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ORIGINAL ARTICLE

Saikosaponin B2 Suppresses Inflammatory Responses Through IKK/IκBα/NF-κB Signaling Inactivation in LPS-Induced RAW 264.7 Macrophages

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Abstract— *Bupleurum falcatum* (Umbelliferae) have been widely used to treat inflammatory diseases as traditional medicines in East Asian region. Although saikosaponins are main bioactive molecules of *B. falcatum*, there is little information on bioactivity of saikosaponin B_2 (SSB2). This study was conducted to assess the anti-inflammatory activities and the involved mechanisms of SSB2 in LPS-induced RAW 264.7 macrophages. SSB2 suppressed the releases of nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor α (TNF-α), interleukins (IL)-6, and IL-1β by suppressing mRNA levels of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-1β, and IL-6 in LPS-induced macrophages. SSB2 blocked LPS-induced DNA binding and nuclear factor kappa B (NF-κB) transcriptional activity by inhibiting nuclear translocation p65 and p50, inhibitory κBα (IκBα) degradation, and IκB kinase β (IKKβ) phosphorylation and activity. In IKKβ-overexpressing cells, SSB2 significantly suppressed IKKβ-dependent NF-κB transcriptional activity. Moreover, SSB2 reduced phosphorylation of p38 and extracellular signal-regulated kinase1/2 (ERK1/2). SSB2 effectively inhibits LPS-induced pro-inflammatory mediator releases by interfering with IKKβ and IκBα activation, thus preventing NF-κB activation. Our data indicates that SSB2 could be a potential therapeutic application for inflammation-associated diseases.

KEY WORDS: saikosaponin B2; NF-κB; IKK; macrophage.

INTRODUCTION

Inflammation is a pivotal protection system in the body against various pathogens including viruses, bacteria, and fungi. This response is finely tuned by the interactions of various immune cells to maintain the body's homeostasis. However, improper activation of inflammatory processes is an underlying contributor to many pathological conditions including infectious diseases, inflammatory bowel disease, arthritis, arteriosclerosis, and cancer. Therefore, controlling unbalanced inflammation is a potentially important strategy for preventing inflammatory conditions. Active macrophages are in charge of the development of inflammatory reactions by secreting many pro-inflammatory molecules, such as cytokines, leukotrienes, nitric oxide (NO), reactive oxygen species (ROS), and lipid autacoids, which have been shown to be crucial components in tissue destruction and following pathological

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proceeding. Regarding the gene regulation of pro-inflammatory molecules, nuclear factor-κB (NF-κB) has been identified to bind the *cis*-acting elements in the promoters of these genes and thus to regulate their transcription [3]. Under rest state, NF-κB dimer comprising p65 and p50 is located in the cytoplasm combined with inhibitory kappa B (IκB). Recognition of lipopolysaccharide (LPS) by toll-like receptor 4 (TLR4) in the cell membranes activates a Myd88-TAK1 signaling axis [3, 13]. The activation of TAK1/TAK1-binding proteins (TABs) phosphorylates the IκB kinase (IKK) complex and then phosphorylates IκBs, which in turn degrades IκBs for liberation of NF-κB. A series of course permits NF-κB to translocate into nuclear and to bind to *cis*-acting elements in target genes to modulate the pro-inflammatory gene transcription [1, 16].

Saikosaponins are main bioactive molecules of *Bupleurum falcatum* (Umbelliferae) that have been widely used to treat chronic hepatitis, kidney disease, inflammatory diseases, and ulcers of digestive organs as traditional oriental medicines in Korea, Japan, and China [42]. Saikosaponins are pentacyclic triterpene saponins and are predicted to possess some steroid-related pharmacological activities [26]. Many scientific reports have demonstrated that saikosaponins have anti-inflammatory, anti-allergic, immunomodulatory, antiviral, and anticancer effects [5, 28, 38, 41]. For instance, saikosaponin A suppressed the secretion of inflammatory mediators and improved survival during lethal endotoxemia [41]. In addition, saikosaponin D has been reported to induce hepatic stellate cell apoptosis through caspase-3-dependent, caspase-3-independent, and mitochondrial pathways [7]. However, little study was made on the anti-inflammatory properties of saikosaponin B_2 (SSB2, Fig. 1a) and its molecular mechanisms. RAW 264.7 macrophages, which has been one of the most commonly used monocyte derived line, can produce high amounts of pro-inflammatory mediators through TLR signaling activation upon stimulation with microbial ligands [2, 37]. In this study, thus, we explored the anti-inflammatory properties of SSB2 in LPS-induced RAW 264.7 macrophages.

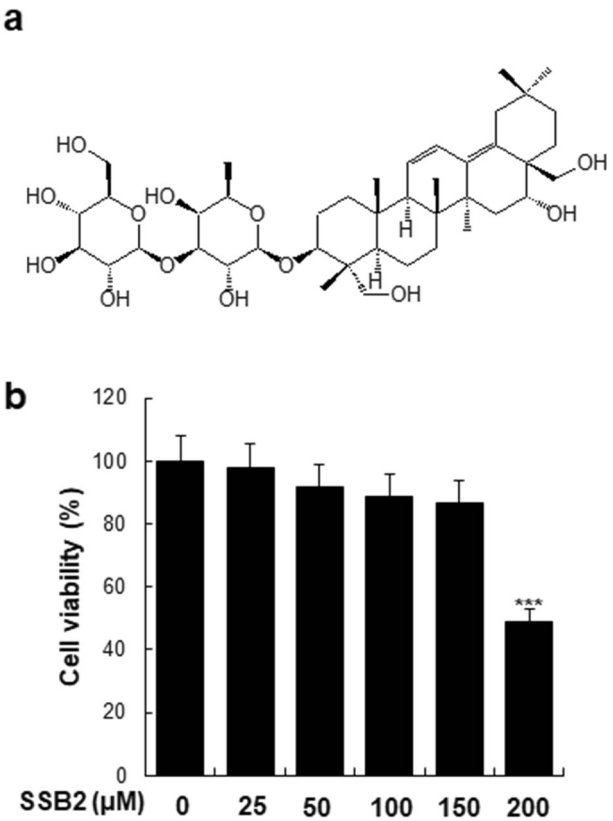

Fig. 1. Chemical structure of SSB2 (a) and its effects on cell viability in RAW 264.7 macrophages (b). Cells were incubated with SSB2 (0–200 μM) for 24 h. Cell viability was estimated by MTT assay. ***$P < 0.001$ compared with the control group.

MATERIALS AND METHODS

Chemicals

SSB2 (purity 98%) was obtained from Biopurify Phytochemicals Ltd. (Chengdu, Sichuan, China). Dulbecco's modified eagle's medium (DMEM) was obtained from Life Technologies Inc. (NY, USA). Fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Hyclone™ (UT, USA). Horseradish peroxidase (HRP)-conjugated Goat anti-mouse IgG, Goat anti-rabbit IgG, and Rabbit anti-Goat IgG secondary antibodies were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), p50, p65, p-extracellular signal-regulated kinase1/2 (ERK1/2), p-cJun N-terminal kinase (JNK), ERK, JNK, p38 MAPK, poly(ADP ribose)polymerase-1 (PARP-1), and β-actin primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). pIκBα, pp38 MAPK, IκB, and IKKβ antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). pIKKβ antibody was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Cell Culture and Sample Treatment

RAW 264.7 murine macrophages (TBI-71™) was purchased from ATCC (Manassas, VA, USA) and maintained in DMEM medium containing 10% FBS, streptomycin sulfate, penicillin, HEPES, and sodium bicarbonate in a 5% CO_2 atmosphere at 37 °C. RAW 264.7 macrophages were incubated with SSB2 (50, 100, and 150 μM) for 1 h and then activated with LPS (1 μg/ml) for the indicated time. SSB_2 were dissolved in DMSO and added to the culture media in serial dilution (the final concentration of DMSO in all experiments did not exceed 0.05%).

Nitrite Determination

RAW 264.7 macrophages were treated with SSB2 and/or LPS for 24 h. Nitrite concentration in culture supernatant were estimated using the Griess reaction as described by Kim et al. [20]. The standard curve was made using $NaNO_2$.

Determination of PGE_2, TNF-α, IL-6, and IL-1β

RAW 264.7 macrophages were treated with SSB2 and/or LPS for 24 h. PGE_2, TNF-α, IL-6, and IL-1β levels in cell culture supernatant were estimated by ELISA kits (R&D Systems, MN, USA).

Western Blot Analysis

Proteins were isolated from SSB2-treated cells as previously described [36]. Cellular proteins were resolved on 10–12% polyacrylamide gels and transferred onto a PVDF membrane. Membranes were treated with 5% skim milk for 1 h and treated with primary antibodies overnight at 4 °C. Membranes were washed using Tris-buffered saline/Tween20 three times and were treated with HRP-conjugated Goat anti-mouse IgG, Goat anti-rabbit IgG, or Rabbit anti-Goat IgG secondary antibodies for 2 h at room temperature. Membranes were again washed using Tris-buffered saline/Tween20, and then developed using immunoCruz™ Western Blotting Luminol reagent (Santa Cruz, CA, USA).

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Total cellular RNA from SSB2-treated cells was isolated using Easy Blue® kits (Intron Biotechnology, Seoul, Korea) and cDNA was prepared using random oligonucleotide primers (Promega, Madison, WI, USA) and TOPscript™ cDNA synthesis kit (Enzynomics, Daejeon, Korea). cDNA aliquots were mixed with 1 units of Taq DNA polymerase, × 10 reaction buffer, 0.2 mM dNTP, and target gene primers and then amplified. PCR primers for iNOS, COX-2, TNF-α, IL-6, IL-1β, and β-actin were listed in the previous study [36]. β-actin was used internal control gene. After amplification, PCR products were electrophoresed on EtBr-stained agarose gel and detected under UV irradiation.

Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts from SSB2-treated cells were prepared using hypotonic buffer and high salt buffer as described previously [36]. Nuclear extracts (10 μg) were applied to DNA binding reaction with [α-^{32}P]-labeled double-stranded NF-κB oligonucleotide using Amersham Megaprime DNA Labeling System (GE Healthcare Life Sciences, Marlborough, MA). DNA-protein mixtures were resolved on native 5% polyacrylamide gels in Tris-boric acid-EDTA buffer. The polyacrylamide gel was dried on vacuum dry for 1 h at 60 °C. Radioactive DNA–protein complexes were detected by exposure to an X-ray film at − 70 °C for 24 h.

Plasmid, Transient Transfection, and Luciferase Assay

Cells were treated with Lipofectamine LTX™ (Invitrogen, CA, USA) containing the pNF-κB-Luc (Clontech, Shiga, Japan) vector and phRL-TK vector (Promega, WI, USA) as an internal luciferase vector. After 4 h of transfection, each well was washed with PBS and incubated with SSB2. After 1 h, cells were stimulated with LPS (1 μg/ml) for 6 h and washed with PBS. Luciferase activity was estimated in cell lysates using the dual-luciferase® reporter assay system (Promega, WI, USA). In other experiment, cells were transfected with expression vector encoding IKKβ (Addgene, MA, USA), pNF-κB-Luc vector and phRL-TK vector. After 24 h, cells were treated with SSB2 for 6 h.

Immunoprecipitation and IKK Kinase Assay

Total cellular proteins were prepared as described previously [34]. Cellular proteins were treated with anti-IKKβ monoclonal antibody and protein A/G–sepharose beads to immunoprecipitate IKKβ protein for 4 h at 4 °C. The protein complex were resuspended in a kinase assay buffer containing GST-IκB-α (1–317) fusion protein (Santa Cruz Biotechnology, Inc., CA, USA) and 5 μCi [γ-^{32}P] ATP and were incubated for 30 min at 37 °C.

Each protein was mixed with Laemmli's loading buffer, heat-denaturated for 10 min, and resolved on 10% polyacrylamide gels. Gels were dried and exposed to X-ray film.

Statistical Analysis

Results are expressed as the mean ± SD of triplicate experiments with similar patterns. The band density was estimated using Quantity One® program (Bio-Rad Laboratories, CA, USA). Statistically significant values were compared using ANOVA and Dunnett's post hoc test, and P values of less than 0.05 were considered statistically significant. $\#P < 0.05$ compared with the control group, and $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ compared with the LPS-stimulated group.

RESULTS

SSB2 Attenuated the NO and PGE_2 Release by Reducing iNOS and COX-2 Expression in LPS-Activated RAW 264.7 Macrophages

Prior to estimating the anti-inflammatory effects of SSB2, we tested its cytotoxicity using MTT assay in SSB2-exposed RAW 264.7 macrophages for 24 h. SSB2 did not show obvious cytotoxic effects at concentrations of 25–150 μM, but it did at 200 μM (cell viability 48.99%) in RAW 264.7 macrophages (Fig. 1b). Therefore, we set the test concentration of SSB2 to 150 μM. To assess the inhibitory effects of SSB2 on LPS-induced NO and PGE_2 releases, cells were pretreated with SSB2 and then activated with LPS (1 μg/ml) for 24 h. Activated cells with LPS (1 μg/ml) highly released NO and PGE_2 compared with control cells (Fig. 2a, b). However, SSB2 significantly suppressed this LPS-induced NO and PGE_2 production (IC_{50} 124.20 μM and 51.94 μM, respectively). Because iNOS responsible for NO production by catalyzing L-arginine [31], we determined the effects of SSB2 on the LPS-induced iNOS expressions at protein and mRNA levels. iNOS protein expression was significantly increased, and SSB2 concentration-dependently inhibited this up-regulation in LPS-activated RAW 264.7 macrophages (Fig. 2c). Similarly, the increased iNOS mRNA expressions by LPS were inhibited by SSB2 (Fig. 2d). We determined whether the suppression of PGE_2 by SSB2 was mediated by regulating COX-2 expression, a main enzyme responsible for large amounts of PGE_2 [32]. LPS-induced COX-2 expression was significantly reduced by SSB2 at protein and mRNA levels in RAW 264.7 macrophages (Fig. 2c, d).

SSB2 Suppressed the Release and mRNA Expressions of TNF-α, IL-6, and IL-1β in LPS-Activated RAW 264.7 Macrophages

We further examined the effects of SSB2 on LPS-induced pro-inflammatory cytokines by ELISAs. LPS-activated cells released large quantity of TNF-α, IL-6, and IL-1β compared with controls, but treatment with SSB2 considerably reduced these increases (TNF-α IC_{50} 76.66 μM, IL-6 IC_{50} 42.84 μM, and IL-1β IC_{50}150.79 μM, respectively, Fig. 3a–c). We then investigated whether the suppression of cytokine release by SSB2 was related to their mRNA expression using RT-PCR. Consistent with the decreased release of TNF-α, IL-6, and IL-1β, SSB2 concentration-dependently reduced the LPS-induced mRNA expression of TNF-α, IL-6, and IL-1β (Fig. 3d). Taken together, our results indicated that SSB2 inhibits the LPS-induced releases of pro-inflammatory mediators by down-regulating gene transcription.

SSB2 Suppressed the NF-κB Activation by Inhibiting NF-κB Nuclear Translocation and IκBα Degradation

Because NF-κB is a key transcription factor in regulating the expression of pro-inflammatory mediators induced by LPS [3], we then investigated the involvement of NF-κB in anti-inflammatory properties of SSB2 in macrophages. A reporter gene assay was used to estimate whether SSB2 suppresses NF-κB transcriptional activity. LPS-treated cell showed 5.85 times greater NF-κB-dependent luciferase activity than control cells, and SSB2 concentration-dependently inhibited these increases (Fig. 4a). In control state, inactive NF-κB protein is combined with IκB and retained in the cytosol. LPS-mediated activation signal initiates the liberty of NF-κB from IκB. Free NF-κB can translocate to the nucleus, binds to the -κB site of the target gene, and consequently induces gene transcription [35]. Accordingly, we analyzed the regulatory effects of SSB2 on the DNA-binding activity of NF-κB. As shown in Fig. 4b, SSB2 potently inhibited NF-κB binding to DNA. Next, we investigated the effects of SSB2 on nuclear translocation of the p65 and p50 NF-κB. Nuclear levels of p65 and p50 were very low in control cells, but LPS markedly increased these

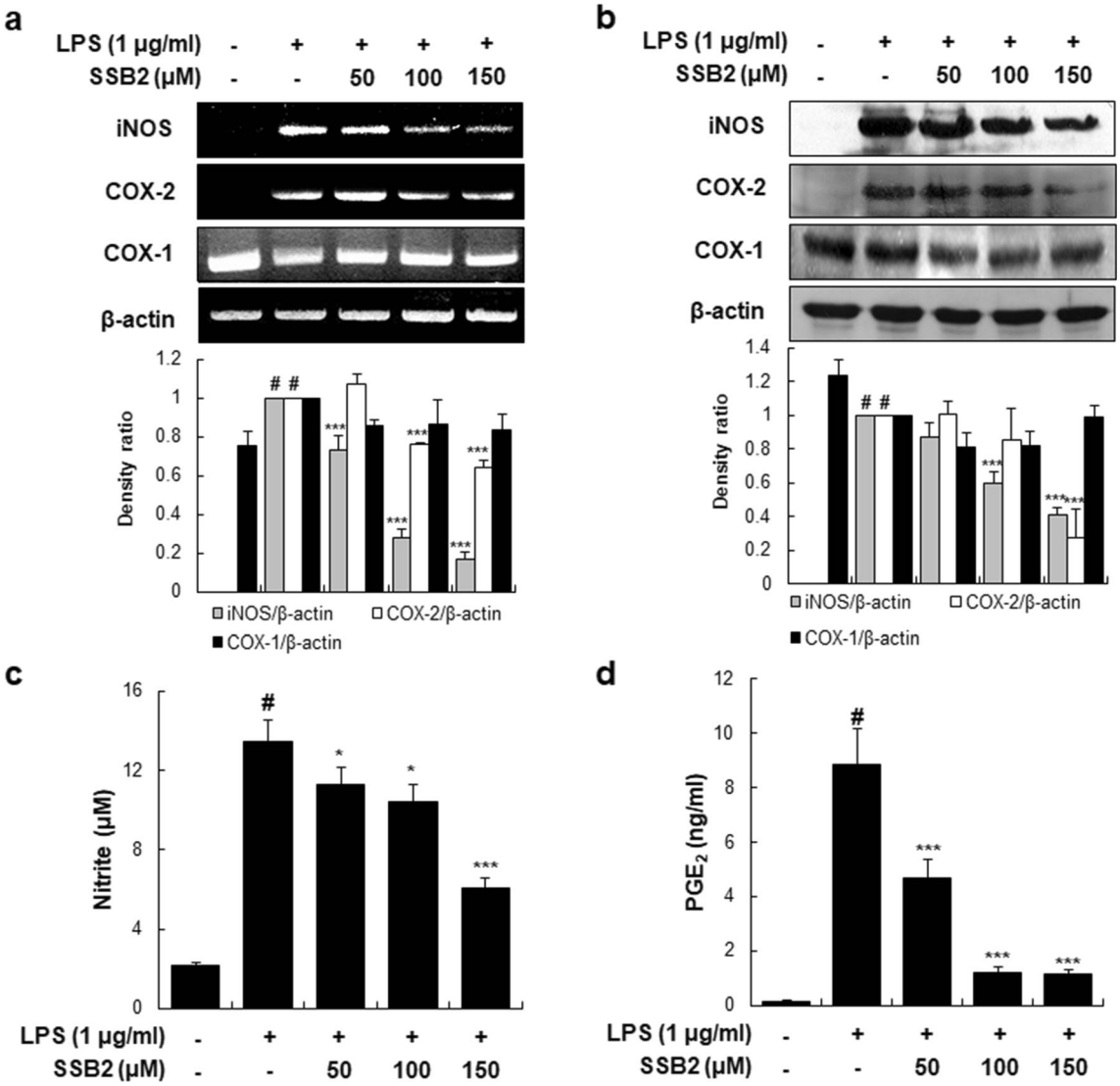

Fig. 2. SSB2 suppressed the NO and PGE_2 releases and iNOS and COX-2 expression in LPS-activated RAW 264.7 macrophages. **a** The mRNA levels of iNOS, COX-2, and COX-1 were estimated by RT-PCR. Cells were treated with SSB2 in presence of LPS (1 μg/ml) for 4 h. **b** Total cellular proteins were prepared the cell treated with SSB2 in presence of LPS (1 μg/ml) for 24 h. The expression levels of iNOS, COX-1, and COX-2 were estimated by Western blotting. **c**, **d** Cells were incubated with SSB2 in presence of LPS (1 μg/ml) for 24 h. The levels of NO and PGE_2 were estimated in supernatant by Griess reaction and ELISAs, respectively. #$P < 0.05$ compared with the control group, and *$P < 0.05$, ***$P < 0.001$ compared with the LPS-stimulated group.

proteins; SSB2 suppressed the LPS-induced nuclear translocation of p65 and p50 (Fig. 4c). Because NF-κB translocation is controlled by IκB, we determined the influence of SSB2 on phosphorylation and degradation of IκB. LPS-activated cells significantly increased the phosphorylated IκBα and decreased the IκBα expression. We observed the significantly reduced phosphorylation and degradation of IκBα in SSB2-pretreated cells (Fig. 4d).

SSB2 Inhibited IKKβ-Mediated NF-κB Activation and Mitogen-Activated Protein Kinases (MAPKs) Phosphorylation

IKK activation is responsible for NF-κB activation by phosphorylating IκB [14, 34]. In an attempt to explore the effects of SSB2 on inhibiting IKK activity in RAW 264.7 macrophages, we performed Western blotting to determine the IKKβ phosphorylation after treatment with

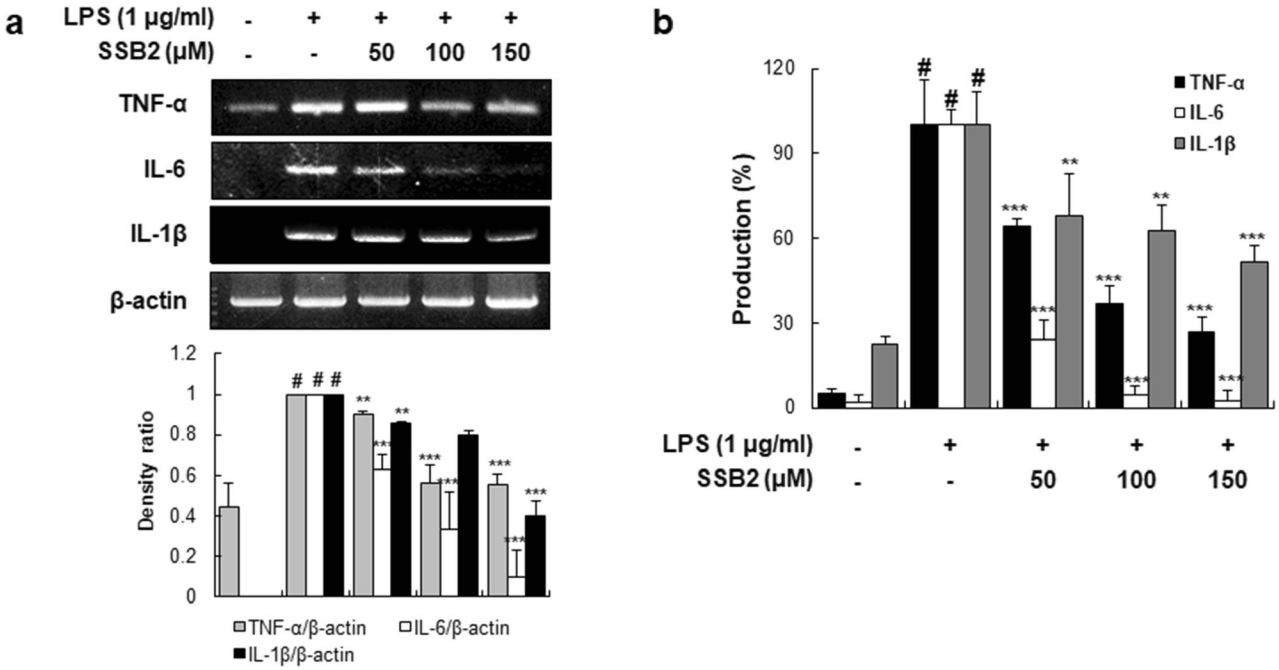

Fig. 3. SSB2 inhibited the TNF-α, IL-6, and IL-1β releases and their mRNA expression in LPS-activated RAW 264.7 macrophages. **a** The mRNA levels of TNF-α, IL-6, and IL-1β were estimated by RT-PCR. Cells were treated with SSB2 in presence of LPS (1 μg/ml) for 4 h. **b** Cells were incubated with SSB2 in presence of LPS (1 μg/ml) for 24 h. The levels of TNF-α, IL-6, and IL-1β were estimated in supernatant by ELISAs. #$P < 0.05$ compared with the control group, and **$P < 0.01$, ***$P < 0.001$ compared with the LPS-stimulated group.

SSB2. As shown in Fig. 5a, we found that SSB2 markedly reduced the LPS-induced IKKβ phosphorylation. In addition, to determine whether SSB2 exerts an inhibitory effect on NF-κB activation by suppressing IKK function, IKKβ was immunoprecipitated from LPS-induced cells and its kinase activity was estimated after treatment of SSB2. LPS-induced IKKβ kinase activity was significantly suppressed at 150 μM SSB2 (Fig. 5b). To confirm the inhibitory effects of SSB2 on IKKβ-dependent NF-κB activation, cells were transfected with IKKβ expression vector. As shown in Fig. 5c, IKKβ over-expression increased NF-κB-dependent luciferase activity without LPS. SSB2 significantly inhibited IKKβ-elicited NF-κB transcriptional activity, suggesting that SSB2 suppresses inflammatory responses through down-regulation of IKK-mediated NF-κB activation in LPS-induced RAW 264.7 macrophages.

It has been known that activation of MAPKs including p38 MAPK, ERK1/2, and JNK through LPS-TLR4 signaling can activate various transcription factors, such as NF-κB, and consequently regulate the expression of inflammatory molecules [15, 18, 39]. In an attempt to reveal the influence of SSB2 on MAPKs activity, we examined whether SSB2 can inhibit the phosphorylation of MAPKs. JNK, p38, and ERK1/2 phosphorylations increased in cells activated by LPS. SSB2 markedly decreased LPS-induced phosphorylated p38 and ERK1/2 (Fig. 5d), but did not inhibit JNK phosphorylation.

SSB2 Inhibited TLR-2 or TLR-3 Ligands-Mediated NO and PGE_2 Release in RAW 264.7 Macrophages

To explore the inhibitory activity of SSB2 on various TLRs-mediated inflammatory responses, the effects of SSB2 on TLR2 (peptidoglycan, PGN)- or TLR3 (poly(I:C))-mediated NO and PGE_2 releases were evaluated in RAW 264.7 macrophages. Upon stimulation with PGN or poly(I:C) for 24 h, the levels of NO were increased, but SSB2 attenuated these increases (IC_{50} 94.68 μM and 116.03 μM, respectively, Fig. 6a). SSB2 significantly suppressed PGN or poly(I:C)-induced PGE_2 production (inhibitory activity; 50.17% and 58.85% at 25 μM, respectively, Fig. 6b). Therefore, these results indicated that SSB2 inhibits TLR-induced inflammatory responses regardless of TLR ligands.

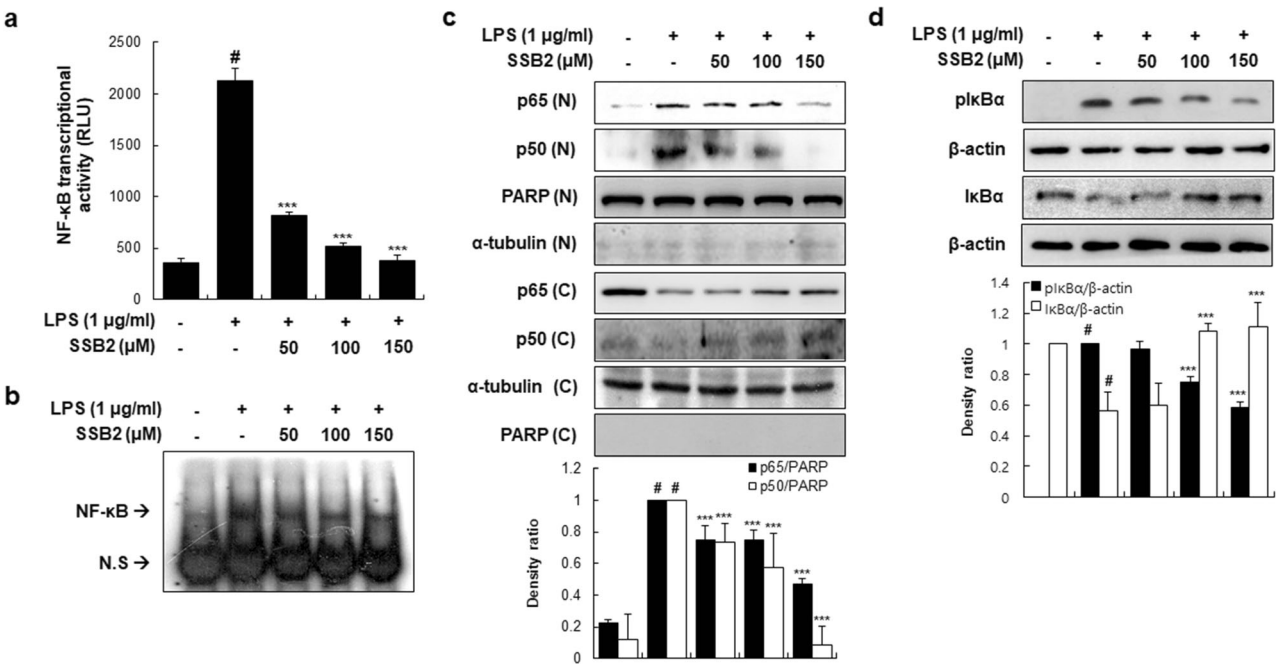

Fig. 4. SSB2 inhibited the DNA-binding, transcriptional activity, nuclear translocation of NF-κB in LPS-activated RAW 264.7 macrophages. **a** pNF-κB-luc reporter vector was inserted to RAW 264.7 macrophages using lipofectamine LTX. Transfected cells were treated with SSB2 for 1 h and then activated with LPS for 3 h. Luciferase activity was estimated in cell supernatant using a Promega luciferase assay kit. **b** Nuclear extract were prepared from cells treated with SSB2 in presence of LPS (1 μg/ml) for 1 h. The binding between [α-^{32}P]-labeled double-stranded NF-κB oligonucleotide and NF-κB in nuclear extract was estimated by EMSA. The arrow indicates the NF-κB band. NS non-specific band. **c** Nuclear and cytosol extract were prepared from cells treated with SSB2 in presence of LPS (1 μg/ml) for 1 h and applied to Western blotting to detect the levels of p65 and p50. α-tubulin and PARP-1 were used as loading control for cytosol and nuclear fraction, respectively. **d** Total cellular proteins were prepared the cell treated with SSB2 in presence of LPS (1 μg/ml) for 15 min. The levels of IκBα and pIκBα were estimated by Western blotting. #$P < 0.05$ compared with the control group, and ***$P < 0.001$ compared with the LPS-stimulated group.

DISCUSSION

SSB2 is known to have a variety of biological functions. It was reported that SSB2 can act as a natural antagonist of hepatitis C virus by inhibiting viral attachment and preventing viral entry/fusion neutralization [26]. SSB2 also can act as a sensitizer to etoposide-induced cell death in melanoma cells by inducing apoptosis [30]. However, the anti-inflammatory properties of SSB2 are poorly understood. We found for the first time the anti-inflammatory activity of SSB2 in LPS-induced macrophages. SSB2 inhibited the activations of IKKβ/IκBα/NF-κB signaling and the subsequent increases of the pro-inflammatory molecules, NO, PGE_2, and cytokines.

LPS is a structural part of endotoxin in the outer membrane of Gram-negative bacteria. LPS is one of the most powerful immune stimulator and induces release of inflammatory mediators [8]. Excessive releases of NO, PGE_2, TNF-α, IL-6, and IL-1β is demonstrated in many acute and chronic inflammatory diseases. The inhibition of these molecules can be an efficient treatment strategy to prevent inflammatory diseases [19]. It has been reported that saikosaponin A (12.5 μM) and D (50 μM) diminish the LPS-activated NO, PGE_2, TNF-α, and IL-6 in RAW 264.7 macrophages, whereas saikosaponin A and saikosaponin D showed obvious cytotoxic effects at 25 μM and 100 μM, respectively [27]. In our study, SSB2 suppressed LPS-induced NO releases with the inhibitory rates 10.41% at 25 μM, 19.22% at 50 μM, 26.63% at 100 μM, and 65.43% at 150 μM, respectively, and SSB2 did not show nonspecific cytotoxicity up to 150 μM. And these inhibitions were resulted from the inhibition of iNOS gene expression. It was found that SSB2 significantly suppresses LPS-induced PGE_2 releases by suppressing COX-2 transcription and thus their protein expression. However, there was discordant phenomenon on between the PGE_2 release and COX-2 expression in SSB2-treated RAW 264.7 macrophages. The effect of SSB2 on the

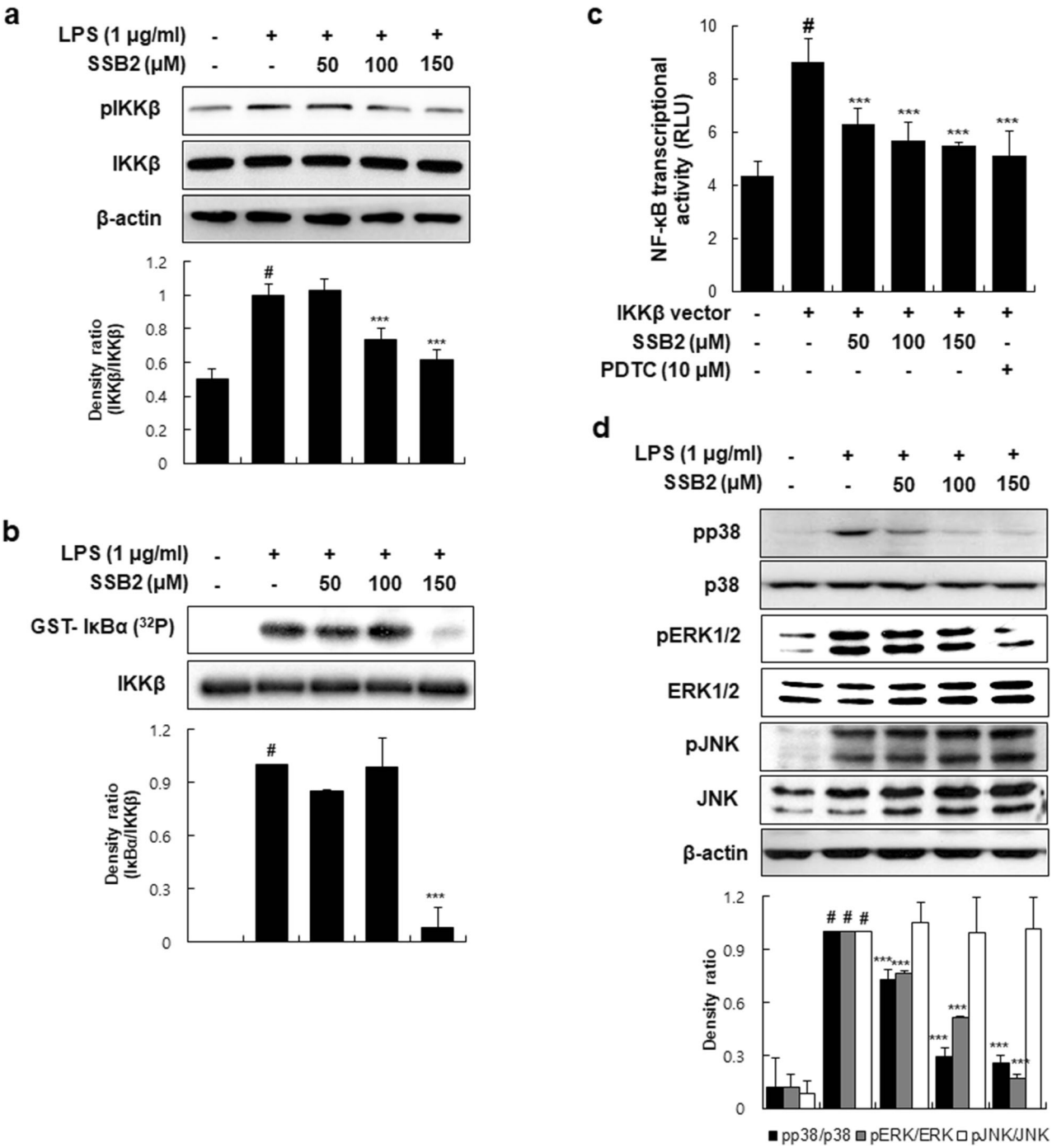

Fig. 5. SSB2 inhibited the activation of IKKβ, p38, and ERK in LPS-activated RAW 264.7 macrophages. **a**, **d** Total cellular proteins were prepared the cell treated with SSB2 in presence of LPS (1 μg/ml) for 15 min. The expressions of pIKKβ, pp38, pERK1/2, pJNK, IKKβ, p38, ERK1/2, and JNK were estimated by Western blotting. **b** IKKβ protein was immunoprecipitated from cells activated by LPS for 10 min. IKKβ protein reacted with [γ-^{32}P]ATP and GST-IκBα fusion protein in presence of SSB2. **c** IKKβ-overexpressing cells were treated with SSB2 for 6 h. Luciferase activity was estimated in cell supernatant using a Promega luciferase assay kit. #$P < 0.05$ compared with the control group, and ***$P < 0.001$ compared with the LPS-stimulated group or IKK-overexpressed group.

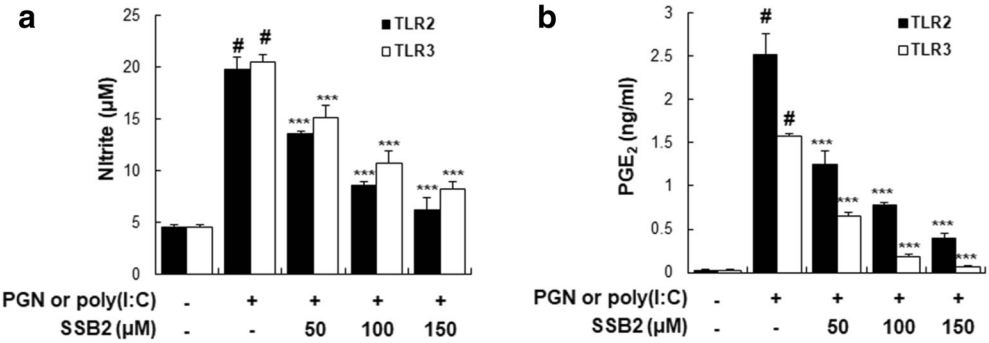

Fig. 6. SSB2 inhibited TLR2- or TLR-3-induced NO and PGE_2 production in RAW 264.7 macrophages. **a**, **b** Cells were pretreated with SSB2 for 1 h and then simulated with peptidoglycan (TLR2 agonist, 20 μg/ml) or poly(I:C) (TLR3 agonist, 100 μg/ml). After 24 h, the level of NO and PGE_2 in cell supernatant were measured using Griess reaction and ELISAs, respectively. #$P < 0.05$ compared with the control group, and ***$P < 0.001$ compared with the LPS-stimulated group.

COX-2 expression did not appear to be sufficient, compared with a decline in PGE_2. PGE_2 are produced in inflammatory environment *via* three sequential enzymes, (i) phospholipase A_2 (PLA_2), (ii) COX-2, and (iii) microsomal PGE_2 synthases (mPGES-1) [33]. Further study is likely to be needed into the deterrent effect of SSB2 on PLA_2 and/or mPGES-1 in LPS-activated RAW 264.7 macrophages. The anti-inflammatory properties of SSB2 can be supported by its inhibitory effects on pro-inflammatory cytokines namely, TNF-α, IL-6, and IL-1β. These cytokines are well known as very important immune elements that can lead to tissue damage, shock, and death through overwhelming immune response activation [9]. SSB2 decreased the release and mRNA expressions of TNF-α, IL-6, and IL-1β. Among them, SSB2 had a noticeable inhibitory effects to IL-6 at both release and gene expression.

Accumulating evidence indicates that NF-κB plays a critical role in the transcription of the pro-inflammatory genes because the promoters of these genes possess active NF-κB binding sites [43]. In addition, successful targeting of the IKK/NF-κB pathway has been reported to be effective in chronic inflammatory diseases [43]. Different compounds from traditional herbal medicine have been addressed to block NF-κB activity *in vitro* and *in vivo* [21, 25, 40, 44]. Inhibitory activities of saikosaponin A and saikosaponin D on inflammatory mediators were attributable to NF-κB inactivation in macrophages [27, 41]. Importantly, SSB2 effectively inhibited etoposide-induced NF-κB activation [30]. On the basis of these reports, we hypothesized that suppression of pro-inflammatory molecules by SSB2 might be involved with NF-κB inactivation. In this study, we found that SSB2 successfully attenuated NF-κB transcriptional activity and DNA binding. DNA binding of NF-κB could be controlled by suppressing translocation of NF-κB into the nucleus or by inhibiting direct association between NF-κB and DNA. Referencing from previous studies demonstrating that saikosaponin A and saikosaponin D suppressed the phosphorylation-dependent IκB degradation [22, 29], we determined the effects of SSB2 on the IκB-controlled NF-κB activation. SSB2 suppressed nuclear levels of p65 and p50 and phosphorylation and degradation of IκBα. These results indicated that SSB2 suppresses the LPS-induced NF-κB release from IκBα by decreasing phosphorylation of IκBα.

It has been understood that NF-κB activation is controlled by IKK complex and MAPKs. The IKK complex activates NF-κB by regulating its nuclear translocation and transactivation. IKKα and IKKβ mediate Ser536 phosphorylation in the p65 transactivation domain. IKKβ also phosphorylates IκBα (at Ser32), which leads to its degradation and subsequent NF-κB release [10, 35]. SSB2 reduced phosphorylation and activity of IKKβ, suggesting that SSB2 decreases NF-κB activation both by intervening in IKKβ catalytic activity and by reducing phosphorylation-dependent IKKβ activation. We verified the inhibitory potency of SSB2 on IKK-dependent NF-κB activation in IKKβ-overexpressed cells. SSB2 significantly inhibited IKKβ-mediated NF-κB transcriptional activity. Our data indicated that IKK protein is one of cellular targets of SSB2 for its anti-inflammatory activity.

LPS-induced MAPK signaling cascades are involved in up-regulating inflammatory mediators by activating cellular kinases and transcription factors including NF-κB [11, 24]. Our previous studies also indicated that MAPKs indeed participate in regulating LPS-induced pro-inflammatory mediators and that some natural compounds exhibit anti-inflammatory activity by modulating MAPK activation [23, 36, 45]. To examine whether MAPKs are

required to anti-inflammatory properties of SSB2, we detected the LPS-induced phosphorylations of p38 MAPK, JNK, and ERK. SSB2 significantly decreased the LPS-mediated phosphorylation of p38 MAPK and ERK1/2. Cumulative data showed that NF-κB-dependent inflammatory gene expression and NF-κB activation are down-regulated by specific inhibition of p38 MAPK or ERK [12, 36, 45]. In particular, p38 MAPK leads to phosphorylation and activation of MSK1/2. Activated MSK1 can phosphorylate p65 (Ser 276), causing a subsequent increase in the transcriptional activity of NF-κB [24]. The ERK blockade by pharmacological inhibitor or gene knockdown revealed that ERK activity is involved in the IκBα degradation and IKK activity [4, 6]. Intriguingly, pharmacological inhibitor of p38 MAPK did not affect the LPS-mediated IκBα degradation and IKK activity [4]. Based on these reports, we assumed that SSB2-mediated p38 MAPK inhibition is related to suppression of NF-κB transcriptional activity, and SSB2-mediated ERK inhibition is in line with the suppression of IKK-mediated IκBα degradation. These simultaneous restraints of multiple signaling might enhance the anti-inflammatory activity of SSB2.

To estimate whether the anti-inflammatory activity of SSB2 is specific to TLR4 signaling, we used TLR2 and TLR3 ligand-stimulated RAW 264.7 macrophages. SSB2 pretreatment significantly inhibited PGN (TLR2 ligand) and poly(I:C)(TLR3 ligand)-induced NO and PGE_2 releases, similar to the observations with LPS-exposed cells. Unlike the TLR4 and TLR2, TLR3 is an intracellular receptor recognizing viral double-stranded RNA (dsRNA) and self-RNAs derived from damaged cells [17]. Our finding implied the anti-inflammatory properties of SSB2 are created by suppressing the intracellular signaling of TLRs, not by interrupting extracellular intercourse between TLRs and their ligands.

In summary, SSB2 possessed inhibitory activity on the LPS-mediated releases of NO, PGE_2, TNF-α, IL-6, and IL-1β. These inhibitions were followed by reductions in iNOS, COX-2, TNF-α, IL-1β, and IL-6 expression at the transcription level. Molecular data revealed that inhibitory properties of SSB2 on inflammatory mediators were resulted from down-regulation of IKKβ/IκBα/NF-κB and MAPK signaling. All results in this study suggest anti-inflammatory protective effects of SSB2 in macrophages. Therefore, further efforts should be made to explore anti-inflammatory properties of SSB2 in animal models so that possible therapeutic approaches to inflammatory diseases can be established in future.

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COMPLIANCE WITH ETHICAL STANDARDS

All data generated or analyzed during this study are included in this published article. The data and materials were available to the corresponding author.

Conflict of Interest. The authors declare that they have no conflict of interest.

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