

## Ginsenoside Rg1 attenuates adjuvant-induced arthritis in rats via modulation of PPAR- $\gamma$ /NF- $\kappa$ B signal pathway

Leiming Zhang<sup>1,\*</sup>, Maojing Zhu<sup>1,\*</sup>, Minmin Li<sup>1,\*</sup>, Yuan Du<sup>1</sup>, Sijin Duan<sup>1</sup>, Yanan Huang<sup>1</sup>, Yongying Lu<sup>1</sup>, Jianqiao Zhang<sup>1</sup>, Tian Wang<sup>1</sup> and Fenghua Fu<sup>1</sup>

<sup>1</sup>School of Pharmacy, Key Laboratory of Molecular Pharmacology and Drug Evaluation (Yantai University), Ministry of Education, Collaborative Innovation Center of Advanced Drug Delivery System and Biotech Drugs in Universities of Shandong, Yantai University, Yantai, P.R. China

\*These authors contributed equally to this work

**Correspondence to:** Leiming Zhang, **email:** zhangleiming2009@126.com  
Fenghua Fu, **email:** fufenghua@sohu.com

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### ABSTRACT

**Ginsenoside Rg1, the main active compound in *Panax ginseng*, has already been shown to have anti-inflammatory effects. However, the protective effects of Rg1 on rheumatoid arthritis (RA) remain unclear. The aim of the present study was to investigate the effects and mechanisms of Rg1 on adjuvant-induced arthritis (AIA) in rats. AIA rats were given Rg1 at doses of 5, 10, and 20 mg/kg intraperitoneally for 14 days to observe the anti-arthritic effects. The results showed that Rg1 significantly alleviated joint swelling and injuries. Rg1 can also significantly reduce the level of TNF- $\alpha$  and IL-6, increase PPAR- $\gamma$  protein expression, inhibit I $\kappa$ B $\alpha$  phosphorylation and NF- $\kappa$ B nuclear translocation in the inflammatory joints of AIA rats and RAW264.7 cells stimulated by lipopolysaccharide (LPS). The results indicate that Rg1 has therapeutic effects on AIA rats, and the mechanism might be associated with its anti-inflammatory effects by up-regulating PPAR- $\gamma$  and subsequent inhibition of NF- $\kappa$ B signal pathway.**

### INTRODUCTION

Rheumatoid arthritis (RA) is a chronic destructive disease of the joints. It is characterized by chronic proliferative synovitis, infiltration of inflammatory cells into the synovial tissue of joints, and cartilage destruction. It is here reported that RA affects around 0.5–1% of adults in developed countries with 5–50 per 100,000 new cases each year [1, 2].

Non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are conventional anti-inflammatory drugs that have been widely used in the treatment of RA. However, in response to recent evidence, NSAIDs have lost their prominent role as first-line treatment because of concerns about their limited effectiveness, inability to alter the long-term course of disease or gastrointestinal or cardiac toxic effects [3, 4]. The adverse effects of long-term glucocorticoid treatment are common and include immunosuppression, osteoporosis, and metabolic disorders [5, 6]. In recent years, biological agents especially tumor

necrosis factor (TNF) inhibitors are widely used in the treatment of RA [7]. However, the infection rate and high costs restrict the prescription of biological agents [8, 9].

In the past few years, PPARs have also emerged as key regulators of inflammatory and immune responses, opening up a new area for the development of therapeutic drugs useful to the treatment of chronic inflammatory diseases such as atherosclerosis, obesity-induced insulin resistance and arthritis [10, 11]. Cumulative experimental evidence has proved that natural and synthetic PPAR- $\gamma$  ligands can inhibit major signaling pathways of inflammation such as NF- $\kappa$ B, which underlies many aspects of the anti-inflammatory effect of PPAR- $\gamma$ , and reduce the synthesis of cartilage catabolic factors responsible for articular cartilage degradation in arthritis. PPAR- $\gamma$  is now considered a probable molecular target for the treatment of chronic inflammatory diseases, including arthritis [12].

Plants used in traditional medicine currently provide a rich source of candidate drugs for the treatment

of chronic inflammatory diseases. Ginseng, the root of *Panax ginseng* C.A. Meyer, is now one of the most extensively used alternative medicines throughout the world [13–15]. The molecular agents responsible for ginseng's actions are ginsenosides [16, 17], which are triterpene saponins that have rigid steroidal skeletons with sugar moieties [18]. Rg1 (Figure 1), the active ingredient of *Panax ginseng* [19], has already been shown to have anti-inflammatory effects. It can inhibit lipopolysaccharide (LPS)-induced sepsis by competitive binding to Toll-like receptor 4 (TLR4), which was found to improve survival in a murine model [20, 21]. It can also ameliorate chronic inflammatory disease such as colitis by inhibiting the binding of LPS to TLR4 on macrophages [22]. Recently, studies reported that Rg1 can suppress inflammation by activating PPAR- $\gamma$ /HO-1 in rat model of cerebral ischemia-reperfusion injury [23], it can also inhibit the TLR4-NF- $\kappa$ B signaling pathway in the murine neuroglial cell line [24].

However, the protective effects and mechanism of ginsenoside Rg1 on RA are still poorly elucidated. In the present study, we determined whether Rg1 had protective effects on complete Freund's adjuvant (CFA)-induced arthritis in rats. We also carried out an investigation into the mechanisms underlying the therapeutic effect of Rg1 on CFA-induced arthritis, which involve PPAR- $\gamma$ /NF- $\kappa$ B signaling.

## RESULTS

### Effects of Rg1 on paw swelling in AIA rats

Adjuvant-injected animals exhibited marked secondary arthritis of the left paw from day 14. DEX

2.5 mg/kg notably inhibited paw swelling at days 17, 21, 25, and 28. Rg1 10 mg/kg and 20 mg/kg notably inhibited paw swelling at days 21, 25, and 28. Rg1 5 mg/kg produced notable inhibition of paw swelling at days 28 (Figure 2A).

### Effects of Rg1 on arthritis index in AIA rats

Fully developed arthritis including redness and swollen paws were observed from day 14. Rats in the AIA group showed severe inflammation with marked lesions and deformity. Secondary lesions such as inflammation of the left paw and nodule formation were also observed. DEX 2.5 mg/kg produced notable inhibition of the arthritis index at days 21, 25, and 28. Rg1 20 mg/kg produced notable inhibition of the arthritis index at days 25 and 28. Rg1 10 mg/kg produced notable inhibition at day 28 (Figure 2B).

### Effects of Rg1 on radiography of the joints of AIA rats

Compared with the control group rats, bone matrix resorption, osteophyte formation and bone erosion can be observed in the joints of AIA group rats. After Rg1 treatment at doses of 10 and 20 mg/kg, bone erosion and the degree of arthritis were significantly reduced (Figure 3).

### Effects of Rg1 on histopathology of the joints of AIA rats

The ankle joints of rats in the control group showed intact cartilage, bone, and synovium. Histologic evaluation

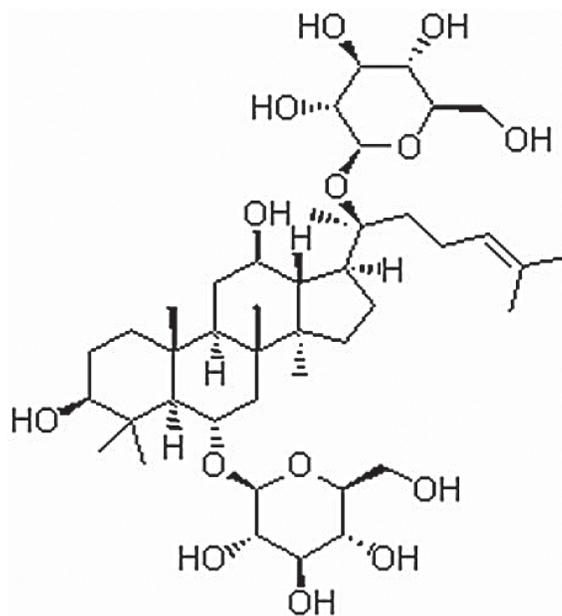


Figure 1: Chemical structure of Rg1.

of the ankle joints in AIA group revealed adipose marrow mesenchymal cells with embedded inflammatory cells, synovium with multiple giant cells, massive periosteal proliferation, periarticular inflammation, and pannus formation. In the rats that received Rg1 at doses of 10 mg/kg and 20 mg/kg, the degree of arthritis was significantly reduced. This indicated a marked decrease of synovial inflammatory cell infiltrate, synovial lining hyperplasia, and bone destruction (Figure 4).

### Effects of Rg1 on TNF- $\alpha$ and IL-6 levels *in vivo* and *in vitro*

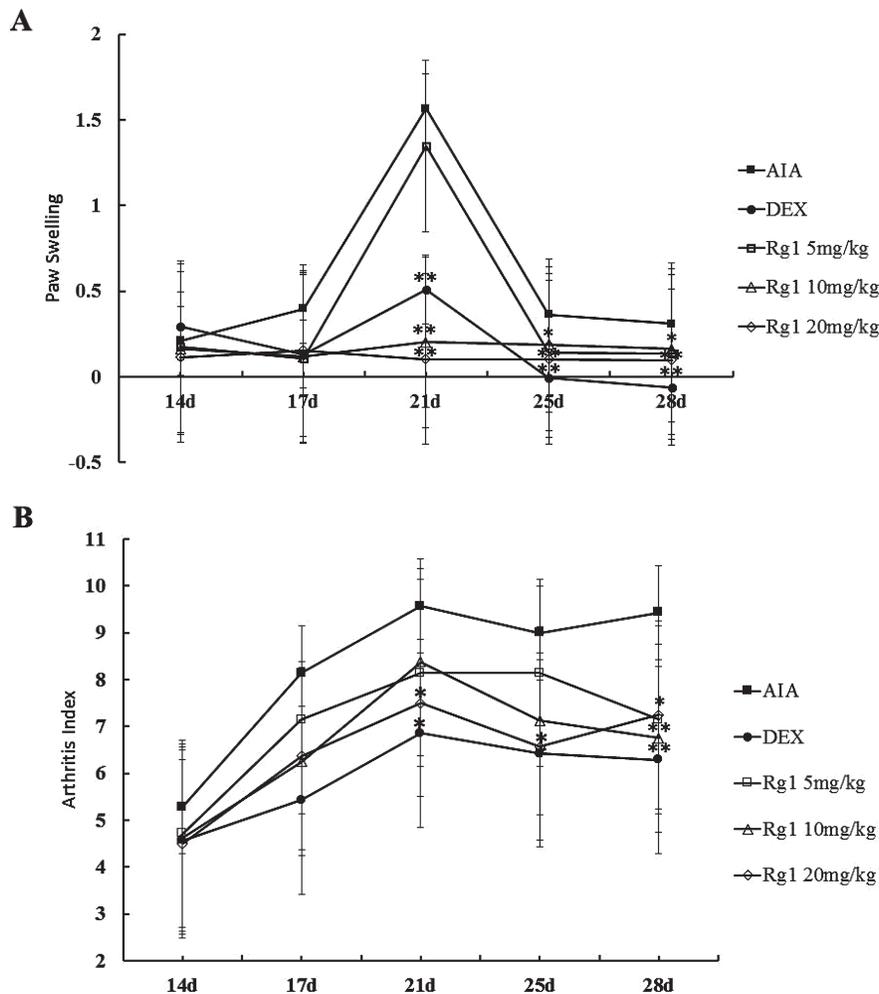
The concentration of TNF- $\alpha$  and IL-6 was significantly higher in AIA rats than in control rats ( $P < 0.01$ ). However, Rg1 appeared to decrease the TNF- $\alpha$  and IL-6 levels significantly ( $P < 0.05$  or  $P < 0.01$ ). The same results were observed in RAW264.7 cells treated with LPS (Figure 5).

### Effects of Rg1 on p-I $\kappa$ B $\alpha$ , p-p65, and PPAR- $\gamma$ protein levels *in vivo* and *in vitro*

In the inflamed joints of AIA rats and RAW264.7 cells stimulated by LPS, the level of p-I $\kappa$ B $\alpha$  and p-p65 were significantly higher than in controls and PPAR- $\gamma$  levels were significantly lower. Treatment with Rg1 *in vivo* and *in vitro* inhibited I $\kappa$ B $\alpha$  phosphorylation, reduced NF- $\kappa$ B nuclear translocation and upregulated PPAR- $\gamma$  expression (Figure 6, Figure 7).

### Effects of Rg1 on index of thymus and spleen in AIA rats

The index of thymus and spleen in DEX administration rats was markedly lower than in AIA rats ( $P < 0.01$ ). However, rats given Rg1 showed no significant differences from AIA rats (Figure 8).

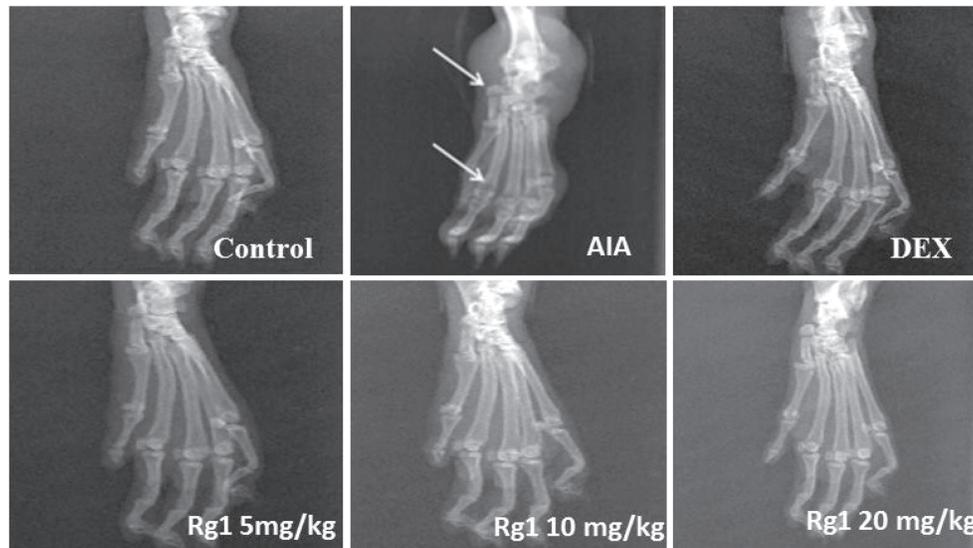


**Figure 2: Effects of Rg1 on paw swelling and the arthritis index in AIA rats.** SD rats were immunized with complete Freund's adjuvant (CFA). The rats were then given Rg1 or DEX from day 15 to day 28. The paw swelling (A) and arthritis indexes (B) were evaluated on days 14, 17, 21, 25, and 28. Data are expressed as mean  $\pm$  SD ( $n = 7$  for AIA group, 7 for DEX group, 7 for Rg1 5 mg/kg group, 8 for the Rg1 10 mg/kg group, 8 for Rg1 20 mg/kg group). \* $P < 0.05$  and \*\* $P < 0.01$  versus AIA.

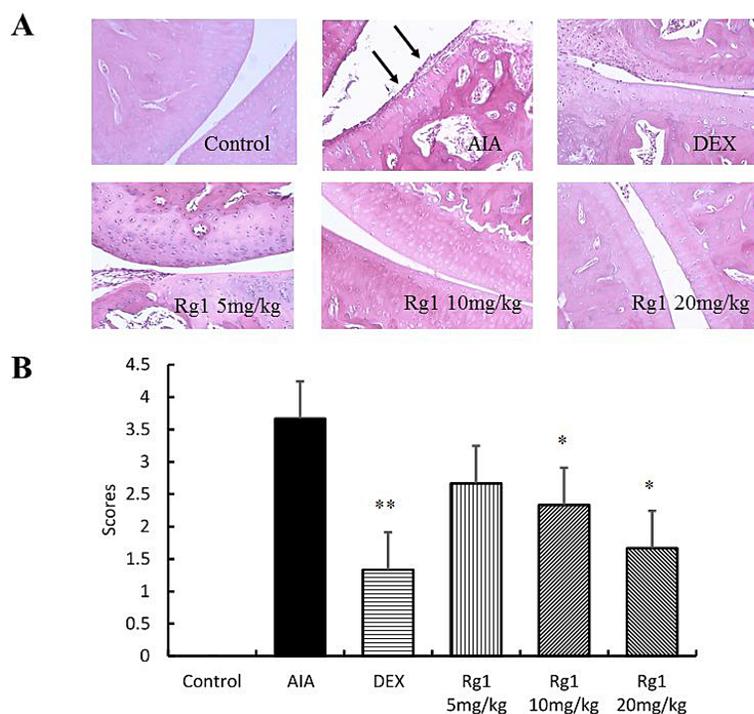
## DISCUSSION

RA is an autoimmune and chronic inflammatory disease; it is pathologically characterized by synovial hyperplasia, inflammation, and angiogenesis in synovial membrane that leads to pannus formation followed by severe cartilage and bone destruction [25].

Adjuvant-induced arthritis is a commonly used animal model for study of the pathogenesis of human RA and also for finding novel drugs for RA management [26]. Complete Freund's-adjuvant-induced arthritis is an important animal model of screening anti-inflammatory drugs due to its histological and immunological similarity to RA in human patients [27].



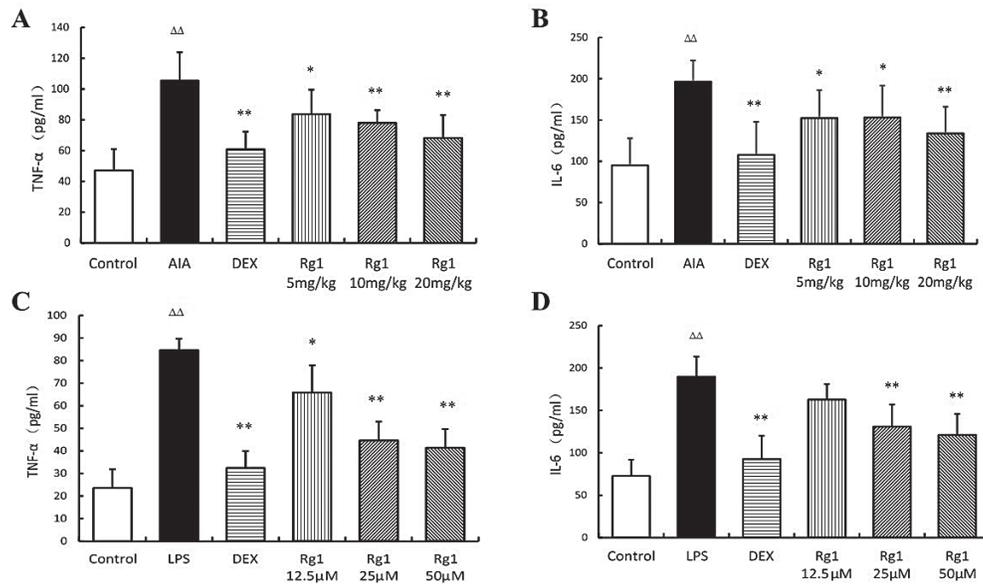
**Figure 3: Effects of Rg1 on radiography of the joints of AIA rats.** SD rats were immunized with complete Freund's adjuvant (CFA). The rats were then given Rg1 or DEX from day 15 to day 28. On day 28, rats were anesthetized with chloral hydrate, then placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiographic analysis were performed by X-ray machine.



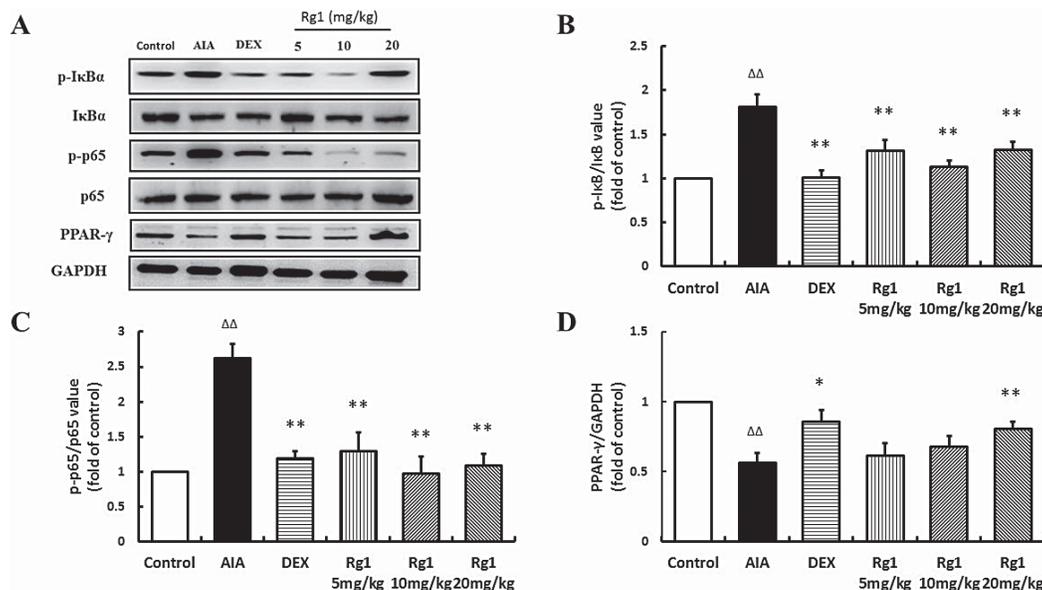
**Figure 4: Effects of Rg1 on joint histopathology of AIA rats.** (A) Representative histopathologies of ankle joints stained with H-E (original magnification, 200×). (B) Histologic scores of the joint were evaluated. Data are expressed as mean ± SD from 3 animals for each group. \* $P < 0.05$  and \*\* $P < 0.01$  versus AIA;  $\Delta P < 0.01$  versus control.

In the present study, adjuvant-injected animals exhibited marked secondary arthritis. Histologic evaluation of the ankle joints revealed massive periosteal proliferation, periarticular inflammation, and pannus formation. Rg1 significantly reduced paw swelling, synovial inflammatory cell infiltration, synovial lining hyperplasia, and bone destruction, which indicates Rg1 has good anti-arthritic effects.

Inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, have proangiogenic activity that promotes synovial inflammation by increasing the expression of vascular growth factors, endothelial mitogenesis, and induction of matrix metalloproteinases [28]. They also supply nutrients and oxygen to increase inflammatory cell mass and facilitate the infiltration of inflammatory cells into the synovial tissues to enhance the inflammatory



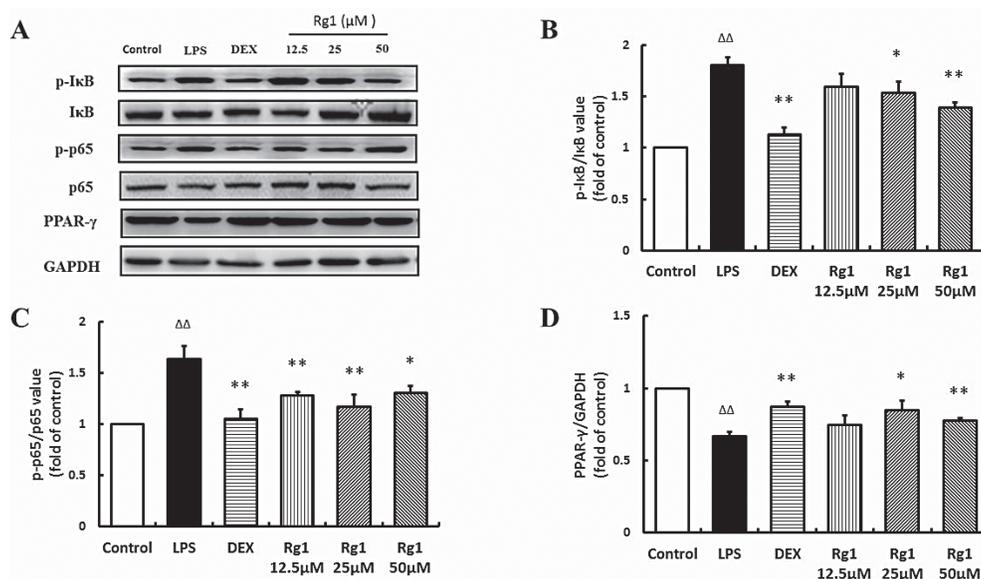
**Figure 5: Effects of Rg1 on TNF- $\alpha$  and IL-6 levels *in vivo* and *in vitro*.** (A–B) Effects of Rg1 on TNF- $\alpha$  and IL-6 levels in AIA rats ( $n = 7$  for each group); (C–D) Effects of Rg1 on TNF- $\alpha$  and IL-6 levels in RAW264.7 cells treated with LPS ( $n = 5$  for each group). Data are expressed as mean  $\pm$  SD. \* $P < 0.05$  and \*\* $P < 0.01$  versus AIA or LPS;  $\Delta\Delta P < 0.01$  versus control.



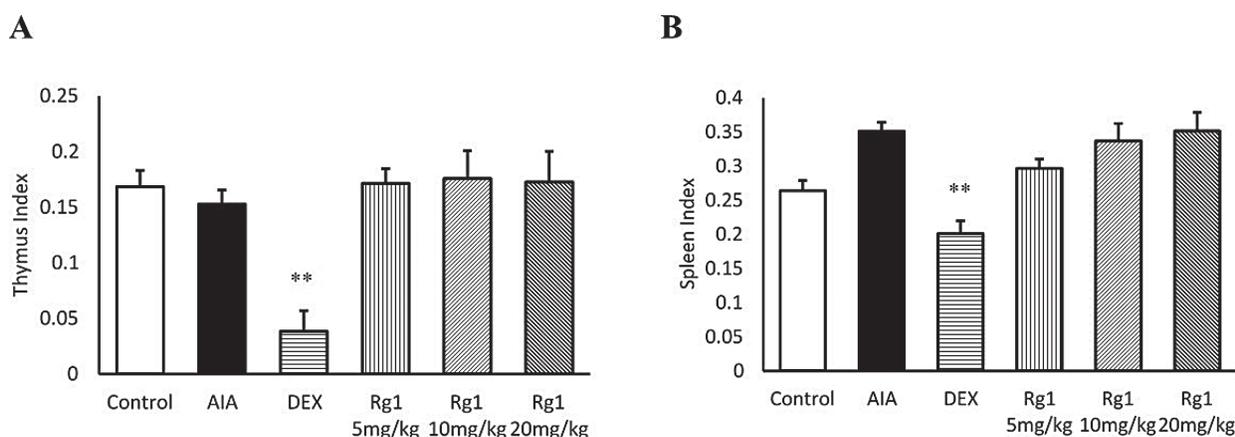
**Figure 6: Effects of Rg1 on p-I $\kappa$ B $\alpha$ , p-p65, and PPAR- $\gamma$  protein expression in AIA rats.** (A) Western blot bands; (B) p-I $\kappa$ B $\alpha$  expression; (C) p-p65 expression; (D) PPAR- $\gamma$  protein expression. Data are expressed as mean  $\pm$  SD,  $n = 3$  for each group. \* $P < 0.05$  and \*\* $P < 0.01$  versus AIA;  $\Delta\Delta P < 0.01$  versus control.

response [29]. In the present study, both DEX and Rg1 significantly inhibited TNF- $\alpha$  and IL-6 concentration in serum of AIA rats and inflammatory cytokine secretion by RAW 264.7 macrophage cells *in vitro*. However, glucocorticoids, as conventional anti-inflammatory drugs, have adverse effects of long-term glucocorticoid treatment including immunosuppression. In the present study, the index of thymus and spleen in DEX administration rats was markedly lower than in AIA rats. But Rg1 showed no significant immunosuppressive effects compared with DEX. The results indicate that Rg1 has good anti-inflammatory effects but no immunosuppressive effects. Currently, the precise mechanisms by which Rg1 exerts its anti-inflammatory effects on AIA rats remain unclear.

Many signal transduction pathways are activated in RA synovial tissue, and the NF- $\kappa$ B pathway is one of the most important to the pathogenesis of RA [30]. The NF- $\kappa$ B family contains 5 related transcription factors. These normally bind to I $\kappa$ B in the cytoplasm. Stress, inflammatory cytokines, and microbial products result in I $\kappa$ B phosphorylation and degradation, allowing NF- $\kappa$ B to translocate to the nucleus and regulate gene transcription. NF- $\kappa$ B plays crucial roles in the regulation of inflammation and immune responses, and inappropriate NF- $\kappa$ B activity has been linked to many autoimmune and inflammatory diseases, including RA [31]. NF- $\kappa$ B is induced by many stimuli, including TNF- $\alpha$  and IL-6, forming a positive regulatory cycle that may amplify



**Figure 7: Effects of Rg1 on p-IκB $\alpha$ , p-p65, and PPAR- $\gamma$  protein expression in RAW264.7 cells treated with LPS.** (A) Western blot bands; (B) p-IκB $\alpha$  expression; (C) p-p65 expression; (D) PPAR- $\gamma$  protein expression. Data are expressed as mean  $\pm$  SD,  $n = 3$  for each group. \* $P < 0.05$  and \*\* $P < 0.01$  versus LPS;  $\Delta\Delta P < 0.01$  versus control.



**Figure 8: Rg1 and DEX on the thymus and spleen index of AIA rats.** The index of thymus (A) and spleen (B) were expressed as the ratio (mg/g) of thymus and spleen wet weight versus body weight, respectively. Data are expressed as mean  $\pm$  SD from 7 animals for each group. \*\* $P < 0.01$  versus AIA.

and maintain RA disease process by producing more inflammatory cytokines. NF- $\kappa$ B and the enzymes involved in its activation may be suitable targets for anti-arthritis treatment [32].

PPAR- $\gamma$  is a transcription factor that acts as an influential pleiotropic regulator of anti-inflammation, antioxidant, and phagocyte mediated cleanup processes. In various experimental models of arthritis, PPAR- $\gamma$  activation has been shown to exhibit an anti-inflammatory effect and reduce the severity of the disease [33, 34]. Apart from its direct genomic effect, PPAR- $\gamma$  has been found to interact negatively with other transcription factors such as NF- $\kappa$ B, which underlies many aspects of the anti-inflammatory effect of PPAR- $\gamma$  [35, 36]. Many reports have documented that activated PPAR- $\gamma$  can inhibit NF- $\kappa$ B signaling pathways to protect against RA [37, 38]. These findings show that treatment with Rg1 *in vivo* and *in vitro* can upregulate PPAR- $\gamma$  expression, inhibit I $\kappa$ B $\alpha$  phosphorylation, and reduce NF- $\kappa$ B nuclear translocation, which has suggested that the anti-inflammatory effects of Rg1 in AIA rats are associated with its ability to modulate PPAR- $\gamma$  and NF- $\kappa$ B transcription factors.

In summary, Rg1 has therapeutic effects on adjuvant-induced arthritis in rats. The mechanism might be associated with its anti-inflammatory effects via upregulation of PPAR- $\gamma$  expression and subsequent inhibition of NF- $\kappa$ B signal pathway.

## MATERIALS AND METHODS

### Animals

Male Sprague–Dawley (SD) rats (weight, 150–170 g) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All animals were acclimated for at least 1 week at temperature of  $24 \pm 1^\circ\text{C}$  and humidity of  $55 \pm 5\%$ . The animals were maintained with free access to standard diet and tap water. The experimental procedures were approved by the Animal Ethics Committee of Yantai University.

### Reagents and drugs

Ginsenoside Rg1 (Formula:  $\text{C}_{42}\text{H}_{72}\text{O}_{14}$ , Figure 1) was purchased from Nanjing Guangrun Biotechnology Co., Ltd. (Nanjing, China) with a purity of  $\geq 99\%$  as determined by HPLC. The dexamethasone sodium phosphate injections were purchased from the Cisen Pharmaceutical Co. Ltd. (China). Complete Freund's adjuvant (CFA) (containing 10 mg/mL of dry, heat-killed *Mycobacterium tuberculosis*) was purchased from Chondrex, Inc. (U.S.). Lipopolysaccharides (LPS) were purchased from Sigma-Aldrich Corporation (U.S.). TNF- $\alpha$  and IL-6 ELISA kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). PPAR- $\gamma$ , I $\kappa$ B $\alpha$ , p-I $\kappa$ B $\alpha$  and NF- $\kappa$ B (p65 and p-p65)

antibodies were obtained from Santa Cruz Biotechnology, Inc. (U.S.). The other chemicals were of analytical grade.

### Induction of adjuvant arthritis

Arthritis was induced by a single intradermal injection of 0.1 ml of CFA (10 mg/ml) emulsion into the right hind metatarsal footpad of SD rats. The control group rats were injected with saline. The day of the first immunization was defined as day 0 [39].

### Drug administration

Rats were divided into 6 groups, including Control, AIA, dexamethasone (DEX, 2.5 mg/kg), Rg1 5 mg/kg, Rg1 10 mg/kg, and Rg1 20 mg/kg (Rg1 doses were selected according to the previous study [20–22]). After the onset of arthritis, rats were given Rg1 and DEX intraperitoneally once daily for 14 days.

### Evaluation of arthritis

To evaluate the severity of arthritis, paw swelling and arthritis indexes were evaluated on days 14, 17, 21, 25, and 28, by 2 observers blind to treatment. Paw swelling was measured with a plethysmometer (YLS-7A, Shandong Academy of Medical Sciences, China). The results were expressed according to the increase in paw volume (mL) calculated by subtracting the basal volume. Inflammation of the 4 paws was graded from 0 to 4 according to previous studies [40]: 0, paws with no swelling or focal redness; 1, swelling of the finger joints; 2, mild swelling of the ankle or wrist joints; 3, severe inflammation of the entire paw; 4, paws with deformity or ankylosis. Each paw was scored separately and the cumulative scores of all 4 paws of each rat served as the polyarthritis index with a maximum value of 16 per rat.

### Radiography

On day 28, rats were anesthetized with chloral hydrate, then placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiographic analysis were performed by X-ray machine.

### Histological examination

After chloral hydrate anesthesia on Day 28, animals were sacrificed by cervical dislocation. Legs were severed under the knee, flayed, and fixed in 10% neutral-buffered formalin for 48 h at room temperature, then decalcified in 5% formic acid and embedded in paraffin. The sections (5  $\mu\text{m}$ ) were stained with hematoxylin and eosin (H-E) and analyzed qualitatively by 2 independent observers in a blind fashion.

The severity of arthritis in the joint was graded from 0 to 4 according to the intensity of lining layer hyperplasia,

mononuclear cell infiltration, and pannus formation, as described previously: 0 = normal ankle joint, 1 = normal synovium with occasional mononuclear cells, 2 = definite arthritis with a few layers of flat to rounded synovial lining cells and scattered mononuclear cells and dense infiltration of mononuclear cells, 3 = clear hyperplasia of the synovium with 3 or more layers of loosely arranged lining cells and dense infiltration with mononuclear cells, 4 = severe synovitis with pannus and erosion of articular cartilage and subchondral bone [41].

### Cytokine determination

Measurement of TNF- $\alpha$  and IL-6 in blood of AIA rats: All rats were sacrificed on day 28 after chloral hydrate anesthesia, and blood was collected from the abdominal aorta for determination of TNF- $\alpha$  and IL-6 levels using ELISA kits according to the manufacturer's instructions.

RAW 264.7 cells culture and cytokine determination: The mouse monocyte-macrophage cell line RAW 264.7 was obtained from the cell library in the Shanghai Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). The cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were kept at 37°C with 5% CO<sub>2</sub> in a fully humidified atmosphere. The medium was routinely changed every day. Cell viability assay: Cells (concentration: 1 × 10<sup>6</sup> cells/ml) were treated with Rg1 at concentrations of 1–100  $\mu$ M. The results showed Rg1 did not exhibit cytotoxicity (data not shown). So this concentration range was used in the following experiments. Cells were treated with LPS (1  $\mu$ g/ml) with or without drugs (Rg1, 12.5  $\mu$ M, 25  $\mu$ M, and 50  $\mu$ M) for 24 h. Then 100  $\mu$ l of the culture supernatant was removed to determine the level of TNF- $\alpha$  and IL-6 using ELISA assay kits according to the manufacturer's instructions.

### Western blot analysis

Tissues of inflamed joints were separated and homogenized on ice in cold lysis buffer (Beyotime, China) plus 1:100 volume of phenylmethyl sulfonyl fluoride (PMSF). RAW 264.7 cells were treated with LPS (1  $\mu$ g/ml) and Rg1 (12.5  $\mu$ M, 25  $\mu$ M, and 50  $\mu$ M) for 24 h and then washed with cold PBS and lysed in ice-cold lysis buffer (Beyotime, China) plus 1:100 volume of PMSF. The supernatant was aliquoted and protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, China). Proteins were segregated by electroblotted and SDS-PAGE into a membrane of nitrocellulose. The blots were probed with antibodies against PPAR- $\gamma$ , I $\kappa$ B $\alpha$ , p-I $\kappa$ B $\alpha$ , NF- $\kappa$ B p65, p-p65 (all antibodies were diluted by 1:1000) at 4°C overnight and later incubated with horseradish peroxidase-conjugated IgG secondary antibody (Beyotime, China; 1:5000 dilution) for 1 h. The expression of every protein was detected by the detection

system of ECL (ChemiDoc™XRS, Bio-Rad, Shanghai, China). The respective bands were quantitated and images were collected utilizing Quantity One Software (Bio-Rad). The fold increase over control was used to express the results [42].

### Index of thymus and spleen

On day 28, the animals were sacrificed via chloral hydrate anesthesia. The thymus and spleen were promptly removed and weighed. The index of thymus and spleen were expressed as the ratio (mg/g) of thymus and spleen wet weight versus body weight, respectively.

### Statistical analysis

The data are expressed as means  $\pm$  SD. Data were analyzed using one-way ANOVA with Bonferroni post-hoc testing for multiple *t*-tests. *P* < 0.05 was considered indicative of statistically significant differences among groups.

### Abbreviations

RA, rheumatoid arthritis; AIA, adjuvant-induced arthritis; DEX, dexamethasone; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6; PPAR- $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; NF- $\kappa$ B, nuclear factor-kappa B; I $\kappa$ B, inhibitor kappa b; LPS, lipopolysaccharide; NSAIDs, non-steroidal anti-inflammatory drugs; TLR4, toll like receptor 4; MAPK, mitogen-activated protein kinase; CFA, complete Freund's adjuvant.

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### CONFLICTS OF INTEREST

The authors state that they have no conflicts of interest.

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