RESEARCH ARTICLE

# MicroRNA-155 inhibitor ameliorates collagen-induced arthritis by modulating the phenotype of pro-inflammatory macrophages in a mouse model

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## ABSTRACT

**Background:** Here, we aimed to illustrate the roles of microRNA (miR)-155 in collagen-induced arthritis (CIA) and its underlying mechanisms.

**Methods:** A mouse model of CIA was first established, and miR-155 inhibitor was intravenously injected. In *in vitro* studies, bone marrow-derived macrophages (BMDMs) were induced to M1 macrophages followed by the treatment of miR-155 inhibitor. RT-qPCR was applied to determine the mRNA expression. The frequency of M1 or M2 macrophages was determined by flow cytometry. Western blotting was employed to detect protein expression. Enzyme-linked immunosorbent assay was employed to measure the production of inflammatory cytokines and anti-collagen antibody.

**Results:** The levels of miR-155 were increased in macrophages from rheumatoid arthritis (RA) patients and M1 macrophages. The treatment of miR-155 inhibitor decreased inflammatory cytokines in M1 macrophages. Besides, treatment of miR-155 inhibitors promoted the differentiation of M0 macrophages into M2 macrophages. *In vivo* studies showed that miR-155 inhibitors ameliorated the RA symptoms by decreasing inflammatory cytokines in the CIA mouse model. Treatment of miR-155 also led to decreased M1 macrophage biomarker and increased M2 macrophage biomarker.

**Conclusion:** MiR-155 inhibitor ameliorates RA symptoms in part by regulating macrophage phenotypes.

**Keywords:** MicroRNA-155 · Arthritis · Collagen-induced arthritis · Macrophages · Macrophage polarization

## Introduction

RA (rheumatoid arthritis), as an autoimmune disease, is characterized by synovial inflammation and the destruction of bones and cartilages (1, 2). The development of RA also induces other tissue damages including the vascular system, lung, heart, and skins (1). The prevalence and incidence of RA are 1% and 0.03%, respectively, worldwide (3). Besides, the medical cost for RA is more than 5,000 dollars per patient per year (3). Although it is reported to be associated with inflammation, the underlying mechanisms of RA is still unclear.

Many studies have demonstrated that macrophages are involved in RA pathology by the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1 (4, 5). The activation of inflammation-related signaling pathways also triggers the release of adhesion molecules, which recruits the leukocytes to the damaged tissues (4). Typical macrophages in activated condition are M1 or M2 types (6), also called classical and alternative activated macrophage, respectively (7). Previous studies have demonstrated that M1 macrophages could produce vast

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inflammatory cytokines, thus accelerate the process of RA, while M2 macrophages could attenuate inflammation and repair the tissues (6, 8). Importantly, M1 macrophages can be shifted into M2 phenotype (7).

MicroRNAs (miRNAs, miRs) have been identified to play crucial roles in the modulation of gene expression (9). Interestingly, macrophage polarization can also be regulated by miRNAs (10). Previous studies have demonstrated that miR-9, miR-127, miR-155, and miR-125b promote M1 polarization, whereas miR-124, miR-223, miR-132, and miR-146a induce M2 polarization (11). Therefore, the regulation of miRNAs is a good strategy for the regulation of macrophage polarization. In 2011, Stephan and colleagues found that miR-155 knockout mice could not develop collagen-induced arthritis (CIA), indicating that miR-155 contributes to the pathogenesis development of CIA (12). Furthermore, Su and colleagues reported that the levels of miR-155 were increased in RA animal models and patients. The knockdown of miR-155 ameliorates the development of RA. All these results demonstrated the important roles of miR-155 in RA (13).

## Materials and Methods

### Patients and healthy volunteers

All participants signed the documents of consent. The study was approved by Haikou Orthopedic and Diabetes Hospital of Shanghai Sixth People's Hospital. Peripheral blood was collected from RA patients and healthy volunteers. The peripheral blood mononuclear cells (PBMCs) were isolated based on the published protocol (14). Macrophages were then separated from PBMCs for further assays.

### Animals

Animal studies were approved by Haikou Orthopedic and Diabetes Hospital of Shanghai Sixth People's Hospital. Male DBA/1 mice were housed under humidity (60 ± 5%) and temperature-controlled (22-24 °C) condition. A CIA mouse model was established by immunizations of bovine type 2 collagen that was emulsified in complete or incomplete Freund's adjuvant at day 0 and day 21.

The mice were divided into phosphate-buffered saline (PBS) group, control inhibitor group, and miR-155 inhibitor group. In the PBS group, the mice were intravenously injected with PBS on day 15 and day 31. In the control inhibitor group and miR-155 inhibitor group, the mice were intravenously injected with control inhibitors or miR-155 inhibitors (50 μg per mouse) that were mixed with Entranster-in vivo at day 15 and day 31.

In the experimental period, the CIA score and ankle thickness were measured every two days. An X-ray was applied to determine the joint damage induced by arthritis at the end of the experiment. After the mice were sacrificed, the joint tissues, blood were collected.

### Histopathology examination

The mice were sacrificed at the end of the experiments. The joint tissues were collected, then fixed in formalin solution (10%) prior to embedding in paraffin. Hematoxylin and eosin staining was conducted. A microscope was used for the observation of the slides.

### Bone marrow-derived macrophage (BMDM) isolation and culture

BMDMs were isolated based on the published protocol (15). Briefly, the collected cells were maintained in DMEM (Dulbecco's modified eagle medium) with M-CSF (macrophage colony-stimulating factor, 10 ng/ml) and 10% fetal bovine serum for 5 days. To generate M1 macrophages, cells were incubated with interferon (IFN)-γ (50 ng/mL) and lipopolysaccharides (LPS) (100 ng/mL) for 24 h. After phenotype examination, M1 macrophages were treated with miR-155 inhibitor or control inhibitor.

### Enzyme-linked immunosorbent assay (ELISA)

Inflammatory cytokine levels including IL-6, MCP-1 (monocyte chemoattractant protein 1), IL-1β, TNF-α, and anti-collagen antibody were determined using ELISA based on the manufacturers' documents.

### Flow cytometry analysis

After the polarized macrophages were transfected with miR-155 inhibitor or control inhibitor, cell suspension was prepared and stained with PE-Cy7 conjugated MHCII and PE-conjugated CD206. FlowJ (BD FACSCalibur) was applied to analyze the results.

### RT-qPCR

RNA was isolated using RNA extraction kit. The melt curves were used to analyze the accuracy. Gene expression was calculated using $2^{-\Delta\Delta Ct}$ values with GAPDH as control.

### Western blotting

The protein was extracted following routine procedures (16, 17). Briefly, the joint tissue was lysed using cold radioimmunoprecipitation assay buffer with protease inhibitor. Then, the extraction buffer was centrifuged (13,000 g) for 15 mins to remove insoluble materials and sample debris. Concentrations of extracted proteins were quantified using BCA protein assay kits. The protein was then loaded, subjected to separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gels were transferred to polyvinylidene fluoride membranes, which were blocked with 5% non-fat milk at room temperature for 2 hours. Primary antibodies against inducible nitric oxide synthase (iNOS) or CD206 were used for incubation with the membranes at 4 °C overnight. Appropriate secondary antibodies conjugated with horseradish peroxidase were used.

### Statistics

Data were shown as mean ± standard deviation (SD). One-way analysis of variance with Student-Newman-Keuls test and multiple comparisons were employed for the data comparison. Statistical significance was

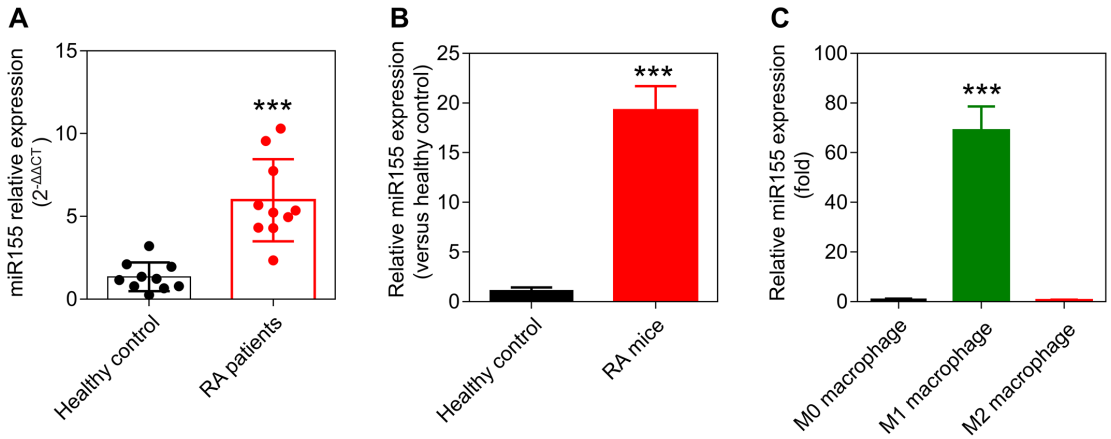


**Figure 1.** (A) qRT-PCR was determined to detect the mRNA levels of miR-155 in the macrophages that were isolated from PBMCs in the RA patients and healthy control (n = 10). (B) The mRNA levels of miR-155 in the macrophages isolated from collagen-induced arthritis (CIA) mouse model (n = 8). (C) The mRNA levels of miR-155 in M0, M1 and M2 macrophages. Data were shown as mean ± SD, ***$p < 0.001$.

determined when p value was less than 0.05.

## Results

### MiR-155 was upregulated in macrophages of RA patients and M1 macrophages

We first investigated the mRNA levels of miR-155 in healthy volunteers and RA patients. mRNA levels of miR-155 in the macrophages from PBMCs of RA patients were remarkably enhanced when compared to the healthy volunteers ($p < 0.001$, **Figure 1A**). Next, we analyzed the mRNA levels of miR-155 in the macrophages from RA mouse model and healthy mice. Interestingly, the mRNA levels of miR-155 in the macrophages of RA mice were significantly increased when compared to the healthy control mice ($p < 0.001$, **Figure 1B**). Finally, to investigate the relationship of miR-155 expression pattern and macrophage phenotype, we determined the mRNA levels of miR-155 in M0 macrophages and polarized M1 and M2 macrophages. mRNA levels of miR-155 were much higher in M1 macrophages in comparison to M0 and M2 macrophages ($p < 0.001$, **Figure 1C**), suggesting that miR-155 expression pattern is associated with the inflammatory status of macrophages.

### MiR-155 inhibitor decreased the production of inflammatory cytokines in M1 macrophages

The effects of miR-155 inhibitor on the production of inflammatory cytokines in M1 macrophages were further examined. To obtain M1 macrophages, BMDMs were stimulated with IFN-γ (50 ng/mL) and LPS (100 ng/mL). We observed the levels of inflammatory cytokines including TNF-α, IL-1β, IL-6, and MCP-1 were obviously decreased in M1 macrophages in the presence of miR-155 inhibitors in a concentration-dependent manner (**Figure 2A-D**).

### Effects of MiR-155 inhibitors on the inflammatory gene expression and macrophages phenotype

We then explored the potential of miR-155 on the expressions of inflammatory genes. To obtain M1 macrophages, BMDMs were stimulated with IFN-γ (50 ng/mL) and LPS (100 ng/mL) followed by the treatment of miR-155 inhibitors (**Figure 3A**). Interestingly, we observed that, in the presence of miR-155 inhibitors, the gene expressions of M1 macrophage-related biomarkers including IL-12 and Nos2 was significantly decreased, whereas the gene expression of M2 macrophage-related biomarkers including Arg1 and Mrc1 was significantly increased (**Figure 3B**). We inferred that miR-155 might affect macrophages polarization. Besides, we also analyze the population of M1 and M2 macrophages. miR-155 inhibitors significantly reduced the percentage of MHC IIhi macrophages ($p < 0.001$, **Figure 3C** and **D**) and increased the percentage of CD206hi macrophages ($p < 0.001$, **Figure 3E** and **F**).

### Effects of miR-155 inhibitors on the RA symptoms of CIA mouse model

To confirm the effects of miR-155 inhibition in vitro, the effects of miR-155 in a CIA mouse model were investigated. As shown in **Figure 4A**, a CIA mouse model was established by immunizations of bovine type 2 collagen emulsified in complete or incomplete Freund's adjuvant. The mice were intravenously injected with control inhibitors or miR-155 inhibitors (50 μg per mouse) that were mixed with Entranster-in vivo at day 15 and day 31. We observed that the miR-155 inhibitors significantly decreased the RA score and mean ankle diameter at day 34 in comparison to the control inhibitor group (**Figure 4B and C**). At the end of the experimental period, X-ray was applied to determine the joint change in each group. We observed that arthritic changes in the PBS and control inhibitor group, whereas the treatment of miR-155 inhibitor ameliorated these changes (**Figure 4D**).

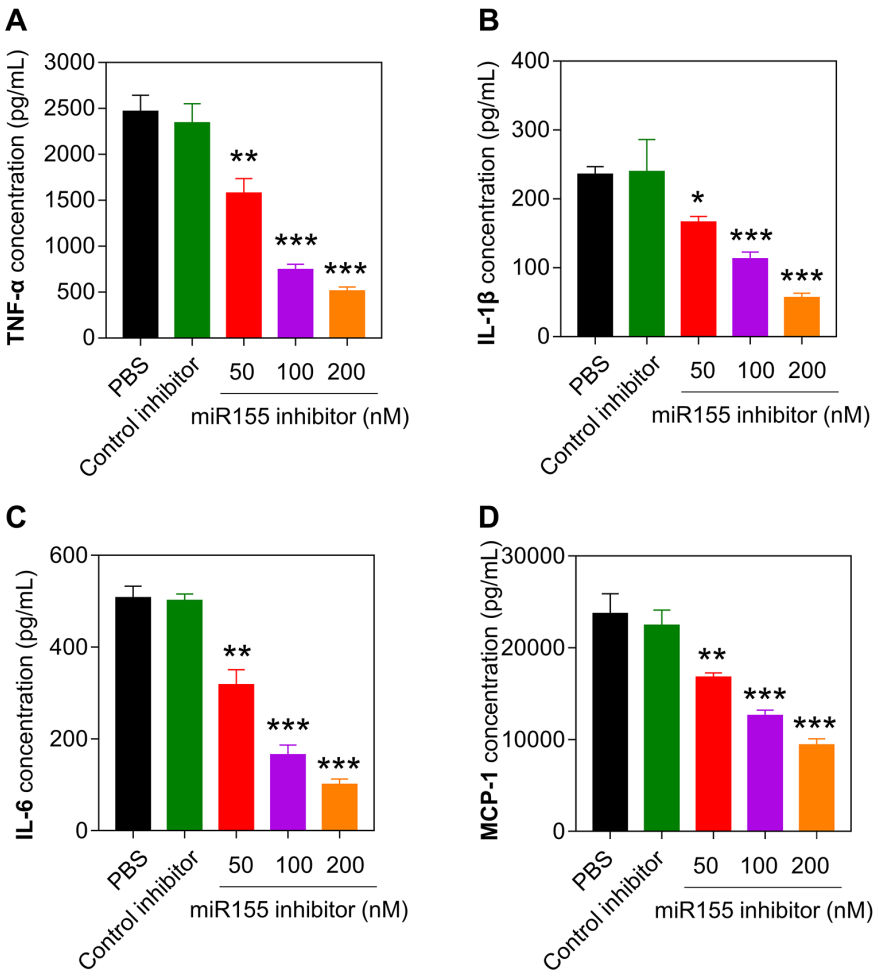


**Figure 2 Inflammatory cytokines including TNF-α (A), IL-1β (B), IL-6 (C), and MCP-1 (D) in M1 macrophages transfected with miR-155 inhibitors were determined using ELISA.** To induce M1 macrophages, BMDMs were incubated with LPS (100 ng/mL) and IFN-γ (50 ng/mL) for 24 and then transfected with miR-155 inhibitors for another 48 h. Data were shown as means ± SD, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs M1 macrophage transfected with control inhibitor.

### Treatment of miR-155 inhibitor attenuated CIA symptoms

Finally, the effects of miR-155 inhibitor on the CIA symptoms were evaluated. Histopathological examination demonstrated synovial lining and bone erosions and proliferation of synovial membrane cells in the PBS and control inhibitor groups, whereas synovial lining and bone erosions were ameliorated and fewer numbers of synovial membrane cells were observed in the miR-155 inhibitor groups (**Figure 5A**). These results demonstrated that the treatment of miR-155 inhibitor reversed the histopathological changes in RA. Additionally, miR-155 inhibitor decreased the levels of anti-collagen IgG in comparison to the PBS and control inhibitor groups (**Figure 5B**).

The expressions of M1/ M2 macrophages biomarkers and inflammatory cytokines were studied. The results demonstrated that miR-155 inhibitor-treated resulted in a decrease of M1 macrophage biomarker iNOS and an increase of M2 macrophage biomarker CD206, suggesting that the levels of miR-155 are associated with the changes of macrophage phenotypes (**Figure 5C**). Furthermore, the results also showed the levels of inflammatory cytokines including TNF-α, IL-1β, and IL-6 were significantly decreased in the presence of miR-155 inhibitor, indicating the inhibition of miR-155 attenuated the expressions of inflammatory cytokines in the joint tissue of CIA mouse (**Figure 5D-F**).

## Discussion

In the present study, the mRNA level of miR-155 was found to be increased in CIA animal models and RA patients. Notably, our results showed a significant increase of miR-155 in M1 macrophages as compared to M0 and M2 macrophages. We further analyzed the relationship between miR-155 and macrophage polarization.

First, we found that miR-155 inhibitor decreased inflammatory cytokines in M1 macrophages. Second, miR-155 inhibitor promoted the polarization of macrophages into M2 phenotypes. The in vivo studies also confirmed the regulatory effects of miR-155 inhibitor on the macrophage phenotype and RA symptoms. Therefore, we speculated that the inhibition of miR-155 might be a

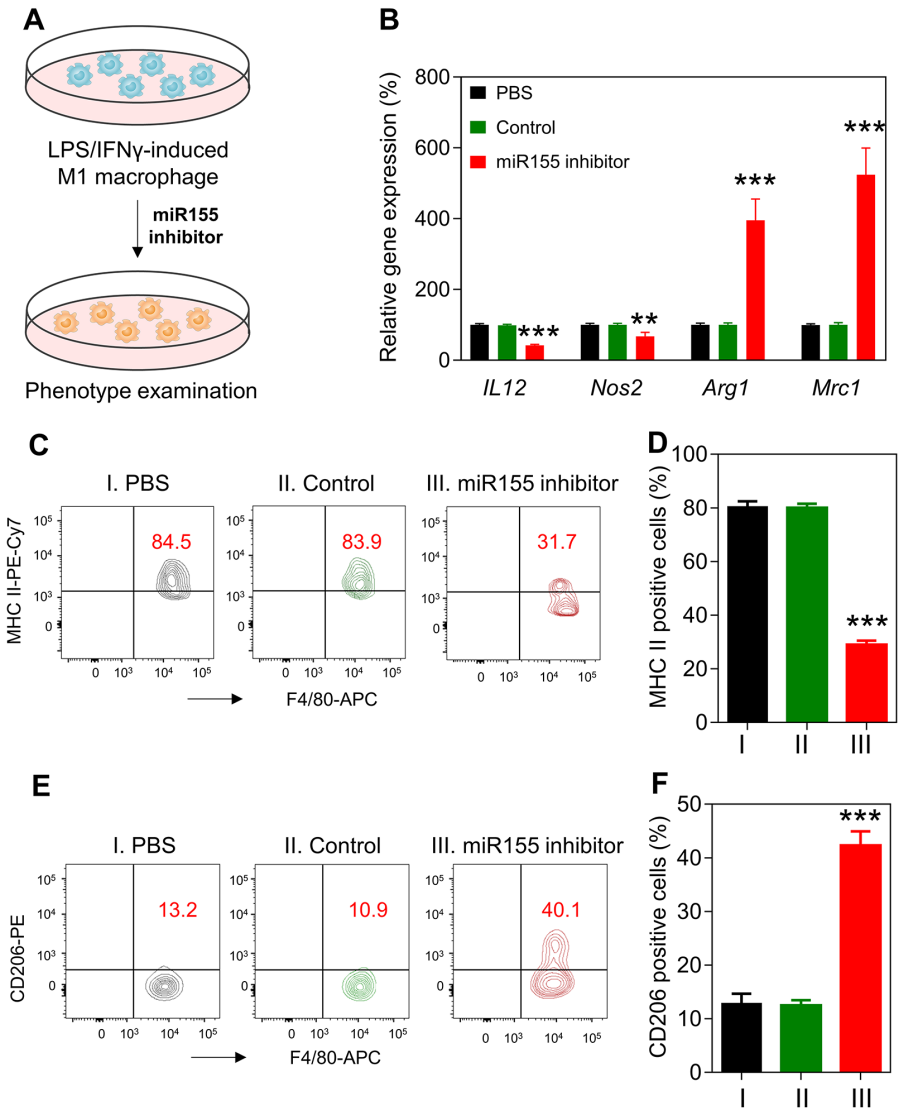


**Figure 3** (A) Diagram of the generation of M1 macrophages. BMDMs were incubated with LPS plus IFN-γ and the phenotype was determined after transfection of miR-155 inhibitor for 48 h. (B) qRT-PCR was determined to detect the mRNA levels of IL12, Nos2, Arg1, and Mrc1 in M1 macrophages after the treatment of miR-155 inhibitor. (C-F) Flow cytometry was applied to determine the percentage of MHCII positive macrophages and CD206 positive macrophages after treatment. Data were shown as means ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$ (vs M1 macrophage treated with control inhibitor).

promising strategy for RA treatment.

The role of macrophages in RA occurrence and development has been well documented (5, 8, 18). The inflammatory mediators and cytokines secreted by macrophages trigger an adaptive immune response in the synovium and contributes to the local joint destruction (4, 19, 20). Here, we specifically determined the expressions of miR-155 in the macrophages. miR-155 was significantly increased in the macrophages from PBMCs of RA patients and in the BMDMs. Our findings are consistent with previous studies, in which an elevation of miR-155 is observed in RA animal models and patients.

Furthermore, activated macrophages in joint tissue are including M1 or M2 phenotypes (7). M1 is called classical activated macrophage whereas M2 is recognized as an alternative activated macrophage (8). M1 phenotypes accelerate the process of RA by the release of large amounts of inflammatory mediators such as iNOS and other pro-inflammatory cytokines, whereas M2 phenotypes could exert anti-inflammatory activities via the secretion of anti-inflammatory cytokines (21, 22). In this study, the expressions of miR-155 in the different types of macrophages including M0, M1, and M2 were all determined. The results showed that the mRNA levels of miR-155 were much higher in M1 macrophages as compared to the other two phenotypes. We then explored the relationship between miR-155 and inflammatory cytokines in the M1 macrophages. Interestingly, we found that the inflammatory cytokines including IL-6, IL-1β, TNF-α and chemokine MCP-1 were significantly decreased in the presence of miR-155 inhibitors. These results demonstrated that inhibition of miR-155 reduced the expressions of inflammatory cytokines and chemokines.

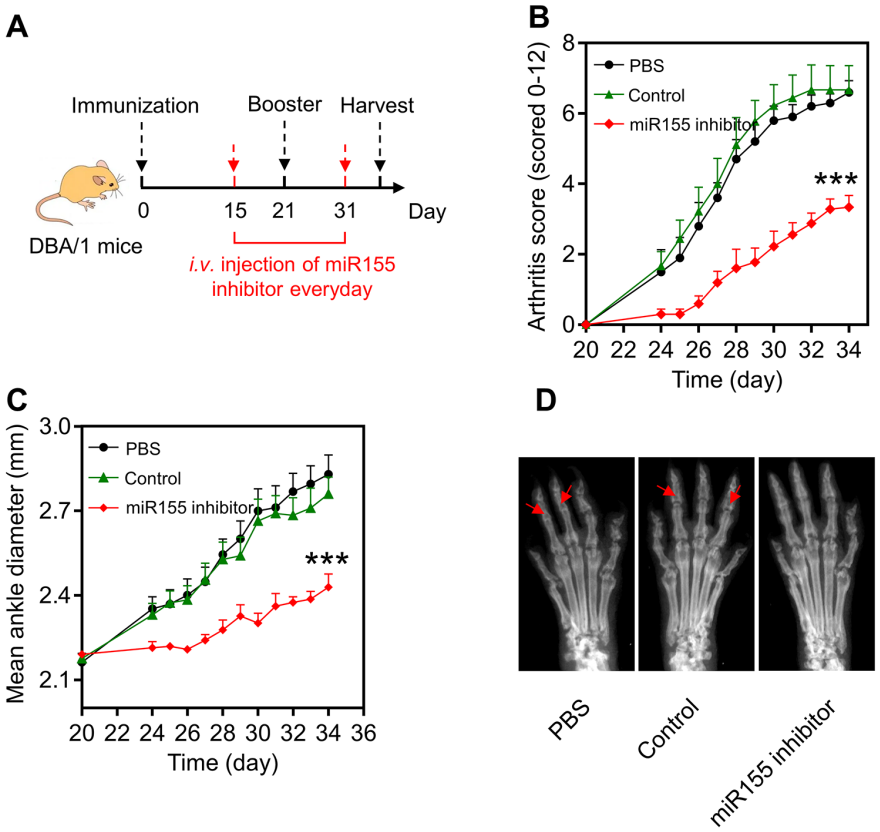


**Figure 4** (A) The schematic diagram showed the process of immunization and treatment methods on the CIA mouse model. To establish a CIA model, the mice were immunized twice with bovine type 2 collagen emulsified in Freund's adjuvant. Next, the mice were intravenously injected with 50 μg miR-155 inhibitor or control inhibitor on 15th and 31th day. In the experimental period, CIA score (B) and ankle thickness (C) were determined every two days. At the end of the experimental period, (D) X-ray was applied to evaluate the joint damage. Data were shown as means ± SD, ***$p < 0.001$ (vs mice injected with control inhibitor).

It has been verified that M1 macrophages can be shifted into M2 phenotypes (23). M1 to M2 transition ameliorates the inflammatory status, leading to the resolution of inflammation and tissue repair in RA (7). We next sought the effects of miR-155 inhibitor on the regulation of macrophages polarization. It is known that M1 macrophages produce iNOS and pro-inflammatory cytokine IL-12, while genes including Arg1 and Mrc1 were highly expressed in the M2 macrophages (24, 25). Our results demonstrated that M1 macrophage-related biomarkers were significantly decreased whereas M2 macrophage-related biomarkers were significantly increased in the presence of miR-155 inhibitor. In addition to detection of the M1 or M2 macrophages related genes, we also explored the effects of miR-155 inhibitor on the frequency of M1 and M2 macrophages. M1 macrophages can be characterized by MHC-IIhi while M2 macrophages can be characterized by CD206hi (26, 27). Interestingly, our results suggested that miR-155 inhibitors promoted the polarization of M2 macrophages.

CIA mouse model was established to determine the effects of miR-155 inhibitor on the RA symptoms, inflammatory cytokine expressions, and macrophages phenotypes. We observed the increase of mean ankle and RA scores in the experimental period. Besides, we also found that arthritic changes including 1) synovial lining and bone erosions, 2) proliferation of synovial membrane cells, 3) an increase of anti-collagen IgG in serum, and 4) the increase of inflammatory cytokines in the joint tissue. These results supported that the CIA mouse model was successfully constructed (28, 29). Interestingly, the treatment of miR-155 inhibitor significantly decreased the RA score and mean ankle diameter. At the end of the experimental period, X-ray and histopathology examinations supported that the treatment of miR-155 inhibitor ameliorated the arthritic changes and pathophysiology of RA.

Finally, we determined the effects of miR-155 inhibitor on the macrophage phenotypes and expressions of inflammatory cytokine. Interestingly, the results demonstrated that the treatment of miR-155 inhibitor promoted the polarization of M2 phenotypes and reduced the polarization of M1 phenotypes. The also showed inflammatory cytokines were remarkably decreased in the presence of miR-155 inhibitor, indicating the inhibition of miR-155 also ameliorated the inflammation in the joint tissues.

## Conclusion

We observed that miR-155 was increased in RA patients and CIA animal models. Treatment of miR-155 inhibitor

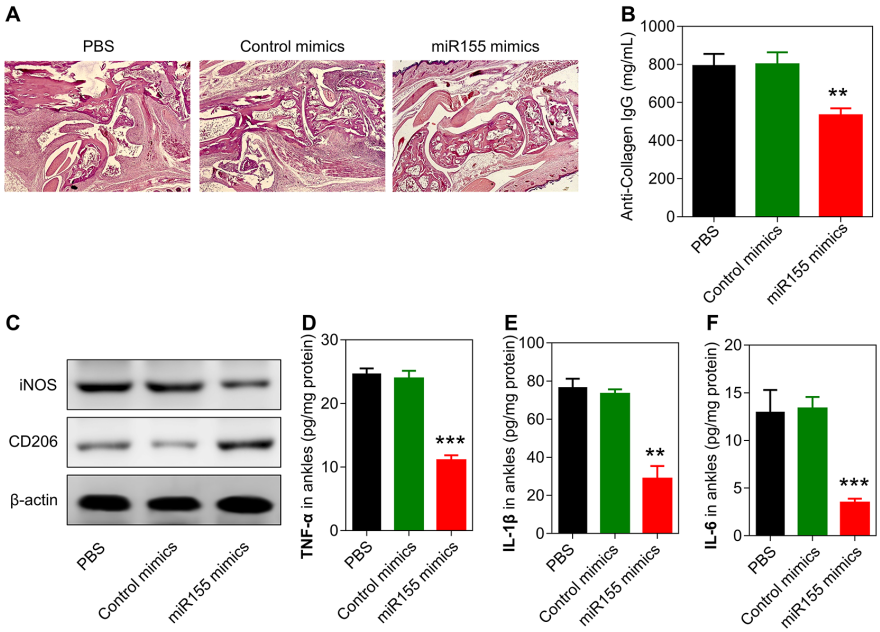


**Figure 5.** Figure 5 (A) Histopathology examination was performed and represented histopathology pictures showed the ankle joints in each group. (B) ELISA was applied to measure the serum anti-collagen antibody. (C) Western blot was applied to determine the protein expressions of iNOS and CD206 in the ankle joint tissues. (D-F) ELISA was applied to determine the levels of TNF-α, IL-1β, and IL-6 in the joint tissues. Data were shown as mean ± SD, $^{**}p < 0.001$, $^{***}p < 0.001$ (vs mice injected with control inhibitor).

decreased inflammatory cytokines in M1 macrophages and promoted M2 macrophages polarization. Additionally, miR-155 inhibitor ameliorated the RA pathological changes and decreased inflammatory cytokines and resulted in the changes of macrophage phenotypes. In summary, microRNA-155 inhibitor ameliorates RA symptoms in part by regulating macrophage phenotypes.

### Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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