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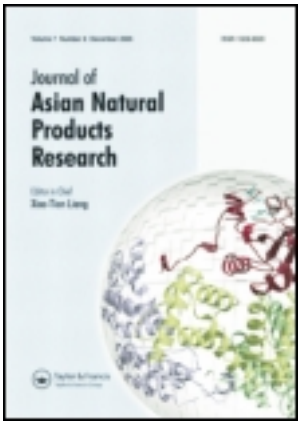

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Glycyrrhetinic acid extracted from *Glycyrrhiza uralensis* Fisch. induces the expression of Toll-like receptor 4 in Ana-1 murine macrophages

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Glycyrrhetinic acid extracted from *Glycyrrhiza uralensis* Fisch. induces the expression of Toll-like receptor 4 in Ana-1 murine macrophages

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Glycyrrhetinic acid (GA) is an active component of licorice root that has long been used as a herbal medicine for the treatment of peptic ulcer, hepatitis, and pulmonary and skin diseases in Asia and Europe. In this study, we analyzed the effect of GA extracted from *Glycyrrhiza uralensis* Fisch. on the expression of Toll-like receptors (TLRs) that play key roles in regulating the innate immune response against invading pathogens. Stimulation of Ana-1 murine macrophages with GA induced a significant dose-dependent expression of TLR-4, and its mRNA expression that increased from 3-h post-treatment was approximately fivefold over the level in the mock-treated cells. No endotoxin contamination contributed to the GA-induced TLR-4 expression, because polymyxin B treatment did not alter the upregulated expression of TLR-4 in GA-treated cells. Several molecules, such as myeloid differentiation factor 88, interferon-β, and interleukin-6, which are involved in the TLR-4 downstream signaling pathway, were upregulated significantly in response to GA stimulation. Our findings demonstrate that GA is able to induce the expression of TLR-4 and activate its downstream signaling pathway.

Keywords: glycyrrhetinic acid; Toll-like receptor 4; Toll-like receptors; innate immunity

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1. Introduction

Licorice roots (*Glycyrrhiza radix*) have long been used as a herbal medicine for the treatment of peptic ulcer, hepatitis, and pulmonary and skin diseases in Asia and Europe [1]. One of the bioactive compounds found in licorice roots is glycyrrhetinic acid (GA, Figure 1), which is derived from glycyrrhizic acid (GL) through the hydrolyzation by glucuronidase of intestinal bacteria, and is absorbed into the blood [2]. Pharmacological studies into the antiviral activities of GA/GL showed that these compounds were able to inhibit the replication of several DNA and RNA viruses that cause respiratory, hepatic, and systemic viral diseases [3]. One of the mechanisms underlying the antiviral effects of GA/GL has been speculated to be the modulation of the antiviral immune response by induction of the expression of interferon (IFN) [4,5].

IFN plays a key role in the antiviral innate immune response, which is activated by several signaling pathways including the Toll-like receptor (TLR) pathway [6]. The TLRs are a family of at least 12 proteins present on the membranes

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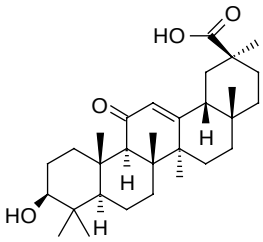

Figure 1. Chemical structure of GA.

of immune cells and collectively recognize lipid, carbohydrate, peptide, and nucleic acid structures widely expressed by different groups of micro-organisms. TLR-2–4, 7, and 9 mainly recognize distinct types of virus-derived nucleic acids and activate signaling cascades that result in the induction of IFN expression [7]. In addition to micro-organisms, some medicinal plants or plant-derived compounds have been showed to trigger the expression of some TLRs and enhance TLR-mediated immune activation [8].

Given that GA/GL has been reported to inhibit virus replication [3] and to induce IFN expression [4,5], we speculated that GA may regulate the expression of TLRs that play a key role in regulating the innate immune response against viral pathogens. In this study, we stimulated a murine macrophage cell line Ana-1 with GA, and found that GA was able to induce the expression of TLR-4 and activate its downstream signaling.

2. Results and discussion

2.1 Detection of the expression of TLRs in Ana-1 murine macrophages

Macrophages express a repertoire of TLRs with functional responses to natural ligands or agonists that play an important role in the TLR-mediated innate immune response. To ensure that Ana-1 murine macrophages are the appropriate cells for analyzing the effect of GA on the expression of TLRs, we examined the constitutive expression of several virus-recognizing TLRs and their downstream genes by a reverse transcription polymerase chain reaction (RT-PCR) analysis. As shown in Figure 2(A), the expression of TLR-2–4, 7, and 9 as well as their downstream genes encoding myeloid differentiation factor 88 (MyD88) and Toll/interleukin 1 receptor (TIR)-domain-containing adaptor inducing IFN (TRIF) was observed. We also stimulated Ana-1 cells with lipopolysaccharide (LPS), an agonist of TLR-4 [9], and detected the expression of TLR-4 and its downstream signaling molecule interleukin-6 (IL-6) [10] by a quantitative real-time RT-PCR (qRT-PCR). Stimulation of Ana-1 cells with LPS induced a significant expression of TLR-4 and IL-6 when compared with the mock-treated cells (Figure 2(B),(C)). Taken together, these data suggested that Ana-1 cells were appropriate for use in our studies.

2.2 GA induced the expression of TLR-4 in Ana-1 murine macrophages

The cytotoxicity of GA on Ana-1 cells was detected by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay (MTT assay). Cell viability was calculated as percentage of dimethyl sulfoxide (DMSO)-treated control. More than 95% of Ana-1 cells survived after 24-h treatment of GA at concentrations up to 80 μM (Figure 3(A)). The effective dose 50 (ED_{50}) value of GA against Ana-1 cells was 117.75 ± 2.72 μM, as calculated with a program of ED_{50} and IC_{50} for Graded Response (http://chiryo.phar.nagoya-cu.ac.jp/javastat/Graded50-j.htm). These results showed that GA has no significant cytotoxicity to Ana-1 cells.

To investigate the effect of GA on the expression of TLRs, Ana-1 cells were treated with GA at a concentration of 40 μM for 12 h, and the expression of TLR-2–4, 7, and 9 was detected by qRT-PCR. In response to GA treatment, the expression of TLR-4 mRNA was found to

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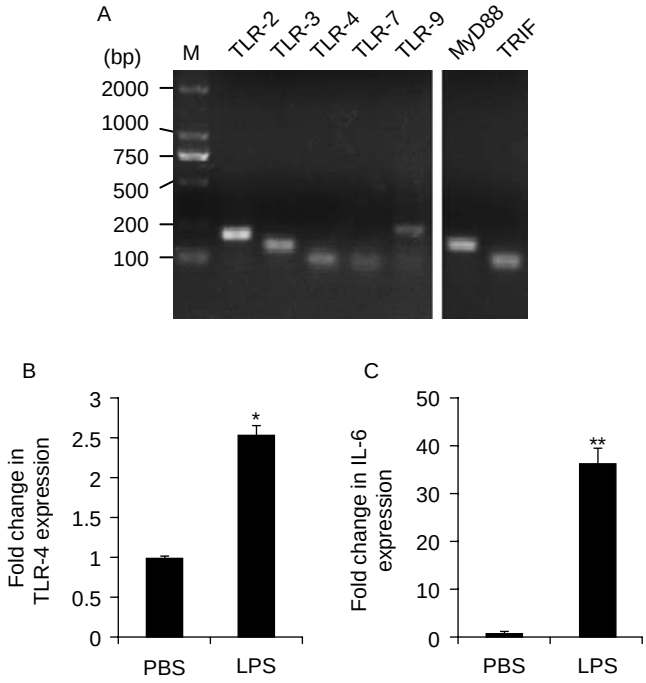

Figure 2. Detection of gene expression in Ana-1 cells. (A) Gene expression in Ana-1 cells was detected by RT-PCR and agarose gel electrophoresis. Lane M, DNA ladder. Ana-1 cells were stimulated with LPS at a concentration of 10 μg/ml for 24 h. The expression of TLR-4 (B) and IL-6 (C) in LPS-stimulated cells was detected by qRT-PCR. PBS was used as a control for LPS. Values represent the mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$ compared with PBS control.

be upregulated significantly when compared with the DMSO-treated cells, whereas the expression of TLR-2, 3, 7, and 9 mRNAs had no significant increase (Figure 3(B)). GA at concentrations ranging from 20 to 80 μM induced a significant expression of TLR-4 mRNA in a dose-dependent manner (Figure 3(C)). The expression of TLR-4 mRNA in GA-treated cells increased from 3-h post-treatment, and reached approximately fivefold over the level in DMSO-treated cells, and declined 24-h post-treatment (Figure 3(D)). This upregulated expression of TLR-4 was further confirmed at protein level using Western blot analysis (Figure 3(C),(D)). Taken together, these observations indicated that GA was able to induce the expression of TLR-4.

2.3 Endotoxin contamination did not contribute to the upregulation of TLR-4

It is known that endotoxin, such as LPS, is an agonist of TLR-4 and induces the upregulation of TLR-4 [11]. The endotoxin contamination in plant-derived compounds has been reported to contribute to the activation of TLR-4 [12]. To rule out the possibility that the upregulated expression of TLR-4 in GA-treated cells results from a potential endotoxin contamination rather than GA stimulation, we pretreated Ana-1 cells with 40 μg/ml polymyxin B, an endotoxin-binding peptide capable of inhibiting endotoxin activity [13], prior to GA treatment. The LPS-treated cells were included as control. As shown in Figure 4, polymyxin B treatment altered neither the basal level of TLR-4 expression in Ana-1 cells nor the

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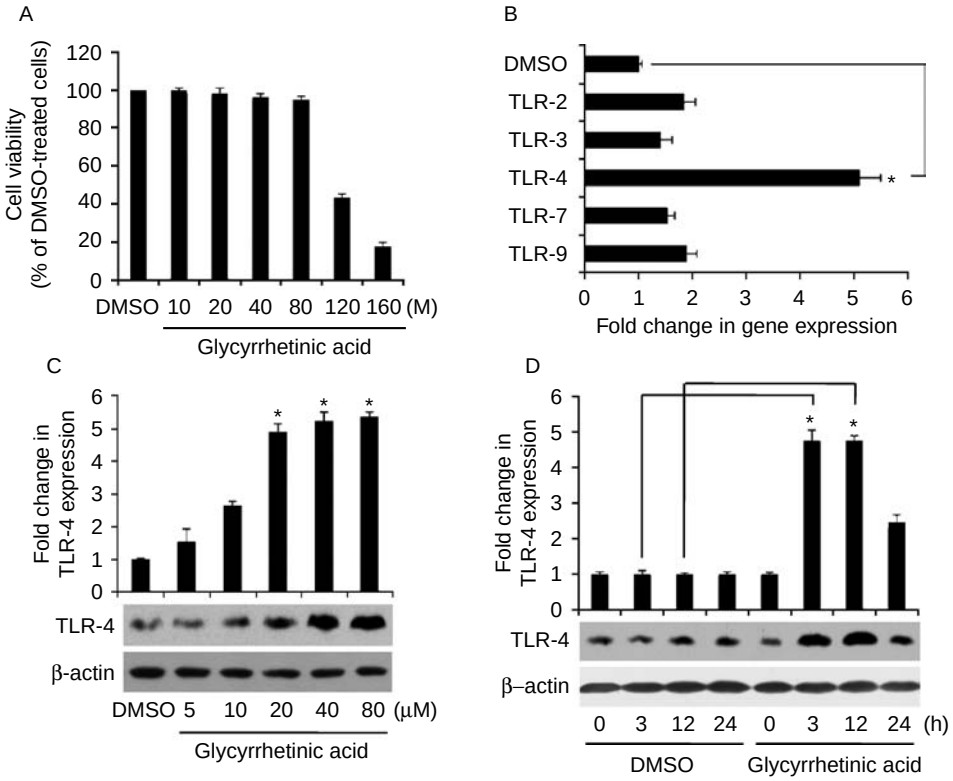

Figure 3. Effect of GA on TLR-4 expression in Ana-1 cells. (A) Analysis of the cytotoxicity of GA. Ana-1 cells were treated with the indicated concentrations of GA for 24 h and the cytotoxicity of GA was analyzed by an MTT assay. Cell viability was calculated as a percentage of total number of viable DMSO-treated control cells. (B) Ana-1 cells were treated with 40 μM GA for 12 h, and the expression of TLR-2–4, 7, and 9 was detected by qRT-PCR. (C) Ana-1 cells were treated with GA at the indicated concentrations and harvested 12-h post-treatment for the analysis of TLR-4 expression by qRT-PCR and Western blot. (D) Ana-1 cells were treated with 40 μM GA and harvested at the indicated times for the analysis of TLR-4 expression by qRT-PCR and Western blot. Values represent the mean ± SD of the three independent experiments. $*p < 0.01$ compared with the DMSO-treated control cells.

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upregulated expression of TLR-4 in GA-treated cells, but decreased the LPS-induced TLR-4 expression significantly, suggesting that GA itself, but not the endotoxin contamination, induces the expression of TLR-4. In addition to TLR-4, endotoxin also induces the expression of TLR-2 [14], however, no significant increase in TLR-2 expression was observed in GA-treated cells (Figure 3(B)), which further demonstrated that no endotoxin contamination contributes to the up-regulation of TLR-4.

2.4 GA induced the expression of downstream signaling molecules of TLR-4 pathway

It is known that the activation of TLR-4 induces the expression of IFN-β and IL-6 through adaptor molecule MyD88 and/or TRIF [15,16]. Given that GA induced the expression of TLR-4, we investigated the expression of several molecules, such as MyD88, TRIF, IFN-β, and IL-6, which are involved in the TLR-4 downstream signaling pathway. In response to GA stimulation, the expression of IFN-β and

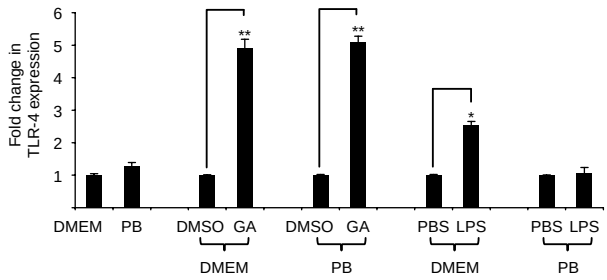

Figure 4. GA-induced TLR-4 expression in an endotoxin-independent manner. Ana-1 cells were pretreated with 40 μg/ml polymyxin B (PB) for 1 h prior to treatment with 40 μM GA or 10 μg/ml LPS. The cells were incubated for 12 h and harvested for analysis of TLR-4 expression by qRT-PCR. Medium DMEM, DMSO, and PBS were used as control for polymyxin B, GA, and LPS, respectively. Values represent the mean ± SD of the three independent experiments. $*p < 0.05$, $**p < 0.01$ compared with the control cells.

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IL-6 was significantly upregulated (Figure 5(A)), which was similar to the results observed previously in the mouse model [4]. The upregulated expression of IFN-β and IL-6 was further confirmed at a protein level using enzyme-linked immunosorbent assay (ELISA; Figure 5(B),(C)). The expression of MyD88, but not TRIF, increased significantly in GA-treated cells (Figure 5(A)), implying that MyD88 is involved in GA-induced activation of TLR-4 signaling.

TLR4-mediated nuclear factor kappa B (NF-κB) activation plays a critical role in the activation and regulation of cytokine expression [17]. To test whether GA is able to activate NF-κB, Ana-1 cells were transiently transfected with a reporter plasmid containing the luciferase reporter gene linked to five repeats of NF-κB binding sites (pNF-κB-Luc) and treated with GA at various concentrations. The effect of GA on the activation of NF-κB was analyzed by the luciferase reporter gene assay. In response to GA stimulation, the NF-κB-driven luciferase activity was increased significantly compared with the DMSO-treated cells (Figure 5(D)), suggesting that NF-κB was activated in GA-treated cells. Taken together, these data suggested that GA was able to activate the downstream signaling pathway of TLR-4.

2.5 Discussion

Although the pharmacological activities of GA, including antiviral infection, regulation of inflammatory responses, antitumor activity and antioxidant effects, have been extensively studied, the mechanisms underlying these pharmacological activities are complex and not well understood. In the context of antiviral infection, GA/GL has been speculated to modulate antiviral immune response by the induction of the expression of IFN [4,5] and by increasing the production of nitric oxide that has an antimicrobial activity against a variety of pathogens including viruses [18]. In this study, we found that GA was able to induce the expression of TLR-4 and its downstream signaling molecules. TLR-4, which is expressed on the cell surface and activated by viral ligands, bacterial LPS, fungal cell wall-associated mannan, and protozoan molecules, plays important roles in pathogen recognition and in regulation of the production of cytokines and chemokines necessary for the development of effective innate and adaptive immunity [19]. To the best of our knowledge, this is the first report to describe the regulatory effect of GA on TLR-4

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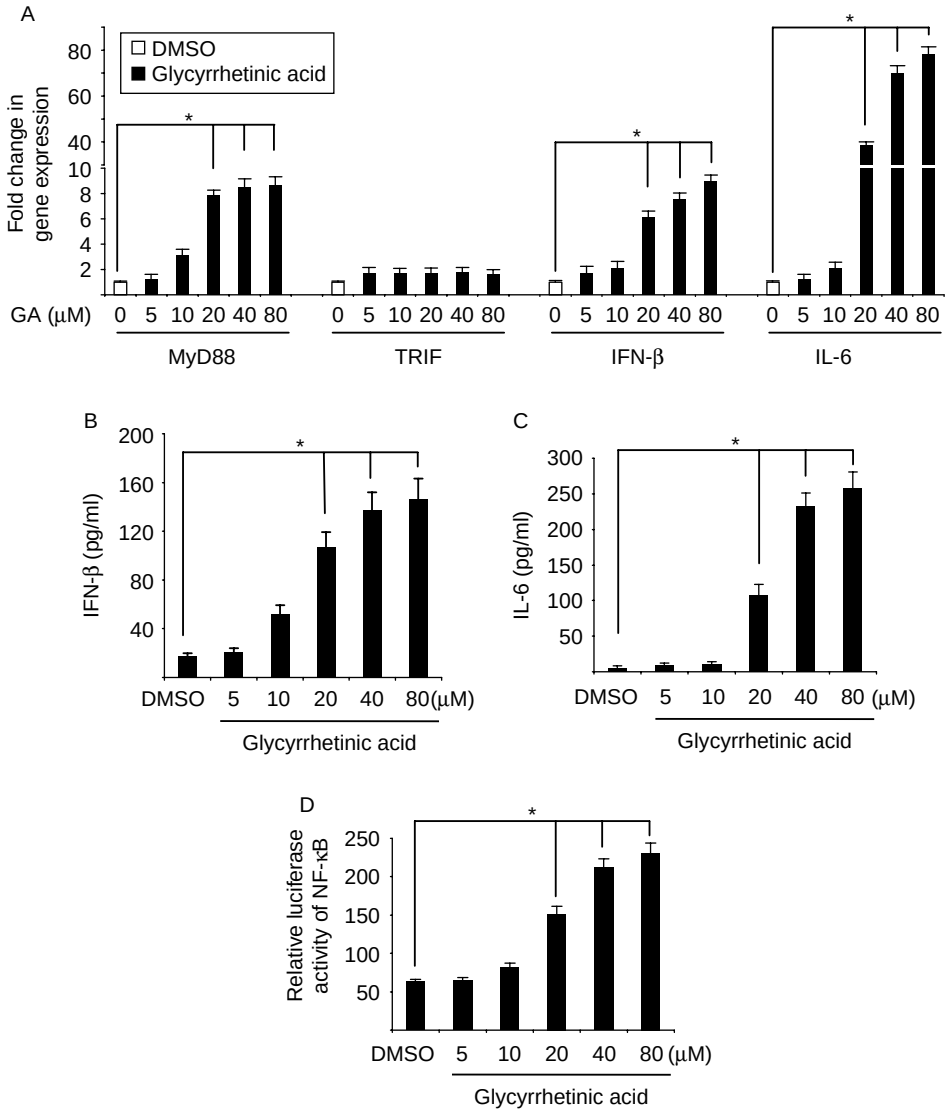

Figure 5. Effect of GA on gene expression in Ana-1 cells. Ana-1 cells were treated with GA at the indicated concentrations and harvested 12-h post-treatment for the analysis of the expression of the genes of interest. (A) The expression of the genes of interest was analyzed by qRT-PCR. (B) The concentrations of IFN-β in the cell supernatants were measured by ELISA. (C) The concentrations of IL-6 in the cell supernatants were measured by ELISA. (D) Ana-1 cells that were co-transfected with the pNF-κB-Luc reporter plasmid and the control plasmid Renilla luciferase pRL-TK were treated with GA at the indicated concentrations and harvested 12-h post-treatment for the analysis of the luciferase activity. The firefly luciferase activity of individual cell lysates was normalized to Renilla luciferase activity. Values represent the mean ± SD of the three independent experiments. $*p < 0.01$ compared with the DMSO-treated control cells.

expression. Our findings provide valuable insight into the molecular basis for the antiviral activity of GA. However, the molecular basis of how GA induces TLR-4 expression is unknown, which is a current topic of investigation in our laboratory.

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3. Materials and methods

3.1 Reagents and cells

GA (Product ID: E-0043, HPLC purity > 98%) extracted from *Glycyrrhiza uralensis* Fisch. was purchased from Shanghai Tauto Biotech Co. (Shanghai, China). GA powder was dissolved in culture-grade DMSO (Sigma, St Louis, MO, USA) at a concentration of 40 mM and then diluted to the indicated concentrations with the medium (final DMSO concentration $\leq 0.4\%$). LPS (serotype 026:B6, *Escherichia coli*) and polymyxin B were purchased from Sigma. An anti-TLR4 polyclonal antibody (sc-16240) and anti-β-actin monoclonal antibody (AC-15) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma, respectively. Ana-1 murine macrophages were obtained from the cell bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2.

3.2 Cytotoxicity assay

Ana-1 cells were treated with GA at various concentrations ranging from 10 to 160 μM at 37°C for 24 h. Cells treated with DMSO alone were used as a control. The cellular toxicity of GA was assessed using MTT assay. Cell viability was calculated as a percentage of total number of viable DMSO-treated control cells.

3.3 Cell treatment

Ana-1 cells were pre-cultured in 35 mm plates for 24 h. The media were replaced with fresh media containing various concentrations of GA. The cells were further incubated at 37°C for the indicated times and then harvested for gene expression analysis. To inhibit endotoxin activity, polymyxin B was added into Ana-1 cells at a concentration of 40 μg/ml and incubated for 1 h prior to GA treatment.

3.4 Reverse transcription polymerase chain reaction

Total RNA was extracted from Ana-1 cells using the RNeasy Plus Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using AMV reverse transcriptase (TaKaRa, Otsu, Japan). RT-PCR analysis of the expression of TLRs, MyD88, and TRIF was carried out using standard techniques. The sequences of the primers used were shown in Table 1.

3.5 Quantitative real-time RT-PCR

RNA isolation and cDNA synthesis were carried out as described above. qRT-PCR for the analysis of TLRs, MyD88, TRIF, IFN-β, and IL-6 was carried out using SYBR Premix Ex Taq™ (TaKaRa) according to the manufacturer's protocol. Briefly, reactions were prepared in 20 μl containing 1 μl of cDNA, 10 μl of SYBR Premix Ex Taq™ (2 ×), and 0.2 μM of specific primers. The amplification parameters were an initial 2 min at 95°C followed by 40 cycles consisting of 15 s at 95°C and 60 s at 60°C. The sequences of the primers used were shown in Table 1. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [20]. Data are presented as the fold change in gene expression normalized to the endogenous β-actin gene (reference gene) and relative to the untreated control.

3.6 ELISA for determination of IFN-β and IL-6

Ana-1 cells were treated with GA at various concentrations at 37°C for 12 h. The cell-free supernatants were collected

Table 1. Primer sequence.

Gene name	Primer sequence (5′–3′)
TLR-2	5′-ATCTTGGAACTGTCGG-3′
	5′-TGATTCGCTTCACCTT-3′
TLR-3	5′-GAGTTTGAAGCGAGC-3′
	5′-CAATCCCGTGAAGGT-3′
TLR-4	5′-GGCATCATCTTCATTG-3′
	5′-ATTCCAGGTAGGTGTTT-3′
TLR-7	5′-GGTTTGTCTGGTGGGT-3′
	5′-TTTGTGTGCTCCTGG-3′
TLR-9	5′-ATCTCCAACCGTATCCA-3′
	5′-GCAGTTCCCGTCCAT-3′
MyD88	5′-GCCTATCGCTGTTCTTG-3′
	5′-TGTGGGACACTGCTTTC-3′
TRIF	5′-GAGGTGCTCAAGACGC-3′
	5′-AGGTAGGATGCCCAGA-3′
IFN-β	5′-CTGCGTTCCTGCTGTGCTTC-3′
	5′-CGCCCTGTAGGTGAGGTTGA-3′
IL-6	5′-TTGCCTTCTTGGGACTGAT-3′
	5′-TTGCCATTGCACAACTCTT-3′
β-actin	5′-CCATCTACGAGGGCTAT-3′
	5′-TCACGCACGATTTCC-3

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and subjected to ELISA detection. The levels of IFN-β and IL-6 in the supernatants were measured using the Mouse IFN Beta ELISA Kit (PBL Biomedical Laboratories, Piscataway, NJ, USA) and the BD OptEIA™ Mouse IL-6 ELISA Kit (BD Bioscience, Franklin Lakes, NJ, USA), respectively, according to the manufacturer's protocol.

3.7 Reporter gene assay

Ana-1 cells were co-transfected with the pNF-κB-Luc reporter plasmid (Clontech, Palo Alto, CA, USA) and the control plasmid Renilla luciferase pRL-TK (Promega, Madison, WI, USA) using the Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) and incubated at 37°C for 24 h. The transfectants were treated with GA at various concentrations at 37°C for 12 h. The luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The firefly luciferase activity of individual cell lysates was normalized to Renilla luciferase activity.

3.8 Western blot analysis

Western blot analysis was carried out as described previously [21].

3.9 Statistical analysis

All measured values are expressed as the mean ± SD of three independent experiments. The significance of the results was analyzed using the Student's two-tailed *t*-test. p values less than 0.05 were considered significant.

Acknowledgements

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