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Original Article

MiR-363 downregulates in CD4$^+$ T cells from arthritis

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Abstract: CD4$^+$ T cells participate in regulating immune response and inflammation. MiRNA is a type of small non-coding RNA that can regulate gene expression at posttranscriptional level. This study investigated miR-363 expression and the role in CD4$^+$ T cells from arthritis. CD4$^+$ T cells were isolated from the synovia of 26 arthritis patients and healthy control using immunomagnetic beads to test miR-363 expression. MiR-363 target gene was predicted by bioinformatics and confirmed by luciferase reporter gene assay. PDPN level in CD4$^+$ T cells were detected by Western blot. MiR-363 or inhibitor was transfected to change miR-363 expression in Jurkat cells. Cell cycle was evaluated by flow cytometry. PDPN and inflammatory cytokines expressions were tested by Western blot. MiR-363 expression downregulated in CD4$^+$ T cells from arthritis patients compared with healthy control ($P < 0.05$). PDPN was jianhgleconfirmed as the target gene of miR-363 by bioinformatics and luciferase reporter gene assay. MiR-363 downregulation in CD4$^+$ T cells obviously shortened cell cycle, enhanced cell proliferation, promoted PDPN expression, and facilitated inflammatory cytokines release including TNF-α and MMP-3 ($P < 0.05$). MiR-363 expression reduced in CD4$^+$ T cells from synovia of arthritis patients, thus to enhance CD4$^+$ T cells proliferation, elevate PDPN expression, and promote inflammatory cytokines release.

Keywords: Arthritis, CD4$^+$ T cells, miR-363, PDPN, inflammatory cytokine

Introduction

Arthritis refers to a chronic inflammatory disease occurred in the joint and its surrounding tissues. It has a variety of classifications, while osteoarthritis and rheumatoid arthritis are the most common types [1]. There are about 100 million arthritis patients in our country with a rising trend [2]. Osteoarthritis causes degenerative pathological changes and affects normal joint structure, leading to joint deformities and even disability [1]. The pathogenesis of arthritis is complex, which is related to inflammation, immune reaction, infection, and metabolic disorder [3]. T helper cell (Th) plays an important role in regulating immune response and inflammation. Th cells can be divided into four subgroups as Th0, Th1, Th2, and Th3 based on the differences of cytokines secretion [4]. Since all of Th cells express CD4, CD4$^+$ T cell belongs to the Th cells [5]. Abnormal activation of CD4$^+$ T cells is thought to be associated with many diseases. It was found that CD4$^+$ T cell response obviously exists in the synovial fluid of arthritis patients, thus speculating that CD4$^+$ T cells are closely related to arthritis [5].

MiRNAs is a kind of small non-coding RNA at the length of 21-25 bp with highly conservative structure [6]. It was showed that miRNAs play a critical role in cell division and metabolism through regulating multiple gene expressions upon the mechanism of RNA interference [7]. It was demonstrated that a variety of miRNAs have a key role in the process of inflammation, including miR-21, miR-155, and miR-181 [8-10]. Other studies found that miR-363 abnormally expressed in CD4$^+$ T cells isolated from synovial tissue of patients with rheumatoid arthritis, thus speculating that miR-363 may participate in the process of CD4$^+$ T cell mediated inflammatory response [11] However, there is still lack of reports about the expression and function of miR-363 in CD4$^+$ T cells from arthritis patients. This study intended to explore miR-363 expression in CD4$^+$ T cells from arthritis and analyze its function.

Materials and methods

Object of study

A total of 26 osteoarthritis patients between Jun 2014 and Dec 2015 in Hong-Hui Hospital,

Table 1. Primers sequences

Name	Sequence
MiR-363-F	5' CATAGTTGCACTACAAGAAG 3'
MiR-363-R	5' GCACAACTACATTCTTCTTG 3'

Xi'an Jiaotong University college of Medicine were enrolled. There were 10 males and 16 females with mean age at 56.3 ± 10.2 (46-72) years old. All the patients were diagnosed based on synovial fluid examination and X-ray inspection. No other complications were found among the patients. No patients received anti-inflammation or immune response inhibition treatment before enrollment. Another 15 healthy volunteers at the same time were selected as normal control. There were 6 males and 6 females with average age at 55.6 ± 8.3 (41-73) years old. This study was approved by the ethics committee and all the objects had signed informed consent.

$CD4^+$ T cell isolation

Knee joint synovial fluid was extracted through surgery in experimental group and control. $CD4^+$ T cells were isolated using immunomagnetic beads. The synovial joint fluid was diluted by equal volume of normal saline, and then added to the surface of Ficoll. After centrifuged at 2000 rpm for 20 min, the monolayer cells were obtained and washed by PBS for $CD4^+$ T cells isolation.

Mononuclear cells were resuspended in cell sorting buffer and then incubated in biotin labeled complex antibody, including CD8, CD14, CD16, CD19, CD36, CD56, TCRγ/δ, and glycophorin A (Proteintech, Wuhan, China) at 4°C for 30 min. Next, the mixture was incubated in biotin antibody coupled immune magnetic beads (Miltenyi Biotec, Germany) at 4°C for 30 min, and then washed by cell sorting buffer. After centrifuged at 1000 rpm for 10 min, the cells were resuspended in 500 μl sorting buffer. MidiMACS classifier (Miltenyi Biotec, Germany) was added with LD sorting column and washed by 2 ml sorting buffer. The cells were separated through the column and added with FITC labeled $CD4^+$ T antibody for immune response. $CD4^+$ T cells were isolated through flow cytometry [12].

qRT-PCR

Sorted $CD4^+$ T cells were used to extract total RNA upon RNAprep pure Tissue Kit (QIAGEN, Germany). qRT-PCR was applied to test miR-363 expression in $CD4^+$ T cells. The primers of miR-363 were designed based on its sequence (GeneBank: NR_029489) and listed in **Table 1**. qRT-PCR was performed using mirVanat qRT-PCR miRNA detection kit. U6-RNA was selected as internal reference. The data was analyzed by $2^{-\Delta\Delta Ct}$ method.

MiR-363 target prediction

TargetScan Release 5.1 (www.targetscan.org) was used to predict the target gene of miR-363. Luciferase reporter gene assay was applied to test the potential target of miR-363. The primer sequences of 3'UTR of PDPN (GeneBank: NM_006474) were as follows: 5'-AAAAAGCACGTATCGGCGAGGATGATCTCTATC-3' and 5'-AGAAAGCTGG- ACCAACACAGGTGTGACCAAGAA-3'. The 3'UTR of PDPN mRNA was amplified by PCR and inserted to the downstream of firefly luciferase gene coding region in pmirGLO to construct pmirGLO-PDPN vector. pmirGLO-PDPN vector and pmirGLO vector were transfected to HEK293 cells, respectively. Then the cells were further transfected with miR-363 mimics to enhance miR-363 activity using INTERFERin™ transfection kit (Polyplus transfection, France). HEK293 cell line was purchased from the cell bank, Chinese academy of sciences. The cells were resuscitated and cultured to logarithmic phase. After digested by trypsin, the cells were seeded in 96-well plate for transfection. Fluorescence intensity was analyzed at 48 h after transfection. Dual luciferase reporter gene analysis system (Promega, USA) and MicroLumatPlus LB96V photometer (Berthold, Germany) were used for luciferase intensity analysis [13].

Cell transfection

To investigate the impact of miR-363 expression on $CD4^+$ T cells, miR-363 mimics and inhibitor were designed based on its sequence. They were transfected to human leukemia T cell line Jurkat cells. Jurkat cells were purchased from Bioleaf (Shanghai, China).

Flow cytometry

Flow cytometry was applied to detect $CD4^+$ T cell cycle after miR-363 mimics or miR-363 inhibitor transfection. The cells were washed by PBS and fixed in 90% ethanol at 4°C overnight. After treated by RNase at 37°C for 30 min, the cells were stained by PI and tested on flow

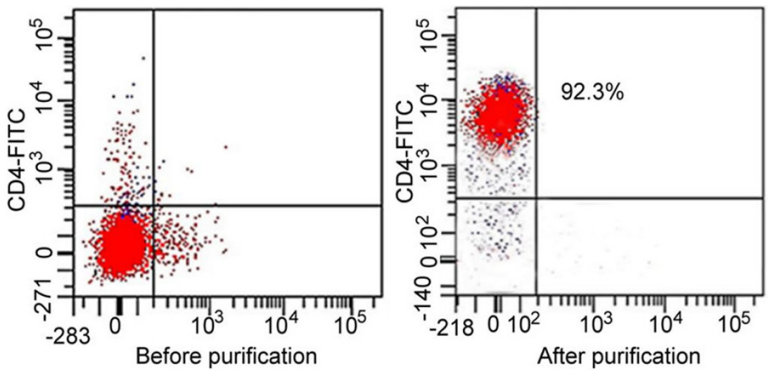

Figure 1. CD4+ T cells obtained from synovial fluid in arthritis patients using magnetic activated cell sorting method.

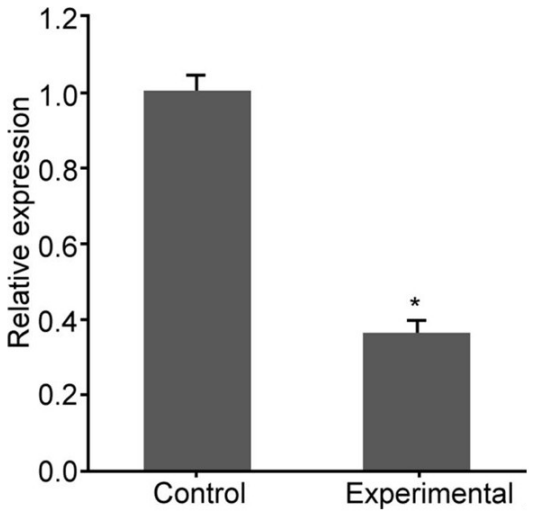

Figure 2. MiR-363 expression in CD4+ T cells from arthritis. *P < 0.05, compared with control.

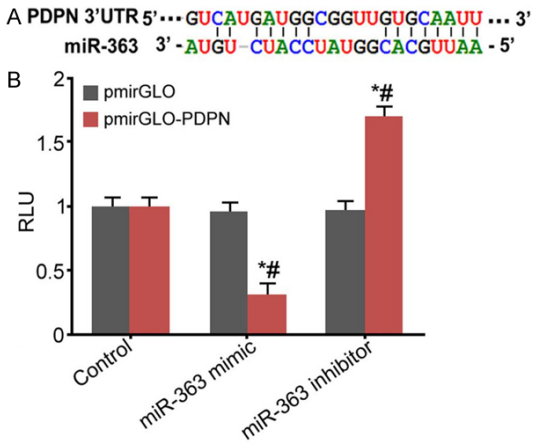

Figure 3. The relationship between miR-363 and PDPN gene. A: The homology between miR-363 and 3'UTR of PDPN. B: The impact of miR-363 on PDPN gene expression.

cytometry (Becton Dickinson, USA). The excitation wavelength was 488 nm, while the emission wavelength was 630 nm. 10,000 fluorescence signals were collected in each sample. FL-2 area and DNA histogram were analyzed using Modifit software. Each test was repeated for three times.

Western blot

Total protein was extracted from CD4+ T cells for Western blot. The primary antibody used included rabbit anti human PDPN, IL-6, MMP-3, and β-actin, whereas the secondary antibody contained HRP labeled mouse anti rabbit IgG (1:1000) (Proteintech, Wuhan, China). The membrane was developed in DAB for 10 min after washed by TBST. The image was analyzed by gel document system to calculate the relative expression [14].

Statistical analysis

SPSS 20.0 software was applied for data analysis. The data was presented as mean ± standard deviation and analyzed by One-Way ANOVA or t test. $P < 0.05$ was depicted as statistical significance.

Results

The yield and purification rate of isolated CD4+ T cells

CD4+ T cells were isolated from synovial fluid in arthritis patients using magnetic activated cell sorting method. The number of mononuclear cells before sorting was 8.6×10^7, while the obtained CD4+ T cells were 1.6×10^7. The CD4+ T cell yield rate was 18.6%, and the purification rate was 92.3% (**Figure 1**).

MiR-363 expression in CD4+ T cells from arthritis

Total RNA was extracted from CD4+ T cells. qRT-PCR was used to detect miR-363 expression in CD4+ T cells. MiR-363 expression in CD4+ T cells from arthritis patients was significantly lower than the healthy control ($P < 0.05$) (**Figure 2**).

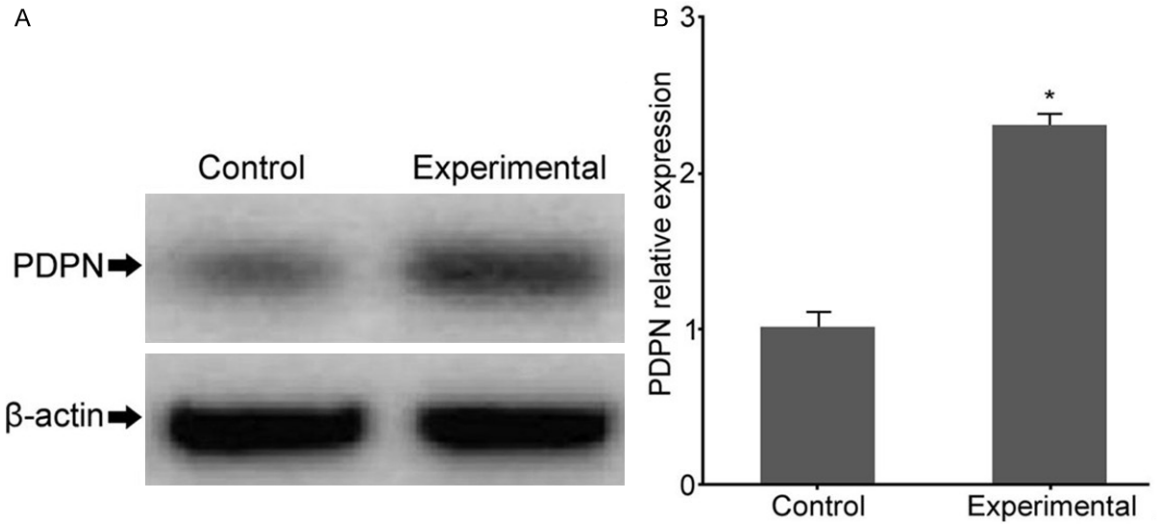

Figure 4. PDPN expression in CD4+ T cells. A: Western blot detection of PDPN. B: PDPN relative expression. *P < 0.05, compared with control.

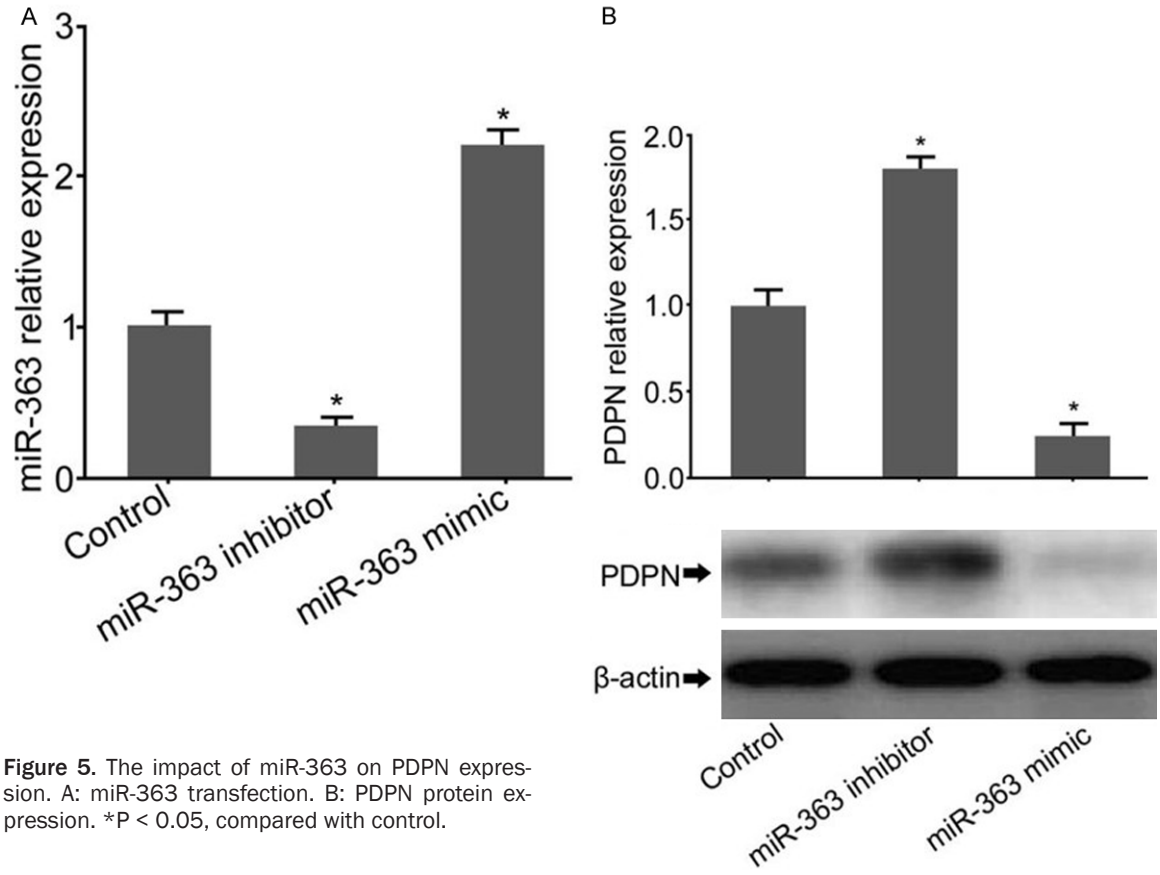

Figure 5. The impact of miR-363 on PDPN expression. A: miR-363 transfection. B: PDPN protein expression. *P < 0.05, compared with control.

The relationship between miR-363 and PDPN gene

TargetScan Release 5.1 predicted that miR-363 showed homology with 3'UTR of PDPN (**Figure 3A**), speculating that PDPN might be a target gene of miR-363. Dual luciferase reporter gene assay revealed that cell fluorescent intensity obviously declined after miR-363 mimic transfection, whereas it enhanced after

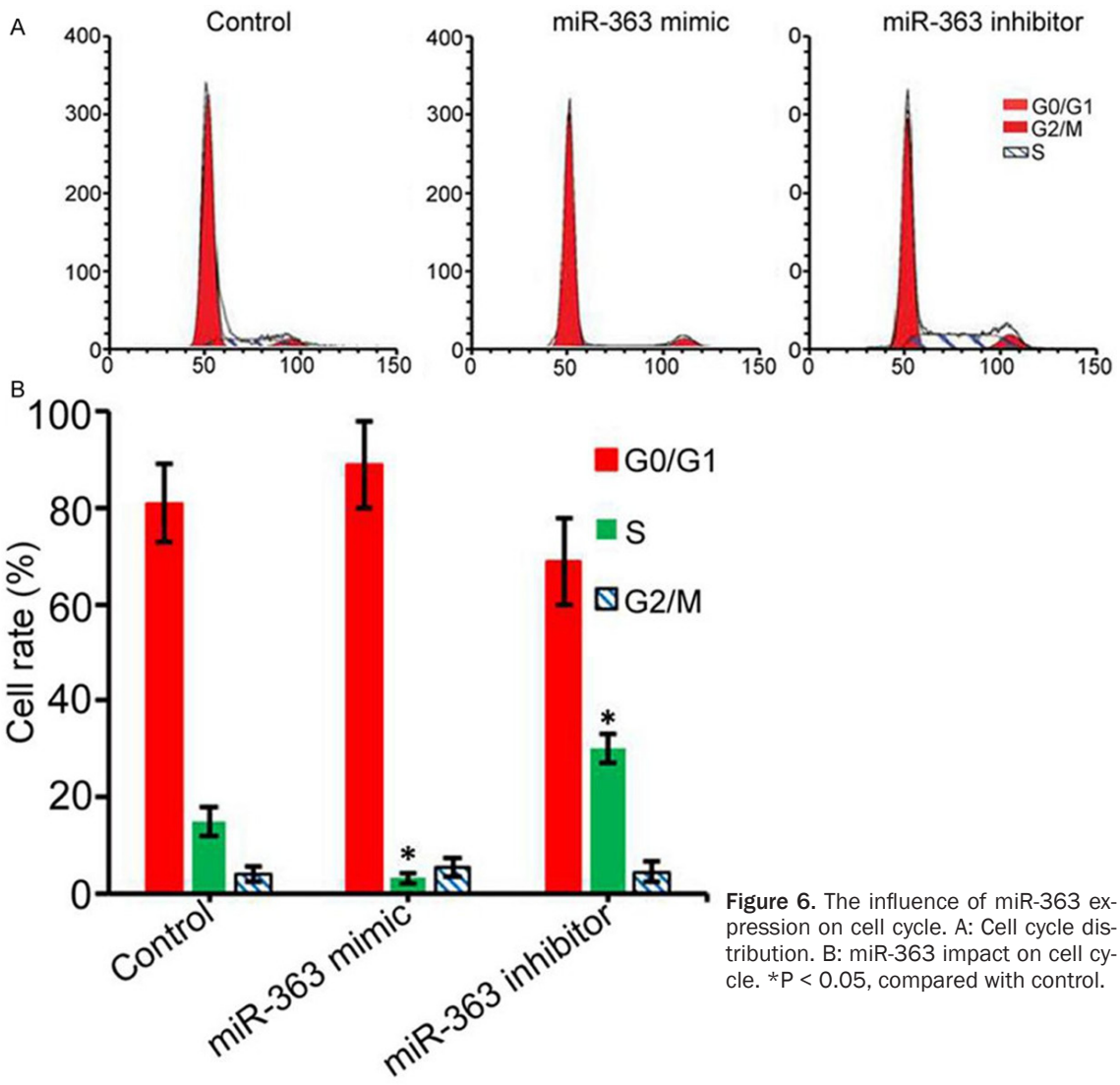

Figure 6. The influence of miR-363 expression on cell cycle. A: Cell cycle distribution. B: miR-363 impact on cell cycle. *P < 0.05, compared with control.

miR-363 inhibitor transfection (**Figure 3B**). It suggested that 3'UTR of PDPN was the target of miR-363.

PDPN expression

Western blot was performed to test PDPN protein expression in CD4+ T cells (**Figure 4**). It was showed opposite to miR-363, PDPN protein expression markedly elevated in CD4+ T cells in synovial fluid from arthritis (P < 0.05).

Cell transfection

MiR-363 mimic and miR-363 inhibitor were transfected to Jurkat cells to change miR-363 expression. Total RNA was extracted to test miR-363 expression (**Figure 5A**). MiR-363 expression increased by 2.2 times after miR-363 mimic transfection, while it obviously reduced after miR-363 inhibitor transfection (P < 0.05).

Western blot was adopted to test PDPN protein expression in Jurkat cells after transfection. As shown in **Figure 5B**, PDPN level declined by 76% in Jurkat cells after miR-363 mimic transfection, whereas it elevated by 1.8 times after miR-363 inhibitor transfection (P < 0.05).

Jurkat cell cycle changes

Flow cytometry was performed to test Jurkat cell cycle transfected by miR-363 mimic and miR-363 inhibitor. Modifit software was used to analyze FL-2 area and DNA histogram (**Figure 6**). It was showed that cell number in S phase from Jurkat cells transfected by miR-363 mimic

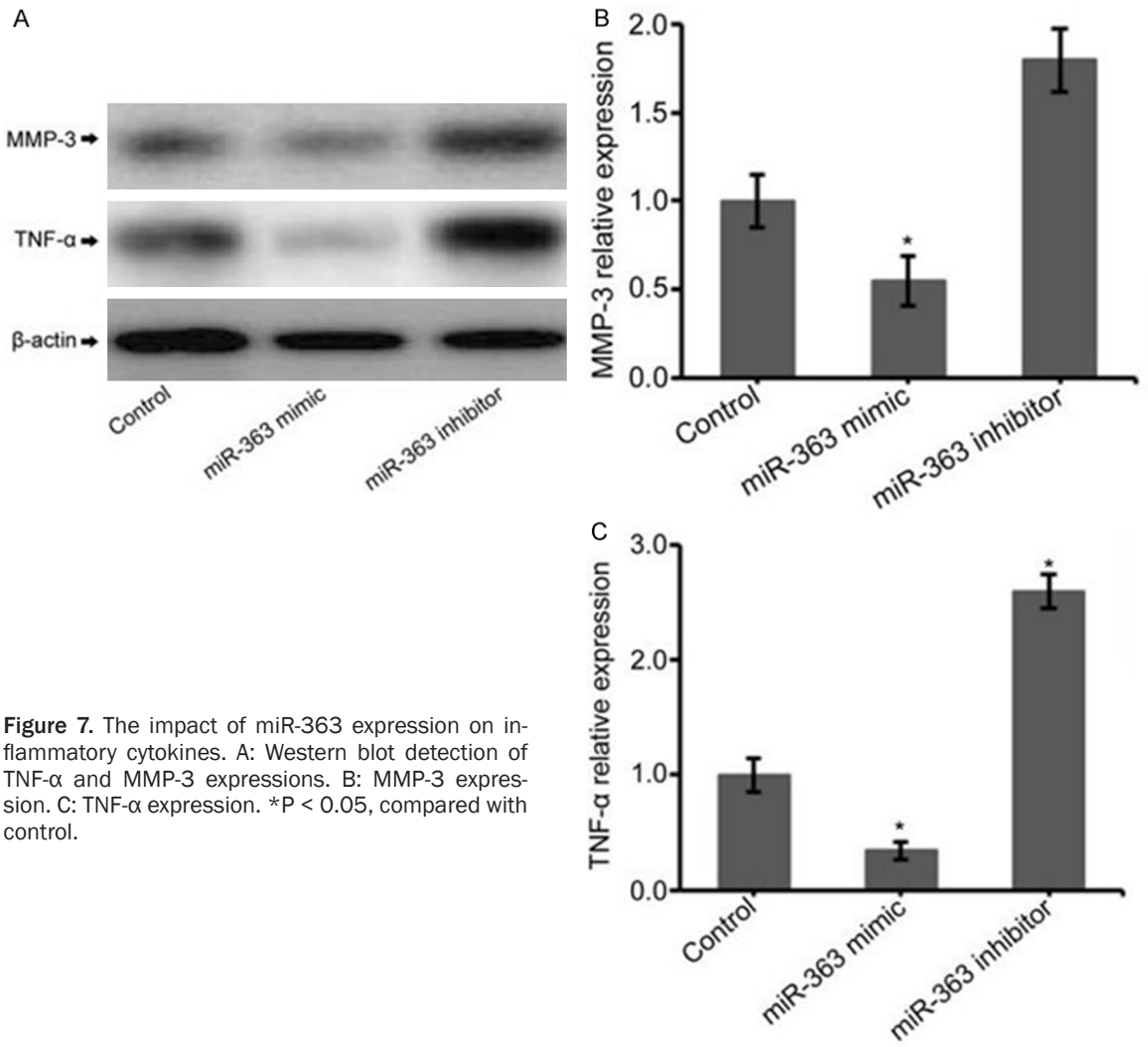

Figure 7. The impact of miR-363 expression on inflammatory cytokines. A: Western blot detection of TNF-α and MMP-3 expressions. B: MMP-3 expression. C: TNF-α expression. *P < 0.05, compared with control.

was obviously reduced (P < 0.05), while cell number in G0/G1 phase and G2/M phase showed no significant changes (P > 0.05), indicating cell cycle prolonged and cell proliferation weakened. On the contrary, miR-363 inhibitor transfection markedly increased cell number in S phase (P < 0.05), suggesting that cell cycle was shortened and cell proliferation was enhanced.

Inflammatory cytokines expression

After transfection for 2 days, inflammatory cytokines expressions in Jurkat cells were detected by Western blot. As shown in **Figure 7**, TNF-α and MMP-3 protein expressions in Jurkat cells after miR-363 mimic transfection were obviously declined (P < 0.05), while they significantly upregulated after miR-363 inhibitor transfection compared with control (P < 0.05).

Discussion

CD4+ T cells play an important role in the regulation of the immune response and inflammation. It was found that CD4+ T cells participate in arthritis disease and inflammation. Meanwhile, it was also reported that miRNA expression was different in CD4+ T cells under the process of inflammation compared with normal CD4+ T cells. Therefore, it was considered that miRNA has a critical role in CD4+ T cells involved inflammatory response [14]. This study found that miR-363 expression in CD4+ T cells in synovial fluid from arthritis patients was significantly lower compared with normal con-

trol, speculating that miR-363 may be involved in CD4+ T cells regulation of arthritis inflammatory response.

MiRNAs are a kind of small non-coding RNA molecules conservative in evolution. They can regulate normal cell behavior and metabolic process by RNA interference [14]. Zhu discovered that miR-23b can suppress autoimmune inflammatory response through inhibiting TAB2, TAB3, and IKK-α expression [15]. It suggested that miRNAs may have a key role in other autoimmune inflammatory disease including arthritis. We found that miR-363 expression downregulated in CD4+ T cells from arthritis patients. Further bioinformatics analysis and dual luciferase report gene assay confirmed that miR-363 may exert its biological function by targeting PDPN.

PDPN, also called GP38, highly expresses on lymphatic vessel endothelial cell membrane and participates in lymphatic endothelial generation [16]. Recent studies showed that PDPN also expresses in T cells and may be involved in T cell response. Peters reported that PDPN positively expressed in effector T cells in inflammation part, and can affect the allergic reaction triggered by effector T cells by mice experiment [17].

At the same time, we found that miR-363 overexpression prolonged cell cycle in T cells upon cell transfection and flow cytometry. Research indicated that PDPN plays a critical role in cell division and metabolism by participating in PI3K-AKT signaling pathway activation and mTOR phosphorylation [18, 19].

At present, it is still unclear about how CD4+ T cells regulate miRNA expression. Some scholars considered that miRNA expression changed upon regulating methylation level in the transcriptional promoter of miRNA [20]. In addition, it was also proposed that the targeted protein product can reverse regulate miRNA expression level [21]. More evidences were needed to confirm how CD4+ T cells changed miR-363 expression.

The pathological process of arthritis is often accompanied by bone destruction and joint deformity, eventually serious influencing movement with high morbidity. This study confirmed the role of miR-363 in CD4+ T cells from arthritis patients by investigating clinical patients and cells in vitro. It has certain guide significance to clarify the role and mechanism of immune cells in the pathogenesis of arthritis. It also provides theoretical basis to treat rheumatoid arthritis and other autoimmune diseases via RNA interference.

Conclusion

MiR-363 expression declined in CD4+ T cells from arthritis patients, thus to regulate cell cycle and inflammatory cytokines release through targeting PDPN.

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Disclosure of conflict of interest

None.

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