

Original Research

SIRT6 Reduces Rheumatoid Arthritis Injury by Inhibiting MyD88-ERK Signaling Pathway

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Abstract

Background: Rheumatoid arthritis (RA) is an autoimmune disease characterized by destruction of synovial joints, abnormal immune responses and chronic inflammatory manifestations, which seriously affects patients' well-being. We explored this study to ascertain the effect and mechanism of silent information regulator 6 (SIRT6) on RA. **Methods:** Genes of RA patients and normal volunteers were analyzed using Gene Expression Omnibus (GEO), Kyoto-Encyclopedia of Genes and Genomes (KEGG) and Disconet databases. Serum samples of RA patients and normal subjects were collected before detection of myeloid differentiation factor-88 (MyD88)-extracellular signal-regulated kinase (ERK) pathway proteins expression with Western blot. *In vitro* RA fibroblast-like synoviocytes (FLS) cell model (RA-FLS) was established by treating RSC-364 with recombinant rat IL-1 β (10 ng/mL) after which SIRT6 and MyD88 adenoviruses treatment was carried out. The enzyme linked immunoassay (ELISA), real time polymerase chain reaction (RT-PCR) and Western blot were respectively used to measure inflammatory factors, related messenger ribonucleic acid (mRNA) and protein expressions. Also, we constructed RA rat model with bovine type II collagen (BIIC) and complete Freund's adjuvant, before treatment with SIRT6 and MyD88 adenoviruses. **Results:** Low expression of *SIRT6* gene were detected in RA patients. Also, levels of MyD88, ERK and phosphorylated extracellular signal-regulated protein kinase (p-ERK) protein expressions in RA patients were increased, whilst that of SIRT6 protein decreased. Compared to FLS cells in Control group, inflammatory factors levels of rats in Model batch increased significantly. SIRT6 adenovirus treatment potentially and significantly inhibited inflammation including suppression of increased inflammatory factors induced by MyD88. In comparison with FLS cells in Control group, Model batch cells' MyD88, interleukin (IL)-1 β , IL-21, IL-22, IL-6, IL-17, tumor necrosis factor-alpha (TNF- α) and monocyte chemo-attractant protein-1 (MCP-1) mRNA expressions increased but *SIRT6* gene treatment could reduce mRNA expression of the aforesaid factors, even after MyD88 adenovirus treatment. Besides, overexpressed SIRT6 negatively regulated levels of MyD88, ERK and p-ERK proteins expressions. SIRT6 demonstrated anti-RA effect by regulating MyD88-ERK pathway and inhibiting inflammatory response in RA rats. **Conclusions:** SIRT6 could potentially inhibit the inflammatory response of RA via a regulatory mechanism mainly relating to MyD88-ERK signal pathway. Thus, SIRT6 and its agonists may serve as new targets for developing drugs that can potentially treat RA.

Keywords: rheumatoid arthritis; SIRT6; MyD88-ERK signaling pathway; ultrasonic; western blot

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by destruction of synovial joints [1], abnormal immune responses [2] and inflammatory manifestations [3]. In southern countries, RA should be properly controlled [4] since its expected prevalence is ranged from 0.2 to 0.3%. Promotion of microvessels formation in joints by inflammation may cause joint damage. With regards to pathological mechanism of RA, rheumatoid factors [5] and several inflammatory processes [6] have been implicated. Through fibroblast-like synoviocytes

(FLS), scientists have observed the promotion of vascular-endothelial growth factor (VEGF) release by proinflammatory mediators like monocyte chemo-attractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF- α), nitric-oxide, prostaglandins, interleukin (IL)-1, IL-6, IL-17 and IL-18 [7,8]. Thus, main invasive proliferative cell involved in RA pathogenesis is FLS, which plays a crucial role in inflammation of RA [9,10]. During RA onset, FLS and macrophages release increased the levels of factors like IL-6, IL-1 β , matrix metalloproteinase (MMP-1/3) and other inflammatory mediators by migrating and invading bone and joint tissues, thus inducing excessive intra-articular



synovial tissue proliferation which further aggravated intra-articular inflammatory response of RA patients [11].

The silent information regulator (SIRT) family is third type of human histone deacetylase dependent on nicotinamide adenine dinucleotide (NAD⁺) [12] with seven members [13]. SIRT6 is expressed as ADP ribosyltransferase in nucleus [14]. Relevant works have found that SIRT6 associates with the regulation of body modifications for environmental adaptation. Particularly, SIRT6 regulates genome stability, DNA repair and inflammatory response through deacetylation of histones and various transcription factors, especially in immune inflammation [15–17]. SIRT6 knockout in endothelial cells of human umbilical vein could lead to increased levels of IL-1 β , IL-6, IL-8, MMP-2 and -9. Contrarily, overexpressed SIRT6 inhibited expression of these factors [18]. Thus, SIRT6 expression inhibition especially in macrophages could aggravate inflammation [19,20]. Contrastingly, ectopically overexpressed SIRT6 in knockout cells decreased inflammation. The SIRT6 plays a crucial role in inflammation suppression by inhibiting nuclear-factor kappa-B (NF- κ B) pathway activation via removal of histone-3 lysine-9 (H3K9) acetylation levels of downstream target genes of NF- κ B [21]. Thus, SIRT6 has an anti-inflammatory function, which can inhibit inflammatory responses and progress of RA [22]. Available literature has described the potential of SIRT6 to control a smoke induced signaling in synovial fibroblasts of RA [23]. Likewise, another study affirmed that SIRT6 could decrease inflammation and release of proinflammatory factors in collagen induced RA mouse model [24]. Besides, a lower SIRT6 expression in PBMC and monocytes/macrophages of RA patients was observed compared to osteoarthritic patients. Thus, SIRT6 may be explored as a treatment target of RA. Nevertheless, actual mechanism of SIRT6 in RA treatment has not been clarified. Bioinformatics prediction has shown multiple sites binding of SIRT6 promoter to transcription factors, including Ahr, SPIB, Pdx1, and Prrx2.

Chronic immune inflammatory diseases such as RA [25] have devastating effect on the well-being of patients. As proteins that are associated with immune system, toll-like receptors (TLRs) widely exist in various innate immune cells, namely neutrophils, monocyte macrophages, dendritic cells and natural killer (NK) cells [26,27]. They (except TLR3) activate myeloid differentiation factor-88 (MyD88), which is most important adaptor protein in TLRs signal transduction pathway [28]. Literature has confirmed that defective *MyD88* gene in TLR signal transduction pathway resulted in substantially reduced joint synovitis and bone tissue damage [29]. An extracellular signal-regulated kinase (ERK) pathway is a vital MAPKs family member. A close linkage of abnormal activation of ERK with pathological process of RA joint destruction has been observed. The ERK is significantly activated in T lymphocytes, FLS and macrophages in RA synovium, thus suggesting its in-

volvement in transduction of pathological signals [30]. It has been shown that ERK and its inhibitors could alleviate RA symptoms and even block disease progress [31].

Herein, we found that MyD88-ERK is an important signal pathway that promotes inflammation in RA pathogenesis. Increased expressions of MyD88-ERK and IL-1 β , IL-21, IL-22, IL-6, IL-17, tumor necrosis factor-alpha (TNF- α) and monocyte chemo-attractant protein-1 (MCP-1) and concomitant decreased expression of Sirt6 were observed in RA patients. Sirt6 treatment inhibited MyD88-ERK signal pathway which decrease inflammatory response via reduced expression and levels of the above-mentioned inflammatory mediators (especially IL-1, IL-6 and TNF- α) in RA patients, thus improving treatment of RA. Hence, this study sought to clarify SIRT6 role in RA inflammatory response, and explore its molecular mechanism in reducing RA inflammatory injury through inhibition of MyD88-ERK signaling pathway in RA patient serum, rat synovial cell RA model and rat arthritic model as well as appropriate techniques.

2. Methods

2.1 Materials

Fetal bovine serum (FBS) and RPMI 1640 culture medium were bought from Hyclone (Logan, UT, USA), while TRIzol reagent was obtained from Invitrogen (Carlsbad, CA, USA). The BCA kit was bought by Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Sigma Co., (St. Louis, MO, USA) supplied Freund's complete adjuvant, Bovine type II collagen (BIIC) and RIPA lysate. Mitogen extracellular kinase (MEK), phosphorylated mitogen extracellular kinase (p-MEK), ERK, phosphorylated extracellular signal-regulated protein kinase (p-ERK), SIRT6 and MyD88 antibody were provided by Abcam corporation (Cambridge, MA, USA). Lentivirus expression vector was bought from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant rat IL-1 β was purchased from peprotech (Rocky, NJ, USA). The IL-1 β , IL-6, IL-17, IL-21, IL-22, MCP-1, lactate dehydrogenase (LDH), glutathione (GSH), superoxide dismutase (SOD) malondialdehyde (MDA) and TNF- α enzyme linked immunoassay (ELISA) kits were bought from Abcam (Cambridge, MA, USA).

2.2 Study Population Recruitment

Between January 2020 and October 2021, we recruited 22 RA patients (10 females and 12 males of 25–65 years) and 22 healthy controls (HC, 22 females and 10 males of 24–63 years) from Jiangsu University affiliated Wujin Hospital. Those with American College of Rheumatology European League against Rheumatism (ACR_EULAR) classified RA [32] were used for this study, wherein they did not receive any treatment. All the subjects that were included in this study did not have autoimmune diseases, blood system, cardiovascular and cerebrovascular system and other systemic diseases.

2.3 Cells and Animals

Rat FLS cell line 364 (RSC-364) was obtained from the Wujin Hospital Affiliated with Jiangsu University and cultured in RPMI-1640 complete medium at 37 °C and 5% CO₂ humidification. Mycoplasma contamination of the cell line was detected by phase contrast microscopy. The cells were inoculated on a cover slide placed in the culture bottle, and removed 24 hours later, before we observed with phase contrast microscope. Mycoplasma is a dark microscopic particle located on the cell surface and between the cells. The results showed that no dark microscopic particles were observed, thus indicating that the mycoplasma contamination test of the cell line was negative. In addition, immunofluorescence staining was used to identify the cell lines. The cells were inoculated on a 24-well plate with a cover slide. After the cell growth confluence was about 60%, the cells were washed twice with PBS (pH 7.4). Later, we fixed them with 4% paraformaldehyde at room temperature for 15 min, and rinsed 3 times with PBS for 5 min each time. Afterwards, 5% goat serum sealing solution (containing 0.3% Triton X-100) was used for sealing at room temperature for 60 min. Vimentin antibody (1:200) and Fibronectin (1:200) were added overnight at 4 °C and rinsed 3 times with PBS for 5 min each time. Alexa Flour 647 (1:500) was incubated at room temperature away from light for 1 h and rinsed 3 times with PBS for 5 min each time. The tablets were sealed with DAPI, while fluorescence staining was observed via fluorescence microscope. The expression of Vimentin and fibronectin was observed to be positive after immunofluorescence identification, which could be used for subsequent experiments.

Sprague-Dawley (SD) rats (female, 6–7 weeks, 200 ± 20 g) were supplied by Jiangsu University laboratory center (Zhenjiang, China). The rats were exposed to 24 ± 1 °C temperature and 50 ± 10% humidity for a light-dark cycle of 12 h. Food and water were available *ad libitum*. All procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee of Jiangsu University. A total of 30 healthy mice were selected for this experiment. They were randomly divided into 5 groups with 6 animals in each group.

2.4 Information Analysis

We downloaded gene data related to RA from databases such as Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>), Kyoto-Encyclopedia of Genes and Genomes (KEGG) (<https://www.kegg.jp/kegg/>) and diskenet (<https://www.disgenet.org/>) as well as screened for distinctly expressed genes (1923 including 752 up-regulated genes and 1171 down regulated genes) in RA and normal groups with GEO2R online tool (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>). The genes with the top 36 differentially expressed values were analyzed with heat map analysis.

2.5 Study on Serum of clinical RA Patients

2.5.1 Serum Samples Collection

In the morning after overnight fasting, we withdrew whole venous blood (7–8 mL) from 22 RA patients and 22 healthy volunteers (as normal control). Then, the blood samples were anti-coagulated with heparin, and centrifuged at 3700 rpm for 10 min. After that, serum was collected into a 2 mL EP tube and stored in a refrigerator at –80 °C for subsequent use.

2.5.2 Detection of Related Protein Expression by Western Blot

The protein of the sample to be tested was extracted by protein extraction lysate. The protein concentration was determined via BCA protein quantitative kit. Later on, 50 µg protein solution was transferred to the PVDF membrane after 12% SDS- polyacrylamide gel electrophoresis. The membrane was sealed with 5% skimmed milk at room temperature for 1.5 h. The corresponding primary antibody was added and incubated at 4 °C overnight. The PVDF membrane was taken out and washed with PBS solution (3 times) for 5 min each time. Fluorescent labeled secondary antibodies were added to the PVDF membrane and incubated at room temperature for 2 h. Then, ECL solution (Thermo Fisher Scientific, Waltham, MA, USA) was added for full reaction to take place. Excess liquid was removed with filter paper and the samples were covered with the film. The gray value of the protein strip was analyzed with the gel image processing system. The relative expression of protein was expressed by the ratio of gray value of target protein to internal reference protein (GAPDH).

2.6 In Vitro Cell Experiments

2.6.1 Cells Culture

RSC-364 cells were selected to establish rat RA cell model *in vitro*. The cells were cultured in RPMI 1640 medium (containing 10% FBS) under humidified CO₂ (5%) and 37 °C. Then, the cells were digested with 0.25% trypsin (containing 0.02% EDTA) and press 5 × 10⁶/L was inoculated in a 25 cm² culture flask. The culture medium was discarded when the cells were fused to 90% and in the logarithmic growth phase. The cells were resuspended with RPMI 1640 medium, in which the cells (1 × 10⁴) were inoculated in 96 well plates with a medium volume of 200 µL (in each well). Later, 96 well plates were cultured at 37 °C, CO₂ (5%) incubator and saturated humidity for 24 h.

2.6.2 Adenovirus Construction

SIRT6 adenovirus construction method was as follows: after the whole coding sequence (CDS) of rat *SIRT6* gene was synthesized, the multiple cloning sites (MCS) of pAdeasy-EF1-MCS-CMV-mcherry were constructed and packaged as adenovirus. The vector map is shown in Fig. 1A, in which the SIRT6 promoter was EF1a, and the virus had red fluorescence.

Table 1. The sequences of the primers employed in the RT-PCR.

Genes		Sequences of primer (5' to 3')
<i>SIRT6</i>	Forward	CTTTATTGTTCCCGTGC GGC
	Reverse	ACCTTGCAATTCAGCTCCTC
<i>MyD88</i>	Forward	CTACAGAGCAAGGAATGTGACT
	Reverse	ACCTGATGCCATTGCTGTCC
<i>IL-6</i>	Forward	AGAGACTTCCAGCCAGTTGC
	Reverse	AGTCTCCTCTCCGGACTTGT
<i>TNF-α</i>	Forward	GGCTTTCGGAACACTCACTGGA
	Reverse	GGGAACAGTCTGGGAAGCTC
<i>IL-1β</i>	Forward	TTGAGTCTGCACAGTTCCCC
	Reverse	TCCTGGGGAAGGCATTAGGA
<i>IL-17</i>	Forward	G TTCAGTGTGTCCAAACGCC
	Reverse	AGGGTGAAGTGGAAACGGTTG
<i>IL-21</i>	Forward	CTAAAGCGGGAAGGACGTGT
	Reverse	CTGGAGGTGAGCGCTACAAA
<i>IL-22</i>	Forward	TCAGCGGTGATGACCAGAAC
	Reverse	TCAGACGCAAGCGTTTCTCA
<i>MCP-1</i>	Forward	AGCCAAC TCTACTGAAGCC
	Reverse	AACTGTGAACAACAGGCCCA

RT-PCR, real time polymerase chain reaction.

with the specific operation method of Western blot being the same as “2.5.2 Detection of related protein expression by Western Blot”.

2.7 In Vivo Rats' Experiments

2.7.1 Establishment of RA Rat's Model

According to previous studies [33,34], BIIC (10 mg) and complete Freund's adjuvant (5 mL) were mixed into emulsifier. Then, 0.1 mL mixed emulsifier (100 mg collagen/rat) was injected into rat tail vein for 21 days to construct the RA rats model.

2.7.2 Animal Grouping and Administration

Healthy SD rats were randomly divided into 5 groups: ① Blank control group (Control) without any treatment. ② Model group (Model): The RA rat model was established by the above modeling method. ③ Adenovirus loaded SIRT6 + model group (Ad-SIRT6-Model): The rats with high SIRT6 expression were treated with SIRT6 adenovirus, before treatment with the above modeling method. ④ Blank adenovirus + model group (Ad-Model): After treating the rats with empty adenovirus, we also treated them with the above modeling method. ⑤ Rats in the adenovirus loaded SIRT6 experimental group (Ad-SIRT6-Model-MyD88) treated with MyD88 adenovirus, after which we treated them with SIRT6 adenovirus, followed by treatment with the above modeling method and MyD88 adenovirus.

2.7.3 Ultrasonic Examination

Two-dimensional ultrasound was used to observe the synovial hyperplasia of rat limb joints. The blood flow in proliferative synovium was observed via the superb microvascular imaging (SMI) technique. The grade of synovial hyperplasia and blood flow were evaluated using ultrasonic grade 4 semi quantitative scoring method, and the disease activity of limb arthritis was evaluated at the imaging level. The specific inspection steps are as follows: Toshiba aplio500 ultrasonic diagnostic instrument and linear array probe (dynamic range 60 dB, frequency 7–18 mhz) were used. The joints of rats' limbs were examined horizontally and longitudinally. The synovial hyperplasia was observed by two-dimensional ultrasound and evaluated with semi quantitative scoring method. The section with thick synovium at the focus was taken to fix the probe position. Later, the SMI mode was started, before we adjusted the appropriate color gain and blood flow sampling frame. The blood flow of synovium was observed under SMI mode and could be evaluated through semi quantitative scoring method.

2.7.4 Sample Collection

After the experiment, blood (0.4 mL) samples were collected via retro-orbital route into EP tubes containing heparin. Then, the rats were sacrificed before the synovium, liver and kidney were collected, wherein blood on the tissue surface was washed with PBS. Briefly, blood samples were centrifuged at 3700 rpm for 10 min. Then, serum was collected at –80 °C for standby. A part of rats' liver and kidney were homogenated and centrifuged at 10,000 rpm for 10 min. After that, the supernatant was taken and stored in the refrigerator at –80 °C. Synovium, extra part of the rats' liver and kidney were fixed in 10% para-formaldehyde solution for 4 h, before embedment in paraffin and sliced (about 4 μ m) for subsequent experiments.

2.7.5 Histopathological Examination

Paraffin sections of synovium, liver and kidney tissues were routinely dewaxed, hydrated and stained with hematoxylin eosin (HE). Finally, the pathological changes in the prepared samples were observed under the microscope (Nikon, Tokyo, Japan).

2.7.6 Molecular Biological Detection

The levels of IL-6, IL-17, IL-21, IL-22, MCP-1, TNF- α , GSH, IL-1 β and MDA as well as SOD in serum of the rats were detected strictly according to the standard operating procedures of the ELISA kits.

2.7.7 Real-Time PCR

SIRT6, MyD88, IL-1 β , IL-6, IL-17, IL-21, IL-22, MCP-1 and TNF- α mRNA expressions were detected with Real-time PCR. Specific operation method of Real-time PCR was the same as described in section “2.6.5 Real-time PCR”.

2.7.8 Western Blot

The protein expressions of SIRT6, MyD88, p-ERK, ERK, MEK and p-MEK were detected with Western blot. The specific operation method of Western blot was the same as depicted in section “2.5.2 Detection of related protein expression by Western Blot”.

2.8 Statistical Analysis

Data were presented in the form of mean \pm standard deviation (SD). We performed student's *t*-test or analysis of variance (ANOVA) to determine the significant difference between two groups. Analysis of experimental results was accomplished with GraphPad prism (version 8, GraphPad Software Inc., San Diego, CA, USA). Statistically, we accepted $p < 0.05$ as significant differences between groups.

3. Results

3.1 Analysis of SIRT6 Gene Expression in RA Patients

We analyzed the sera of RA patients to ascertain their level of SIRT6 expression. Of note, red color indicates reduced gene expression (Z score), while green color shows increased gene expression with color depth suggesting difference in distinct expression levels. We observed from the analysis that *SIRT6* gene expression decreased significantly in RA patients (Fig. 2), which may be due to the increased expressions of inflammatory factors such as IL-1 β , IL-6, TNF- α and MCP-1 or high expression of LncRNA plasmacytoma variant translocation 1 (PVT1), which lowers Sirt6 expression via methylation of the deacetylase protein in RA fibroblast-like synoviocytes (RA-FLS) and synovial tissues of RA patients [35,36].

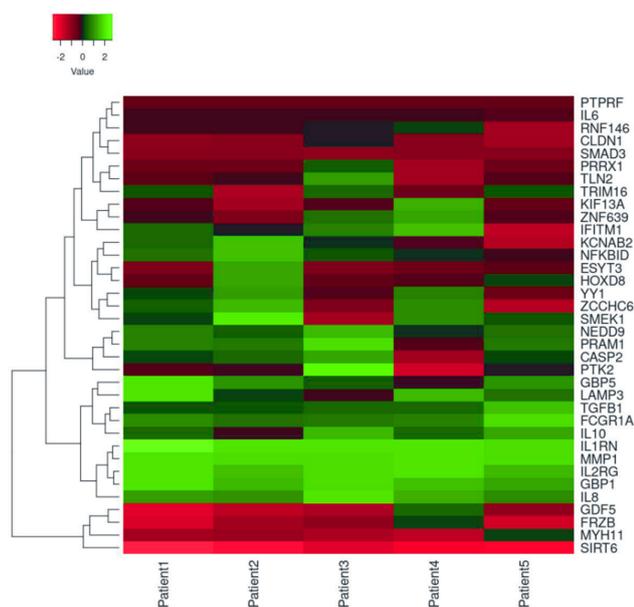


Fig. 2. Analysis of 36 gene expressions in rheumatoid arthritis (RA) patients (relative to healthy subjects).

3.2 Detection of SIRT6 and MyD88-ERK Protein Expression in RA Patients' Sera

Expression of MyD88, ERK and p-ERK proteins in RA patients was investigated with western blot technique. It was discovered that the aforementioned proteins increased significantly (Mean \pm SD for each protein in RA group: MyD88: 0.96 ± 0.37 ; ERK: 1.18 ± 0.18 ; p-ERK: 1.24 ± 0.08 , $p < 0.05$ or $p < 0.01$) compared to normal group (Mean \pm SD for each protein in normal control group: MyD88: 0.06 ± 0.02 ; ERK: 0.43 ± 0.01 ; p-ERK: 0.53 ± 0.19) (Fig. 3). In comparison with normal group, level of SIRT6 protein expression in RA patients decreased substantially (Mean \pm SD for SIRT6 protein in RA group: 1.10 ± 0.03 ; in normal control group: 1.60 ± 0.12 , $p < 0.01$), which may be ascribed to increased expression of MyD88, ERK and p-ERK proteins in RA patients. Collectively, we observed increased of MyD88, ERK and p-ERK proteins expressions with concomitant decreased Sirt6 protein expression in RA patients.

3.3 Effect of SIRT6 on Inflammatory Markers and MyD88-ERK Pathway in RA-RSC Cells in Vitro

ELISA testing was employed to measure levels of IL-1 β , SOD, IL-21, LDH, IL-6, MDA, IL-22, TNF- α , IL-17 and MCP-1 in RA-FLS cells. It was observed that Model group cells increased significantly with decreased SOD (Mean \pm SD for each inflammatory markers in model group: IL-1 β : 417.16 ± 25.63 ; SOD: 19.83 ± 1.41 ; IL-21: 17.05 ± 1.27 ; LDH: 33.05 ± 2.15 ; IL-6: 107.1 ± 5.68 ; MDA: 9.80 ± 0.62 ; IL-22: 41.24 ± 2.93 ; TNF- α : 762.9 ± 58.32 ; IL-17: 81.07 ± 6.40 ; MCP-1: 2.41 ± 0.17 ; $p < 0.01$) compared with control group (Mean \pm SD for each inflammatory markers in control group: IL-1 β : 71.22 ± 2.57 ; SOD: 44.96 ± 3.01 ; IL-21: 5.07 ± 0.37 ; LDH: 8.08 ± 0.51 ; IL-6: 18.33 ± 0.97 ; MDA: 1.17 ± 0.08 ; IL-22: 11.78 ± 0.77 ; TNF- α : 381.6 ± 25.5 ; IL-17: 15.84 ± 1.04 ; MCP-1: 0.79 ± 0.05 ; $p < 0.01$) (Fig. 4). Adenovirus SIRT6 and blank adenovirus vectors exhibited no effect on normal FLS cells because Ad-SIRT6-Control and Ad-Control were unchanged or changed slightly. Compared with Model cells, levels of aforesaid inflammatory mediators and SOD in Ad-Model batch were unchanged, which suggests that blank adenovirus vector demonstrated no effect on inflammation. Compared with group Model, the contents of IL-1 β , IL-21, IL-22, IL-6, IL-17, LDH, MDA, TNF- α , MCP-1 and SOD in group Ad-Model were basically unchanged, which indicates that the blank adenovirus vector had no effect on inflammation. In comparison with group Model, the levels of IL-1 β , IL-21, IL-22, IL-6, IL-17, LDH, MDA, TNF- α and MCP-1 in group Ad-SIRT6-Model decreased significantly (Mean \pm SD for each inflammatory markers in Ad-SIRT6-Model group: IL-1 β : 171.14 ± 8.46 ; IL-21: 7.14 ± 0.46 ; LDH: 15.25 ± 1.04 ; IL-6: 25.8 ± 3.34 ; MDA: 2.84 ± 0.21 ; IL-22: 20.16 ± 1.36 ; TNF- α : 478.75 ± 34.21 ; IL-17: 30.77 ± 2.03 ; MCP-

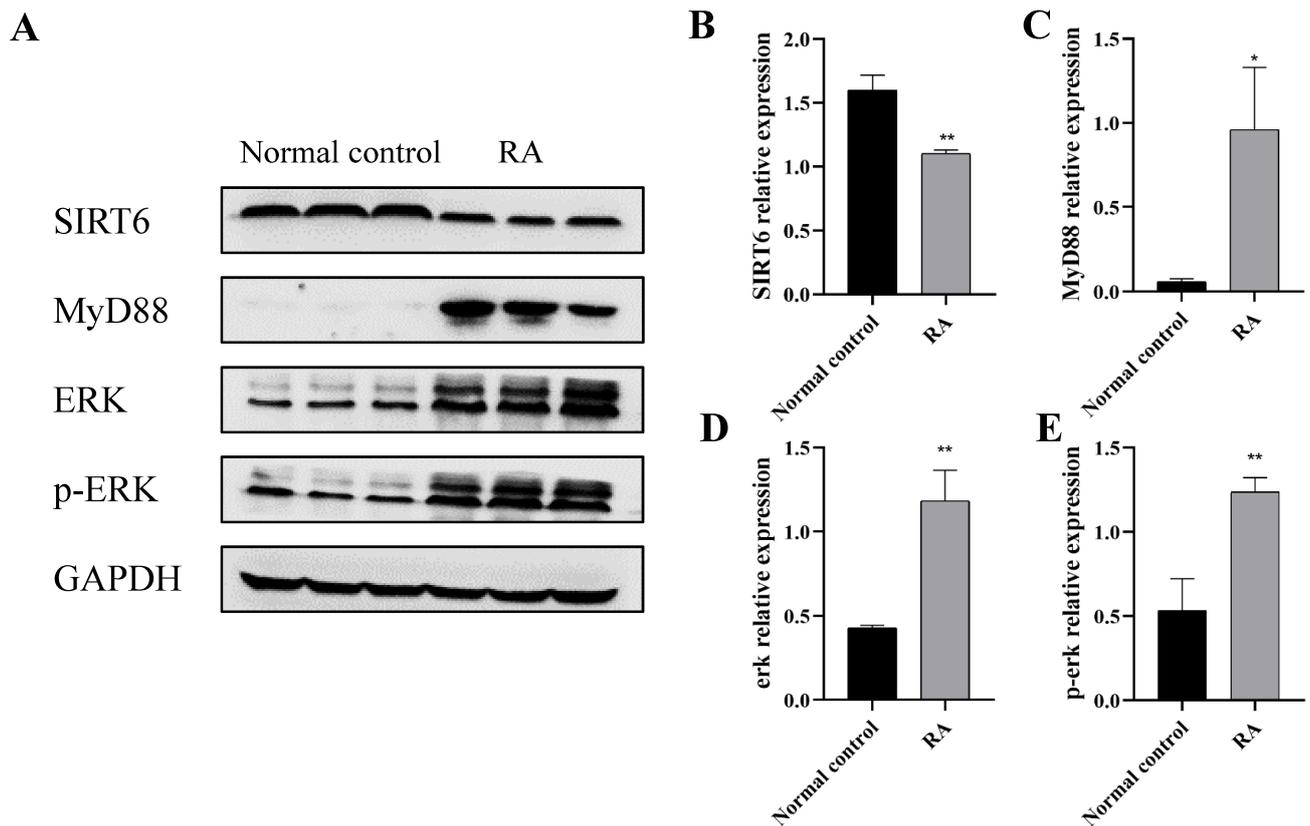


Fig. 3. Expressional levels of SIRT6, MyD88, extracellular signal-regulated kinase (ERK) and phosphorylated extracellular signal-regulated protein kinase (p-ERK) protein of normal control and RA patient groups. (A) Western blot analysis of SIRT6, MyD88, ERK and p-ERK levels. Gray value of target protein to internal reference protein (GAPDH) was used as a loading control. Quantitative densitometry of SIRT6 (B), MyD88 (C), ERK (D) and p-ERK (E) levels relative to GAPDH. * $p < 0.05$, ** $p < 0.01$, compared to Model control group. Experimental results are expressed as mean \pm SD, $n = 3$. SD, standard deviation.

1: 1.18 ± 0.07 ; $p < 0.01$), while the level of SOD in group Ad-SIRT6-Model increased significantly (Mean \pm SD of SOD in Ad-SIRT6-Model group: 31.05 ± 1.98 ; $p < 0.01$). In addition, compared with group Model, the levels of IL-1 β , IL-21, IL-22, IL-6, IL-17, LDH, MDA, TNF- α and MCP-1 in group Ad-SIRT6-Model-MyD88 decreased significantly (Mean \pm SD for each inflammatory markers in Ad-SIRT6-Model-MyD88 group: IL-1 β : 365.21 ± 44.77 ; IL-21: 15.11 ± 1.49 ; LDH: 29.82 ± 2.01 ; IL-6: 98.99 ± 4.36 ; MDA: 8.55 ± 1.44 ; IL-22: 36.65 ± 4.64 ; TNF- α : 640.95 ± 73.43 ; IL-17: 72.12 ± 8.75 ; MCP-1: 2.03 ± 0.47 ; $p < 0.05$ or $p < 0.01$), while the level of SOD increased significantly (Mean \pm SD of SOD in Ad-SIRT6-Model-MyD88 group: 25.03 ± 3.60 , $p < 0.05$). The results suggest that the RA cells treated with SIRT6 adenovirus could significantly inhibit inflammation. On this basis, SIRT6 could regulate the activities of IL-1 β , IL-21, IL-22, IL-6, IL-17, LDH, MDA, TNF- α , MCP-1 and SOD induced by MyD88 to a certain extent.

3.4 Detection of SIRT6 Expression at mRNA Level

The RT-PCR technique was explored to detect the expression of SIRT6 at mRNA level. It can be discov-

ered (Fig. 5) that compared with control group, while the Ad-SIRT6-Control showed increased relative SIRT6 expression at mRNA (Mean \pm SD for SIRT6 in Ad-SIRT6-Control group: 3.10 ± 0.34 ; control group: 1.00 ± 0.18 , model group: 0.89 ± 0.10 , $p < 0.01$), but demonstrated relatively decreased expression of nuclear protein in Model batch (Mean \pm SD for SIRT6 in model group: 0.89 ± 0.10 , $p < 0.05$). In a similar fashion, comparative analysis of the Model with Ad-SIRT6-Model and Ad-SIRT6-Model-MyD88 groups demonstrated increased (Mean \pm SD for SIRT6 in Ad-SIRT6-Model group: 2.62 ± 0.34 ; Ad-SIRT6-Model-MyD88 group: 2.47 ± 0.44 , $p < 0.01$) relative expression of SIRT6 mRNA in the latter than former. This implies that *SIRT6* gene loaded by adenovirus vectors in Ad-SIRT6-Control, Ad-SIRT6-Model and Ad-SIRT6-Model-MyD88 groups can be carried into cells with high expression. In model group, we observed disturbed and decreased relative expression of SIRT6 mRNA in normal FLS cells that were treated with recombinant rat IL-1 β (10 ng/mL). In addition, compared with control group, the relative expression of IL-1 β , IL-21, IL-22, IL-6, IL-17, TNF- α and MCP-1 mRNA in model group increased significantly ($p < 0.01$) with that of Ad-SIRT6-Control and Ad-Control groups re-

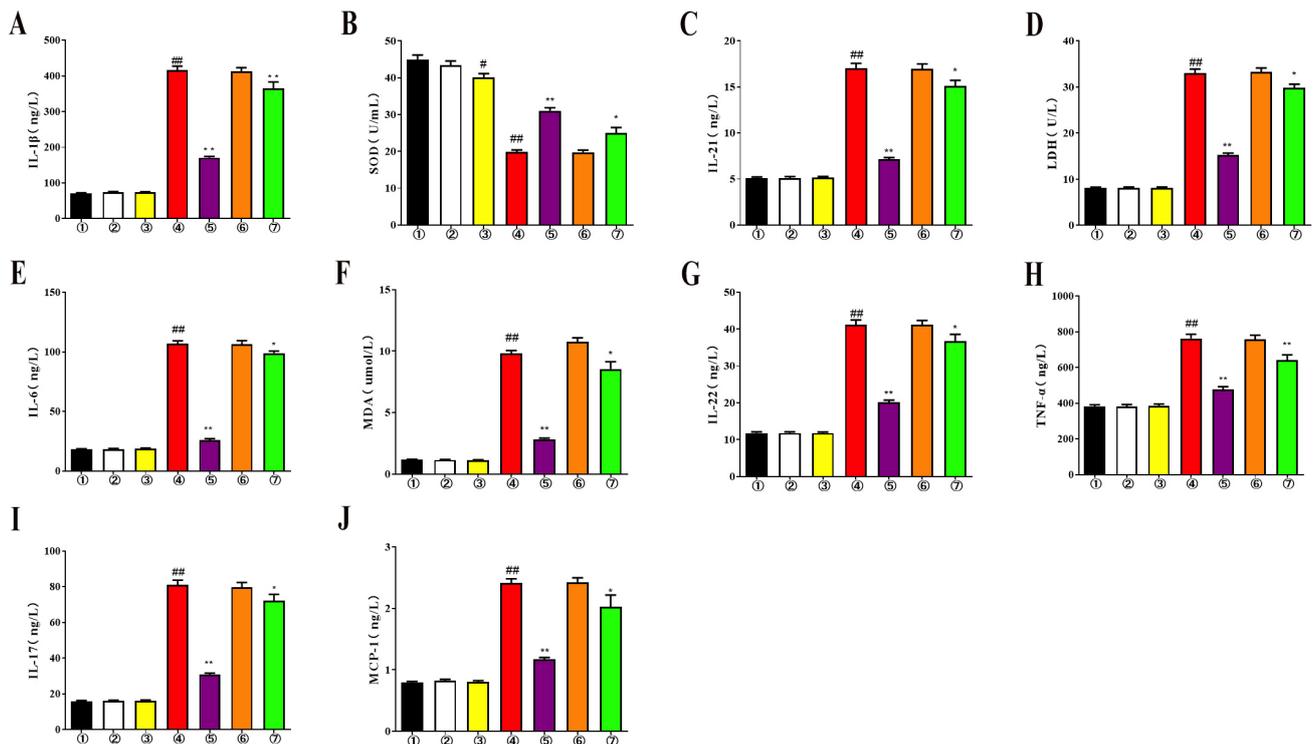


Fig. 4. ELISA results of RSC-364 cells after different treatments. (A) IL-1 β . (B) Superoxide dismutase (SOD). (C) IL-21. (D) Lactate dehydrogenase (LDH). (E) IL-6. (F) Malondialdehyde (MDA). (G) IL-22. (H) TNF- α . (I) IL-17. (J) Monocyte chemo-attractant protein-1 (MCP-1). Experimental results were expressed as mean \pm SD, n = 6. # p < 0.05, ## p < 0.01 compared to control group; * p < 0.05, ** p < 0.01 compared to Model group. ①: Control group; ②: Ad-SIRT6-Control; ③: Ad-Control; ④: Model group; ⑤: Ad-SIRT6-Model; ⑥: Ad-Model; ⑦: Ad-SIRT6-Model-MyD88.

maintaining basically unchanged. Also, compared with Model batch, the relative expression of IL-1 β , IL-21, IL-22, IL-6, IL-17, TNF- α and MCP-1 mRNA in Ad-SIRT6-Model and Ad-SIRT6-Model-MyD88 groups decreased significantly (p < 0.01 and p < 0.05, respectively), while the relative expressions of above genes (at mRNA level) in Ad-Model group remained basically unchanged. The results showed that the expression of IL-1 β , IL-21, IL-22, IL-6, IL-17, TNF- α and MCP-1 mRNA in normal FLS cells that were treated with recombinant rat IL-1 β (10 ng/mL) increased significantly, but their expressions could be reduced substantially after treatment with *SIRT6* gene. Of note, the expression of IL-1 β , IL-21, IL-22, IL-6, IL-17, TNF- α and MCP-1 mRNA was markedly reduced after administration of MyD88 adenovirus (p < 0.05), which verified that MyD88 could regulate inflammation related process of RA. Meanwhile, compared with Control, the relative expression of MyD88 mRNA in Model group increased significantly (p < 0.01), while the relative expression of MyD88 mRNA in Ad-SIRT6-Control and Ad-Control groups remained basically unchanged. In comparison with Model group, the relative expression of MyD88 mRNA in Ad-SIRT6-Model and Ad-SIRT6-Model-MyD88 groups decreased significantly (p < 0.01 and p < 0.05, respectively) decreased, but the relative expression of the same gene in Ad-Model

group did not basically change. The results showed that the expression of MyD88 mRNA in normal recombinant rat IL-1 β (10 ng/mL) treated FLS cells increased significantly, wherein the expression of the same gene was decreased substantially after treatment with *SIRT6* gene. This finding affirms that the importance of MyD88 pathway as target for *SIRT6* gene in RA regulation.

3.5 Detection of *SIRT6* Protein Expression Levels

The expression of *SIRT6* at protein level was measured with western blot technique. In comparison with control, relative expression of *SIRT6* protein in Ad-SIRT6-Control group increased substantially (Mean \pm SD of *SIRT6* protein expression in control group: 0.42 ± 0.02 ; in Ad-SIRT6-Control group: 0.90 ± 0.01 ; p < 0.01), whilst those expressions in Ad-Control, Model and Ad-Model groups (Fig. 6) remained relatively unchanged (Mean \pm SD of *SIRT6* protein expression in Ad-Control group: 0.48 ± 0.01 ; in Model group: 0.46 ± 0.02 ; in Ad-Model group: 0.42 ± 0.01). Also, we observed similar expression of *SIRT6* protein in Ad-SIRT6-Control, Ad-SIRT6-Model and Ad-SIRT6-Model-MyD88 groups (Mean \pm SD of *SIRT6* protein expression in Ad-SIRT6-Control: 0.90 ± 0.01 ; in Ad-SIRT6-Model: 1.01 ± 0.02 ; in Ad-SIRT6-Model-MyD88: 0.83 ± 0.02). Inferably, *SIRT6* gene that was

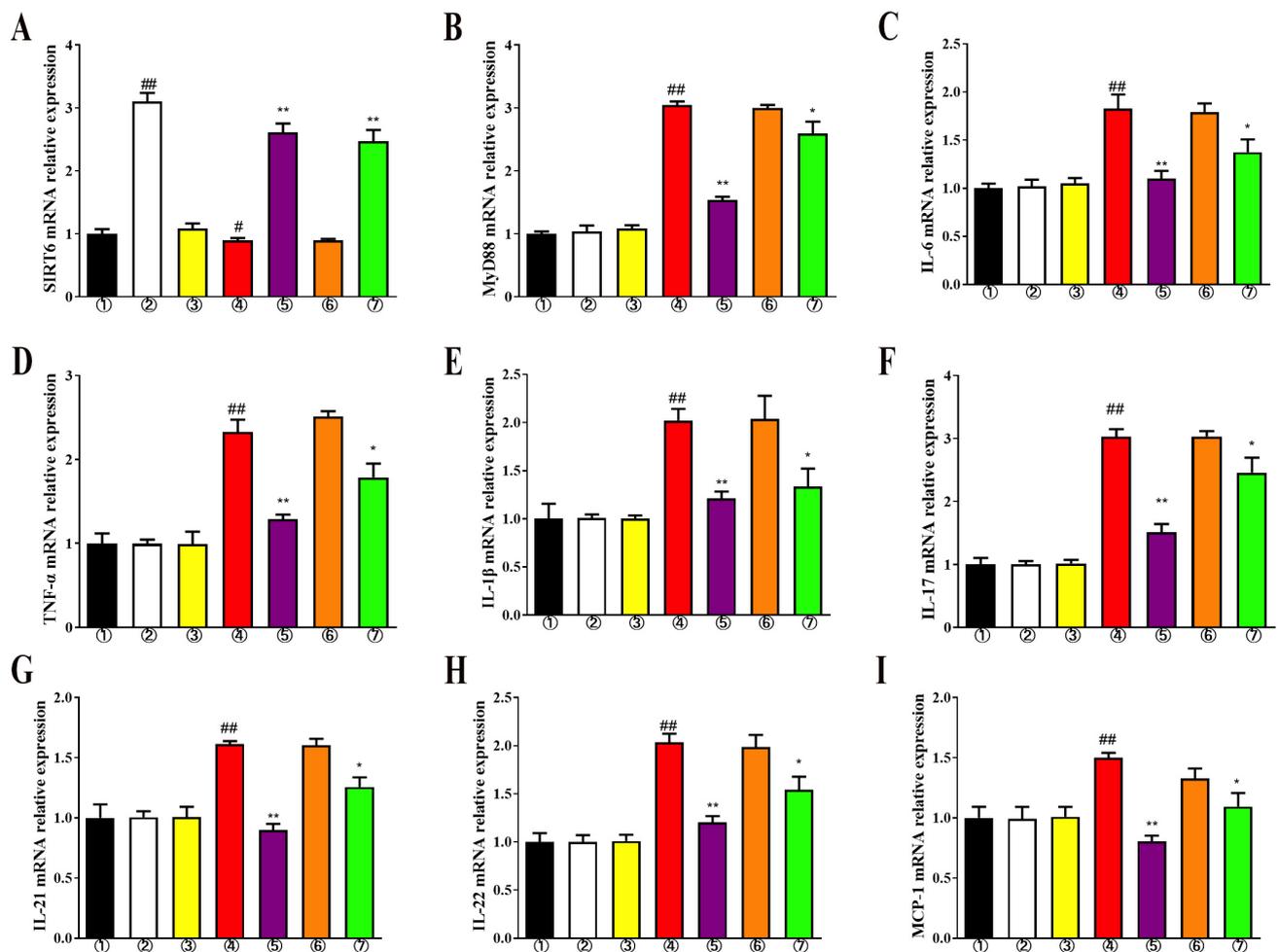


Fig. 5. Relative expression of related mRNA in RSC-364 cells after different treatments. RT-PCR analysis of relative expression of (A) SIRT6, (B) MyD88, (C) IL-6, (D) TNF- α , (E) IL-1 β , (F) IL-17, (G) IL-21, (H) IL-22, (I) MCP-1. In all experiments, GAPDH was used as an internal control. Experimental results were expressed as mean \pm SD, n = 6. # $p < 0.05$, ## $p < 0.01$ compared to control group; * $p < 0.05$, ** $p < 0.01$ compared to Model group. ①: Control group; ②: Ad-SIRT6-Control; ③: Ad-Control; ④: Model group; ⑤: Ad-SIRT6-Model; ⑥: Ad-Model; ⑦: Ad-SIRT6-Model-MyD88.

loaded with adenovirus vectors in Ad-SIRT6-Control, Ad-SIRT6-Model and Ad-SIRT6-Model-MyD88 could be introduced into cells with high protein expression. Compared with Control group, the relative expression of MyD88, ERK and p-ERK proteins in Model group increased significantly (Mean \pm SD of MyD88 protein expression in control group: 0.41 ± 0.01 ; in model group: 0.73 ± 0.03 ; Mean \pm SD of ERK protein expression in control group: 0.63 ± 0.02 ; in model group: 1.07 ± 0.06 ; Mean \pm SD of p-ERK protein expression in control group: 0.45 ± 0.02 ; in model group: 0.90 ± 0.04 ; $p < 0.01$), while the relative expression of the same proteins in Ad-SIRT6-Control and Ad-Control groups remained basically unchanged. In comparison to Model group, the relative MyD88, ERK and p-ERK protein expressions decreased significantly in Ad-SIRT6-Model (Mean \pm SD of MyD88 protein expression in Ad-SIRT6-Model group: 0.54 ± 0.01 ; Mean \pm SD of ERK protein expression in Ad-SIRT6-Model group: 0.70

± 0.03 ; Mean \pm SD of p-ERK protein expression in Ad-SIRT6-Model group: 0.63 ± 0.02 ; $p < 0.01$) and Ad-SIRT6-Model-MyD88 groups (Mean \pm SD of MyD88 protein expression in Ad-SIRT6-Model-MyD88 group: 0.68 ± 0.05 ; Mean \pm SD of ERK protein expression in Ad-SIRT6-Model-MyD88 group: 0.95 ± 0.06 ; Mean \pm SD of p-ERK protein expression in Ad-SIRT6-Model-MyD88 group: 0.75 ± 0.01 ; $p < 0.05$), but remained basically unchanged in Ad-SIRT6-Model group. These results demonstrate a significantly increased expression of MyD88, ERK and p-ERK protein in normal FLS cells that were treated with recombinant rat IL-1 β (10 ng/mL), which was reduced markedly after treatment with SIRT6 gene. On this account, expression of MyD88, ERK and p-ERK protein increased significantly after treatment with MyD88 adenovirus, which confirms the significance of MyD88-ERK pathway as target for regulating RA by SIRT6 gene.

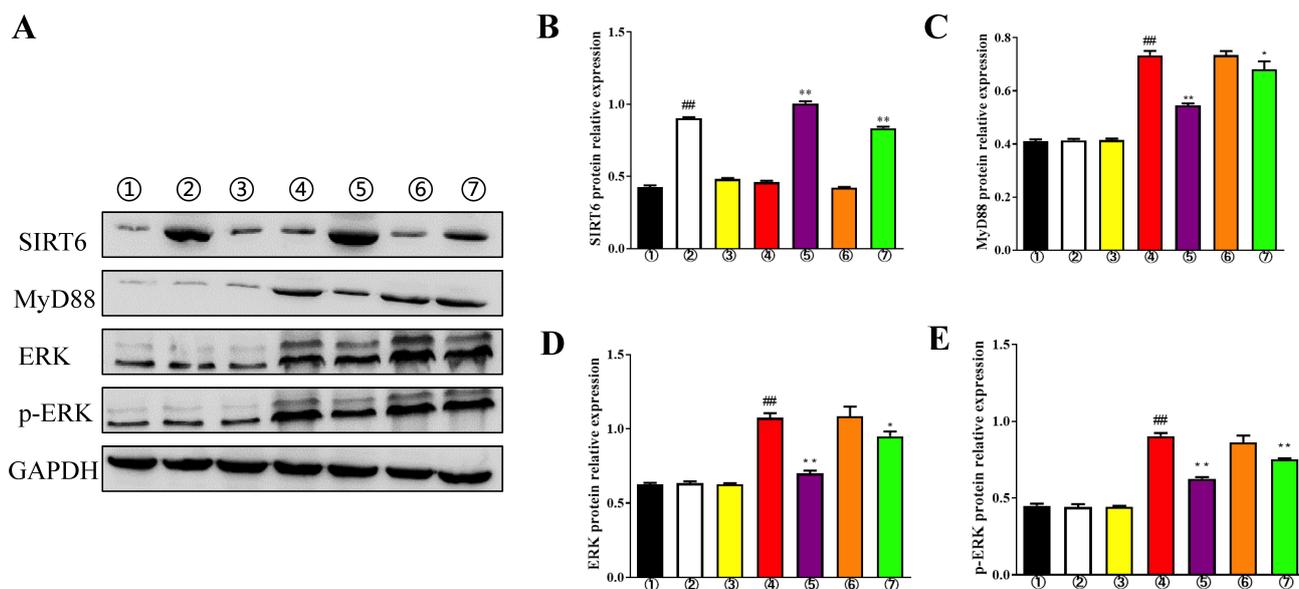


Fig. 6. Relative expression of related protein in RSC-364 cells after different treatments. (A) Western blot analysis of SIRT6, MyD88, ERK and p-ERK levels. GAPDH was used as a loading control. Quantitative densitometry of SIRT6 (B), MyD88 (C), ERK (D) and p-ERK (E) levels relative to GAPDH. ^{##} $p < 0.01$ compared to control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ compared to Model group. Experimental results were expressed as mean \pm SD, $n = 3$. ①: Control group; ②: Ad-SIRT6-Control; ③: Ad-Control; ④: Model group; ⑤: Ad-SIRT6-Model; ⑥: Ad-Model; ⑦: Ad-SIRT6-Model-MyD88.

3.6 Effect of SIRT6 on MyD88-ERK Pathway in RA Rat Model in Vivo

3.6.1 Ultrasonic Test Results

To understand the effect of SIRT6 on MyD88-ERK signaling pathway, we performed ultrasonic test with the results displayed in Fig. 7A. Ultrasound examination of rats' ankle joint in control showed no obvious hyperplasia of synovium and no blood flow signal. Proliferative synovium and punctate blood flow signals were seen in the ankle of Model rats, thus indicating successful construct of RA model. In overexpressed SIRT6 group (Ad-SIRT6 Model), the proliferative synovium of ankle joint was not significant when compared with RA group, amid no obvious blood flow signal, which therefore indicates that *SIRT6* gene overexpression showed a good effect on RA. After the RA rats in group Ad-model were given blank carrier, the proliferative synovium and short linear blood flow signals could be seen in their ankles. In Ad-SIRT6-Model-MyD88 rats, we observed no obvious hyperplasia of synovium and no blood flow signal, which suggests the symptoms of RA were significantly relieved, and hence SIRT6 could potentially inhibit MyD88 pathway and inflammation with concomitant treatment of RA.

3.6.2 Thickness Test Results at the Thickest Part of Synovium

Thickness test was also performed, wherein the result is presented in Fig. 7B. Thickness of joint synovium of Model rats increased substantially (Mean \pm SD of Thickness at the thickest part of synovium in control group: 1.33

± 0.58 ; in model group: 6.80 ± 1.20 ; $p < 0.01$) compared to control, which indicates successive replication of RA rat model with obvious inflammatory reaction. Remission of synovial thickness of rats in Ad-SIRT6-Model group markedly decreased (Mean \pm SD of Thickness at the thickest part of synovium in Ad-SIRT6-Model group: 3.77 ± 0.92 ; $p < 0.01$) compared to model batch, which indicates improvement in the inflammation of RA rats after they have received *SIRT6* gene overexpression treatment. This finding demonstrates that SIRT6 could play an anti RA role. At the same time, compared with the Model group, the synovial thickness of rats in Ad-Model group did no change, which implies that blank adenovirus had no therapeutic effect on RA. In comparison with the Model group, we observed substantial decrease (Mean \pm SD of Thickness at the thickest part of synovium in Ad-SIRT6-Model-MyD88 group: 4.50 ± 0.56 ; $p < 0.05$) in the synovial thickness of rats in Ad-SIRT6-Model-MyD88 group, which suggests that the inflammatory symptoms of RA in rats were obviously alleviated, wherein SIRT6 could regulate MyD88 pathway to inhibit RA inflammation.

3.6.3 HE Staining

Through HE staining, we observed that the synovial morphology of rats in control was normal with cells arranged orderly, amid no synovial thickening and angiogenesis (Fig. 8A). Model group rats displayed severe hyperplasia of synovium, obvious cell infiltration of inflammation, visible defect and formation of pannus. The morphology of synovium in SIRT6 overexpression group (Ad-SIRT6-

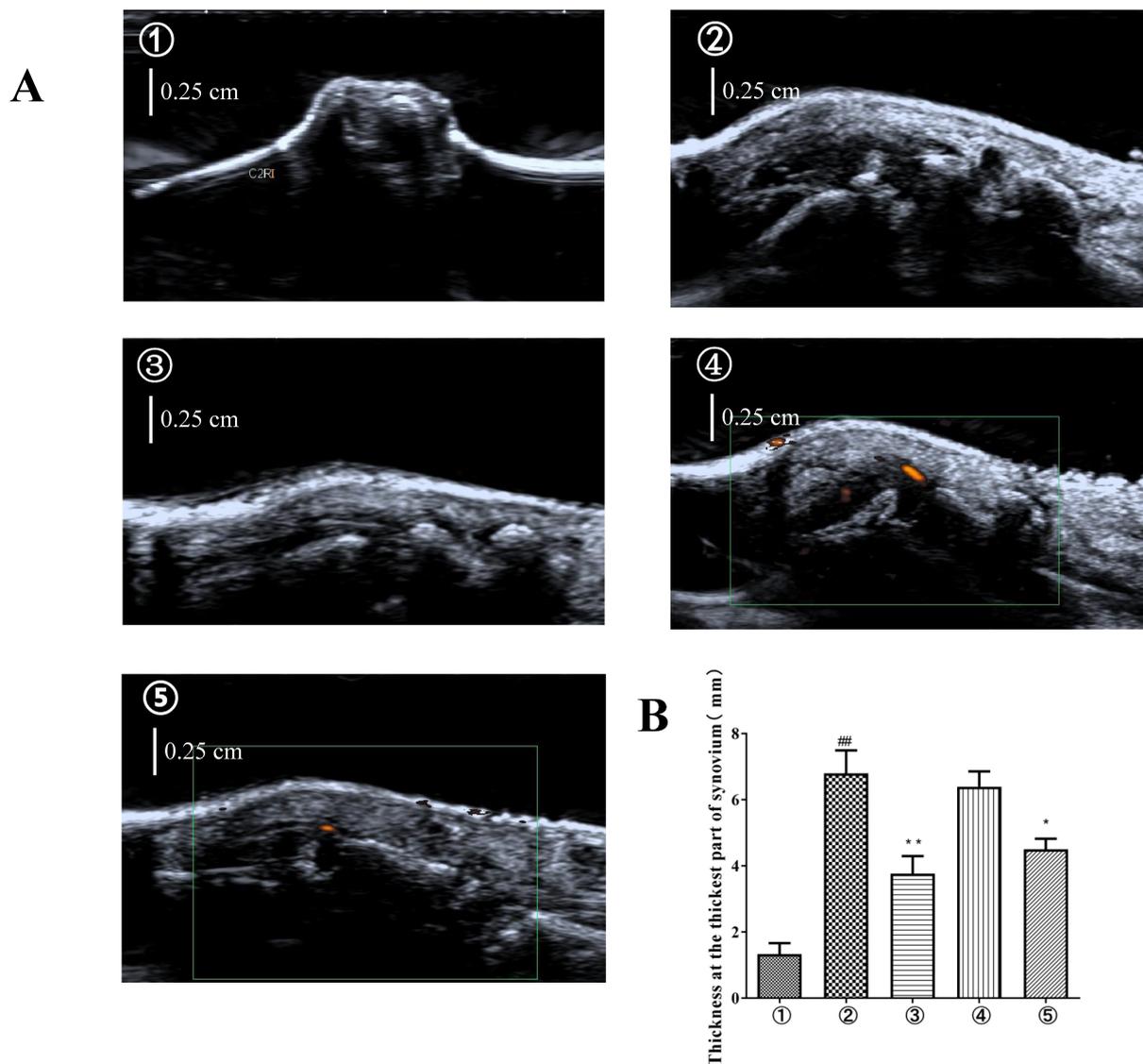


Fig. 7. Ultrasonic Test Results. (A) Ultrasonic test results of each group. (B) Thickness measurement results of the thickest joint synovium of rats in different groups. Scale bars = 0.25 cm. ^{##} $p < 0.01$ compared to control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ compared to Model group. Experimental results were expressed as mean \pm SD, $n = 3$. ①: Control group; ②: Model group; ③: Ad-SIRT6-Model; ④: Ad-Model group; ⑤: Ad-SIRT6-Model-MyD88.

Model) tended to be normal, whereas the cells were arranged orderly with thickened synovium but no obvious angiogenesis. Obviously, these results imply that SIRT6 overexpression had a clear therapeutic effect on RA. Synovial morphology of rats in group Ad-Model was consistent with the Model group. In Ad-SIRT6-Model-MyD88 group, it could also be found that the synovial tissue of rats was significantly better than that of the Model group.

Pathological examination suggested that renal tissues of rats in Control group showed no obvious pathological changes with normal nuclear morphology and no inflammatory infiltration (Fig. 8B). However, renal tubular epithelial cells of those in Model group displayed some vacuolar degeneration, cell edema, damaged cell membranes and nuclei in varying degrees, coupled with obvious inflammatory

cell infiltration. Cell morphology of rat kidney in overexpressed SIRT6 can be observed in Fig. 8B. Likewise, we observed similar trend when liver tissue of rats in all the groups were stained with HE after treatments (Fig. 8C).

3.6.4 Effect of SIRT6 on Levels of Biomarkers in Sera of RA Rats

Levels of biomarkers in sera of RA rats was studied with ELISA testing. It was discovered that the levels of serum IL-1 β , IL-6, IL-17, IL-21, IL-22, GSH, MDA, TNF- α and MCP-1 in Model rats increased substantially (Mean \pm SD for each inflammatory markers in Model group: IL-1 β : 409.48 \pm 21.72; SOD: 39.72 \pm 3.00; IL-21: 12.13 \pm 0.80; IL-6: 236.53 \pm 18.86; MDA: 29.73 \pm 2.25; IL-22: 212.09 \pm 15.38; GSH: 78.95 \pm 5.89; TNF- α : 302.74 \pm

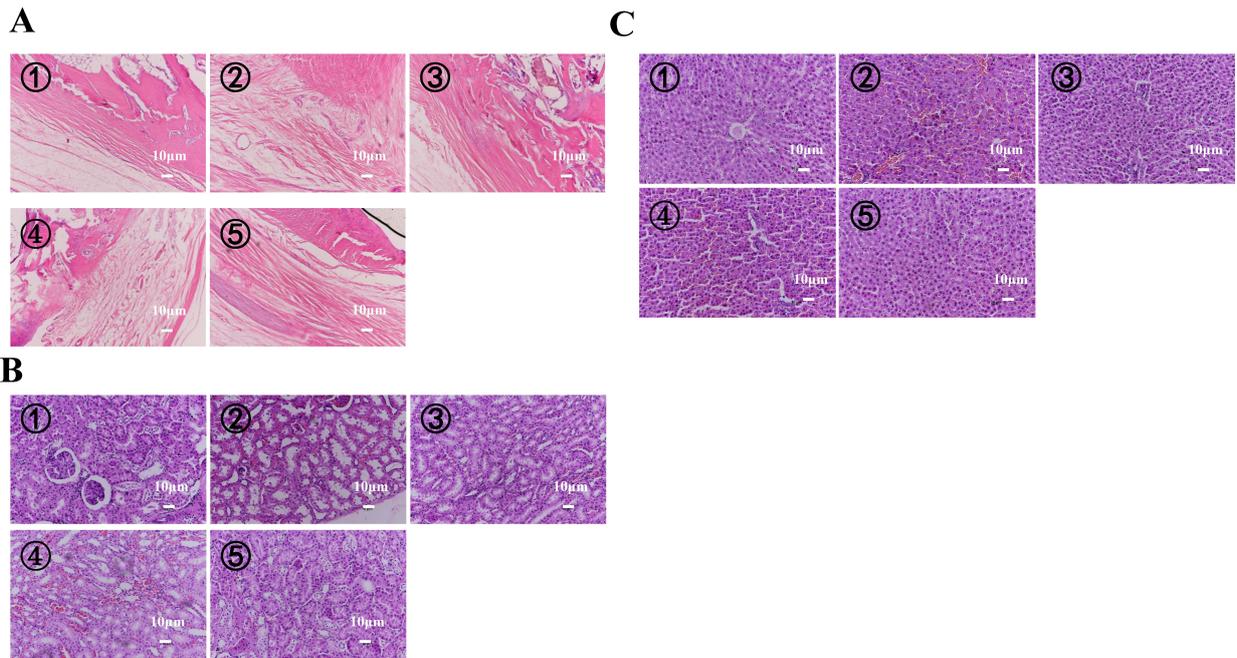


Fig. 8. HE staining results of rats' tissues in different groups. Scale bars = 10 μm . (A) Synovial tissues. (B) Kidney tissues. (C) Liver tissues. ①: Control group; ②: Model group; ③: Ad-SIRT6-Model; ④: Ad-Model group; ⑤: Ad-SIRT6-Model-MyD88.

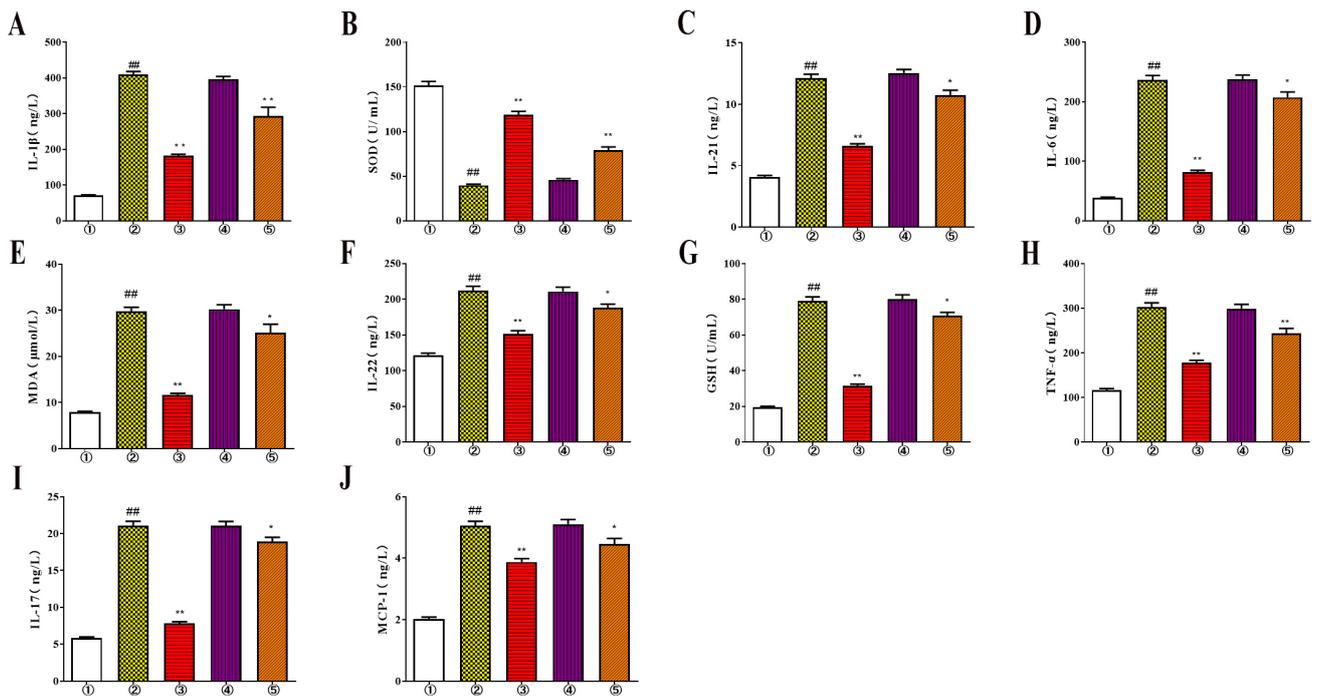


Fig. 9. ELISA results of serum from different groups of rats. (A) IL-1 β . (B) SOD. (C) IL-21. (D) LDH. (E) IL-6. (F) MDA. (G) IL-22. (H) TNF- α . (I) IL-17. (J) MCP-1. Experimental results were expressed as mean \pm SD, n = 6. ^{###} $p < 0.01$ compared to control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ compared to Model group. ①: Control group; ②: Model group; ③: Ad-SIRT6-Model; ④: Ad-Model group; ⑤: Ad-SIRT6-Model-MyD88. ELISA, enzyme linked immunoassay.

22.79; IL-17: 21.09 \pm 1.44; MCP-1: 5.06 \pm 0.34; Mean \pm SD for each inflammatory markers in control group: IL-1 β : 71.14 \pm 3.72; SOD: 151.41 \pm 11.66; IL-21: 4.08 \pm 0.31; IL-6: 38.76 \pm 2.52; MDA: 7.86 \pm 0.51; IL-22: 121.35

\pm 7.91; GSH: 19.45 \pm 1.59; TNF- α : 116.83 \pm 8.14; IL-17: 5.83 \pm 0.38; MCP-1: 2.03 \pm 0.14; $p < 0.01$) compared to Control (Fig. 9), whilst SOD level decreased in serum of Model rats. This showed that the constructed

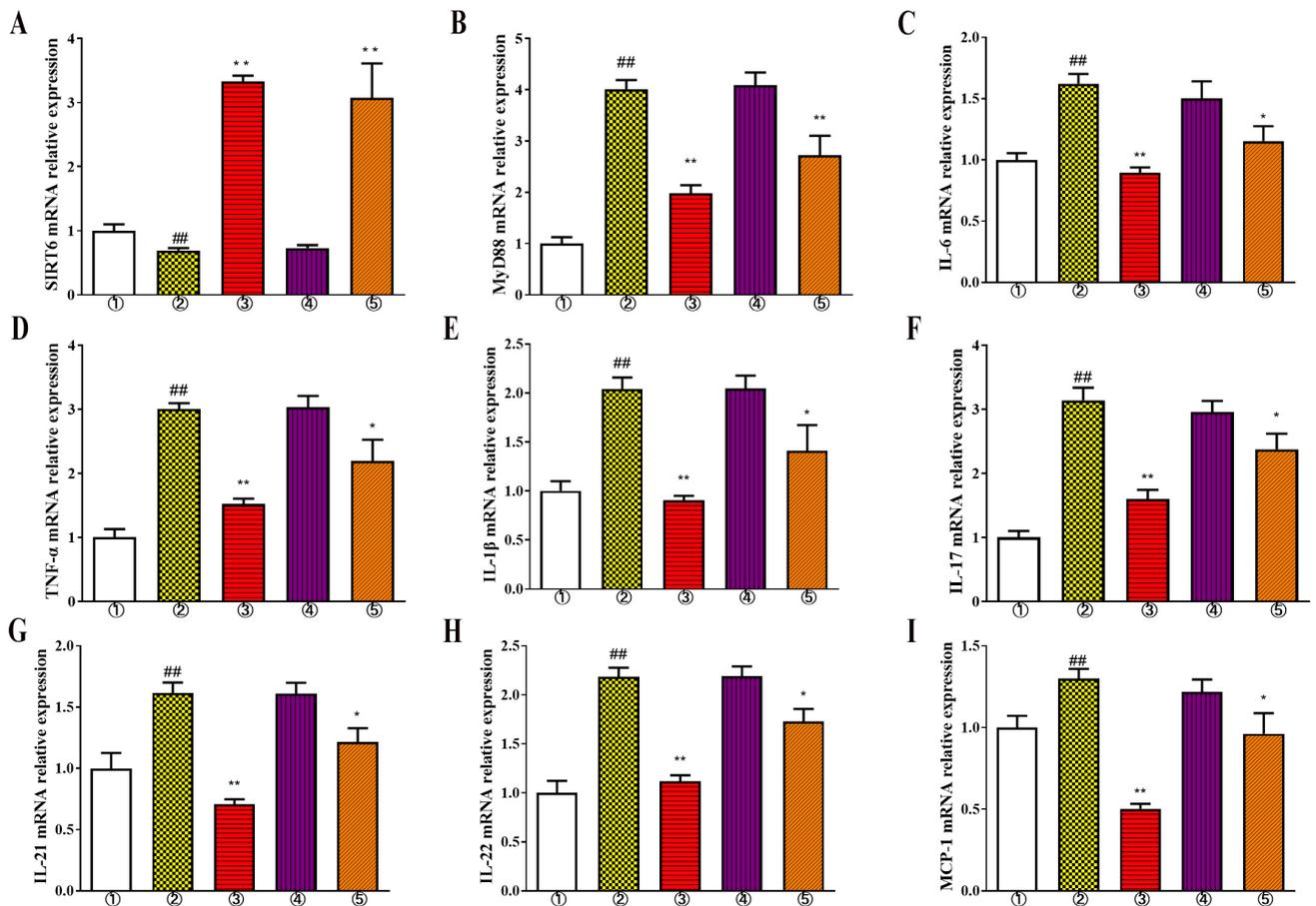


Fig. 10. Levels of related SIRT6 and MyD88 mRNA expressions in different groups of rats. RT-PCR analysis of relative expression of (A) SIRT6, (B) MyD88, (C) IL-6, (D) TNF- α , (E) IL-1 β , (F) IL-17, (G) IL-21, (H) IL-22, (I) MCP-1. In all experiments, GAPDH was used as an internal control. Experimental results were expressed as mean \pm SD, n = 6. ##*p* < 0.01 compared to control group; **p* < 0.05, ***p* < 0.01 compared to Model group. ①: Control group; ②: Model group; ③: Ad-SIRT6-Model; ④: Ad-Model group; ⑤: Ad-SIRT6-Model-MyD88.

model method could successfully establish RA model. In comparison with Model group, serum levels of the above-mentioned inflammatory mediators and SOD in Ad-Model remained basically unchanged, thus indicating blank adenovirus vector had no effect on RA inflammation. Compared with Model group, we found that the levels of serum IL-1 β , IL-21, IL-22, IL-6, IL-17, GSH, MDA, TNF- α and MCP-1 in group Ad-SIRT6-Model decreased substantially (Mean \pm SD for each inflammatory markers in Ad-SIRT6-Model group: IL-1 β : 182.45 \pm 9.75; IL-21: 6.63 \pm 0.41; IL-6: 82.06 \pm 6.75; MDA: 11.64 \pm 0.83; IL-22: 151.37 \pm 11.31; GSH: 31.48 \pm 2.56; TNF- α : 178.08 \pm 12.43; IL-17: 7.83 \pm 0.56; MCP-1: 3.88 \pm 0.28; *p* < 0.01), while the level of serum SOD in Ad-SIRT6-Model group increased markedly (Mean \pm SD of SOD in Ad-SIRT6-Model group: 118.9 \pm 8.94, *p* < 0.01). In addition, serum levels of IL-1 β , IL-21, IL-22, IL-6, IL-17, GSH, MDA, TNF- α and MCP-1 in Ad-SIRT6-Model-MyD88 group decreased significantly (Mean \pm SD for each inflammatory markers in Ad-SIRT6-Model-MyD88 group: IL-1 β : 293.61 \pm 60.05;

IL-21: 10.75 \pm 0.96; IL-6: 207.50 \pm 22.45; MDA: 25.13 \pm 4.50; IL-22: 188.13 \pm 12.27; GSH: 70.70 \pm 4.79; TNF- α : 243.52 \pm 27.47; IL-17: 18.96 \pm 1.35; MCP-1: 4.46 \pm 0.46; *p* < 0.05 or *p* < 0.01) compared to Model, while SOD level in Ad-SIRT6-Model-MyD88 rat serum increased significantly (Mean \pm SD of SOD in Ad-SIRT6-Model-MyD88 group: 79.18 \pm 8.93, *p* < 0.01). The results collectively affirm that treatment of RA rats with SIRT6 adenovirus could significantly inhibit inflammation. Consequently, SIRT6 could still regulate MyD88 induced activities of serum IL-1 β , IL-21, IL-22, IL-6, IL-17, GSH, MDA, TNF- α , MCP-1 and SOD to a certain extent.

3.6.5 Detection of SIRT6 and MyD88 mRNA Expressions in RA Rats

Relative expression of SIRT6 mRNA in the sera of rats in Model group decreased (Mean \pm SD of SIRT6 in control group: 1.00 \pm 0.24; in Model group: 0.69 \pm 0.10; *p* < 0.01) compared to control (Fig. 10), which suggests disturbed and decreased relative expression of SIRT6 mRNA

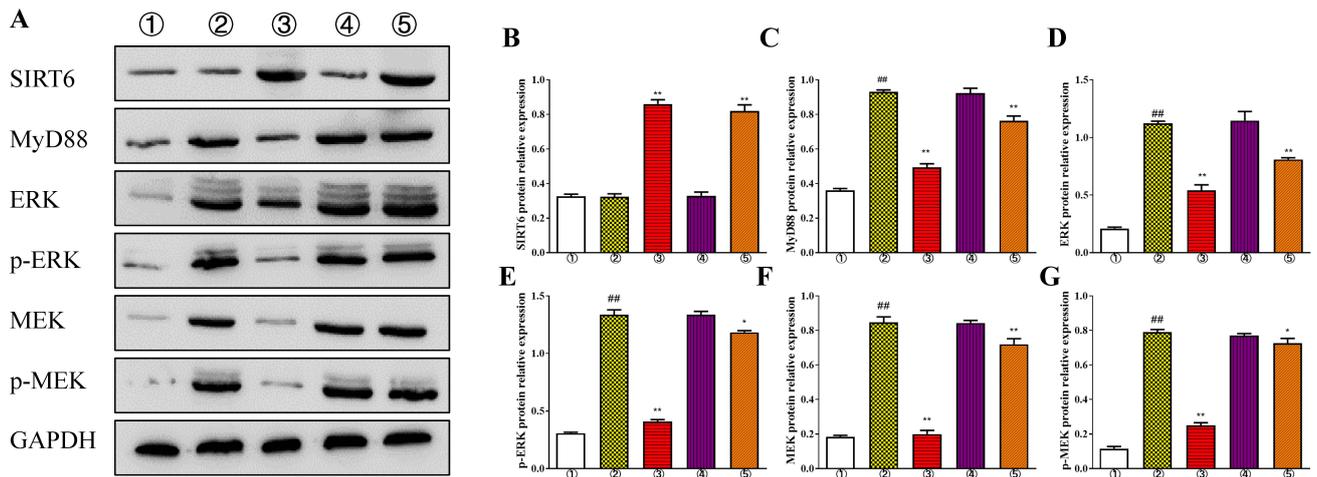


Fig. 11. Expression levels of related proteins in distinct groups of rats. (A) Related expression levels of protein in different groups; Quantitative densitometry of SIRT6 (B), MyD88 (C), ERK (D), p-ERK (E), MEK (F) and p-MEK (G) levels relative to GAPDH. ## $p < 0.01$ compared to control group; * $p < 0.05$, ** $p < 0.01$ compared to Model group. Experimental results were expressed as mean \pm SD, $n = 3$. ①: Control group; ②: Model group; ③: Ad-SIRT6-Model; ④: Ad-Model group; ⑤: Ad-SIRT6-Model-MyD88.

in serum of RA rats. Through comparison with Model rats, we observed substantially (Mean \pm SD of SIRT6 in Ad-SIRT6-Model group: 3.33 ± 0.23 ; in Ad-SIRT6-Model-MyD88 group: 3.07 ± 1.32 ; $p < 0.01$) increased relative expression of SIRT6 mRNA in Ad-SIRT6-Model and Ad-SIRT6-Model-MyD88 groups. It was showed that *SIRT6* gene loaded by adenovirus vectors in Ad-SIRT6-Model and Ad-SIRT6-Model-MyD88 groups can be carried into rats with high expression. In addition, compared to Control, the relative expression of serum IL-1 β , IL-21, IL-22, IL-6, IL-17, TNF- α and MCP-1 mRNA in Model group increased significantly (Mean \pm SD of related genes in Control group: IL-6: 1.00 ± 0.13 ; TNF- α : 1.00 ± 0.32 ; IL-1 β : 1.00 ± 0.24 ; IL-17: 1.00 ± 0.24 ; IL-21: 1.00 ± 0.31 ; IL-22: 1.00 ± 0.30 ; MCP-1: 1.00 ± 0.18 ; in model group: IL-6: 1.62 ± 0.19 ; TNF- α : 3.00 ± 0.23 ; IL-1 β : 2.04 ± 0.29 ; IL-17: 3.14 ± 0.48 ; IL-21: 1.61 ± 0.22 ; IL-22: 2.19 ± 0.22 ; MCP-1: 1.30 ± 0.14 ; $p < 0.01$). Also, we observed substantial decreased relative expression of serum IL-1 β , IL-21, IL-22, IL-6, IL-17, TNF- α and MCP-1 mRNA in Ad-SIRT6-Model (Mean \pm SD of related genes in Ad-SIRT6-Model group: IL-6: 0.89 ± 0.11 ; TNF- α : 1.52 ± 0.21 ; IL-1 β : 0.91 ± 0.11 ; IL-17: 1.60 ± 0.35 ; IL-21: 0.71 ± 0.10 ; IL-22: 1.12 ± 0.15 ; MCP-1: 0.50 ± 0.07 ; $p < 0.01$) and Ad-SIRT6-Model-MyD88 groups (Mean \pm SD of related genes in Ad-SIRT6-Model-MyD88 group: IL-6: 1.15 ± 0.30 ; TNF- α : 2.19 ± 0.82 ; IL-1 β : 1.41 ± 0.65 ; IL-17: 2.38 ± 0.60 ; IL-21: 1.21 ± 0.28 ; IL-22: 1.73 ± 0.31 ; MCP-1: 0.96 ± 0.31 ; Mean \pm SD of related genes in Ad-Model group: IL-6: 1.50 ± 0.35 ; TNF- α : 3.03 ± 0.43 ; IL-1 β : 2.05 ± 0.32 ; IL-17: 2.96 ± 0.42 ; IL-21: 1.61 ± 0.22 ; IL-22: 2.19 ± 0.24 ; MCP-1: 1.22 ± 0.19 ; $p < 0.05$ or $p < 0.01$) compared to Model group with their relative expressions in Ad-Model group remaining basically unchanged.

These findings indicate that the expression of serum IL-1 β , IL-21, IL-22, IL-6, IL-17, TNF- α and MCP-1 mRNA could be reduced significantly after treatment with *SIRT6* gene. Meanwhile, the expression of the above-mentioned genes at mRNA level was still significantly reduced after administration of MyD88 adenovirus ($p < 0.05$), which verified that MyD88 could regulate inflammation in RA. Meanwhile, compared with group Control, the relative expression of serum MyD88 mRNA in Model group increased significantly (Mean \pm SD of MyD88 in Control group: 1.00 ± 0.30 ; in Model group: 4.01 ± 0.44 ; $p < 0.01$). However, the relative expression of serum MyD88 mRNA in Ad-SIRT6-Model (Mean \pm SD of MyD88 in Ad-SIRT6-Model group: 1.98 ± 0.39 ; $p < 0.01$) and Ad-SIRT6-Model-MyD88 decreased significantly (Mean \pm SD of MyD88 in Ad-SIRT6-Model-MyD88 group: 2.72 ± 0.92 ; $p < 0.01$) compared to Model group, but remained basically unchanged in Ad-Model group. Altogether, expression of serum MyD88 mRNA could be substantially reduced after treatment with SIRT6, which affirm the importance of MyD88 pathway to the regulatory role of *SIRT6* gene in RA.

3.6.6 Detection of SIRT6 and MyD88 Proteins Expression in RA Rats

Through comparative analysis, we observed that relative SIRT6 protein expression in serum of rats in Ad-SIRT6-model increased significantly (Mean \pm SD of SIRT6 protein expression in control group: 0.33 ± 0.02 ; in Ad-SIRT6-Model group: 0.86 ± 0.04 ; $p < 0.01$, Fig. 11) compared to control, but that of Ad-Model remained unchanged. Likewise, relative expression of serum SIRT6 protein in Ad-SIRT6-Model and Ad-SIRT6-Model-MyD88 groups increased substantially (Mean \pm SD of SIRT6 protein expression in Ad-SIRT6-Model-MyD88 group: 0.82

± 0.06 ; $p < 0.01$). Also, upon comparison with Control, we discovered that MyD88, ERK, p-ERK, MEK and p-MEK protein expressions in serum of Model rats relatively increased (Mean \pm SD of related proteins expression in control group: MyD88: 0.36 ± 0.02 ; ERK: 0.21 ± 0.02 ; p-ERK: 0.31 ± 0.02 ; MEK: 0.18 ± 0.02 ; p-MEK: 0.11 ± 0.03 ; in Model group: MyD88: 0.93 ± 0.02 ; ERK: 1.12 ± 0.03 ; p-ERK: 1.34 ± 0.07 ; MEK: 0.85 ± 0.06 ; p-MEK: 0.79 ± 0.03 ; $p < 0.01$). In compared with Model group, the relative expression of serum MyD88, ERK, p-ERK, MEK and p-MEK protein in Ad-SIRT6-Model (Mean \pm SD of related proteins expression in Ad-SIRT6-Model group: MyD88: 0.49 ± 0.04 ; ERK: 0.54 ± 0.09 ; p-ERK: 0.41 ± 0.03 ; MEK: 0.20 ± 0.04 ; p-MEK: 0.25 ± 0.03 ; $p < 0.01$) and Ad-SIRT6-Model-MyD88 groups decreased significantly (Mean \pm SD of related proteins expression in Ad-SIRT6-Model-MyD88 group: MyD88: 0.76 ± 0.05 ; ERK: 0.81 ± 0.03 ; p-ERK: 1.18 ± 0.03 ; MEK: 0.72 ± 0.06 ; p-MEK: 0.73 ± 0.05 ; $p < 0.05$), but did not basically change in Ad-SIRT6-Model group. Hence, the expression of serum MyD88, ERK and p-ERK, MEK and p-MEK protein could be markedly reduced after SIRT6 treatment. Overall, the expression of serum MyD88, ERK and p-ERK, MEK and p-MEK protein increased significantly after treatment with MyD88 adenovirus, which confirms the significance of MyD88-ERK pathway in the regulation of RA by SIRT6.

4. Discussion

As a common joint disease of chronic inflammation, RA exhibits characteristics like synovitis and hyperplasia of synovium, cartilage degeneration and bone destruction [37], which seriously affects patients' well-being. Incidence rate of RA is mainly high in the thirtieth to fiftieth years of age [38], albeit occurring at any age. Increased production of inflammatory cytokines by synovial cells, namely TNF- α and IL-1 β , which play an important role in RA pathogenesis and are centrally link to RA [39]. Currently, prevention and alleviation of RA has been successful because of sustained disease modifying anti-rheumatic drugs (DMARDs) development [40]. However, these DMARDs are not effective in some RA patients and as a result there is unmet need for novel therapeutic targets. In this regard, this work sought to explore the mechanistic effect of SIRT6 on RA. The RA has a complex pathogenesis which involves genes interacting with the environment, and consequently culminates in immune tolerance collapse coupled with inflammation of the synovia [41,42]. Notwithstanding, intracellular signaling like MyD88 and ERK has been implicated in the pathogenesis of RA. As an important component of downstream cascade signaling of interactive IL-1 β /IL-1 receptor, Myd88 is reported to converge with NF- κ B pathway (canonical) ultimately and result in promotion of amplified production of inflammatory factor in RA [43]. On the other hand, the involvement of ERK

in RA pathogenesis is mainly through the activation of the pathway in RA-FLS [44]. Of note, ERK activation has been described to increase in RA patients, thereby affirming the significance of the pathway in the pathological process of the disease [45]. Besides, existing studies have proven that SIRT6 is a principal regulatory protein of inflammation and aging diseases, which plays crucial roles in regulating multiple pathways of inflammation, wherein it has the potential to inhibit inflammation development [46]. In RA, SIRT6 has been reported to exhibit anti-inflammatory activity by blocking NF- κ B pathway wherein it can reduce inflammation and tissue destruction. In agreement to the above phenomenon, we observed a significant decrease in SIRT6 expression in RA patients compared to normal volunteers. This phenomenon can be ascribable to the increased expressions of pro-inflammatory factors like TNF- α or high expression of long-noncoding RNAs (lncRNA)-plasmacytoma-variant translocation-1 (PVT-1), which lowers Sirt6 expression via methylation of the deacetylase protein in RA-FLS and synovial tissues of RA patients [35,36]. Existing literature has posited that TNF- α -activated endothelial cells (ECs) demonstrated decreased Sirt6 expression in a dose dependent fashion [35]. Besides, the activation of ECs in RA by increased levels of pro-inflammatory mediators has also been described by other works [47]. These observations affirm that inflammation in RA comprised of complex cascade of pro-and anti-inflammatory factors [48]. Further, other authors discovered that Sirt6 expression was reduced by increased lncRNA-PVT-1 expression in synovial tissue and RA-FLS of RA rats [36]. In that work, low expression of Sirt6 in RA rats was as a result of recruitment of DNA methyl transferases to promoter region of Sirt6 and subsequent methylation of the protein by PVT-1. Based on the above findings, our future studies will comprehensively investigate the potential pathomechanistic roles of PTV-1 and the actual mechanisms underlying the effect of TNF- α on Sirt6 expression in RA-related cells.

In support of the anti-inflammatory activity of Sirt6, other works have shown that treatment with SIRT6 adenovirus could significantly reduce concentrations of pro-inflammatory mediators at local and systemic levels [49]. Herein, we observed increased level of MCP-1, IL-1 β , IL-21, IL-22, IL-6, IL-17, LDH, MDA and TNF- α expressions in RA model group compared to control group, albeit decreased SOD expression. Notably, the reverse of aforementioned results was observed when RA-FLS cells were treated with overexpressed SIRT6. It is obvious that pro-inflammatory mediators will increase in a typical disease of chronic inflammation like RA. Also, a decreased SOD levels in RA may be ascribed to overproduction of superoxide anion ($O_2^{\bullet-}$) in the inflammatory disease, wherein an attempt to physiologically defend against the oxidative stress culminate in reduced antioxidant protein [50]. Further, overexpression of SIRT6 reduced the above-mentioned pro-inflammatory markers, which affirms the potential of the

protein to suppress inhibition of such factors [51]. Previous studies have shown that MyD88 dependent pathway plays a major role in TLR4 mediated pathway [28], which has been shown to potentially promote joint inflammation via binding of various endogenous ligands [52]. Also, existing work has discovered the activation of ERK pathway in the RA-FLS and its subsequent involvement in the pathological process and destruction of RA [44]. Also, overexpressed Sirt6 increased ERK phosphorylation in Huh cells [53]. Of note, the therapeutic role of SIRT6 in RA via MyD88-ERK pathway regulation has not been reported yet. Hence, we decided to detect the expression of MyD88-ERK pathway at mRNA and protein levels to predict the potential role of SIRT6 in RA. MyD88 deregulation and its downstream cascade are associated with chronic inflammation in RA [54, 55]. Another work [54] has reported that streptococcal cell wall (SCW)-induced arthritis mainly depended on MyD88 and TLR2 in mice that lacked MyD88 did not develop SCW-induced arthritis, wherein local pro-inflammatory cytokines levels in the mouse synovial tissue decreased significantly. Similar report [55] also detected synovial cells from RA tissue, wherein overexpressed MyD88 substantially down-regulated cytokines in RA. Aside, ERK pathway, as a representative member of MAPK family, has also been proven to associate closely with inflammation regulation [56]. MEK (MAPkinasekinase) is a member of map2k and an upstream ERK1/2 signal [57]. An ERK activation by MEK phosphorylation is core element downstream of the pathway. Thus, phosphorylated ERK (p-ERK) is one of the active cell function biomarkers [58]. Blocking of ERK activation by administering MEK inhibitor (PD184352) to CIA rats could improve joint lesions [59]. Another study [60] found that overexpressed SIRT6 could inhibit the level of ERK1/2 phosphorylation. Thus, suppression of MyD88-ERK pathway in inflammation may contribute to RA progress. Also, it seems relationship between SIRT6 and MyD88 has not been clearly established. Notwithstanding, a relationship exists between overexpressed SIRT6 and NF- κ B pathway [61], wherein the latter is activated by TLR4 via MyD88-dependent pathway [62]. Therefore, we therefore assumed that SIRT6 may have an association with MyD88-ERK pathway. In this regard, we observed increased of MyD88, ERK and p-ERK proteins expressions with concomitant decreased Sirt6 protein expression in RA patients. Also, *in vitro* cell study demonstrated significantly increased MyD88 mRNA expression in RA-FLS cells. These findings may affirm the inflammatory role of MyD88-ERK pathway in contributing to development and destructive processes of human RA [63]. Available literature has suggested the potential of overexpressed SIRT6 to suppress inflammation and damaged bone in mouse model of collagen-induced arthritis [24]. Thus, SIRT6 overexpression can substantially reduce MyD88 expression, and inhibit expression and phosphorylation of ERK and MEK proteins, which ultimately

suppress MyD88-ERK pathway and alleviate inflammation in RA. Despite the above promising findings, this study is limited to some extent. In the first place, the actual mechanism underlying the interaction between SIRT6 and MyD88-ERK pathway is not known. Also, the downstream and upstream of the constituents of MyD88-ERK pathway have not been established. Besides, the effect of SIRT6 on the supposed components of MyD88-ERK pathway was also not studied. Hence, future works will seek to comprehensively investigate the underlying prospects to fully understand the beneficial role of SIRT6 in RA treatment.

5. Conclusions

In conclusion, we successfully studied the effect and mechanism of SIRT6 on RA. We established that SIRT6 was lowly expressed in RA patients' sera with an increase in related inflammatory factors in RA-FLS cells and RA rats. But our study discovered that overexpressed SIRT6 substantially decreased the above factors. Also, we observed increased mRNA and protein expression of MyD88 in RA-FLS cells and RA rats, which was reversed after SIRT6 was overexpressed. Additionally, we found that SIRT6 expression could inhibit ERK pathway by inhibiting the activities of ERK and MEK. Likewise, SIRT6 overexpression reduced inflammatory injury in RA through inhibition of MyD88-ERK signaling pathway. Based on the aforementioned findings, we intend to focus our not-too distant future research works on activation and up-regulation of SIRT6 expression, coupled with exploration of natural agonists or chemically synthesized activators of SIRT6 for suppression of RA progression via MyD88-ERK signal pathway.

Availability of Data and Materials

Upon reasonable request, data supporting findings of this work are available from corresponding author.

Author Contributions

XY, ZJ, FR, and JX designed the research study. XY, ZJ, PZ, and JWu performed the research. FR and JWa provided help and advice on the experiments. MR analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Human experiments were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the ethics committee of Jiangsu University affiliated Wujin Hospital (No. 2021[25]). Animal experiments were performed comply with the ARRIVE guidelines and carried out in accordance with the National Research Council's Guide for the Care

and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Jiangsu University (UJS-IACUC-2022010801). Each participant was informed their right to have their information kept confidential. All participants provided written consent prior to participation.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2901005>.

References

- [1] Chen M, Li MH, Zhang N, Sun WW, Wang H, Wang YA, *et al.* Pro-angiogenic effect of exosomal microRNA-103a in mice with rheumatoid arthritis via the downregulation of hepatocyte nuclear factor 4 alpha and activation of the JAK/STAT3 signaling pathway. *Journal of Biological Regulators and Homeostatic Agents.* 2021; 35: 629–640.
- [2] Satoh K, Kikuchi S, Sekimata M, Kabuyama Y, Homma MK, Homma Y. Involvement of ErbB-2 in rheumatoid synovial cell growth. *Arthritis and Rheumatism.* 2001; 44: 260–265.
- [3] Zlatanović G, Veselinović D, Cekić S, Zivković M, Dorđević-Jocić J, Zlatanović M. Ocular manifestation of rheumatoid arthritis-different forms and frequency. *Bosnian Journal of Basic Medical Sciences.* 2010; 10: 323–327.
- [4] Darmawan J, World Health Organization-International League of Associations for Rheumatology Community Oriented Program for Control of Rheumatic Disease. Recommendations from the Community Oriented Program for Control of Rheumatic Disease for data collection for the measurement and monitoring of health in developing countries. *Clinical Rheumatology.* 2007; 26: 853–857.
- [5] Hwang SY, Kim JY, Kim KW, Park MK, Moon Y, Kim WU, *et al.* IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-kappaB- and PI3-kinase/Akt-dependent pathways. *Arthritis Research & Therapy.* 2004; 6: R120–8.
- [6] Li X, Wu Z, He B, Zhong W. Tetrandrine alleviates symptoms of rheumatoid arthritis in rats by regulating the expression of cyclooxygenase-2 and inflammatory factors. *Experimental and Therapeutic Medicine.* 2018; 16: 2670–2676.
- [7] Cho ML, Jung YO, Moon YM, Min SY, Yoon CH, Lee SH, *et al.* Interleukin-18 induces the production of vascular endothelial growth factor (VEGF) in rheumatoid arthritis synovial fibroblasts via AP-1-dependent pathways. *Immunology Letters.* 2006; 103: 159–166.
- [8] Ryu S, Lee JH, Kim SI. IL-17 increased the production of vascular endothelial growth factor in rheumatoid arthritis synovio-cytes. *Clinical Rheumatology.* 2006; 25: 16–20.
- [9] Song B, Li X, Xu Q, Yin S, Wu S, Meng X, *et al.* Inhibition of BMP3 increases the inflammatory response of fibroblast-like synoviocytes in rheumatoid arthritis. *Aging.* 2020; 12: 12305–12323.
- [10] Bustamante MF, Garcia-Carbonell R, Whisenant KD, Guma M. Fibroblast-like synoviocyte metabolism in the pathogenesis of rheumatoid arthritis. *Arthritis Research & Therapy.* 2017; 19: 110.
- [11] Xiao Y, Sun M, Zhan Z, Ye Y, Huang M, Zou Y, *et al.* Increased phosphorylation of ezrin is associated with the migration and invasion of fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Rheumatology (Oxford, England).* 2014; 53: 1291–1300.
- [12] Chen J, Chen H, Pan L. SIRT1 and gynecological malignancies (Review). *Oncology Reports.* 2021; 45: 43.
- [13] Ansari A, Rahman MS, Saha SK, Saikot FK, Deep A, Kim KH. Function of the SIRT3 mitochondrial deacetylase in cellular physiology, cancer, and neurodegenerative disease. *Aging Cell.* 2017; 16: 4–16.
- [14] Kugel S, Sebastián C, Fitamant J, Ross KN, Saha SK, Jain E, *et al.* SIRT6 Suppresses Pancreatic Cancer through Control of Lin28b. *Cell.* 2016; 165: 1401–1415.
- [15] Gertler AA, Cohen HY. SIRT6, a protein with many faces. *Biogerontology.* 2013; 14: 629–639.
- [16] Mao Z, Hine C, Tian X, Van Meter M, Au M, Vaidya A, *et al.* SIRT6 promotes DNA repair under stress by activating PARP1. *Science (New York, N.Y.).* 2011; 332: 1443–1446.
- [17] Toiber D, Erdel F, Bouazoune K, Silberman DM, Zhong L, Mulligan P, *et al.* SIRT6 recruits SNF2H to DNA break sites, preventing genomic instability through chromatin remodeling. *Molecular Cell.* 2013; 51: 454–468.
- [18] Lappas M. Anti-inflammatory properties of sirtuin 6 in human umbilical vein endothelial cells. *Mediators of Inflammation.* 2012; 2012: 597514.
- [19] Jang HY, Gu S, Lee SM, Park BH. Overexpression of sirtuin 6 suppresses allergic airway inflammation through deacetylation of GATA3. *The Journal of Allergy and Clinical Immunology.* 2016; 138: 1452–1455.e13.
- [20] Woo SJ, Noh HS, Lee NY, Cheon YH, Yi SM, Jeon HM, *et al.* Myeloid sirtuin 6 deficiency accelerates experimental rheumatoid arthritis by enhancing macrophage activation and infiltration into synovium. *EBioMedicine.* 2018; 38: 228–237.
- [21] Kawahara TLA, Michishita E, Adler AS, Damian M, Berber E, Lin M, *et al.* SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell.* 2009; 136: 62–74.
- [22] Liu G, Chen H, Liu H, Zhang W, Zhou J. Emerging roles of SIRT6 in human diseases and its modulators. *Medicinal Research Reviews.* 2021; 41: 1089–1137.
- [23] Engler A, Niederer F, Klein K, Gay RE, Kyburz D, Camici GG, *et al.* SIRT6 regulates the cigarette smoke-induced signalling in rheumatoid arthritis synovial fibroblasts. *Journal of Molecular Medicine (Berlin, Germany).* 2014; 92: 757–767.
- [24] Lee HS, Ka SO, Lee SM, Lee SI, Park JW, Park BH. Overexpression of sirtuin 6 suppresses inflammatory responses and bone

- destruction in mice with collagen-induced arthritis. *Arthritis and Rheumatism*. 2013; 65: 1776–1785.
- [25] Chang WJ, Toledo-Pereyra LH. Toll-like receptor signaling in liver ischemia and reperfusion. *Journal of Investigative Surgery: the Official Journal of the Academy of Surgical Research*. 2012; 25: 271–277.
- [26] Liu G, Zhang L, Zhao Y. Modulation of immune responses through direct activation of Toll-like receptors to T cells. *Clinical and Experimental Immunology*. 2010; 160: 168–175.
- [27] Banerjee S, Thompson WE, Chowdhury I. Emerging roles of microRNAs in the regulation of Toll-like receptor (TLR)-signaling. *Frontiers in Bioscience (Landmark Edition)*. 2021; 26: 771.
- [28] Brown J, Wang H, Hajishengallis GN, Martin M. TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. *Journal of Dental Research*. 2011; 90: 417–427.
- [29] Choe JY, Crain B, Wu SR, Corr M. Interleukin 1 receptor dependence of serum transferred arthritis can be circumvented by toll-like receptor 4 signaling. *The Journal of Experimental Medicine*. 2003; 197: 537–542.
- [30] Schett G, Tohidast-Akrad M, Smolen JS, Schmid BJ, Steiner CW, Bitzan P, *et al.* Activation, differential localization, and regulation of the stress-activated protein kinases, extracellular signal-regulated kinase, c-JUN N-terminal kinase, and p38 mitogen-activated protein kinase, in synovial tissue and cells in rheumatoid arthritis. *Arthritis and Rheumatism*. 2000; 43: 2501–2512.
- [31] Ohori M. ERK inhibitors as a potential new therapy for rheumatoid arthritis. *Drug News & Perspectives*. 2008; 21: 245–250.
- [32] Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, *et al.* 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis and Rheumatism*. 2010; 62: 2569–2581.
- [33] Cao J, Lv X, Zhang Y, Xu A, Jiang T. LncRNA GAS5 activates the AMPK pathway in peripheral blood mononuclear cells derived from rheumatoid arthritis patients. *International Journal of Rheumatic Diseases*. 2020; 23: 1318–1327.
- [34] Adefegha SA, Bottari NB, Leal DB, de Andrade CM, Schetinger MR. Interferon gamma/interleukin-4 modulation, anti-inflammatory and antioxidant effects of hesperidin in complete Freund's adjuvant (CFA)-induced arthritis model of rats. *Immunopharmacology and Immunotoxicology*. 2020; 42: 509–520.
- [35] He Y, Yang G, Sun L, Gao H, Yao F, Jin Z, *et al.* SIRT6 inhibits inflammatory response through regulation of NRF2 in vascular endothelial cells. *International Immunopharmacology*. 2021; 99: 107926.
- [36] Zhang CW, Wu X, Liu D, Zhou W, Tan W, Fang YX, *et al.* Long non-coding RNA PVT1 knockdown suppresses fibroblast-like synoviocyte inflammation and induces apoptosis in rheumatoid arthritis through demethylation of *sirt6*. *Journal of Biological Engineering*. 2019; 13: 60.
- [37] Chen H, Pan T, Liu P, Wang P, Xu S. Baihu Jia Guizhi Decoction Improves Rheumatoid Arthritis Inflammation by Regulating Succinate/SUCNR1 Metabolic Signaling Pathway. *Evidence-based Complementary and Alternative Medicine: ECAM*. 2019; 2019: 3258572.
- [38] Shrivastava AK, Pandey A. Inflammation and rheumatoid arthritis. *Journal of Physiology and Biochemistry*. 2013; 69: 335–347.
- [39] McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *The New England Journal of Medicine*. 2011; 365: 2205–2219.
- [40] Huang J, Fu X, Chen X, Li Z, Huang Y, Liang C. Promising Therapeutic Targets for Treatment of Rheumatoid Arthritis. *Frontiers in Immunology*. 2021; 12: 686155.
- [41] Smolen JS, Aletaha D, Barton A, Burmester GR, Emery P, Firestein GS, *et al.* Rheumatoid arthritis. *Nature Reviews. Disease Primers*. 2018; 4: 18001.
- [42] Firestein GS, McInnes IB. Immunopathogenesis of Rheumatoid Arthritis. *Immunity*. 2017; 46: 183–196.
- [43] Chen L, Zheng L, Chen P, Liang G. Myeloid Differentiation Primary Response Protein 88 (MyD88): The Central Hub of TLR/IL-1R Signaling. *Journal of Medicinal Chemistry*. 2020; 63: 13316–13329.
- [44] Dong X, Zhang Q, Zeng F, Cai M, Ding D. The protective effect of gentisic acid on rheumatoid arthritis via the RAF/ERK signaling pathway. *Journal of Orthopaedic Surgery and Research*. 2022; 17: 109.
- [45] de Launay D, van de Sande MGH, de Hair MJH, Grabiec AM, van de Sande GPM, Lehmann KA, *et al.* Selective involvement of ERK and JNK mitogen-activated protein kinases in early rheumatoid arthritis (1987 ACR criteria compared to 2010 ACR/EULAR criteria): a prospective study aimed at identification of diagnostic and prognostic biomarkers as well as therapeutic targets. *Annals of the Rheumatic Diseases*. 2012; 71: 415–423.
- [46] Kanfi Y, Naiman S, Amir G, Peshti V, Zinman G, Nahum L, *et al.* The sirtuin SIRT6 regulates lifespan in male mice. *Nature*. 2012; 483: 218–221.
- [47] Totoson P, Maguin-Gaté K, Prati C, Wendling D, Demougeot C. Mechanisms of endothelial dysfunction in rheumatoid arthritis: lessons from animal studies. *Arthritis Research & Therapy*. 2014; 16: 202.
- [48] Megha KB, Joseph X, Akhil V, Mohanan PV. Cascade of immune mechanism and consequences of inflammatory disorders. *Phytomedicine: International Journal of Phytotherapy and Phytomedicine*. 2021; 91: 153712.
- [49] Harty LC, Binińska M, O'Sullivan J, Fox E, Mulhall K, Veale DJ, *et al.* Mitochondrial mutagenesis correlates with the local inflammatory environment in arthritis. *Annals of the Rheumatic Diseases*. 2012; 71: 582–588.
- [50] Di Cesare Mannelli L, Bani D, Bencini A, Brandi ML, Calosi L, Cantore M, *et al.* Therapeutic effects of the superoxide dismutase mimetic compound MnIIme2DO2A on experimental articular pain in rats. *Mediators of Inflammation*. 2013; 2013: 905360.
- [51] Li Y, Jin J, Wang Y. SIRT6 Widely Regulates Aging, Immunity, and Cancer. *Frontiers in Oncology*. 2022; 12: 861334.
- [52] Pierer M, Wagner U, Rossol M, Ibrahim S. Toll-like receptor 4 is involved in inflammatory and joint destructive pathways in collagen-induced arthritis in DBA1J mice. *PLoS One*. 2011; 6: e23539.
- [53] Zhang C, Yu Y, Huang Q, Tang K. SIRT6 regulates the proliferation and apoptosis of hepatocellular carcinoma via the ERK1/2 signaling pathway. *Molecular Medicine Reports*. 2019; 20: 1575–1582.
- [54] Joosten LAB, Koenders MI, Smeets RL, Heuvelmans-Jacobs M, Helsen MMA, Takeda K, *et al.* Toll-like receptor 2 pathway drives streptococcal cell wall-induced joint inflammation: critical role of myeloid differentiation factor 88. *Journal of Immunology (Baltimore, Md.: 1950)*. 2003; 171: 6145–6153.
- [55] Sacre SM, Andreacos E, Kiriakidis S, Amjadi P, Lundberg A, Giddins G, *et al.* The Toll-like receptor adaptor proteins MyD88 and Mal/TIRAP contribute to the inflammatory and destructive processes in a human model of rheumatoid arthritis. *The American Journal of Pathology*. 2007; 170: 518–525.
- [56] Song ZM, Liu F, Chen YM, Liu YJ, Wang XD, Du SY. CTGF-mediated ERK signaling pathway influences the inflammatory factors and intestinal flora in ulcerative colitis. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*. 2019; 111: 1429–1437.
- [57] Yousefi B, Darabi M, Baradaran B, Shekari Khaniani M, Rahbani M, Darabi M, *et al.* Inhibition of MEK/ERK1/2 Signaling

Affects the Fatty Acid Composition of HepG2 Human Hepatic Cell Line. *BioImpacts: BI*. 2012; 2: 145–150.

- [58] Mao LM, Wang HH, Wang JQ. Antagonism of Muscarinic Acetylcholine Receptors Alters Synaptic ERK Phosphorylation in the Rat Forebrain. *Neurochemical Research*. 2017; 42: 1202–1210.
- [59] Thiel MJ, Schaefer CJ, Lesch ME, Mobley JL, Dudley DT, Teele H, *et al.* Central role of the MEK/ERK MAP kinase pathway in a mouse model of rheumatoid arthritis: potential proinflammatory mechanisms. *Arthritis and Rheumatism*. 2007; 56: 3347–3357.
- [60] Zhang ZG, Qin CY. Sirt6 suppresses hepatocellular carcinoma cell growth via inhibiting the extracellular signal regulated kinase signaling pathway. *Molecular Medicine Reports*. 2014; 9: 882–888.
- [61] Dzidek A, Czerwińska-Ledwig O, Żychowska M, Pilch W, Piotrowska A. The Role of Increased Expression of Sirtuin 6 in the Prevention of Premature Aging Pathomechanisms. *International Journal of Molecular Sciences*. 2023; 24: 9655.
- [62] Tian J, Zhao Y, Wang L, Li L. Role of TLR4/MyD88/NF- κ B signaling in heart and liver-related complications in a rat model of type 2 diabetes mellitus. *The Journal of International Medical Research*. 2021; 49: 300060521997590.
- [63] Gomes da Silva IIF, Lima CAD, Silva JEA, Rushansky E, Mariano MHQA, Rolim P, *et al.* Is there an Inflammation Role for MYD88 in Rheumatoid Arthritis? *Inflammation*. 2021; 44: 1014–1022.