BMDMs (Fig. 3D and E). PECs isolated from NS-fed mice presented reduced transcription levels of TLR2, TLR3, TLR6, and TLR9, whereas there was no increase in TLR transcription (Fig. 6A). NS treatment in BMDMs downregulated TLR5, TLR8, and TLR9 gene expression but upregulated mRNA expression of TLR1, TLR2, TLR4, and TLR6 (Fig. 6B). Although NS differentially regulated TLR expression between PECs and BMDMs, NS constantly downregulated mRNA expression of TLR4 and TLR9. Taken together, we conclude that the inhibitory effects of NS on cytokine secretion in peritonitis (Figs. 1A, 2A and 2B) can be attributed to downregulation of TLR4 because MSU and LPS are TLR4 ligands. In addition, reduction of TLRs expression in PECs might attenuate cytokine production stimulated by invading bacteria.

4. Discussion

Based on the results of this study, we conclude that NS intake downregulated TLR4 expression in inflammatory cells, resulting in attenuation of cytokine production in response to peritonitis. Mice fed RGE or NS presented lower peritoneal IL-1β and IL-6 secretions in peritonitis (Figs. 1A, 2A and 2B) can be attributed to downregulation of TLR4 because MSU and LPS are TLR4 ligands. In addition, reduction of TLRs expression in PECs might attenuate cytokine production stimulated by invading bacteria.

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Fig. 6. Effects of NS pretreatment on LPS- or HKST-mediated cytokine expression. (A) Mice (n = 6 per group) fed Non (200 μL of water) or NS (1.1 mg/mouse/day) for 7 days and then ip injected with MSU (10 mg/mouse) to induce peritonitis. PECs were isolated from mice, and TLR mRNA expression was analyzed by qPCR. (B) BMDMs were treated with NS for 3 h, and expression levels of TLR mRNAs were measured using RT-PCR and qPCR. Bar graph presents the mean ± SD. All data shown are representative of at least two independent experiments. MW, molecular weight (100 bp ladder); BMDM, bone marrow-derived macrophages; HKST, heat-killed Salmonella typhimurium. IL, interleukin; ip, intraperitoneally; LPS, lipopolysaccharide; MSU, monosodium urate; NS, nonsaponin fraction; PEC, peritoneal exudate cell; qPCR, quantitative real-time polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation.
resulting from MSU, alum, and LPS injection. However, inflammasomes were not involved in NS-mediated cytokine inhibition because peritoneal IL-1β secretion was unchanged in NS-fed mice, and NS treatment did not alter IL-1β or caspase-1 secretion in BMDMs. In addition to being inflammasome triggers, MSU and LPS are ligands for TLR4. Expression of TLR4 in PECs isolated from peritonitis mice was inhibited by NS ingestion, and TLR4 expression was also attenuated by NS treatment in BMDMs. Cytokine secretion in peritonitis mice was inhibited by NS ingestion, and TLR4 expression in mice was also reduced by NS treatment. Moreover, NS attenuated TLR4 expression in vivo, suggesting that NS treatment inhibits cytokine secretion in vivo.

Red ginseng contains several saponins, such as Rg1, Re, Rb1, Rc, Rb2, and Rd [25]. These saponin contents are regarded as major pharmaceutical effectors on biological activities. However, Red ginseng also has nutritional components such as saccharides and amino acids [25]. Among them, arginine with sugars such as argi- 

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