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Korean Red Ginseng Saponin Fraction Downregulates Proinflammatory Mediators in LPS Stimulated RAW264.7 Cells and Protects Mice against Endotoxic Shock

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Korean red ginseng has shown therapeutic effects for a number of disease conditions. However, little is known about the anti-inflammatory effect of Korean red ginseng saponin fraction (RGSF) *in vitro* and *in vivo*. Therefore, in this study, we showed that RGSF containing 20(S)-protopanaxadiol type saponins inhibited nitric oxide production and attenuated the release of tumor necrotic factor (TNF)-α, interleukin (IL)-6, granulocyte monocyte colony stimulating factor (GMCSF), and macrophage chemo-attractant protein-1 in lipopolysaccharide (LPS) stimulated murine macrophage RAW264.7 cells. Moreover, RGSF down-regulated the mRNA expressions of inducible nitric oxide synthase, cyclooxyginase-2, IL-1β, TNF-α, GMCSF, and IL-6. Furthermore, RGSF reduced the level of TNF-α in the serum and protected mice against LPS mediated endotoxic shock. In conclusion, these results indicated that ginsenosides from RGSF and their metabolites could be potential sources of therapeutic agents against inflammation.

Keywords: *Panax ginseng*, 20(S)-protopanaxadiol saponins, Pro-inflammatory cytokines, Endotoxic shock, Macrophages

INTRODUCTION

Macrophages play a central role in inflammatory processes through the release of proinflammatory mediators and cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, macrophage chemo-attractant protein (MCP)-1 and granulocyte macrophage colony stimulating factor (GMCSF), nitric oxide (NO) and prostaglandin E_2 (PGE_2) [1-3]. Lipopolysaccharide (LPS) triggers inflammation through the release of the aforementioned inflammatory mediators [4-7], which could be employed for the design, development and study of new anti-inflammatory agents.

Recently, a great deal of interest has been growing on

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the use of natural products to develop potential candidate drugs for the treatment of chronic diseases like rheumatoid arthritis (RA) [8,9]. Phytotherapeutic agents with the capacity to modulate the inflammatory response and reduce the subsequent tissue injury are required, while minimizing side effects of long-term applications. In this regard, anti-inflammatory agents of plant origin such as *Panax ginseng* could be considered as alternative candidates.

Previous works have indicated that the root of *P. ginseng* is the major oriental folk medicine that has been used for the treatment of a variety of ailments in Asia [10,11]. Ginseng contains many active components such as ginsenosides, polysaccharides, peptides, fatty acids, and mineral oils [12], of which ginsenosides (steroidal saponins) are believed to be the main components responsible for most of the pharmacological and immunological actions [13,14]. Jia et al. [10] extensivly reviewed the anti-hyperglycemic, aphrodisiac, cancer cell apoptotic, anti-oxidant and anti-inflammatory effects of ginseng. Saponin components of ginsenosides are divided into two different classes, 20(S)-protopanaxdiol (PPD) type saponins (e.g., Rb1, Rb2, Rc, and Rd) and 20(S)-protopanaxtriol (PPT) type saponins (e.g., Re, Rf, Rg1, and Rg2) [15]. Previous investigations reported that ginsenosides of 20(S) PPT type saponins have shown anti-cancer [16] and anti-inflammatory [17] effects. However, little is known about the *in vitro* and *in vivo* anti-inflammatory effects of PPD-rich fraction of ginseng. In this study, therefore, we showed that PPD-rich red ginseng saponin fraction (RGSF) inhibits the release of proinflammatory mediators *in vitro* and protects mice against endotoxin mediated shock.

MATERIALS AND METHODS

Materials

Korean red ginseng was kindly provided by the Research Institute of Technology, Korea Ginseng Corporation (Daejeon, Korea). RAW264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Reverse transcription (RT) and polymerase chain reaction (PCR) premixes were from Bioneer Co. (Daejon, Korea). LPS was from Sigma (St Louis, MO, USA). All other reagents were obtained from Sigma unless indicated.

Red ginseng saponin fraction extraction and preparation

In brief, the Korean red ginseng was extracted with ethanol and the extract was air-dried at 60°C for 2 d. The powder was then subjected to three time's aqueous extraction at 95°C to 100°C. The resultant water extracts were ultrafilterd with a pore size of 100,000 μm. Finally, the filtrate was harvested and stored as RGSF for further identification of major chemical components (PPD saponins) by HPLC profile analysis (Fig. 1).

Cell culture

RAW264.7 cells were maintained in Dulbecco's modified Eagle medium supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 5% fetal bovine serum. Cells were grown at 37°C and 5% CO_2 in humidified air.

Nitric oxide assay and cell viability test

NO and cell viability assays were performed as described during our previous work [18]. Briefly, RAW264.7 cells (1×10^6 cells/mL) were pre-incubated with RGSF (25, 50, 100, and 200 μg/mL) or vehicle for 30 min and then stimulated with LPS (100 ng/mL) for 18 h. One-hundred microliter of cell supernatant from each well were transferred into 96-well microplates and mixed with an equal volume of Griess reagent at room temperature. The absorbance at 540 nm was determined by a Spectramax 250 microplate reader. For cell viability test, 30 μL of 5 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent was added to the culture plates and cell viability test was performed based on the reduction of MTT reagent into an insoluble, dark purple formazan product in viable cells.

Total RNA isolation and semi-quantitative reverse transcriptase polymerase chain reaction

Total RNA was isolated from LPS treated and untreated-RAW264.7 cells using Easy Blue Reagent (iNtRON Biotechnology Co., Daejeon, Korea), according to the manufacturer's protocol. The extracted total RNA was then used for semi-quantitative RT-PCR using RT premix (Bioneer Co., Daejeon, Korea). Briefly, 2 μg of total RNA was incubated with oligo-dT_{18} at 70°C for 5 min and cooled on ice for 3 min, and then the reaction mixture containing RT premix was incubated for 90 min at 42.5°C with a final inactivation of reverse transcriptase at 95°C for 5 min. The PCR reaction was continued using a PCR premix (Bioneer Co.) with appropriate sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense primer, 5'-CAC TCA CGG CAA ATT CAA CGG C-3'; antisense primer, 5'-CCT TGG CAG CAC CAG TGG ATG CAG G-3'), inducible nitric oxide synthase (iNOS; sense primer, 5'- CCC

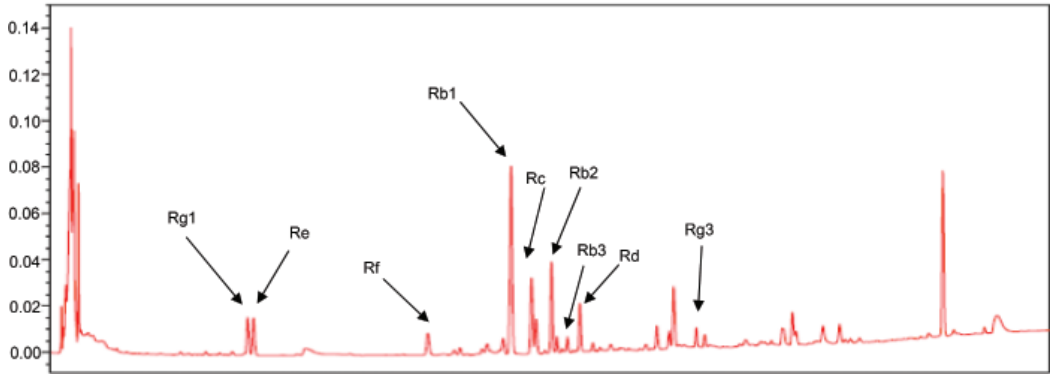

Fig. 1. HPLC analysis of ginsenoside contents from red ginseng saponin fraction.

TTC CGA AGT TTC TGG CAG CAG C-3'; antisense primer, 5'- GGC TGT CAG AGC CTC GTG GCT TTG G-3'), cyclooxyginase-2 (COX-2; sense primer, TCT-CAGCACCCACCCGCTCA; anti-sense, GCCCCG-TAGACCCTGCTCGA), IL-1β (sense primer, 5'- CAG GAT GAG GAC ATG AGC ACC-3'; antisense primer, 5'- CTC TGC AGA CTC AAA CTC CAC-3'), IL-6 (sense primer, GCTGGAGTCACAGAAGGAGTGGC; anti-sense primer, GGCATAACGCACTAGGTTT-GCCG), GMCSF (sense primer, ACTCTGCTCAC-GAAGGAACTCAGC; anti-sense primer, CACAGCTC-GGAAGAGCATCGCA), and TNF-α (sense primer, 5'- TTG ACC TCA GCG CTG AGT TG -3'; antisense primer, 5'- CCT GTA GCC CAC GTC GTA GC-3'), under incubation conditions of 95°C predenaturation for 5 min and 35 cycles of '95°C denaturation for 45 sec, 55 and 60°C annealing for 45 s, 72°C extension for 45 s,' and a final elongation period of 10 min at 72°C. Next, PCR products were separated using 1% agarose gel electrophoresis (BioRad Co.) and relative band intensity levels were determined by Eagle Eyes Image Analysis software (Stratagene Co., La Jolla, CA, USA,). The resulting density levels were calculated relative to the corresponding density level of GAPDH (housekeeping gene) from the same RNA samples to make a bar graph of gene expressions.

Enzyme-linked immunosorbent assay

RAW264.7 cells were preincubated with RGSF for 30 min before LPS stimulation for 24 h, and cytokine contents in the culture medium were measured by enzyme-linked immunosorbent assay (ELISA) using anti-mouse TNF-α, IL-6, GMCSF and MCP-1 antibodies and biotinylated secondary antibodies following the manufacturer's instruction (Millipore Milliplex Mouse Cytokine/Chemokine kit; Millipore, St. Charles, MO, USA).

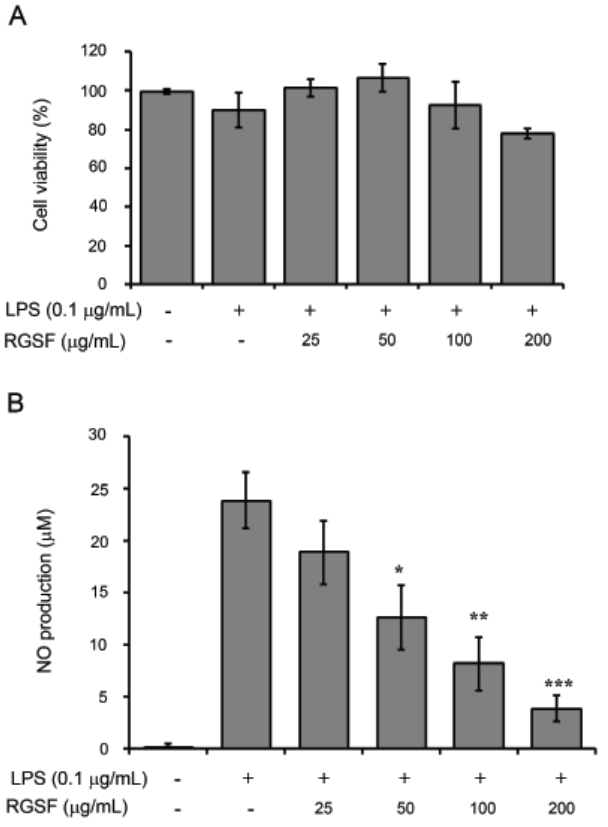

Fig. 2. Effect of red ginseng saponin fraction (RGSF) on cell viability and nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. RAW cells were pre-treated with RGSF (25, 50,100, 200 μg/mL) or vehicle and stimulated by LPS for 24 h. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay was carried out to check the cytotoxic effect of RGSF (A), and then its inhibitory effect on the release of NO was determined (B). Each bar graph represents mean±SE of at least 4 independent experiments. *p<0.05, **p<0.01, ***p<0.005 *vs*. LPS.

Endotoxin induced shock in mice

LPS was used to induce endotoxic shock in ICR mice (male, 6-8 weeks old) in accordance with Guidelines for the Care and Use of Laboratory Animals. Three groups of mice (n=10) were pretreated for 1 h orally with or without

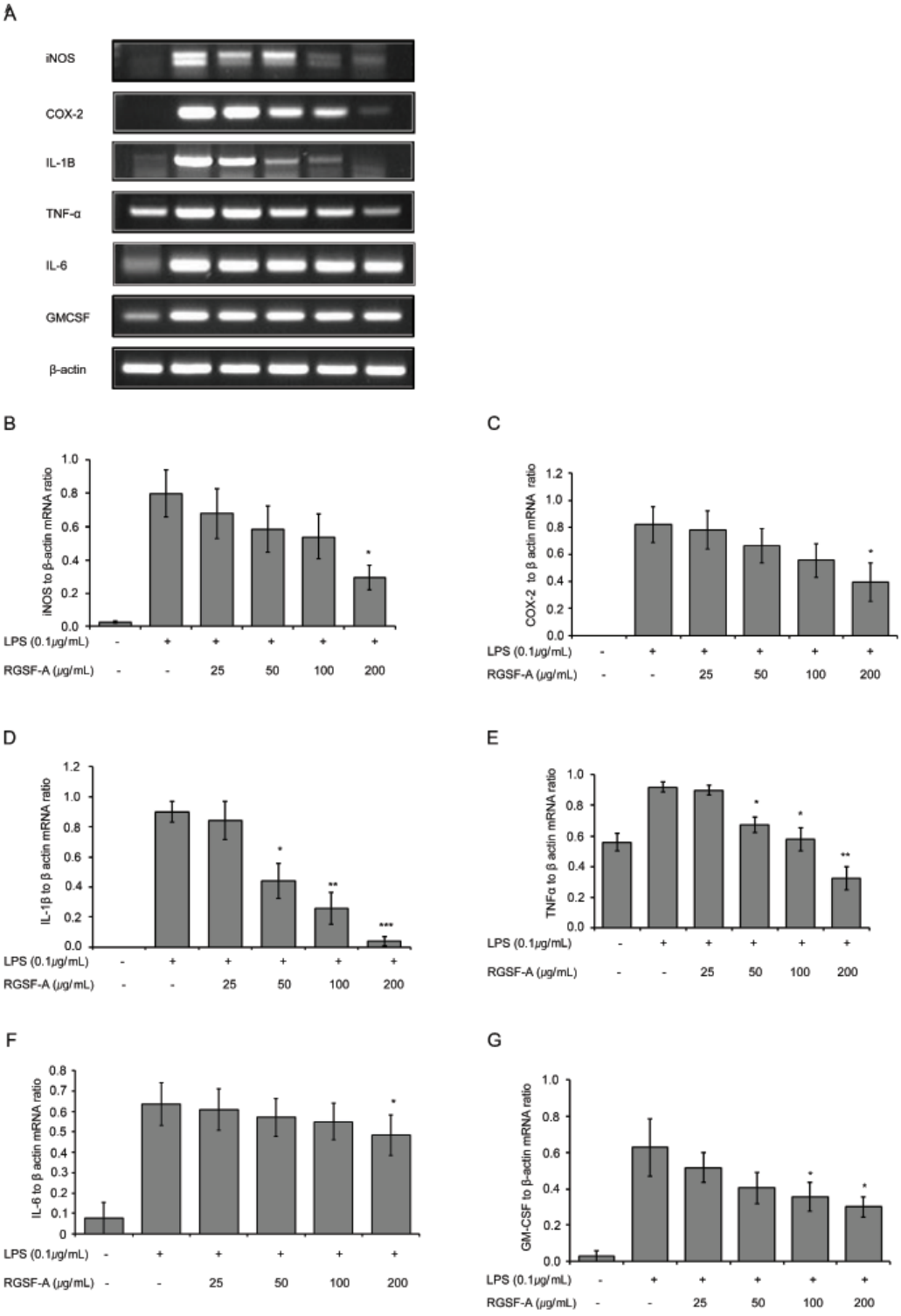

Fig. 3. Effect of red ginseng saponin fraction (RGSF) on proinflammatory mediators mRNA expressions in lipopolysaccharide (LPS) stimulated RAW264.7 cells. Cells were pretreated for 30 min with the indicated concentrations of RGSF, followed by stimulation with LPS for 24 h. RGSF dose-dependently inhibited mRNA expression of inducible nitric oxide synthase (iNOS) (A,B), cyclooxyginase (COX)-2 (A,C), interleukin (IL)-1β (A,D), tumor necrosis factor (TNF)-α (A,E), IL-6 (A,F), and granulocyte macrophage colony stimulating factor (GMCSF) (G) levels as assessed by reverse transcriptase polymerase chain reaction. The mRNA band density ratios were determined relative to the loading control (β-actin). Each bar graph represents mean±SE of at least four independent experiments. ***p*<0.01, ****p*<0.005 *vs*. LPS.

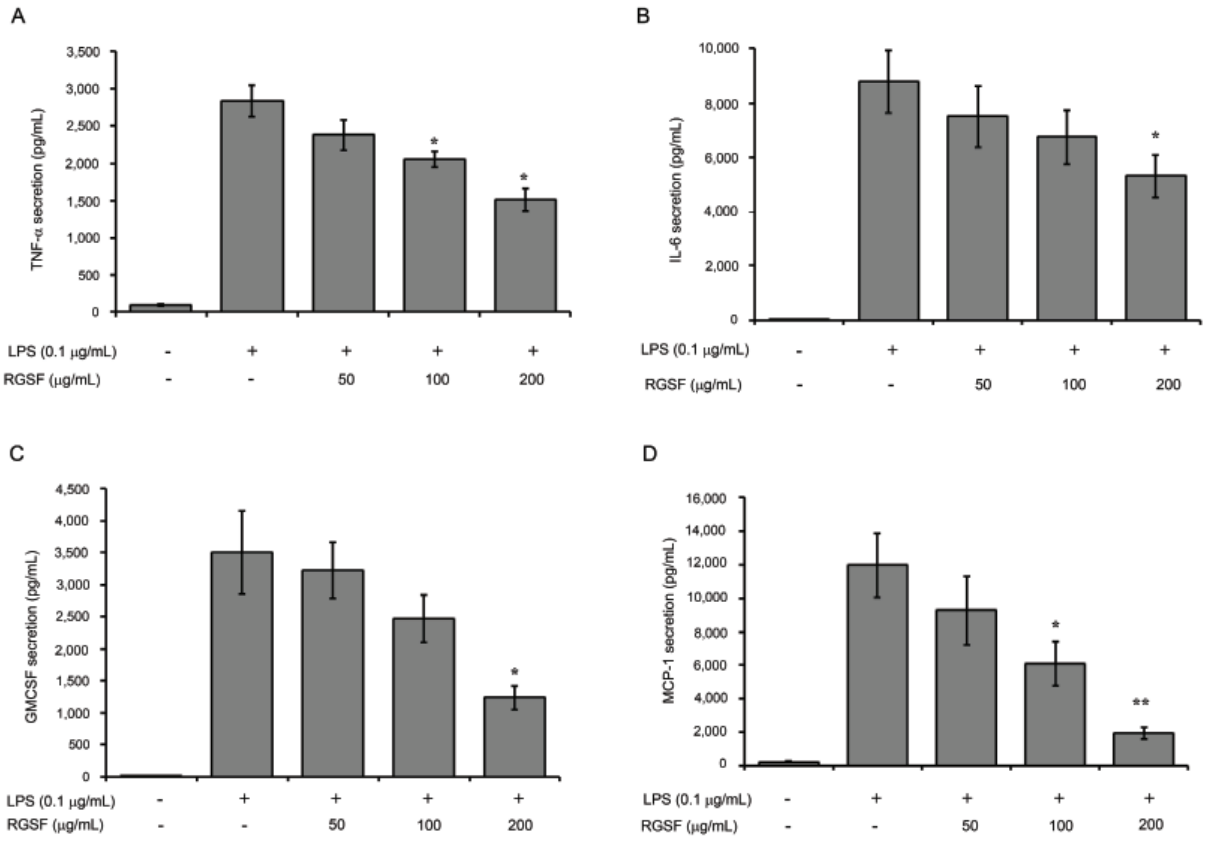

Fig. 4. Effect of red ginseng saponin fraction (RGSF) on the production of pro-inflammatory cytokines in lipopolysaccharide (LPS) stimulated RAW264.7 cells. After pretreatment of cells with various concentration of RGSF, the amount of tumor necrosis factor (TNF)-α (A), interleukin (IL)-6 (B), granulocyte macrophage colony stimulating factor (GMCSF) (C), and chemo-attractant protein (MCP)-1 (D) were determined using enzyme immunoassay. Each bar graph represents mean±SE of at least four independent experiments. *p<0.05, **p<0.01 *vs*. LPS.

RGSF (50 and 200 mg/kg dissolved in water) followed by a single dose of intraperitoneal LPS (10 mg/kg) was administered at day one and then mice were routinely examined for 1 wk to determine the survival rate. For TNF-α assay, blood was collected from each group of mice using retro-orbital bleeding after 6 h of LPS treatment, and the level of TNF-α in the serum was determined by ELISA using specific antibodies (Biosource International Inc.) according to the manufacturer's instructions.

Statistical analysis

Data were represented as the means±SEM of three independent experiments, conducted in triplicate. A p-value less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Red ginseng saponin fraction inhibits nitric oxide production in LPS-stimulated RAW264.7 murine macrophage cells

To assess whether RGSF attenuates overproduction of NO which is a potent mediator of cellular damage in a wide range of pathological conditions [19], RAW264.7 cells were pre-treated with different concentrations of RGSF (25, 50, 100, and 200 μg/mL) and then stimulated with LPS (0.1 μg/mL) to induce inflammation. RGSF at the indicated concentrations exhibited inhibitory effect on the production of NO in murine RAW264.7 macrophage cell line (Fig. 2B). Since overproduction of NO is produced by iNOS, we further checked if this saponin fraction of ginseng had its effect at the transcriptional level of this inducible enzyme. As shown in Fig. 3A and 3B, RGSF not only showed its downregulating effect on NO in LPS stimulated RAW264.7 cells but also revealed its inhibitory role on iNOS gene at the transcriptional level. Previously, it was reported that cross talks between iNOS and COX-2 exist at different pathological conditions [20], suggesting that this PPD rich fraction of Korean RGSF could also possess inhibitory effect at the transcriptional level of COX-2 (Fig. 3A, C), which is a key enzyme involved in the synthesis of another inflammatory mediator (PGE_2) that exists in cancer related inflammation [21-23].

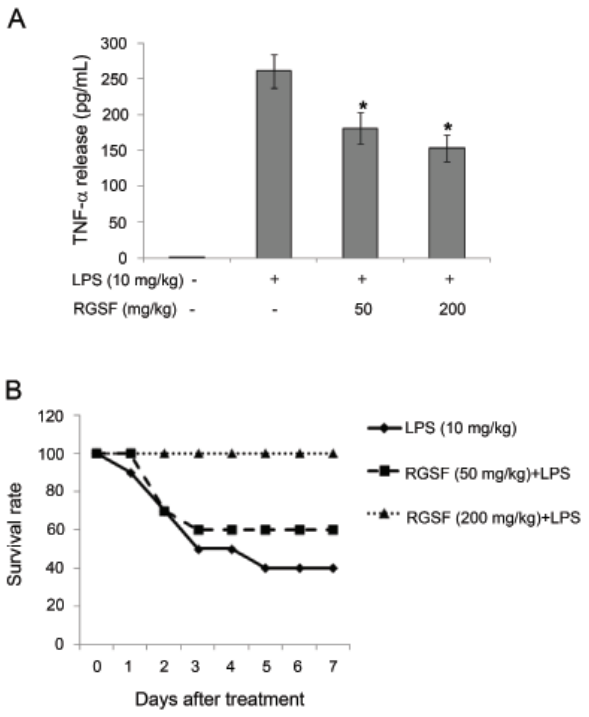

Fig. 5. Effect of red ginseng saponin fraction (RGSF) in lipopolysaccharide (LPS) induced endotoxic shock in mice. Mice were pretreated with or without RGSF (50-200 mg/kg) orally for 1 h and then administered with LPS (10 mg/kg) for 7 d. After 6 h of LPS stimulation, sera were collected for tumor necrosis factor (TNF)-α assay (A), and then mice were followed for their survival for a week (B). **p*<0.05 vs. LPS considered as significant.

Interestingly, all these observed inhibitory effects were attributed to the effect of PPD rich RGSF since the cytotoxic effect of this extract was excluded at the beginning of our work using MTT assay (Fig. 2A).

Red ginseng saponin fraction attenuates the release of TNF-α, IL-6, GMCSF, and MCP-1 in LPS-induced RAW264.7 cells

Since black Korean ginseng rich in Rh2 and Rg3 revealed its inhibitory effect on the release of TNF-α in LPS treated peritoneal macrophages obtained from mice [24]. Moreover, topical application of RGSF endowed with Rh2 and Rg3 has shown anti-inflammatory effect *in vivo* through the abrogation of TNF-α and IL-4 in an animal model of acute dermatitis [25]. In line with these reports, we found that RGSF (50, 100, and 200 μg/mL) rich in Rb1, Rb2, Rg3, and Rc (Fig. 1) showed dose dependent attenuation of TNF-α mRNA expression (Fig. 3A, E) and secretion (Fig. 4A) in LPS stimulated RAW264.7 cells. Likewise, RGSF obviously inhibited mRNA expression of IL-1β (Fig. 3A, D), IL-6 (Fig. 3A, F), and GMCSF (Fig. 3A, G) with a concentration above 50 μg/mL. Furthermore, RGSF moderately inhibited the release of GMCSF (Fig. 4C) and strongly abrogated MCP-1 (Fig. 4D) secretion in LPS treated RAW264.7 cells, suggesting that ginsenosides of PPD type acquired from Korean red ginseng might have pleiotropic effects on the secretion of proinflammatory cytokines from macrophages exposed to LPS. These results could be further supported by the fact that different ginsenosides acquired from Korean red ginseng posses various pharmacological effects [26].

Red ginseng saponin fraction attenuates the level of TNF-α in the serum and improves the survival rate of mice in LPS induced shock

To determined if the *in vitro* anti-inflammatory effects of RGSF on LPS stimulated RAW264.7 cells coincides with the *in vivo* experimental study, we determined the level of TNF-α and the survival rate of mice administered with RGSF and/or LPS (Fig. 5A). RGSF diminished the level of TNF-α *in vivo* as well, and improved the survival rate of mice by 20% at a dose of 50 mg/kg, and further rescued mice from endotoxin triggered death when 200 mg/kg RGSF was used (Fig. 5B). A previous study revealed that ginsenosides showed anti-inflammatory effects *in vitro* [17,27] and, yet there was scarcity of data pertaining to their effects *in vivo*. This study, therefore, showed that the *in vitro* anti-inflammatory effect of RGSF was consistent with the *in vivo* protective effect against endotoxin mediated shock, suggesting the effect of ginsenosides was not limited to *in vitro*. This could also be further supported by our observation on the anti-arthritic effect of RGSF in collagen type II induced rheumatoid arthritis in mice (unpublished result).

In conclusion, our work showed that PPD rich RGSF attenuates the release of NO and proinflammatory cytokines in LPS stimulated murine macrophage RAW264.7 cells. Furthermore, RGSF protected mice against endotoxin mediated shock. However, data pertaining to the associated receptors and cellular signaling mechanisms during inflammation remained elusive. Thus, further work may warrant for the detailed cellular signaling mechanisms of individual ginsenosides.

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