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Original Paper

MiR-125b Inhibits LPS-Induced Inflammatory Injury via Targeting MIP-1α in Chondrogenic Cell ATDC5

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Key Words
Osteoarthritis • MicroRNA-125b • Inflammatory injury • Macrophage inflammatory protein-1 alpha • NF-κB/JNK signaling pathways

Abstract
Background/Aims: Chondrocyte apoptosis is largely responsible for cartilage degeneration in osteoarthritis (OA). MicroRNAs (miRNAs) play an important role in chondrogenesis and cartilage remodeling. This study explored the effect of miR-125b on inflammatory injury in chondrogenic cells. ***Methods:*** LPS was used to simulate inflammatory injury in murine chondrogenic ATDC5 cell lines. Targeting effect of miR-125b on MIP-1α 3'UTR was assessed by dual luciferase activity assay. Regulatory effect of miR-125b on MIP-1α expression and the potential regulatory mechanism on inflammatory injury were assessed by Western blot. ***Results:*** miR-125b expression was decreased in LPS-induced ATDC5 cells and overexpression of miR-125b inhibited LPS-induced cell viability decline, the rise of apoptosis and inflammatory factors' productions. MIP-1α expression was negatively related to miR-125b, and miR-125b directly targeted with 3'UTR of MIP-1α. Knockdown of miR-125b promoted LPS-induced inflammatory response via upregulation of MIP-1α. miR-125b expression in LPS-induced ATDC5 cells was negatively related with activations of NF-κB and JNK signaling pathways. Overexpression of miR-125b inhibited LPS-induced inflammation injury via suppressing MIP-1α expression and inhibiting activations of NF-κB and JNK signaling pathways. ***Conclusion:*** miR-125b could play an important role in inflammatory injury of chondrogenic cells and miR-125b affected inflammatory injury of ATDC5 cells via regulating expression of MIP-1α and regulating NF-κB and JNK signaling pathways.

Introduction

Osteoarthritis (OA) is one of the most common form of chronical degenerative joint disease which is mainly related to inflammation, autoimmune response, infection, metabolic disorders, trauma, degenerative and other factors. The incidence of OA increases with age,

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becoming to be one of the major problem in the aged persons [1]. The pathological change of OA mainly involves synovium and articular cartilage degradation injury, subchondral bone reactive hyperplasia and etc. due to aging, obesity, trauma, joint congenital anomalies, joint deformities and many other factors. The most significant symptom is the degeneration or destruction of the articular cartilage tissue in which the extracellular matrix (ECM) serves as the primary target [2]. While the articular chondrocytes only comprise 2-3% of total cartilage volume, it could regulate the fate of articular cartilage by synthesizing components of ECM and matrix-degrading proteases [3]. Therefore, chondrocyte becomes the preferred cells in OA researches, and studying the mechanism of inflammatory injury in chondrocytes is of great significance to understanding the pathogenesis and clinical treatment of OA.

MicroRNAs (miRNAs) are a class of small non-coding RNAs (about 17-24 nucleotides), which are proved to be acting as important regulators in gene expression at post-transcriptional level [4]. miRNAs pose an exciting emerging research area because of their involvement in various basic cell functions via imperfect base paring with the 3' untranslated region (UTR) of the target mRNA, leading to the reduced translation and/or degradation [5]. A growing evidence indicates that miRNAs have an important role in the process of chondrogenesis and cartilage remodeling [6, 7]. miRNAs serve as the important regulator in inflammatory response and OA progression [8, 9]. Aberrant expression profiles of miRNAs have been demonstrated to be associated with OA, including miRNA-483 (miR-483) [10], miR-140, miR-455 [11], and etc.

miR-125b has been proved to be involved in multiply of cancers, and the presumed regulation mechanisms of miR-125b in cancer was deciphered [12-14]. Meanwhile, miR-125b was reported to be related with key inflammatory chemokines and cytokine genes in nondiabetic cells [15]. Aberrant expression of miR-125b might contribute to persistent inflammation [16, 17]. miR-125b inhibited cell biological progression of Ewing's sarcoma by suppressing the PI3K/AKT signaling pathway [18]. It is unclear whether miR-125b plays a role in regulation of inflammation response of chondrocyte.

This study was aimed to investigate the role of miR-125b in inflammatory injury of chondrogenic cells by using lipopolysaccharides (LPS)-induced cell damage model *in vitro*. The mechanism researches were also performed to investigate the target gen by which miR-125b regulated functional proteins and affected inflammatory response of chondrogenic cells, thus involved in OA progression.

Materials and Methods

Cell culture and administration

The murine chondrogenic ATDC5 cell line, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Thermo Scientific, Rockford, IL, USA), supplemented with 2 mM Glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 10% (v/v) (for cell growth) or 2% (v/v) (for cell maintaining) fetal bovine serum (FBS, HyClone, Logan, UT, USA), and incubated at 37°C in a humidified CO2 (5%) incubator. Cells with more than 75% confluence were split 1:2 by using 0.25% trypsin (Ameresco, Framingham, MA, USA) for subculture.

For LPS administration, culture medium of stable cultured ATDC5 cells with 80% confluence treated were changed by serum-free medium and cells were cultured for another 24 h in in a humidified atmosphere with 5% CO_2 at 37°C. After that LPS (from *Escherichia coli* 055:B5) purchased from Sigma (St. Louis, Mo, USA) was added into each well with a series of concentrations (0, 1, 5, and 10 μg/mL). Cells were cultured for 5 h, and then harvested and processed for analyses. Cells treated with normal culture medium without LPS administration was served as negative control.

Cell viability assay

Cell viability was analyzed by using Cell counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan). In brief, ATDC5 cells after corresponding administration were seeded in six-well plates at a density of 5×10^3 cells/ well, and been cultured for 24 h. Following incubation, 20 μL of CCK-8 solution

(5 g/L) in phosphate-buffered saline (PBS, Sigma-Aldrich) was added. Cells were incubated for another 3 h at 37°C. The absorbance at 450 nm was measured by the microplate reader (Bio-Rad Laboratories, Tokyo, Japan). Three independent experiments each in triplicate were performed.

Apoptosis assay

To identify and quantify the apoptotic cells after administrations, apoptosis analysis was performed by using Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) followed by flow cytometry. Briefly, after administrations, cells were cultured for 48h, total of 1×10^6 cells were stained by 5 μL Annexin V-FITC from the kit for 20 min at room temperature in the dark. Then, 10 μL PI (5 μg/mL) in 1 × binding buffer was added into each sample and incubated for 15 min in the dark. Subsequently, relative apoptosis of cells was determined by flow cytometry with FCM (FacsCalibur; Becton-Dickinson, Franklin Lakes, NJ, USA) and Cell Quest software (Becton-Dickinson) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from administrated cells was extracted by Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions and been purified by RNeasy Mini kit (Qiagen, Germany). The extracted RNA samples were reversed by TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA), and TaqMan MicroRNA Assay supplemented with the TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) were used for qRT-PCR analysis of miR-125b. For the mRNA measurement, Multiscribe™ Reverse transcription Kit (Applied Biosystems, CA, USA) supplemented with random hexamers or oligo (dT) and Fast START Universal SYBR Green Master (ROX) (Roche, Heiman, Germany) were used for qRT-PCR on the ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). All primers were obtained from the NCBI GeneBank database and were synthesized by GenePharma (Shanghai, China). The reactions were performed in triplicate for each sample at least three independent runs. Data were analyzed according to the classic $2^{-\triangle\triangle Ct}$ method, and normalized to *U6* snRNA or *GAPDH* expression in each sample.

Enzyme-linked immunosorbent assay (ELISA)

After corresponding administration on cells, cell culture supernatant was collected from 24-well plates and content of inflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α were measured by using ELISA kit (R&D Systems, Abingdon, UK) according to the manufacturers' protocols. Three independent experiments each in triplicate were performed. Data was normalized and calculated on the basis of linear calibration curves generated by standard solutions.

Cell transfection

The testing cell samples were seeded into 6-well plate at density of 5×10^4/well and incubated in a humidified atmosphere with 5% CO_2 at 37°C for 24 h to get about 70% confluence. Then cells were respectively transfected with miR-125b mimic, scramble control, miR-125b inhibitor, inhibitor control, siRNA special against macrophage inflammatory protein-1 alpha (MIP-1α) (si-MIP-1α), or negative control, which were all synthesized by GenPharma Co. (Shanghai, China). All the transfection were performed 48h by using Lipofectamine 3000 reagents (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Then transfected cells were incubated in the culture medium containing 0.5 mg/mL G418 (Sigma-Aldrich, St Louis, MO, USA) for 4 to 5 weeks, and stable cultured clones were isolated and selected for the following experiments [19].

Dual luciferins activity assay

The constructed pMiR-luciferase report vector (Promega, Madison, WI, USA) with 3'UTR sequence of MIP-1α carrying the putative miR-125b binding sites was used to assess binding effect between miR-125b and MIP-1α. Cells were co-transfected with the constructed vector and miR-125b mimic by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The empty vector and scramble control were used as negative control, respectively. Reporter analyses were performed by using the dual-luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's information.

Western blot assay

Cell after corresponding administration were treated by PIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitor (Roche, Guangzhou, China) to extract total protein. The protein samples were quantified by using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Western blot system was established by using Bio-Rad Bis-Tris Gel system and polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA) according to the manufacture's instruction. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1000 andincubated with the membranes at 4°C overnight. After that, membranes were washed and incubated with secondary antibodies marked by horseradish peroxidase (HRP) at a dilution of 1: 500 for 1 h at room temperature. After been rinsed, the membranes carried blots and antibodies were transfected into the Bio-Rad ChemiDoc™ XRS system (Bio-Rad, Laboratories, Inc. USA), and then 200 μL Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of each band was quantified by Image Lab™ Software (Bio-Rad, Shanghai, China). The following primary antibodies were used in western blotting: B cell lymphoma 2 (Bcl-2, sc509), Bax (sc20067), pro-Caspase 3 (sc7272), pro-Caspase 9 (sc17784), MIP-1α (sc166942), p65(sc71675), phosphorylated p65 (p-p65, sc136548), IκBα (sc373893), p-IκBα (sc8404), c-Jun N-terminal kinase (JNK, sc7345); p-JNK (sc293136); c-Jun (sc166540); p-c-Jun (sc53182) (Santa Cruz, USA); Cleaved Caspase 3 (ab2302), Cleaved Caspase 9 (ab2324), and GAPDH (ab8245) purchased from Abcam (Abcam, USA).

Statistical analysis

All experiments were repeated three times. All data of independent experiments at least in triplicate were expressed as mean ± standard deviation (SD). Statistical analyses were performed by Graphpad Prism 6.0 statistical software (GraphPad Software Inc., La Jolla, USA). The P-values were calculated by using a two-way analysis of variance (ANOVA) followed by Bonferroni test (for more than two groups), or two-tailed student's *t*-test (within two groups). *P*-value of < 0.05 was considered to indicate a statistically significant result.

Author Contributions

Jinling Jia and Bin Zhao conceived and designed the experiments;, Jingyu Wang, Mingxing Cui and Qingjiang Li performed the experiments; Junlei Zhang and Xiaohui Sun analyzed the data; Bin Zhao contributed reagents/materials/analysis tools; Jinling Jia wrote the paper.

Results

LPS induced inflammatory injury of ATDC5 cells

After LPS administration with different concentrations (1, 5, or 10 μg/mL), cell viability and apoptosis of ATDC5 cells were assessed, respectively. The CCK-8 analysis results in Fig. 1A showed that LPS (5 and 10 μg/mL) significantly decreased cell viability compared with control group without LPS treatment ($P < 0.01$, or $P < 0.001$). Flow cytometry assay results showed that apoptosis of ATDC5 cells were significantly promoted by LPS treatment (1, 5, or 10 μg/mL) ($P < 0.05$, $P < 0.01$, or $P < 0.001$, Fig. 1B). Western blotting results in Fig. 1C also showed that expression of Bcl-2 was decreased in ATDC5 cells after LPS administrations, while expression of Bax, cleaved Caspase 3 and cleaved Caspase 9 were all increased by LPS, suggesting LPS induced cell apoptosis via regulating apoptosis-related factors' expressions. As the decreased cell viability and prompted apoptosis by LPS treatment, it could be inferred that LSP induced cell damage in ATDC5 cells. And 5 μg/mL of LPS was used in the following experiments. To determine the LPS-induced inflammatory reaction in ATDC5 cells, the expressions of inflammatory factors including IL-1β, IL-6, IL-8 and TNF-α were measured by qPCR. Results showed that LPS induced increased expressions of these cytokines in mRNA level ($P < 0.001$, Fig. 1D). And then we detected the expression level of miR-125b in ATDC5 cells after LPS induction by qPCR. Results shown in Fig. 1E showed that miR-125b was significantly decreased in LPS-induced cells compared with control ($P < 0.05$), suggesting downregulation of miR-125b induced by LPS in ATDC5 cells.

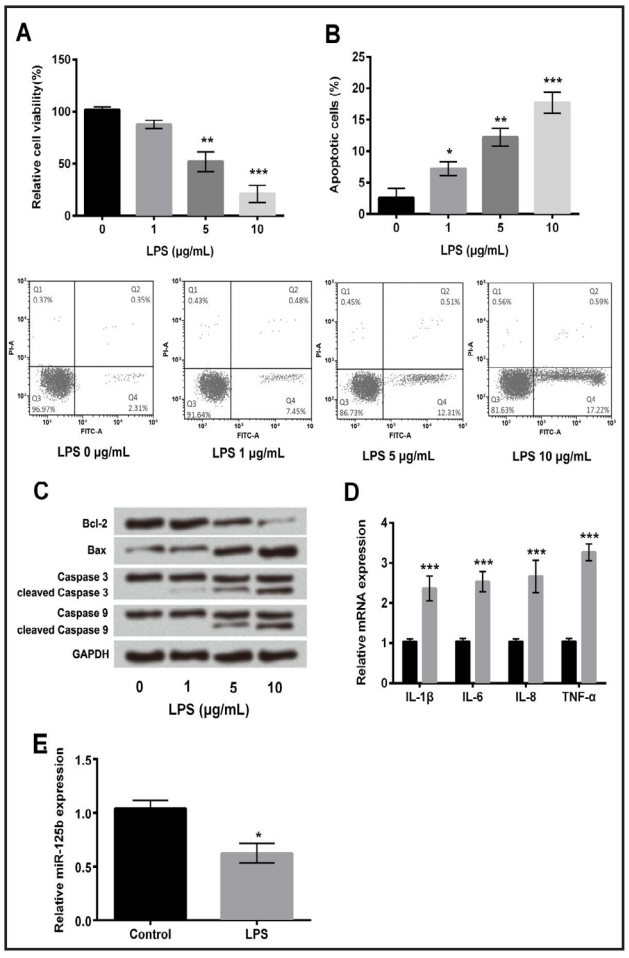

Fig. 1. LPS induced inflammatory injury of ATDC5 cells. The murine chondrogenic ATDC5 cell line was administrated with different contents of LPS (1, 5, 10 μg/mL) to simulate inflammatory lesions. Cells without LPS administration (0 μg/mL) was used as control. (A) CCK-8 was performed to assess cell viability. (B) Relative apoptotic cells was measured by flow cytometry. Viable cells are Annexin V- and PI-, apoptotic cells are Annexin V + and PI-, and necrotic cells are Annexin V- and PI+, as well as Annexin V+ and PI+. (C) The protein immunoblots of apoptotis related factors were measured by western blotting. GAPDH acted as internal control. (D) The mRNA expression levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) were measured by qPCR. (E) Relative miR-125b expression was measured by qPCR. *, P<0.05; **, P<0.01; or ***, P<0.001 compared with control.

According to ELISA analysis, the contents of IL-1β, IL-6, IL-8 and TNF-α in these LPS-induced cells were all increased compared with that in control groups without LPS treatment (P < 0.001, Fig. 2A-D). By combining all above results, it could be deduced that LPS induced inflammatory injury in ATDC5 cells *in vitro*.

Overexpression of miR-125b alleviated LPS-induced inflammatory injury of ATDC5 cells

The effects of miR-125b on LPS-induced inflammatory injury in ATDC5 cells were next studied. miR-125b was aberrantly expressed in ATDC5 cells by transfections with miR-125b mimic or miR-125b inhibitor. The qPCR analysis results showed that miR-125b mimic transfection increased miR-125b expression compared with the scramble control ($P < 0.001$, Fig. 3A), and miR-125b inhibitor transfection significantly decreased expression level of miR-125b in ATDC5 cells compared with inhibitor control group ($P < 0.01$, Fig. 3A), suggesting the transfection effectively affected miR-125b expression in ATDC5 cells

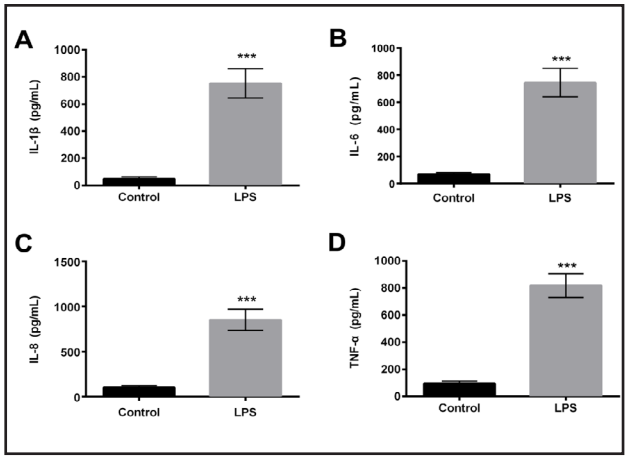

Fig. 2. LPS enhanced expressions of some pro-inflammatory factors in LPS-treated ATDC5 cells. ATDC5 cells were administrated by LPS (5 μg/mL). Cells without LPS administration was used as negative control. ELISA was performed to measure the productions of IL-1β (E) IL-6 (F), IL-8 (G), and TNF-α (H). ***, P<0.001 compared with control.

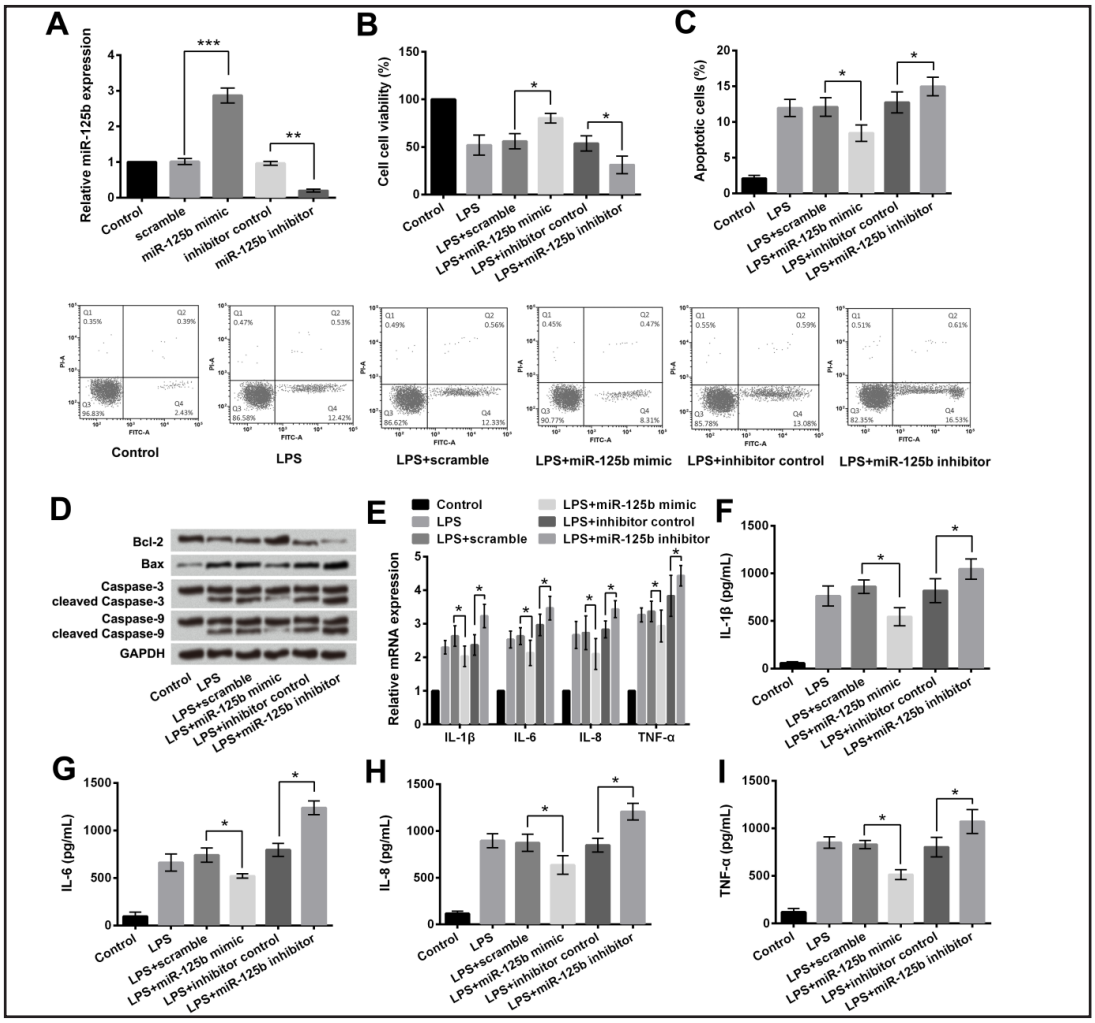

Fig. 3. miR-125b inhibited LPS-induced cell inflammatory injury. (A) Relative miR-125b expression was measured by qPCR after TDC5 cells were transfected with miR-125b mimic, scramble control, miR-125b inhibitor, or inhibitor control, respectively. ATDC5 cells were administrated by LPS (5 μg/mL), and then been transfected with miR-125b mimic, scramble control, miR-125b inhibitor, or inhibitor control, respectively. (B) CCK-8 was performed to assess cell viability. (C) Relative apoptotic cells was measured by flow cytometry. Viable cells are Annexin V- and PI-, apoptotic cells are Annexin V + and PI-, and necrotic cells are Annexin V- and PI+, as well as Annexin V+ and PI+. (D) The protein immunoblots of apoptotic related factors were measured by western blotting. GAPDH acted as internal control. (E) The mRNA expression levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) were measured by qPCR. ELISA was performed to measure the productions of IL-1β (F) IL-6 (G), IL-8 (H), and TNF-α (I). *, P<0.05; **, P<0.01; or ***, P<0.001 compared with control.compared with corresponding negative control.

in vitro. ATDC5 cells were administrated with LPS and then transfected with miR-125b mimic or inhibitor to investigate the effect of miR-125b on LPS-induced inflammatory injury. Cell viability assay results showed that after LPS administration, miR-125b mimic transfection increased cell viability which was decreased by LPS administration, while it was further decreased by miR-125b inhibitor compared with that decreased by LPS in inhibitor control group ($P < 0.05$, Fig. 3B). Meanwhile, relative apoptotic cells were significantly decreased in miR-125b mimic transfection group, and increased in miR-125b inhibitor transfection group compared with scramble/inhibitor control ($P < 0.05$, Fig. 3C). Western blot assay results in Fig. 3D showed that miR-125b mimic transfection increased expression of Bcl-2, while miR-125b inhibitor further decreased Bcl-2 expression compared with LPS-induced decreasing

expression. Meanwhile, expressions of Bax, cleaved Caspase 3 and cleaved Caspase 9 were all affected by miR-125b transfection. Interestingly, expressions of them in miR-125b mimic transfection group were decreased compared with LPS administration, suggesting the inhibitory effect of miR-125b on LPS-induced cell apoptosis..

The mRNA expression levels of inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α were all decreased after miR-125b mimic transfection compared with LPS-induced scramble control group, while they were increased in ATDC5 cells after miR-125b inhibitor transfection ($P < 0.05$, Fig. 3E). The increased contents of these cytokines in LPS-induced ATDC5 cells were decreased after miR-125b mimic transfection, and further more increased by miR-125b inhibitor transfection ($P < 0.05$ compared with corresponding control, Fig. 3F-3I), suggesting inhibiting effect of miR-125b on LPS-induced inflammatory response in ATDC5 cells.

miR-125b negatively regulated MIP-1α expression

MIP-1α is a chemokine that has pro-inflammatory and stem cell inhibitory activities [20]. In the present study, qPCR and western blot assay results showed that expression of MIP-1α in both mRNA level (Fig. 4A) and protein level (Fig. 4B) were decreased in miR-125b mimic-transfected cells compared with that in scramble control ($P < 0.05$ in Fig. 4A), while it was increased in miR-125b inhibitor-transfected cells relative to inhibitor control ($P < 0.05$ in Fig. 4A), suggesting that miR-125b was negatively related with MIP-1α expression. Dual luciferase activity assay was performed to assess binding effect of miR-125b on 3'UTR of MIP-1α. Results in Fig. 4C showed that miR-125b mimic transfection significantly decreased luciferase activity of MIP-1α 3'UTR group ($P < 0.05$), suggesting binding effect of miR-125b on 3'UTR of MIP-1α. These results suggested that miR-125b could negatively regulated expression of MIP-1α inATDC5 cells via targeting MIP-1α.

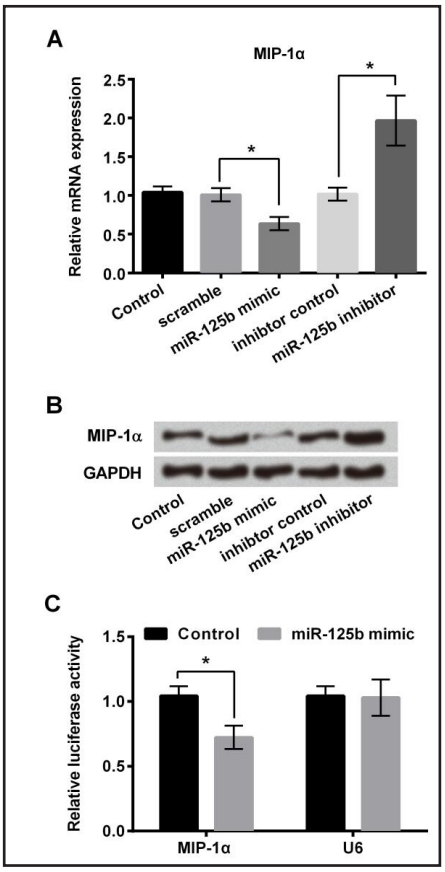

Fig. 4. miR-125b negatively regulated expression of MIP-1α and MIP-1α was a directly target of miR-125b. Murine chondrogenic cell line ATDC5 cells were transfected with miR-125b mimic, scramble control, miR-125b inhibitor, or inhibitor control, respectively. (A) The mRNA expression level of MIP-1α was measured by qPCR. (B) Protein expression of MIP-1α was measured by western blotting. GAPDH acted as internal control. (C) Cells were co-transfected with miR-125b mimic and MIP-1α 3'UTR recombinant vector. The U6 recombinant vector was used as positive control. Targeting effect of miR-125b on the 3'UTR of MIP-1α was measured by dual-luciferase activity assay. *, P<0.05 compared with corresponding negative control.

Knockdown of miR-125b promoted LPS-induced inflammatory injury via upregulating MIP-1α

ATDC5 cells were administrated with LPS, and then been transfected with miR-125b inhibitor, or miR-125b inhibitor+si-MIP-1α to investigate role of MIP-1α in inflammatory injury-regulatory effect of miR-125b. Cell viability assay results in Fig. 5A showed that miR-125b inhibitor decreased cell viability of ATDC5 cells, co-transfection with si-MIP-1α significantly increased cell viability compared with miR-125b inhibitor transfection alone ($P < 0.05$). The increased number of relative apoptotic cells in miR-125b inhibitor transfection group was significantly decreased by si-MIP-1α co-transfection ($P < 0.05$, Fig. 5B). Western blot assay results in Fig. 5C also showed

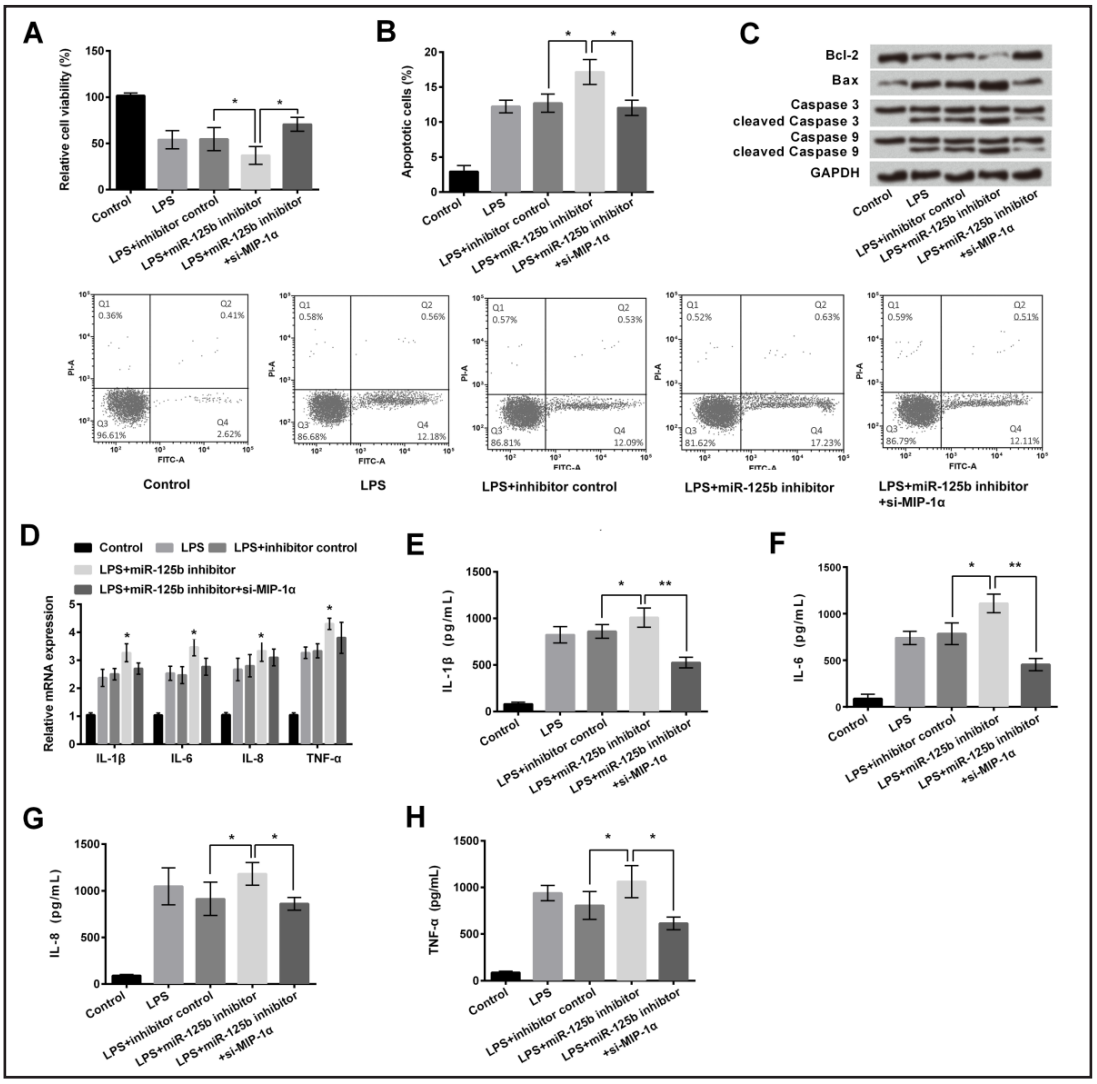

Fig. 5. miR-125b regulated ATDC5 cell inflammatory injury via MIP-1α. ATDC5 cells were administrated by LPS (5 μg/mL), and then been transfected with inhibitor control, miR-125b inhibitor, or/and specific siRNA for MIP-1α (si-MIP-1α), respectively. (A) CCK-8 was performed to assess cell viability. (B) Relative apoptotic cells was measured by flow cytometry. Viable cells are Annexin V- and PI-, apoptotic cells are Annexin V + and PI-, and necrotic cells are Annexin V- and PI+, as well as Annexin V+ and PI+. (C) The protein immunoblots of apoptotic related factors were measured by western blotting. GAPDH acted as internal control. (D) The mRNA expression levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) were measured by qPCR. ELISA was performed to measure the productions of IL-1β (E) IL-6 (F), IL-8 (G), and TNF-α (H). *, P<0.05; or **, P<0.01 compared with corresponding negative control.

the decreased expression of Bcl-2 and increased expressions of Bax, cleaved Caspase 3, and cleaved Caspase 9 in miR-125b inhibitor-transfected cells, while these effects were all inhibited after si-MIP-1α co-transfection, suggesting the pro-apoptotic effect of miR-125b inhibition on ATDC5 cells might be via upregulating of MIP-1α.

Additionally, analysis about inflammatory cytokines showed that miR-125b inhibitor transfection increased mRNA expressions (Fig. 5D) and protein contents (Fig. 5E-5H) of IL-1β, IL-6, IL-8 and TNF-α, while co-transfected with si-MIP-1α decreased their expressions and contents in ATDC5 cells compared with miR-125b inhibitor alone transfection ($P < 0.05$). All the above results suggested that miR-125b knockdown might aggravate LPS-induced inflammation in ATDC5 cells via upregulating MIP-1α.

miR-125b was negatively related to the activations of NF-κB and JNK signaling pathways via regulating MIP-1α expression

Western blot assay results showed that expression of MIP-1α, as well as phosphorylation levels of p65 and IκBα in NF-κB pathway (Fig. 6A), and phosphorylation levels of JNK and c-Jun in JNK signaling pathway (Fig. 6B) were all decreased in miR-125b mimic-transfected cells compared with those in LPS-treated cells. Meanwhile, in miR-125b inhibitor-transfected ATDC5 cells, all these proteins expression were increased compared with those in LPS-treated groups. These results suggested that expression of miR-125b might be negatively related with activation of NF-κB and JNK signaling pathways, which might be related with regulating effect on MIP-1α expression.

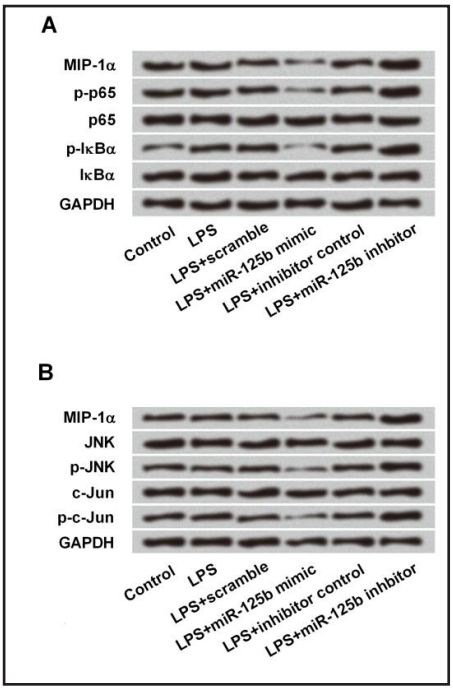

Fig. 6. miR-125b was negatively related with activations of NF-κB and JNK signaling pathways via regulating MIP-1α expression. ATDC5 cells were administrated by LPS (5 μg/mL), and then been transfected with miR-125b mimic, scramble control, miR-125b inhibitor, or inhibitor control, respectively. Expression of MIP-1α, phosphorylated (p-) p65, p65, IκBα, p-IκBα (A); p-JNK, JNK, c-Jun, p-c-Jun (B) were measured by western blotting. GAPDH acted as internal control.

Discussion

The important pathological feature of OA is articular cartilage degeneration. Chondrocyte is the only cell type in mature cartilaginous, and the decreased cell structure is the ultimate reason for matrix degradation and OA occurrence [21]. Researches have shown that apoptosis of chondrocytes was largely responsible for the cartilage degeneration in OA [22]. Therefore, Studies about the basically mechanisms of chondrocytes in gene and cytological level contributed to the understanding and therapy treatment of OA. In the present study, we used LPS to induce the inflammatory injury of chondrogenic cell ATDC5 and investigated the effect of miR-125b on LPS-induced ATDC5 cells. And we found that LPS administration effectively suppressed cell viability and promoted apoptosis of ATDC5 cells *in vitro*, as well as increased the productions of inflammatory factors, including IL-1β, IL-6, IL-8, and TNF-α that were widely used as the chondrocyte apoptosis-inducing agent [23]. The *E.coli*-derived LPS has been known to be able to stimulate the production of interleukins and tumor necrosis factors in host cells, activate intracellular signaling pathway, and also induce the productions and releases of numerous pro-inflammatory mediators, leading to multiple organ damage [24]. The present study results showed that LPS was effective in inducing ATDC5 cell inflammatory injury *in vitro* experiment.

We also found that expression of miR-125b in LPS-induced ATDC5 cells was downregulated, suggesting that miR-125b might be involved in the inflammatory responses. Results also showed that aberrant expression of miR-125b in LPS-induced ATDC5 cells affected cell viability and apoptosis, as well as the production of inflammatory cytokines, suggesting that miR-125b upregulation might reduce the chondrocytes inflammation injury. The mechanisms by which miRNAs are regulated and expressed under normal or disease conditions are currently of great interest. Although miR-125b has been implicated in various cancers [12, 25-27], and might contribute to persistent inflammation [28, 29], this is the first indication about the role of miR-125b in chondrocytes inflammatory response.

MiRNAs modulate the expression of multiple target genes at the post-transcriptional level and affect protein-encoding genes to regulate cell bioactivity. Following the determination of inhibiting effect of miR-125b on LPS-induced inflammatory response, the mechanism studies were performed to investigate target gene and the regulation mechanism of miR-125b in chondrocytes. MIP-1α is a chemokine which has pro-inflammatory and stem cell-inhibitory activities, and it is an important mediator of virus-induced inflammation *in vivo* [30]. In the present study, we found that expression of MIP-1α was negatively regulated by miR-125b, and acted as a directly target of miR-125b in ATDC5 cells. Additionally, *in vitro* experiments also demonstrated that miR-125b affected inflammatory injury of ATDC5 cells via regulating expression of MIP-1α, suggesting that the inhibiting effect of miR-125b on chondrocytes inflammation response might be closely related with negatively regulatory effect on MIP-1α.

The transcription factor NF-κB is a key regulator of inflammation. The activated NF-κB was detected in OA synovial tissue, which contributed to the initiation and maintenance of chronic inflammation [31]. In addition, it has been proved that NF-κB activation promoted synovial hyperplasia via promoting cell proliferation and inhibiting c-myc–induced apoptosis [32]. The c-Jun NH2-terminal Kinase (JNK) pathway represents one sub-group of the mitogen-activated protein (MAP) kinases. There was significant progress towards about function of JNK signaling pathway during the past few years, and it was proved that JNK pathway play an important role in various inflammatory diseases [33]. A recent study about IL-7-inflicted neuroinflammation showed that expression of MIP-1α was significantly inhibited by suppression of IL-17-mediated ERK, p38, and JNK MAPKs and PI3K/AKT pathway activations [34]. In the present study, we found that miR-125b expression was negatively related with activations of NF-κB and JNK signaling pathways which was also negatively regulated MIP-1α expression. Therefore, we deduced that miR-125b overexpression might alleviate inflammatory injury in chondrocytes via negative regulation of MIP-1αthrough NF-κB and JNK signaling pathways.

In summary, these results might provide new insights into the cross-talk and relationships between miR-125b and protein-coding genes leading to inflammatory gene expression in chondrocytes, especially in an inflammatory disease relevant model. It must be admitted that more *in vivo* or animal researches will be helpful to understand the pathogenesis of inflammation response of chondrocytes and uncover more miRNAs as potential biomarkers which contribute to clinical treatment of OA.

Disclosure Statement

No conflict of interests exists.

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