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RESEARCH ARTICLE

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Ginsenoside Rg3 suppresses the NLRP3 inflammasome activation through inhibition of its assembly

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Abstract

Ginsenoside Rg3 is one of the main constituents of *Panax ginseng*. Compelling evidence has demonstrated that ginsenoside Rg3 is capable of inhibiting inflammation. However, the mechanism mediating its anti-inflammatory effects remain unclear. Here we show that ginsenoside Rg3 blocks IL-1β secretion and caspase-1 activation through inhibiting LPS priming and the NLRP3 inflammasome activation in human and mouse macrophages. Rg3 specifically inhibits activation of NLRP3 but not the NLRC4 or AIM2 inflammasomes. In addition, Rg3 has no effect on upstream regulation of NLRP3 inflammasome, such as K^+ efflux, ROS production, or mitochondrial membrane potential. Mechanistically, Rg3 abrogates NEK7-NLRP3 interaction, and subsequently inhibits NLRP3-ASC interaction, ASC oligomerization, and speckle formation. More importantly, Rg3 can reduce IL-1β secretion induced by LPS in mice and protect mice from lethal endotoxic shock. Thus, our findings reveal an anti-inflammatory mechanism for Rg3 and suggest its potential use in NLRP3-driven diseases.

KEYWORDS

anti-inflammation, ginsenoside Rg3, inflammasome, NLRP3 inflammasome

Abbreviations: PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; ASC, apoptosis-associated speck-like protein containing a CARD; NEK7, NIMA-related kinase 7; ROS, reactive oxygen species; LPS, lipopolysaccharides; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; BMDM, bone marrow derived macrophage; LDH, lactate dehydrogenase.
Yuhua Shi and Huanan Wang contributed equally to this work.

1 | INTRODUCTION

Ginsenoside Rg3 is one of the most effective ingredients isolated from a traditional medicinal herb *Panax ginseng* and has been used as a traditional Chinese medicine.[1] To date, a wide range of protective roles have been demonstrated for Rg3, including anti-cancer, anti-obesity activities, antioxidant as well as anti-inflammation.[2-4] Because of its therapeutic and pharmacological properties, Rg3 was approved by the Chinese Food and Drug Administration for treating nonsmall cell lung cancer in 2002.[2] Recently, Rg3 was shown to abolish IL-1β production through inhibiting inflammasomes activation,[5] but the mechanism is still not well understood.

The inflammasomes are multimeric protein complexes that regulate caspase-1 activation and induce inflammation to against pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs).[6] To date, several inflammasomes have been described, including NLRP1, NLRC4, AIM2, and NLRP3 inflammasome, and the NLRP3 inflammasome is the most characterized one. The NLRP3 inflammasome is composed of innate immune sensor NLRP3, the adaptor protein ASC, and pro-caspase-1. In addition, a newly identified protein NEK7 (NIMA-related kinase 7), which can directly bind to NLRP3 protein, is also thought to be the component of NLRP3 inflammasome.[7,8] Assembly of the NLRP3 inflammasome leads to activation of caspase-1 and subsequently cleaves pro-IL-1β and pro-IL-18 into mature and functional IL-1β and IL-18.[9,10] It is reported that NLRP3 inflammasome can be activated by many different stimuli including PAMPs and DAMPs.[11] It seems that these stimuli activate NLRP3 inflammasome through three main processes: ROS production, ionic fluxes, and lysosomal damage,[11] but the precise mechanism is still not well clarified. The aberrant activation of the NLRP3 inflammasome is related to a variety of diseases, including Prion diseases,[12,13] Alzheimer's diseases[14], type 2 diabetes, and other inflammatory diseases.[15,16] Given the large number and diversity of NLRP3 inflammasome stimuli, searching for inhibitory agents targeting NLRP3 inflammasome itself, but not its upstream or downstream signaling pathways, might be a better choice for the treatment of NLRP3 inflammasome-related diseases.

Ginsenoside Rg3 has been reported to inhibit NF-κB activation and pro-inflammatory cytokine IL-1β secretion.[5,17] These findings promoted us to investigate the possible mechanism of Rg3 in controlling inflammasome activation. In this study, we found that Rg3 exerted its anti-inflammatory activity through inhibiting NLRP3 inflammasome assembly. Furthermore, we showed that Rg3 prevented inflammation in LPS-induced peritonitis and endotoxic shock in vivo. These findings identified Rg3 as a potential inhibitory agent for the treatment of NLRP3-driven diseases.

2 | MATERIALS AND METHODS

2.1 | Reagents

Ginsenoside Rg3 (purity ≥98%, SML0184; reconstituted in DMSO at 5 mg/mL, diluted in culture medium at indicated concentrations), LPS (O11:B4, L2630; reconstituted in sterile endotoxin-free water at 5 mg/mL, diluted to 1 μg/mL in culture medium), MCC950 (PZ0280; reconstituted in sterile endotoxin-free water at 1 mM, diluted to 10 μM in culture medium), anti-Flag M2 (F1804), anti-Myc (M5546) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-ASC (sc-514414) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-IL-1β (AF-401-NA) was obtained from R&D Systems (Minneapolis, MN, USA). Anti-caspase-1 (2225), anti-NLRP3 (15101), anti-β-actin (3700) and secondary antibodies used in western blot and immunofluorescence were from Cell Signaling Technology (Beverly, MA, USA). ATP (tlrl-atpl; reconstituted in sterile endotoxin-free water at 200 mM, diluted to 5 mM in culture medium), Nigericin (tlrl-nig; reconstituted in 100% ethanol at 6.7 mM, diluted to 10 μM in culture medium), Alum crystals (tlrl-alk; reconstituted in sterile endotoxin-free water at 20 mg/mL, diluted to 300 μg/mL in culture medium), Pam3CSK4 (tlrl-pms; reconstituted in sterile endotoxin-free water at 1 mg/mL, diluted to 500 ng/mL in culture medium), CL097 (tlrl-c97; reconstituted in sterile endotoxin-free water at 1 mg/mL, diluted to 15 μg/mL in culture medium), purified flagellin from *Salmonella typhimurium* (tlrl-epstfla; reconstituted in sterile endotoxin-free water at 500 μg/mL, diluted to 500 ng/mL in culture medium), and Poly(dA:dT) (tlrl-patn; reconstituted in sterile endotoxin-free water at 1 mg/mL, diluted to 4 μg/mL in culture medium) were from InvivoGen (San Diego, CA, USA). Imiquinod (S1211; reconstituted in DMSO at 12.5 mM, diluted to 100 μM in culture medium) was purchased from Selleck (Shanghai, China). Murine M-CSF (315-02; reconstituted in sterile endotoxin-free water at 100 μg/mL, diluted to 20 ng/mL in culture medium) was from PeproTech (Rocky Hill, NJ, USA). ELISA kits for human and mouse TNF-α, IL-6, and IL-1β were from MultiSciences (Hangzhou, Zhejiang, China). CytoTox 96 LDH-release assay kit (G1780) was from Promega (Madison, WI, USA).

2.2 | Cell culture and stimulation

J774.A1, RAW264.7, and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in a humidified incubator at 37°C with 5% CO_2, supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin. Mouse bone marrow-derived macrophages (BMDMs), human monocyte cell line THP-1, and NLRP3 deficient cell line THP-1-defNLRP3 (thp-dnlp, InvivoGen) were cultured

in RPMI 1640 supplemented with 10% FBS, 100 μg/mL Normocin, 100 μg/mL streptomycin, and 100 U/mL penicillin.

For inducing NLRP3 inflammasome activation, 5×10^5 cells were plated overnight in 24-well plates, and then the cells were primed with 1 μg/mL LPS for 4 hours. After that, Rg3 was added into the culture for another 1 hour. Cells were then stimulated with ATP (5 mM, 1 hour), Nigericin (10 μM, 1 hour), or Alum crystals (300 μg/mL, 4 hours). For AIM2 and NLRC4 inflammasomes activation, cells were primed with 1 μg/mL LPS for 4 hours and then transfected with 4 μg/mL Poly(dA:dT) or 500 ng/mL flagellin for 8 hours through the use of Lipofectamine 2000. For non-canonical inflammasome activation, cells were primed with 500 ng/mL Pam3CSK4 for 4 hours, after which the medium was replaced and cells were transfected with 2 μg/mL LPS using lipofectamine 2000 for 6 hours. The cell lysates and supernatants were collected and analyzed for TNF-α, IL-6, IL-18, and IL-1β by ELISA and caspase-1 activation by western blot.

2.3 | Isolation and differentiation of BMDMs

BMDMs were isolated from femur and tibia of female 6- to 8-week-old C57BL/6J mice. Mice were sacrificed by cervical dislocation and both femurs and tibias were collected. After removing of muscles and adherent soft tissue, the bones were washed in 70% ethanol. The bone ends were cut and bone marrow was flushed out into a Petri dish using a syringe filled with RPMI 1640 medium. Pipette the bone marrow cells up and down to obtain a single cell suspension. After depleting of red blood cells using RBC lysing buffer and two times washing, 2×10^6 cells were cultured on 10 cm^2 tissue culture dishes in RPMI 1640 medium supplemented with 10% FBS, 20 ng/mL M-CSF, 100 μg/mL streptomycin and 100 U/mL penicillin. The cells were cultured in a humidified incubator at 37°C with 5% CO_2, and medium were replaced on day 3 and day 6 with RPMI 1640 medium supplemented with 10% FBS, 20 ng/mL M-CSF, 100 μg/mL streptomycinm and 100 U/mL penicillin. On day 7, cells were harvested and used in subsequent experiments.

2.4 | ELISA

Supernatants from cell culture, mouse serum, or peritoneal lavage fluid were assayed for mouse TNF-α, IL-6, IL-18, IL-1β, or human TNF-α and IL-1β in accordance with the manufacturer's instructions. Because the large variation in different cell lines as well as primary cells, the values were transformed to percent change. For example, in LPS+nigricin/ATP stimulated cells each mean value in pg/mL was set at 100%, and other values of percent change was calculated.

2.5 | Western blot analysis

Supernatants from treated cells were concentrated with by using Amicon Ultra-0.5 mL Centrifugal Filters (C82301, Millipore, Billerica, MA, USA). Cell lysates were lysed by RIPA buffer. The samples were separated by SDS-PAGE on 12% gels. The separated proteins were then transferred onto PVDF membrane and hybridized with primary antibodies. After incubation with HRP-conjugated secondary antibody, the membranes were detected by an image system (Clinx Science Instruments, Shanghai, China).

2.6 | Plasmid construction

The cDNAs encoding mouse *NLRP3, ASC, Pro-caspase-1,* and *IL-1β* were obtained by reverse transcription of total RNA from J774.A1 cells. Specific primers were used for PCR amplifying, and the products were subcloned into p3Flag-CMV-7.1, pCMV-Myc, pEFGP-C1, and pCI-neo vectors. The primers used in this study are shown in Supplementary Table S1.

2.7 | Quantitative PCR (qPCR)

Total RNA was extracted using Total RNA Isolation Kit (Aidlab Biotechnology, Beijing, China) according to the manufacturer's instructions. Reverse transcription was carried out using cDNA Synthesis Kit (Fermentas, MD, USA). Relative expression levels were determined by the comparative CT method ($2^{-\Delta\Delta CT}$). All samples were performed in triplicate. Primers used in for qPCR are shown in Supplementary Table S2.

2.8 | Reconstitution of the NLRP3 inflammsome in HEK293T cells

HEK293T cells grown to approximately 80% confluence in 24-well plates were transfected with 100 ng of p3XFlag-NLRP3, 20 ng of pEGFP-C1-ASC, 200 ng of pCI-Casp-1-HA, and 400 ng of pEGFP-C1-IL-1β using lipofectamine 2000 (Invitrogen, CA, USA). Ginsenoside Rg3 was added into the cell culture 6 hours after transfection. The supernatants were collected 24 hours after transfection and analyzed for IL-1β by ELISA.

2.9 | ASC oligomerization assay

THP-1 cells were seeded at 1×10^6 per well in 6-well plates. The following day, the cells were primed with 1 μg/mL

LPS for 4 hours and then treated with Rg3 for 1 hour. After stimulation with nigericin for 1 hour, the supernatants were removed, and cells were lysed in 0.5 mL ice-cold NP-40 by shearing 20 times through a 21-gauge needle. Cell lysates were then centrifuged at 6000× *g* for 15 minutes, and the pellets were resuspended in 0.5 mL PBS, crosslinked with fresh disuccinimidyl suberate (DSS, 2 mM, Sigma-Aldrich) at 37°C for 30 minutes. The crosslinked pellets were re-suspended in 35 μl SDS loading buffer for western blot analysis.

2.10 | Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was detected by using mitochondrial membrane potential assay kit with JC-1 (C2006, Beyongtime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, THP-1 cells were seeded at a density of 1×10^6 per well in six-well plates. After stimulation, cells were incubated with JC-1 staining solution for 20 minutes at 37°C. After washing twice with PBS, the fluorescence was determined by a flow cytometer.

2.11 | Protein purification

HEK293T cells were transfected for 48 hours with p3XFlag-NLRP3 vector. Cells were lysed and FLAG proteins were purified using the FLAG M purification kit (Catalog Number: CELLMM2, Sigma-Aldrich) according to the manufacturer's instructions. Purified FLAG target proteins were dialyzed against PBS to remove the 3xFLAG elution peptide.

2.12 | NLRP3 ATPase activity assay

Purified mouse recombinant NLRP3 (1.5 ng/μl) was incubated with Rg3 at 37°C for 15 minutes in the reaction buffer. Ultra-pure ATP (25 μM) was then added for another 40 minutes at 37°C. The amount of ATP converted into ADP was detected using ADP-Glo Kinase Assay (Promega, Madison, MI, USA) according to the manufacturer's instruction.

2.13 | Measurement of mitochondrial ROS production

Mitochondrial ROS production was detected by MitoSOX Red mitochondrial superoxide indicator kit (M36008, Invitrogen). Cells were stained with MitoSOX at a final concentration of 5 μM at 37°C for 30 minutes. MitoSOX fluorescence was detected by flow cytomery.

2.14 | Confocal microscopy

HEK293T cells or BMDMs were plated at 1×10^5 per well in 24-well plates overnight on coverslips. The following day, cells were stimulated or treated with Rg3 at indicated concentration. Cells were then fixed in 4% paraformaldehyde for 30 minutes at room temperature followed by incubation with antibodies and DAPI. The cells were examined by a laser scanning microscope (Zeiss LSM510 META).

2.15 | Co-immunoprecipitation assay

HEK293T cells were transfected with different plasmids as indicated. Twenty-four hours later, cells were collected and lysed with NP-40 lysis buffer containing a protease inhibitor cocktail (5871, Cell Signaling Technology). Extracts were immunoprecipitated with control mouse IgG or anti-Flag antibody with Protein-A Sepharose (P9424, Sigma-Aldrich) and then assessed by immunoblot analysis.

2.16 | In vivo LPS challenge

For LPS-induced peritonitis, C57BL/6J mice were pretreated with Rg3 (10 mg/kg body weight, i.p. injection) for 3 days. One hour after the last injection, the mice were injected i.p. with 200 μl LPS (L2630, *E. coli* O11:B4, Sigma-Aldrich) at 10 mg/kg. After 6 hours, the mice were sacrificed and 0.5 mL ice-cold PBS were used to wash them peritoneally. Cytokines in peritoneal lavage fluid and serum were determined by ELISA.

For LPS septic shock model, mice aged 8-10 weeks were injected i.p. 20 mg/kg LPS. Rg3 (10 mg/kg body weight) were injected i.p. every 24 hours for 72 hours, and mice were monitored every 12 hours for 72 hours.

2.17 | Statistical analysis

The values are presented as mean ± SD. Data were analyzed using GraphPad Prism 6.0. Difference between an experimental group and a control was analyzed with Student's *t* test or one-way ANOVA. All assays were performed at least three times independently. P values $< .05$ were considered significant. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

3 | RESULTS

3.1 | Ginsenoside Rg3 blocks NLRP3 inflammasome activation in macrophages

The structure of ginsenoside Rg3 is depicted in Figure 1A. The effect of ginsenoside Rg3 on NLRP3 inflammsome

activation was first detected in human monocytic THP-1 cells. Cells were first primed with LPS, then pretreated with ginsenosdie Rg3, and lastly treated with nigericin. Rg3 exhibited dose-dependent inhibitory effects on IL-1β and IL-18 secretion (Figure 1B,C), but had no effects on inflammasome-independent cytokines TNF-α and IL-6 production (Figure 1D,E). Rg3 could also significantly reduce lactate dehydrogenase (LDH) release during LPS/nigericin-induced cells pyroptosis (Figure 1F). By western blot, mature IL-1β and active caspase-1 p20 subunit were reduced in the supernatants, but the precursors proteins pro-IL-1β and pro-caspase-1 in the cell lysates did not change (Figure 1G). In addition, Rg3 also inhibited noncanonical NLRP3 inflammasome-mediated IL-1β secretion (Figure 1H), which is activated by cytosolic LPS, but had no effect on TNF-α production (Figure 1I).

As found with nigericin, Rg3 blocked NLRP3 inflammasome-mediated IL-1β secretion in LPS-primed THP-1 cells by ATP and Alum (Figure 2A,B), and the inhibition effect was not due to Rg3 cytotoxicity (Figure 2C). In LPS-primed BMDMs, Rg3 also abrogated nigericin-induced IL-1β production (Figure 2D), but had no effect on TNF-α secretion (Figure 2E). Similar results were found in the murine macrophages cell line J774A.1 (Figure 2F). Thus, these findings demonstrate that Rg3 can inhibit the NLRP3 inflammasome activation in both human and mice macrophages.

3.2 | The inhibition effect of Rg3 is specific for NLRP3 inflammasome

To further determine the inhibitory effect of Rg3 on NLRP3 inflammasome activation, we performed studies in NLRP3-deficient THP-1 cells (THP-1-def). As shown in Figure 3A, Rg3 significantly decreased IL-1β release in response to LPS/nigericin stimulation in wild-type THP-1 cells, but had no effect in NLRP3-deficient THP-1 cells. Similar results were found in cytosolic LPS-induced noncanonical inflammasome activation in wild-type and NLRP3-deficient THP-1 cells (Figure 3B). Rg3 exerted a slight inhibitory effect on Poly(dA:dT)-induced AIM2 inflammasome activation in wild-type THP-1 cells, and it did not affect AIM2 inflammasome activation when NLRP3 is absent (Figure 3C). In addition, there were no differences in flagellin-induced NLRC4 inflammasome activation between wild-type and NLRP3-deficient cells (Figure 3D). By western blot, Rg3 could slightly inhibit mature IL-1β secretion in the supernatants upon Poly(dA:dT)-induced AIM2 inflammasome activation (Supporting Information Figure S1A). However, mature IL-1β and active caspase-1 p20 subunits were not changed upon flagellin-induced NLRC4 inflammasome activation (Supporting Information Figure S1B).

It was reported that a murine macrophage cell line RAW264.7 lacks ASC gene expression, but it resembles BMDMs when ASC gene was introduced into it.[18-20] We ectopically introduced GFP tagged ASC into RAW264.7 and termed it as RAW264.7-ASC (Figure 3E). As expected, RAW264.7 released negligible IL-1β upon LPS/nigericin and LPS/ATP stimulation (Figure 3F,G). In contrast, RAW264.7-ASC secreted robust IL-1β upon NLRP3 inflammasome agonists treatment (Figure 3F,G). Rg3 significantly decreased IL-1β production in LPS-primed RAW264.7-ASC stimulated with nigericin and ATP (Figure 3F,G). Therefore, these results further demonstrate that the inhibition effect of Rg3 is specific for NLRP3 inflammasome.

It was suggested that Rg3 could inhibit LPS-induced nuclear factor-κB (NF-κB) activation.[21] Therefore, we examined whether Rg3 had an impact on LPS-induced priming for NLRP3 inflammasome activation. When THP-1 cells were treated with Rg3 before or after LPS priming, Rg3 decreased pro-IL-1β expression at a dose of 10 μg/mL, however, Rg3 had no effect on LPS-induced NLRP3 and pro-caspase-1 expression (Figure 3H). Analysis of mRNA expression further confirmed the inhibition effect of Rg3 on pro-IL-1β (Supporting Information Figure S2A). In addition, Rg3 treatment before LPS also decreased IL-1β production, but the inhibition effect was not as strong as the NLRP3 inflammasome inhibition (Figure 3I). Similarly, Rg3 treatment before LPS priming also decreased Poly(dA:dT)- and flagellin-induced IL-1β production (Supporting Information Figure S2B). Therefore, these results indicate that Rg3 can inhibit IL-1β secretion by suppressing both LPS priming and inflammasome activation.

3.3 | Ginsenoside Rg3 has no effect on upstream regulation of NLRP3 inflammasome activation

Several cellular events have been proposed as upstream of NLRP3 inflammasome activation, including postassium (K^+) efflux and ROS production. To determine whether Rg3 abrogated NLRP3 inflammasome activation by inhibiting K^+ efflux, we used two NLRP3 inflammasome agonists, Imiquinod and CL097, which could induce K^+ efflux-independent NLRP3 inflammasome activation.[22] As shown in Figure 4A and 4B, Rg3 significantly decreased IL-1β production both in Imiquinod and CL097-induced NLRP3 inflammasome activation, which indicated that Rg3 exerted its inhibitory effect in a K^+ efflux-independent manner. Rg3 also had no effect on mtROS production in nigericin-induced LPS-primed THP-1 cells (Figure 4C).

Autophagy is a negative regulator for NLRP3 inflammasome activation,[23] and induction of autophagy has been linked to Rg3.[24,25] Indeed, inhibition of autophagy by 3-MA enhanced nigericin-induced IL-1β secretion in LPS-primed

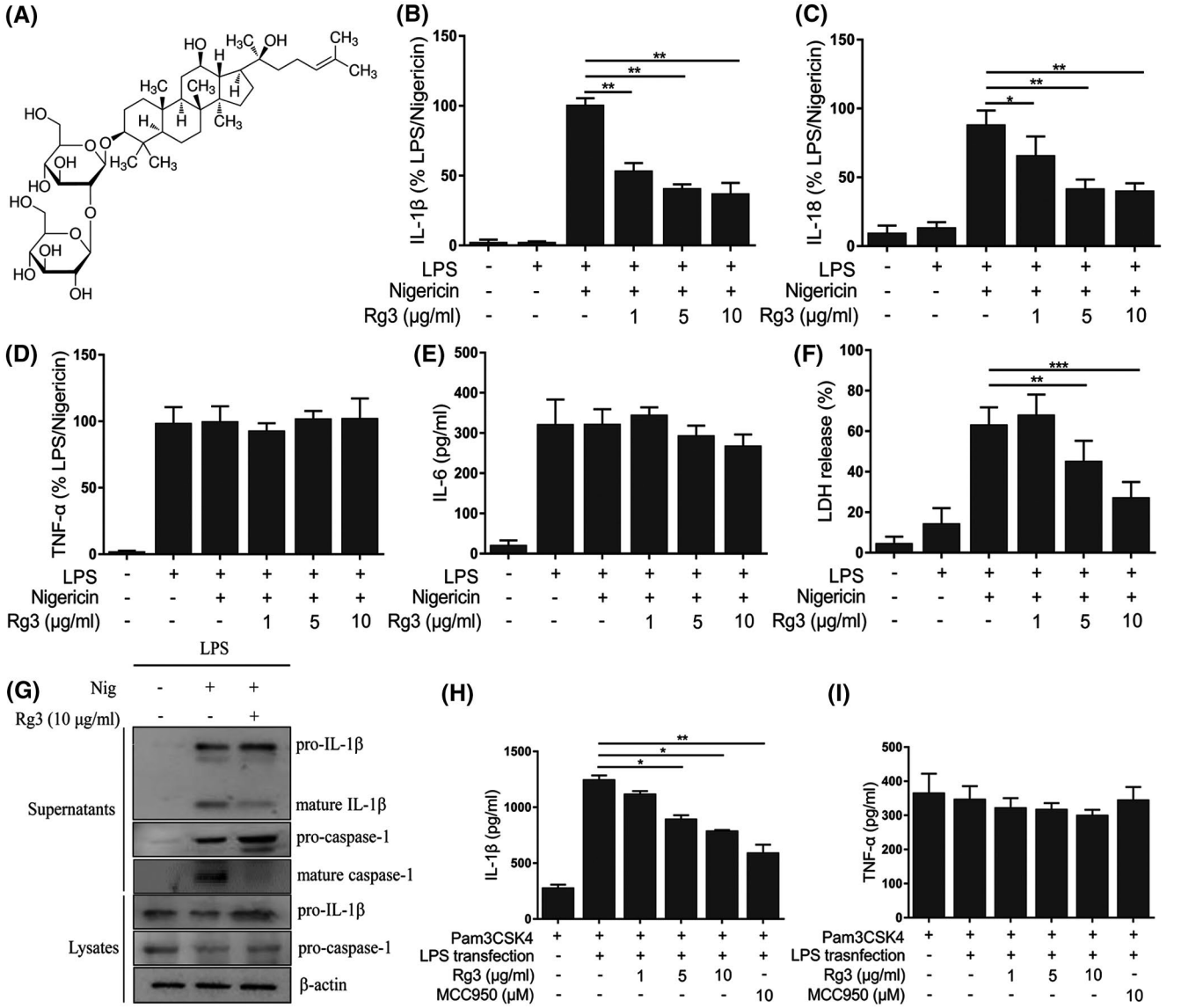


FIGURE 1 Rg3 blocks caspase-1 activation and IL-1β secretion. A, Rg3 structure. B-D, LPS-primed THP-1 cells were treated with Rg3 for 1 hour and then stimulated with nigericin for another 1 hour. IL-1β, IL-18, and TNF-α were determined by ELISA and then expressed as percentage change. E, Supernatants were also analyzed for IL-6 by ELISA. F, LDH release assay in supernatants treated with various dose of Rg3. G, Immunoblot analysis of IL-1β and active caspase-1 in supernatants and cell lysates of LPS-primed THP-1 cells treated with Rg3 and then stimulated with nigericin. H,I, Secretion of IL-1β and TNF-α from Pam3CSK4-primed THP-1 cells transfected with LPS and treated with Rg3. All the real numerical value for the production of cytokines was shown in Supporting Information Figure S3. Data are representative of three independent experiments. Statistics were analyzed using unpaired Student's test

THP-1 cells, but this enhancement did not change in the presence of Rg3 (Figure 4D). Furthermore, Rg3 treatment did not significantly induce autophagy in THP-1 cells (Figure 4E), which ruled out the possible role for autophagy in the inhibitory effect of Rg3 on the NLRP3 inflammasome activation.

Mitochondrial damage is another upstream regulator which links to NLRP3 inflammasome activation. We next tested the effect of Rg3 on mitochondrial membrane potential. As shown in Figure 4F, nigercin lead to significantly mitochondrial membrane potential depolarization in LPS primed THP-1 cells, and Rg3 did not affect this depolarization. The percentage of green fluorescent cells in LPS+Nig group is comparable with LPS+Rg3+Nig group. Therefore, these results suggest that Rg3 does not interfere upstream signal pathways during NLRP3 inflammasome activation.

3.4 | Ginsenoside Rg3 inhibits ASC oligomerization and NLRP3-ASC interaction

During NLRP3 inflammasome activation, ASC oligomerization and NLRP3-ASC interaction are critical events for the subsequent caspase-1 activation and IL-1β secretion. Consistent with the inhibitory effect of Rg3 on IL-1β

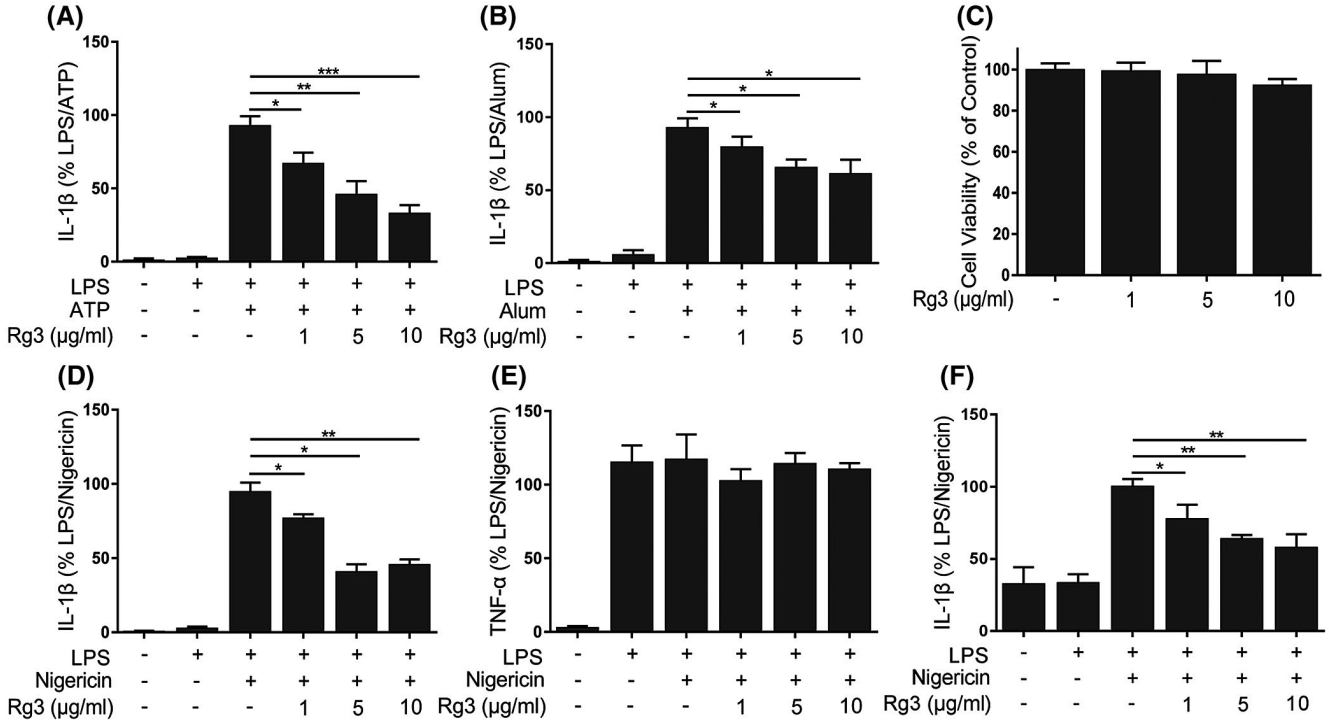


FIGURE 2 Rg3 inhibits NLRP3 inflammasome activation. A,B, LPS-primed THP-1 cells were treated with different doses of Rg3 for 1 hour and then stimulated with ATP or Alum. Percentage changes of secreted IL-1β were calculated. C, The cytotoxicity of Rg3 was examined by MTT assay. D,E, Percentage changes of IL-1β and TNF-α in supernatants from LPS-primed mouse BMDMs treated with various doses of Rg3 and then stimulated with nigericin. F, Percentage change of IL-1β in supernatants from LPS-primed J774.A1 cells treated with Rg3 and then stimulated with nigericin. Data are representative of three independent experiments. All the real numerical value for the production of cytokines was shown in Supporting Information Figure S3. Statistics were analyzed using unpaired Student's test

secretion, Rg3 significantly decreased nigericin-induced ASC oligomerization (Figure 5A). We also detected the effect of Rg3 on ASC speckles formation using immunofluorescence. Following LPS and ATP treatment, ASC oligomerization was observed in the speck-like structure in the cytosol of BMDMs. Treatment with Rg3 markedly reduced the formation of ASC speckles induced by ATP (Figure 5B).

As we had observed that Rg3 could abrogate ASC oligomerization, we next tested whether Rg3 could prevent NLRP3-ASC interaction. HEK293T cells were co-transfected with Flag-tagged NLRP3 and Myc-tagged ASC and treated with different concentrations of Rg3, and then co-immunoprecipitation assay was performed. Rg3 markedly decreased the interaction between NLRP3 and ASC at all tested concentrations (Figure 5C). We further determined the effect of Rg3 on the co-localization of ASC and NLRP3. HEK293T cells were co-transfected with Flag-tagged NLRP3 and GFP-tagged ASC and treated with Rg3, and then immunofluorescence assay was performed. As shown in Figure 5D, when two proteins presented, NLRP3 and ASC were co-localized in the cytoplasm to form "ring-like" structures, but in the presence of Rg3, thiese structures were disturbed. Thus, these results indicate that Rg3 acts upstream of ASC oligomerization and inhibits NLRP3-ASC interaction to block NLRP3 inflammasome activation.

3.5 | Ginsenoside Rg3 blocks NEK7-NLRP3 interaction

Based on the above observations, we speculated that the inhibitory effect of Rg3 on NLRP3 inflammasome activation may target NLRP3 inflammasome itself directly. To test this, we established an in vitro IL-1β cleavage cell system in HEK293T cells, in which the NLRP3 inflammasome is deficient and can be reconstituted.[26-28] HEK293T cells were co-transfected with plasmids encoding p3XFlag-NLRP3, pEGFP-C1-ASC, pCI-Casp-1-HA and pEGFP-C1-IL-1β. Rg3 was added into the cell culture 6 hours post transfection. As shown in Fig 6A, increased IL-1β production was detected in the cell culture after 24 hours co-transfection of the NLRP3 inflammasome components. Interestingly, all tested concentrations of Rg3 could markedly decrease IL-1β secretion (Figure 6A).

NLRP3 oligomerization is a critical step for NLRP3 inflammasome complex formation and recruitment of ASC to NLRP3.[11] We then examined whether Rg3 could inhibit direct NLRP3-NLRP3 interaction. Indeed, Rg3 treatment significantly prevented the interaction of Flag-tagged NLRP3 and Myc-tagged NLRP3 in transfected HEK293T cells (Figure 6B), which suggest that Rg3 blocks NLRP3 oligomerization. Because Rg3 inhibited NLRP3 oligomerization,

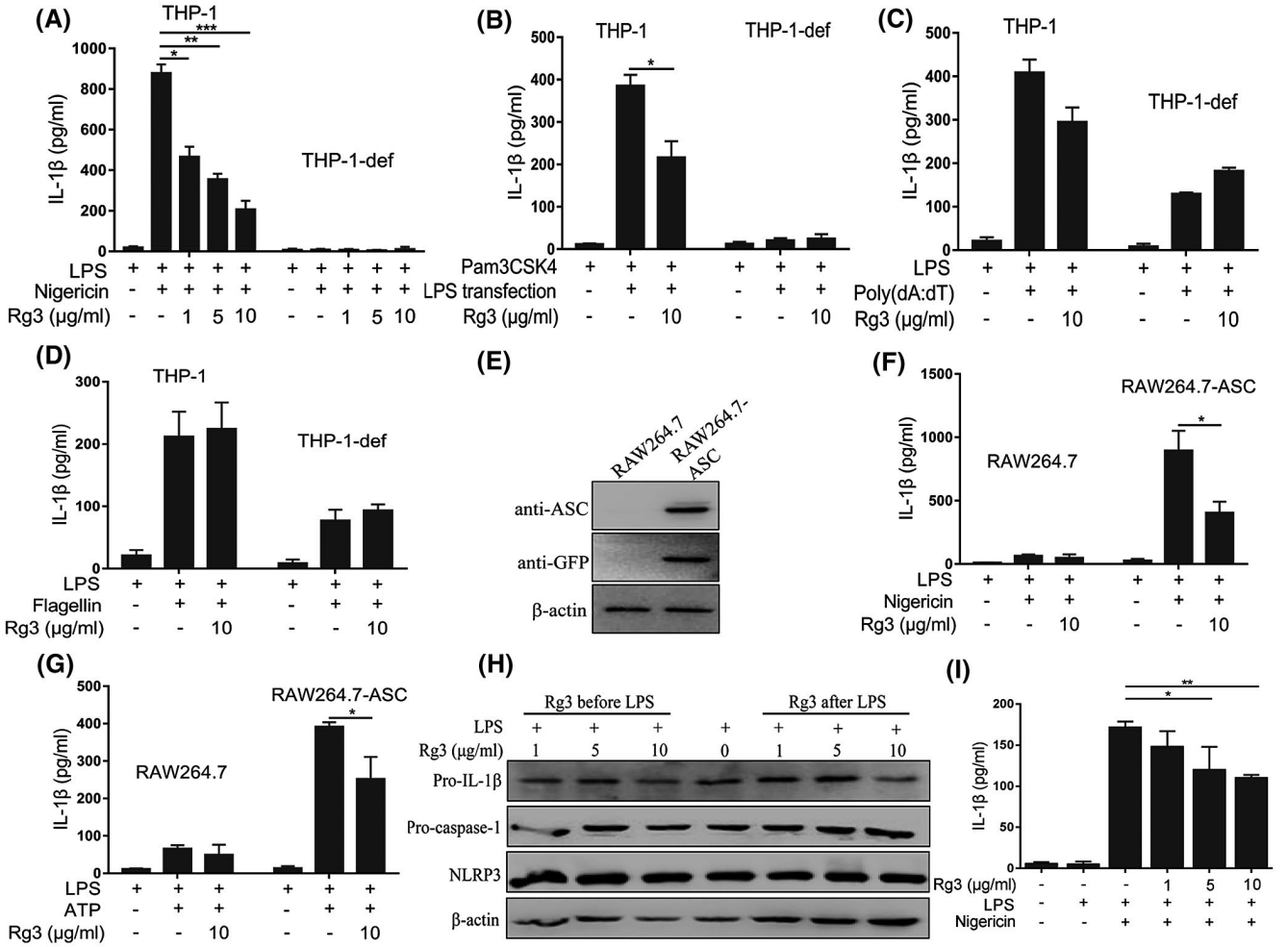


FIGURE 3 Rg3 specifically inhibits NLRP3 inflammasome activation. A, LPS-primed wild-type THP-1 and NLRP3-deficient THP-1 (THP-1-def) cells were treated with Rg3 for 1 hour and then stimulated with nigericin for another 1 hour. IL-1β production were determined by ELISA. B, Secretion of IL-1β from Pam3CSK4-primed THP-1 and THP-1-defecient cells transfected with LPS and treated with Rg3. C,D, LPS-primed THP-1 and THP-1-defecint cells were transfected with 4 μg/mL Poly(dA:dT) or 500 ng/mL flagellin for 8 hours in the presence of Rg3, respectively. Secretion of IL-1β was assayed by ELISA. E, Immunoblot analysis of ASC expression in RAW264.7 cells ectopically introduced into GFP-tagged ASC gene (RAW264.7-ASC). F,G, LPS-primed RAW264.7 and RAW264.7-ASC cells were treated with Rg3 for 1 hour and then stimulated with nigericin or ATP. IL-1β productions were determined by ELISA. H, Immunoblot analysis of the indicated proteins from THP-1 cells treated with Rg3 for 1 hour and then stimulated LPS for 4 hours (Rg3 before LPS) or THP-1 cells treated with LPS for 4 hours and then stimulated with Rg3 for 1 hour (Rg3 after LPS). I, Production of IL-1β from THP-1 cells treated with Rg3 for 1 hour and then LPS primed for 4 hours following nigericin treatment for another 1 hour. Data are representative of three independent experiments. Statistics were analyzed using unpaired Student's test

we next tested whether Rg3 abrogated NLRP3 oligomerization by interrupting the ATPase activity. As previous studies have suggested that the ATPase activity of NLRP3 is critical for the NLRP3 oligomerization.[29] The results showed that Rg3 did not affect the ATPase activity of purified NLRP3 as measured by the release of free phosphate (Figure 6C). These results indicate that Rg3 may inhibit NLRP3 oligomerization by targeting other signal pathways.

Another essential step for NLRP3 oligomerizatioin is the interaction between NEK7 and NLRP3. NEK7 is a newly identified NLRP3 inflammasome component, and the formation of the high-molecular mass NEK7-NLRP3 complex is responsible for the subsequent ASC oligomerization and ASC speck formation.[7,8] We then examined the inhibitory effect of Rg3 on NEK7-NLRP3 interaction. HEK293T cells were co-transfected with Flag-tagged NLRP3 and HA-tagged NEK7 and treated with different concentrations of Rg3, and then co-immunoprecipitation assay was performed. We found that Rg3 treatment abrogated NEK7-NLRP3 interaction in HEK293T cells (Figure 6D). In addition, co-immunoprecipitation analysis of interaction between endogenous NEK7 and NLRP3 in THP-1 cells further confirmed the results (Supporting Information Figure S4B). Taken together, these results suggest that Rg3 can interrupt NEK7-NLRP3 interaction and prevent NLRP3 oligomerization.

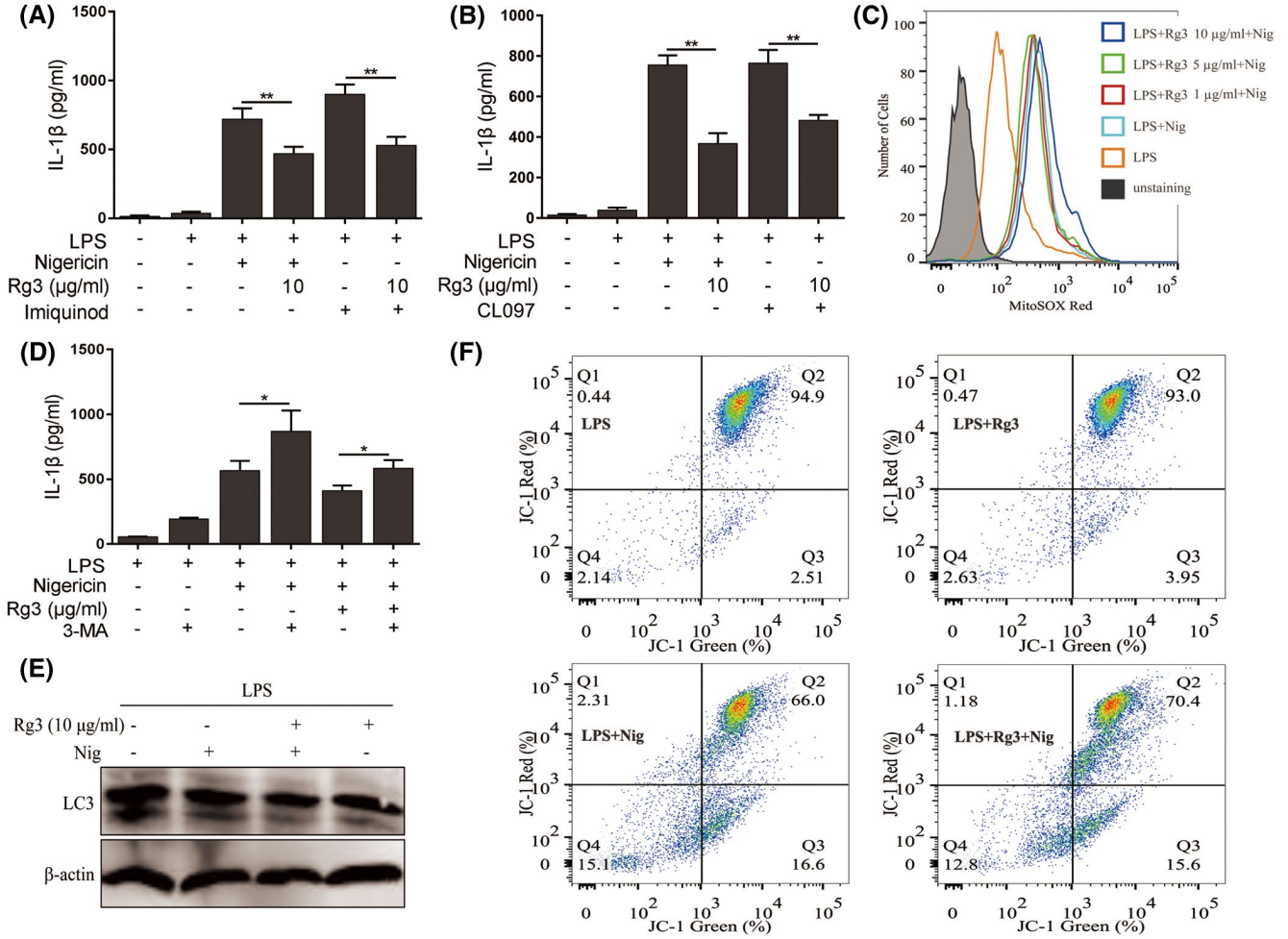


FIGURE 4 The effect of Rg3 on upstream regulation of NLRP3 inflammasome activation. A,B, Secretion of IL-1β from LPS-primed THP-1 cells treated with imiquinod or CL097 in the presence of Rg3. C, LPS-primed THP-1 cells were treated with various doses of Rg3 for 1 hour and then stimulated with nigericin. Cells were then stained with MitoSOX and the fluorescence was detected by flow cytometry. D, Release of IL-1β in supernatants from LPS-primed THP-1 cells pretreated with 3-MA (10 mM) for 30 minutes and then stimulated with nigericin in the presence of Rg3. E, Immunoblot analysis of LC3 in cell lysates from LPS-primed THP-1 cells stimulated with nigericin. F, LPS-primed THP-1 cells were treated with Rg3 for 1 hour and then stimulated with nigericin for another 1 hour. Cells were then stained with JC-1 and the fluorescence was examined by flow cytometry. All data are representative of three independent experiments

3.6 | Ginsenoside Rg3 reduces the severity of LPS-induced systemic inflammation in vivo

We next tested the efficacy of Rg3 treatment on the NLRP3 inflammasome activation in vivo. LPS induced IL-1β secretion by intraperitoneal (i.p.) injection was shown to be NLRP3 inflammasome dependent.[30,31] C57BL/6J mice were i.p. injected with Rg3 (10 mg/kg body weight) for 3 days before LPS injection. After 1 hour of the last injection of Rg3, LPS (10 mg/kg bodyweight) was i.p. injected. After 6 hours, serum and peritoneal lavage samples were collected and the cytokines were determine by ELISA. We found that Rg3 significantly reduced the level of IL-1β (Figure 7A) and slightly inhibited TNF-α (Figure 7B) in serum, whereas no effect on IL-6 secretion was observed (Figure 7C). In the peritoneal lavage fluid, Rg3 markedly decreased IL-1β and TNF-α production compared with the LPS treatment group (Figure 7D,E). Rg3 had no effect on LPS-induced IL-6 release in peritoneal lavage (Figure 7F). In addition, Rg3 attenuated LPS-induced splenomegaly and accumulation of immune cells in the spleen (Figure 7G).

High dose of LPS injection can induce lethal endotoxic shock in mice in a NLRP3 inflammasome-dependent manner.[32,33] To test whether Rg3 can rescue mice from lethal endotoxic shock, C57BL/6J mice pre-injected Rg3 (10 mg/kg body weight) were i.p. injected with LPS at 20 mg/kg bw. Rg3 was injected i.p. every 24 hours for 72 hours.

As shown in Figure 7H, the survival was improved in mice treated with Rg3. Taken together, these results suggest that Rg3 can inhibit the activity of the NLRP3 inflammasome in vivo.

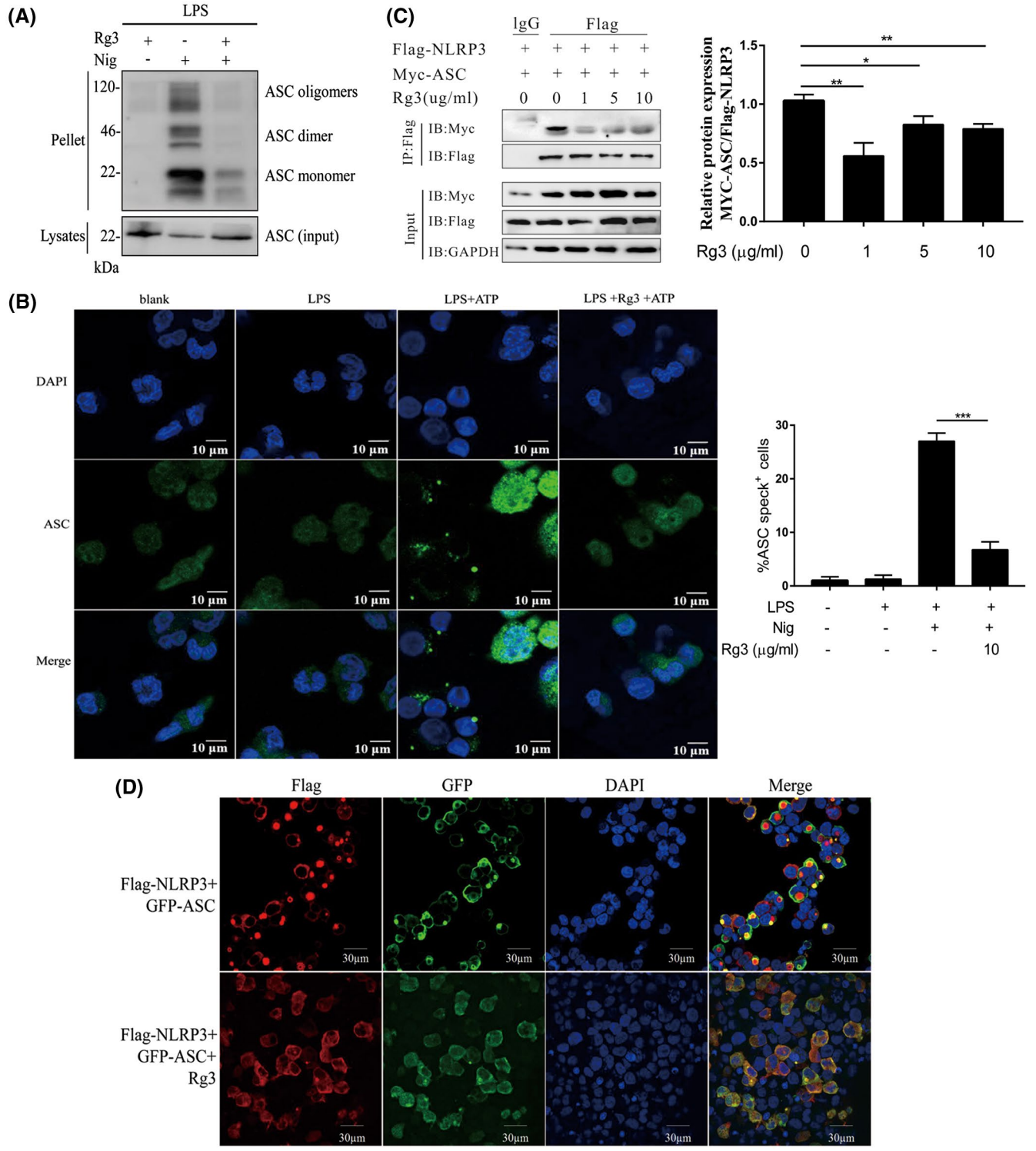


FIGURE 5 Rg3 blocks ASC oligomerization and NLRP3-ASC interaction. A, THP-1 cells were primed with LPS for 4 hours, treated with Rg3 for 1 hour and finally stimulated with nigericin for 1 hour. Cells were lysed with NP-40 and then centrifuged. The supernatants (lysates) and disuccinimidyl suberate-crosslinked pellets were subjected to immunoblot analysis. The results are representative of three independent experiments. B, BMDMs were primed with LPS for 4 hours, treated with Rg3 for 1 hour, and finally stimulated with ATP for 1 hour. ASC speck-like structures (green) and nuclei (blue) were visualized using a confocal fluorescence microscope. C, Western blot of cell lysates and Flag-immunoprecipitation (IP) samples from HEK293T cells transfected with Flag-NLRP3, Myc-ASC plasmids and treated with various doses of Rg3. The results are representative of three independent experiments. D, HEK293T cells were transfected with Flag-NLRP3 (red) and GFP-ASC (green) in the presence or absence of Rg3, and then the co-localization of NLRP3-ASC were examined by confocal microscopy

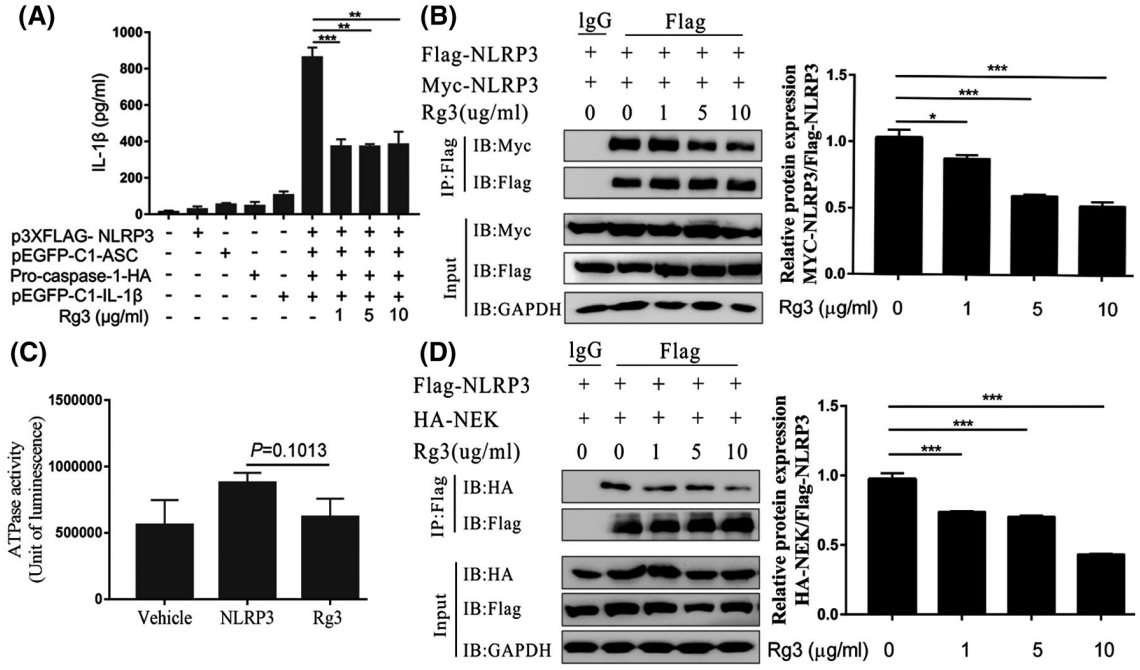


FIGURE 6 Rg3 inhibits NLRP3 inflammasome assembly. A, HEK293T cells were co-transfected with plasmids encoding p3XFlag-NLRP3, pEGFP-C1-ASC, pCI-Casp-1-HA, and pEGFP-C1-IL-1β using lipofectamine 2000. Rg3 was added into the cell culture 6 hours after transfection. IL-1β release in the supernantants was determined by ELISA. B, Western blot of cell lysates and Flag-immunoprecipitation samples from HEK293T cells transfected with Flag-NLRP3, Myc-NLRP3 plasmids and treated with various doses of Rg3. C, ATPase activity assay of recombinant NLRP3 in the presence of Rg3. D, Immunoblot analysis of cell lysates and Flag-immunoprecipitation samples from HEK293T cells transfected with Flag-NLRP3, HA-NEK7 plasmids and treated with various doses of Rg3. The results are representative of three independent experiments. Statistics were analyzed using unpaired Student's test

4 | DISCUSSION

In the present study, we identified ginsenoside Rg3 as a common NLRP3 inflammasome activation inhibitor that was active both in mice in vivo and in human macrophage cells in vitro. Rg3 inhibited both pro-IL-1β expression and NLRP3 inflammasome activation pathways (Figure 8). The inhibitory effect of Rg3 was specific to the NLRP3 inflammasome and had no effect on the AIM2 or NLRC4 inflammasomes activation. Furthermore, Rg3 attenuated LPS-induced NLRP3 inflammasome-dependent systemic inflammation and endotoxic shock in mice model.

Importantly, as a main active component, ginsenoside Rg3 is being used in clinical practice for treating cancers, which indicate its relatively high safety. Taken together, our results reveal a new use for Rg3 as a potential inhibitory agent for treating NLRP3 inflammasome-related diseases.

A lot of molecules have shown potent inhibitory effect for the NLRP3 inflammasome activation and have been tested in animal models. However, most of these inhibitory agents exert broad anti-inflammatory activity or have low efficacy. The broad anti-inflammatory activity may lead to immunosuppressive effects and increase the risk for infection. For instance, a recombinant IL-1β receptor antagonist, anakinra, can target IL-1β to decrease its secretion.[34] However, IL-1β is not the only cytokine of NLRP3 inflammasome activation. Other proinflammatory cytokines, such as IL-18 and HMGB1, also participate in the progress of these diseases. In addition, IL-1β can be released through other inflammasomes, such as AIM2 or NLRC4 inflammasomes, or by other inflammsome-independent manner.[10] Therefore, inhibitory agents specific to NLRP3 inflammasome itself, but not upstream or downstream of NLRP3 inflammsome activation may be the best choice for treatment of NLRP3-driven diseases. To date, few of compounds have been shown to target NLRP3 inflammasome itself, including MCC950,[35] OLT1177,[36] Tranilast,[37] Oridonin,[38] and CY-09.[39] Here we identified Rg3 as another inhibitor specific to NLRP3 inflammasome by interrupting NLRP3 inflammasome assembly.

Rg3 has shown significantly antitumor properties in several kinds of cancers both in vitro and in vivo. Rg3 can be used alone or as an adjuvant to minimize chemotherapeutic resistance and improve the therapeutic efficacy.[40] Rg3 also possess anti-inflammation activities. For instance, Rg3 can ameliorate LPS-induced acute lung injury in mice by inhibiting NF-κB signaling pathway.[21] Indeed, our results indicated that Rg3 could interrupt the priming stage of NLRP3 inflammsome by inhibiting pro-IL-1β expression and subsequently decrease IL-1β release. But the inhibitory effect on priming was not as strong as the NLRP3 inflammasome

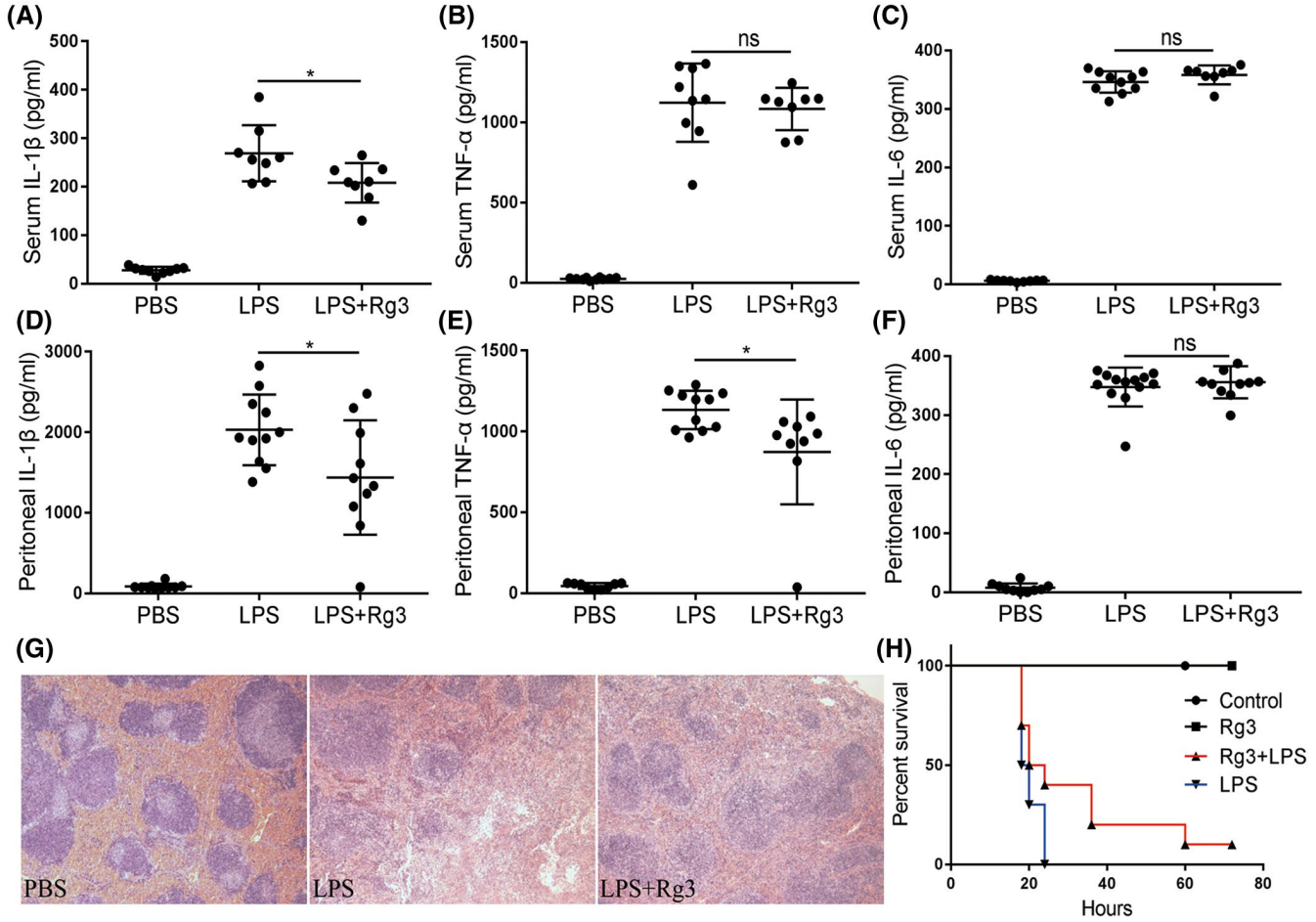
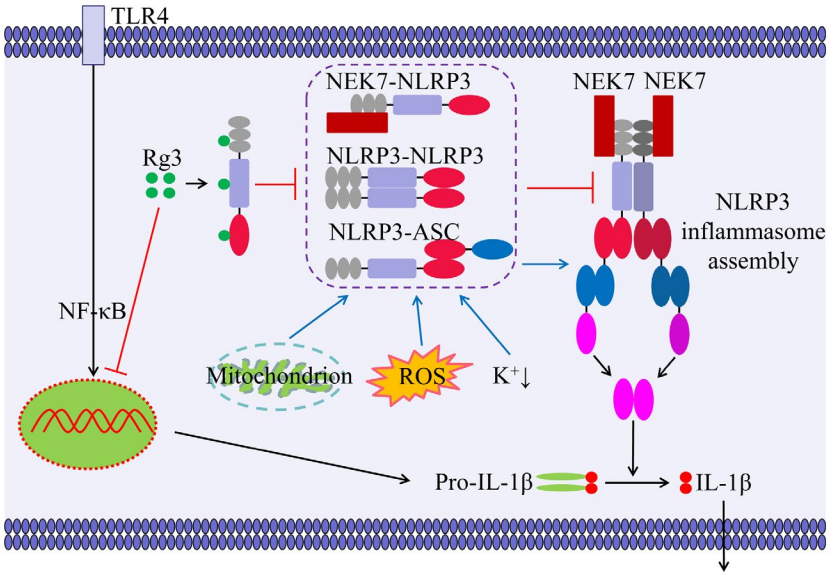


FIGURE 7 Rg3 ameliorates LPS-induced systemic inflammation and lethal endotoxic shock in vivo. A-C, Serum levels of IL-1β, TNF-α, and IL-6 from C57BL/6J mice pretreated with Rg3 or PBS control as assessed by ELISA 6 hours after i.p. LPS injection (n = 10 per group). D-F, levels of IL-1β, TNF-α and IL-6 in peritoneal fluid were measured by ELPSA (n = 10 per group). G, Representative images of H&E staining of spleen sections from PBS control, LPS-treated and LPS plus Rg3-treated mice. H, Survival of mice i.p. injected with LPS (20 mg/kg body weight) with or without Rg3 (n = 10 per group). All data are representative of three independent experiments

FIGURE 8 Schematic diagram illustrates the inhibitory effect of Rg3 on NLRP3 inflammasome activation. Rg3 inhibits IL-1β release by decreasing pro-IL-1β transcription at priming stage. On the other hand, Rg3 blocks NLRP3 inflammasome assembly by interrupting NEK7-NLRP3, NLRP3-NLRP3, and NLRP3-ASC interactions



inhibition. Thus, the focus of the present study was to investigate how Rg3 inhibits NLRP3 inflammasome activation. We first confirmed the inhibitory effect of Rg3 on suppressing NLRP3 inflammasome activation by various agonists, including ATP, nigericin, and Alum. Then, we focused on investigation the role of Rg3 on upstream regulations of NLRP3 inflammasome activation, such as ROS production, K^+ efflux, antophage and mitochondrial membrane potential. The ROS production was one of the first identified common signaling pathway for NLRP3 inflammasome activation.[41] However, many conflicting studies also described the unnecessary role of ROS in the NLRP3 inflammasome activation.[42] Our results showed that Rg3 did not affect ROS production during LPS/nigericin-induced NLRP3 inflamamsome activation. In addition, K^+ efflux and mitochondrial membrane potential also did not participate in the process of Rg3 inhibitory effect on NLRP3 inflammasome activation. It should be noted that although Rg3 abrogates NLRP3 inflammasome activation induced by K^+-independent agonists (Imiquimod and CL097), Rg3 can significantly inhibit NEK7-NLRP3 interaction, which is dependent on K^+ efflux.[7] Thus, we cannot entirely rule out the possibility that Rg3 inhibit K^+ efflux. These data suggest that Rg3 has no effect on upstream regulation of NLRP3 inflammasome activation. We next investigated the role of Rg3 on NLRP3 inflammasome assembly. In order to better describe the relationship between Rg3 and NLRP3 inflammasome, we reconstituted NLRP3 inflammasome in HEK293T cells, in which the NLRP3 inflammasome is deficient. The inhibitory effect of Rg3 on reconstituted NLRP3 inflammasome suggests that Rg3 may directly interrupt NLRP3 inflamamsome assembly. Indeed, subsequent investigation confirmed our hypothesis that Rg3 could inhibit NLRP3 inflammasome assembly through abrogating NEK7-NLRP3, NLRP3-NLRP3, and NLRP3-ASC interactions. Based on these observations, we speculate that Rg3 may target one or more than one sites on NLRP3 protein and then inhibit the interaction of NLRP3 with NEK7, ASC, and NLRP3 oligomerization (Figure 8). Thus, further studies are needed to identify the precise site(s) of Rg3 on NLRP3 protein.

Previous studies have shown that Rg3 can inhibit NLRP3 inflammasome activation, but the underlying mechanism is not well clarified.[5,43] It should be noted that Kim et al reported that Rg3 could inhibit both NLRP3 and AIM2 inflammasome activation.[5] The discrepancy of the inhibitory role of Rg3 on AIM2 inflammasome activation may be due to the different concentrations of transfected Poly(dA:dT), the duration time of transfection, or the different cell types we used. In addition, we also confirmed the results on NLRP3 deficient THP-1 cells, in which the AIM2 and NLRC4 inflammasome activation are not affected. Our data revealed that Poly(dA:dT) and flagellin could induce AIM2 and NLRC4 inflammasome activation in NLRP3-deficient THP-1 cells, respectively. However, Rg3 also could not prevent AIM2 and NLRC4 inflamamsomes activation in NLRP3-deficient THP-1 cells. Therefore, additional investigations are needed to further confirm the role of Rg3 on AIM2 inflammasome activation.

Collectively, our findings demonstrate the inhibitory effect of Rg3 on NLRP3 inflammasome activation in macrophages. Our results further show that Rg3 can reduce the severity of LPS-induced systemic inflammation and lethal endotoxic shock in mice model. Considering the high safety and low-cost of Rg3 in clinic, our study provides a new pharmacological approach for treating NLRP3 inflammasome-related diseases.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Y. Shi, H. Wang, M. Zheng, W. Xu, and Y. Yang performed the experiments. F. Shi conceived the overall scope of the project and wrote the manuscript. Y. Yang interpreted the data and contributed in writing the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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