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Original article

Genistein promotes M1 macrophage apoptosis and reduces inflammatory response by disrupting miR-21/TIPE2 pathway

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ABSTRACT

Cardiovascular diseases are a major cause of mortality, and vascular injury, a common pathological basis of cardiovascular disease, is deeply correlated with macrophage apoptosis and inflammatory response. Genistein, a type of phytoestrogen, exerts cardiovascular protective activities, but the underlying mechanism has not been fully elucidated. In this study, RAW264.7 cells were treated with genistein, lipopolysaccharide (LPS), nuclear factor-kappa B (NF-κB) inhibitor, and/or protein kinase B (AKT) agonist to determine the role of genistein in apoptosis and inflammation in LPS-stimulated cells. Simultaneously, high fat diet-fed C57BL/6 mice were administered genistein to evaluate the function of genistein on LPS-induced cardiovascular injury mouse model. Here, we demonstrated that LPS obviously increased apoptosis resistance and inflammatory response of macrophages by promoting miR-21 expression, and miR-21 downregulated tumor necrosis factor-α-induced protein 8-like 2 (TIPE2) expression by targeting the coding region. Genistein reduced miR-21 expression by inhibiting NF-κB, then blocked toll-like receptor 4 (TLR4) pathway and AKT phosphorylation dependent on TIPE2, resulting in inhibition of LPS. Our research suggests that miR-21/TIPE2 pathway is involved in M1 macrophage apoptosis and inflammatory response, and genistein inhibits the progression of LPS-induced cardiovascular injury at the epigenetic level via regulating the promoter region of Vmp1 by NF-κB.

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide and pose an enormous financial health-care burden, chronic inflammation of arterial wall is a major underlying factor of CVDs (Evans et al., 2020). Macrophages actively participate in all stages of cardiovascular injury, occurrence of inflammation and lesion regression are strongly dependent upon them (Gonzalez and Trigatti, 2017, Back et al., 2019), especially the progression of macrophages to foam cells by uptaking oxidized lipoproteins, which is an important link in the development of intima-media inflammatory CVD (Back et al., 2019). Notably, recent studies suggest a central role for epigenetic mechanisms about the pro-inflammatory M1-like macrophages, and the balance of pro-/anti-inflammatory mechanism may determine the clinical outcome (Back et al., 2019, Yang et al., 2022). Furthermore, pro-inflammatory macrophage apoptosis in early lesions contributes to depressed local inflammatory reactions and secondary cell necrosis, suppressing the progression of CVDs (Martinet et al., 2019). Therefore, promoting macrophage apoptosis and reducing inflammatory reactions provide a novel pharmacological strategy to resolve cardiovascular injury.

Abbreviations: 3′ UTR, 3′ untranslated region; ABCG1, ATP-binding cassette transporter G1; AKT, protein kinase B; AP-1, activating protein-1; BAX, BCL-2 associated X; BCL-2, B cell lymphoma-2; CCK-8, cell counting kit-8; ChIP, chromatin immunoprecipitation; CHOP, C/EBP homologous protein; COX-2, cyclooxygenase-2; CVDs, cardiovascular diseases; DMEM, Dulbecco's modified eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GEN, genistein; HSP90, heat shock protein 90; IF, immunofluorescence; IGF-1, insulin-like growth factor-1; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MUT, mutant; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor-kappa B; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PDCD4, programmed cell death 4; PDTC, pyrrolidine dithiocarbamate; PMφs, peritoneal macrophages; qRT-PCR, quantitative real-time polymerase chain reaction; STAT3, signal transducer and activator of transcription 3; TiO2 NPs, titanium dioxide nanoparticles; TIPE2, tumor necrosis factor-α-induced protein 8-like 2; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN-β; WT, wild type.

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Genistein (4′,5,7-trihydroxy isoflavone) is a naturally bioactive phytoestrogen derived mainly from the Leguminosae family, its chemical structure is 4,5,7-trihydroxyisoflavone and 5,7-dihydroxy-3-(4-hydroxyphenyl) chromen-4-one (Mukund et al., 2017). Genistein can promote apoptosis, inhibit inflammation, and modulate metabolic pathways (Xin et al., 2019). For example, genistein causes U937 cells to arrest at G2/M phase and significantly induces apoptosis associated with procaspase-3 (Hasan et al., 2020); treatment of wild-type mice with genistein attenuates concanavalin A-induced inflammatory mediator expression (Xu et al., 2018). Interestingly, genistein also influences epigenetic regulation in macrophage. Emerging evidence supports that the histone methylation and acetylation are related to macrophage apoptosis (Cong et al., 2017, Wang et al., 2020), and genistein recovers Klotho by reversing promoter histone 3 hypoacetylation and relieving hypermethylation to mitigate renal fibrosis in mice (Li et al., 2019). In addition, miR-451 is downregulated in various types of liver inflammation, and genistein ameliorates liver inflammation by increasing miR-451 expression in normal liver cells and macrophages (Gan et al., 2019). Furthermore, studies have confirmed that genistein prevents fat deposition in the cardiovascular system, limits the inflammatory response, prevents myocardial ischemia and reperfusion injury, and contributes to a reduction in the incidence of CVDs (Thangavel et al., 2019). It is also becoming apparent that the proapoptotic and anti-inflammatory activities of genistein are related to epigenetic modulation, and clarification of mechanism in the process of vascular injury is urgently required.

miRNAs are endogenous small noncoding RNAs that post-transcriptionally regulate gene expression, acting as epigenetic modulators (Lan et al., 2021), and miRNAs are intimately associated with macrophage apoptosis and inflammation. For instance, miR-23a is downregulated and directly binds to heat shock protein 90 (HSP90) mRNA 3′ untranslated region (3′ UTR), resulting in suppression of M1 macrophage apoptosis (Qiao et al., 2020), and miR-29b-3p reinforces RAW264.7 cells apoptosis after titanium dioxide nanoparticles (TiO_2 NPs) exposure (Xu et al., 2020). miR-21 is highly expressed in activated macrophages and emerged as an important regulator in the anti-inflammatory response of macrophages (Sheedy, 2015). Deletion of miR-21 in macrophages directly targets the activation of MKK3-p38-CHOP and c-Jun N-terminal kinase (JNK) signaling, increases proinflammatory phenotype, and also enhances the degradation of ATP-binding cassette transporter G1 (ABCG1) to facilitate foam cell formation, thus explaining the adverse cardiovascular plaque remodeling in miR-21- deficient mice (Canfran-Duque et al., 2017). In another study on nickel nanoparticle-induced lung inflammation and fibrosis, knockdown of miR-21 is observed to reduce neutrophils and macrophages in the lung, possibly by upregulating Smad7 to alleviate pulmonary toxicity (Mo et al., 2020). Evidence of miRNAs involvement in macrophage apoptosis and inflammation suggests that miRNAs play a vital and complex role in vascular injury closely related to macrophages, and the specific functions and molecular mechanisms merit further research.

miR-21 is involved in apoptosis and inflammation in the pathogenesis of several conditions, including aging, obesity and CVDs (Kura et al., 2020, Gao et al., 2021). nuclear factor-kappa B (NF-κB) is a transcription factor that plays an important role in inflammatory response, cell proliferation and apoptosis (Yi et al., 2019). miR-21 maintains the function of hematopoietic stem cells via NF-κB and is regulated by direct NF-κB binding to the promoter (Wang et al., 2016, Mengjia et al., 2020). Moreover, apoptosis promoter programmed cell death 4 (PDCD4) (Correia de Sousa et al., 2019), zinc finger protein A20 (Xue et al., 2019), and mitogen-activated protein kinase 3 (Canfran-Duque et al., 2017) are miR-21 target genes related to macrophage apoptosis and inflammation. Here, we mainly focus on tumor necrosis factor-α-induced protein 8-like 2 (TIPE2), a death effector domain protein and a negative regulator of immune response, that provides a molecular bridge between miR-21 and apoptosis (Ruan et al., 2014). It is suggested that the relationships between NF-κB, miR-21 and TIPE2 exert significant effects on macrophage apoptosis and inflammation in cardiovascular injury. Protein kinase B (AKT) is a core participant in promoting proliferation against apoptosis (Shariati and Meric-Bernstam, 2019). Studies have shown that TIPE2 overexpression significantly inhibits the phosphorylation of AKT, thereby inhibiting cell proliferation and promoting apoptosis (Lin et al., 2018). In addition, our previous work proved that inflammatory protein toll-like receptor 4 (TLR4) is one of the downstream target proteins of TIPE2 in macrophages (Cong et al., 2021). Hence, elucidating gene crosstalk would be of great help in clarifying the pharmacological mechanism of genistein.

Genistein, as a phytoestrogen, specifically plays an endogenous estrogen-like role in humans (Jaiswal et al., 2019). Yan et al discovered that estrogen significantly downregulated miR-21 in polymyositis patients and rats (Yan et al., 2017). Jiang et al. found that genistein reduced apoptosis and inflammation via TLR4/NF-κB pathway in rats and BV2 microglia cells (Jiang et al., 2021). Furthermore, our previous studies indicated that genistein inhibits chronic cardiovascular inflammation in mice via miR 21/NF κB p65 pathway (Xie et al., 2021). Overall, these findings suggest that genistein exhibits epigenetic modulation characteristics in macrophage apoptosis and inflammation by dominating miR-21, but the detailed mechanism remains elusive. In this study, we explored the epigenetic regulatory role and molecular mechanisms of miR-21 in the protective effect of genistein on blood vessels.

2. Materials and methods

2.1. Cell culture

RAW264.7 cells were obtained from Shanghai Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, China. The cells were cultured in Dulbecco's modified eagle's medium (DMEM, Bioind, Israel) supplemented with 10% (v/v) fetal bovine serum (FBS, Bioind, Israel), 1,000 U/mL penicillin and 100 U/mL streptomycin in cell incubator (Thermo Fisher Scientific, USA) at 37 °C with 5% CO_2. In different cell culture experiments, RAW264.7 cells were treated with lipopolysaccharide (LPS, 1 μg/mL, 24 h), genistein (indicated concentration and time), insulin-like growth factor-1 (IGF-1, 100 ng/mL, 2 h), pyrrolidine dithiocarbamate (PDTC, 10 μM, 0.5 h) alone or in combination.

2.2. Animal models and sample collection

C57BL/6 mice was derived from the C57BL line, which was established in 1921 by C. C. Little and separated into sublines C57BL/10 and C57BL/6 in 1973 (Mekada and Yoshiki, 2021). C57BL/6 mice (male, 6 weeks, 20 ± 2.0 g) used in this study were purchased from Hunan SJA Laboratory Animal Co., Ltd. (certificate no. 43004700059037) and housed in SPF units (22 ± 2 °C, 50–60% relative humidity, with a 12 h light/dark cycle) in Animal Centre at Hunan Normal University. After 7 d of acclimatization, the mice were randomly divided into 3 groups (n = 10 each group). Ctrol group: fed a normal diet; LPS group: fed a high-fat diet and treated with LPS (2 mg/kg·2d, intraperitoneal injection, Solarbio, China); LPS + genistein group: fed a high-fat diet and treated with LPS (2 mg/kg·2d, intraperitoneal injection) and genistein (10 mg/kg·d, intragastric administration, Winherb, China). Drug treatment was determined by referencing previous studies (Kim et al., 2011, Cong et al., 2021, Xie et al., 2021). After 20 weeks, the mice were

sacrificed by cervical dislocation and peripheral blood mononuclear cells (PBMCs), aortas and peritoneal macrophages (PMφs) were harvested (Wolfson et al., 2017, Xie et al., 2021). All procedures strictly agreed with National Institutes of Health guidelines for care and use of laboratory animals and were approved by Ethics Committee of Hunan Normal University.

2.3. Cell viability assays

Cells (1×10^4/well) were seeded in 96-well plates with five replicate wells. After overnight incubation, the cells were treated with drugs. Then, the mediums were discarded, 10 μL Cell Counting Kit-8 reagent (CCK-8, Vazyme, China) and 90 μL DMEM were added into each well, and the cells were incubated at 37 °C with 5% CO_2 for 2 h. Finally, absorption values were measured at 450 nm with a multilabel plate reader (EL800, Bio-tek, USA).

2.4. Cytotoxicity assays

Lactate dehydrogenase (LDH) is an important enzyme in glycolysis pathway, and extracellular LDH is used to assess cytotoxicity. The cells (2×10^5/well) were seeded in 6-well plates, incubated for 24 h, and then treated with different concentrations of genistein. The supernatants were collected, centrifuged at 300 g, 4 ℃ for 10 min and analysed according to the instruction (BC0685, Solarbio, Beijing, China). Afterwards, the absorption value at 450 nm was detected with a multilabel plate reader (EL800, Bio-tek, USA).

2.5. Enzyme-Linked immunosorbent assay (ELISA)

The cells (2×10^5/well) were seeded in 6-well plates overnight and then treated with drugs. TNF-α and Interleukin 6 (IL 6) in the supernatants were quantified using ELISA kits (MultiSciences, Hangzhou, China). In brief, the supernatants were collected, precipitates were removed via centrifugation at 300 g for 10 min, and the clarified supernatants were incubated according to the manufacturer's protocol. Absorbance was examed at 450 nm on a multilabel plate reader (EL800, Bio-tek, USA), and the protein concentration was calculated.

2.6. Flow cytometry to assess cell apoptosis

The cells (2×10^4/well) were seeded in 12-well plates, incubated for one night, then treated with genistein and LPS. Cells were centrifuged at 300 g, 4 °C for 7 min and washed with phosphate buffered saline (PBS, Bioind, Israel) and resuspended in buffer at a density of 3×10^5/mL. Apoptosis was determined using Annexin V-FITC/PI (Vazyme, China) and Annexin V-APC/7-AAD (BD Biosciences, USA) Apoptosis Detection kits, and immediately analyzed by flowcytometry (FACSCCantoII, BD, USA).

2.7. miRNA miroarray analysis

RAW264.7 cells were treated with genistein for 2 h and then stimulated by LPS for another 24 h. After that, total RNA was collected from the cells using TRIzol reagent (R401-01, Vazyme, Nanjing, China) and sent to Oebiotech (China) for miRNA enrichment and detection. miRNA expression profiles were investigated using Agilent Mouse miRNA V21.0 ($|\log_2 FC|>1$, $P < 0.05$). Student's *t*-test was performed to evaluate the differential expression of miRNAs. Differentially expressed miRNAs between two groups were identified through fold change values.

2.8. Quantitative Real-Time polymerase chain reaction (qRT-PCR)

Total RNA from cells and aortas were extracted with TRIzol reagent (R401-01, Vazyme, Nanjing, China), and the purity and concentration were measured using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA). Reverse transcription was performed using HiScript® Ⅱ Q RT SuperMix for qPCR Kit (+gDNA wiper, Vazyme, China) and miRNA 1st Strand cDNA Synthesis Kit (by stem-loop, Vazyme, China). qPCR was carried out with AceQ® qPCR SYBR® Green Master Mix kit (Vazyme, China) using a commercial thermal cycler (CFX Connect, Bio Rad, USA). The qPCR amplification procedure was as follows: initial denaturation at 95 °C for 5 min; 39 cycles of 10 sec at 95 °C, 10 sec at 60 °C, and 30 sec at 72 °C. U6 and 18S were used as housekeeping genes, and all gene expression was analyzed according to the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Supplementary Table 1.

2.9. Western blotting

Cells and aortas were lysed in RIPA buffer (Beyotime, Guangzhou, China), and 40 μg total protein was resolved in 12% or 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membrane, which was then blocked with 5% skimmed milk. Primary antibodies against iNOS (1:2,000, AF0199, Affinity, USA), COX-2 (1:2,000, AF7003, Affinity), CHOP (1:1,000, 2895, CST, USA), BCL-2 (1:1,000, YT0470, ImmunoWay, USA), BAX (1:1,000, YT0455, ImmunoWay), cleaved Caspase 3 (1:1,000, AF7022, Affinity), cleaved Caspase 8 (1:1,000, AF5267, Affinity), TIPE2 (1:1,000, DF3326, Affinity), TLR4 (1:2,000, AF7017, Affinity), TRIF (1:2,000, DF6289, Affinity), MyD88 (1:2,000, AF5195, Affinity), AKT (1:1000, YM3618, ImmunoWay), p-AKT^{Ser473} (1:1000, YP0006, ImmunoWay), and β-actin (1:10,000, AF7018, Affinity) were used. HRP-conjugated corresponding secondary antibodies (1:10,000, S0001 and S0002, Affinity) were used, and antigen–antibody reactions were visualized using an enhanced chemiluminescence detection kit (E411-05, Vazyme, China). ImageJ 1.48 (NIH, Maryland, USA) was used to quantify the intensities of each band. β-actin served as an internal reference.

2.10. Immunofluorescence (IF)

RAW264.7 cells on round coverslips and aortic paraffin sections (4 μm) were incubated with 0.3% hydrogen peroxide in methanol for 20 min and heated in EDTA buffer at 150 °C for 30 min to retrieve antigenic activity. Subsequently, cells on coverslips and tissue sections were blocked with goat serum for 30 min, incubated with antibodies specific for TLR4 (1:100) or TIPE2 (1:100) at 4 °C overnight, and then incubated with DyLight™ 594 goat anti-rabbit IgG secondary antibody (1:1,000, A23430, Abbkine, USA) for 2 h at room temperature in dark. Cells were stained with DAPI for 5 min at room temperature. The stained cells and sections were visualized using a fluorescence microscope (DMi8-M, Leica, Germany).

2.11. Luciferase reporter analysis

To verify miR-21 target gene, RAW 264.7 cells were transfected with miR-21 mimic and pmiR-RB-REPORT™ vector (RiboBio, China) with cloned wild type or mutant miR-21 targeting TIPE2 coding region in the downstream of hRluc, using Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific, USA). After 48 h, Dual-Luciferase® Reporter Assay System was used to analyze luciferase activity value following the manufacturer's instruction.

For the promoter activity reporter assay, vectors (RiboBio, China), containing pGL3-Basic or pGL3-miR-21 Promoter, pcDNA3.1 p65 and pRL-TK were transfected into RAW264.7 cells

by Lipofectamine™ 3000 transfection reagent according to the protocol, followed by the indicated treatments. pRL-TK was applied to correct for variations in transfection efficiency. Then, the cells were harvested, and luciferase activity was detected by Dual-Luciferase® Reporter Assay System (Promega). The data represented the mean ± standard deviation of three independent experiments performed in triplicates.

2.12. Chromatin immunoprecipitation (ChIP)

ChIP assays of miR-21 promoter in RAW264.7 cells were performed using EZ-Magna ChIP Chromatin Immunoprecipitation kit (Millipore, USA) according to the instruction. The cells treated with LPS and genistein were fixed in 1% formaldehyde at room temperature for 10 min. Fixation was stopped by addition of 1/10 vol 1.25 M glycine, and then the cells were incubated for 5 min at room temperature, lysed and sonicated (3 min, 10 s on, 20 s off) to 200∼500 bp fragments. The crosslinked chromatin DNA was immunoprecipitated with anti-NF-κB p65 and Normal IgG. qPCR was applied to detect ChIP DNA. Primers for detecting p65 enrichment at miR-21 promoter were as follows, Forward: TCTCCAAGCCAGAACAAGGA; Reverse: ATTTCGGTTCGGCTTTCTCG.

2.13. Statistical analyses

SPSS 20.0 (Illinois, USA) and GraphPad Prism 6.0 (California, USA) were used for all statistical analyses. The data are presented as means ± standard deviation (SD). Student's *t*-test was applied to compare differences between two groups. One-way analysis of variance with Dunnett, Bonferroni and Dunnett's T3 post hoc test was used for comparisons between multiple groups. Correlation analysis was performed to assess the association between two variables. All statistical analyses were executed using two-tailed *P* values. $P < 0.05$ was taken as the cutoff value.

3. Results

3.1. Genistein promotes M1 macrophage apoptosis and reduces inflammation

To explore the role of genistein in regulating macrophage apoptosis and inflammatory response, we first used genistein (2.5, 5.0, 10.0, 20.0, 40.0 μM) to treat RAW264.7 cells for assessing the safety. After 2 h, compared with the control group, the cell viability and LDH content in the supernatants of macrophages treated with genistein showed no significant changes (Supplementary Fig. 1 A and B). Therefore, the effects of genistein on macrophages within the experimental concentration were due to the biological activities rather than cytotoxicity. LPS, a type of endotoxin, stimulates macrophage M1 polarization (Jiang et al., 2020, Wang et al., 2020); thus we used LPS (1 μg/mL, 24 h) to induce M1 macrophage among RAW264.7 cells and found that the levels of TNF-α, IL-6, iNOS and COX-2 increased significantly (Fig. 1 A-C), which are markers of M1 macrophage. In addition, we observed genistein reversed inflammation and apoptosis resistance of M1 macrophages (Fig. 1 A-E and Supplementary Fig. 1 C), which is consistent with the previous reports (Park et al., 2019, Ortega-Santos et al., 2020). More importantly, genistein promoted M1 macrophage apoptosis in a time-dependent manner (Fig. 1 D and E, and Supplementary Fig. 1 C). These findings suggest that genistein may serve as an inhibitor of apoptosis resistance and inflammation, consequently displaying a vital biological function in cardiovascular injury.

3.2. miR-21 is involved in the increased apoptosis of M1 macrophage and inhibition of inflammatory response induced by genistein

miRNAs have been reported to play a crucial role in mediating apoptosis and inflammation. Hence, we first detected miRNAs expression in macrophages incubated with LPS and genistein via microarray analysis and found that miR-155, miR-21 and miR-125a were the top three downregulated miRNAs (Fig. 2 A). qRT-PCR analysis confirmed miR-155, miR-21 and miR-125a were decreased due to genistein in M1 macrophage (Fig. 2 B). Besides, among them, miR-21 expression was the highest in untreated RAW264.7 cells (Fig. 2 C). Our previous studies showed that genistein alleviates chronic cardiovascular inflammatory response through miR 21/NF κB p65 axis in LPS treated mice (Xie et al., 2021). After combining the microarray data and previously reported results, we mainly focused on miR-21 for further study of the mechanism by which genistein protects blood vessels.

To determine whether the increased macrophage apoptosis and depressed inflammatory response were related to miR-21, we performed stable overexpression and knockdown of miR-21 in RAW264.7 cells by lentiviral infection, respectively. Subsequently, overexpression and knockdown efficiency were detected by qRT-PCR (Fig. 2 D). After that, we treated the cells with genistein and LPS, and cell viability, flow cytometry, Western blot, qRT-PCR analyses illustrated that miR-21 significantly conferred cell sensitivity to LPS, which was disrupted by genistein (Fig. 2 E-H, and Supplementary Fig. 2 A-E). Moreover, we observed that cell viability and inflammation protein levels were decreased and apoptosis was increased in LPS-induced RAW264.7 cells with knockdown of miR-21. More interestingly, repressing miR-21 dramatically enhanced the function of genistein in M1 macrophages (Fig. 2 E-H, and Supplementary Fig. 2 A-E). Taken together, our data showed that knockdown of miR-21 not only accelerated macrophage apoptosis but also reduced inflammatory response, and miR-21 was closely related to proapoptosis and anti-inflammatory effects of genistein. Although miR-21 may be a target of genistein, the detailed molecular mechanism remains elusive.

3.3. TIPE2 is a direct target gene of miR-21

TIPE2, highly expressed in immune cells and organs, induces apoptosis of macrophage. It has been reported that miR-21 suppresses activated $CD4^+$ T cells apoptosis at least in part through directly targeting TIPE2 (Ruan et al., 2014). To better define the correlation between them, we performed qRT-PCR, Western blot and IF assays to evaluate TIPE2 in RAW264.7 cells incubated with genistein and LPS (Fig. 3 A and Supplementary Fig. 3). Considering the above results (Fig. 2 B), we found genistein remarkably enhanced TIPE2 expression, which was reduced by LPS, but miR-21 changes exhibited opposite trends *in vitro*. Consistently, the inverse association was further confirmed by quantification in PBMCs and aortas from mice (Fig. 3 B and C). Combined with the website prediction results (Fig. 3 E), we speculated that miR-21 targets TIPE2.

To further address how miR-21 suppresses endogenous TIPE2 expression, RAW264.7 cells were transfected with miR-21 mimic and inhibitor, and then, TIPE2 was examined by qRT-PCR and Western blot. Notably, we discovered that miR-21 mimic significantly reduced TIPE2 mRNA and protein expression levels, and the opposite results were observed with the inhibitor (Fig. 3 D). These findings suggest that miR-21 negatively regulates endogenous TIPE2 via mRNA degradation and protein translation inhibition. To clarify the mechanisms, we predicted and found binding sites in TIPE2 coding sequence using bioinformatics software. Immediately, we

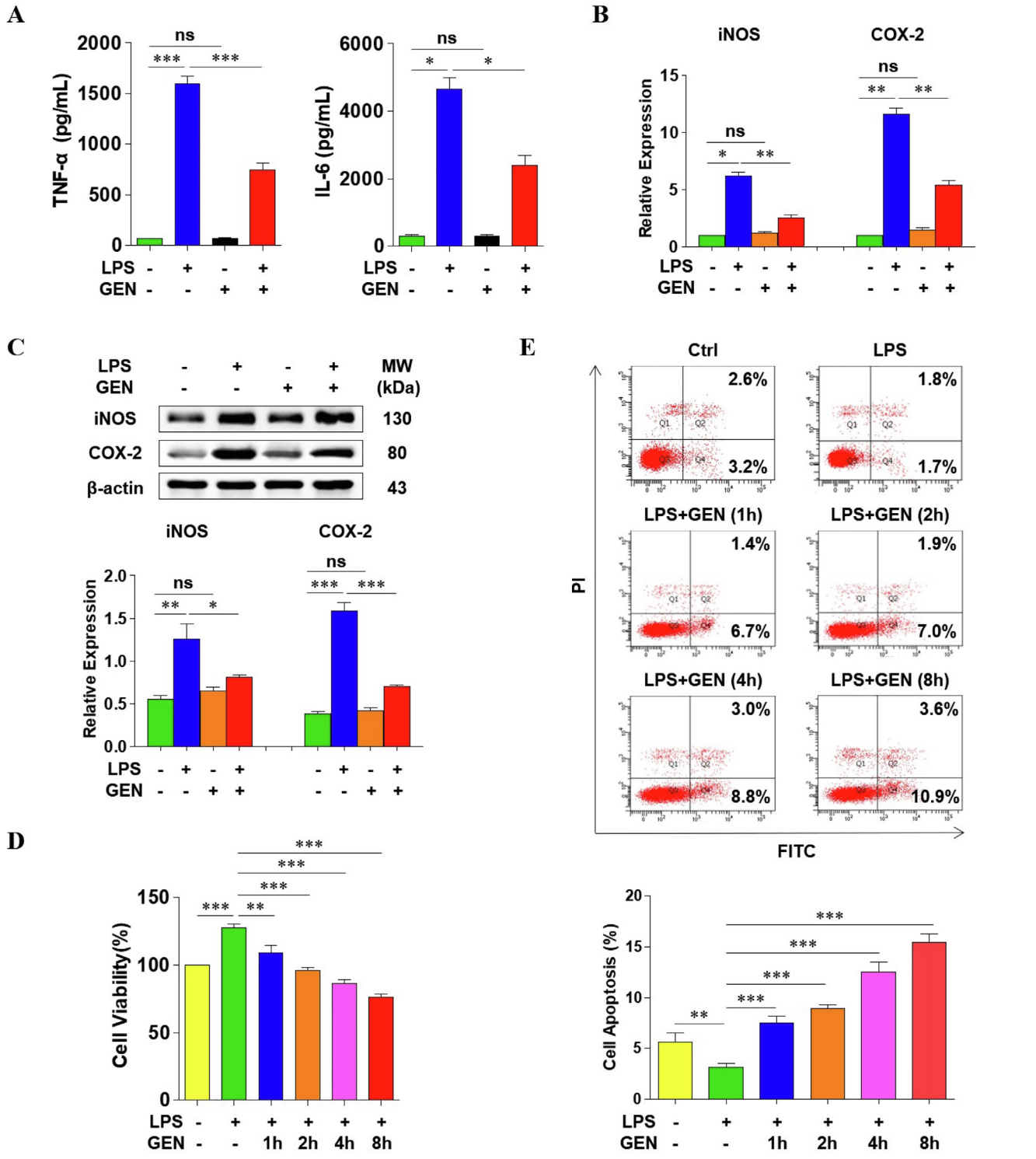

Fig. 1. Genistein promotes M1 macrophage apoptosis and reduces inflammation. (A-C) ELISA of TNF-α and IL-6 content, qRT-PCR of iNOS and COX-2 mRNA expression, Western blot of iNOS and COX-2 protein levels in RAW264.7 cells treated with LPS (1 μg/mL, 24 h) or genistein (10 μM, 2 h). (D) Statistical analysis of cell viability in RAW264.7 cells treated by LPS (1 μg/mL, 24 h) or genistein (10 μM, indicated time). (E) Flow cytometry analysis for Annexin V-FITC/PI staining in RAW264.7 cells with the treatment of LPS or genistein (10 μM, indicated time). Data are shown as mean ± SD of three independent experiments. LPS, lipopolysaccharides; GEN, genistein; ns, nonsignificant ($P > 0.05$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cloned wild type (WT) and mutant (MUT) miR-21 binding sites in TIPE2 coding sequence downstream of firefly luciferase gene and co-transfected RAW264.7 cells with miR-21 mimic and WT or MUT reporter gene vector. Then, we observed a distinctive decrease of luciferase activity in WT group transfected with miR-21 mimic, but a significant change was not observed in MUT group (Fig. 3 E). Therefore, we firmly confirmed that miR-21 targets TIPE2 in a coding region-dependent manner, but not in a non-coding manner.

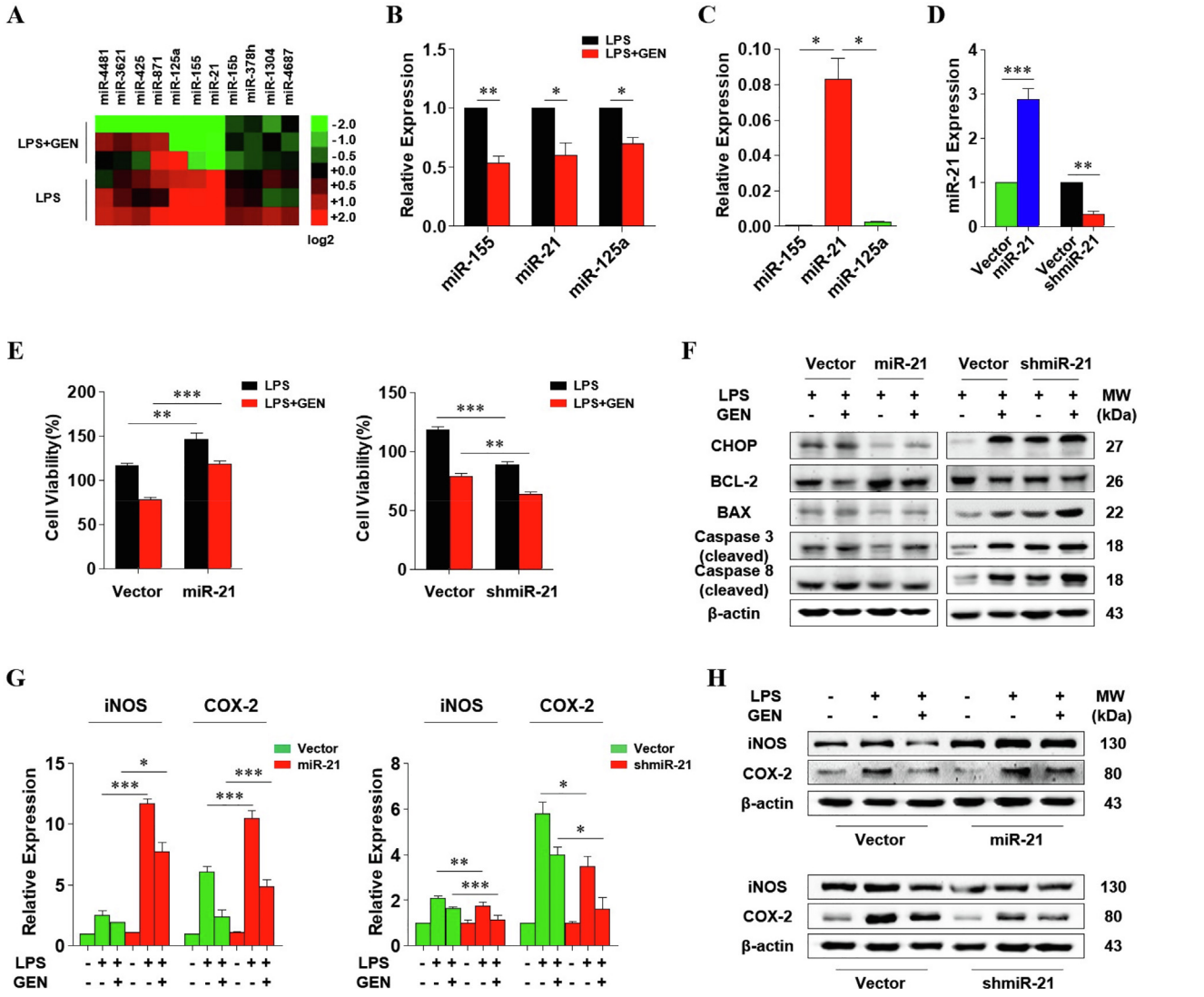

Fig. 2. miR-21 is involved in the increased apoptosis of M1 macrophage and inhibition of inflammatory response induced by genistein. (A) Heatmap of miRNA microarray results in RAW264.7 cells treated with LPS and genistein. (B) qRT-PCR of miR-155, miR-21 and miR-125a expression in RAW264.7 cells with the treatments of LPS and genistein. (C) qRT-PCR of miR-155, miR-21 and miR-125a expression in RAW264.7 cells. (D) qRT-PCR of miR-21 in stably expressing miRNA RAW264.7 cells. (E and F) Cell viability and Western blot of pro-apoptotic proteins CHOP, BAX, cleaved Caspase 3, cleaved Caspase 8 and anti-apoptotic proteins BCL-2 in RAW264.7 cells with stable miR-21 overexpression or repression treated by LPS and genistein (10 μM, 2 h). (G and H) qRT-PCR and Western blot of iNOS and COX-2 in RAW264.7 cells with the indicated treatments after miR-21 overexpression or knockdown. Data are shown as mean ± SD of three independent experiments. LPS, lipopolysaccharides; GEN, genistein; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.4. Genistein enhances M1 macrophage apoptosis through TIPE2/AKT pathway

M1 macrophage apoptosis not only inhibits inflammatory cascade effect but also decreases the formation of macrophage-derived foam cells (Linton et al., 2019). AKT is a serine/threonine protein kinase, that impacts pro-/anti-inflammatory cytokine, phagocytosis, autophagy and apoptosis through multiple signaling (Yang et al., 2020). To reveal the relationship between TIPE2 and AKT, we constructed stable overexpression or knockdown TIPE2 in RAW264.7 cells by lentiviral infection, respectively. Subsequently, qRT-PCR and Western blot were performed to detect the efficiency of overexpression and knockdown (Fig. 4 A and B, and Supplementary Fig. 4 A). Afterwards, we observed that TIPE2 remarkably decreased p-AKT^{Ser473} protein level, and TIPE2 repression promoted AKT phosphorylation in LPS-induced RAW264.7 cells (Fig. 4 C and Supplementary Fig. 4 B). Next, we used AKT phosphorylation agonists (insulin-like growth factor-1, IGF-1, 100 ng/mL, 2 h) to culture the cells with stable TIPE2 overexpression or knockdown, and cell viability, flow cytometry and Western blot analyses illustrated that M1 macrophage apoptosis was decreased in genistein-treated cells with TIPE2 knockdown, and IGF-1 significantly repressed macrophage apoptosis, which was promoted by TIPE2 (Fig. 4 D and E, and Supplementary Fig. 4 C-E). Taken together, our data showed that AKT phosphorylation played an important role in the pharmacological mechanism of genistein, which enhances M1 macrophage apoptosis through TIPE2/AKT pathway.

3.5. Genistein decreases inflammation via miR-21/TIPE2 axis associated with TLR4 pathway

Accumulating research has shown that TLR4 pathway is associated with blood vessel injury (Ye et al., 2019, Singh et al., 2020), and modulation of TLR4 signaling is a potential strategy to specifically target inflammation. To further explore if the anti-

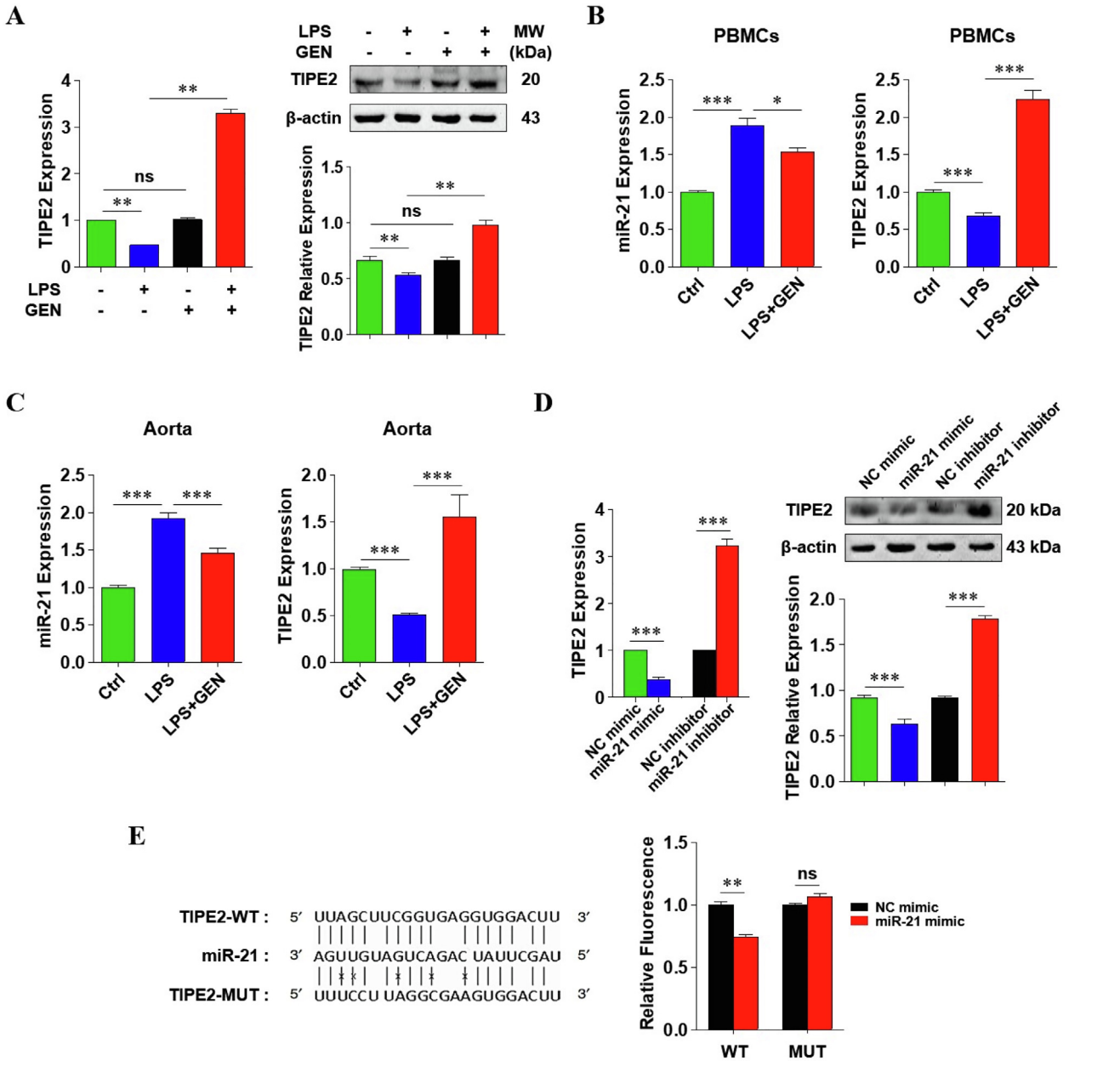

Fig. 3. TIPE2 is a direct target gene of miR-21. (A) qRT-PCR and Western blot of TIPE2 in RAW264.7 cells with the treatments of genistein and LPS. (B and C) qRT-PCR of miR-21 and TIPE2 in mice PBMCs and aortas. n = 10. (D) qRT-PCR and Western blot of TIPE2 in RAW264.7 cells transfected with miR-21 mimic or inhibitor. (E) The predicted miR-21 binding sites in murine TIPE2 coding region. 'X' indicates the mutated site. Wild type and miR-21 binding site-mutated TIPE2 coding regions were analyzed with dual luciferase reporter assay in RAW264.7 cells transfected with miR-21 mimic. Data are shown as mean ± SD of three independent experiments. LPS, lipopolysaccharides; GEN, genistein; PBMCs, peripheral blood mononuclear cells; ns, nonsignificant ($P > 0.05$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

inflammatory effect of genistein is related to TLR4, we used immunofluorescence staining and qRT-PCR to detect TLR4 and TIPE2 in mice aortas, and we discovered that TLR4 was higher in high fat diet-fed mice injected with LPS than it in normal diet-fed mice, but TIPE2 was attenuated. In addition, the effects of LPS on TLR4 and TIPE2 were availably reversed by genistein administration (Fig. 5 A and Supplementary Fig. 5 A). Therefore, we evaluated the relationship between them, and the results showed a negative correlation (n = 10, r = −0.931, $P = 0.0000$, Fig. 5 A). Additionally, Western blot analysis showed that genistein remarkably inhibited TLR4, TRIF, MyD88, iNOS and COX-2 proteins levels in aortas, which were enhanced by LPS administration (Fig. 5 B and C, and Supplementary Fig. 5 B and C). At the same time, we collected the peritoneal macrophages of mice, in which the protein changes were consistent with them in aortas (Fig. 5 B and C, and Supplementary Fig. 5 B and C), which further confirmed that genistein may decrease cardiovascular inflammatory response via miR-21/TIPE2/TLR4 signaling.

Since anti-inflammatory genistein presents the aforementioned molecular mechanism *in vivo*, what is the situation *in vitro*? We verified the functional relationships among miR-21, TIPE2 and TLR4 through rescue experiments using miR-21 mimic and derepressed TIPE2. We first transfected RAW264.7 cells stably overexpressing TIPE2 with miR-21 mimic, and 24 h later, the cells were incubated with genistein and LPS. Subsequent qRT-PCR indicated that miR-21 mimic significantly counteracted the effects of genistein on TLR4, MyD88 and TRIF expression induced by LPS, and TIPE2 dramatically decreased the expression of genes regulated

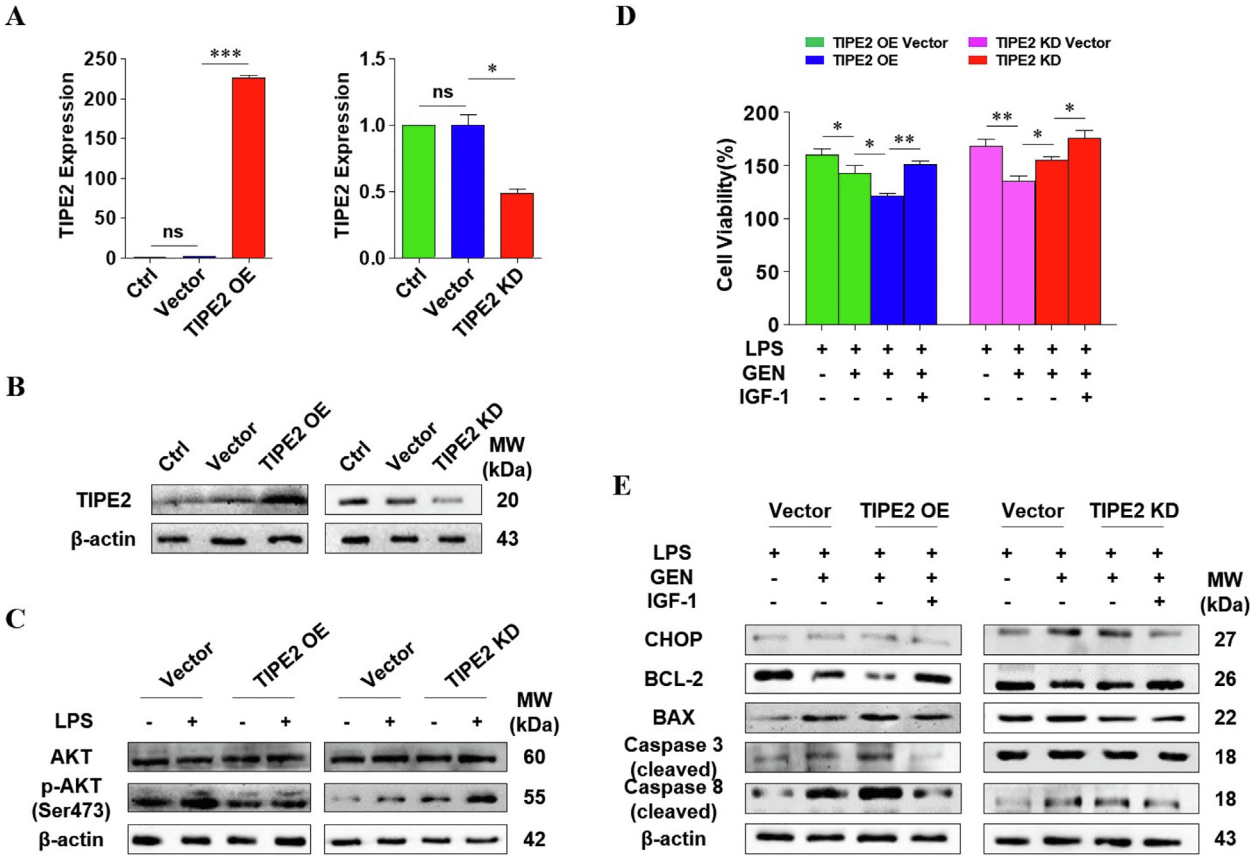

Fig. 4. Genistein enhances M1 macrophage apoptosis via TIPE2/AKT pathway. (A and B) qRT-PCR and Western blot of TIPE2 in RAW264.7 cells with stable TIPE2 expression. (C) Western blot of AKT and p-AKT^{Ser473} in RAW264.7 cells with stable TIPE2 expression and treated with LPS. (D and E) Cell viability and Western blot of pro-apoptotic proteins CHOP, BAX, cleaved Caspase 3, cleaved Caspase 8 and anti-apoptotic proteins BCL-2 in RAW264.7 cells stably expressing TIPE2 and treated with LPS, genistein, IGF-1 (100 ng/mL, 2 h). Data are shown as mean ± SD of three independent experiments. OE, overexpression; KD, knockdown; LPS, lipopolysaccharides; GEN, genistein; IGF-1, insulin-like growth factor-1 (AKT phosphorylation agonists); ns, nonsignificant ($P > 0.05$); $^{*}P < 0.05$, $^{***}P < 0.001$.

by miR-21 mimic in RAW264.7 cells exposed to LPS and genistein (Fig. 5 D). Furthermore, the protein changes related to TLR4 pathway were consistent with the results from qRT-PCR (Fig. 5 E and Supplementary Fig. 5 D). Overall, these data integrated with above studies revealed that miR-21 efficiently alleviates the effects of genistein on TIPE2 and TLR4, further demonstrating that genistein inhibited inflammatory response to protect against cardiovascular damage by miR-21/TIPE2 axis and associated with TLR4 pathway.

3.6. Genistein regulates the promoter region of Vmp1 via NF-κB

miRNA expression is mediated by transcription factors, RNA binding proteins, lncRNAs, et al, but we were most concerned about the regulation of miR-21 by NF-κB in macrophage. qRT-PCR indicated that NF-κB inhibitor PDTC (10 μM, 0.5 h) strongly downregulated miR-21 (Fig. 6 A), which suggests that NF-κB may be the upstream nuclear transcription factor. Using University of California Santa Cruz (UCSC) Genome Browser, we found that pre-mir-21 was completely located in the 3′ UTR of miR-21 host gene Vmp1 with consistent transcription direction. Then, we predicted that the binding site of NF-κB was within −3,847∼−3,837 bp upstream of Vmp1 transcription start site by FIMO software (Fig. 6 B).

To determine if the inhibitory effect of genistein on miR-21 in M1 macrophage depends on NF-κB, we cloned Vmp1 promoter region (−4,000∼+100 bp) and constructed promoter reporter gene pGL3-Basic and reference reporter gene vector pRL-CMV, and then co-transfected RAW264.7 cells with them. We observed that LPS increased the promoter activity by 2.5 times, which was remarkably depressed by PDTC (Fig. 6 C). After that, we co-transfected RAW264.7 cells with pGL3-Basic, pRL-CMV and NF-κB overexpression vector pcDNA3.1 for 24 h, and then treated the cells with genistein and LPS. The relative fluorescence value of reporter gene was detected and showed that overexpression of NF-κB significantly promoted promoter activity stimulated by LPS and effectively reversed the effect of genistein (Fig. 6 D). In addition, we carried out chromatin immunoprecipitation experiments and found that the enrichment of NF-κB in miR-21 promoter region was decreased in RAW264.7 cells after combined treatment with LPS and genistein (Fig. 6 E). These results suggest that NF-κB is involved in the regulation of miR-21 by genistein; in other words, genistein blocks the promoter region of miR-21 to prevent NF-κB-mediated upregulation in LPS-induced macrophage.

4. Discussion

Chronic vascular inflammation is an important pathophysiological basis of numerous accelerated cardiovascular diseases, including atherosclerosis, polyarteritis nodosa, myocardial infarction, and stroke (Aksentijevich et al., 2020). In recent years, several evidences showed that altered miRNA expression profiles are linked to cardiovascular injury, consisting of endothelial damage, macrophage apoptosis and inflammatory response (Iuliano et al., 2019). For example, endothelial miR-21 promotes the pathogenesis of atherosclerosis in apolipoprotein E-deficient mice via inhibited DDAH1-ADMA-eNOS-NO pathway (Yang et al., 2020), and exoso-

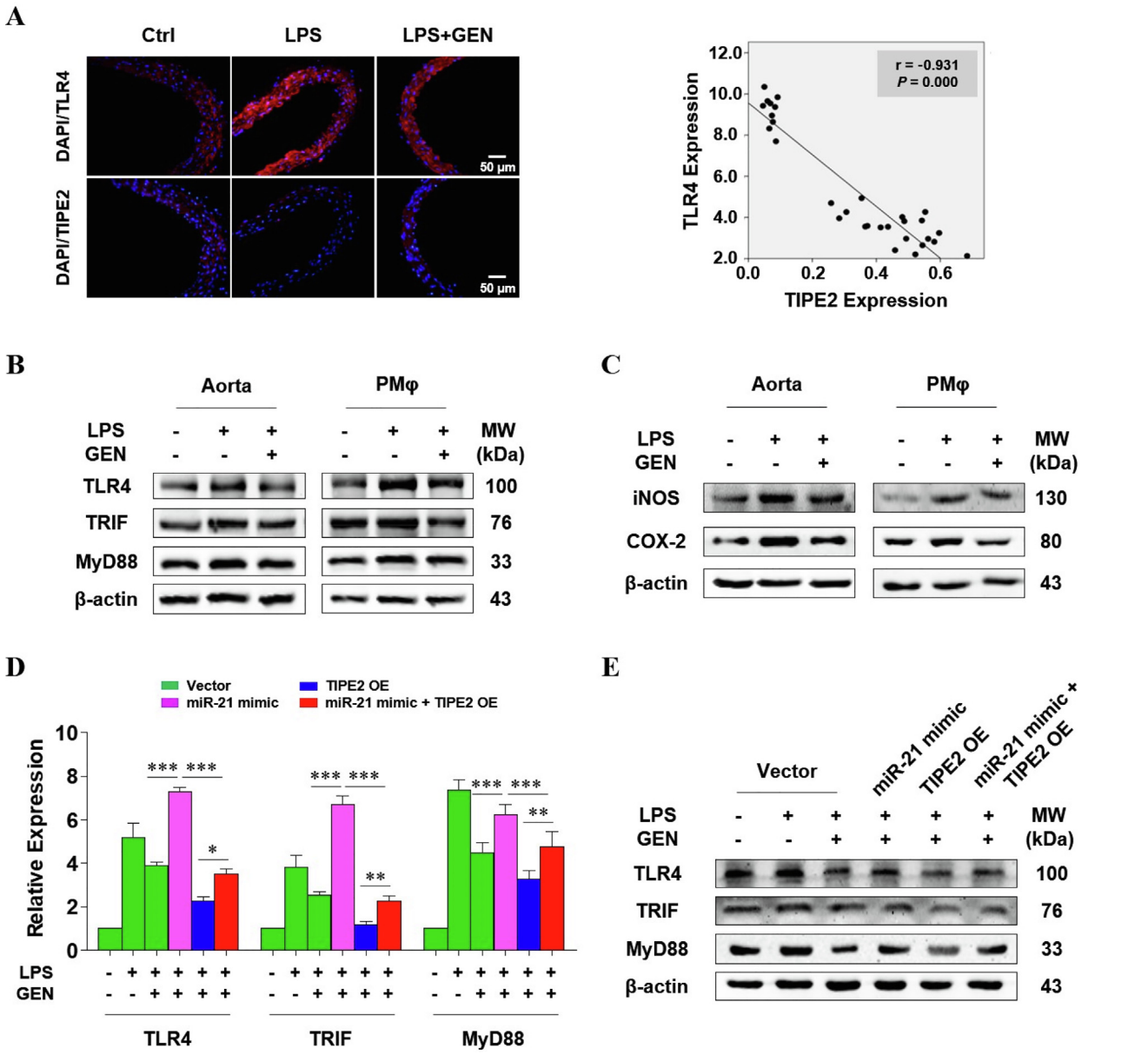

Fig. 5. Genistein decreases inflammation via miR-21/TIPE2 axis associated with TLR4 pathway. (A) Representative images (left) of immunofluorescence staining and correlation analysis (right) for TIPE2 and TLR4 in mouse aortas. n = 10, scale bars = 50 μm. (B and C) Western blot of TLR4, TRIF, MyD88, iNOS and COX-2 protein levels in mouse aortas and PMφs. (D and E) qRT-PCR and Western blot of TLR4, TRIF, MyD88 expression in RAW264.7 cells stably overexpressing TIPE2 and treated with miR-21 mimic, LPS and genistein. Data are shown as mean ± SD of three independent experiments. LPS, lipopolysaccharides; GEN, genistein; PMφ, peritoneal macrophage; OE, overexpression; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

mal miR-21 from nicotine-treated macrophage accelerates the development of vascular injury by PTEN-mediated smooth muscle cells migration and proliferation (Zhu et al., 2019). Apparently, miR-21 is increasingly regarded as a key regulator with the potential to improve sensitivity to vascular injury protection therapy. Here, we discovered that vascular-related miR-21 was highly depressed in LPS-induced M1 macrophage after treatment with genistein, suggesting that miR-21 may participate in the inhibitory mechanism of genistein against LPS-induced cardiovascular injury.

As important epigenetic modifiers, miRNAs are closely associated with macrophage apoptosis and exert critical regulatory roles in inflammation. Accumulating studies have revealed that LPS-induced inflammatory response cooperating with apoptosis inhibition seriously disturbs long-term vascular well-being (Grylls et al., 2021). Consequently, we intraperitoneally injected LPS to establish a vascular injury model in mice. As expected, gastric infusion of genistein inhibited LPS-induced increase of iNOS and COX-2 protein levels, which showed a strong protective effect on vasculature. Besides, in LPS-stimulated macrophage, we observed that genistein pretreatment efficiently reduced antiapoptotic action and inflammatory response. More interestingly, *in vitro*, using gene overexpression technology, we found that miR-21 evidently resisted genistein effects. Our results demonstrated that the protective effect of genistein on blood vessel depends on miR-21; however, the reason miR-21 connects genistein to vascular injury requires further investigation.

miRNAs are small non-coding RNAs, the major mechanisms of regulating protein expression are mRNA degradation and protein translation inhibition, and the binding sites are located in both coding sequences and 3′ UTRs (Correia de Sousa et al., 2019). TIPE2

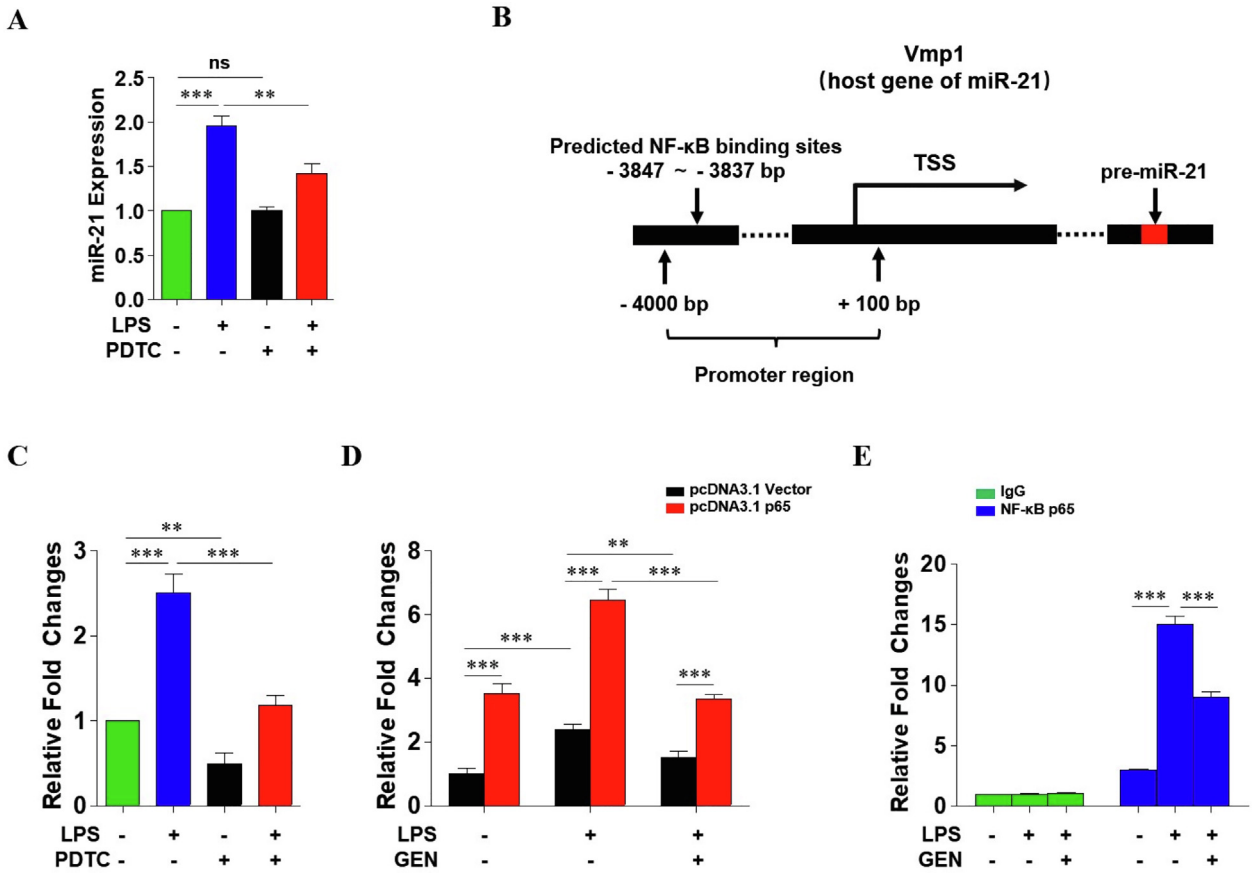

Fig. 6. Genistein regulates the promoter region of Vmp1 via NF-κB. (A) qRT-PCR of miR-21 expression in RAW264.7 cells treated with LPS and PDTC (10 μM, 0.5 h). (B) The binding sites for NF-κB in Vmp1 promoter region. (C) RAW264.7 cells were co-transfected with the promoter and reference reporter gene vector (pGL3-Basic and pRL-CMV) and treated with LPS and PDTC (10 μM, 0.5 h), and then, the luciferase activity was measured. (D) RAW264.7 cells were transfected with NF-κB p65 overexpression vector (pcDNA3.1) following LPS and genistein exposure, and the luciferase value was detected. (E) ChIP-qPCR assays indicated that NF-κB was recruited to the promoter region of miR-21 in RAW264.7 cells after LPS treatment, but the enrichment decreased with genistein exposure. Data are shown as mean ± SD of three independent experiments. LPS, lipopolysaccharides; PDTC, Pyrrolidine Dithiocarbamate, inhibitor of NF-κB; TSS, transcription start site; GEN, genistein; ns, nonsignificant ($P > 0.05$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

promotes Fas-induced apoptosis in immune cells and is a potential therapeutic target. Here, to explore the regulatory mechanism of miR-21, we paid more attention to the relevance between TIPE2 and miR-21. *In vivo*, we detected that genistein significantly enhanced the expression of TIPE2 in mice with damaged blood vessels, which contrasted with its effect on miR-21, and these changes were also observed in LPS-stimulated macrophage. Moreover, genistein accelerated M1 macrophage apoptosis in a manner dependent on activated TIPE2 and dephosphorylated AKT, and TIPE2 inhibited AKT phosphorylation, strengthening effects of genistein on macrophage. In addition, we found that miR-21 binding sites were within TIPE2 coding region instead of TIPE2 3′-UTR, which was consistent with previous studies in T cells (Ruan et al., 2014). In brief, our results showed that genistein enhances M1 macrophage apoptosis to reduce vascular injury through miR-21/TIPE2/AKT pathway.

The majority of miRNAs have been shown to be intergenic. miR-21 is the first miRNA to be identified as transcribed by RNA polymerase II with its own promoter region, but the actual size, transcriptional start site and minimal promoter region of pri-miR-21 are still subject to debate. Multiple transcription factors, such as signal transducer and activator of transcription 3 (STAT3), NF-κB, and activating protein-1 (AP-1), can regulate miR-21. Our data further confirmed that NF-κB regulates miR-21 expression by directly binding to its promoter, and genistein depresses the promoter in a manner dependent upon NF-κB. In addition, TIPE2, a target gene of miR-21, has been reported as a marker of environmental hormones in mammals, not only maintaining immune homeostasis but also regulating inflammation. Our preliminary research showed that TIPE2/TLR4 axis had a major part to play in promoting plaque stability (Cong et al., 2021). Here, we illustrated that genistein inhibits TLR4/TRIF- and TLR4/MyD88-mediated inflammatory response in a TIPE2-dependent manner; in other words, genistein reduces inflammation by blocking miR-21/TIPE2 axis associated with TLR4 pathway.

5. Conclusions

In conclusion, we provided pieces of evidence that miR-21 was involved in genistein-mediated inhibition of cardiovascular injury, which is artificially induced by LPS *in vitro* and *in vivo*. Specifically, genistein suppressed the promoter region of miR-21 host gene Vmp1 by NF-κB. As a result, miR-21 downstream target protein TIPE2 is upregulated, which not only promotes M1 macrophage apoptosis by depressing AKT phosphorylation but also reduces inflammatory response via inhibiting inflammatory proteins TRIF and MyD88, thereby exerting a protective effect on blood vessels (Fig. 7). This study further verified the pharmacological functions of genistein and provides more potential targets for the development of health products and drugs to protect blood vessels. However, the epigenetic regulation mechanism is very complex. This

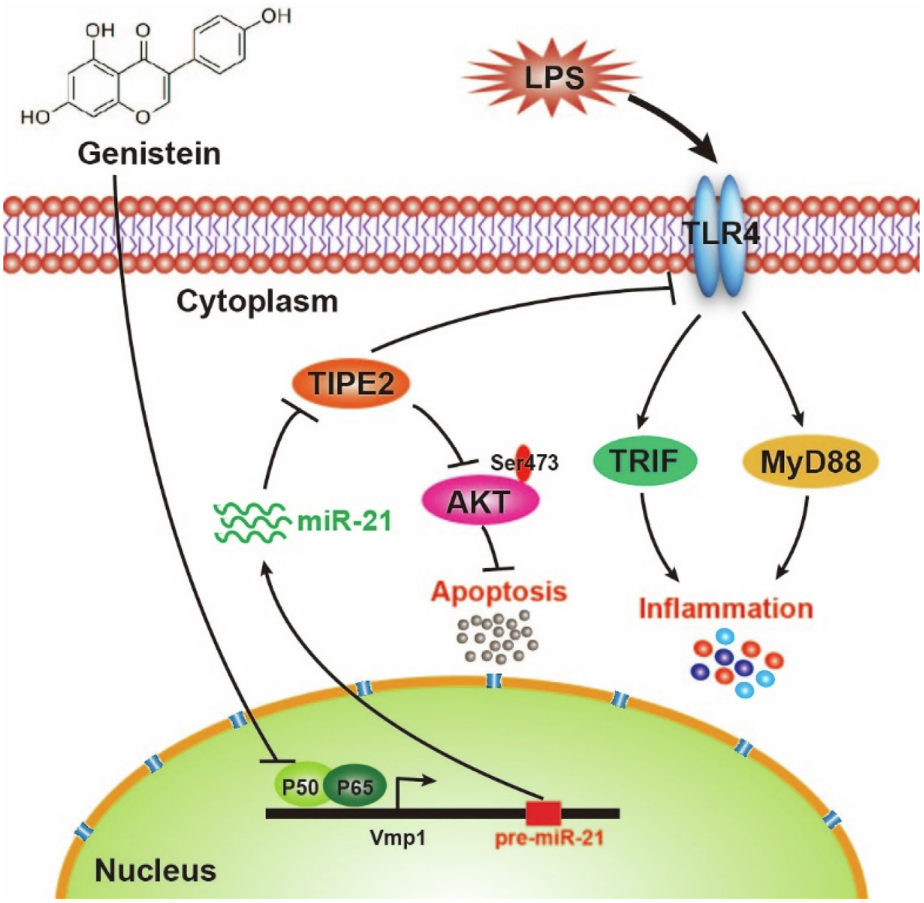

Fig. 7. Working model for genistein promotes M1 macrophage apoptosis and reduces inflammatory response in LPS-induced cardiovascular injury. Genistein regulates NF-κB/miR-21/TIPE2 signaling pathway, which on the one hand leads to inhibition of downstream AKT phosphorylation and promotes apoptosis of M1 macrophages, and on the other hand down-regulates TRIF and MyD88 expressions to reduce inflammation. As a result, it can resist LPS-induced cardiovascular injury.

study mainly focuses on nuclear transcription factor NF-κB regulating miR-21 to explain the mechanism of genistein protecting cardiovascular, while DNA methylation and chromatin remodeling have not been studied.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2022.05.009.

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