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Research article

Ginsenoside Rc from *Panax ginseng* exerts anti-inflammatory activity by targeting TANK-binding kinase 1/interferon regulatory factor-3 and p38/ATF-2

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ABSTRACT

Background: Ginsenoside Rc (G-Rc) is one of the major protopanaxadiol-type saponins isolated from *Panax ginseng*, a well-known medicinal herb with many beneficial properties including anticancer, anti-inflammatory, antiobesity, and antidiabetic effects. In this study, we investigated the effects of G-Rc on inflammatory responses *in vitro* and examined the mechanisms of these effects.
Methods: The *in vitro* inflammation system used lipopolysaccharide-treated macrophages, tumor necrosis factor-α/interferon-γ-treated synovial cells, and HEK293 cells transfected with various inducers of inflammation.
Results: G-Rc significantly inhibited the expression of macrophage-derived cytokines, such as tumor necrosis factor-α and interleukin-1β. G-Rc also markedly suppressed the activation of TANK-binding kinase 1/IκB kinase ε/interferon regulatory factor-3 and p38/ATF-2 signaling in activated RAW264.7 macrophages, human synovial cells, and HEK293 cells.
Conclusion: G-Rc exerts its anti-inflammatory actions by suppressing TANK-binding kinase 1/IκB kinase ε/interferon regulatory factor-3 and p38/ATF-2 signaling.

1. Introduction

Although the inflammatory response is employed by the innate immune system to protect the host against various infectious microorganisms, this response can also cause serious problems under chronic conditions. Representative inflammatory diseases include cancer, diabetes, atherosclerosis, and rheumatoid arthritis [1]. These diseases are primarily caused by hyperactivated macrophages, which cause tissue damage to the inflamed organs or tissues in chronic inflammatory conditions. Therefore, achieving effective modulation of macrophages is considered to be a therapeutic goal for the control of many inflammatory diseases [2,3].

To control the immunopathological activities of macrophages in disease states effectively, it is important to understand the cellular events that occur in hyperactivated macrophages. The cellular responses observed in activated macrophages are initiated by interactions between various surface receptors such as toll-like receptors (TLRs) and their corresponding ligands, including the TLR4 ligand lipopolysaccharide (LPS), or between cytokine receptors and their corresponding cytokines [4,5]. After such events, various

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signaling enzymes such as the protein serine/threonine kinases extracellular signal-regulated kinase (ERK), p38, c-Jun N-kinase (JNK), TANK-binding kinase 1 (TBK1), and IκB kinase ε (IKKε) contribute to the activation and translocation of transcription factors such as nuclear factor (NF)-κB, interferon regulatory factor (IRF)-3, and activator protein (AP)-1 [6–11]. Thus, the therapeutic targeting of one or more of these enzymes could effectively suppress macrophage-mediated chronic diseases such as rheumatoid arthritis.

The root of *Panax ginseng* Meyer (ginseng) is a valuable ethnomedicinal herb that has been prescribed for more than 2,000 yr in many Asian countries, including Korea, China, and Japan. Currently, air-dried white ginseng and steamed red ginseng are widely consumed to revitalize and stabilize bodily functions [12]. In addition, ginseng is widely used as both a preventive and therapeutic treatment against various diseases because of its antioxidative, anti-inflammatory, anticancer, and antidiabetic effects [13,14]. However, although the pharmacological activities of ginseng have been well studied, the specific proteins and/or macromolecules targeted by the ginsenosides have not been fully elucidated. In particular, the mechanisms by which ginseng exerts its antiarthritic activities and the various cellular responses involved in these activities are not yet known. From a therapeutic perspective, it is important to understand its mechanisms of action. It is also necessary to determine which signaling enzymes are targeted in arthritic inflammation. To answer these questions, we examined the anti-inflammatory and antiarthritic activities of a diol-type ginsenoside (G-Rc) and identified the target by which G-Rc exerts its anti-inflammatory effects.

2. Materials and methods

2.1. Materials

Ginsenosides (GG-Rb1, G-Rb2, and G-Rc) were purchased from Ambo Institute (Daejeon, Korea). Phorbol 12-myristate 13-acetate, concanavalin A (Con A), and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fetal bovine serum and RPMI 1640 medium were obtained from Gibco (Grand Island, NY, USA). RAW264.7 and HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). All other chemicals were from Sigma-Aldrich and were of analytical grade. Antibodies against the total or phosphorylated forms of the signaling proteins p65, p50, c-Jun, ATF2, Fra-1, IRF-3, TBK1, IKKε, extracellular signal-related kinase (ERK), JNK, p38, γ-tubulin, and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorochrome-labeled monoclonal antibodies (mAbs) against CD11b [phycoerythrin (PE)-Cy 7-labeled) and TCRαβ (PE-labeled) were purchased from BD Biosciences (San Jose, CA, USA).

2.2. Constructs

Constructs driving the expression of signaling proteins (CFP-TRAM, FLAG-TBK1-wild type, and FLAG-TBK1-K38A) and luciferase constructs containing the binding promoters for IRF3 were obtained from Addgene (Cambridge, MA, USA). All constructs were confirmed by automated DNA sequencing. Sequences of the oligonucleotides used for mutagenesis are available upon request.

2.3. Cell culture

Murine macrophage RAW264.7 cells, mouse splenocytes, and human embryonic kidney 293 (HEK293) cells were maintained in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. The cells were grown at 37°C and 5% CO_2 in a humidified atmosphere.

Table 1
Real-time polymerase chain reaction primers

Name		Sequence (5′ to 3′)
Mouse		
Tumor necrosis factor-α	F	TGC CTA TGT CTC AGC CTC TT
	R	GAG GCC ATT TGG GAA CTT CT
GAPDH	F	CAA TGA ATA CGG CTA CAG CAA C
	R	AGG GAG ATG CTC AGT GTT GG
Human		
Tumor necrosis factor-α	F	CCTCTCTCTAATCAGCCCTCTG
	R	AGGACCTGGGAGTAGATGAG
β-actin	F	CCTTCCTTCCTGGGCATGGAG
	R	CTCAGGAGGAGCAATGATCTTGAT

2.4. Preparation of spleen cell suspensions

Mice were sacrificed on Day 19 after booster immunizations, and single-cell suspensions were prepared from the spleens as reported previously [15]. The splenocytes were stimulated with 50 μg/mL anti-collagen type II peptide in the absence or presence of drug treatments. The cytokine levels in the cell culture supernatants were determined after 48 h of culture.

2.5. Synovial cell preparation from the synovial fluid of patients with rheumatoid arthritis

Synovial fluid was obtained from patients with rheumatoid arthritis treated at Korea University Guro Hospital (Guro, Korea). All experiments were performed with the approval of the Korea University Guro Hospital Institutional Review Board, and all patients provided informed consent for the academic usage of their tissue. To separate cells from the synovial fluid, phosphate-buffered saline (PBS) was added to the fluid and the mixture was centrifuged at 630 × g for 5 min. After depleting the red blood cells with Gey's solution, impurities were filtered from the cell suspensions with a 70-μm cell strainer. The separated cell suspensions were used for cytokine mRNA, flow cytometric, and immunoblot analyses.

2.6. mRNA analysis by real-time polymerase chain reaction

To determine the cytokine mRNA expression levels under various conditions, total RNA was isolated from LPS-treated RAW264.7 cells or IFN-γ/TNF-α-treated synovial cells in the presence or absence of G-Rc (6 h). RNA was isolated with TRIzol Reagent (Gibco) according to the manufacturer's instructions. Purified RNA was stored at −70°C until use. mRNA levels were quantified by real-time reverse transcription–polymerase chain reaction using SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. Thermocycling was performed on a real-time Bio-Rad instrument (Hercules, CA, USA) as reported previously [16,17]. All results are given as expression ratios relative to the housekeeping gene GAPDH. Primers were obtained from Bioneer (Daejeon, Korea) and are listed in Table 1.

2.7. Flow cytometry analysis

The percentages of macrophages ($TCR\alpha\beta^-/CD11^+$ cells) in the synovial cells were determined by flow cytometry. Cells (10^5) were washed with PBS containing 2% FCS and 0.1% sodium azide and then incubated in 50 μL of staining buffer containing 10% rabbit serum for 10 min on ice. Next, cells were incubated with primary antibodies for 45 min. After washing three times with staining buffer, cells were incubated with TCRαβ-PE and CD11b-PE-Cy (1:20 dilution) for 45 min, washed three times with staining buffer, and analyzed on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA, USA).

2.8. Luciferase reporter activity assay

HEK293 cells (1×10^6 cells/mL) were transfected with 1 μg of reporter plasmid driving the expression of β-galactosidase and either IRF3-Luc or TBK1-Luc. Cells were transfected in 12-well plates according to the polyethylenimine (PEI) method [18] and used for experiments at 48 h post-transfection. The luciferase assays were performed with the Luciferase Assay System (Promega, Madison, WI, USA) as reported previously [19].

2.9. Preparation of cell lysates and nuclear fractions, immunoblotting, and immunoprecipitation

RAW264.7 cells (5×10^6 cells/mL) were washed three times in cold PBS with 1 mM sodium orthovanadate. Washed cells were then lysed with either a sonicator or a Tissuemizer in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycotetraacetic acid, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 1 mM benzimide, and 2 mM PMSF) for 30 min with rotation at 4°C. The lysates were clarified by centrifugation at 16,000 *g* for 10 min at 4°C and stored at −20°C until use.

Nuclear lysates from RAW264.7 cells were prepared in a three-step procedure [20]. For the immunoprecipitation experiments, lysates from RAW264.7 cells (1×10^7 cells/mL) treated with or without LPS (1 μg/mL) for 2.5 min were precleared with 10 μL of Protein A-coupled Sepharose beads (50% v/v; Amersham, Buckinghamshire, UK) for 1 h at 4°C. All lysates contained equal amounts of protein (500 μg). The precleared samples were incubated overnight at 4°C with 5 μL of antibodies against p38, TBK1, IRF-3, or IKKε. Next, the immune complexes were mixed with 10 μL of Protein A-coupled Sepharose beads (50% v/v) and rotated for 3 h at 4°C.

2.10. Kinase assay

To evaluate the effects of G-Rc on kinase activity, immunoprecipitated TBK1, IKKε, and p38 were incubated in reaction buffer in the presence or absence of G-Rc. The reactions were initiated by the addition of Mg-ATP. After a 30 min incubation at 30°C, the reactions were stopped by the addition of sample buffer and the samples were boiled. Kinase activity was assessed by immunoblotting with antibodies against the phospho-forms of IKKε, IRF-3, and ATF-2.

2.11. Statistical analysis

All *in vitro* data are expressed as means ± standard deviations of experiments performed with six samples. All other data presented are representative of three different experiments that yielded similar results. Similar experimental data were also obtained in an additional independent set of *in vitro* experiments that were performed with the same numbers of samples. For statistical comparisons, results were analyzed with analysis of variance and Scheffe's *post hoc* test and the Kruskal–Wallis and Mann–Whitney tests. A p-value < 0.05 was considered statistically significant. All statistical tests were performed with the SPSS software package (version 22.0, 2013; IBM Corp., Armonk, NY, USA).

3. Results

3.1. Effect of ginseng-derived ginsenosides on the expression of proinflammatory cytokines

Since TNF-α is a representative proinflammatory cytokine that promotes rheumatoid arthritis, we first determined whether G-Rc

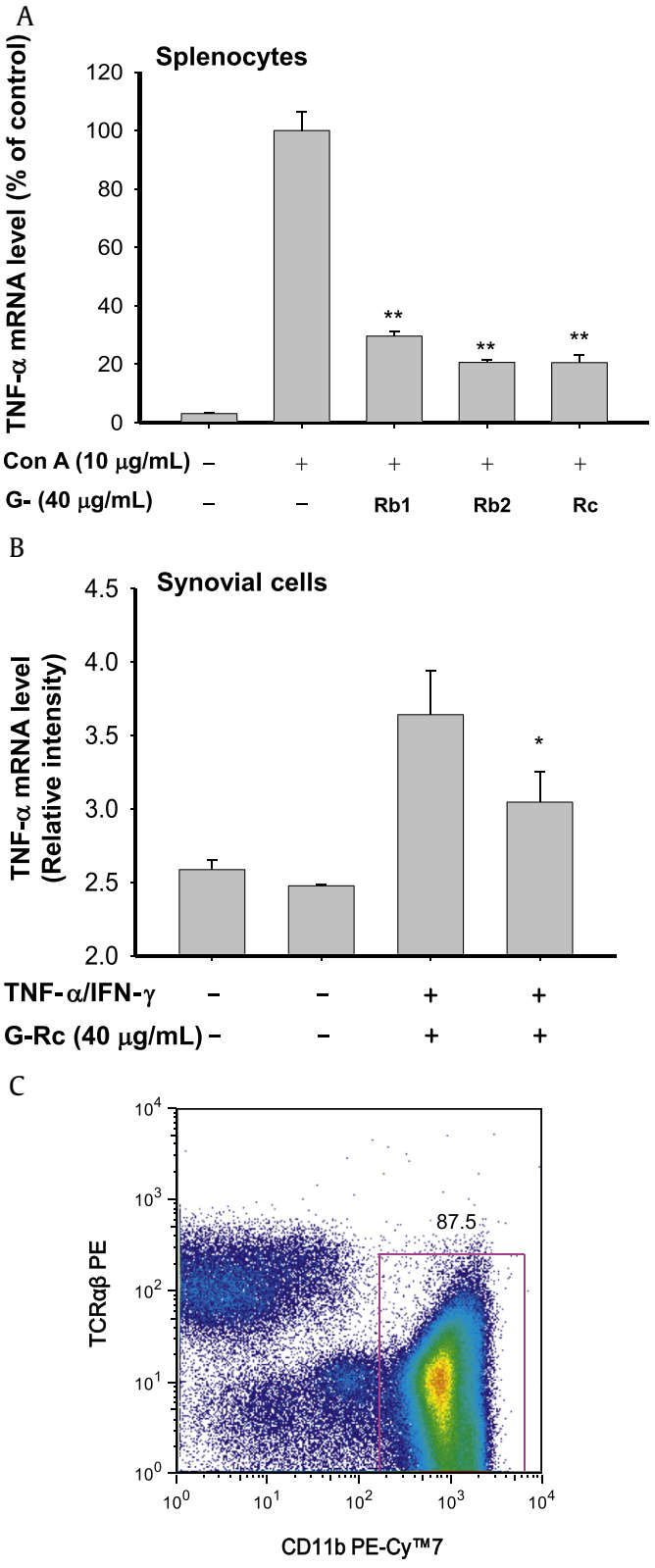

Fig. 1. Effect of ginsenoside-Rc (G-Rc) on tumor necrosis factor (TNF)-α mRNA expression in splenocytes and synovial cells. (A, B) Splenocytes (5×10^6 cells/mL) or synovial cells were incubated with G-Rc in the presence or absence of concanavalin A (10 μg/mL) or TNF-α/interferon-γ (10 ng/mL each) for 6 h. The levels of TNF-α and GAPDH mRNA were determined by real-time polymerase chain reaction. (C) Flow cytometric analysis of macrophages isolated from the synovial fluid of a patient with rheumatoid arthritis. Cells (5×10^6 cells/mL) from the synovial fluid were prepared and costained with fluorochrome-conjugated CD11b and TCRαβ. $^*p < 0.05$ and $^{**}p < 0.01$ compared with the control group.

could block the generation of TNF-α in splenic lymphocytes. We observed that G-Rc, as well as other diol-type ginsenosides (G-Rb1 and G-Rb2), strongly inhibited the expression of TNF-α in Con A-treated splenocytes (Fig. 1A). These results suggest that G-Rc is the constituent of ginseng that mediates its strong anti-inflammatory activity. Importantly, G-Rc was nontoxic at the concentrations used here (data not shown). As predicted, G-Rc also significantly suppressed TNF-α expression in synovial cells (Fig. 1B), which were 87.5% macrophages according to flow cytometric analysis (Fig. 1C). The anti-inflammatory activity of G-Rc prompted us to evaluate its mechanisms of action further.

3.2. Effect of G-Rc on AP-1 and NF-κB activation

To determine which transcription factors are regulated by G-Rc, we analyzed the nuclear fractions of LPS-treated macrophage-like RAW264.7 cells by Western blotting. Time-course analyses were used to determine the levels of translocation for each transcription factor so that the optimal time points could be analyzed. G-Rc suppressed the nuclear translocation of phospho-ATF-2 and phospho-FRA-1 (Fig. 2A), whereas the translocation of p65 at its peak time points (30 and 60 min) was not decreased by G-Rc treatment (data not shown). These results indicate that G-Rc regulates the expression of the proinflammatory cytokine TNF-α, which is produced by macrophages, by suppressing AP-1 activation.

Since G-Rc suppressed ATF-2 phosphorylation, we next investigated which upstream enzyme is targeted by G-Rc. Unexpectedly, G-Rc did not prevent MAPK phosphorylation (Fig. 2B), implying that G-Rc does not inhibit the kinase upstream of MAPK. Since p38 is known to phosphorylate ATF-2 [21], we next used a p38 kinase assay kit to evaluate if G-Rc directly blocks p38 kinase activity. As expected, G-Rc treatment markedly suppressed ATF-2 phosphorylation (Fig. 2C), suggesting that p38 is a target of G-Rc.

3.3. Effect of G-Rc on IRF-3 activation

Since IRF-3 is another important transcription factor that affects the expression of proinflammatory cytokines, we next investigated whether G-Rc suppresses IRF-3 activation. Of all the tested ginsenosides, G-Rc most strongly inhibited IRF-3-driven luciferase expression (Fig. 3A). Furthermore, LPS-mediated activation of IRF-3 in RAW264.7 cells was also decreased by G-Rc in our reporter assay (Fig. 3B). In agreement with these findings, the level of translocated p-IRF-3 was reduced by a 5-min treatment with G-Rc (Fig. 3C).

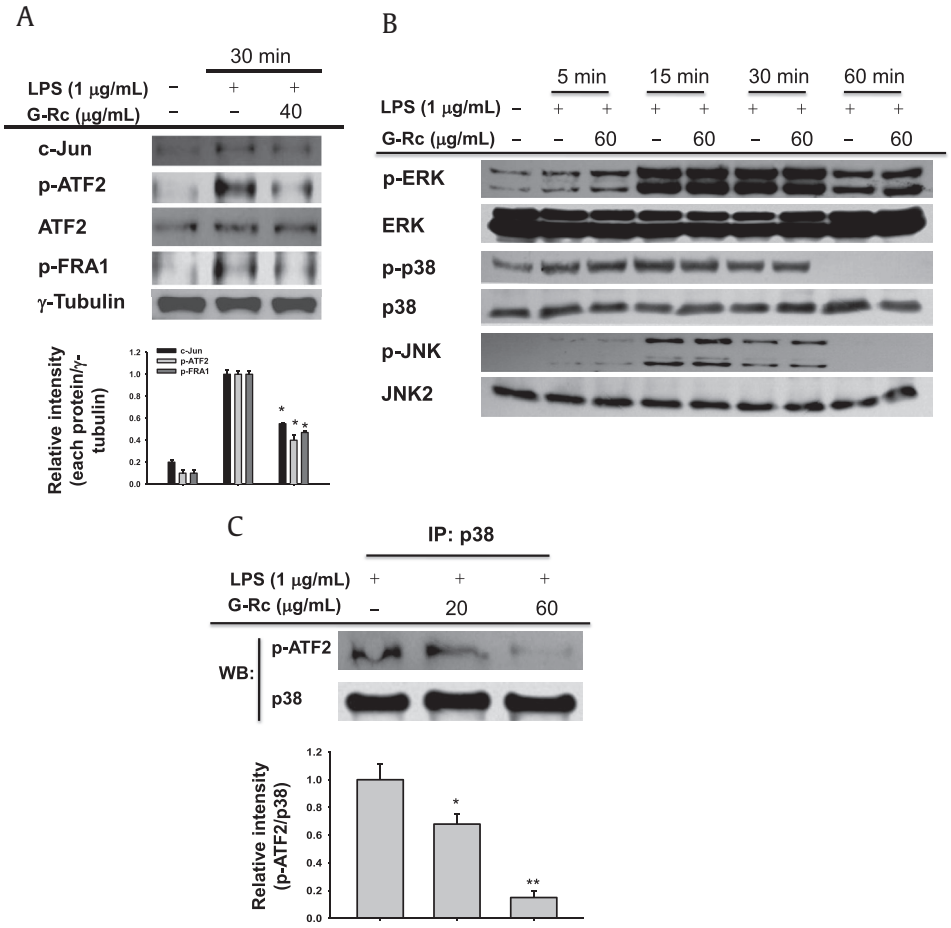

Fig. 2. Effects of ginsenoside-Rc (G-Rc) on activator protein-1 and nuclear factor-κB activation in RAW264.7 cells. (A) RAW264.7 cells (5×10^6 cells/mL) were incubated with G-Rc in the presence or absence of LPS (1 μg/mL) for 30 min. Nuclear fractions were prepared and the levels of the translocated total and phospho-forms of various transcription factors (c-Jun, ATF-2, FRA-1, and γ-tubulin) were analyzed by immunoblotting. (B) RAW264.7 cells (5×10^6 cells/mL) were incubated with G-Rc in the presence or absence of lipopolysaccharide (LPS; 1 μg/mL) for the indicated times. After preparing total lysates, the total and phospho-forms of ERK, JNK, and p38 were analyzed by immunoblotting. (C) An *in vitro* kinase assay was performed using G-Rc, purified ATF-2 substrate, and p38 enzyme. The p38 was immunoprecipitated from RAW264.7 cells that had been treated with LPS for 30 min. The kinase activity was determined by measuring the levels of phospho-ATF-2 with a phospho-specific antibody. Relativeintensities were calculated by normalizing to the total levels using the DNR Bio-imaging system. Kinase activity in the control reaction (LPS alone) was set to 1. Data are presented as means ± standard deviation of one biological experiment performed with six technical replicates ($n = 6$). $^*p < 0.05$ and $^{**}p < 0.01$ compared with the control group.

Since IRF-3 phosphorylation requires the activation of several upstream enzymes such as IKKε and TBK1, we next determined if G-Rc inhibits these enzymes. LPS-treated RAW264.7 cells and TBK1-transfected HEK293 cells were used to test this hypothesis. As shown in the left panel of Fig. 3D, G-Rc significantly blocked the LPS-induced phosphorylation of TBK1 at 5 min and 15 min post-stimulation. This inhibitory activity of G-Rc (40 μg/mL and 60 μg/mL) was also observed under conditions in which TRAM and TBK1 were co-overexpressed (Fig. 3D, right panel), suggesting that a TRAM-induced signaling event upstream of TBK1 activation is

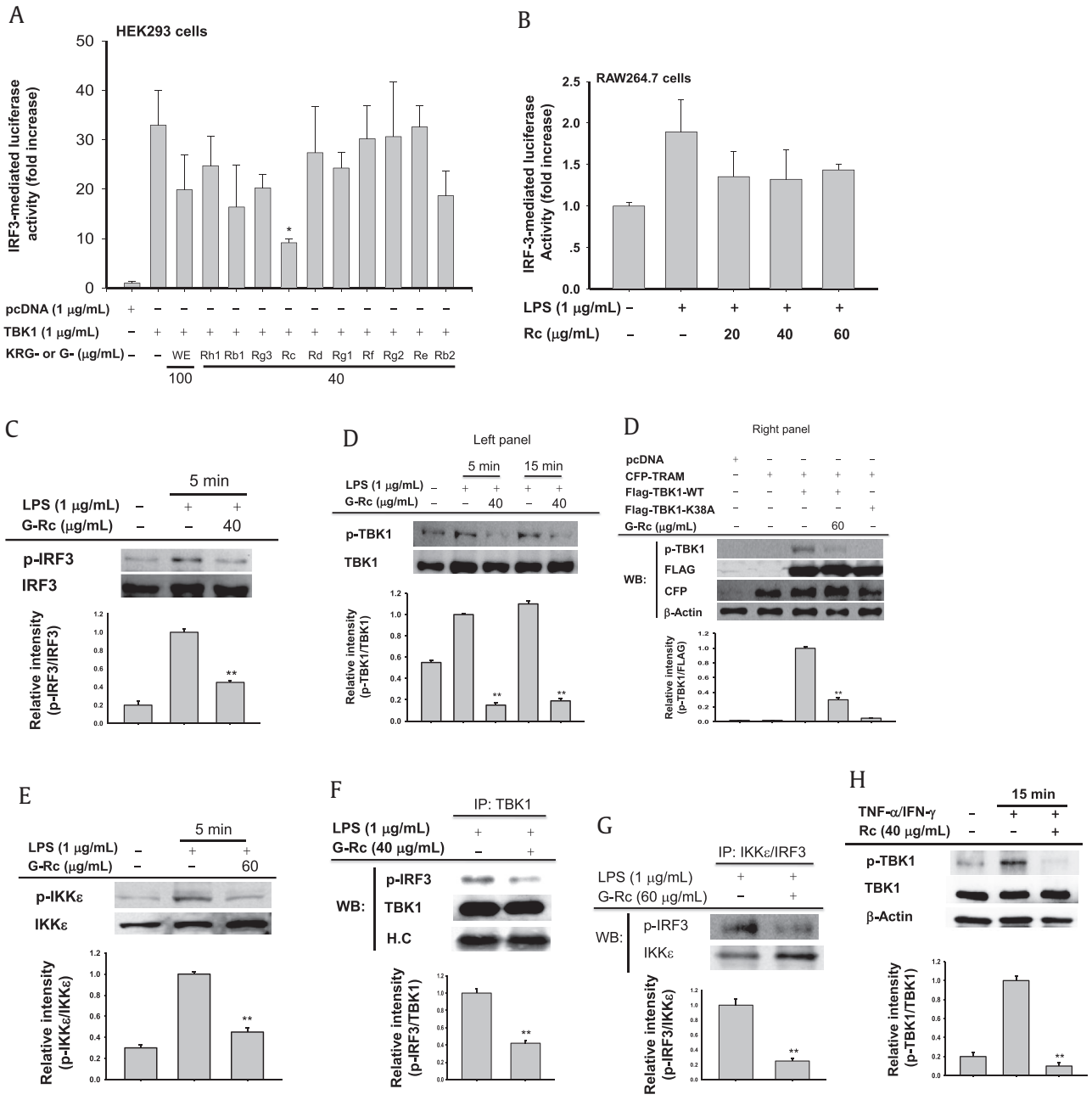

Fig. 3. Effect of ginsenoside-Rc (G-Rc) on interferon regulating factor (IRF)-3 activation in RAW264.7 and HEK293 cells. (A, B) The promoter binding activity of IRF-3 in the presence or absence of TANK-binding kinase 1 (TBK1) or lipopolysaccharide (LPS) was determined by a luciferase reporter assay. HEK293 or RAW264.7 cells (5×10^6 cells/mL) were treated with various ginsenosides, including G-Rc and KRG-WE, and the levels of luciferase were measured. (C) RAW264.7 cells (5×10^6 cells/mL) were incubated with G-Rc in the presence or absence of LPS (1 μg/mL) for 5 min. Nuclear fractions were prepared and the levels of the translocated total and phospho-forms of IRF-3 were analyzed by immunoblotting. (D, left panel and E) RAW264.7 cells (5×10^6 cells/mL) were incubated with G-Rc in the presence or absence of LPS (1 μg/mL) for the indicated times. Total cell lysates were prepared and the total and phospho-forms of TBK1 and IκB kinase ε (IKKε) were analyzed by immunoblotting. (D, right panel) HEK293 cells were transfected with CFP-TRAM and TBK1 constructs (wild type or K38A) and incubated for 12 h. After an additional treatment with G-Rc for 24 h, cell lysates were prepared. The phospho- and total levels of TBK1, FLAG, and CFP were assessed by immunoblotting. (F and G) An *in vitro* kinase assay was performed using G-Rc, IRF-3 substrate, and TBK1 or IKKε enzyme. The IRF-3 substrate had been immunoprecipitated from RAW264.7 cells (5×10^6 cells/mL), whereas the enzymes had been immunoprecipitated from RAW264.7 cells treated with LPS for 30 min. Kinase activities were determined by measuring the levels of phospho-IRF-3 with a phospho-specific antibody. (H) Synovial cells (5×10^6 cells/mL) were incubated with G-Rc in the presence or absence of tumor necrosis factor-α/interferon-γ (10 ng/mL each) for 15 min. Total lysates were prepared and the total and phospho-forms of TBK1 were analyzed by immunoblotting. Intensities were calculated relative to the total levels using the DNR Bio-imaging system. The intensity in the control group (LPS alone) was set to 1. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control group.

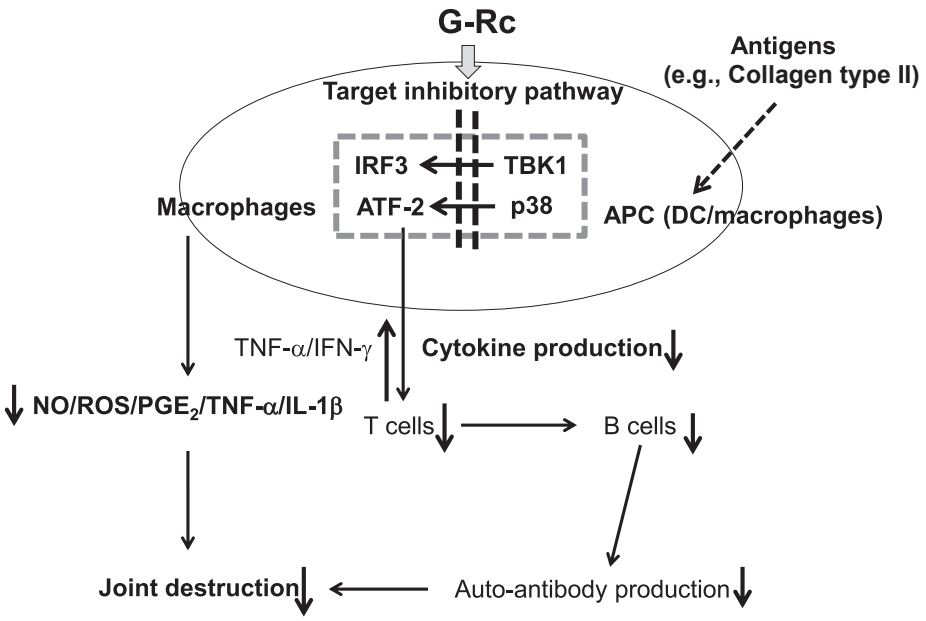

Fig. 4. Putative mechanism of ginsenoside-Rc (G-Rc)-mediated anti-inflammatory activity in arthritis. G-Rc is proposed to simultaneously target both the TANK-binding kinase 1/IκB kinase ε/interferon regulating factor 3 and p38/ATF-2-mediated inflammatory pathways.

targeted by G-Rc. We also investigated whether G-Rc inhibits signaling downstream from TBK1 activation. To this end, we assessed the levels of phosphorylated IKKε, a downstream kinase activated by TBK1 [22], and the integrity of the TBK1/IKKε/IRF3 complex by immunoprecipitation and immunoblotting, respectively. G-Rc inhibited the phosphorylation of IKKε in a manner consistent with its effects on TBK1 (Fig. 3E). Furthermore, G-Rc decreased the formation of the TBK1/IKKε/AKT/IRF-3 signaling complex (Fig. 3F). Finally, we examined the ability of G-Rc to suppress TBK1 phosphorylation in synovial cells. As shown in Fig. 3H, TBK1 phosphorylation induced by cotreatment with TNF-α and IFN-γ was remarkably reduced by a 15-min treatment with G-Rc. This finding suggests that G-Rc-mediated inhibition of TBK1 and IKKε phosphorylation affects the proinflammatory signaling cascade required for IRF-3 activation in macrophages.

4. Discussion

Ginseng has long been used as a medicinal herb to prevent or treat various diseases such as diabetes and cancer, as well as neurodegenerative diseases. Although ginseng has been used traditionally for centuries, no ginseng-derived components have been identified for curative purposes. Therefore, in this study we investigated the potential of G-Rc as an antiarthritic agent.

In our analysis, G-Rc displayed significant anti-inflammatory activity in splenocytes and synovial cells that were stimulated with Con A and TNF-α/IFN-γ (Fig. 1). Moreover, we have clearly demonstrated that TBK1/IRF-3 and p38/ATF-2 are targets of G-Rc (Figs. 2 and 3), strongly suggesting that these proteins are important pharmacological targets of G-Rc. Indeed, pivotal roles for TBK1 and p38 have been reported by several groups, and these proteins were subsequently proposed as putative therapeutic targets for the treatment of arthritis and other inflammatory diseases [23,24]. In particular, TBK1 is known to be an important enzyme regulating virus-induced inflammatory symptoms [25]. Our recent study found that inhibitory plant extract (ethanol extract of *Dryopteris crassirhizoma*) on this enzyme is able to display curative activity against gastritis symptoms stimulated by HCl/EtOH [26]. In case of p38, it was reported that this enzyme is critical in managing various inflammatory diseases such as asthma and inflammatory bowel disease [27,28]. Therefore, based on previous reports, inhibitory properties of G-Rc against the activation of TBK1 and p38 will be expanded to be applied for the treatment of various inflammatory diseases. Indeed, broad-spectrum therapeutic activities of ginseng toward various inflammatory diseases might be in part contributed to the pharmacological activity of G-Rc.

In conclusion, here we have demonstrated that the protopanaxadiol-type ginseng derivative G-Rc reduces inflammatory responses by suppressing the TBK1/IRF-3 and p38/ATF-2 pathways. These findings are summarized in Fig. 4. Moreover, we identified the molecular targets of protopanaxadiol-type ginsenosides, thus shedding light on the mechanism by which G-Rc exerts its antiarthritic activity.

Conflicts of interest

The authors have no financial conflicts of interest.

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