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Ginsenoside Re Ameliorates Inflammation by Inhibiting the Binding of Lipopolysaccharide to TLR4 on Macrophages

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ABSTRACT: Ginseng (the root of *Panax ginseng* C.A. Meyer, family Araliaceae), which contains protopanaxadiol ginsenoside Rb1 and protopanaxatriol ginsenoside Re as main constituents, is frequently used to treat cancer, inflammation, and stress. In the preliminary study, protopanaxatriol ginsenoside Re inhibited NF-κB activation in lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages. Therefore, we investigated its anti-inflammatory effect in peptidoglycan (PGN)-, LPS-, or tumor necrosis factor-α (TNF-α)-stimulated peritoneal macrophages and, in addition, in LPS-induced systemic inflammation and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. Ginsenoside Re inhibited IKK-β phosphorylation and NF-κB activation, as well as the expression of proinflammatory cytokines, TNF-α and IL-1β, in LPS-stimulated peritoneal macrophages, but it did not inhibit them in TNF-α- or PG-stimulated peritoneal macrophages. Ginsenoside Re also inhibited IRAK-1 phosphorylation induced by LPS, as well as IRAK-1 and IRAK-4 degradations in LPS-stimulated peritoneal macrophages. Ginsenoside Re inhibited the binding of Alexa Fluor 488-conjugated LPS to TLR4 on peritoneal macrophages. Furthermore, ginsenoside Re inhibited the binding of LPS to TLR4 on peritoneal macrophages transiently transfected with MyD88 siRNAs. Orally administered ginsenoside Re significantly inhibited the expression of IL-1β and TNF-α on LPS-induced systemic inflammation and TNBS-induced colitis in mice. Ginsenoside Re inhibited colon shortening and myeloperoxidase activity in TNBS-treated mice. Ginsenoside Re reversed the reduced expression of tight-junction-associated proteins ZO-1, claudin-1, and occludin. Ginsenoside Re (20 mg/kg) inhibited the activation of NF-κB in TNBS-treated mice. On the basis of these findings, ginsenoside Re may ameliorate inflammation by inhibiting the binding of LPS to TLR4 on macrophages.

KEYWORDS: *ginsenoside Re, macrophage, inflammation, Toll-like receptor 4, lipopolysaccharide*

INTRODUCTION

Inflammation is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. In order to remove the injurious stimuli and to initiate the healing process, inflammation is a highly regulated defense process characterized by the release of cytokines, chemokines, and growth factors and by the transmigration of inflammatory cells, such as neutrophils, monocytes, and lymphocytes, from the blood to the affected tissue.[1] Of these inflammatory mediators, proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β are activated through nuclear factor-κB (NF-κB), and they also activate NF-κB.[2,3] These result in the production of proinflammatory cytokines and other inflammatory mediators, causing inflammatory reactions via signaling pathways through Toll-like receptors (TLRs) and/or cytokine receptors.[4] TLRs, which are a major connection between innate and adaptive mucosal immune responses, act as transmembrane coreceptors with CD14 in the cellular response to onslaughts, such as lipopolysaccharides (LPS).[5,6] Among this family of receptors, TLR4, which is associated with the activation of transcription factor NF-κB via IL-1 receptor-associated kinases (IRAKs), may serve as the primary mediator of LPS signaling.[6,7] The TLR4 receptor complex is comprised of TLR4 and MD-2 (TLR4/MD-2), a small extracellular glycoprotein that links with the extracellular domain of TLR4. Following the recognition of pathogen-associated molecular patterns such as LPS by TLR4,[8,9] a signaling cascade is initiated through the Toll/IL-1R domain of its cytoplasmic tail, which recruits the adaptor protein myeloid differentiation factor 88 (MyD88), allowing for subsequent activation of IL-1R-associated kinases (IRAKs). All IRAK members form multimeric receptor complexes.[10] Phosphorylated IRAK-1 activates a multimeric protein complex composed of TRAF6, TAK1, TAB1, and TAB2, leading to activation of NF-κB and mitogen-associated MAPK pathways as well as induction of proinflammatory cytokines. Regulating expression of these inflammatory mediators can therefore be beneficial in decreasing inflammatory diseases. Thus, to prevent chronic inflammatory diseases, the application of dietary ingredients has recently become a focus of interest.[11−15]

Ginseng (the root of *Panax ginseng* C.A. Meyer, family Araliaceae), which contains protopanaxadiol ginsenoside Rb1 and protopanaxatriol ginsenoside Re as main constituents, is frequently used for treatment of cancer, inflammation, stress, and diabetes. These ginsenosides have been reported to exhibit anti-inflammatory effects.[16,17] Of these ginsenosides, protopanaxadiol ginsenoside Rb1 and its metabolite, compound K, exhibit anti-inflammatory effects in LPS-stimulated macrophages and ameliorate LPS-stimulated inflammation by inhibiting IRAK-1.[18] However, the anti-inflammatory mechanism of protopanaxatriol ginsenoside Re has not been thoroughly studied.

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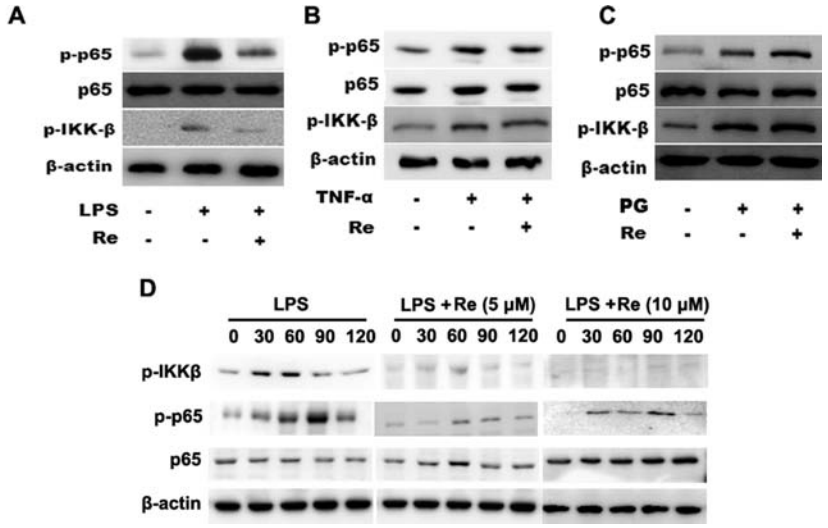

Figure 1. Effect of ginsenoside Re on the activation of IKK-β and NF-κB on peritoneal macrophages stimulated with lipopolysaccharide (LPS) (A), peptidoglycans (PG) (B), or tumor necrosis factor (TNF)-α (C). The peritoneal macrophages were incubated with LPS (50 ng/mL), TNF-α 50 (ng/mL), or PG (50 ng/mL) in the absence or presence of ginsenoside Re (Re, 10 μM) for 30 min. (D) The peritoneal macrophages were also incubated with 50 ng/mL LPS in the absence or presence of ginsenoside Re (Re, 5 or 10 μM) for 0, 30, 60, and 120 min. Protein expressions of p-IKK-β and p-p65 levels were assayed by immunoblotting.

Therefore, to evaluate the anti-inflammatory effect of protopanaxatriol ginsenoside Re isolated from ginseng, we investigated its anti-inflammatory mechanism in LPS-stimulated peritoneal macrophages and its anti-inflammatory effect against LPS-induced systemic inflammation and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice.

MATERIALS AND METHODS

Materials. RPMI 1640, penicillin−streptomycin, lipopolysaccharide (LPS) purified from *Escherichia coli* O111:B4, peptidoglycan (PG) purified from *Streptococcus aureus*, fetal bovine serum (FBS), TNBS, and TNF-α were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for IRAK-4, IRAK-1, p-IRAK-1, COX-2, iNOS, ZO-1, claudin-1, occludin, myeloperoxidase (MPO), and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for IκB-α, p-IKK-β, p65, and p-p65 were purchased from Cell Signaling Technology (Beverly, MA). Enzyme-linked immunosorbent assay (ELISA) kits for cytokines were purchased from R&D Systems (Minneapolis, MN). 3,3′-Diaminobenzidine tetrahydrochloride substrate was purchased from Thermo Scientific (Rockford, IL). Ginsenoside Re (purity, >95%) was isolated according to the previously published method of Bae et al.[19]

Animals. The male ICR mice (20−25 g, 4.5 weeks old) were supplied from Orient Animal Breeding Center (Sungnam-si, Korea). All animals were fed standard laboratory chow (Samyang Co., Seoul, South Korea), housed in wire cages at 20−22 °C and 50 ± 10% humidity, and allowed water ad libitum. All experiments were performed in accordance with the NIH and Kyung Hee University guides for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

Isolation and Culture of Peritoneal Macrophages. Mice were intraperitoneally injected with 2 mL of 4% thioglycolate solution and sacrificed 4 days after injection, and the peritoneal cavities were swilled with 10 mL of RPMI 1640. The peritoneal lavage fluids were centrifuged at 300*g* for 10 min, and the cells were resuspended with RPMI 1640 and plated. After incubation for 2 h at 37 °C, the cells were washed three times, and nonadherent cells were removed by aspiration. Cells were cultured in 24-well plates (0.5×10^6 cells/well) at 37 °C in RPMI 1640 with 10% FBS. The attached cells were used as peritoneal macrophages.[20] To examine the anti-inflammatory effects of ginsenoside Re (5 and 10 μM), peritoneal macrophages were incubated in the absence or presence of ginseonside Re with LPS (or PG or TNF-α).

ELISA and Immunoblot Analysis in Peritoneal Macrophages. The cells (0.5×10^6 cells) were stimulated with LPS (50 ng/mL, Invitrogen, CA) for 30 min or 20 h in the presence or absence of ginsenoside Re (5 and 10 μM), lysed, and centrifuged at 2000*g* for 10 min. The cell supernatant extracts prepared from macrophages were transferred to 96-well ELISA (IL-1β, TNF-α) plates and separated by 10% SDS−PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dried milk proteins in 0.05% PBST and then probed with COX-2, iNOS, IRAK-4, IRAK-1, p-IKK-β, IκB-α, p65, p-p65, or β-actin antibody. After washing with PBST, proteins were detected with HRP-conjugated secondary antibodies for 50 min. Bands were visualized with enhanced chemiluminescence reagent.[21]

Immunofluorescent Confocal Microscopy. For the assay of LPS/TLR4 complex formation, peritoneal macrophages plated on cover slides were incubated at 37 °C overnight. Macrophages were stimulated with Alexa Fluor 594-conjugated LPS (100 ng/mL, Invitrogen) for 30 min in the presence or absence of ginsenoside Re. The cells were fixed with 4% formaldehyde and 3% sucrose for 20 min.[3] The cells were stained with rabbit polyclonal anti-pTLR4 antibody for 90 min at 4 °C and then incubated with secondary antibodies conjugated with Alexa Fluor 488 for 1 h.[22] The stained cells were observed by confocal microscopy.

Flow Cytometry. Mouse peritoneal macrophages were incubated with or without Alexa Fluor 448-conjugated LPS (10 μg/mL) for 30 min. The cells were then fixed in PBS containing 4% paraformaldehyde and 3% sucrose for 20 min. The cells were stained with rabbit polyclonal anti-TLR4 antibody for 2 h at 4 °C and then incubated with secondary antibodies conjugated with TRITC for 1 h and then analyzed by flow cytometry (C6 Flow Cytometer System, Ann Arbor, MI).

Transient Transfection of Small Interfering RNA (siRNA). Peritoneal macrophages were seeded at 3×10^5 cells/well in 24-well plates and allowed to rest for 1 day prior to the transfection. TLR4 small interfering RNA was purchased from Dharmacon (Chicago, IL) and MyD88 small interfering RNA was purchased from Santa Cruz (Santa Cruz, CA). The cells were transfected with 100 nM siRNA for TLR4 and/or MyD88 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. At 48 h after transfections, cells were treated with or without ginsenoside Re (10 μM) and/or LPS (100 ng/mL).

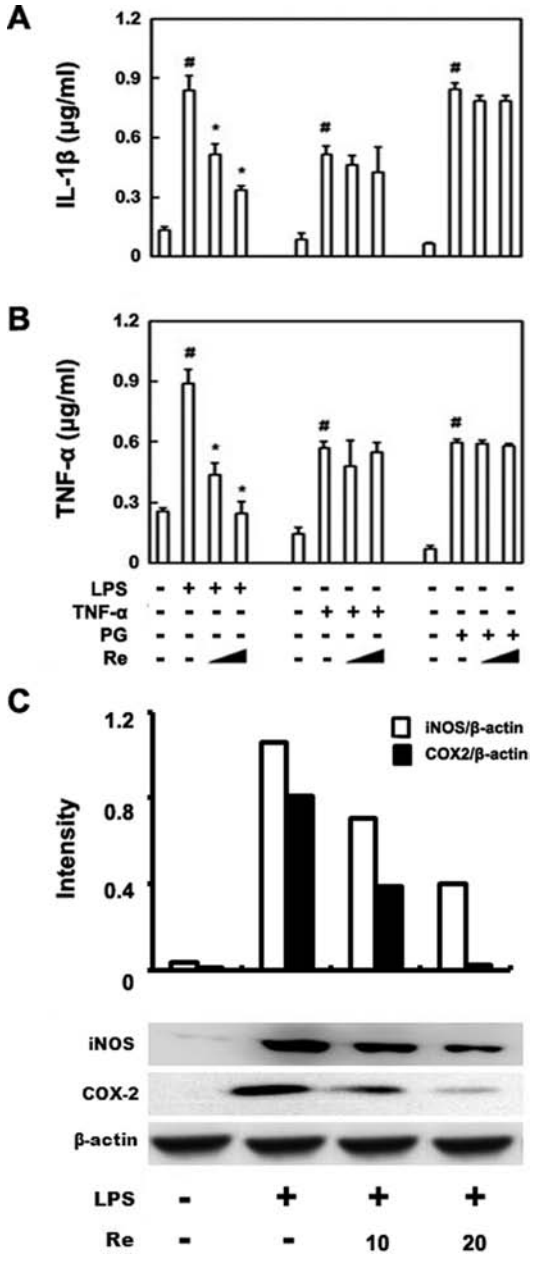

Figure 2. Effect of ginsenoside Re on proinflammatory cytokines IL-1β (A) and TNF-α (B) and inflammatory enzymes iNOS and COX-2 (C) in peritoneal macrophages stimulated with lipopolysaccharide (LPS), peptidoglycans (PG), or TNF-α. The peritoneal macrophages were incubated with LPS (50 ng/mL), TNF-α (50 ng/mL), or PG (50 ng/mL) in the absence or presence of ginsenoside Re (Re, 5 or 10 μM) for 30 min. Proinflammatory cytokines were assayed by ELISA. Protein expressions of iNOS and COX-2 were assayed by immunoblotting. All values are the mean ± SD (n = 3). #Significantly different vs normal group (P < 0.05). *Significantly different vs control group (P < 0.05).

Preparation of Systemic Inflammation in Mice. ICR mice were intraperitoneally injected with LPS (4 mg/kg), and then ginsenoside Re (10 and 20 mg/kg) was orally administered. Normal control was treated with vehicle alone instead of LPS and ginsenoside Re. Mice were sacrificed 4 h after the administration of ginsenoside Re, whole blood was obtained by cardiac puncture, and serum was obtained by centrifugation at 12 000g for 20 min. The serum levels of IL-1β and TNF-α were measured using an ELISA kit.

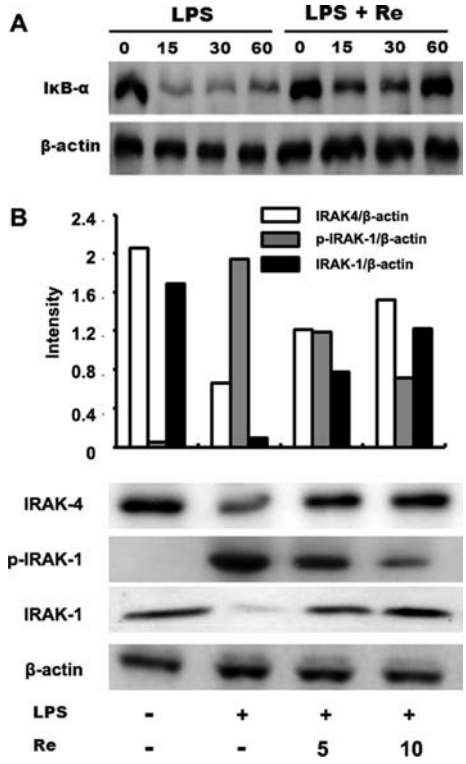

Figure 3. Effect of ginsenoside Re on the degradation of IκB-α, IRAK-1, and IRAK-4 and the phosphorylation of IRAK-1 in lipopolysaccharide (LPS)-stimulated peritoneal macrophages. (A) Effect on degradation of IκB-α. The peritoneal macrophages isolated from mice were incubated with 50 ng/mL LPS in the absence or presence of ginsenoside Re (Re, 10 μM) for 0, 15, 30, and 60 min. IκB-α was assayed by immunoblotting. (B) Effect on the degradation of IRAK-1 and IRAK-4 and the phosphorylation of IRAK-1. Peritoneal macrophages were incubated with LPS (50 ng/mL) in the absence or presence of ginsenoside Re (Re, 5 and 10 μM) for 30 min. IRAK-1, p-IRAK-1, and IRAK-4 were measured by immunoblotting.

Preparation of Experimental Colitis in Mice. To investigate the curative effect of ginsenoside Re against colitis in mice, the mice were divided into five groups: normal and TNBS-induced colitic groups treated with or without ginsenoside Re (10 or 20 mg/kg) or sulfasalazine (50 mg/kg). TNBS-induced colitis was induced by the administration of 2.5% (w/v) TNBS solution (100 μL) in 50% ethanol into the colon of lightly anesthetized mice via a thin round-tip needle equipped with a 1 mL syringe.[20] The normal group was treated with vehicle alone. The needle was inserted so that the tip was 3.5−4 cm proximal to the anal verge. To distribute the agents within the entire colon and cecum, mice were held in a vertical position for 30 s after the injection. Using this procedure, >96% of the mice retained the TNBS enema. If an animal quickly excreted the TNBS−ethanol solution, it was excluded from the remainder of the study. Ginsenoside Re (10 and 20 mg/kg) or sulfasalazine (50 mg/kg) was orally administered once daily from the first day after TNBS treatment for 3 days. The mice were sacrificed 18 h after the final administration of test agents. The colon was quickly removed, opened longitudinally, and gently cleared of stool by PBS. Macroscopic assessment of the disease grade was scored according to a previously reported scoring system (0, no ulcer and no inflammation; 1, ulceration and without hyperemia; 2, ulceration and local hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending more than 2 cm),[16] and the colon tissue was then used for immunoblotting and ELISA analysis.

For the histological exam, the colons were fixed in 10% buffered formalin solution, embedded in paraffin using standard methods, cut into 5-μm sections, stained with hematoxylin−eosin, and then assessed by light microscopy.

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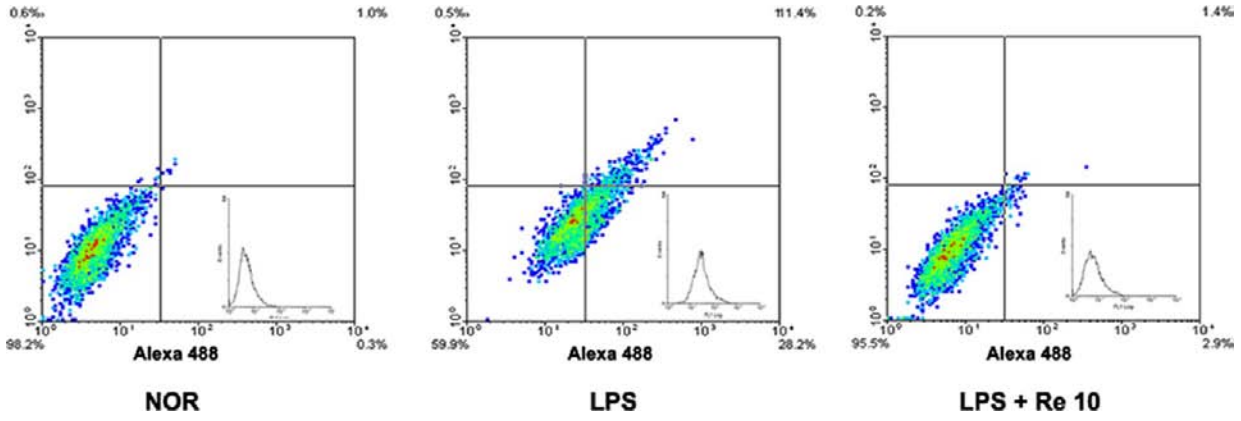

Figure 4. Inhibitory effect of ginsenoside Re on the binding of LPS to TLR4 in peritoneal macrophages. Peritoneal macrophages isolated from mice were incubated with Alexa Fluor 488-conjugated LPS for 30 min in the absence or presence of ginsenoside Re (Re, 10 μM), and the cells were stained with rabbit polyclonal anti-TLR4 antibody for 2 h at 4 °C and then incubated with secondary antibodies conjugated with TRITC for 1 h and then analyzed by flow cytometry. Normal group (NOR) was treated with vehicle alone instead of LPS and ginseoside Re.

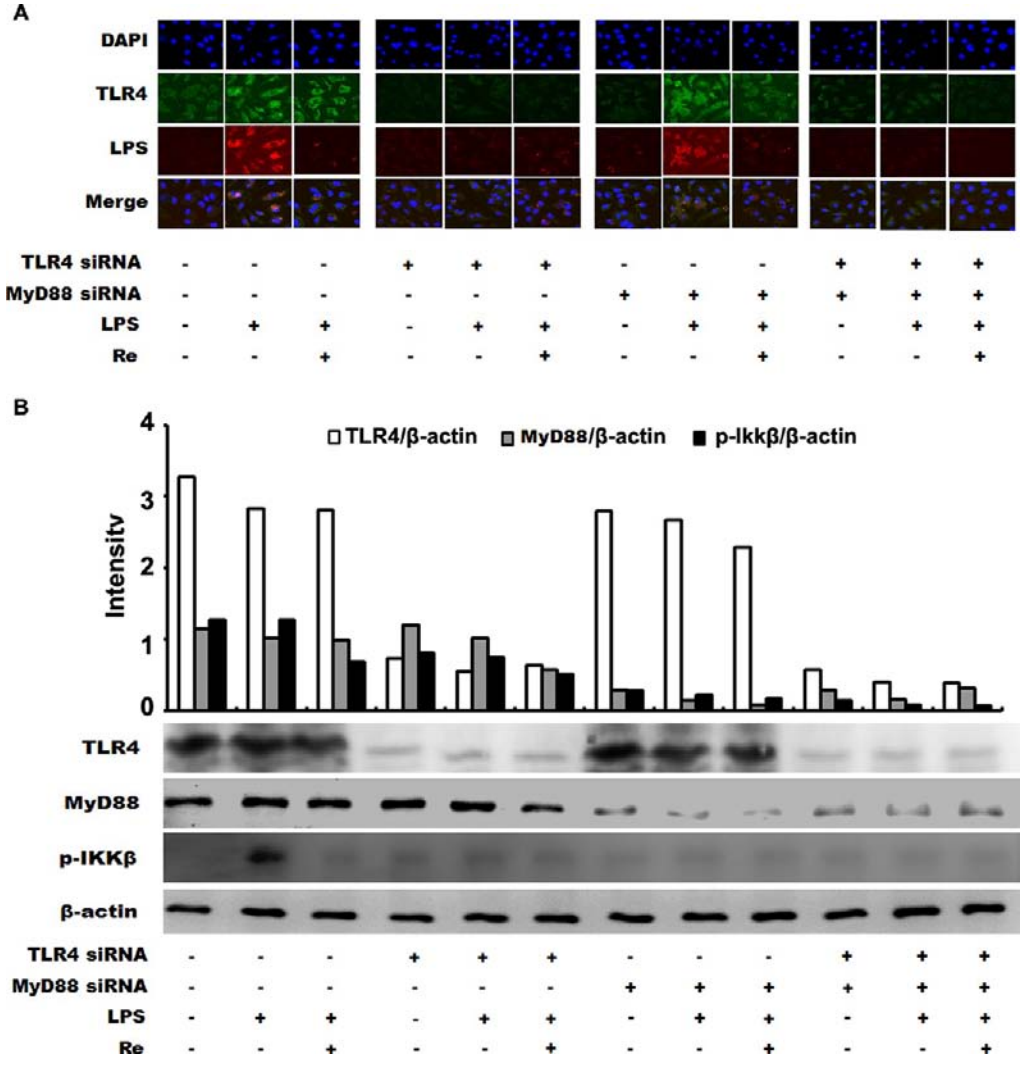

Figure 5. Effect of ginsenoside Re on the expression of TLR4 and MyD88 and the activation of NF-κB in peritoneal macrophages transfected with or without TLR4-siRNA and/or MyD88-siRNA. Peritoneal macrophages isolated from mice were incubated with Alexa Fluor 594-conjugated LPS (100 ng/mL) for 20 min in the absence or presence of ginsenoside Re (10 μM), and then LPS binding on the surface of TLR4 and/or MyD88 siRNA-transfected peritoneal macrophages was measured by confocal microscopy (A). (B) Effect on IKK-β phosphorylation. TLR4, MyD88, p-IKKβ, and β-actin proteins were assayed by immunoblotting.

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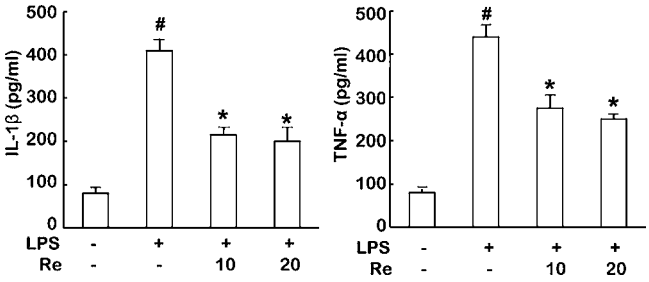

Figure 6. Inhibitory effect of ginsenoside Re on the production of inflammatory cytokines IL-1β (A) and TNF-α (B) in LPS-injected mice. Mice were intraperitoneally injected with or without ginsenoside Re (10 and 20 mg/kg) and LPS (4 mg/kg). Normal group was treated with vehicle alone instead of LPS and ginsenoside Re. Mice were sacrificed 4 h after LPS injection, whole blood was obtained by cardiac puncture, and serum was obtained by centrifugation at 12 000*g* for 20 min. The serum levels of IL-1β and TNF-α were measured using ELISA kit. All data shown are the mean ± SD (n = 6). #Significantly different vs normal group treated without LPS and ginsenoside Re (P < 0.05). *Significantly different vs control group treated without LPS alone (P < 0.05).

Immunohistochemical Staining for Mouse Colonic Myeloperoxidase. Immunolocalization of neutrophils was analyzed using a three-step staining procedure consisting of sequential incubation with first and second antibodies and streptavidin−biotin complex with horseradish peroxidase. Inflammatory cell profiles in the colonic tissues were investigated using neutrophil/monocytes (myeloperoxidase) antibodies. The serial sections were subjected to this procedure. Horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbazole.

Assay of Myeloperoxidase Activity. Colons were homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7.0) and then centrifuged for 20 min at 20 000*g* and 4 °C. An aliquot (50 μL) of the supernatant was added to a reaction mixture of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2 and incubated at 37 °C; the absorbance was obtained at 650 nm over time. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol/mL of peroxide at 37 °C and expressed in unit per milligram protein. The protein content was assayed by the Bradford method.[23]

ELISA and Immunoblot for the Colons of Mice. For the ELISA of IL-1β, TNF-α, IL-6, and IL-10, colons were homogenized in 1 mL of ice-cold RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The lysate was centrifuged (15 000*g*, 4 °C) for 10 min, and the supernatant was transferred to 96-well ELISA plates. For immunoblotting of ZO-1, claudin-1, occludin, COX-2, iNOS, phosphor-NF-κB (p-p65), NF-κB (p65), and β-actin, the colon tissue was carefully homogenized to obtain viable single cells, which were resuspended in 1 mL of RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The protein from collected cells was subjected to electrophoresis on 10% sodium dodecyl sulfate−polyacrylamide gel and then transferred to nitrocellulose membrane. Immunodetection was performed using an enhanced chemiluminescence detection kit.

Statistical Analysis. The data are presented as the means ± standard deviation of at least four replicates. ANOVA was used for comparisons between multiple groups. The Student's t-test was used for the statistical analysis of the difference noted. P values of 0.05 or less were considered statistically significant.

RESULTS

Inhibitory Effect of Ginsenoside Re on COX-2 and iNOS Expressions in Peritoneal Macrophages Stimulated with LPS, PG, or TNF-α. To evaluate the anti-inflammatory effect of ginsenoside Re, we investigated its inhibitory effect on the phosphorylation of IKKβ and p65 in peritoneal macrophages

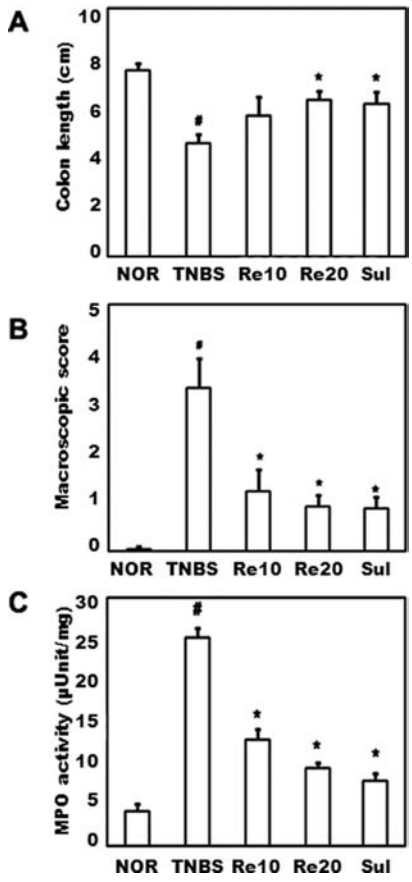

Figure 7. Effect of ginsenoside Re on colon length (A), macroscopic score (B), and colonic myeloperoxidase activity (C) in TNBS-induced colitic mice. TNBS (2.5% TNBS in 50% ethanol) was intrarectally administered in mice. Test agents (TNBS, vehicle alone; Re10, 10 mg/kg of ginsenoside Re; Re20, 20 mg/kg of ginsenoside Re; and Sul, 50 mg/kg sulfasalazine) were orally administered. Normal group (NOR) was treated with vehicle alone in normal mice. Test agents (suspended in 2% Tween 80) were orally administered once daily for 3 days after TNBS treatment. The mice were sacrificed 18 h after the final administration of test agents. All values are the mean ± SD (n = 10). #Significantly different vs normal group (P < 0.05). *Significantly different vs control group (P < 0.05).

activated with LPS, PG, or TNF-α (Figure 1). Exposure to LPS, PG, or TNF-α increased IKKβ phosphorylation and NF-κB activation in these cells. Furthermore, these stimulations increased the expression of proinflammatory cytokines IL-1β (Figure 2A) and TNF-α (Figure 2B). Ginsenoside Re (10 μM) potently inhibited IKKβ phosphorylation and NF-κB activation, as well as these proinflammatory cytokine expressions in LPS-stimulated macrophages, but it did not inhibit them in TNF-α- or PG-stimulated macrophages. Furthermore, we examined the effect of ginsenoside Re on the protein level of COX-2 and iNOS in peritoneal macrophages stimulated with LPS (Figure 2C). LPS induced the expression of COX-2 and iNOS. However, treatment with LPS in the presence of ginsenoside Re significantly inhibited the expression of these enzymes.

Phosphorylation of IKKβ and IκBα (through ubiquitination and proteolytic degradation) lead to the nuclear translocation of NF-κB, which exists as a complex of NF-κB·IκB-α in the cytoplasm.[24] Therefore, to investigate the inhibitory effect of ginsenoside Re on the degradation of IκBα in activated peritoneal macrophages, the cells were treated with LPS in the presence

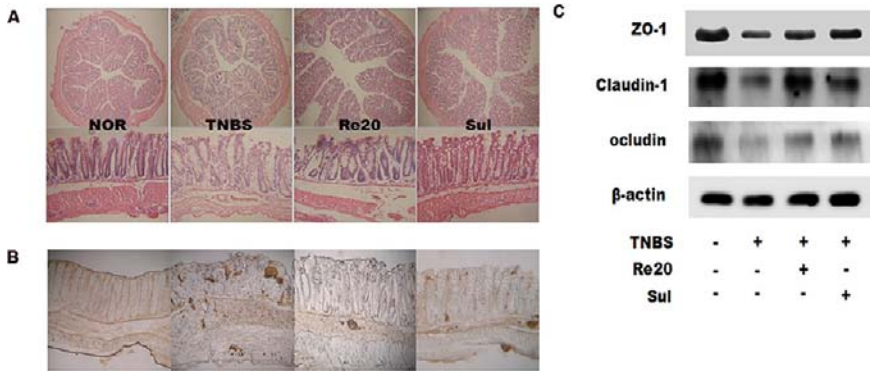

Figure 8. Effect of ginsenoside Re on histology (A), immunohistochemical staining (B), and tight junction protein expression (C) in TNBS-induced colitic mice. TNBS (2.5% TNBS in 50% ethanol) was intrarectally administered in mice. Test agents (TNBS, vehicle alone; Re20, 20 mg/kg of ginsenoside Re; and Sul, 50 mg/kg sulfasalazine) were orally administered. Normal group (NOR) was treated with vehicle alone in normal mice. Test agents (suspended in 2% Tween 80) were orally administered once daily for 3 days after TNBS treatment. The mice were sacrificed 18 h after the final administration of test agents. The colons were stained with hematoxylin−eosin and then assessed by light microscopy.

or absence of ginsenoside Re (Figure 3). LPS induced IκBα degradation, but treatment with LPS in the presence of ginsenoside Re inhibited IκBα degradation. Furthermore, ginsenoside Re also inhibited IRAK-1 phosphorylation, as well as IRAK-1 and IRAK-4 degradations, in LPS-stimulated peritoneal macrophages.

Inhibitory Effect of Ginsenoside Re on the Binding of LPS to TLR4. Treatment with ginsenoside Re (10 μM) inhibited LPS-induced IRAK-1 activation. Therefore, we examined whether ginsenoside Re could inhibit the binding of LPS to TLR4 on peritoneal macrophages using flow cytometry (Figure 4). Treatment with Alexa Fluor 488-conjugated LPS to peritoneal macrophages significantly shifted them by flow cytometry. However, treatment with ginsenoside Re significantly inhibited the shift of macrophages by Alexa Fluor 488-conjugated LPS.

To further confirm whether ginsenoside Re inhibits the binding of LPS to TLR4 on peritoneal macrophages, the macrophages were transiently transfected with TLR4 or/and MyD88 siRNAs for 48 h, and then protein expression of TLR4 and MyD88 was detected. Knockdown efficiencies of TLR4, MyD88, and both TLR4/MyD88 were 87.9%, 85.1%, and 85.9/80.6%, respectively, as determined by an immunoblot analysis (Figure 5). When LPS was treated in these cells, LPS did not bind to peritoneal macrophages transfected with TLR4 or TLR4/MyD88 siRNA and did not activate IKKβ. However, LPS bound to MyD88 siRNA-transfected peritoneal cells, but did not activate IKKβ. When ginsenoside Re (10 μM) was treated prior to stimulation with LPS (100 ng/mL) for 4 h in these transfected peritoneal macrophages, LPS did not bind to TLR4 on these cells and did not activated IKKβ.

Effect of Ginsenoside Re on the Expression of Serum Proinflammatory Cytokines IL-1β and TNF-α in LPS-Stimulated Mice. To evaluate the anti-inflammatory effect of ginsenoside Re in vivo, we investigated the inhibitory effect of ginsenoside Re in LPS-induced systemic inflammation in mice. Treatment with LPS significantly induced the expression of proinflammatory cytokines IL-1β and TNF-α in the blood of mice. Orally administered ginsenoside Re (10 and 20 mg/kg) significantly inhibited the expression of these proinflammatory cytokines after LPS-induced inflammation (Figure 6).

Inhibitory Effect of Ginsenosides Re on the Expression of Proinflammatory Cytokines and the Activation of NF-κB in TNBS-Induced Colitic Mice. Intrarectal injection of TNBS in mice caused severe inflammation, manifested by shortened, thickened, and erythematous colons. Treatment with ginsenoside Re in TNBS-treated mice ameliorated colon shortening and macroscopic score (Figure 7). Ginsenosides Re (20 mg/kg) inhibited colonic myeloperoxidase activity by 63% compared with that in the group treated with TNBS alone ($P < 0.05$). Histologic examination of the colon in TNBS-induced colitic mice showed the increase of neutrophils, massive bowel edema, dense infiltration of the superficial layers of the mucosa, and epithelial cell disruption by large ulcerations. Orally administered ginsenoside Re suppressed the massive bowel edema and epithelial cell disruption (Figure 8). Ginsenoside Re reversed the reduced expression of tight-junction-associated proteins, ZO-1, occludin, and claudin, in the colon of TNBS-treated mice. We next measured the levels of proinflammatory cytokines, IL-1β, IL-6, and TNF-α, and an anti-inflammatory cytokine, IL-10, in the colons of TNBS-treated mice (Figure 9). TNBS increased the expression of TNF-α, IL-1β, and IL-6 by 20.1-, 6.3-, and 3.8-fold, respectively, but inhibited IL-10 expression by 76%. Treatment with ginsenoside Re in TNBS-treated mice inhibited the expression of these proinflammatory cytokines, although β-actin expression was not affected. Thus, ginsenoside Re (20 mg/kg) inhibited the expression of these cytokines by 59% for IL-1β ($P < 0.05$), 87% for TNF-α ($P < 0.05$), and 49% for IL-6 ($P < 0.05$), respectively. However, ginenoside Re increased IL-10 expression by 58% ($P < 0.05$). Treatment with TNBS also increased the expression of COX-2 and iNOS and the activation of NF-κB (p-p65). Ginsenoside Re also blocked the expression of COX-2 and iNOS and the activation of NF-κB.

DISCUSSION

Pathogens, damaged cells, or irritants cause inflammation, which induces the release cytokines, chemokines, and growth factors and the transmigration of inflammatory cells, such as neutrophils, monocytes, and lymphocytes, from the blood to the affected tissue.[25] Among these inflammatory mediators, proinflammatory cytokines such as TNF-α and IL-1β are expressed through the NF-κB signaling pathway, but they also activate NF-κB.[2,3] Thus, these inflammatory mediators arouse the innate immune response, but their overexpressions cause acute phase endotoxemia, leading to organ failure, shock, tissue injury, and even death.[26] In order to regulate the expression of these inflammatory mediators, the application of many herbal ingredients has recently been investigated.[11−15]

Many ginsenosides isolated from ginseng exhibit anti-inflammatory effects in vitro and in vivo.[27−29] Among them,

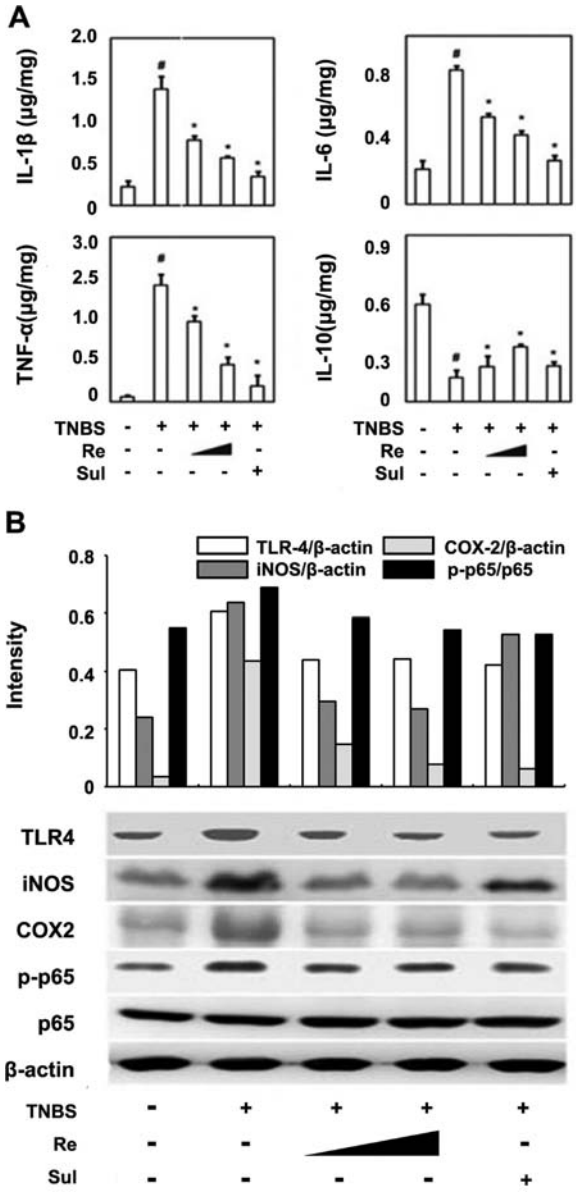

Figure 9. Effect of ginsenoside Re on the expression of inflammatory cytokines IL-1β, IL-6, and TNF-α, and iNOS and COX-2 and the activation of NF-κB in TNBS-induced colitic mice. TNBS was intrarectally administered in control (2.5% TNBS in 50% ethanol), ginsenoside Re (Re, 10 or 20 mg/kg), and sulfasalazine (Sul, 50 mg/kg) groups; the normal group was treated with vehicle alone. The mice were sacrificed 18 h after the final administration of test agents. (A) Effect on proinflammatory cytokines. These cytokines were assay by ELISA. (B) Effect on the expression of TLR-4, iNOS, and COX2 and the activation of NF-κB. These were determined by immunoblotting. All values are the mean ± SD (n = 10). #Significantly different vs normal group (P < 0.05). *Significantly different vs control group (P < 0.05).

compound K ameliorates inflammation by inhibiting IRAK1 phosphorylation in macrophages. Ginsenoside Re also showed inhibitory effects against carrageenan-induced inflammation or oxazolone-induced dermatitis in mice. Furthermore, ginsenoside Re also inhibits the expression of inflammatory markers COX-2, iNOS, TNF-α, and IL-1β in LPS-stimulated RAW264.7 cells. Nevertheless, the target molecule that ginsenoside Re regulates in inflammatory reaction has not been studied. In the present study, we also found that ginsenoside Re inhibited IKKβ phosphorylation and NF-κB activation, as well as TNF-α, IL-1β, COX-2, and iNOS expressions in LPS-stimulated peritoneal macrophages, but it did not inhibit them in PG- or TNF-α-stimulated peritoneal macrophages. Furthermore, ginsenoside Re inhibited IRAK-1 phosphorylation and IRAK-4 degradation in LPS-stimulated peritoneal macrophages. Ginsenoside Re inhibited the binding of LPS to TLR4 on peritoneal macrophages. Ginsenoside Re also inhibited the binding of LPS to TLR4 in MyD88 siRNA-transfected peritoneal macrophages. However, LPS in the absence or presence of ginsenoside Re did not bind to TLR4 on TLR4 siRNA- and TLR4/MyD88 siRNA-transfected peritoneal macrophages. These macrophages attenuated NF-κB activation, even if stimulated with LPS, as the previously reported.[30] On the basis of these findings, ginsenoside Re may suppress the expression of proinflammatory cytokines and the activation of their transcription factor NF-κB by inhibiting the binding of LPS to TLR4 receptor on immune cells, such as macrophages. Some saponins, such as echinocystic acid and glycyrrhizin, also inhibit the binding of LPS to TLR4 on macrophages.[31,32] However, protopanaxadiol ginsenoside compound K did not inhibit the binding of LPS to TLR4 on peritoneal macrophages.[18] These results suggest that 6-O-β-D-glucosyl and 20-hydroxyl moieties of protopanaxatriol may be important in inhibiting the binding of LPS to TLR4.

Ginsenoside Re inhibited LPS-induced systemic inflammation and TNBS-induced colitis in mice. Particularly, ginsenoside Re inhibited the expression of proinflammatory cytokines TNF-α and IL-1β, but ginsenoside Re increased the expression of anti-inflammatory cytokine IL-10 reduced by TNBS. These results suggest that ginsenoside Re may inhibit the activation of TH1 rather than TH2 cells. Furthemroe, ginsenoside Re inhibited the expression of inflammatory enzymes COX-2 and iNOS, as well as the activation of their transcription factor NF-κB, in TNBS-stimulated colitic mice. These results suggest that ginsenoside Re may inhibit inflammations, such as systemic inflammation and colitis, by regulating the NF-κB signaling pathway, like compound K.[18]

In conclusion, ginsenoside Re also inhibited the phosphorylation of IRAK-1 and IKK-β phosphorylation and the activation of NF-κB in LPS-stimulated peritoneal macrophages, as well as the binding of Alexa Fluor 488-conjugated LPS to TLR4 on peritoneal macrophages transiently transfected with or without MyD88 siRNAs. Furthermore, ginsenoside Re significantly inhibited the expression of IL-1β and TNF-α on LPS-induced systemic inflammation and TNBS-induced colitis in mice. On the basis of these findings, ginsenoside Re may ameliorate inflammatory diseases by inhibiting the binding of LPS to TLR4 on macrophages.

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Notes

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ABBREVIATIONS USED

CD, cluster determinant; COX, cyclooxygenase; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IKK-β, inhibitor of nuclear factor κ-B kinase subunit beta; IL, interleukin; iNOS, inducible NO synthetase; IRAK, interleukin 1 receptor-associated kinase; LPS, lipopolysaccharide; MPO, myeloperoxidase; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; PGN, peptidoglycan; TLR, Toll-like receptor; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNF, tumor necrosis factor.

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