MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 4892-4900
DOI: 10.12659/MSM.915858

Received: 2019.02.23
Accepted: 2019.05.08
Published: 2019.07.02

MicroRNA-149 Suppresses Inflammation in Nucleus Pulposus Cells of Intervertebral Discs by Regulating MyD88

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: This research was supported by a grant from the Natural Science Foundation of Guangdong Province (grant no. 2016A030313607)

Background: Intervertebral disc degeneration (IDD) is associated with low back and neck pain, but the mechanisms underlying its pathogenesis are unclear. In this study, we explored the function of microRNA-149 (miR-149) in inflammatory response mediated by lipopolysaccharide (LPS) in nucleus pulposus (NP) cells.

Material/Methods: Quantitative real-time PCR was used to detect miRNA and mRNA levels, while Western blotting was utilized to determine protein levels. ELISA was used to examine chemokine production. The correlation between miR-149 and MyD88 was assessed by reporter assay. Apoptosis was examined by flow cytometry.

Results: miR-149 expression was significantly decreased after LPS exposure in NP cells. Overexpression of miR-149 reversed LPS-induced inhibition in aggrecan and collagen II expression and attenuated LPS-mediated promotion in the levels of MMP3, ADAMTS4, and inflammatory cytokines. Moreover, we found that miR-149 exerted its function by targeting MyD88 in NP cells.

Conclusions: miR-149 can inhibit the inflammatory response mediated by LPS in NP cells, and might be a potential target for the treatment of IDD.

MeSH Keywords: Inflammation Mediators • MicroRNAs • Myeloid Differentiation Factor 88

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/915858

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Background

Intervertebral disc degeneration (IDD) causes chronic pain in the lower back and neck, but mechanisms underlying its pathogenesis are not fully understood. It has been suggested that maintenance of a homeostatic environment is important for healthy discs. When it is disrupted, metabolic disorders occur, resulting in a loss of matrix protein, increased degradative enzymes, and upregulation of inflammatory cytokines, and subsequent development of IDD [1,2].

Inflammatory cytokines have been shown to be important in the pathophysiological process of IDD [3–5]. For example, IL-1β is upregulated in degenerated discs, which increases the levels of MMPs and ADAMTs, and decreases synthesis of aggrecan and collagen II in NP cells [3]. Similarly, increased production of TNF-α is observed in patients with IDD [6]. TNF-α increases aggrecan degradation and enhances MMP/ADAMT expression, thus contributing to the progression of IDD [7–9]. Additionally, IL-6, IL-10, and IL-21 are also reported to be associated with IDD [5,10]. However, the exact mechanism of inflammation involved in IDD remains unclear.

miRNAs are a class of small noncoding RNAs that are 21–25 nucleotides in length. miRNAs regulate gene expression by targeting specific mRNAs for degradation or translation repression [11]. Inflammatory response regulation is mediated by controlling gene expression in participating immune system and tissue cells. Studies have demonstrated that miRNAs are important in various biological processes, including inflammatory response [12–14]. Moreover, miRNAs have been reported to be implicated in the pathogenesis of IDD. For example, Lv et al. showed that microRNA-146a (miR-146a) promotes IDD by ameliorating inflammation via the TRAF6/NF-κB pathway [15]. miR-194 has been reported to inhibit inflammatory response by TRAF-6 in nucleus pulposus (NP) cells [16]. Similarly, Zhang et al. demonstrated that overexpression of miR-140-5p inhibits LPS-induced human intervertebral disc inflammation and degeneration [17]. miR-149 has been shown to play a critical role in inflammatory response [18,19]; however, whether miR-149 has a function in IDD is not yet known.

Lipopolysaccharide (LPS) has been used as an inflammatory mediator to trigger IDD [16,17]. In this study, we investigated the role of miR-149 in LPS-mediated inflammation in NP cells of intervertebral discs and explored the underlying mechanism.

Material and Methods

Antibodies

Anti-aggrecan (ab36861), anti-Collage II (ab185430), anti-GAPDH (ab9484), anti-ADAMTS4 (ab185722), anti-Bcl2 (ab196495), and anti-MyD88 (ab2064) were obtained from Abcam (Cambridge, MA, USA). Anti-TLR4 (sc-293072) was obtained from Santa Cruz (USA). Antibodies against MMP3 (#14351), capase3 (#9662), caspase9 (#9508), and Bax (#2772), p65 (#3033) and HRP-conjugated second antibodies were purchased from Cell Signaling Technology (Danvers, USA).

Cell culture

Sprague-Dawley rats (6 weeks old) were obtained from the Experimental Animal Center of Shanghai (Shanghai, China). The lumber vertebrae of rats were extracted using a scalpel, and then NP was extracted under a microscope. NP was digested with trypsin for 30 min and then with collagenase II for 3 h. NP cells were filtered with a 100-μm filter and centrifuged at 2000 rpm for 5 min. NP cells were cultured in DMEM medium containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. All animal experiments were approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University.

Cell transfection and LPS treatment

MiR-149 mimics were obtained from Genepharma (Shanghai, China). The full-length cDNA of MyD88 was cloned into pcDNA3.1 with a Myc-tag at the C-terminal. Transfection was performed using Liposome 2000 (Invitrogen, USA). LPS (10 μg/ml, Sigma) was used to induce inflammation in NP cells. For co-transfection experiments, NP cells were transfected with miR-149 mimics together with pcDNA 3.1 vector or pcDNA3.1-MyD88 plasmid, and then treated with LPS.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from NP cells with Trizol reagent and transcribed into first-strand cDNA using the PrimeScript RT reagent kit (Takara, China). qRT-PCR was applied to determine mRNA expression by using the SYBR Premix Ex Taq kit (Takara, China). The reaction procedure was as follows: 95°C for 30 s and 40 cycles of 95°C for 30 s, then 60°C for 30 s. GAPDH was utilized as an internal control. For detection of miR-149, reverse transcription and qRT-PCR were performed using the bulge-loop miRNA qPCR primer set (RiboBio, China) according to the manufacturer's instructions, and a human U6 small nuclear RNA was used for normalization. Gene expression was calculated by $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in Supplementary Table 1.

ELISA analysis

Levels of inflammatory cytokines were determined by ELISA kits (R&D Systems, USA) following the manufacturer's instructions.

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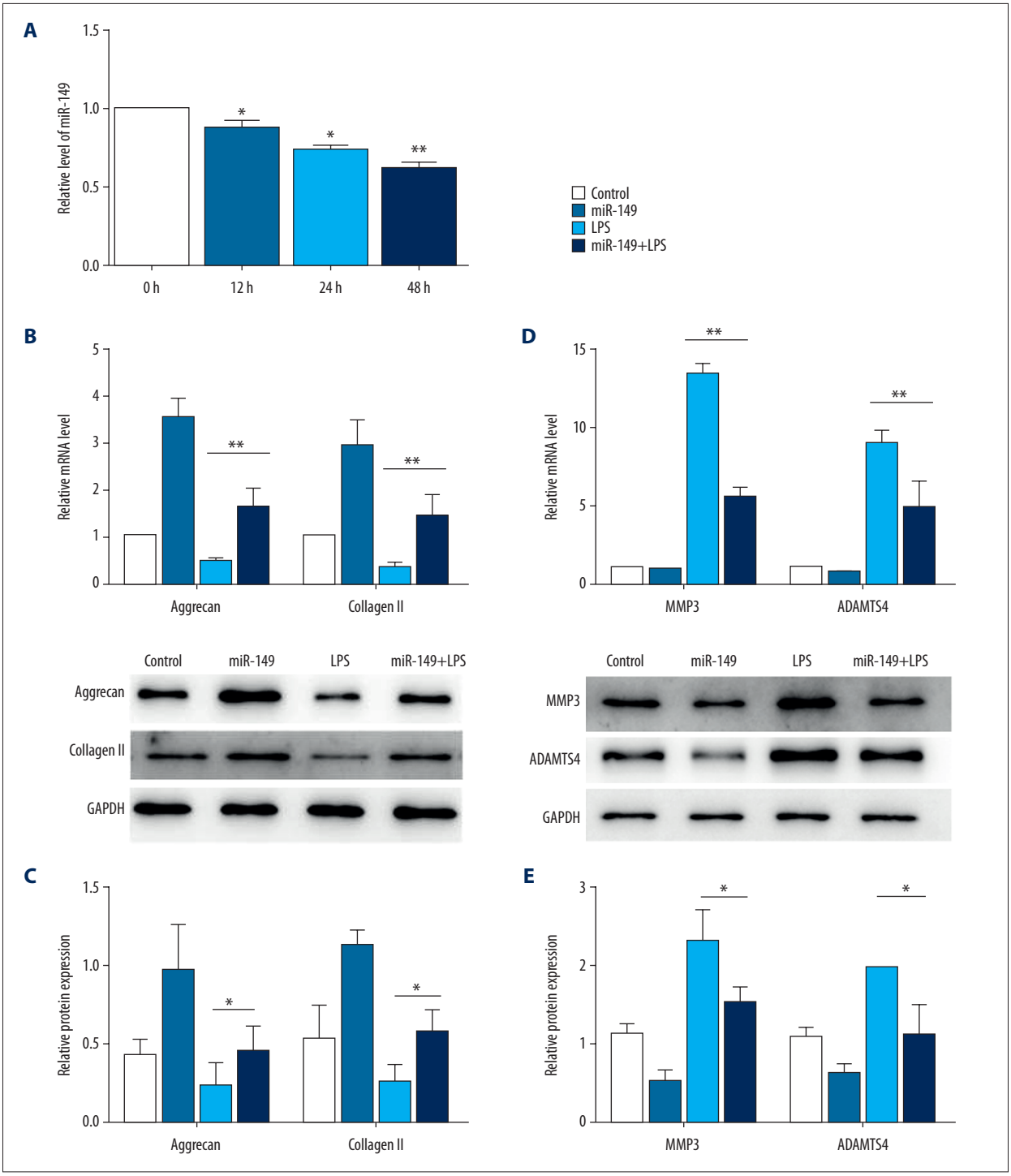

Figure 1. Effects of miR-149 on the expression of ECM-related genes. (**A**) miR-149 expression was detected by qRT-PCR in NP cells after LPS treatment. * P<0.05, ** P<0.01 *vs.* 0 h. (**B, C**) Effects of miR-149 on the mRNA levels (**B**) and protein (**C**) levels of aggrecan and collagen II in LPS-treated NP cells. * P<0.05, ** P<0.01. (**D, E**) Effects of miR-149 on the mRNA levels (**D**) and protein (**E**) levels of MMP3 and ADAMTS4 in LPS-treated NP cells. * P<0.05, ** P<0.01.

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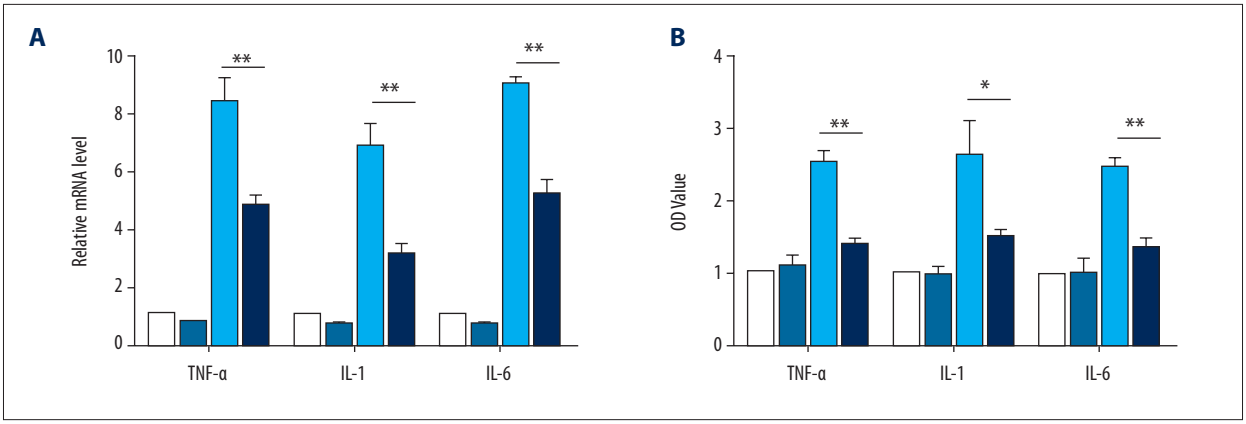

Figure 2. Effects of miR-149 on the production of LPS-induced inflammatory cytokines. (**A**) Effects of miR-149 on the mRNA levels of TNF-α, IL-1, and IL-6 in NP cells after LPS stimulation, as determined by qRT-PCR. ** P<0.01. (**B**) Effects of miR-149 on the production of TNF-α, IL-1, and IL-6 in NP cells after LPS stimulation, as detected by ELISA. * P<0.05, ** P<0.01.

Western blotting

Total protein was isolated from NP cells. Protein concentration was measured using the BCA method. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, USA). After blocking with 5% skim milk at room temperature for 1 h, the membrane was incubated with primary antibody at 4°C overnight. After washing with TBS-Tween, the membrane was incubated with HRP-conjugated secondary antibody for 1 h. Protein bands were determined by use of an enhanced chemiluminescence assay kit (Thermo Scientific, USA). The gray value of bands was analyzed using Image J software.

Luciferase assay

Target genes of miR-149 were predicted using miR-Base (*http://www.mirbase.org/*) and TargetScan Human 7.0 (*http://www.targetscan.org/*) databases. MyD88 3'UTR and 3'UTR mutants that contain miR-149 mutant binding site were amplified by PCR and then subcloned into pMIR-REPORT vector (Applied Biosystems, USA). The firefly luciferase plasmid and Renilla vector (Promega Corp., Madison, Wisconsin, USA) were co-transfected into NP cells together with miR-149 mimics or negative controls. The luciferase activity was examined by use of a dual-luciferase reporter assay system kit (Promega, USA).

Apoptosis detection

The apoptosis of NP cells was examined with a FITC Annexin V apoptosis Detection kit (BD, USA) according the manufacturer's protocol. Briefly, after washing twice with PBS and staining with Annexin V- FITC and propidium iodide, cells were analyzed using a flow cytometer (BD Biosciences, USA).

Statistical analysis

All data are shown as mean ±SD. Analysis was performed using SPSS 20.0 software (USA). Statistical comparisons were performed using the *t* test or one-way ANOVA. A *P* value of <0.05 was considered statistically significant.

Results

miR-149 regulates ECM-related gene expression

First, we detected whether LPS treatment affected the expression of miR-149. qRT-PCR result showed that miR-149 expression was significantly reduced after LPS treatment for 12 h in NP cells, and the expression levels were further decreased after treatment for 24 h and 48 h ($P < 0.05$, Figure 1A). It has been reported that LPS exposure decreased aggrecan and collagen expression [16,17]. Consistent with previous results, we found that LPS treatment significantly reduced aggrecan mRNA expression and collagen II mRNA level in NP cells. Interestingly, transfection of miR-149 restored the mRNA expression of aggrecan and collagen II inhibited by LPS (P<0.01, Figure 1B). Similar to the qRT-PCR result, LPS stimulation decreased the protein levels of aggrecan and collagen II as determined by Western blotting, and overexpression of miR-149 abolished the promotional effects of LPS (P<0.05, Figure 1C). Then, we determined the impacts of miR-149 on the protein levels of ECM degradation-related enzymes MMP3 and ADAMTS4. The results showed that LPS significantly induced the mRNA levels of MMP3 and ADAMTS4, and miR-149 transfection inhibited this induction (P<0.05, Figure 1D). Consistently, miR-149 overexpression significantly suppressed the increased protein levels of MMP3 and ADAMTS4 promoted by LPS (P<0.01, Figure 1E).

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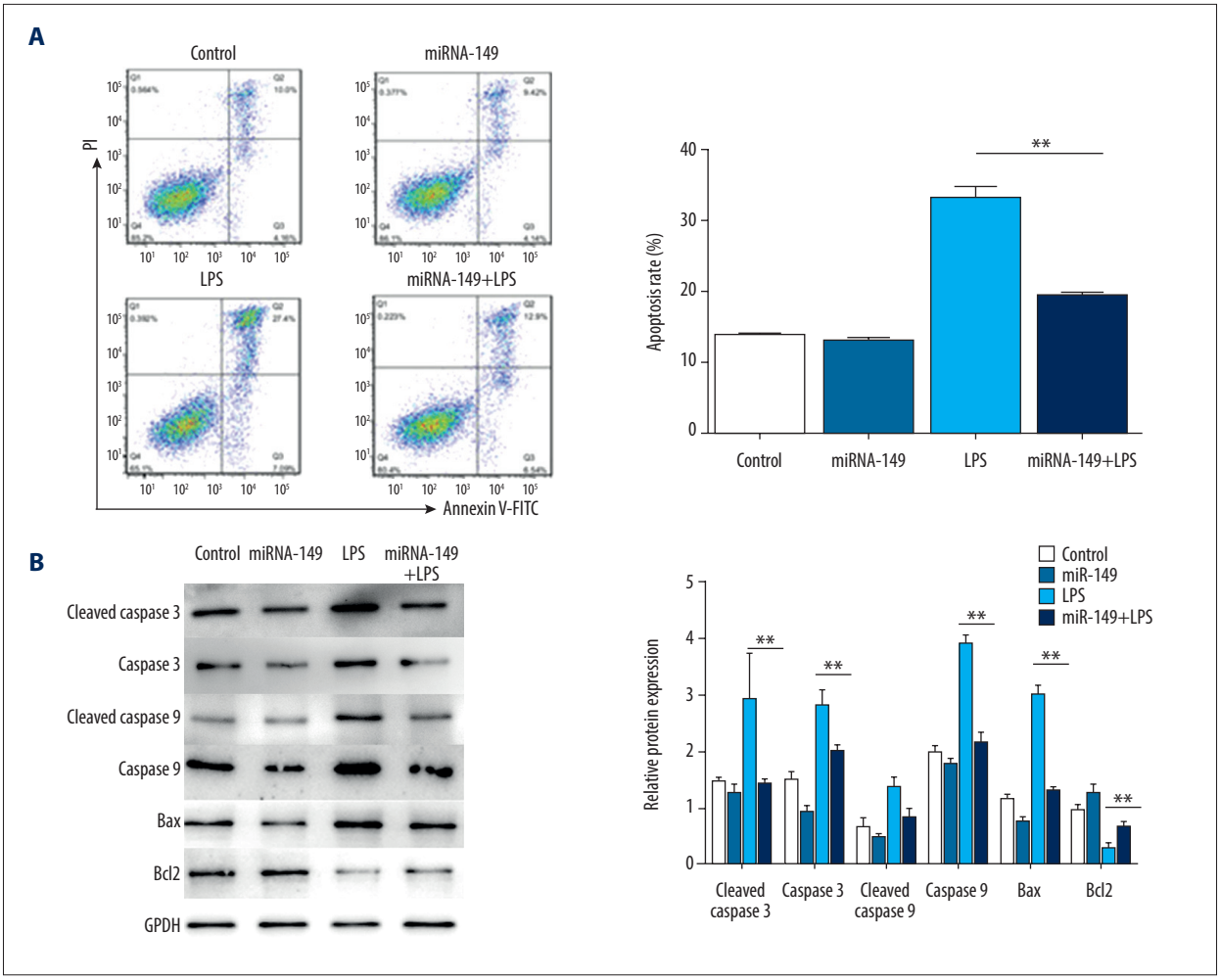

Figure 3. The effect of miR-149 on LPS-triggered apoptosis in NP cells. (**A**) The effect of miR-149 on LPS-promoted apoptosis in NP cells was determined by flow cytometry. ** P<0.01. (**B**) The levels of apoptosis-related proteins were analyzed by Western blotting. * P<0.05, ** P<0.01.

miR-149 inhibits the production of inflammatory cytokines

We then examined the effect of miR-149 on the production of inflammatory cytokines in NP cells treated with LPS. LPS exposure markedly increased the mRNA levels of TNF-α, IL-1, and IL-6 as determined by qRT-PCR, whereas transfection with miR-149 suppressed the promotional effects of LPS on these cytokines (P<0.01, Figure 2A). In agreement with this result, ELISA analysis showed the overexpression of miR-149 restrained the enhanced production of TNF-α, IL-1, and IL-6 stimulated by LPS (P<0.05, Figure 2B).

miR-149 represses LPS-induced apoptosis in NP cells

Given the important role of apoptosis in IDD, we examined the impact of miR-149 on LPS-mediated apoptosis in NP cells. Flow cytometry analysis showed that administration of LPS significantly induced apoptosis in NP cells, and transfection of miR-149 suppressed this induction (P<0.01, Figure 3A). In line with this result, Western blotting analysis showed that LPS treatment markedly enhanced the levels of caspase 3, caspase 8, and Bax, but decreased the level of anti-apoptosis protein Bcl2. However, upregulation of miR-149 suppressed the LPS-mediated enhancement in the levels of caspase 3, caspase 8, and Bax, and restored the level of Bcl2 inhibited by LPS (P<0.05, Figure 3B).

miR-149 regulates MyD88 expression

miRNAs usually function via regulating the transcription or degradation of target genes. Through online target prediction, MyD88, which has been reported to play a vital role in IDD [20], was predicted as a potential target of miR-149. To confirm that MyD88 is a target of miR-149, we constructed reporter vectors consisting of the luciferase coding sequence followed by the WT or mutant 3'UTR of MyD88. Cotransfection experiments

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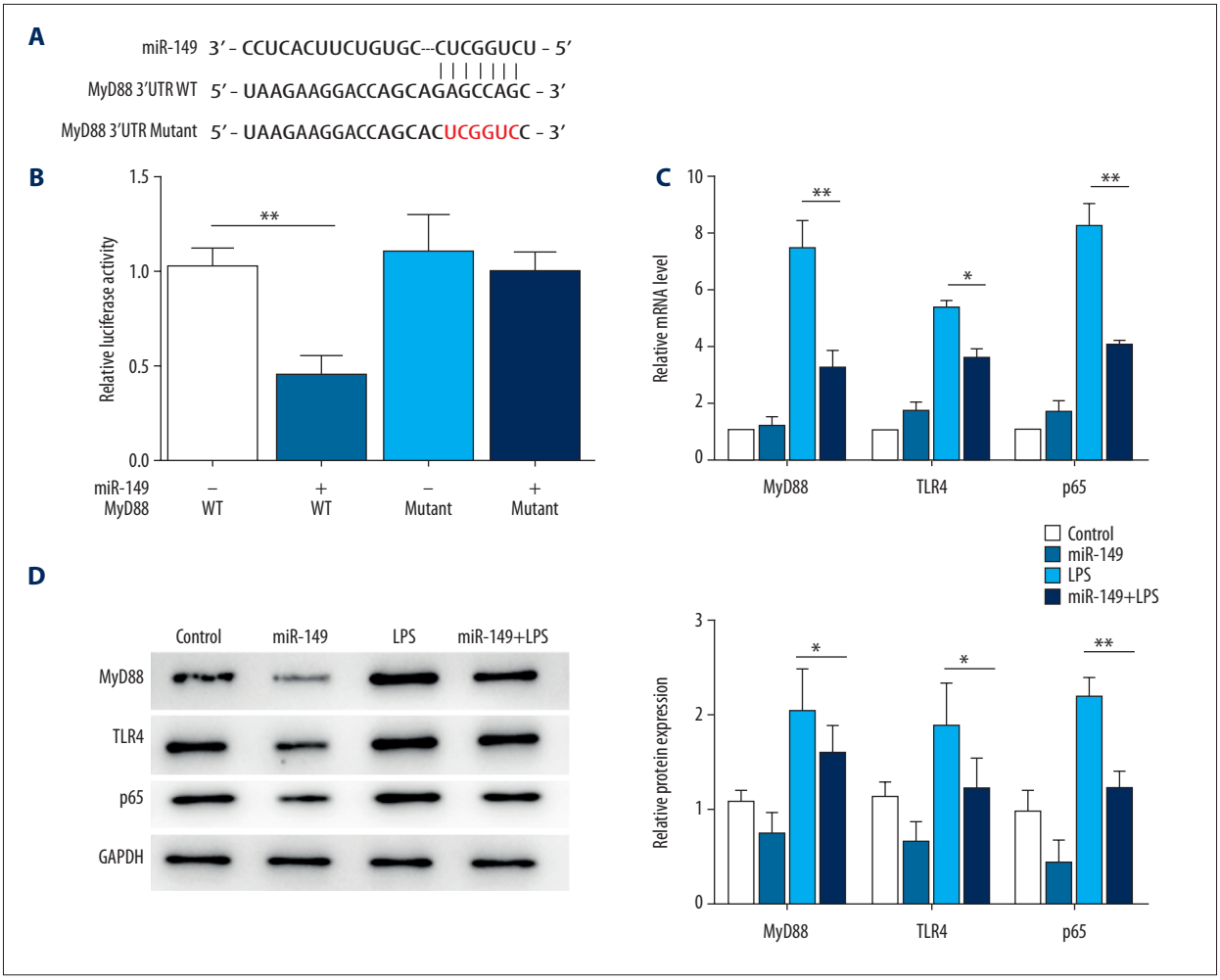

Figure 4. MyD88 is a target of miR-149. (**A**) Sequence of potential binding sites of miR-149 in MyD88 3'-UTR. (**B**) miR-149 repressed the luciferase activity of MyD88 3'UTR, but had no significant effect on the activity of the mutant. ** P<0.01. (**C**) Effects of miR-149 on LPS-mediated mRNA expression of MyD88, TLR4, and p65 in NP cells. * P<0.05, ** P<0.01. (**D**) Effects of miR-149 on LPS-mediated protein expression of MyD88, TLR4, and p65 in NP cells. * P<0.05, ** P<0.01.

showed that miR-149 decreased the luciferase activity of MyD88 3'UTR WT, but had no significant effect on MyD88 3'UTR mutant (Figure 4A, 4B). Moreover, in NP cells, LPS treatment significantly enhanced the expression of MyD88, as well as the levels of its receptor TLR4 and downstream effector p65, and these effects of LPS was inhibited by transfection with miR-149 (P<0.01, Figure 4B, 4C).

miR-149 regulates LPS-induced ECM degradation and inflammation response via targeting MyD88 in NP cells

Next, we investigated whether miR-149 regulates ECM degradation and inflammation response by targeting MyD88. Overexpression of MyD88 further reduced the expression levels of aggrecan and collagen II decreased by LPS, and enhanced the levels of MMP3, ADAMTS4, TNF-α, IL-1, and IL-6 stimulated by LPS treatment (P<0.01, Figure 5A–5E). While miR-149 abolished LPS-induced inhibition in aggrecan and collagen II expression and suppressed LPS-mediated promotion in the levels of MMP3, ADAMTS4, TNF-α, IL-1, IL-6, overexpression of MyD88 reversed these effects of miR-149 (P<0.01, Figure 5A–5E). Together, these results suggest that miR-149 modulates LPS-induced ECM degradation and inflammation response through MyD88 in NP cells.

Discussion

Over 90% of individuals aged over 50 years have common chronic IDD, but the pathological mechanism of IDD remains largely unknown. In recent years, increasing studies suggest that miRNAs are involved in the progression of IDD. Intriguingly, in this study, we identified that miR-149 has an important function in the process of IDD. We showed that miR-149 expression

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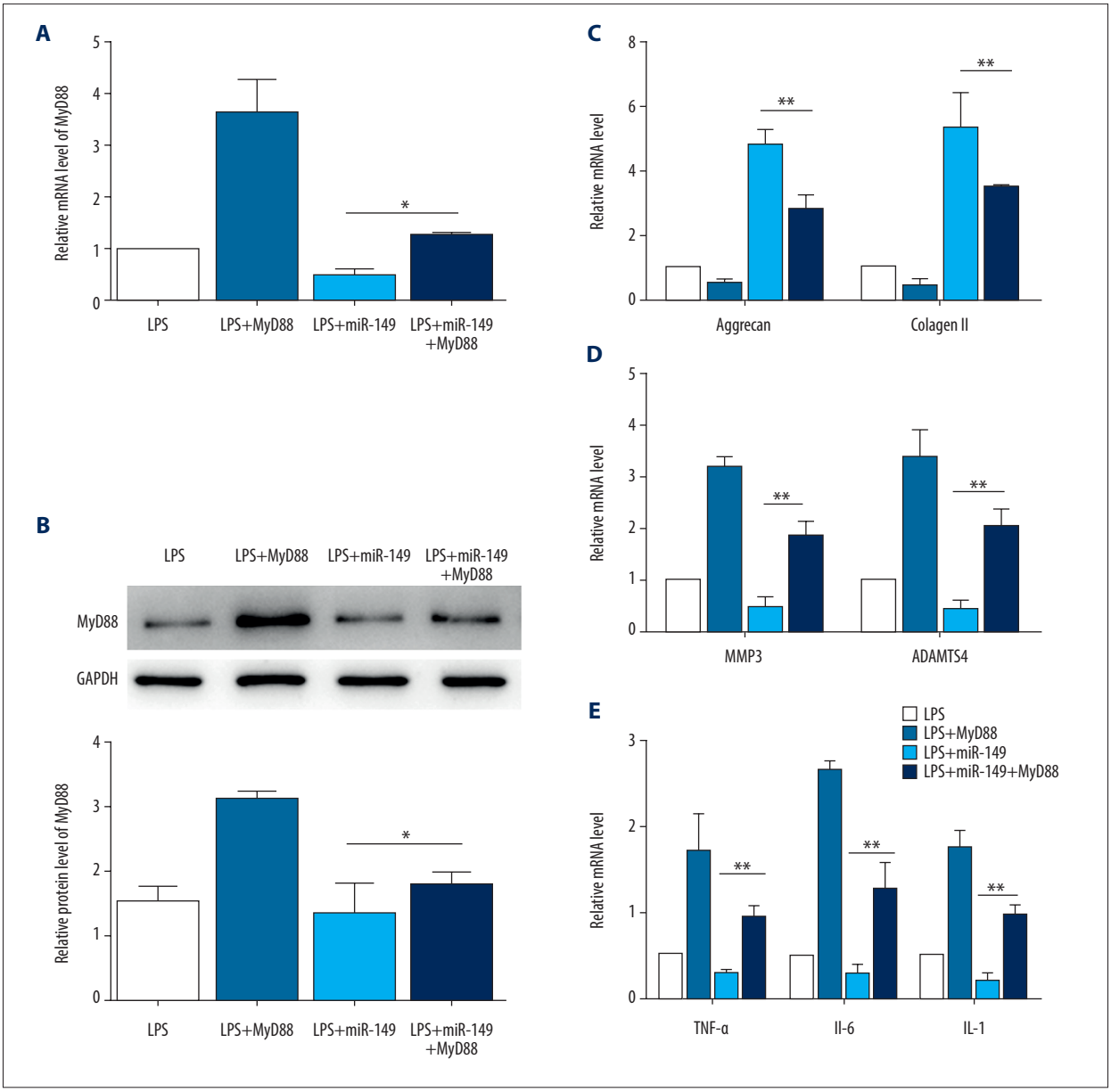

Figure 5. miR-149 inhibits LPS-induced ECM degradation and inflammation response by targeting MyD88. (**A**) The mRNA expression of MyD88 in LPS-stimulated NP cells transfected with miR-149 and/or MyD88. * P<0.05. (**B**) The protein expression of MyD88 in LPS-stimulated NP cells transfected with miR-149 and/or MyD88. * P<0.05. (**C**) MyD88 transfection suppressed the levels of aggrecan and collagen II promoted by miR-149 in LPS-treated NP cells. ** P<0.01. (**D**) MyD88 transfection restored the levels of MMP3 and ADAMTS4 repressed by miR-149 in LPS-treated NP cells. ** P<0.01. (**E**) MyD88 transfection rescued the levels of TNF-α, IL-1, and IL-6 inhibited by miR-149 in LPS-treated NP cells. ** P<0.01.

was decreased in NP cells of intervertebral discs after LPS treatment. Overexpression of miR-149 attenuated LPS-induced ECM degradation, apoptosis, and inflammatory response in NP cells. Our findings imply that miR-149 might be potential target for the treatment of IDD.

Development of IDD involves a multi-step process, in which a major step is degradation of ECM. NP is reported to be important for the maintenance of the biomechanical performance of intervertebral discs, which undergoes ECM changes during IDD [21]. Collagen II and proteoglycan, particularly aggrecan, are the major components of ECM in NP cells and are vital to the normal function of the disc. Loss of collagen II and aggrecan is associated with IDD generation [1]. Here, we found that miR-194 significantly attenuated LPS-induced inhibition in the levels of aggrecan and collagen II. MMPs are primary mediators

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of ECM degradation, and play a critical role in the destruction of the matrix during IDD [22]. Previous studies have shown that MMP3 and MMP7 are responsible for the degradation of collagen and the core protein of aggrecan [23,24]. In addition, ADAMTS4 is an autocrine factor of the nucleus pulposus cells and is crucial for degrading proteoglycans in IDD [25]. Here, we showed that miR-194 markedly decreased LPS-increased MMP3 and ADAMTS4 expression. Together, our data suggest that miR-194 is critical in LPS-induced ECM degradation.

Accumulating studies have demonstrated that inflammatory cytokines play pivotal roles during the development of IDD. It has been reported that inflammatory cytokines exert their function in IDD via modulating the levels of ECM-degrading enzymes [1]. TNF-α and IL-1β are the 2 most important inflammatory factors. Johnson et al. demonstrated that accumulation of TNF-α and IL-1β in intervertebral discs increases the expression of MMPs [26]. Similarly, Le Maitre et al. reported that activation of IL-1β upregulates MMPs and downregulates collagen II and aggrecan in NP cells [3]. Tian et al. have also shown that treatment with TNF-α and IL-1β significantly increases the expression of ADAMTS4 in NP cells [9]. In this study, we found that overexpression of miR-149 suppressed the production of inflammatory cytokines induced by LPS, including TNF-α, IL-1, and IL-6, indicating that miR-149 has a suppressive effect on LPS-mediated inflammation in NP cells.

To further understand the mechanism underlying the function of miR-149, we searched the potential target of miR-149. We defined that MyD88 is a target of miR-149. MyD88 is an adapter protein that links Toll-like receptors and interleukin-1 receptors with downstream signaling molecules (e.g., NF-kB) and plays an important role in immune response and inflammation response [27]. Previous studies have indicated that MyD88-mediated signaling is critical in the pathogenesis of IDD [20,28]. Consistently, we found that the effects of miR-149 on the levels of aggrecan, collagen II, MMP3, ADAMTS4, TNF-α, IL-1, and IL-6 induced by LPS were reversed by overexpression of MyD88, indicating that miR-149 regulates LPS-induced ECM degradation and inflammation response via targeting MyD88.

Conclusions

miR-149 inhibits the TLR4 signal pathway by targeting MyD88, which suppresses LPS-induced inflammatory responses, leading to decreased ECM degradation and NP cell apoptosis. Therefore, miR-149 may be a possible target for IDD therapy development.

Conflict of interest

None.

Supplementary Table

Supplementary Table 1. Primers for qRT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
miR-149	CATCCTTTCTGGCTCCGTGT	GCGTGATTCGTGCTCGTATATC
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
Aggrecan	CAGTGAGTTGGACAGTAGTG	CAGATGTTTCTCCACTGACA
Collagen II	GGCCCTCAGGGACCTGCCGG	CCAGGGGTACCAGGTTCTCC
MMP3	CCTGGACAAGCAGTTCCAAA	TTCACAATCCTGTAGGAGAT
ADAMTS4	GGAGGCGCCCTTAACTCTGC	GGGCTCCCAGAAGGAGCCTT
TNFα	CATCTGCTGGTACCACCAGTT	TGAGCACAGAAAGCATGATC
IL-1	TTGTACAAGGAGAGACAAGC	CAGCTGCAGGGTGGGTGTGC
IL-6	GTGTGA AAGCAGCAAAGAGGC	CTGGAGGTACTCTAGGTATAC
MyD88	GAGATCCGCGAGTTTGAGAC	CTGTTTCTGCTGGTTGCGTA
TLR4	GAGGACTGGGTGAGAAACGA	AGATACACCAACGGCTCTGG
p65	CAAGATCAATGGCAACACGG	CAAGATCAATGGCAACACGG
GAPDH	ATGGGAAGCTGGTCATCAAC	GTGGTTCACACCCATCACAA

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