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Geniposide regulates the miR-101/MKP-1/p38 pathway and alleviates atherosclerosis inflammatory injury in *ApoE*$^{-/-}$ mice

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ABSTRACT

Atherosclerosis (AS) is the common pathological basis of chronic cardiovascular diseases and is associated with inflammation and lipid metabolism dysfunction. Geniposide, the main active ingredient of *Gardenia jasminoides Ellis* fruit, exhibits a variety of anti-inflammatory and anti-oxidative functions; however, its role in AS remains unclear. The aim of this study was to investigate the mechanisms of geniposide in alleviating inflammation and thereby attenuating the development of AS. *ApoE*$^{-/-}$ mice were fed a high fat diet to induce AS and were treated with geniposide (50 mg/kg) for 12 weeks. Blood glucose and lipid levels were measured by biochemical analysis. H&E, Masson and Oil red O staining were performed to observe morphological changes and lipid deposition in the aorta and liver. Serum inflammatory cytokines were detected by ELISA. Dual-luciferase reporter gene assay was used to verify the target relationship between microRNA-101 (miR-101) and mitogen-activated protein kinase phosphatase-1 (MKP-1). The levels of miR-101, p-p38, and MKP-1 in the aorta were detected by *q*PCR and western blotting. The anti-inflammatory effect of geniposide *in vitro* was investigated in the RAW264.7 macrophage cell line. A miR-101 mimic and an inhibitor were used to study the effect of miR-101 on regulating the expression of the target MKP-1 and the downstream inflammatory cytokines. Geniposide treatment reduced lipid levels and plaque size in the mouse model of AS. Geniposide downregulated miR-101 to upregulate MKP-1 and suppress the production of inflammatory factors *in vitro and* in vivo. Geniposide suppressed the levels of inflammatory factors in the presence of the miR-101 mimic, whereas no obvious effect was observed in the miR-101 inhibitor group. We concluded that geniposide reduced the plaque size and alleviated inflammatory injury in *ApoE*$^{-/-}$ mice and RAW264.7 cells. The specific anti-inflammatory mechanism was related to the miR-101/MKP-1/p38 signaling pathway.

1. Introduction

Atherosclerosis (AS), a chronic inflammatory disease, is a major contributor to morbidity and mortality worldwide and is closely related to vascular inflammation and lipid metabolism disorders (Mannarino and Pirro, 2008). The occurrence and development of AS involve inflammatory responses, which are associated with the cardiovascular risk factors for AS and its complications (Feng et al., 2011). Increasing evidence confirms that statins effectively reduce the incidence of cardiovascular events by modulating blood lipids and inhibiting inflammation (Katsumoto et al., 2005). Therefore, inhibiting inflammation is important in the treatment of AS.

Activated p38 mitogen-activated protein kinase (MAPK) promotes vascular inflammation associated with the occurrence and development of AS and induces the secretion of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-10 (IL-10) (Chi et al., 2006). The phosphorylation level of p38 MAPK is enhanced and results in the production of inflammatory cytokines in TNF-α-induced endothelial cells (Chen and Goeddel, 2002). Mitogen-activated protein kinase phosphatase-1 (MKP-1), is a dual-specificity phosphatase that dephosphorylates both threonine/serine and tyrosine residues (Guan et al., 1991). It is a critical negative regulator that targets MAPKs and

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suppresses inflammation by removing phosphate groups and inhibiting the production of pro-inflammatory cytokines (Zhao et al., 2005). MKP-1 protects arteries from inflammatory injury by inactivating p38 MAPK in the vascular endothelium (Zakkar et al., 2008). The anti-atherogenic effects of MKP-1 are related to the inhibition of vascular smooth muscle cell growth and vasoconstriction, as well as the promotion of endothelial cell migration (Lai et al., 1996).

MiRNAs are involved in the regulation of inflammation and cholesterol homeostasis. MicroRNA-101 (miR-101) is a pro-inflammatory miRNA that inhibits the expression of MKP-1, increases the secretion of inflammatory factors, and plays a significant role in the development of AS and non-alcoholic fatty liver disease (NAFLD) (Elfimova et al., 2013). MiR-101 upregulation promotes the phosphorylation of MAPK in chronic obstructive pulmonary disease patients, and the mechanism is related to the inhibition of MKP-1 expression (Hassan et al., 2012). A previous study showed that miR-101 downregulated MKP-1 to modulate the activation of MAPK thereby affecting the production of downstream inflammatory factors in the innate immune system (Zhu et al., 2010).

Geniposide, an iridoid glycoside compound isolated from *Gardenia jasminoides Ellis*, exhibits a broad spectrum of anti-inflammatory and anti-oxidative effects (Fu et al., 2012). Geniposide decreases the activation of p38 MAPK and the degradation of inhibitory factor-κB-α induced by lipopolysaccharide (LPS) in N9 murine microglial cells (Zhang et al., 2012) and blocks the p38 MAPK signaling pathway to suppress the production of IL-6 and IL-8 in human umbilical vein endothelial cells (Liu et al., 2010). In addition, geniposide suppresses the release of inflammatory mediators by inhibiting the nuclear factor-κB (NF-κB) and MAPK signaling pathways in macrophages (Shi et al., 2014). However, the anti-inflammatory mechanism underlying the effect of geniposide in AS remains to be fully elucidated.

In the present study, we hypothesized that geniposide modulated the miR-101/MKP-1/p38 signaling pathway to suppress the production of inflammatory cytokines and alleviate AS inflammatory injury. The principal aim of the study was to investigate the anti-inflammatory mechanism of geniposide *in vitro* and *in vivo* to identify novel potential treatments for AS.

2. Materials and methods

2.1. Animals and drug administration

Thirty eight-week-old *ApoE*$^{-/-}$ mice obtained from Charles River Laboratories (SCXK 2012-0001) were bred at Laboratory Animal Center of Guangzhou University of Chinese Medicine (SYXK 2013-0001). All mice were housed four per cage at 23 ± 2℃ under a 12 h/12 h light/dark cycle. After 1 week of acclimation, body weight was measured as initial body weight, and mice were randomly divided into three groups. Group 1 (HFD group, $n = 10$) was fed a high fat diet containing 21% pork lard and 0.15% cholesterol (Guangdong Medical Laboratory Animal Center, SCXK 2013-0002), and received saline by gavage feeding once daily for 12 weeks. Group 2 (Simvastatin group, $n = 10$) was fed a high fat diet and received simvastatin (5 mg/kg/day) by gavage feeding once daily for 12 weeks. Group 3 (Geniposide group, $n = 10$) was fed a high fat diet and received geniposide (50 mg/kg/day) by gavage feeding once daily for 12 weeks. Ten wild-type C57BL/6 J mice (WT group, $n = 10$) of the same age (SCXK 2011-0015) that were fed a standard diet served as a normal control group. All mice were given free access to water. All animal experiments were approved by animal experimental ethics committee of Guangzhou University of Chinese Medicine.

2.2. Reagents

Geniposide (B21661) and LPS (S11060) were purchased from Yuanye Bio-Technology Co., Ltd (Shanghai, China). The purity of geniposide was > 98% as detected by HPLC. Simvastatin (J20130068) was obtained from Merck Sharp & Dohme B.V. (Netherlands). Total cholesterol (TC, YZB0397-2012), triglyceride (TG, YZB0422-2012), low density lipoprotein-cholesterol (LDL-C, YZB0596-2012), high density lipoprotein-cholesterol (HDL-C, YZB0402-2012), Masson's Trichrome stain kit (D026) and Oil Red O stain kit (D027) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mouse TNF-α (CSB-E04741 m) and IL-6 (CSB-E04639 m) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Cusabio Biotech Co., Ltd (Wuhan, China). Primary antibodies including anti-MKP-1 (rabbit monoclonal antibody, ab195261), anti-p38 (rabbit monoclonal antibody, ab170099), anti-p38 (phospho T180 + Y182) (rabbit monoclonal antibody, ab195049), and anti-GAPDH (rabbit monoclonal antibody, ab181602) were purchased from Abcam (Cambridge, MA, USA). SAPK/JNK antibody (#9252), phospho-SAPK/JNK (Thr183/Tyr185) rabbit mAb (#4668), p44/42 MAPK (Erk1/2) rabbit mAb (#4695) and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit mAb (#4370) were purchased from Cell Signaling Technology (Danvers, MA, USA). HRP-conjugated goat anti-rabbit antibody (GB23303) was provided by Servicebio Technology Co., Ltd (Wuhan, China). PrimeScript™RT Master Mix (RR036 A) and SYBR® Premix Ex Taq™II (RR820 A) were purchased from Takara Bio Inc. (Tokyo). Lipofectamine® 2000 was obtained from Invitrogen (Carlsbad, CA, USA). Mimic and inhibitor for mmu-miR-101a were purchased from Ribobio Co., Ltd (Guangzhou, China). All other chemicals were of reagent grade.

2.3. Serum biochemical assays

After 12 weeks of treatment, mice were fasted for 12 h and anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Then, the serum was collected and stored at −80 °C for later biochemical analysis. The levels of TC, TG, LDL-C, and HDL-C were measured by specific assay kits in accordance with the manufacturer's instructions.

2.4. Enzyme-linked immunosorbent assay

The serum and cell culture supernatant levels of inflammatory cytokines, including TNF-α and IL-6, were determined using ELISA kits according to the manufacturer's instructions. The optical density of each well was read at 450 nm.

2.5. Histological examination

Mice were perfused with PBS through the left ventricle. The aortic arch and liver tissues were immediately isolated, fixed in 10% neutral buffered formalin overnight, embedded in paraffin, and sectioned at a thickness of 5 μm. Hematoxylin and eosin (H&E) and Masson's Trichrome staining were performed according to standard procedures. The pathological changes in the aortic arch and liver were observed under an optical microscope.

2.6. Oil red O staining

To evaluate the atherosclerotic lesions in aortic sinus, the proximal aorta attached to heart was collected rapidly and fixed in 4% paraformaldehyde solution. Then the tissues were embedded in optimum cutting temperature compound (OCT), and serial 8-μm cryosections of the aortic sinus were cut using a Leica CM1900 cryostat (Leica Biosystems GmbH, Wetzlar, Germany). The lipid deposition was analyzed using Oil red O stain kit following the manufacturer's instructions.

2.7. Small RNA sequencing analysis

After assessment of total RNA samples from cardiovascular tissues,

total RNA was fragmented and fragments of 18–30 nt were collected using gel separation techniques. The 3′ adaptor and 5′ adaptor were connected to the separated small RNA fragments, and PCR amplification was used to establish a sequencing library. Finally, Illumina high-throughput sequencing was performed using the SE50 sequencing strategy. For each sample, the number of total clean reads matching known miRNAs and the reads per million total reads (RPM) values were calculated.

2.8. Cell culture

The murine macrophage cell line RAW264.7 was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a humidified incubator containing 5% CO_2. Cells (80%–90% confluent) were pretreated with geniposide for 4 h, and then stimulated with 100 ng/ml LPS for different times. The culture supernatants and cells were collected for further experiments.

2.9. Cell viability assay

To determine the effect of geniposide on RAW264.7 macrophages and the proliferation of cells induced by LPS, cell viability was measured using the Cell Counting Kit-8 (Dojindo, Japan). Cells were seeded on 96-well plates at a density of 1×10^4 cells/well and further incubated with various concentrations (2.5, 5, 10, 20, 40 and 80 μM) of geniposide for 24 h, or pretreated with different concentrations of geniposide for 4 h and then co-incubated with LPS (100 ng/ml) for 24 h. After incubating the cells with CCK-8 solution at 37 °C for 2.5 h, absorbance was detected at 450 nm using a microplate reader. The relative cell viability was expressed as a percentage of the control from three independent experiments.

2.10. Cell transfection

Cells were seeded on 6-well plates at a density of 2×10^5 cells/well. When cells reached 50% confluence, miR-101 mimic (50 nM) and miR-101 inhibitor (100 nM) were transfected into cells in antibiotic- and serum- free medium using Lipofectamine™ 2000 reagent according to the manufacturer's protocol. The medium was replaced with DMEM containing 10% FBS after 6 h, and the cells were cultured for another 48 h. Protein and supernatants were collected for further analysis.

2.11. Dual-luciferase reporter gene assay

The target gene of miR-101a was predicted by TargetScan software, and the dual-luciferase reporter gene assay was used to verify whether MKP-1 was the direct target of miR-101a. The wild-type (WT) or mutant (Mut) MKP-1 3′-UTR containing miR-101 binding site was inserted into psiCHECK™-2 plasmids. Then the constructs were co-transfected with miR-101a mimics or mimic-NC into 293 T cells using Lipofectamine®2000. After transfection for 48 h, the luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega Madison, WI, USA) according to the manufacturer's instructions. The results were represented as the ratio of renilla to firefly luciferase activity. The experiment was repeated in triplicates.

2.12. Western blot analysis

RIPA lysis buffer containing phosphatase inhibitors was added to aortic tissues and cells to obtain protein samples for western blotting. The homogenate was agitated on ice for 30 min and then centrifuged. Proteins in supernatants were collected and quantified. Equivalent amounts of 40 μg of total protein were loaded and separated by 10% SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. After blocking, membranes were incubated with primary antibodies at 4 °C overnight, including anti-MKP-1 (1:1000), anti-p38 (1:3000), anti-p38 (phospho T180 + Y182) (1:1000), anti-GAPDH (1:10,000), SAPK/JNK (1:1000), phospho-SAPK/JNK (Thr183/Tyr185) (1:1000), p44/42 MAPK (Erk1/2) (1:1000), and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:2000). Subsequently, the membranes were washed three times with TBST, followed by incubation with HRP-conjugated secondary anti-rabbit antibodies (1:3000). Protein bands were detected by enhanced chemiluminescence (ECL-plus, Thermo Scientific, USA). The intensity of blots was analyzed and compared using the Gel-Pro analyzer program.

2.13. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from cardiovascular tissues and cells using Trizol, and the same amount of RNA was reverse-transcribed and amplified into cDNA with PrimeScript™ RT Master Mix kit according to the manufacturer's instructions. Gene expression was quantified with the SYBR® Premix Ex Taq™ II kit. The primers used were as follows: TNF-α forward, 5′-TATGGCTCAGGGTCCAACTC-3′ and reverse, 5′−CCCATT TGAGTCCTTGATGG-3′; IL-6 forward, 5′-AGTTGCCTTCTTGGGAC TGA-3′ and reverse, 5′−CCTCCGACTTGTGAAGTGGT-3′; MKP-1 forward, 5′-AGGATATGCTTGACGCCTTG-3′ and reverse, 5′-GTCTGCCTT GTGGTTGTC CT-3′; miR-101 RT, 5′-GTCGTATCCAGTGCAGGGTCCGA GGTATTCGCACTGGATACGACGC ATCA, forward, 5′-TGCGCGTCAGTT ATCACAGTGC-3′ and reverse, 5′-ATCCAGTGCAGGGTC CGAGG-3′; β-actin forward, 5′-GTCCCTCACCCTCCCAAAAG-3′ and reverse, 5′-GCT GCCTCA ACACCTCAACCC-3′. *q*PCR was performed on a Stratagene Mx3005*P* Quantitative PCR System according to the following protocol: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 30 s. All samples were assayed in triplicate. The relative expression of target genes was normalized to that of β-actin and control groups using the $2^{-\Delta\Delta Ct}$ method.

2.14. Data analysis and statistics

Results were presented as the mean ± SD. SPSS 20.0 software (Chicago, IL, USA) was used for data analysis. The statistical significance was conducted with the Student's *t*-test for two groups comparison. For multiple groups comparison, the data were analyzed using one-way analysis of variance. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Geniposide inhibits the development of AS in $ApoE^{-/-}$ mice

To examine the anti-atherogenic effect of geniposide, lipid levels in the serum were measured and histological changes in the aorta and liver were observed. Weight, blood glucose, TC, TG, LDL-C, HDL-C, and body fat percentage were higher in the HFD mice than in the WT mice. Geniposide significantly reduced blood glucose, TC, LDL-C, and body fat percentage, whereas weight gain and the levels of TG and HDL-C did not differ significantly between the geniposide-treated and the HFD mice (Table 1).

H&E, Oil red O and Masson's Trichrome staining demonstrated that the endangium of the WT mice was smooth and the endothelial cells were arranged in a regular pattern. No obvious atherosclerotic plaque was observed. Moreover, the morphological structure of the hepatic lobule was complete and liver cells had a consistent and regular size. All HFD mice showed typical AS pathological changes. The intima of the HFD mice was significantly thickened. Lipid deposition, basement membrane destruction and a fibrous plaque were observed. In addition, the liver tissue in the HFD mice showed obvious liver injuries characterized by loss of cellular boundaries, irregular shape, and fatty degeneration. Geniposide reduced the ratio of the plaque area to the lumen area (PA/LA; Fig. 1A and B) and ameliorated hepatocyte fatty

Table 1
Effects of geniposide on blood glucose and lipid metabolism in *ApoE*$^{-/-}$ mice.

	WT	HFD	SV	GP
Weight gain (g)	7.58 ± 0.92	10.04 ± 1.68$^{\#\#}$	7.98 ± 1.33**	8.54 ± 1.25
Blood glucose (mmol/L)	5.18 ± 0.81	7.54 ± 1.21$^{\#\#}$	5.76 ± 1.12**	5.22 ± 0.78**
Total cholesterol (mmol/L)	3.69 ± 0.88	21.95 ± 4.37$^{\#\#}$	15.20 ± 3.04**	16.24 ± 3.84*
Triglycerides (mmol/L)	0.66 ± 0.16	0.95 ± 0.29$^{\#}$	0.68 ± 0.15*	0.86 ± 0.23
LDL-C (mmol/L)	0.77 ± 0.21	17.08 ± 3.42$^{\#\#}$	10.66 ± 2.58**	12.60 ± 3.16*
HDL-C (mmol/L)	2.09 ± 0.51	3.69 ± 1.08$^{\#\#}$	3.19 ± 0.80	3.04 ± 0.50
Body fat percentage (%)	2.88 ± 0.77	4.38 ± 1.35$^{\#}$	2.97 ± 1.00*	3.08 ± 0.83*

degeneration (Fig. 1C). Collagen fibers in the atherosclerotic plaque were increased to stabilize the plaque in response to geniposide treatment (Fig. 1D and E). In addition, the result of Oil red O staining showed that geniposide reduced the size of the atherosclerotic lesions (Fig. 1F).

Body weight was measured using an electronic balance and the blood glucose was determined using an Accu-Chek glucometer before anesthesia. Weight gain was calculated (final minus initial weight). Lipid levels and body fat percentage were also measured ($n = 10$). Data are presented as the mean ± SD; # vs. WT group, * vs. HFD group, # and *$P < 0.05$, ## and **$P < 0.01$. SV, simvastatin- treated group; GP, geniposide-treated group.

3.2. Geniposide suppresses the production of inflammatory cytokines and decreases p38 MAPK activity

The effect of geniposide on the production of inflammatory cytokines was investigated by measuring the levels of TNF-α. The production of inflammatory cytokines in the serum and the transcriptional levels of inflammatory cytokines in the aorta were higher in the HFD mice than in the WT mice. Geniposide decreased TNF-α levels (Fig. 2A and B). Western blotting showed that the p38 MAPK phosphorylation level was higher in the HFD mice than in the WT mice and the activation of p38 MAPK was attenuated after geniposide treatment (Fig. 2C and D).

3.3. MKP-1 is a target of miR-101 and geniposide regulates miR-101/MKP-1 in *ApoE*$^{-/-}$ mice

To examine the mechanisms underlying the anti-inflammatory effects of geniposide, we detected miRNAs associated with inflammation. The RPM and total count of miR-101 were higher in the HFD and simvastatin-treated mice than in the WT mice. Geniposide significantly decreased the RPM of miR-101 (Fig. 3A). Then we used TargetScan to evaluate the potential relationship between miR-101 and MKP-1. The result suggested that 3′-UTR of MKP-1 mRNA contained a putative miR-101 binding site and MKP-1 might be a target for miR-101 (Fig. 3B). To confirm whether miR-101-3p directly binds to MKP-1 3′-UTR, we performed dual-luciferase reporter gene assay. The results indicated that transfection of miR-101-3p mimics significantly decreased the luciferase activity of the constructed reporter containing the WT MKP-1 3′-UTR. However, there was no change regarding the luciferase activity of the constructed reporter containing the Mut MKP-1 3′-UTR after transfection (Fig. 5C and D).

To determine the potential role of miR-101/MKP-1 in AS development, the expression of these molecules was examined in the mouse AS model. Compared with the HFD mice, miR-101 was downregulated and MKP-1 protein expression was upregulated in the geniposide-treated mice. However, geniposide had no effect on the mRNA levels of MKP-1 (Fig. 3E–H).

3.4. Geniposide reduces the production of inflammatory cytokines in RAW264.7 cells

Geniposide did not affect cell viability (Fig. 4A). Furthermore, cell proliferation was increased in the presence of LPS, and geniposide inhibited the proliferation of cells induced by LPS except for 2.5 and 5 μM concentrations (Fig. 4B). Assessment of the levels of inflammatory factors in the supernatants of LPS-induced cells showed that geniposide decreased the production and mRNA levels of TNF-α and IL-6 (Fig. 4C–F).

3.5. Geniposide downregulates miR-101 and upregulates MKP-1 in RAW264.7 cells induced by LPS

The effect of geniposide on inflammatory pathway was examined in RAW264.7 cells. Geniposide increased the expression of MKP-1, suppressed the activation of p38 MAPK and JNK, but little affected ERK1/2 phosphorylation (Fig. 5A–D). Further assessment of the transcriptional level of miR-101 and MKP-1 showed that geniposide downregulated the mRNA expression of miR-101, whereas it had no effect on the transcriptional level of MKP-1 (Fig. 5E and F).

3.6. Geniposide regulates the signaling pathway induced by miR-101 to inhibit inflammation in RAW264.7 cells

To clarify whether geniposide inhibited inflammation through the modulation of miR-101, cells were transfected with a specific miR-101 mimic and inhibitor. First, we investigated the effect of the miR-101 mimic or inhibitor on MKP-1 protein expression in RAW264.7 cells. MKP-1 was downregulated or upregulated in the presence of the miR-101 mimic or inhibitor, respectively (Fig. 6A and B). Assessment of the levels of inflammatory factors in the cell supernatants showed that geniposide reduced the production of TNF-α and IL-6 in the presence of the miR-101 mimic, whereas there was no effect in the inhibitor group (Fig. 6C–F).

4. Discussion

The present study demonstrated, for the first time, a protective mechanism of geniposide against AS inflammatory injury both *in vitro* and *in vivo*. The underlying mechanism involved the downregulation of miR-101 and the upregulation of MKP-1, leading to decreased activation of p38 MAPK and production of inflammatory cytokines.

Inflammation plays a central role in the initiation and development of AS. Substantial evidence suggests that circulating acute-phase reactants triggered by inflammation increase the risk of cardiovascular diseases and contribute to the pathogenesis of the disease. Persistent inflammation leads to an increase in the number of macrophages and lymphocytes. Activation of these cells results in the release of cytokines and chemokines, which induces further vascular damage and the development of AS (Ross, 1999). Treatments that reduce the risk of coronary disease mainly involve inhibition of inflammation. Therefore, effective anti-inflammatory therapies need to be identified.

Geniposide, the main active ingredient of the *Gardenia jasminoides*

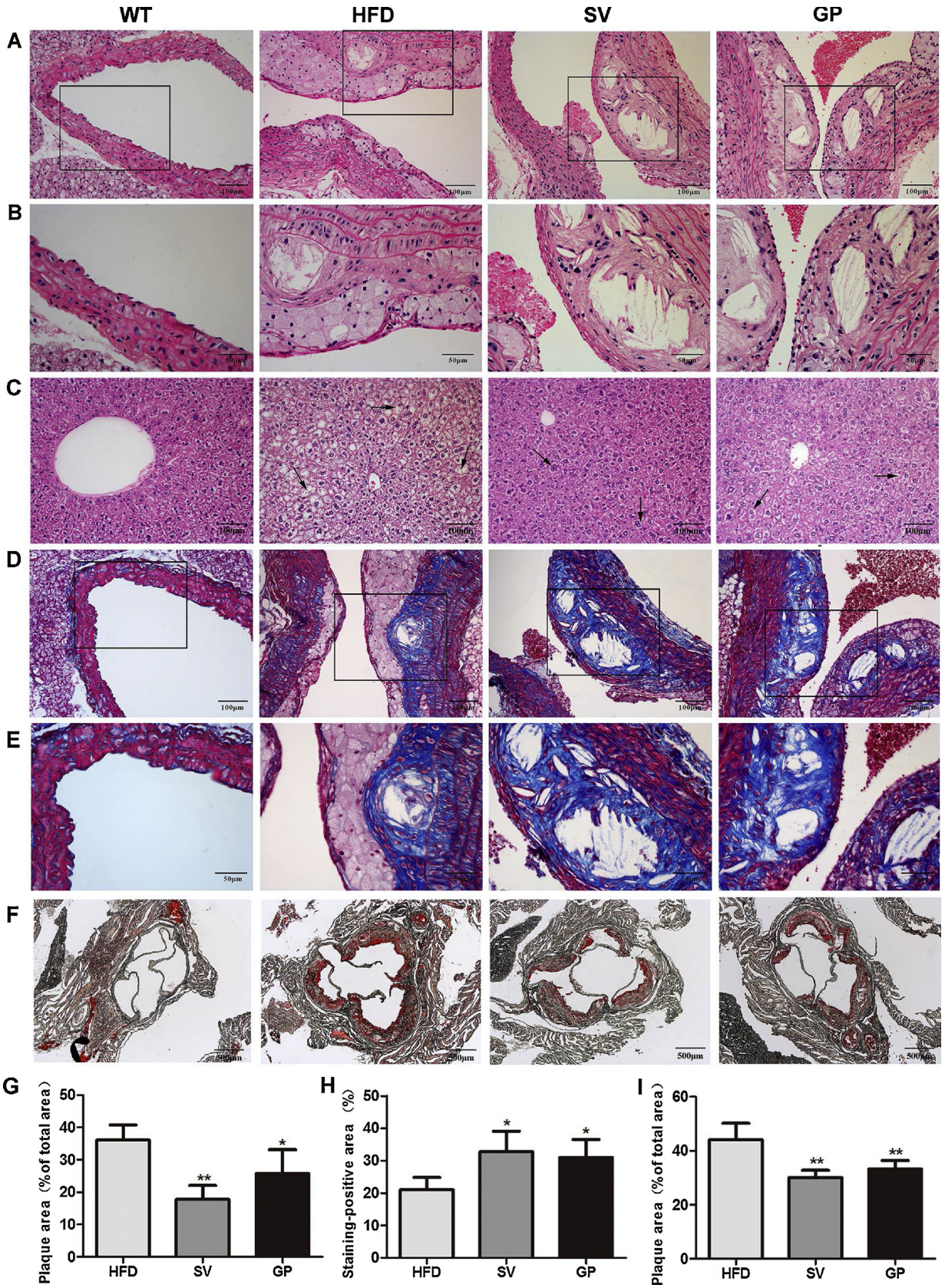


Fig. 1. Geniposide reduced plaque size and ameliorated hepatocyte fatty degeneration in *ApoE*$^{-/-}$ mice. (A, B) Pathological changes in the aorta were observed by H &E staining. (C) H&E staining was used to observe the pathological changes in the liver. (D, E) Masson's trichrome staining was used to evaluate the content of collagen fibers in the atherosclerotic plaque. (F) Representative light photomicrographs of Oil red O staining sections from the aortic root. (G, I) Plaque area was measured using Adobe Photoshop software and was expressed as the percentage of the total area. (H) Positive Masson's trichrome staining areas were analyzed using Image Pro-Plus software ($n = 5$). Data are presented as the mean ± SD; * vs. HFD group, # and *$P < 0.05$.

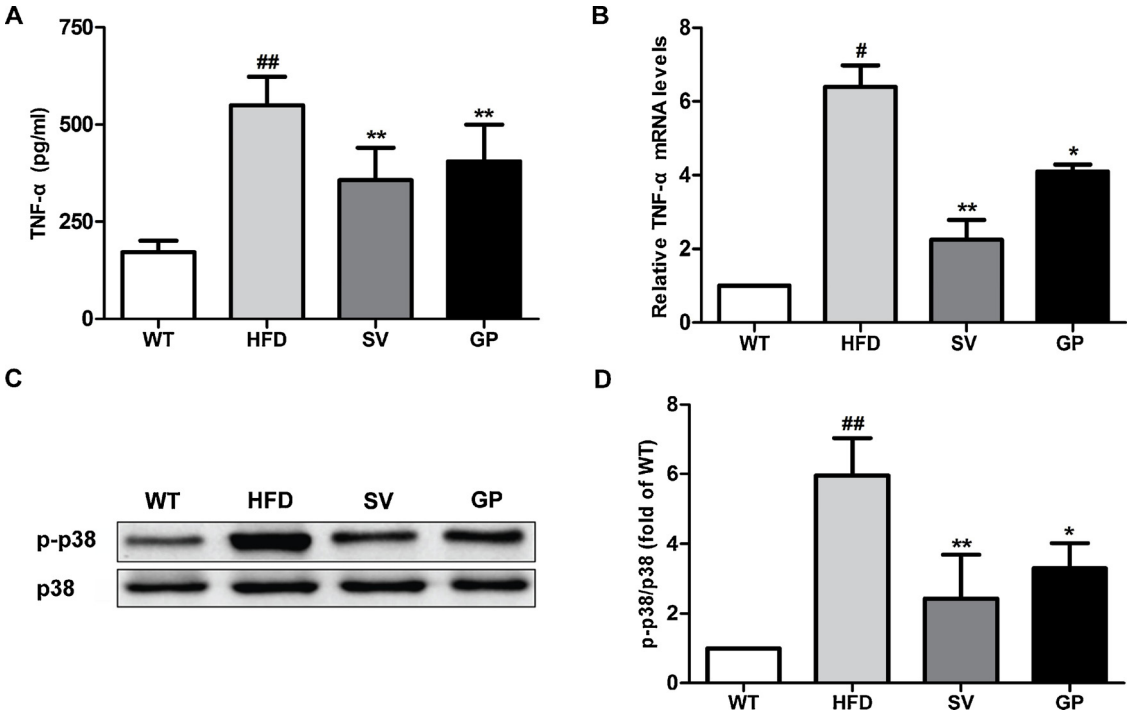


Fig. 2. Geniposide suppressed the production of inflammatory cytokines and attenuated p38 MAPK activity in *ApoE*$^{-/-}$ mice. (A) The level of serum TNF-α was detected by ELISA (n = 10). (B) TNF-α transcriptional level was determined by *q*PCR in isolated aortas. (C, D) The phosphorylation level of p38 MAPK in aortas was measured by western blotting, and the data of panel C were quantitatively analyzed (n = 3). Data are presented as the mean ± SD; # vs. WT group, * vs. HFD group; # and *$P < 0.05$, ## and **$P < 0.01$.

Ellis fruit, has anti-inflammatory and anti-oxidative effects and is used as an anti-inflammatory and antipyretic agent (Koo et al., 2004). Geniposide reduces blood glucose and food intake in diabetic rats induced by streptozotocin (Hu et al., 2017). The protective effect of geniposide in rats with hepatic steatosis is associated with the regulation of lipid metabolism by increasing the expression of peroxisome proliferator-activated receptor-α (PPARα) (Ma et al., 2011). In the present study, we showed that geniposide decreased blood glucose, TC, LDL-C, and body fat percentage in *ApoE*$^{-/-}$ mice but there was no effect on TG and HDL-C. Furthermore, pathological examination showed that geniposide ameliorated atheromatous lesions and stabilized the plaque by reducing plaque size and increasing collagen fiber content.

The important members of the MAPK family include extracellular signal-regulated kinases, p38 MAPKs, and c-Jun NH_2-terminal protein kinases. Accumulating evidence suggests that p38 MAPK regulates vascular inflammation and plays a critical role in the secretion of inflammatory cytokines in AS. Phosphorylated p38 MAPK promotes the secretion of inflammatory cytokines to activate NF-κB under inflammatory conditions. Meanwhile, NF-κB activates p38 MAPK signaling pathways through the production of inflammatory cytokines to mediate inflammation and participate in the development of AS (Zhang et al., 2014). Previous research has demonstrated that the inhibitory effect of geniposide on the expression of inflammatory mediators in osteoarthritis is associated with the suppression of the p38 MAPK signaling pathways (Chen et al., 2018). Here, p38 MAPK activity and the production of inflammatory cytokines were decreased in response to geniposide treatment both in vivo *and in vitro*, indicating that inhibition of p38 MAPK activation contributes to the anti-inflammatory effect of geniposide in the AS model.

MicroRNAs are small non-coding RNAs that play a crucial role in biological processes by regulating gene expression at the post-transcriptional level. Alterations in miRNA expression are involved in the initiation and development of multiple cardiovascular diseases including AS and arrhythmia. To examine the mechanisms underlying the anti-inflammatory effects of geniposide, we focused on the detection of certain microRNAs associated with inflammation through small RNA sequencing analysis. In our study, we found that geniposide significantly modulated the transcriptional levels of certain miRNAs in the cardiovascular system, including miR-101, miR-16, and miR-21. MiR-101 is a pro-inflammatory miRNA that increases the secretion of inflammatory factors and is closely related to the development of various inflammatory diseases. MiR-101 overexpression under inflammatory conditions negatively regulates ATP-binding cassette transporter A1 (ABCA1) expression and cholesterol efflux, and promotes intracellular cholesterol retention by directly targeting the ABCA1 3′-untranslated region (3′-UTR) (Zhang et al., 2015). Meanwhile, upregulation of miR-101 activates p38 MAPK and increases the inflammatory factors to induce inflammation in macrophages stimulated with LPS (Gao et al., 2014). Therefore, we focused on miR-101 and its mechanism of regulating inflammatory injury in AS.

MKP-1 is a pivotal negative regulatory factor of MAPKs and plays a key role in the modulation of cell proliferation and inflammatory responses. MKP-1 decreases the phosphorylation level of p38 MAPK rapidly and the production of inflammatory cytokines in RAW264.7 cells induced by LPS (Sartori et al., 2009). In our experiment, geniposide increased the expression of MKP-1 and attenuated the phosphorylation of JNK and p38 MAPK, but little affected ERK1/2 phosphorylation in LPS-induced cells. The results suggested that MKP-1 preferentially dephosphorylated phospho JNK and phospho p38 MAPK to regulate the expression of inflammatory genes and transcription factors (Liu et al., 2007). Furthermore, the expression of MKP-1 is modulated by gene silencing mediated by miRNAs. Through TargetScan database and the dual-luciferase reporter gene assay, we confirmed that miR-101 directly targeted and bound to MKP-1 3′-UTR. MKP-1 translation was attenuated or increased in the presence of a miR-101 mimic or inhibitor in RAW264.7 cells, respectively, also indicating that miR-101 acted as a positive regulatory factor in inflammation. MKP-1 was a direct target of miR-101 and miR-101 had a negative regulatory effect on MKP-1.

In vivo experiment, we found that geniposide significantly

A

RPM

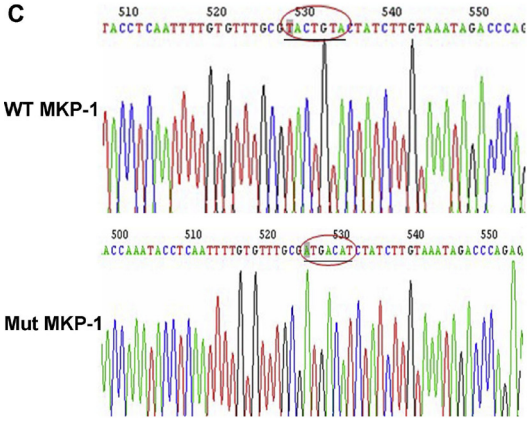
	WT	HFD	SV	GP
miR-101	3655.58	6231.15	7110.79	2827.12
miR-16	43440.59	17598.05	16875.55	62007.42
miR-17	155.82	71	109.14	188.09
miR-21	5486.6	2978.38	4614.8	4632.97
miR-126	42770.5	22597	49765.94	29221.61
miR-142	8920.57	1534.59	2210.96	6209.71
miR-146	1568.46	1935.34	733.78	1738.59
miR-155	589.72	950	712.64	961.83
miR-210	185.18	181.02	161.04	110.71
miR-499	3093.11	4331.09	4617.88	1757.59

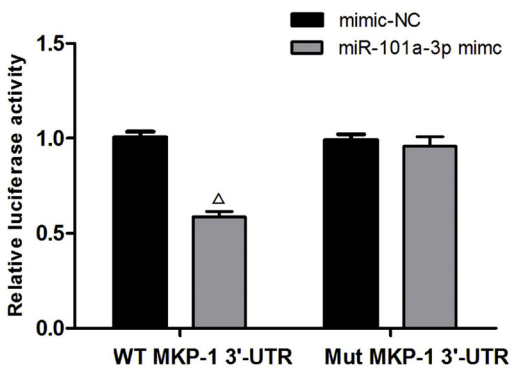
COUNT

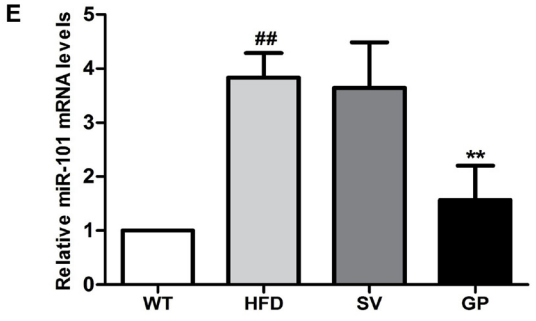
	WT	HFD	SV	GP
miR-101	24281	43443	53163	18744
miR-16	288540	122692	126168	411115
miR-17	1035	495	816	1247
miR-21	36443	20765	34502	30717
miR-126	284089	157544	372069	193742
miR-142	59252	10699	16530	41171
miR-146	10418	13493	5486	11527
miR-155	3917	6623	5328	6377
miR-210	1230	1262	1204	734
miR-499	20545	30196	34525	11653

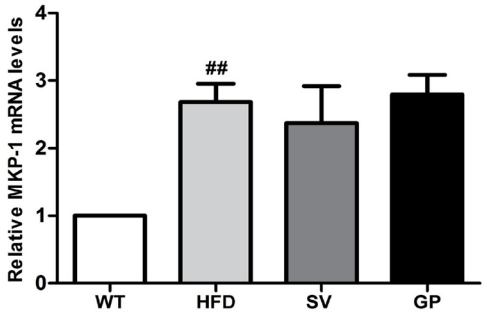
B

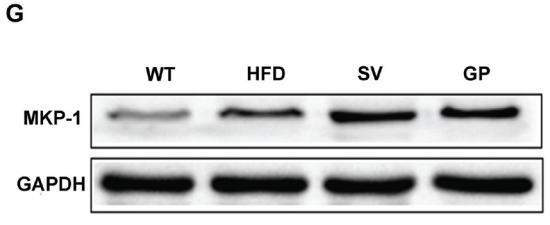
	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 482-488 of DUSP1 3' UTR mmu-miR-101a-3p.2	5' ...AAUUUUGUGUUUGCGUACUGUAC... 3' AGUCAAUAGUGUCAUGACAUG	7mer-m8	-0.12	88	-0.12	7.974	0.46











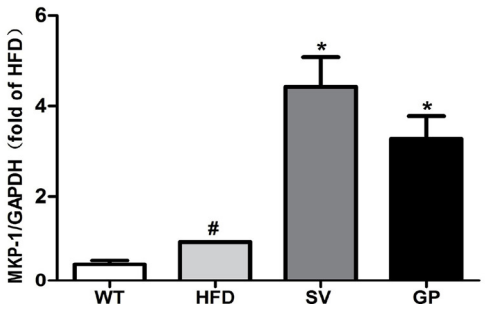


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Fig. 3. MKP-1 was a target of miR-101 and geniposide regulated miR-101/MKP-1 in *ApoE*$^{-/-}$ mice. (A) The expression of miRNAs associated with inflammation was detected by small RNA sequencing. The RPM and total clean read values were calculated. (B) TargetScan indicated that MKP-1 3′-UTR contained a putative binding site for miR-101-3p. (C) Cloned target fragments in psiCHECK-2-MKP-1-WT and psiCHECK-2-MKP-1-Mut were confirmed by sequencing. Mutant sites were represented by underlined sequence. (D) Dual-luciferase reporter gene assay showed that miR-101-3p bound to MKP-1 3′-UTR directly. MKP-1 3′-UTR constructs were co-transfected with miR-101-3p mimics or mimic-NC into 293 T cells and relative luciferase activity was measured. (E) The transcriptional level of miR-101 was analyzed by *q*PCR. (F) The mRNA level of MKP-1 was analyzed by *q*PCR. (G, H) MKP-1 protein expression in the aorta was measured by western blotting, and the data were quantitatively analyzed ($n = 3$). Data are presented as the mean ± SD. △ vs. mimic-NC group, # vs. WT group, * vs. HFD group. △, # and * $P < 0.05$, ## and ** $P < 0.01$.

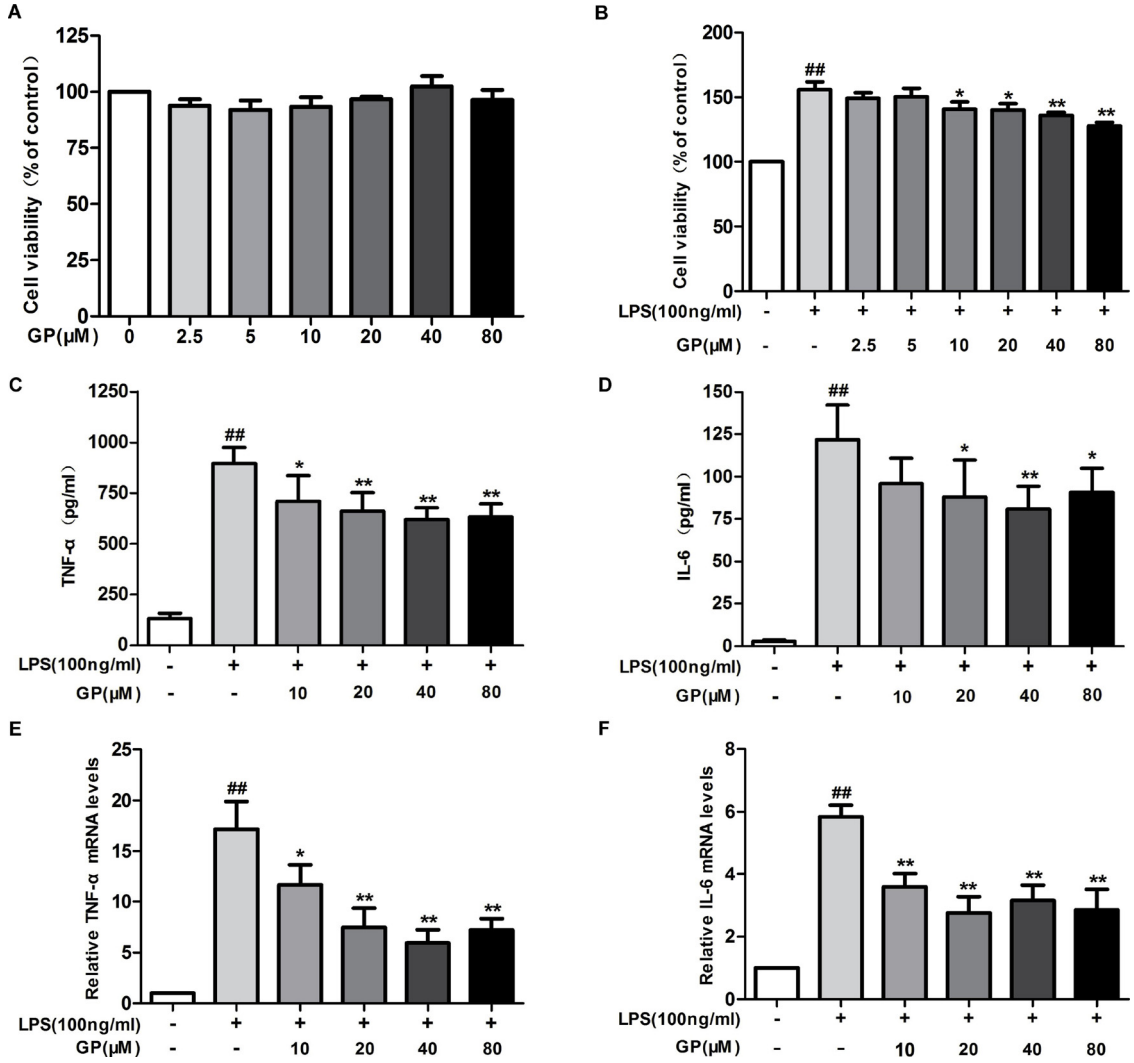

Fig. 4. Geniposide regulation of inflammatory factors in RAW264.7 cells induced by LPS. (A) Cells were treated with geniposide for 24 h, and cell viability was measured using the CCK-8 assay. (B) Cells were pretreated with different concentrations of geniposide for 4 h and then co-incubated with LPS (100 ng/ml) for 24 h. Cell viability was measured using the CCK-8 assay. (C, D) Cells were pretreated with different concentrations of geniposide for 4 h and then co-incubated with LPS (100 ng/ml) for 24 h. ELISA was used to measure inflammatory factors in the supernatant. (E, F) Cells were pretreated with different concentrations of geniposide for 4 h and then co-incubated with LPS (100 ng/ml) for 1 h. The transcriptional level of inflammatory factors was determined by *q*PCR. Data are presented as the mean ± SD, $n = 3$. # vs. control group, * vs. LPS alone. # and * $P < 0.05$, ## and ** $P < 0.01$.

downregulated the transcriptional levels of miR-101. However, simvastatin had no effect on miR-101, suggesting that the anti-inflammatory effect of simvastatin associated with MKP-1 upregulation did not involve the regulation of miR-101, but might involve other mechanisms. Unlike simvastatin, geniposide significantly downregulated miR-101, increased the expression of MKP-1, and decreased the phosphorylation level of p38 MAPK in *ApoE*$^{-/-}$ mice. In addition, according to Zhang's findings (Zhang et al., 2015), we speculate that the pharmacological mechanism of geniposide in lowering blood lipids is related to the downregulation of miR-101, leading to increased expression of ABCA1 to promote cholesterol efflux.

Macrophages play a key role in releasing pro-inflammatory cytokines in response to various harmful stimuli and regulating inflammation, as well as immune responses. Activated macrophages that produce excessive amounts of inflammatory mediators are closely associated with a variety of inflammatory diseases, including AS and rheumatoid arthritis (Medvedev et al., 2000). When macrophages are stimulated with LPS, the toll-like receptor-4 (TLR4) signaling pathway is initiated, leading to the phosphorylation of MAPK and the activation of NF-κB (Verstrepen et al., 2008). Moreover, PI3K and its downstream target Akt can be activated by TLR4. The activation of PI3K/Akt upregulates miR-101 and inhibits the expression of MKP-1 to prolong the

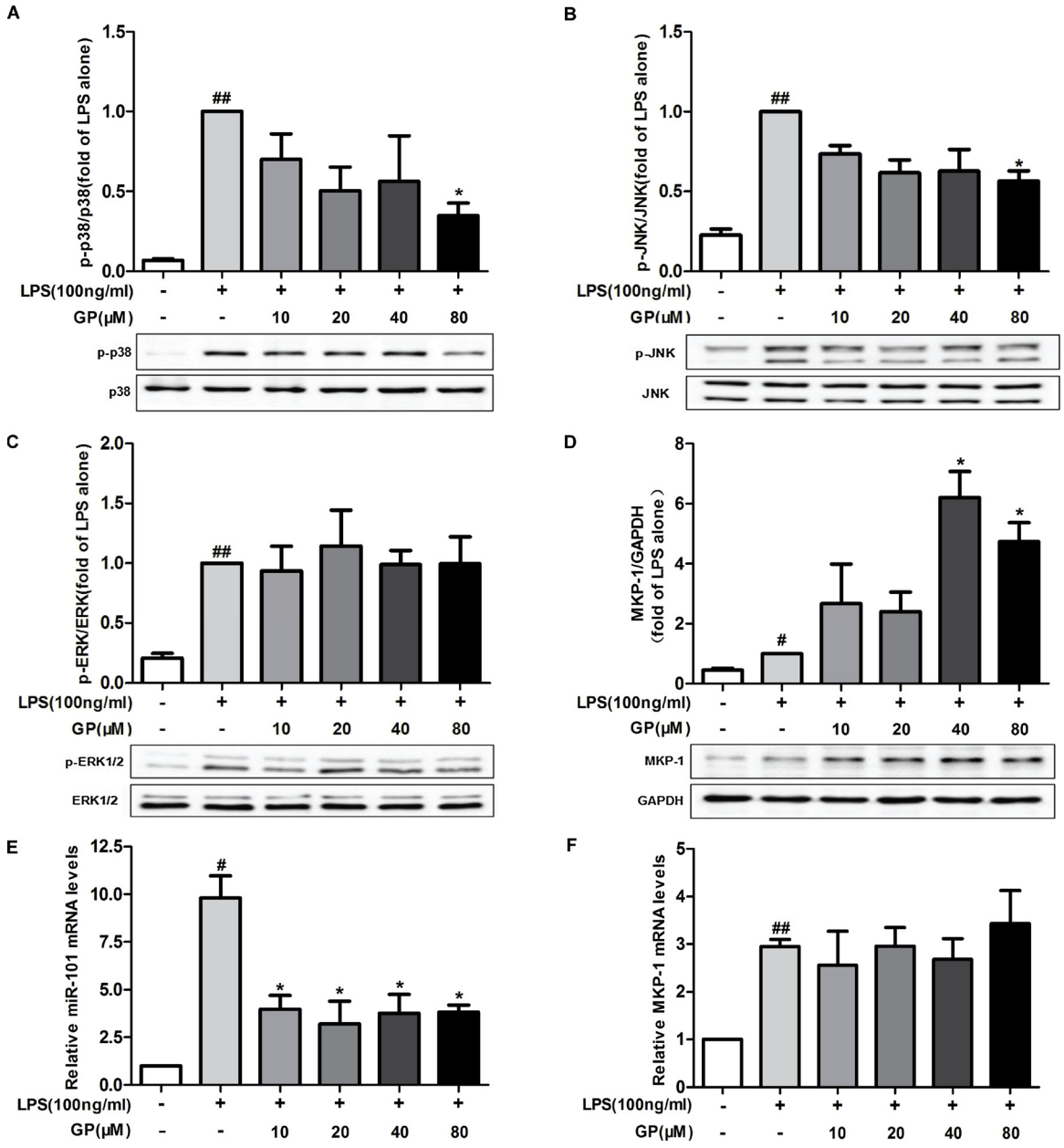

Fig. 5. Geniposide regulation of miR-101, MKP-1 and MAPKs in RAW264.7 cells (A–D) Cells were pretreated with different concentrations of geniposide for 4 h and then co-incubated with LPS (100 ng/ml) for 1 h. The phosphorylation levels of p38 MAPK, JNK, ERK and MKP-1 were determined by western blotting, and the data were quantitatively analyzed. (E, F) *q*PCR was used to detect the transcriptional levels of miR-101 and MKP-1. Data are presented as the mean ± SD, $n = 3$. # vs. control group, * vs. LPS alone. # and * $P < 0.05$, ## and ** $P < 0.01$.

phosphorylation of MAPKs (Zhu et al., 2010). Geniposide inhibits the expression of TLR4, blocking the downstream MAPK signaling pathways and reducing the levels of pro-inflammatory cytokines to exert its anti-inflammatory effects (Song et al., 2014). Geniposide inhibits the activation of PI3K/Akt/NF-κB signaling pathway to suppress inflammation and apoptosis induced by IL-1β, and plays a key role in the treatment of osteoarthritis (Pan et al., 2018). Therefore, we concluded that the mechanisms of geniposide in downregulating the transcriptional level of miR-101 was related to inhibiting the expression of TLR4 and the activation of PI3K/Akt signaling pathway. The step will be studied in our next project.

In LPS-stimulated macrophages, MKP-1 shows a transient expression pattern with induction and then returns to basal levels (Chi et al., 2006). In addition, LPS-induced expression of MKP-1 reached the peak at 60 min, but transfection with miR-101 mimic suppressed MKP-1 expression and prolonged the activation of p38 MAPK to 120 min (Zhu et al., 2010). In vitro experiment, miR-101 was upregulated and the expression of MKP-1 was increased in LPS-induced cells. Our results indicated that MKP-1 expression was induced by stimulation with LPS, and factors in the atherosclerotic milieu, leading to feedback control of dephosphorylation of MAPKs. Our study also showed that geniposide significantly downregulated miR-101 and increased the expression of MKP-1. Moreover, geniposide suppressed the levels of inflammatory factors in the presence of the miR-101 mimic. The results indicated that geniposide exerted anti-inflammatory effects mainly through reducing miR-101 production.

In summary, geniposide reduced the size of the atherosclerotic plaque and alleviated inflammatory injury in $ApoE^{-/-}$ mice and RAW264.7 cells. The miR-101/MKP-1/p38 signaling pathway was responsible for the anti-inflammatory effects of geniposide, suggesting its

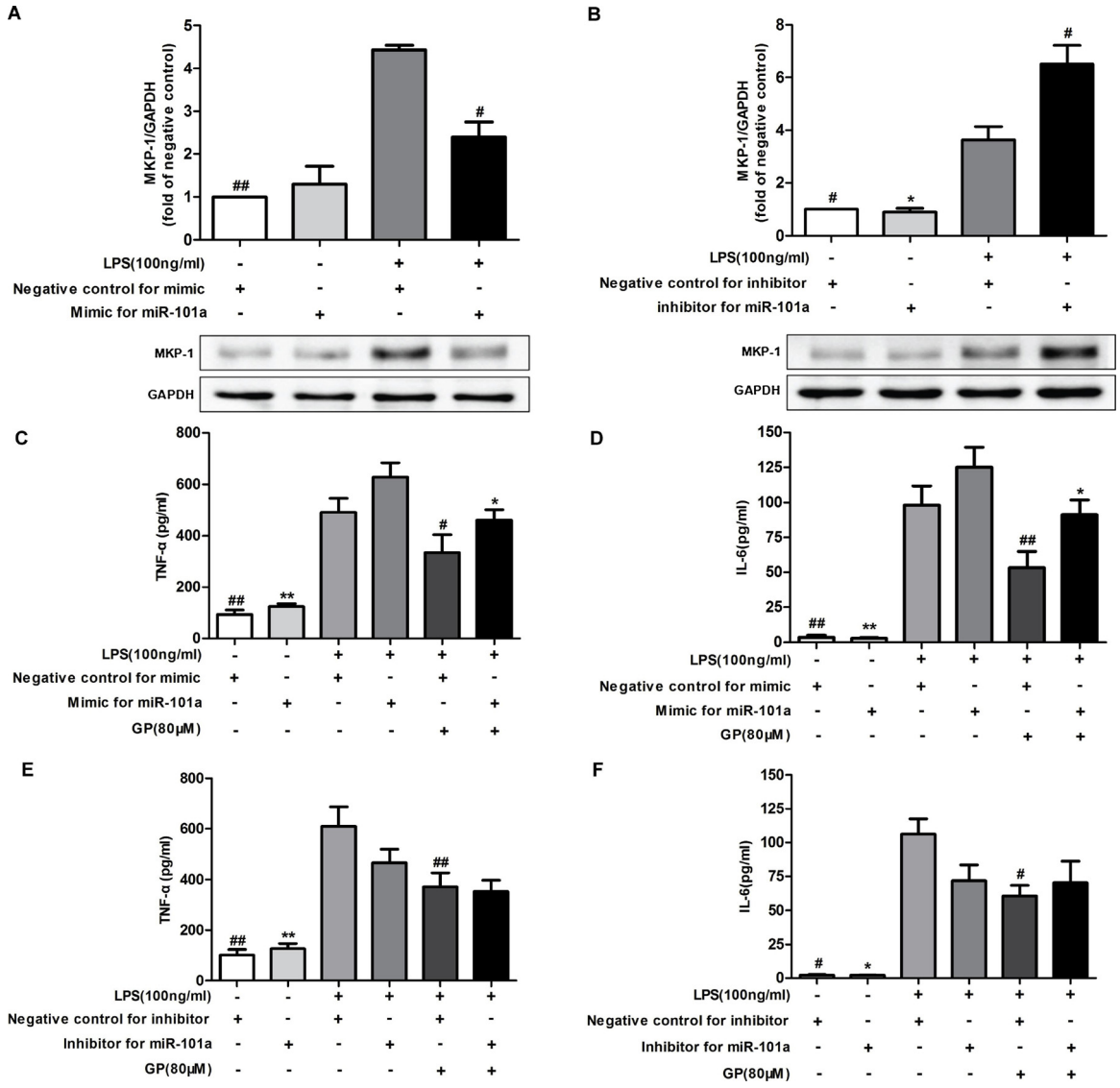

Fig. 6. Geniposide directly targeted miR-101 to suppress inflammation in RAW264.7 cells. (A, B) Cells were transfected with miR-101 mimic/inhibitor or their negative controls for 48 h and then stimulated with LPS (100 ng/ml) for 1 h. The expression of MKP-1 was determined by western blotting, and the data of panels A and B were quantitatively analyzed. (C–F) Cells were transfected with miR-101 mimic/inhibitor or their negative controls and then treated with geniposide (80 μM) and LPS (100 ng/ml) for 24 h. ELISA was used to measure the levels of inflammatory factors in the supernatants. Data are presented as the mean ± SD, $n = 3$. # vs. LPS-activated cells transfected with negative controls, * vs. LPS-activated cells transfected with miR-101mimic/inhibitor, # and * $P < 0.05$, ## and ** $P < 0.01$.

crucial role in the occurrence and development of AS. One limitation of the present study is that the miR-101 mimic and inhibitor were not used in animal experiments; this will be examined in further studies. Our findings provide potential targets and a theoretical basis for the clinical treatment of AS.

Conflict of interest

The authors declare that they have no competing interests.

Funding

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