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ORIGINAL RESEARCH

α-Mangostin Inhibited M1 Polarization of Macrophages/Monocytes in Antigen-Induced Arthritis Mice by Up-Regulating Silent Information Regulator 1 and Peroxisome Proliferators-Activated Receptor γ Simultaneously

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Background: α-Mangostin (MG) showed the potentials in alleviating experimental arthritis, inhibiting inflammatory polarization of macrophages/monocytes, and regulating peroxisome proliferators-activated receptor γ (PPAR-γ) and silent information regulator 1 (SIRT1) signals. The aim of this study was to analyze the correlations among the above-mentioned properties.

Methods: Antigen-induced arthritis (AIA) was established in mouse, which was treated with MG in combination with SIRT1/PPAR-γ inhibitors to clarify the role of the two signals in the anti-arthritic actions. Pathological changes were systematically investigated. Phenotypes of cells were investigated by flow cytometry. Expression and co-localization of SIRT1 and PPAR-γ proteins in joint tissues were observed by the immunofluorescence method. Finally, clinical implications from the synchronous up-regulation of SIRT1 and PPAR-γ were validated by experiments in vitro.

Results: SIRT1 and PPAR-γ inhibitors (nicotinamide and T0070097) reduced the therapeutic effects of MG on AIA mice, and abrogated MG-induced up-regulation of SIRT1/PPAR-γ and inhibition of M1 polarization in macrophages/monocytes. MG has a good binding affinity to PPAR-γ, and MG promoted the co-expression of SIRT1 and PPAR-γ in joints. Synchronously activating SIRT1 and PPAR-γ was revealed to be necessary by MG to repress inflammatory responses in THP-1 monocytes.

Conclusion: MG binds PPAR-γ and excites this signaling to initiate ligand-dependent anti-inflammatory activity. Due to certain unspecified signal transduction crosstalk mechanism, it then promoted SIRT1 expression and further limited inflammatory polarization of macrophages/monocytes in AIA mice.

Keywords: rheumatoid arthritis, mangosteen, metabolism, inflammation, immune

Introduction

The successful treatment of rheumatoid arthritis (RA) remains a great challenge worldwide. Immune cells play a key role in RA. Disruption of immune homeostasis directly induces systemic inflammation, and leads to synovitis and tissue damages.[1] It has been confirmed that a large number of macrophages are infiltrated in synovium of RA patients. This phenomenon is regarded as an important sign of early RA, and the count of infiltrated macrophages is closely related to

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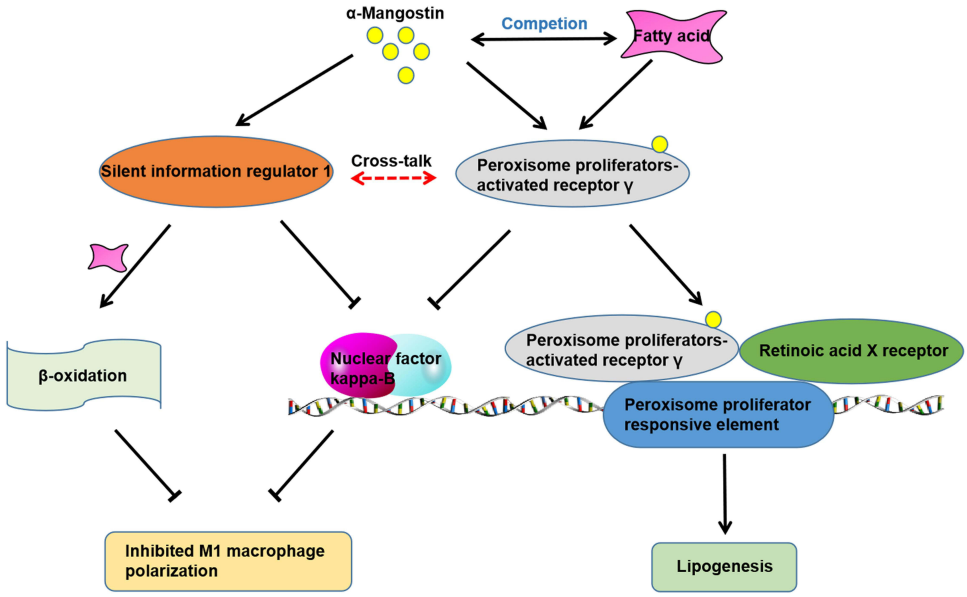
Graphical Abstract



disease activity.[2] Unsurprisingly, the imbalanced M1/M2 polarization of macrophages plays an essential role in the etiology of RA.[3] Reversing this situation will curb RA progression. Successful application of certain traditional anti-rheumatic drugs and biological agents with the above-mentioned activities has fully demonstrated the feasibility of this strategy.[4]

Meanwhile, the clinical performance of conventional anti-RA drugs is far away from satisfaction. Both nonsteroidal anti-inflammatory drugs and disease-modifying anti rheumatic drugs can only partially alleviate symptoms, but cannot totally abrogate tissue degradation. Besides, they have obvious side effects.[5] Comparatively, the emerging biotherapy has specific targets, and notable short-term clinical effects. But their safety concerns are not thoroughly resolved. Also, we should be aware that regulating one isolated signal cannot completely reverse the development of systemic diseases, and the unaffordable cost is another important factor limiting its wide application.[6] Therefore, further researches on some well-known compounds with low toxicity and good therapeutic potentials are necessary. It will provide us with more options.

The accumulating knowledge about immune-metabolism feedback gives us some inspiring clues to efficiently screen out reagents potentially affecting macrophages. Hyperlipidemia occurs in 42% and 31% of patients with early and long-term RA, respectively.[7] Under this circumstance, inhibiting fat accumulation and promoting β-oxidation may benefit anti-RA therapies, and macrophage/monocyte polarization is also affected.[8,9] Key players underneath these clinical phenomena typically include peroxisome proliferators-activated receptor γ (PPAR-γ) and silent information regulator 1 (SIRT1). PPAR-γ controls adipogenesis, lipid anabolism, as well as macrophage/monocyte polarization.[10] Many PPAR-γ agonists like thiazolidinediones and rosiglitazone can effectively alleviate tissue damages and inflammation of RA.[11] SIRT1 is similarly an important regulator of energy metabolism by promoting glucose and fatty acid utilization.[12] It favors M2 polarization of macrophage/monocyte by deacetylating nuclear factor kappa-B (NF-κB) and some other downstream targets.[13] It is worthy to be noted PPAR-γ and SIRT1 are a pair of functional rivals from the metabolic perspective, despite both having anti-inflammatory properties.

We previously investigated the anti-rheumatic activity and relevant mechanisms of xanthone derivatives.[14,15] α-Mangostin (MG, an isoprenyl-substituted xanthone isolated from mangosteen) is a representative of them. It is separated from the pericarp of mangosteen, and has been massively produced thanks to its high abundance in nature. This versatile compound shows antibacterial, antioxidant, anti-cancer, anti-inflammatory and many other physiological activities.[16]

Besides, we confirm that it is endowed with good anti-RA potentials. It produced remarkable curative effects on adjuvant-induced arthritis (AA) and collagen-induced arthritis (CIA) models.[17,18] In addition to restorative changes of some conventional arthritis indicators, the immune microenvironment was improved by MG too. We found that MG can inhibit lipopolysaccharide (LPS)-induced M1 polarization and inflammatory responses of macrophages/monocytes.[19,20] Meanwhile, it brought significant metabolic changes, and affected relevant pathways like nicotinamide phosphoribosyltransferase (NAMPT)/nicotinic adenine dinucleotide (NAD).[17] Another research group revealed that MG cured hepatic steatosis in obese mice through SIRT1-adenosine 5'-monophosphate-activated protein kinase (AMPK) and PPAR-γ pathways.[21] These collective evidences demonstrate that MG could regulate metabolic pathways and consequently affect macrophages when treating inflammatory diseases like RA. To confirm this hypothesis, we performed this study. It selectively focused on SIRT1 and PPAR-γ, because they are potent regulators in both energy metabolism and macrophages/monocytes polarization. Relevant results will not only further clarify anti-rheumatic mechanism of MG, but also provide a therapeutic option selectively targeting macrophages/monocytes.

Materials and Methods

Materials and Reagents

The MG (purity >98%) were purchased from SanHerb Bioscience (Chengdu, Sichuan, China). Carboxyl methyl cellulose sodium (CMC-Na) was obtained from Aladdin (Shanghai, China). Nicotinamide (NAM, an endogenous SIRT1 antagonist), T007097 (a PPAR-γ specific antagonist) and sirtinol (a SIRT1 specific antagonist) were provided by Sigma-Aldrich (St Louis, MO, USA), Selleck Selleck Chemicals LLC (Houston, TX, USA) and Apexbio (Houston, TX, USA), respectively. Bacillus Calmette-Guerin (BCG), complete Freund's adjuvant (CFA) and LPS (from the Gram-negative bacterium E. coli 055:B5) were purchased from Sigma (St Louis, MO, USA). Methylated bovine serum albumin (mBSA) was purchased from Millipore Sigma (Burlington, MA, USA). Roswell Park Memorial Institute (RPMI) 1640 medium was bought from Hyclone (Logan City, UT, USA). Fetal bovine serum (FBS) was purchased from Gibco (CA, USA). Lipofectamine 3000 transfection reagent was supplied by Invitrogen (Carlsbad, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor-α (TNF-α) and interleukin-1 beta (IL-1β) were procured from Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Monocytes separation kits were the products of Solarbio (Beijing, China). Anti-mice CD11b-FITC, CD86-APC and CD206-PE antibodies were purchased from BioLegend (San Diego, CA, USA). Anti-human SIRT1, p65, p-p65, PPAR-γ and Arginase-1 (Arg-1) antibodies were the products of Proteintech Inc. (Wuhan, Hubei, China). Biotin-conjugated secondary antibodies were supplied by Beyotime Biotech (Nantong, Jiangsu, China).

Molecular Docking

CB-Dock docking platform was used to predict the binding affinity between MG and PPAR-γ based on previously reported procedures.[22,23] In brief, the chemical structure of MG was downloaded from PubChem website, and the crystal structure of protein PPAR-γ was downloaded from RCSB (https://www.rcsb.org/) (PDB ID: 4YT1).[24] MG structure was converted into a standard delay format (SDF) file, and uploaded as a ligand. The PDB file of PPAR-γ was uploaded as a target protein directly. These files were then checked and converted to *. pdbqt files by OpenBabel and MGLTools. CB-Dock analyzed all cavities of the protein, and predicted possible docking centers and sizes of the top 5 cavities. Afterwards, the molecular simulation docking procedure was initiated by using all the default parameters.[22] Final results are displayed in graphical interfaces. Users can browse binding scores, cavity sizes, and docking parameters of the predicted binding modes in the generated table. Moreover, users can inspect the 3D structures of any binding modes on the web page by clicking the structures.[22]

Cells and Animals

THP-1 monocytes (CL-0233) were provided by Procell Life Co., Ltd. (Wuhan, Hubei, China). Male C57BL/6 mice (8–12 weeks old, SCXK 2020–0005) were bought from Skbex Biotechnology (Zhenzhou, Henan, China). In total, 35 mice were used in the experiments in vivo, which were accommodated in a strictly controlled environment

(temperature: 24 ± 2°C; dark/light circle: 12 h; relative humidity: 50 ± 2%) and had free access to tap water and standard rodent chow. All in vivo experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by the Ethical Committee of The Second Affiliated Hospital of Wannan Medical College (NO. wyefy 2021–036).

Establishment of Antigen-Induced Arthritis (AIA) Models and Treatments

AIA model was adopted in this study, because it shares many pathological commons with RA, and similarly shows high immunoreactivity to collagen. Besides, AIA mice are typically detected with high titers of anti-cyclic citrullinated peptide antibody, and shows severe cartilage injuries.[25] Mice were immunized with 200 mg of mBSA (emulsified in 0.2 mL CFA) by a subcutaneous injection into the flank skin. Seven days after the first injection, the mice were intradermally injected with 100 mg of mBSA in 0.1 mL CFA at the base of the tail. Arthritis was finally induced on day 21 by an intra-articular injection using 50 mg of mBSA (dissolved in 10 mL sterile phosphate buffer saline) into both knee joint cavities.[25] After the second injection, 28 immunized mice were equally divided into four and received treatments according to grouping: AIA model (0.5% CMC-Na), MG treatment (58 mg/kg), MG (58 mg/kg) + NAM (72 mg/kg) treatment, and MG (58 mg/kg) + T007097 (15 mg/kg) treatment groups, respectively. Another 7 healthy mice were taken as normal controls. MG, NAM and T007097 were dissolved in 0.5% CMC-Na with the help of ethanol and Tween 40 in advance. All the reagents were gavaged once a day. The normal controls were treated simultaneously with 0.5% CMC-Na. Since day 7, the diameter of knee joint and arthritis index score were periodically (every 3 days) recorded. Mice were killed by cervical dislocation on day 28. Key tissues and organs were removed and weighed immediately. Weight changes of spleen and thymus were especially highlighted in the manner of organ indexes (organ weight/body weight ×1000), which were used as a gauge of the immune condition in vivo.[26,27]

Histopathological and Immunohistochemical Evaluation

Knee joints, spleen and pararenal fat pad were fixed in 4% formaldehyde solution. After 14 days of decalcification by ethylene diamine tetraacetic acid (EDTA), the joints were transparentized. The specimens were then embedded in paraffin, sectioned and stained with hematoxylin-eosin (HE).[26] Preparation of histological sections for spleen and fat pad was similar to the above procedures, only without decalcification. Pathological changes within these specimens were observed under a light microscope. Some paraffin sections were dewaxed and immersed in methanol-H_2O_2 solution to inactivate endogenous peroxidase. The slices were heated by microwave, and antigens were repaired by citric acid, followed by the incubation with anti- SIRT1 (1:200) or PPAR-γ (1:200) primary antibodies (at 37°C for 60 min) and biotin-conjugated secondary antibody (1:1000, at room temperature for 20 min) in turn. Extensive washing was carried out at every experimental interval. Signals were visualized by diaminobenzidine (DAB) chromogenic solution and hematoxylin (Zsbio, Beijing, China). The stained sections were finally dehydrated, sealed, and observed under a light microscope. Pathological assessment about spleen mainly focused on the density of germinal center, periarteriolar lymphoid sheaths, lymphoid follicles, marginal zone, and red pulp. Pathological scores of spleen from 0 to 3 represented no abnormality to severe pathological changes.[28]

Immunofluorescence

Knee joint slices were obtained from the above described procedures, which were shortly immersed in EDTA (PH 9.0) antigen retrieval solution, and then blocked with 0.5% bovine serum albumin (BSA). The specimens were subsequently incubated with anti-SIRT1 (1:500) or PPAR-γ (1:500) antibody at appropriate concentrations overnight in 4°C. Then, they were treated with HRP-tagged secondary antibodies (1:500) at room temperature. The signals were visualized by FITC-TSA incubation in dark. After that, another blocking procedure was performed, followed by anti-CD86 antibody (1:100) treatment overnight under 4°C. The signal of CD86 was developed by a further incubation with CY3-tagged secondary antibody (1:300). Nucleus of cells was finally dyed by DAPI in dark for 10 min.[28,29] Fluorescent images were taken by a fluorescence microscope (Leica, Wetzlar, Germany).

Harvest of Macrophages from Mouse Spleen

When sacrificed, the spleen of mice was removed immediately. It was washed with 75% ethanol, placed in 5 mL of mouse mononuclear cell separation medium, wrapped with sterile gauze, and then thoroughly grinded with a homogenizer. The obtained cell suspension was transferred to a 15 mL centrifuge tube, and pre-cooled RPMI 1640 was slowly added. Macrophages were separated by the gradient centrifugation according to the manufacturer's instruction.[30]

Flow Cytometry Analysis

CD11b indicates the existence of macrophages, while CD86 and CD206 are usually taken as the markers of M1 and M2 macrophages, respectively. Hence, we analyzed macrophage subsets by using them as indicators here.[31] Anti-CD11b-FITC and anti-CD86-APC antibodies were added to cell suspensions, which were then kept in dark for 30 min. After the cells were fixed, their membrane was permeabilized by the appropriate buffer. Thereafter, anti-CD206 -PE antibody was added, and further incubated for 30 min at room temperature. The stained cells were washed with phosphate buffer saline (PBS), and centrifuged at 2500 rpm for 10 min. The cell sediment was finally re-suspended in PBS, and fed to a flow cytometer (FC500, Beckman) for quantitative analysis. Obtained data were processed by CytExpert (version 2.4).[32] $CD11b^{+}CD86^{+}$ and $CD11b^{+}CD206^{+}$ cells were identified as M1 and M2 cells, respectively.

Treatment of THP-1 Monocytes in vitro

THP-1 cells were cultured in a 6-well plate with a density of 2×10^{5}/well, and RPMI 1640 medium containing 10% FBS was adopted in the culture system.[33] After LPS (2 μg/mL) stimulation for 8 h, the culture medium was discarded, and the fresh complete medium containing different reagents was added. The treatments included MG (5 μg/mL), MG (5 μg/mL) + sirtinol (30 μM) and MG (5 μg/mL) + T007097 (10 μM). After being cultured for 24 h, the cells were washed twice with pre-cooled PBS, and harvested upon the centrifugation at 1000 rpm at 4°C for 10 min. SIRT1 or PPAR-γ in some cells were silenced by siRNA. Gene-specific siRNA was chemically synthesized by Genepharma Co., Ltd. (Shanghai, China) with the sequences as below: negative control, 5'-UUCUCCGAACGUGUCACGUTT-3' (sense), 5'-ACGUGACACGUUCGGAGAATT-3' (anti-sense); si-SIRT1, 5'-CGGGAAUCCAAAGGAUAAUTT-3' (sense); 5'-AUUAUCCUUUGGAUUCCCGTT-3' (anti-sense) ; si-PPAR-γ, 5'-GAGUCCACGAGAUCAUUUATT-3' (sense); 5'-UAAAUGAUCUCGUGGACUCTT-3' (anti-sense). THP-1 cells were seeded into 6-well plates at a density of 5×10^{5}/well. After adaptive culture, the cells were deprived of FBS for 12 h. The freshly prepared transfection reagent solution (200 μL Optin-MEM + 5 μL lipofectamine 3000) was thoroughly mixed with siRNA solution (5 μL siRNA in 200 μL Optin-MEM) when they reach room temperature. The product stood for 20 min, and was then added into cell culture plates. After 4 h, the medium was replaced with normal complete medium, and a further incubation for 48 h was performed to complete the transfection.

Western Blot Assay

Proteins in cells were extracted by radio immunoprecipitation assay (RIPA) lysis buffer. Samples containing qualified amount of protein (10 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to polyvinylidene fluoride membranes, which were then incubated with different primary antibodies at various concentrations (anti-SIRT1 antibody, 1:2000; anti-p65 antibody, 1:1000; anti-p-p65 antibody, 1:500; anti-PPAR-γ antibody, 1:1000; anti-Arg-1 antibody, 1:5000; anti-β-Actin antibody, 1:5000) at 4°C for 12 h. The membranes were blocked with 5% skim milk for 2 h at 37°C, and further incubated with the appropriate secondary antibodies (anti-rabbit or rat HRP-linked IgG antibody, 1:10,000). Finally, signals were developed with an enhanced chemiluminescence (ECL) substrate kit. The bands were photographed and analyzed using ImageJ (version 1.52a, NIH, Bethesda, MD, USA) software.[34]

Statistical Analysis

Quantification data were presented as mean ± standard deviation (SD). Statistical differences among groups were analyzed by GraphPad Prism 8.0 (GraphPad Software, Cary, NC, USA) using a one-way analysis of variance coupled with Tukey post hoc test. Differences were considered statistically significant when $P < 0.05$.

Results

Regulation of PPAR-γ and SIRT1 Was Involved in Anti-Rheumatic Actions of MG

After mBSA injection, all the immunized mice showed signs of arthritis, and the clinical manifestations progressed rapidly, with obvious redness and local swelling around knee joints. It confirmed the successful establishment of AIA in mice. MG improved the conditions a lot. When used in the combination with either NAM or T0070097, its therapeutic effects were impaired (Figure 1A). It is well known that the spleen and thymus are the most representative immune organs of mammals, where numerous immune cells differentiate, develop, and mature. Under this context, it is not surprising that many RA-related studies selectively investigated the two organs.[26,27] In this study,

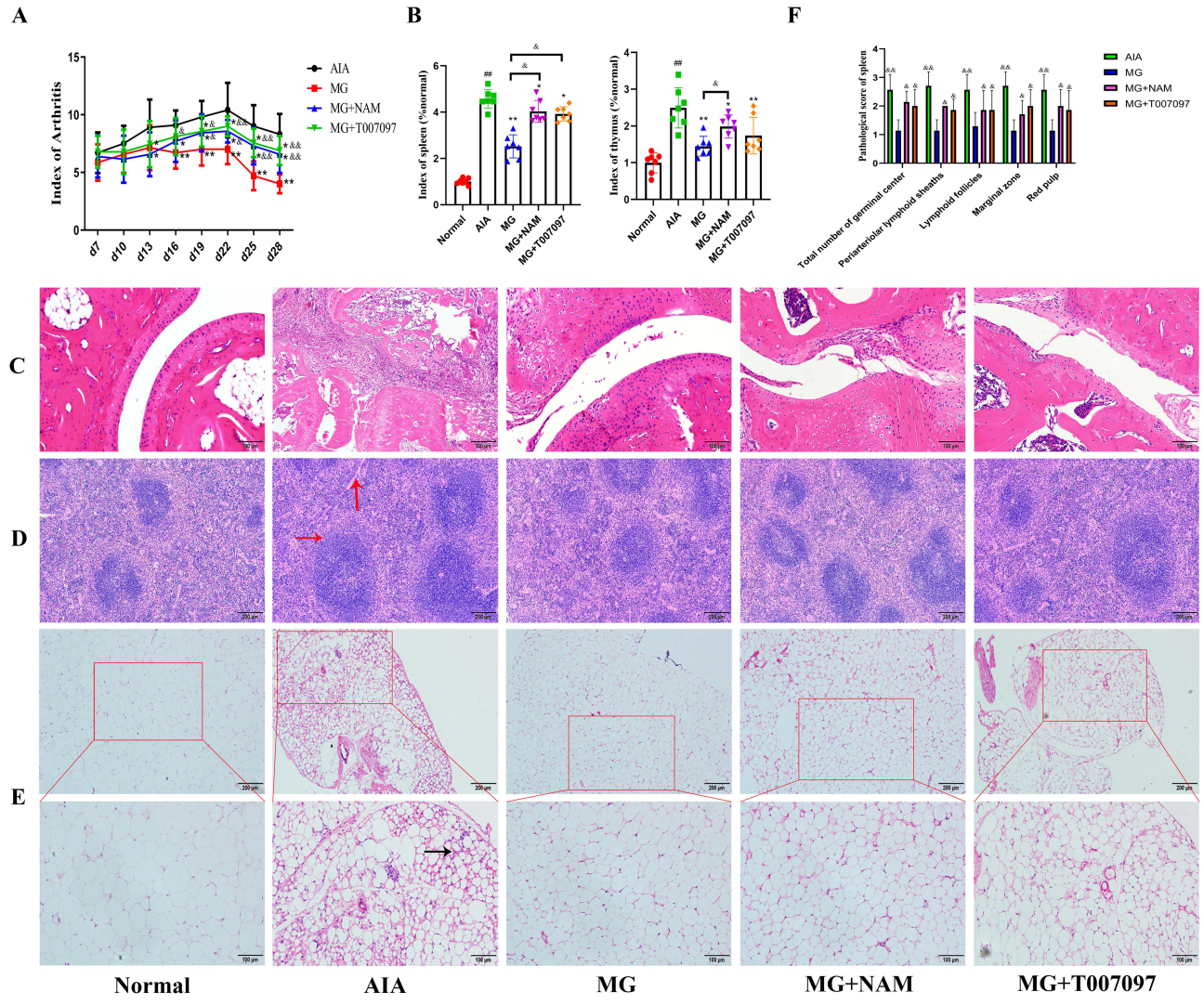


Figure 1 Therapeutic effects of MG on AIA mice. (**A**) Arthritis index score in the whole course of AIA. (**B**) Score of thymus index and spleen relative weight index. Histological examination of joint (**C**), spleen (**D** and **F**) and pararenal adipose tissue (**E**). $^{\#\#}P < 0.01$ compared with normal, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with AIA, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared with MG.

Abbreviations: Red horizontal arrow, blurred boundaries between white pulps and red pulps; red vertical arrow, white pulp hyperplasia; black horizontal arrow, immune cell infiltration within white adipose tissues; MG, α-mangostin; AIA, antigen-induced arthritis.

we emphasized the immune changes brought by MG treatment in AIA mice, and similarly estimated the changes of spleen and thymus. Hyperplasia of thymus and spleen indicates the excessive proliferation of immune cells and hyperactivation of immune system. Because of mBSA-brought stimulation, AIA mice apparently suffer from chronic inflammation and immune intolerance, which is partially evidenced by the expanded volume of organ tissues. MG treatment favored immune rebalancing and therefore attenuated this situation, which reflected in changes of their weight indexes (Figure 1B). NAM and T0070097 weakened MG-brought benefits, as they would aggravate immune abnormalities by inhibiting SIRT1 and PPAR-γ signals. MG significantly eased joint damages in AIA mice, whereas the pathological changes including inflammatory cell infiltration and synovial hyperplasia in joints can still be found in AIA mice receiving combination treatments (Figure 1C). Boundaries between white pulps and red pulps were blurred in the spleen of AIA mice, which was accompanied by white pulp hyperplasia (Figure 1D). AIA-related inflammation was accompanied by a decrease in adipocyte size and increased immune cell infiltration within white adipose tissues. AIA mice treated by MG showed the reduced inflammatory cell infiltration and enlarged adipocytes. These effects were also weakened in the combination treatments (Figure 1E). RA will deplete fat depot and shrink the size of adipocytes. From the metabolic perspective, PPAR-γ governs lipogenesis.[10] Therefore, the enlarged adipocytes indicated the up-regulation of PPAR-γ. It is known monocytes/macrophages are the main immune cells infiltrated in adipose tissues and joints cavity.[35,36] Both PPAR-γ and SIRT1 hamper M1 polarization and the cell enrichment.[37,38] The reduced inflammatory cell infiltration could be resulted from the up-regulation of PPAR-γ and SIRT1. We further highlight the impacts of different treatments on spleen pathological scores, which further support the beneficial immunoregulatory properties of MG on AIA and the antagonistic effects of NAM and T0070097 to MG (Figure 1F).

PPAR-γ governs lipogenesis, and the changed volume of adipocytes indicated the fluctuation of PPAR-γ signaling. Considering the suppressive roles of SIRT1 and PPAR-γ in inflammatory macrophages, above results hinted that SIRT1 could be also involved in the therapeutic effects of MG on AIA.[20,39] To confirm this, we observed the expression of SIRT1 (Figure 2A) and PPAR-γ (Figure 2B) in spleen. Indeed, MG obviously reversed the decrease in their expression caused by AIA (Figure 2C and D). The inhibitors suppressed expression of the corresponding proteins. Taken together, it suggests that both SIRT1 and PPAR-γ could be potential targets of MG.

MG Suppressed M1 Polarization in AIA Mice by Regulating SIRT1 and PPAR-γ

Macrophages that mainly secrete pro-inflammatory factors are defined as M1 subset, which mainly fulfill defensive host immune functions. Macrophages that play a major role in reducing inflammatory response are defined as M2 subset, which promote wound repair.[31] It is observed that macrophages are significantly increased in joints of RA patients, which basically show M1 phenotype. The imbalance of macrophage polarization directly account for the local inflammation and tissue injuries.[40] In this study, the cells expressing both CD11b and CD86 were identified as M1 macrophages (Figure 3A), and those expressing CD11b and CD206 were taken as M2 macrophages (Figure 3B). The results showed that M1 macrophage counts were significantly increased in AIA mice compared to healthy controls. MG reduced its distribution from 28.29% to 13.97%, while this effect was impaired by NAM and T0070097 (Figure 3C). However, we observed that neither MG nor T0070097 exerted significant effect on M2 polarization (Figure 3D). NAM treatment seemed to favor the development of M2 macrophages. M1 macrophages are the main source of many RA-related inflammatory cytokines, such as TNF-α and IL-1β. Consistent to their impacts on M1 polarization, MG significantly reduced the AIA-caused increase in TNF-α and IL-1β levels in spleen homogenates, and NAM and T0070097 partially antagonized this effect (Figure 3E and F).

The polarization of macrophages in joints was also affected by these treatments. We observed the local expression of SIRT1 and PPAR-γ as well as a M1 macrophage marker (CD86) in joints by immunofluorescence method (Figure 4A and B). By the development of inflammation, macrophages tended to polarize towards to the M1 phenotype, evidenced by the greatly increased expression of CD86 (Figure 4C and E). At this condition, SIRT1 and PPAR-γ were unobservable (Figure 4A and B). Whereas MG effectively promoted the expression of both SIRT1 and PPAR-γ, and inhibited the expression of CD86 (Figure 4D and F), suggesting the hampered M1 polarization.

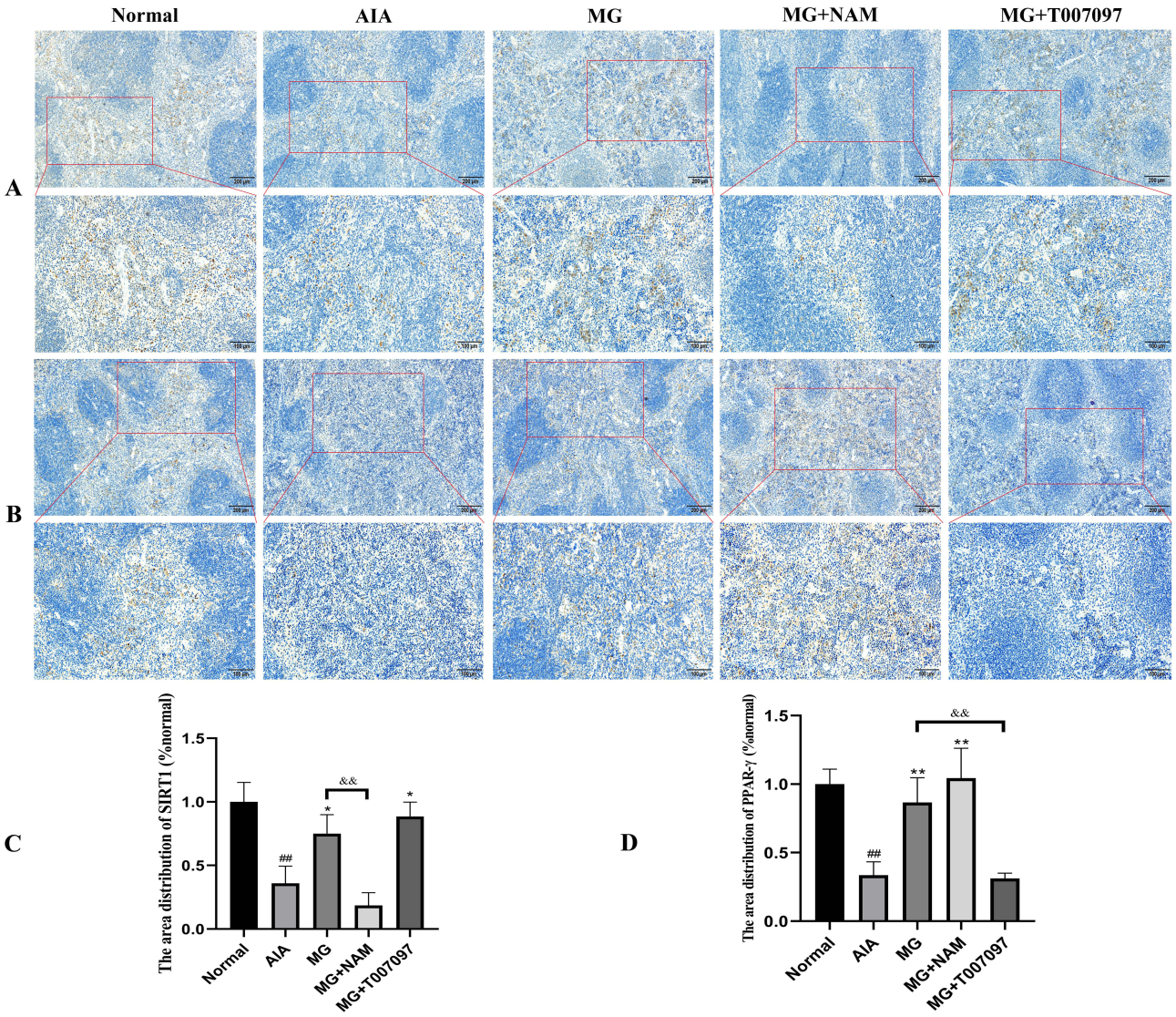


Figure 2 The effects of MG on the expression of SIRT1 and PPAR-γ in AIA mice. Local expression of SIRT1 (**A**) and PPAR-γ (**B**) in the spleen. (**C** and **D**) Quantification results of the immunohistochemical experiment analyzed by ipwin software. $^{\#\#}P < 0.01$ compared with normal, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with AIA, $^{\&\&}P < 0.01$ compared with MG.

Abbreviations: MG, α-mangostin; AIA, antigen-induced arthritis; SIRT1, silent information regulator 1; PPAR-γ, peroxisome proliferators-activated receptor γ.

SIRT1 and PPAR-γ are Required by MG to Reshape Monocytes in vitro

We used LPS to stimulate THP-1 monocytes, and then treated the cells using different reagents (Figure 5A). Results from Western blotting assay are summarized in Figure 5B–E. LPS significantly inhibited the expression of SIRT1, while MG reversed this trend. Interestingly, SIRT1 selective inhibitor sirtinol did not affect SIRT1 expression itself, while PPAR-γ inhibitor T0070097 further promoted its expression (Figure 5B). It vividly demonstrated the negative feedback loop between SIRT1 and PPAR-γ.[41] Similar changes occurred concerning PPAR-γ expression (Figure 5C). NF-κB activation is a driving force for M1 polarization, and deeply implicated in RA-related joint inflammation.[20] As expected, MG inhibited LPS-induced phosphorylation of p65 in THP-1 monocytes. Sirtinol and T007097 weakened this effect to varying degrees (Figure 5D). In addition, we observed that the two inhibitors abrogated MG-induced up-regulation of Arg-1 expression, a well-recognized marker of M2 macrophages (Figure 5E). Next, we silenced SIRT1 (Figure 5F) and PPAR-γ (Figure 5G) by siRNA in THP-1 monocytes. Under these conditions, we solidly confirm that MG possesses the ability to promote the expression of SIRT1 (Figure 5H) and PPAR-γ (Figure 5I). Quantification results of above immunoblotting assays (Figure 5F–I) are shown in Figure 5J and K.

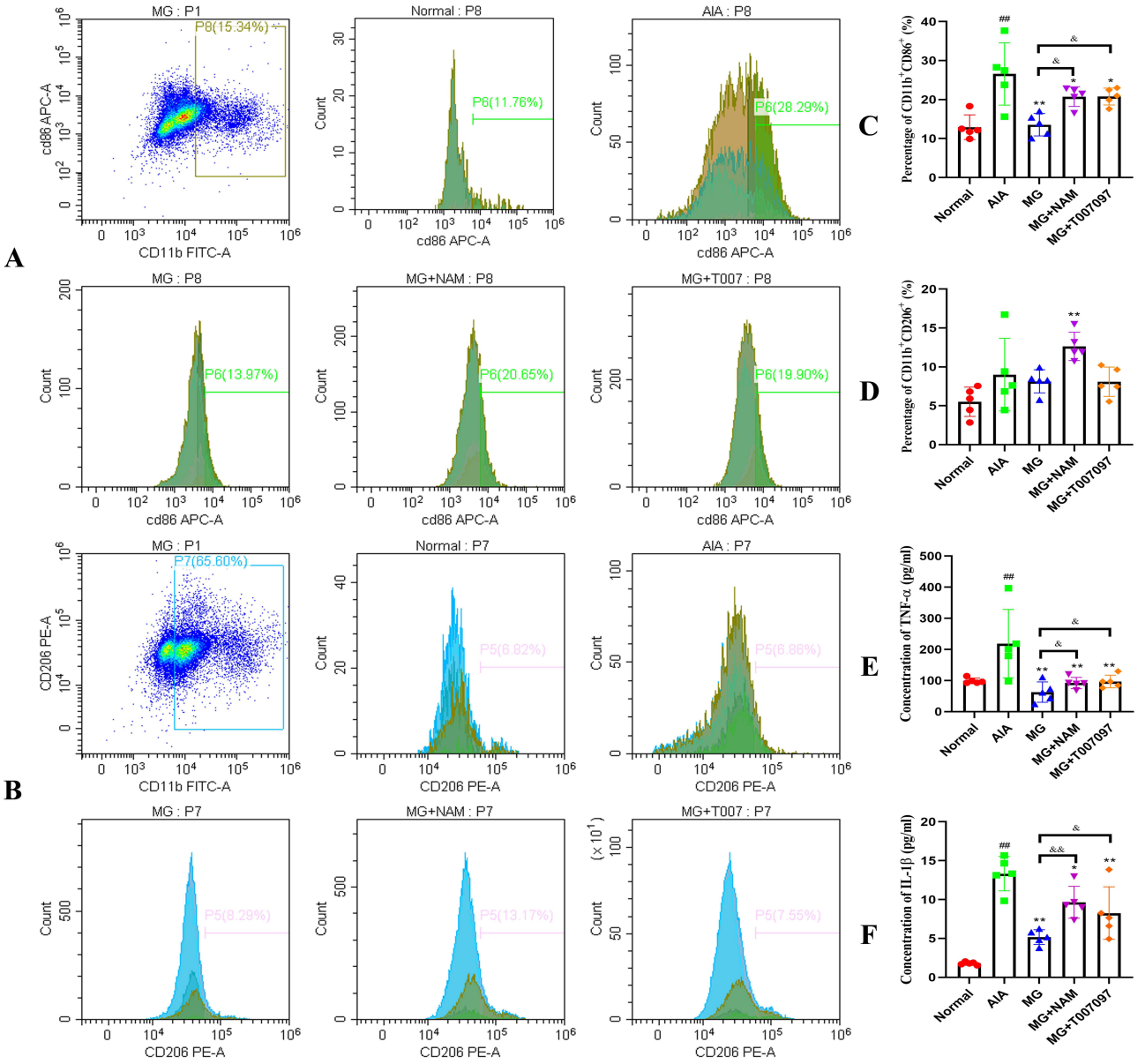


Figure 3 Relevance of SIRT1/PPAR-γ regulation of MG on macrophages polarization in AIA mice. NAM and T007097 attenuated the effect of MG in inhibiting M1 (**A** and **C**) macrophage polarization, with no obvious effect on M2 (**B** and **D**) macrophage polarization; levels of TNF-α (**E**) and IL-1β (**F**) were determined by ELISA. $^{\#\#}P < 0.01$ compared with normal, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with AIA, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared with MG.

Abbreviations: SIRT1, silent information regulator 1; PPAR-γ, peroxisome proliferators-activated receptor γ; MG, α-mangostin; AIA, antigen-induced arthritis; NAM, Nicotinamide; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1 beta; ELISA, enzyme-linked immunosorbent assay.

MG is a Potential PPAR-γ Agonist

It is known that SIRT1 is a marker for M2 macrophages/monocytes. Meanwhile, the nuclear receptor PPAR-γ promotes M2 polarization by acting as a transcription factor and controlling the secretion of many cytokines. That is, PPAR-γ up-regulation favors M2 polarization, and could consequently promote SIRT1 expression. Considering MG-caused enlargement of adipocytes in AIA mice, we speculated that the priority of the MG treatment is to activate PPAR-γ, and test this hypothesis by evaluating the binding ability of the MG to PPAR-γ. The preliminary result shows that MG can bind to PPAR-γ with high affinity (Figure 6A). We further showed its interaction with PPAR-γ in detail, and found that many hydrogen bonds were formed between them (Figure 6B). The detailed results generated by this simulation analysis are included in Table 1. The immunofluorescence experiments further supported our hypothesis. Regardless of the physiological differences, SIRT1 and PPAR-γ were always co-expressed in joints. MG simultaneously promoted the expression of SIRT1 and PPAR-γ in AIA mice (Figure 6C).

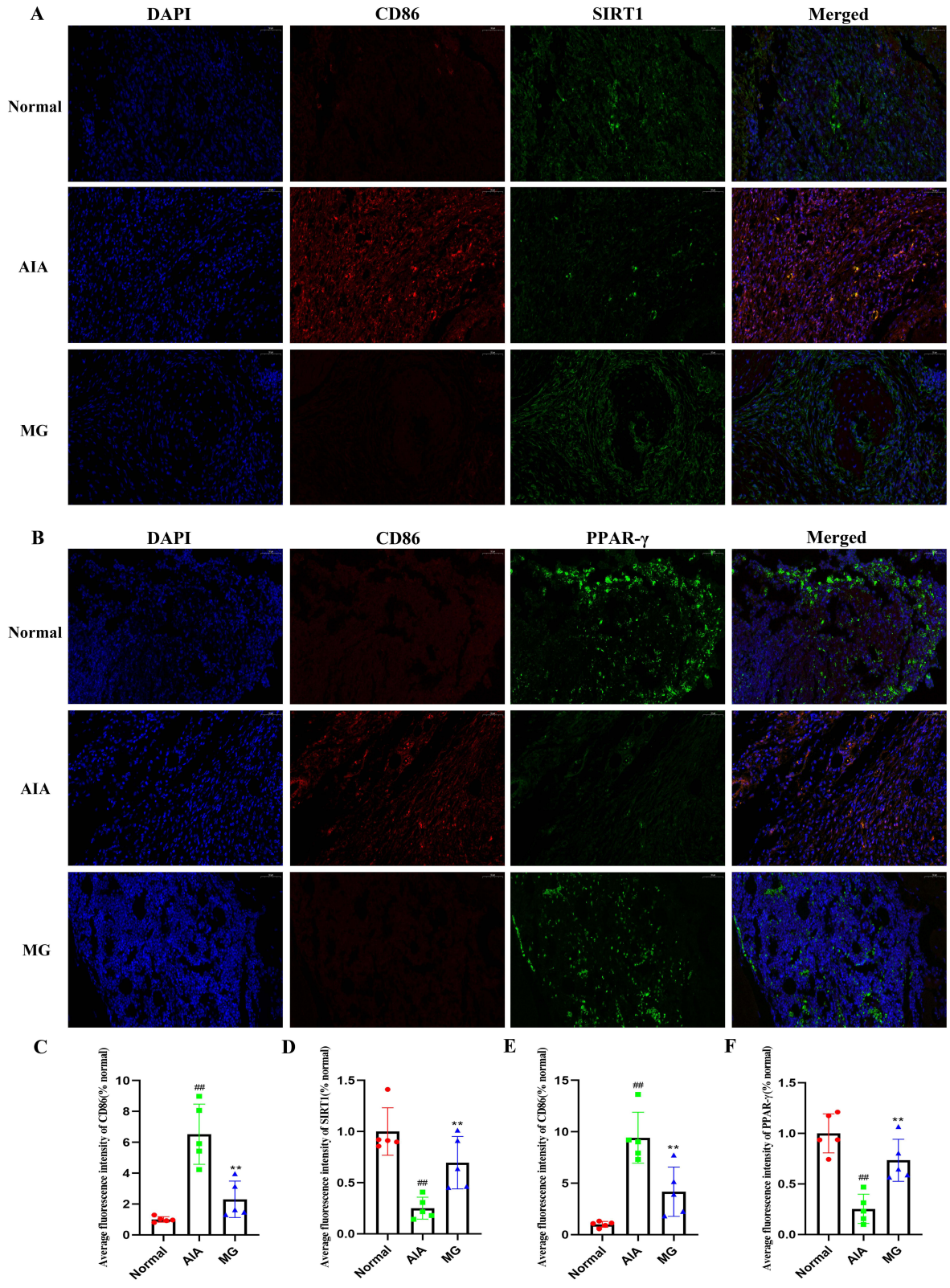


Figure 4 Effects of MG on SIRT1/PPAR-γ in macrophages from joints of AIA mice. Local expression of SIRT1/PPAR-γ and CD86 (**A** and **B**). Expression and co-localization of the proteins were observed by immunofluorescence approach. Scale bar = 50 μm. (**C**–**F**) Quantitative analysis of the average fluorescence intensity. $^{\#\#}P < 0.01$ compared with normal, $^{**}P < 0.01$ compared with AIA.

Abbreviations: SIRT1, silent information regulator 1; PPAR-γ, peroxisome proliferators-activated receptor γ; AIA, antigen-induced arthritis.

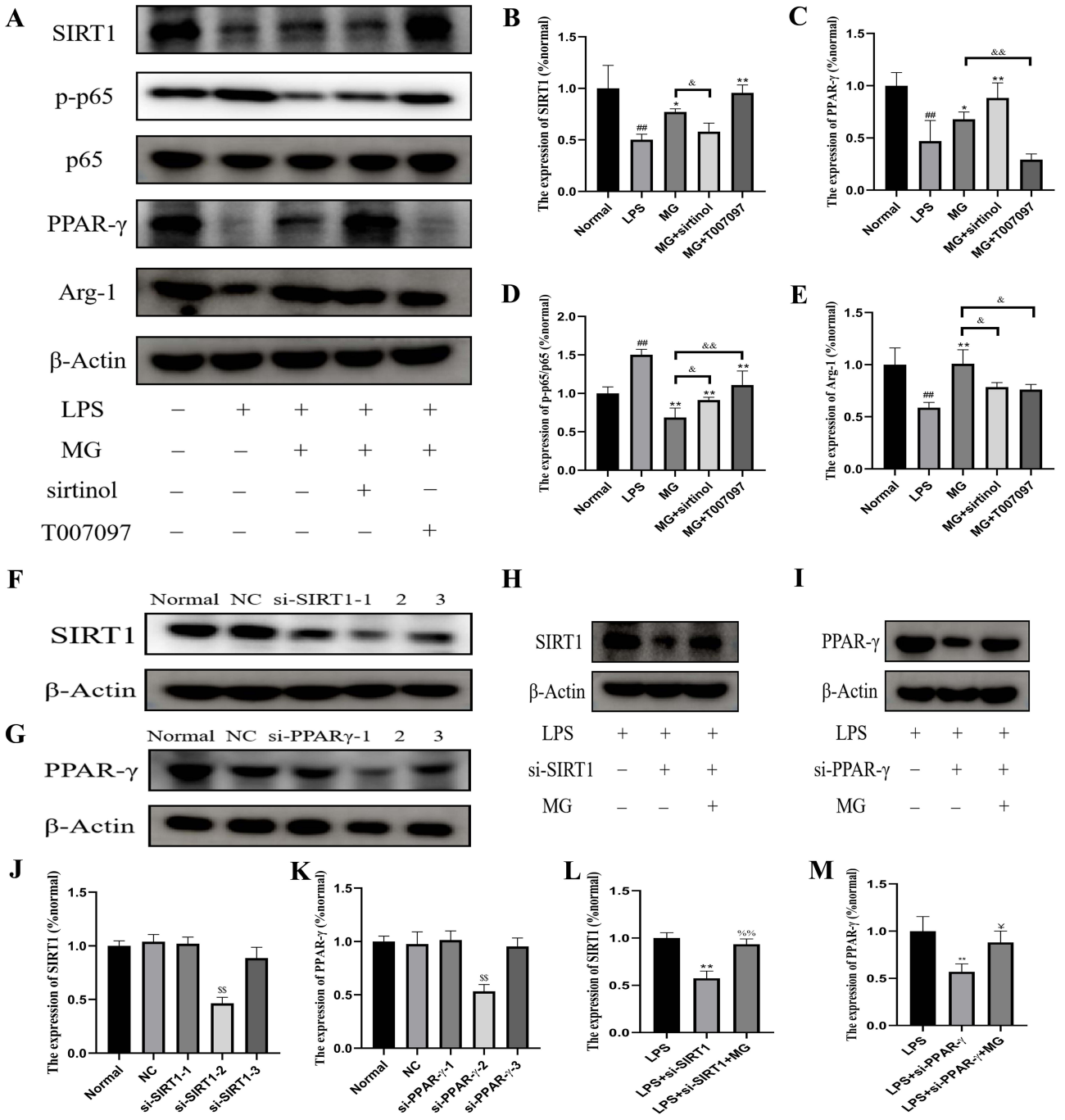


Figure 5 Relevance of MG-caused SIRT1/PPAR-γ regulation to its anti-inflammatory effects on THP-1 cells in vitro. (**A**) Representative image of immunoblotting assays evaluating SIRT1, p-p65, p65, PPAR-γ and Arg-1 expression in cells under different stimulation schemes. (**B**–**E**) Quantification results of immunoblotting assay. Results of assays screening si-SIRT1 (**F**) and si-PPAR-γ (**G**). MG-caused up-regulation of SIRT1 (**H**) and PPAR-γ (**I**) expression in cells. (**J**–**M**) Quantification results of immunoblotting assay above. $^{\#\#}P < 0.01$ compared with normal, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with LPS, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared with MG, $^{\$\$}P < 0.01$ compared with NC, $^{\%\%}P < 0.01$ compared with LPS+si-SIRT1, $^{¥}P < 0.05$ compared with LPS+si-PPAR-γ.

Abbreviations: MG, α-mangostin; SIRT1, silent information regulator 1; PPAR-γ, peroxisome proliferators-activated receptor γ; arginase-1, Arg-1; NC, negative control.

Discussion

In recent years, we focus on anti-rheumatic potentials of naturally derived xanthone derivatives, and reveal that MG is a representative especially worthy of investigations. As a natural polyphenol, MG is mainly distributed in a tropical fruit mangosteen. Because of its significant biological activities and high abundance, better exploiting its medicinal potential is not only of clinical significance but also economically efficient.[42] It showed excellent therapeutic effects on RA both in vivo and

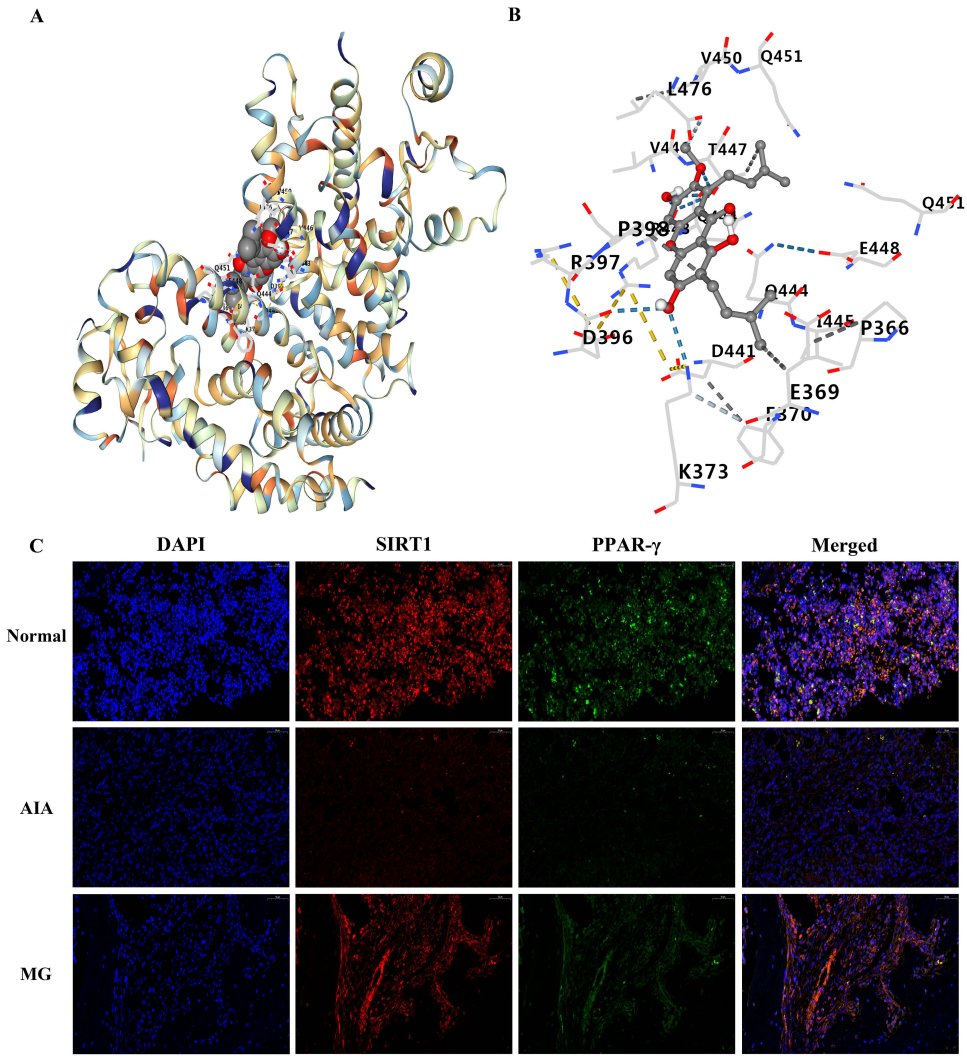


Figure 6 Potential executive of MG on PPAR-γ. (**A**) Overview on 3D structure of MG-PPAR-γ complex. (**B**) Interaction between amino acid residues of PPAR-γ and MG. (**C**) MG promoted the local expression and co-localization of SIRT1 and PPAR-γ in the joints of AIA mice.
Abbreviations: MG, α-mangostin; SIRT1, silent information regulator 1; PPAR-γ, peroxisome proliferators-activated receptor γ; AIA, antigen-induced arthritis.

in vitro.[17,18,20,43] Unfortunately, our knowledge about the relevant mechanisms is limited. Available evidences demonstrate that MG can potently affect innate immune system, including monocytes and macrophages.[20] Inflammatory macrophages infiltration into joint synovium has been regarded as an important marker of early RA.[4] This situation will disrupt immune homeostasis directly, and consequently lead to synovitis and tissue damages.[1] Targeting these cells will affect early manifestations, and even final prognosis of RA.[44,45] Under above contexts, we performed the current study in an attempt to clarify the mechanism involved in the anti-rheumatic effects of MG by selectively investigating macrophages/monocytes.

The pathological link between metabolic disorders and immune abnormalities becomes increasingly clear. Metabolic profiles of RA patients are changed a lot. Hypertriglyceridemia is a common metabolic complication of RA.[39] However, blood lipids tend to be decreased in the early and active stages of RA. The conflicts resulted in the conceptualization of

Table 1 Docking Results of MG with PPAR-γ

Targets	Vina Score	Cavity Score	Center (x, y, z)	Size (x, y, z)
PPAR-γ	−6.7	2263	24, 50, 24	23, 31, 32

Abbreviations: MG, α-mangostin; PPAR-γ, peroxisome proliferators-activated receptor γ.

the so-called "lipid paradox".[46] From this point of view, we still lack sufficient knowledge to clarify RA-caused lipid metabolism alteration and the clinical implications. The signals accountable for metabolism regulation are therefore especially worthy of investigation, as an increasing number of studies have found that the effective anti-rheumatic therapies are usually accompanied by obvious improvement of metabolic disorders.[47] SIRT1 and PPAR-γ are good representatives of them. They are the mostly affected metabolic pathways during anti-RA regimens, and they are key regulators of monocytes/macrophages status.[48,49]

PPAR-γ belongs to the nuclear receptor family. After being stimulated by the ligands, the conformation of PPAR-γ changes, which is conducive to the binding of co-agonist molecules and the release of inhibitory molecules to exert transcriptional functions. With the above mechanism, PPAR-γ controls the expression of many important genes involved in adipogenesis. It should be noted that PPAR-γ possesses effective anti-inflammatory properties independent of the transcriptional activity, and can prevent NF-κB, activator protein-1 (AP-1) and many other transcription factors from activation.[50] SIRT1, a NAD^+-dependent deacetylase, can similarly inhibit NF-κB activation, besides from its well-known pro-catabolic activity.[13] These facts hint that simultaneous up-regulation of SIRT1 and PPAR-γ would reinforce the inhibitory effects against M1 polarization, creating an environment favorable for RA remission.

Monocytes and macrophages are heterogeneous cell groups, which are typically divided into M1 and M2 subsets.[31] Under RA circumstance, M1/M2 polarization is imbalanced, and the increased M1 cells will intensify inflammatory responses and participate in tissue degradation.[3,51] The pathological functions of M1 cells are driven by their immune nature. They do not only eliminate pathogenic microorganisms, but also participate in adaptive immune activation and maturation by presenting antigens and producing a variety of cytokines/chemokines. Hence, one therapeutic goal of anti-RA treatments is to restore M1/M2 balance. In fact, the imbalanced polarization of monocytes/macrophages is also observed in many other immune diseases.[52–54] According to the current understanding, PPAR-γ and SIRT1 promote M2 polarization and repress M1 polarization by affecting both metabolism and immune pathways. In this study, we found that MG can up-regulate the two signals both in vivo and in vitro. It suggests that MG can be used to treat many inflammatory diseases, including RA.

Under this background, it is not surprising to find that MG had satisfying anti-rheumatic effects in AIA mice-based experiments, but its therapeutic efficacy was greatly weakened when SIRT1 or PPAR-γ was unilaterally inhibited (Figure 1). Consistently, MG attenuated SIRT1 and PPAR-γ expression deficiency caused by AIA (Figure 2). Furthermore, the selective inhibitors of SIRT1 and PPAR-γ both reduced the ability of MG inhibiting AIA-induced M1 abnormal polarization (Figures 3 and 4). However, we found that effects of these treatments on M2 macrophage development were not significant (Figure 3). It hints that the M1 polarization is especially sensitive to SIRT1 and PPAR-γ pathway changes. There is another possibility. Those samples were obtained at the later stage of AIA, when the spontaneous inflammation remission had occurred. At this time, distribution of M2 macrophages had already been restored to certain extents. Consequently, therapies can hardly further increase their counts.

It is worthy of noticing that SIRT1 and PPAR-γ construct a negative feedback loop. On the one hand, PPAR-γ recruits SIRT1 to its own promoter, and inhibits the expression of the latter.[55] On the other hand, SIRT1 down-regulates the expression of PPAR-γ via aP2 pathway.[41] These clues explain the phenomenon that sirtinol and T0070097 up-regulated expression of PPAR-γ and SIRT1, respectively (Figure 5). But the phenomena further add to the mystery why MG promoted PPAR-γ and SIRT1 expression simultaneously (Figures 2 and 5). It has been reported that a variety of natural polyphenols can act as PPAR-γ agonists.[19] Unsurprisingly, xanthone derivatives including MG could be also PPAR-γ agonist candidates.[56] PPAR-γ activation can induce the expression itself, and favors the development of M2 macrophages/monocytes.[57] As a result, SIRT1 expression would be increased. Hence, MG could achieve the above effects by acting as a PPAR-γ agonist directly. This speculation was preliminarily confirmed by molecular simulation docking (Figure 6).

Conclusion

This study found that MG induced simultaneous up-regulation of SIRT1 and PPAR-γ in AIA mice, and hampered macrophages to acquire M1 phenotype. Clues from the experiments in vivo and in vitro together imply that MG can bind to PPAR-γ protein and activates this signaling, which then lead to NF-κB inhibition and M1 polarization impairment. Thanks to the reshaped immune phenotype of monocytes/macrophages, SIRT1 expression deficiency was restored. The improved immune milieu eventually eased arthritic manifestations of AIA mice.

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Disclosure

The authors declare no conflicts of interest in this work.

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