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ORIGINAL RESEARCH

Geniposide Stabilized Atherosclerosis Plaque by Induced M2 Polarization via PPARγ Signaling Pathway

Zheng Jin, Qingmin Chu, Zhiyi Du, Junlong Li, Wei Wu, Xiaoxiong Zhou, Huanyi Zhao

First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, 510000, People's Republic of China

Correspondence: Xiaoxiong Zhou; Huanyi Zhao, First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, 510000, People's Republic of China, Email zhouxiaoxiong0825@163.com; zhao_banli@163.com

Objective: The instability of atherosclerotic (AS) plaque constitutes a critical trigger for acute intravascular thrombosis and cardiovascular disease. Prior studies have found that *Geniposide* (Gen) is capable of regulating macrophage polarization and stabilizing AS plaque. However, its potential mechanism is not clear. Given the role of peroxisome proliferator-activated receptor γ (PPARγ) in mediating macrophage polarization, this study aims to investigate the relationship between Gen, PPARγ and macrophage polarization.

Methods: In vitro, RAW264.7 was used to investigate the effects of Gen on polarization phenotype, anti-inflammatory activity, and its correlation with PPARγ. In vivo, ApoE$^{-/-}$ mice were fed with a high-fat diet to induce AS and were used to evaluate the pharmacological effects of Gen. Additionally, the relationship between Gen, PPARγ and M2 polarization in AS was verified.

Results: In vitro, Gen upregulated the expressions of M2 macrophage markers (CD163, IL-10, Arg-1). Moreover, Gen increased the expressions of PPARγ target genes (CD36, ABCG1) and activated PPARγ activity, which could be inhibited by PPARγ antagonist GW9662. In vivo, the intervention of Gen on AS model of ApoE$^{-/-}$ mice played a role in reducing the blood lipid, stabilizing AS plaques and down-regulating the level of inflammatory factors. Consistent with the results in vitro, Gen was able to regulate the expression of macrophage polarization and increase the expression of PPARγ, while GW9662 treatment inhibited the expression of M2 phenotypic markers.

Conclusion: Gen regulates macrophage polarization to M2 phenotype and plays a role in inhibiting inflammation and stabilizing plaque by mediating PPARγ activation, which suggests that Gen may be a promising agent for AS.

Keywords: atherosclerosis, geniposide, macrophage polarization, inflammation, PPARγ

Introduction

Atherosclerosis (AS) is a progressive large-artery disease and constitutes the primary cause of coronary heart disease (CHD). The increase of circulating low-density lipoprotein cholesterol (LDL-c) has been proved to be closely related to the onset and progression of AS. Statins serve as first-line agents for the prevention and treatment of atherosclerotic vascular diseases.[1] By reducing the plasma LDL-c level, statins exert a plaque-stabilizing effect. However, the residual risk still persists in the form of additional vascular events in patients receiving intensive statin therapy. Meanwhile, intensive lipid-lowering therapy is also associated with the risk of side effects, including diabetes, myopathy, hepatotoxicity and nephrotoxicity.[2] The novel lipid-lowering agents, proprotein convertase subtilisin kexin type 9 (PCSK9) inhibitors, can significantly reduce the LDL-c levels and induce plaque regression. Nonetheless, the lack of long-term safety and efficacy data, coupled with the high cost of PCSK9 inhibitors, restricts their clinical use.[3] Therefore, there is an urgent need to explore new targets and therapeutic agents for AS intervention.

AS is a chronic inflammatory disease of arterial wall driven by intimal hyperplasia and immune cell activation. Previous studies have shown that macrophages play a crucial role among immune cells in this process.[4] During the

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development of AS, macrophages exhibit highly heterogeneous and further differentiate into different polarization states, which are defined as M1 and M2 subtypes, after being stimulated by microenvironment factors within plaques.[5] M1 macrophages, as classically activated macrophages, are characterized by secreting pro-inflammatory factors. High expressions of pro-inflammatory proteins promote plaque growth and instability. In contrast, the M2 phenotype is referred to as alternatively activated macrophages. Activated M2 macrophages exert a preventive effect on AS formation, such as reducing plaque size and enhancing plaque stability, and are closely associated with anti-inflammatory expression patterns.[6] Therefore, intervening in macrophage polarization can regulate the progression of AS and has the potential for clinical treatment.

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-dependent nuclear transcription factor involved in the polarization of macrophages from the classically activated M1 phenotype to the alternatively activated M2 phenotype. The importance of PPARγ in regulating the M1/M2 phenotype switch has been demonstrated in previous researches.[7] Cluster of differentiation 36 (CD36) is a highly glycosylated Type B scavenger receptor, and its expression is linked to the enhancement of M2 polarization and phagocytosis.[8] The ATP-binding cassette transporter G1 (ABCG1) is capable of regulating cholesterol efflux in macrophage. Previous studies have suggested that ABCG1 could regulate cholesterol efflux via PPARγ-LXRα-ABCA1 pathway. Consequently, both of them are employed as relevant indicators to evaluate the regulatory role of PPARγ in lipid metabolism researches. There is evidence that confirm Procyanidin B2 can regulate macrophage M2 polarization via activating PPARγ and increased the expressions of PPARγ target genes in macrophages, such as CD36 and ABCG1.[9]

Geniposide (Gen) is the primary active ingredient of *Gardenia jasminoides*, and it is one of the components of traditional Chinese medicine that has been studied extensively. It has been demonstrated that derivatives of Gen can exert inhibitory effects on inflammation and fibrosis in hyperuricemia.[10] Additionally, another research provides valuable insights into the potential neuroprotective of Gen when combined with nanoparticles in Alzheimer's disease.[11] Moreover, Gen alleviates colitis by activating the Nrf2/ARE signaling, while simultaneously preventing colonic redox imbalance and inflammatory damage.[12] While our previous study has confirmed that Gen exerts a stabilizing effect on AS plaque,[13] the specific mechanism of its role in stabilizing plaque is not completely clear. In this study, we further investigates the molecular mechanism through which Gen exerts its therapeutic effects on AS. Results suggest that Gen could induce the polarization of RAW264.7 macrophages toward M2 phenotype by activating PPARγ and increasing the expression of downstream target genes of PPARγ. The workflow of the study is shown in Figure 1.

Methods

Reagents

Geniposide (Gen, HY-N0009) was purchased from MCE (New Jersey, USA). Rosiglitazone (RGZ, S2556) and GW9662 (S2915) were purchased from Selleck (Houston, USA). Antibodies against iNOS (ab178945), Ym-1+Ym-2 (ab192029), Fizz-1 (ab39626), MOMA-2 (ab33451), GAPDH (ab9484), horseradish peroxidase (HRP)-conjugated secondary antibody (ab6721), and Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) antibody (ab150113) were purchased from Abcam (Cambridge, USA). Anti-phospho-PPARγ (bs-4888R) and Anti-PPARγ (bs-0530R) antibodies were purchased from Bioss (Beijing, China). Anti-Arg-1 (93668S) antibodies was from Cell Signaling Technology (Danvers, USA). Elisa kits IL-1β (ab241673), IL-17A (ab199081), and IL-6 (ab203360) were purchased from Abcam (Cambridge, USA).

CCK-8 Assay

Cell viability was assessed using CCK-8 assay according to the manufacturer's instructions (C0039, Beyotime, Shanghai, China). Cells were grown and treated on 96-well plates and were incubated with CCK-8 reagent for 2 hours at 37°C. Optical density (OD) was measured at 450 nm and calculated for cell viability.

Cell Culture

Mouse monocytic cell line (RAW264.7) and human embryonic kidney epithelial cell line (HEK293) were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing

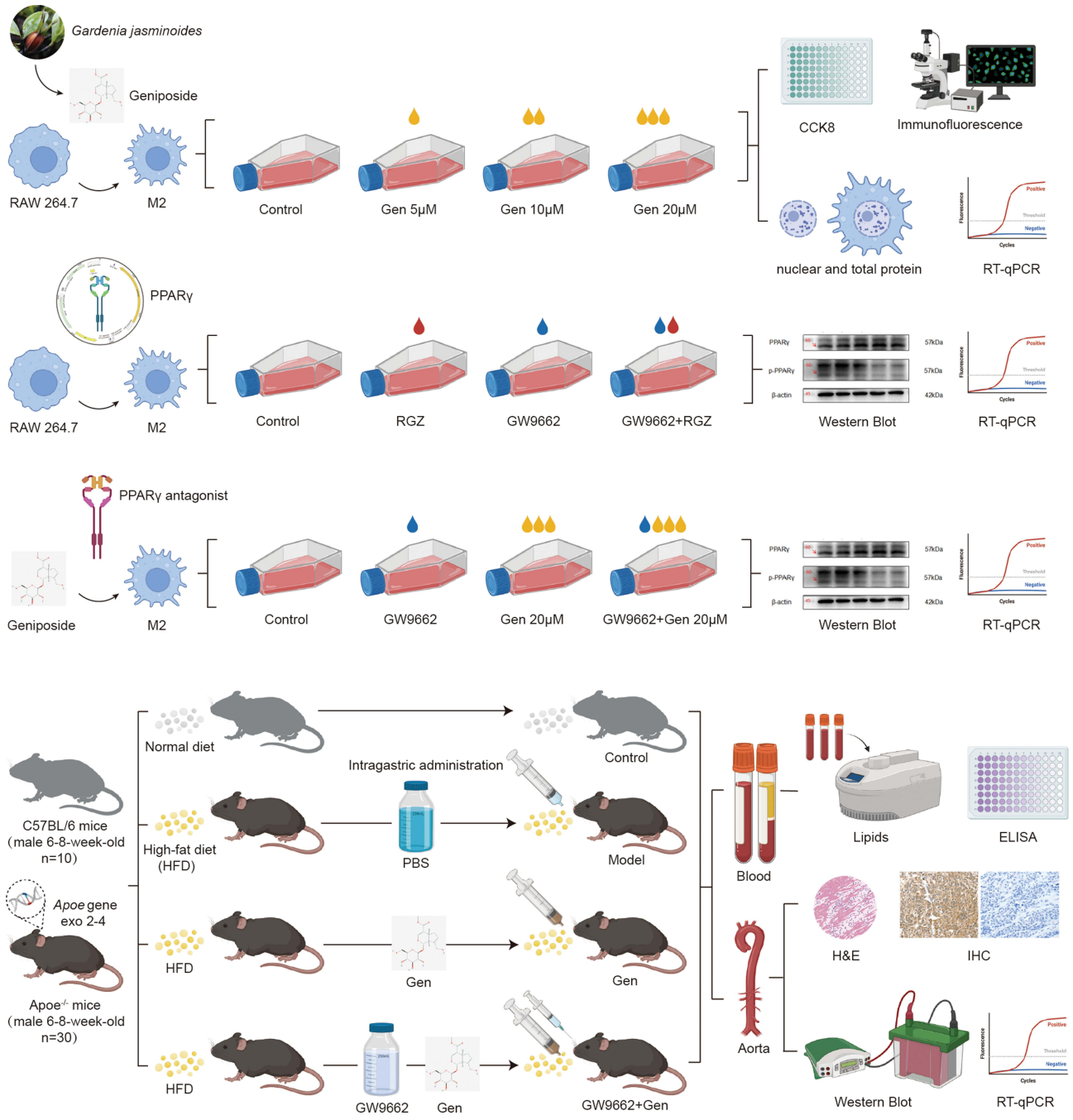

Figure 1 Workflow of the systematic strategies to elucidate the mechanisms of Gen on macrophage polarization and plaque stability.

10% FBS with penicillin (100 U/mL) and streptomycin (100 U/mL) in 37˚C with 5% CO_2. Cells were divided into different groups according to different experimental requirements. In Gen and RGZ groups, RAW264.7 cells were exposed to RGZ (10 μM, as positive control) or different concentration of Gen (5, 10, 20 μM) for 24h. In GW9662 groups, RAW264.7 cells were pre-treated with GW9662 (5 μM) for 1 h and/or no exposed to Gen (20 μM) for 24 h. It should be particularly noted that HEK293 cells were only used in luciferase reporter assay to detect the activity of specific enzymes.

Immunofluorescent Staining

RAW264.7 cells were seeded on 24-well plates. After intervening for 24h, 4% paraformaldehyde was used to fix the cells for 20 min at room temperature. The cells were then washed three times in phosphate-buffered saline (PBS) containing

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0.5% Triton-X for permeabilization. Then cells were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. Primary antibodies for iNOS and Arg-1 were incubated at 4 °C overnight. After sufficient washing, the corresponding secondary antibody was incubated for 1 h at room temperature. The nuclei were stained with DAPI for 5 min, and images were captured using a fluorescence microscope (OLYMPUS IXplore).

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from cells in each group at room temperature and collected for an RT-qPCR assay using the SteadyPure Universal RNA Extraction Kit (AG21017, Accurate Biotechnology, Hunan, China) according to the manufacturer's instructions. RT-qPCR was performed on a CFX Connect Real-Time PCR System with iQ SYBR Green Supermix (Bio-Rad).

Fold-changes of one sample compared to calibration sample was calculated using the $2^{-\Delta\Delta Ct}$ method. The gene primers list in Table 1 and *GAPDH* was used as an endogenous control.

Western Blot

Protein samples were obtained from the lysates of cultured cells or tissues and the protein concentration was determined with the Protein BCA Assay Kit (Beyotime, Shanghai, China). Protein samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a 0.22 μm PVDF for antibody hybridization. The membranes were blocked in 5% non-fat-milk for 2 h at room temperature and incubated with primary antibodies overnight at 4 °C. After sufficient washing with Tris-based Saline Solution with Tween-20 (TBS-T), the membranes were further incubated with HRP-conjugated secondary anti-bodies (Abcam, ab6721, England) for 1 h at room temperature on the next day. The protein samples were visualized using a chemiluminescence, images were quantified by Image-Pro Plus.

Plasmids Transfection and Luciferase Reporter Assay

The expressing PPARγ plasmids were constructed as PPRE-TK-luciferase reporter which was containing three copies of PPAR-response elements (PPRE) from acyl CoA oxidase (ACO) gene and β-galactosidase (β-gal). Then the plasmids were transfected into HEK293 cells according to the manufacturer's instructions using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, USA). After the treatments with Gen (5, 10, 20 μM) or RGZ (10 μM) for 24 h, cell lysates were harvested and subjected to analysis using the Dual-Luciferase Reporter Assay System (Promega, E1910, USA) and a multifunctional enzyme labeling instrument to measure the luciferase activities of luciferases.

Adenoviral Vectors and Infection

The target gene PPARγ and the pcDNA3.1-EGFP vector were double-digested using EcoRI and HandIII restriction endonucleases (Takara, Japan) and then construct the recombinant plasmid. The pcDNA3.1-EGFP/PPARγ recombinant

Table 1 Primer Sequences Used for RT-qPCR

Gene	Forward	Reverse
CCR7	CCTACAGCGAAGCCAAGTCC	ATCCAGATGCCCACACAGGA
CD163	ACGGCTGGAGCATGAATGAA	ATGCTTTCCCCACCCATCAT
IL-10	GGACAACATACTGCTAACCGACTC	ATTTCCGATAAGGCTTGGCAACC
IL-1β	TCGCAGCAGCACATCAACAAG	TCCACGGGAAAGACACAGGTAG
PPARγ	TGTTCGCCAAGGTGCTCCAG	GCTCATGTCTGTCTCTGTCTTCTTG
CD36	GCAGCCTCCTTTCCACCTTTTG	TCCGAACACAGCGTAGATAGACC
ABCG1	TGCTGCTGCCTCACCTCAC	TCTCGTCTGCCTTCATCCTTCTC
Fizz-1	TGCCAATCCAGCTAACTATCCCT	CACAAGCACACCCAGTAGCAG
Ym-1	CTACTATACCAGTTGGGCTAAGGAC	TCAATGCTTCATAGTCACGCAAG
Arg-1	GGGACCTGGCCTTTGTTGAT	GCACCACACTGACTCTTCCA
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

plasmid has been further transfected into RAW264.7 cells. According to the manufacturer's protocol, the RAW264.7 cells grown in 6-well plates were transfected with the recombinant plasmid (pcDNA3.1-EGFP/PPARγ) and empty plasmid (pcDNA3.1-EGFP) as negative control mediated by Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, USA), respectively. The cell lysates were harvested and subjected to the analysis of expression levels of PPARγ using RT-qPCR and Western blot.

Animals and Experimental Protocol

Ten male C57BL/6 mice (6–8 weeks old) were purchased from the Animal Experimental Center of Southern Medical University (Guangzhou, China). Thirty male *ApoE*$^{-/-}$ mice (6–8 weeks old) were purchased from Jicui Pharmachem Biotechnology Co., Ltd (Jiangsu, China). Experiments involving animals had approval from the Animal Ethics Committee of First Affiliated Hospital of Guangzhou University of Chinese Medicine (GZTCMF1-2022002) and performed according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. All mice were housed in standard cages in a temperature-controlled environment. All mice were provided with appropriate food and water daily. C57BL/6 mice were fed with a normal diet as the Control group (n = 10). *ApoE*$^{-/-}$ mice were divided into following three groups randomly (n = 10): including the Model group (high-fat diet (HFD), PBS), Gen group (HFD, 25 mg/kg/d Gen), GW9662 +Gen group (HFD, 25 mg/kg/d Gen +1mg/kg/d GW9662). Except for the Control group, other groups were fed with the HFD used for modelling AS (HFD formula: 21% fat, 0.15% cholesterol, 15.5% protein, and 62% common feed; purchased from the Guangdong Medical Laboratory Animal Center). Doses of 25 mg/kg/d of Gen were administered starting from the end of 8th week onwards by gavage and continued until the end of 16th week. Meanwhile, mice in GW9662+Gen group was intraperitoneally induced with doses of 1mg/kg/d of GW9662. After anaesthesia was intraperitoneally induced with 1% sodium pentobarbital, blood was collected from the abdominal aorta to sacrifice all mice.

Detection of Blood Lipids and Inflammatory Factors

The plasma was separated by centrifugation at 3000 rpm for 20 min at 4°C. Total cholesterol (TC), triglyceride (TG), LDL-c and high-density lipoprotein-cholesterol (HDL-C) were determined by Automatic biochemical analyzer (Myriad, DC-220, USA). Concentrations of IL-1β, IL-6, and IL-17A in the serum samples were quantified by ELISA assay according to the manufacturer's protocol.

Haematoxylin and Eosin Staining, Oil-Red Staining, and Immunohistochemistry

The whole aortas from the thoracic to the abdominal isolated from the mice were dehydrated, embedded in paraffin, and cut into 4 μm sections. The sections were then stained with haematoxylin and eosin (H&E) for the histological assessment of lesion size in the aortic. For Oil-Red staining, the whole tissues were immersed in the stain and then differentiated with ethanol according to the manufacturer's protocol. For immunohistochemistry, paraffin-embedded serial sections were immunostained using primary antibodies against a macrophage marker (MOMA-2) and PPAR*γ*, followed by incubation with the corresponding secondary antibodies. The quantitative data were calculated by Image-Pro Plus (Media Cybernetics, USA).

Statistical Analysis

Data were presented as the mean ± SD (standard of the mean). The data with the number of groups of two groups were compared between the two groups using the Independent Samples *t*-test, while multiple intergroup comparisons was evaluated by one-way ANOVA (more than two groups) with Bonferroni post-test using SPSS 22.0 (SPSS, USA). Values of $p < 0.05$ were considered statistically significant.

Results

Effects of Gen on Macrophage Polarization in RAW264.7

The concentration of Gen on RAW264.7 cells was determined by evaluating the cell viability by CCK8 assay. RAW264.7 cells were incubated with different concentrations (0, 5, 10, 20, 50, 100, 200, 500 μM) of Gen for 24 hours. As shown in

Figure 2A, the viability of RAW264.7 cells was not affected by Gen at concentrations 5–100 μM. Therefore, 5, 10, 20 μM were selected for subsequent experiments to avoid drug-induced toxicity to RAW264.7 cells.

To further investigate the induction of Gen on M2 polarization, RAW264.7 cells were subjected to staining with inducible nitric oxide synthase (iNOS, M1 marker), Arginase-1 (Arg-1, M2 marker), and DAPI (nucleus). The representative figures are shown in Figure 2C. Results showed that there was no significant difference among groups in iNOS ($p > 0.05$). Compared with the Control group, there was no significant difference in fluorescence intensity of Arg-1 between 5 μM and 10 μM Gen groups, while 20 μM Gen group had stronger level of fluorescence ($p < 0.05$) (Figure 2B). The gene expression levels of macrophage phenotype markers determined by RT-qPCR were depicted in Figure 2D. There were no significant differences in the expression of chemokine receptor 7 (*CCR7)* and interleukin-1β (*IL-1β*) among different groups. In contrast, when the concentration of Gen reached 10 μM and 20 μM, the gene expression levels of *CD163* and *IL-10* were significantly increased to varying extents compared with the relevant control ($p < 0.05$).

Geniposide Activated PPARγ

To verify whether Gen activated PPARγ, HEK293 cells were transfected with *PPARγ* expression and the PPRE-driven luciferase reporter plasmids, followed by exposure to Gen. The luciferase reporter assay showed that Gen enhanced the *PPARγ* activity significantly. Among the tested concentrations, 20 μM Gen exerted the most prominent activation effect on *PPARγ*, which was comparable to that of the agonist RGZ ($p < 0.05$)(Figure 3A). Further, we examined the effects of Gen on the expressions of endogenous PPARγ target genes, including cluster of differentiation 36 (*CD36*) and ATP binding cassette subfamily G member 1 (*ABCG1*) in RAW264.7 cells. Results showed that mRNA levels of *CD36* and *ABCG1* were increased by Gen. Notably, GW9662, a selective antagonist of PPARγ, effectively attenuated the induction of *CD36* and *ABCG1* by Gen, suggesting that Gen might have a specific mechanism on PPARγ (Figure 3B). We next investigated the time-dependent in the protein levels of total and phosphorylated PPARγ in the nucleus and cytoplasm, respectively. Gen significantly increased the level of phospho-PPARγ at 120 min and 180 min of intervention, while having no effect on total protein level of PPARγ in the cytoplasm within the observed time periods. In contrast, Gen upregulated total protein level of PPARγ in the nucleus at 120 min and 180 min, with no changes in expression of phosphorylated PPARγ significantly. To evaluate the impact of Gen intervention on PPARγ activation, we calculated the ratio of p-PPARγ/PPAR in both the nucleus and cytoplasm. The results demonstrate that Gen activated the phosphorylation of PPARγ in the cytoplasm while reducing PPARγ activation in the nucleus (Figure 3C and D). Taken together, we speculate that Gen could promote the outward transfer of phosphorylated PPARγ from the nucleus in macrophages.

PPARγ Transcriptionally Activated M2 Polarization in RAW264.7 Cells

To explore the role of PPARγ in macrophage polarization, RAW264.7 cells were treated with RGZ or GW9662 for 24 h according to observe the changes of M2 polarization markers, including found in inflammatory zone 1 (Fizz-1), chitinase-like secretory protein (Ym-1) and Arg-1. To confirm that RGZ exerts its effects through PPARγ, we treated RAW264.7 cells with GW9662 before the exposure to RGZ in the combined group. Expressions of Fizz-1, Ym-1 and Arg-1 were induced in the RGZ group, while abrogated in the RGZ+GW9662 group significantly. Consistent changes of mRNA and protein levels were observed in intervention groups (Figure 4A–C). To further validate the regulatory effects of PPARγ on Fizz-1, Ym-1 and Arg-1, we overexpressed PPARγ in RAW264.7 cells using a recombinant adenovirus. Results demonstrated that overexpression of PPARγ increased the expressions of Fizz-1, Ym-1 and Arg-1 genes at both mRNA and protein levels, which could help further identify the role of PPARγ in our study (Figure 4D–F).

Geniposide Promoted Macrophage M2 Polarization via PPARγ Activation

To investigate whether PPARγ activation is required for the macrophage M2 polarization induced by Gen, RAW264.7 cells were pre-treated with GW9662 to block PPARγ activity prior to exposure to Gen. Compared with Gen group, the gene expressions of *Fizz-1, Ym-1* and *Arg-1* were decreased significantly (Figure 5A), consistent with the trend of protein expression (Figure 5B–D), which suggests that GW9662 attenuated the effects of Gen on mRNA and protein levels. These findings demonstrate that Gen promotes macrophages M2 polarization through a PPARγ-dependent mechanism.

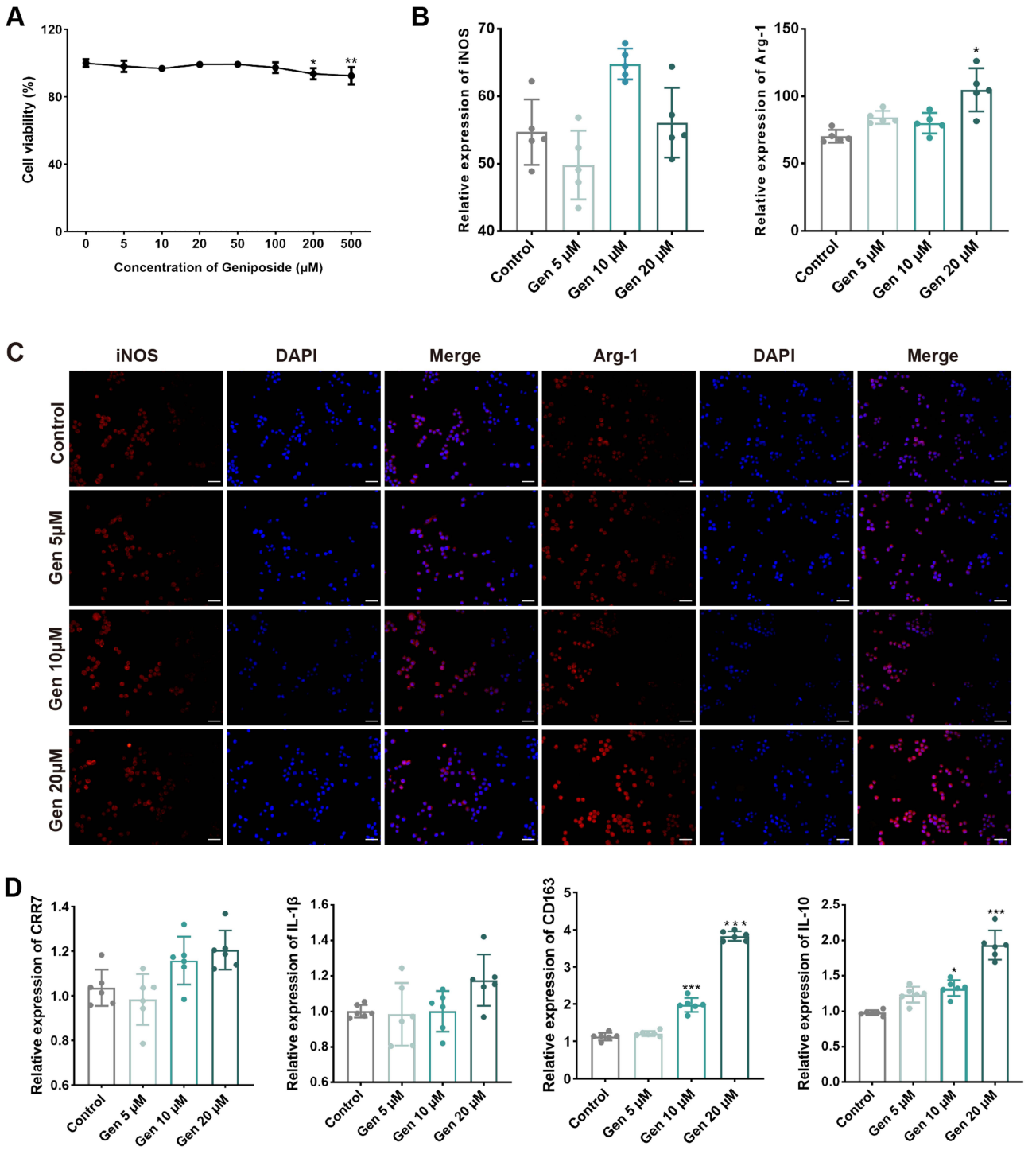

Figure 2 Effects of Gen on macrophage polarization in RAW264.7. (**A**) Cell viability of RAW264.7 which were treated with Gen (0, 5, 10, 20, 50, 100, 200, 500 μM) for 24 h measured by CCK-8 (n=6). (**B**) Immunofluorescent detection of the iNOS (M1 marker, Red fluorophores), Arg-1 (M2 marker, Red fluorophores), and DAPI (nucleus, Blue fluorophores) after treating with Gen (5, 10, 20μM) (n=5). (**C**) Quantitative analysis of Immunofluorescent. Scale bar, 25 μm. (**D**) RT-qPCR results of mRNA markers represents M1 macrophages (*CCR7* and *IL-1β*) and markers represents M2 macrophages (*CD163* and *IL-10*) after treating with Gen (5, 10, 20μM) (n=6). Data were expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs Control group.

Gen Has an Anti-AS Effect via Regulating Macrophage Polarization and PPARγ in vivo

To determine whether Gen can influence AS by improving the lipid metabolism, the TC, TG, LDL-c, and HDL-c of serum were analyzed. The results showed that the serum levels of TC, TG and LDL-c in Model group were significantly higher than the Control group, while there is no significant difference in HDL-c. After 8 weeks of treatment with Gen at a dose of

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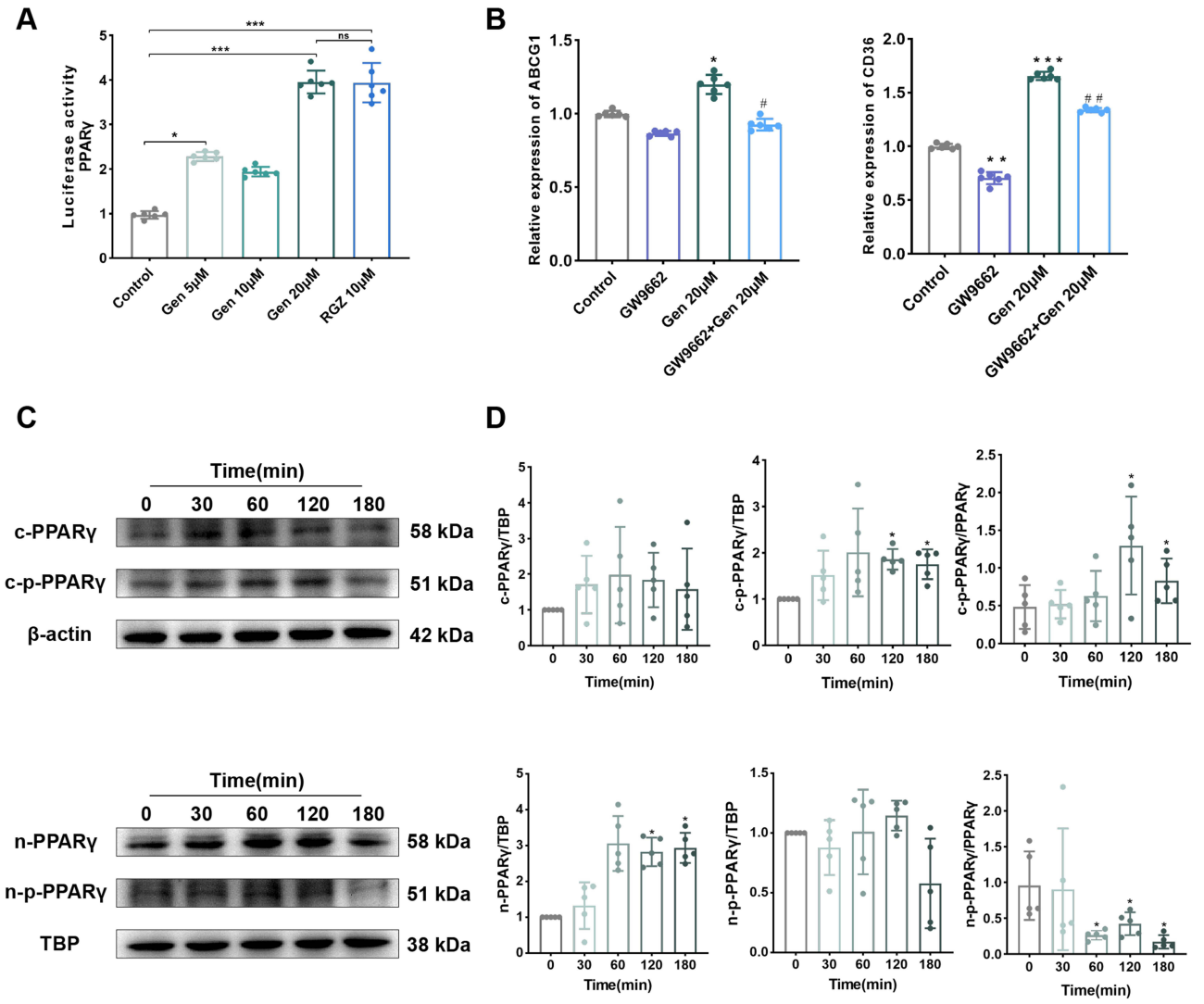

Figure 3 Gen activated PPARγ in RAW264.7 cells. (**A**) HEK293 cells were transfected with PPRE- luciferase reporter plasmids and then treated with Gen (5, 10, 20 μM) or RGZ (10 μM, as positive control) for 24 h(n=6). (**B**) RAW264.7 cells were pre-treated with GW9662 (5 μM) for 1 h and exposed to Gen (20 μM) for 24 h. Gene expressions of *CD36* and *ABCG1* were measured by RT-qPCR (n=6). (**C**) RAW264.7 cells were treated with Gen (20 μM) for 0, 30, 60, 120, 180 min. Total and phosphorylated-PPARγ levels in nuclear and cytoplasm extracts were analyzed using Western Blot (n=5). (**D**) Quantitative analysis of c-PPARγ (total PPARγ in cytoplasm), c-p-PPARγ (phosphorylated-PPARγ in cytoplasm), n-PPARγ (total PPARγ in nuclear), n-p-PPARγ (phosphorylated-PPARγ in nuclear) levels, with TBP as the control. Data were expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs Gen group.

25 mg/kg/d, the serum TC, TG, and LDL-c levels were reduced. Then the therapeutic effect was inhibited after administration of GW9662 (Figure 6A). We further evaluated the impact of Gen on serum inflammation levels in *ApoE*$^{-/-}$ mice. Expression level of inflammatory cytokines IL-1β, IL-6 and IL-17A were down-regulated in the Gen group compared with the Model group and up-regulated in the combined group (Figure 6B). To visually evaluate the aortic plaque, H&E and Oil-Red staining were performed to measure the plaque area in the sections and tissues. Results showed that the lesion area in the Model group was significantly larger than that in the Control group. Compared with the Model group, Gen significantly reduced the plaque area, and this inhibitory effect on plaque formation was reversed by co-treatment with GW9662 (Figure 6C and D). Similarly, quantitative analysis of Oil-Red staining also demonstrated that Gen attenuated AS plaque formation, and this effect was blocked by GW9662 (Figure 6E and F). These findings suggest that the model in vivo induced by high-fat diet promotes AS progression and triggers an inflammatory response. Gen can effectively inhibit this pathological process, and this inhibitory effect can be antagonized by GW9662.

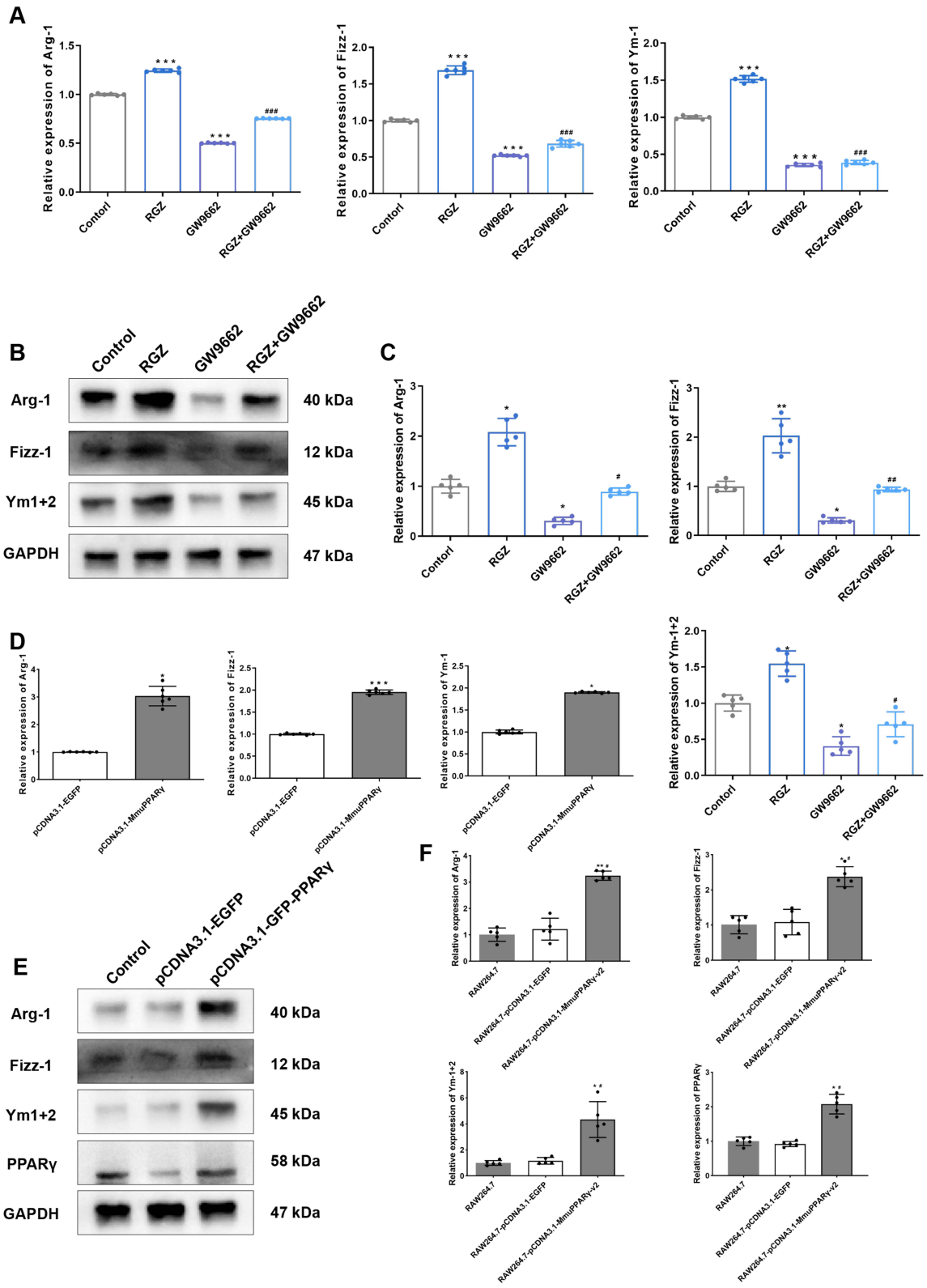

Figure 4 PPARγ transcriptionally activated M2 polarization in RAW264.7 Cells. (**A**) Gene expressions of *Fizz-1, Ym-1* and *Arg-1* were measured by RT-qPCR (n=6) after treating with RGZ (10 μM) or GW9662 (5 μM). (**B**) Expressions of Fizz-1, Ym-1 and Arg-1 were examined at the protein level by using Western Blot (n=5) after treating with RGZ (10 μM) or GW9662 (5 μM). (**C**) Quantitative analysis of Fizz-1, Ym-1 and Arg-1, with GAPDH as control. (**D**) RAW264.7 cells were infected with empty plasmid (pcDNA3.1-EGFP, as negative control) or recombinant plasmid (pcDNA3.1-MmuPPARγ) and then verified (n=6). (**E**) The protein levels of PPARγ, Fizz-1, Ym-1 and Arg-1 were measured by Western Blot (n=5). (**F**) Quantitative analysis of PPARγ, Fizz-1, Ym-1 and Arg-1, with GAPDH as control. Data were expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs GW9662 group in A and C; $^{\#}p < 0.05$ vs pcDNA3.1-EGFP group in F.

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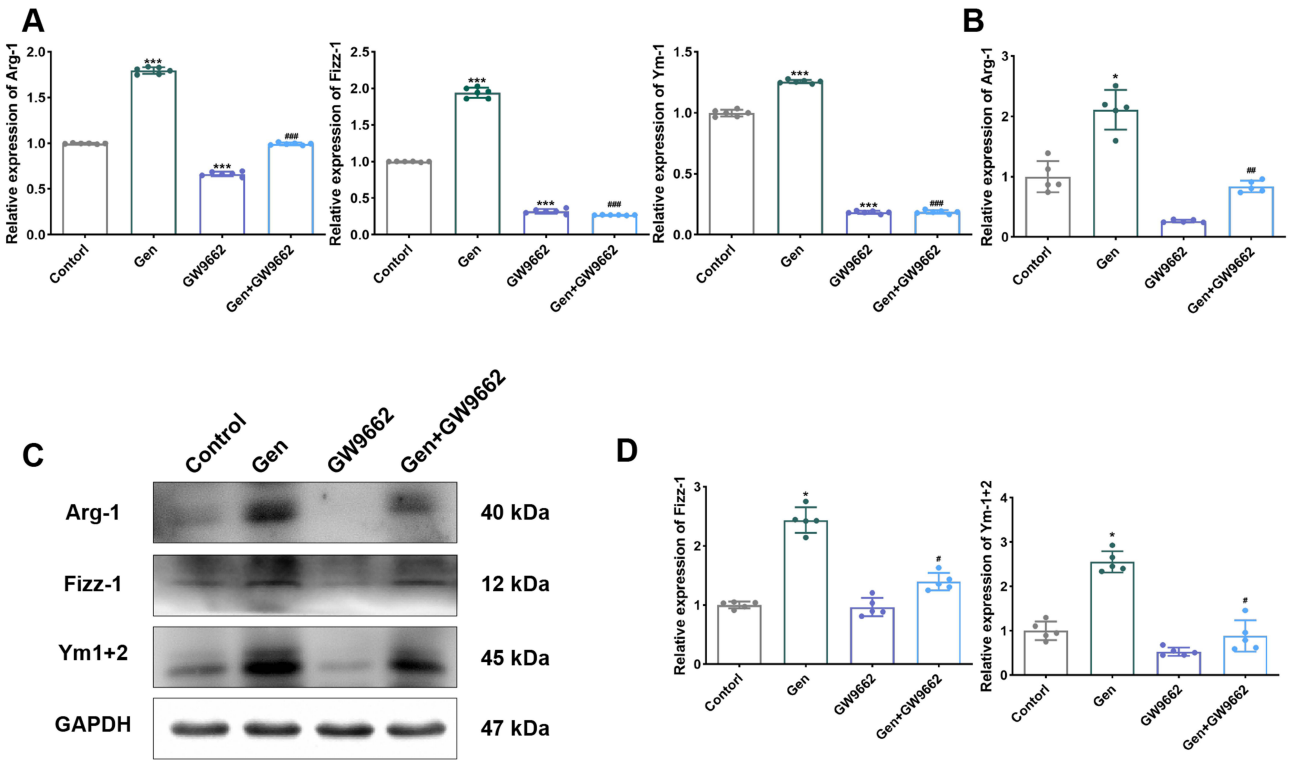

Figure 5 Gen promoted macrophage M2 polarization via PPARγ activation. (**A**) RAW264.7 cells were pre-treated with GW9662 (5 μM) for 1 h and exposed to Gen (20 μM) for 24 h. Total RNA was extracted and subjected to RT-qPCR for the assessments of *Fizz-1, Ym-1* and *Arg-1* levels (n=6). (**B**) Quantitative analysis of Arg-1 with GAPDH as control. (**C**) Expressions of Arg-1, Fizz-1 and Ym-1 were measured by Western Blot (n=5). (**D**) Quantitative analysis of Fizz-1 and Ym-1, with GAPDH as control. Data were expressed as mean ± SD. *$p < 0.05$, ***$p < 0.001$ vs Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs Gen group.

Furthermore, to assess whether Gen exerts its anti-AS effect by affecting macrophage phenotype and the expression of PPARγ, we performed immunohistochemical analysis of MOMA-2 and PPARγ in the aortic tissue. Results showed that the relative expression of MOMA-2 in the Gen group was lower than that in the Model group. Compared with the Gen group, the MOMA-2 was increased significantly in the Gen+GW9662 group (Figure 7A and C). Additionally, quantitative analysis revealed that the expression of PPARγ was opposite to that of MOMA-2 (Figure 7B and D). Similar to PPARγ, Arg-1 positive area in immunofluorescence was decreased in the Model group, while Gen treatment led to an increase in Arg-1 expression in the Gen group (Figure 7E and G). To further evaluate the extent of M2 polarization, we examined the mRNA levels of representative marker (*Fizz-1, Ym-1* and *Arg-1*). Results showed markedly decreased *Fizz-1, Ym-1* and *Arg-1* in Model group compared with Control group, indicating the a low level of M2 polarization. Compared with Model group, the expression of these markers in Gen group was increased significantly (Figure 7F). In summary, these results suggest that Gen primarily exerts its inhibitory effect on AS progression by regulating macrophage phenotype through PPARγ.

Discussion

Coronary heart disease (CHD) has surpassed tumor to become the most significant health problems in the world. As the main cause of CHD, AS has been a research focus in the medical field.[14] It is well established that macrophages play a dominant role,[15] because they are involved in all stages of plaque formation and progression.[16] Stabilizing AS plaque is widely recognized as the most critical strategy for preventing cardiovascular events. Currently, statins and other lipid-lowering drugs serve as first-line therapeutic options; however, their associated side effects and residual risks limit their ability to achieve optimal clinical outcomes.[17]

Traditional Chinese medicine is widely used in the prevention and treatment of atherosclerotic diseases due to its few side effects and long-lasting effects.[18–20] For the monomer components extracted from different herbs, researchers

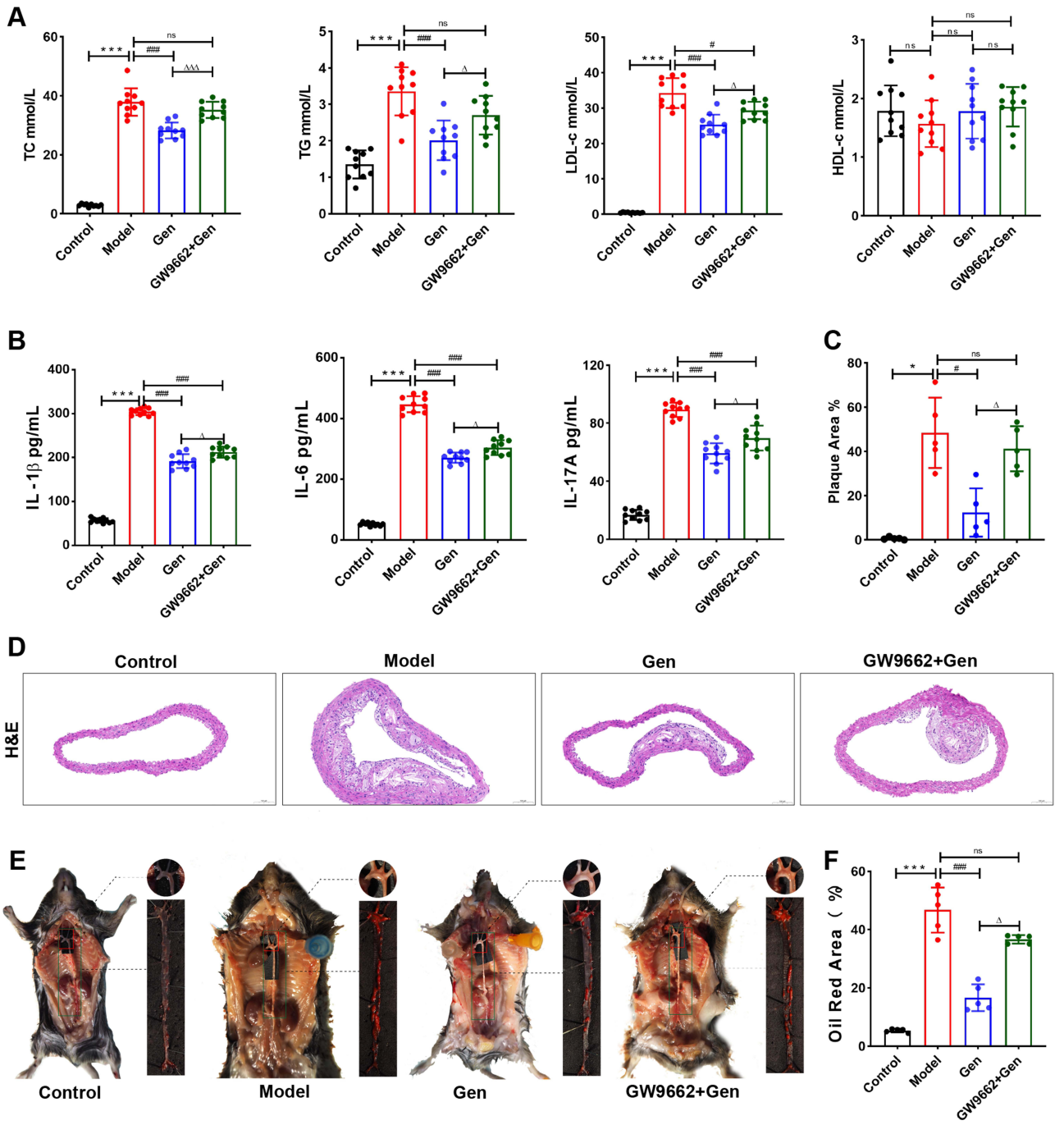

Figure 6 Gen has an anti-AS effect via regulating macrophage polarization and PPARγ in vivo. The ApoE$^{-/-}$ mice were fed a high-fat diet for 16 weeks to induce AS. They also underwent GW9662 (a PPARγ inhibitor, 1mg/kg/d, i.p.) and Geniposide (25 mg/kg/d, i.g.) intervention for eight weeks. (**A**) Levels of TC, TG, LDL-C, HDL-C in serum (n=10). (**B**) The levels of inflammatory factors IL-1β, IL-6 and IL-17A (n=10). (**C**) Quantitative analysis of H&E staining. (**D**) Representative images of H&E staining of plaque in the aortic (n=5). (**E**) Representative images of Oil-Red staining of plaque in the aortic (n=5). (**F**) Quantitative analysis of Oil-Red staining. Data were expressed as mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$ vs Control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs Model group; $^{\Delta}p < 0.05$, $^{\Delta\Delta\Delta}p < 0.001$ vs Gen group.

explored their roles in regulating macrophage polarization. *Saffron sativus* could not only decrease LDL-c in a rat model induced by VD3 but also aggravate the degree of AS lesions by inducing macrophage polarization.[21] *Curcumin* has been shown to inhibit extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 phosphorylation in vitro. Furthermore, *Curcumin* could inhibit AS partly via regulating macrophage polarization, which depends on toll receptor 4 (TLR4) expression.[22] *Ginsenoside Rb1* has been proved to promote stability of atherosclerotic plaque by

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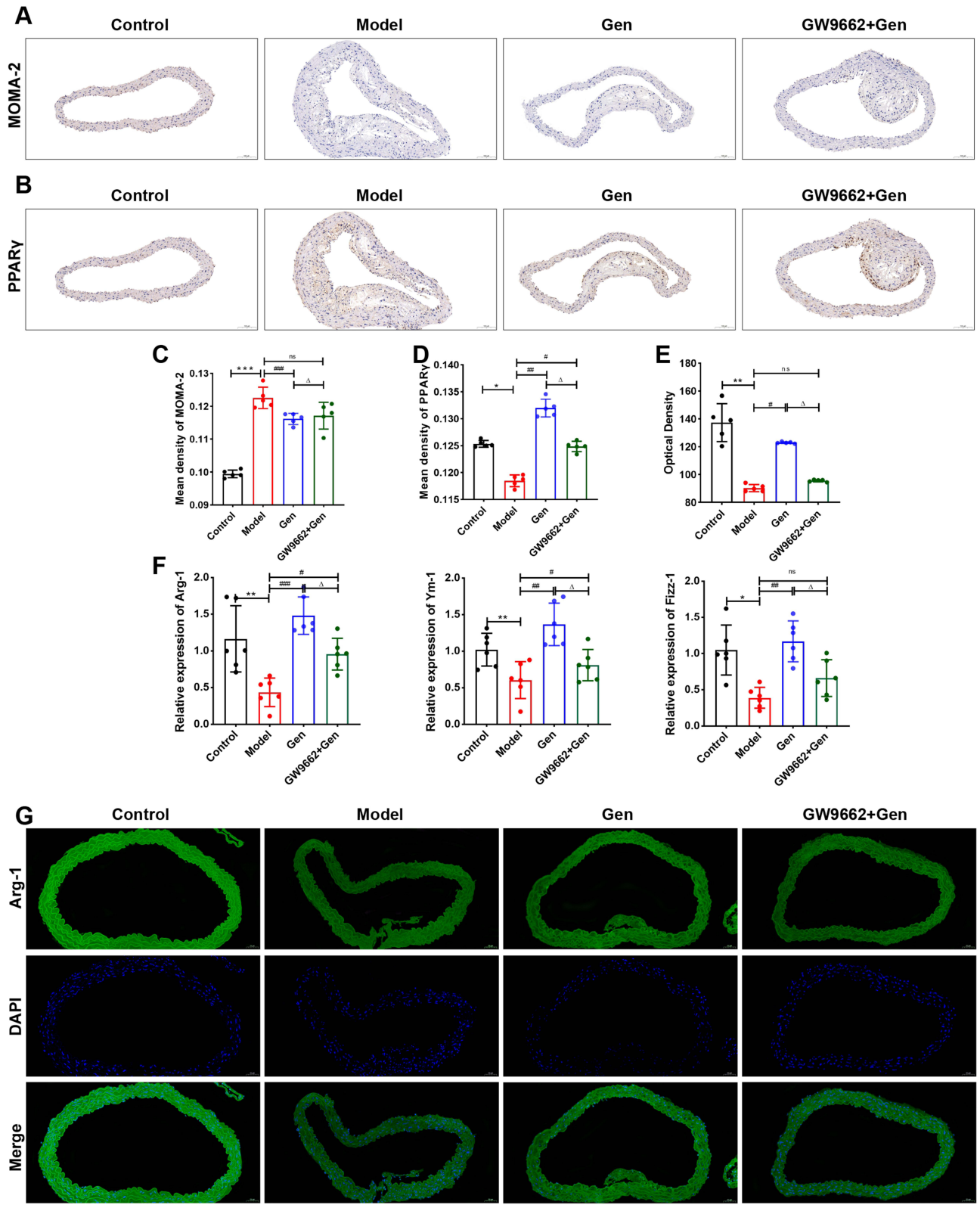

Figure 7 Effects of Gen on macrophage phenotype and PPARγ. (**A**) Representive images of Immunohistochemistry with MOMA-2 (n=5). (**B**) Representive images of Immunohistochemistry with PPARγ (n=5). Scale Bar: 100 μm. (**C**) Quantitative analysis of MOMA-2. (**D**) Quantitative analysis of PPARγ. (**E**) Quantitative analysis of Arg-1. (**F**) Total RNA in aorta tissue was extracted and subjected to RT-qPCR for the assessments of *Fizz-1*, *Ym-1* and *Arg-1* levels (n=6). (**G**) Representive images of Immunofluorescence with Arg-1. Scale Bar: 50 μm. Data were expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs Model group; $^{\Delta}p < 0.05$ vs Gen group.

polarizing macrophages into M2 in *ApoE*$^{-/-}$ mice.[23,24] These pieces of evidence indicate that traditional Chinese herbal ingredients have potential for stabilizing AS plaque to a certain extent by regulating macrophage polarization, even though the experimental models used in these studies differ from each other.

One feature of AS is the chronic inflammation of vessel walls.[25] Macrophages, which are derived from monocytes, could respond to various microenvironmental stimuli, exhibit different polarization procedures, and further mediate inflammation.[26] Several studies have confirmed that the relative proportion of macrophage subsets within plaques could reflect the phenotype characteristics and stability of the plaques.[27] Classically activated M1 macrophages participate in pathogen clearance during infection by activating the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system and the subsequent generation of reactive oxygen species.[28] Local aggregation of M2 macrophages with high ability to clear apoptotic cells helps to eliminate inflammation and prevent the formation of necrotic cores in plaques, which is closely associated with plaque stability.[29] These evidence suggests that different types of macrophage polarization are directly involved in the progression of AS by regulating inflammatory responses. In our study, the degree of aortic plaques showed a trend consistent with changes in typical inflammatory factors, which aligns with previous research findings.

Macrophage populations can be activated by a variety of diverse cytokines and identified by the presence of specific surface markers. In this study, the expression levels of iNOS and Arg-1 were selected as the detection criteria of polarisation levels. The expression of iNOS, regarded as one of markers of M1 macrophages, regulates both the gene expression and the differentiation of M1 macrophages.[30] The high expression of CD86 coenzyme regulatory molecules is another characteristic of M1 macrophages.[31] Another marker with a similar expression trend is CCR7.[32] CCR7 is expressed on several types of immune cells, including semi-mature and mature dendritic cells (DC), monocytes/macrophages, and naïve B cells and T cells. Recent findings have revealed that CCR7, on circulating monocytes is representative of the patient's disease activity score.[33] The expression of CCR7 was found to be the hallmark M1 macrophages and its levels were potentiated in response to M1 mediating factors and curtailed by M2 mediators in naïve macrophages. Arg-1 is a typical marker for M2 macrophage action, and enzyme products in its pathway contribute greatly to tissue repair.[34] Similar to other biomarkers, Fizz-1 and Ym-1 are strongly induced in alternatively activated macrophages both in vivo and *in vitro*.[35] In addition, CD163 and CD206 are also specific markers of M2 subtypes. CD206, a transmembrane glycoprotein of macrophages, is expressed at a low level during inflammation and at a high level during inflammation regression, which could limit secondary inflammation-mediated damage.[36] CD163, a glycoprotein belonging to the cysteine-rich Scavenger receptor superfamily, has the similar expression trend upon macrophage activation.[37] Observing the expression changes of these typical markers can more accurately reflect the differentiation status of macrophages.

Peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor family, is involved in several important physiological processes, such as glucose homeostasis and cell differentiation, as well as lipid metabolism.[38] In addition to signaling pathways mentioned above, the activation of PPARγ pathway also has been proven to be one of the typical pathway to induce to M2 polarization.[39,40] A research has confirmed that PPARγ deficiency exacerbates AS and triggers vascular and systemic inflammation, providing new perspectives for understanding AS and cardiovascular diseases in general.[41] The mechanism by which pigment epithelium-derived factor enhances plaque stability by inhibiting M1 polarization has been demonstrated to involve PPARγ and its downstream MAPK signaling pathway.[42] Oleoylethanolamide improved atherosclerotic plaque stability through regulating macrophage polarization via AMPK-PPARα pathway.[43] In macrophages, anti-inflammatory signals are transmitted via the PPARγ signaling pathway and its target genes, including CD36 and ABCG1, which promote the transcription of anti-inflammatory cytokines, thereby reducing the susceptibility of atherosclerotic plaques to thrombotic events.[9]

Current studies have shown that certain components derived from traditional Chinese medicine can also interfere with PPARγ and its related targets and pathways. A study demonstrates that *Quercetin* regulates ABCA1 protein expression and increases the HDL-C level via the activation of the PPARγ-LXRα pathway, and consequently reduces the risk of atherogenesis.[44] Both QiShenYiQi pill and LXR-α agonist downregulated the CD36 expression and upregulated PPARγ-LXRα/β-ABCA1 expression in atherosclerotic plaque.[45] *Ganoderic acid A* inhibited ox-LDL-induced macrophage inflammation and lipid deposition in human monocyte leukemia cells (THP-1) via Notch1/PPARα/CD36 signaling

pathway. Some researches suggest that the ethanol extract of black mulberry might alleviate AS-related inflammatory reaction via the ox-LDL-PPARγ-CD36 feed-forward cycle.[46]

In conclusion, our in vitro experiments demonstrated that Gen primarily promotes M2 macrophage polarization in a PPARγ-dependent manner. This conclusion is supported by the findings that Gen activates the PPARγ reporter and induces the expression of endogenous PPARγ target genes. More importantly, the effects of Gen on target genes and M2 markers were attenuated by GW9662, a selective PPARγ antagonist. Moreover, we found that Gen increased PPARγ phosphorylation in the cytoplasm, which might be a potential mechanism underlying its regulation of PPARγ activity.

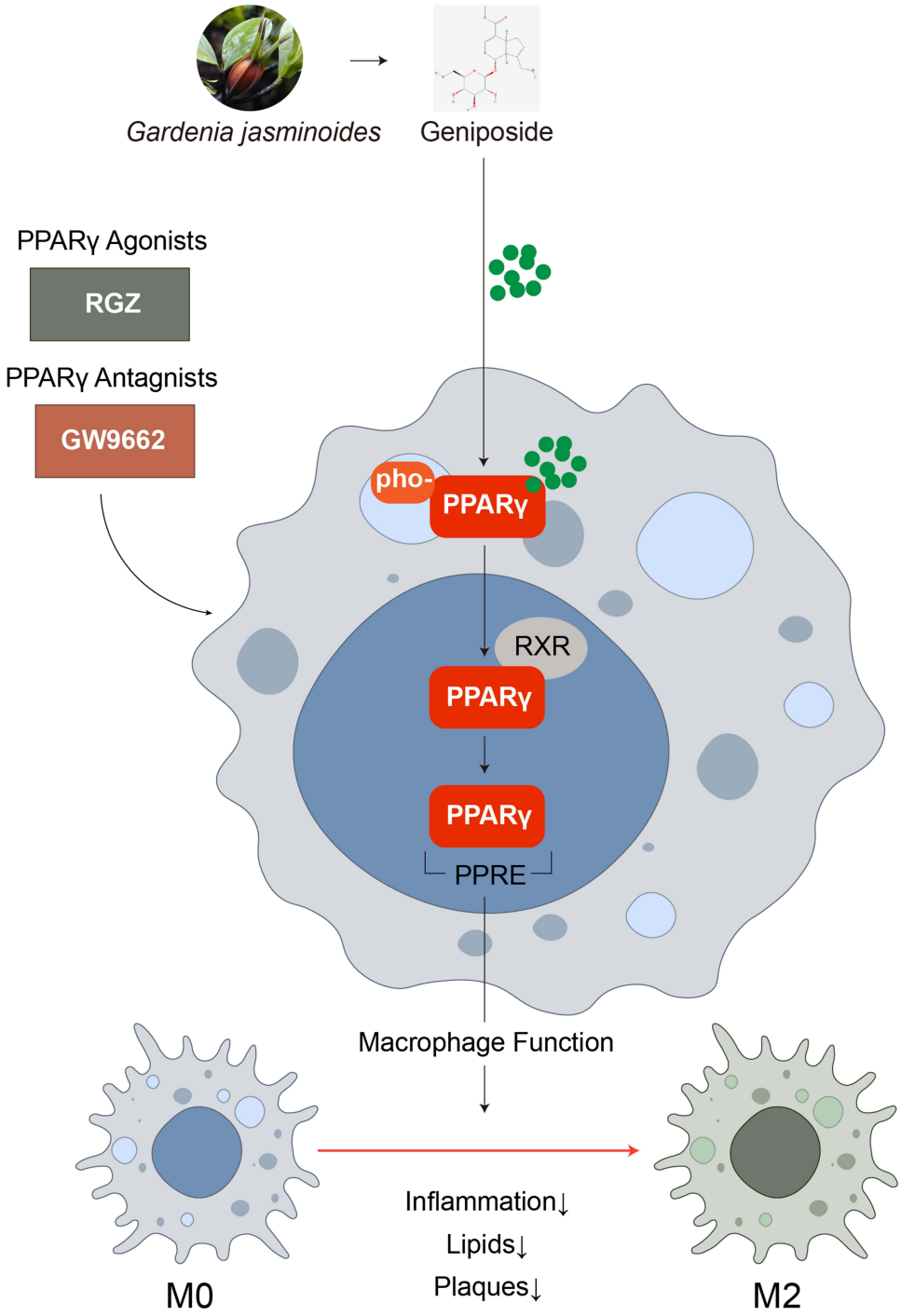

Figure 8 Gen regulates the macrophage polarization to M2 phenotype through PPARγ, which to stabilize plaque and inhibit inflammation.

The present study further confirmed that PPARγ activation is essential for Gen -induced M2 polarization. Specifically, both the PPARγ agonist and transfected PPARγ groups increased the induction of Arg1, Ym1, Fizz1 at both mRNA and protein levels. Conversely, the induced expressions of Arg1, Ym1, Fizz1, which were M2 macrophage phenotypic markers, were attenuated by inhibition of PPARγ. The results, combined with previous published studies, support the inference that the activation of PPARγ may play a remarkable role in macrophage polarization during AS progression. Meanwhile, the expression of gene-induced M2 macrophage markers could be attenuated by PPARγ antagonist. Subsequently, the anti-inflammatory and plaque-stabilizing effects of Gen were verified in vivo. It is confirmed that Gen regulates the macrophage polarization to M2 phenotype through PPARγ, which to stabilize plaque and inhibit inflammation (Figure 8). These results suggest that Gen may serve as a potential therapeutic agent for stabilizing AS plaques and reducing the incidence of malignant cardiovascular events.

It is worth noting that Gen can be converted into a variety of products through metabolism in vivo. Therefore, further study on the pharmacodynamics and pharmacokinetics of Gen will help to clarify the therapeutic effect and pharmacological model of Gen in the prevention of AS. Our current study represents a preliminary exploration on the effect of Gen in the treatment of AS, and more comprehensive and precise mechanistic research still needs to be further investigated.

Conclusions

To sum up, our study confirmed that Gen can mediate PPARγ pathway to regulate the macrophage polarization to M2 phenotype in vitro. Meanwhile, PPARγ is the core target of Gen regulating macrophage polarization to exert anti-inflammatory and stable AS plaque in vivo. This finding reveals a new mechanism of Gen in the treatment of AS-related diseases.

Ethics Approval and Consent to Participate

Experiments involving animals had approval from the Animal Ethics Committee of First Affiliated Hospital of Guangzhou University of Chinese Medicine (GZTCMF1-2022002) and performed according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interests.

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