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ORIGINAL RESEARCH



Downregulation of FPR1 abates lipopolysaccharide-induced inflammatory injury and apoptosis by upregulating MAPK signaling pathway in murine chondrogenic ATDC5 cells

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Abstract

Background and objective: Osteoarthritis is the most common chronic osteoarthrosis disease. There are complex factors that lead to osteoarthritis. Therefore, it is essential to investigate the molecular mechanism of osteoarthritis, especially the mechanism of articular cartilage degeneration. In this study, the mechanism of FPR1 (formyl peptide receptor 1) in LPS (lipopolysaccharide) induced chondrogenic cell ATDC5 was investigated.

Materials and methods: We employed real-time quantitative polymerase chain reaction (RT-qPCR) and western blot assay to analyze the expression level of FPR1 in ATDC5 cell lines induced by LPS at 0, 2.5, 5, and 10 μ g/mL concentrations. Then we constructed the FPR1 knockdown plasmid to transfect the LPS-ATDC5. MTT assay was used to test cell viability in control, LPS, LPS+shNC and LPS+shFPR1 groups. ELISA and RT-qPCR assay were employed to examine the TNF-α (tumor necrosis factor-α) IL-6 and IL-18 expression level. Flow cytometry and western blot assay were employed to analyze the apoptosis of LPS-ATDC5. Finally, we utilized the western blot assay to text related protein expression level of MAPK (mitogen-activated protein kinase) signaling pathway.

Results: In this study, we found the expression level of FPR1 was increased in LPS-ATDC5, downregulation of FPR1 improves the survival rate and alleviates inflammatory response of LPS-ATDC5. Meanwhile, downregulation of FPR1 alleviates apoptosis of LPS-ATDC5. Finally, downregulation of FPR1 inhibits the MAPK signal pathway.

Conclusion: Present study revealed that FPR1 was highly expressed in LPS-induced chondrocytes ATDC5, and the downregulation of FPR1 abated the inflammatory response and apoptosis of LPS-ATDC5 cells by regulating the MAPK signaling pathway.

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Introduction

Osteoarthritis (OA) is the most common chronic osteoarthrosis disease, and globally it is one of the major causes of pain and disability in older adults. As a degenerative disease, the degeneration of the articular cartilage is the main cause of osteoarthritis. There are many factors that contribute to cartilage degeneration, including aging, obesity, strain, trauma, and inflammatory diseases. Because of the complex factors that lead to osteoarthritis, it is essential to investigate the molecular mechanism of articular cartilage degeneration.

Articular cartilage plays an important role in arthrocentesis. Chondrocytes are unique cells in articular cartilage which are responsible for maintaining a balance between degradation and synthesis of extracellular matrix (ECM).⁵ Pro-inflammatory cytokines such as Interleukin-1 (IL-1) family, matrix metalloproteinases (MMP), and cyclooxygenase 2 (COX-2) contribute to the loss of major components of ECM during the degeneration of OA articular cartilage.⁶ In addition, apoptosis is also the direct cause of chondrocyte death.⁷ Therefore, it is necessary to explore the molecular mechanism of inflammatory response and apoptosis of chondrogenic cells.

Formyl peptide receptor 1 (FPR1), a member of the G-protein-coupled pattern-recognition receptor family, is mainly expressed by mammalian phagocytic leukocytes and is one of the major proteins that cause inflammation and tissue damage.8,9 FPR1 is associated with the development of many diseases. It has been reported that FPR1 gene silencing can inhibit the apoptosis of myocardial cells and cardiovascular remodeling in a rat model of ischemia reperfusion by inhibiting the MAPK signaling pathway, thereby alleviating myocardial injury.¹⁰ Wang et al. found that exogenous carbon monoxide could interfere with the activity of FPR1 by inhibiting p38 MAPK, so as to reduce the inflammatory response of progressive multiple sclerosis (PMS)-related neutrophils induced by LPS.11 FPR1 is highly expressed in neuroblastoma and promotes its occurrence by activating the MAPK/Erk, PI3K/Akt, and P38-MAPK pathways.¹² In the case of degenerative disc herniation, FPR1 activity was inhibited by a novel FPR1 antagonist, which attenuated pro-inflammatory factor expression and alleviated neuralgia response in a mouse model.¹³ In osteoarthritis, FPR1 expression was found to be upregulated compared with normal tissue, and it is one of the 10 hub genes that causes arthritis.14 In addition, by targeting FPR1 with CFLFLF-PEG-64cu peptide to detect inflammatory responses associated with the onset of osteoarthritis, FPR1 could be used as a target for the diagnosis and treatment of osteoarthritis.15 However, the role of FPR1 in OA cartilage degeneration of has not been reported, and the specific molecular mechanism of FPR1 is unclear. The objective of this study was to investigate the mechanism of FPR1 in lipopolysaccharide (LPS)-induced chondrogenesis cell ATDC5.

Materials and Methods

Cell culture

The chondrogenic cell line ATDC5 was resuspended in Dulbecco's Modified Eagle Medium (DMEM) culture

(Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10% fetal bovine serum (FBS; Biological Industries), 1% penicillin/streptomycin (Gibco, Grand Island, USA), and 0.025-µg/mL amphotericin B (Sigma-Aldrich, St. Louis, MO, USA). The cells were treated with increasing doses of LPS (0-, 2.5-, 5-, and 10-µg/mL concentrations) at 37°C for 12 h. The cells were cultured in a humidified 5% CO, incubator at 37°C.

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from collected cells by adding trizol reagent (Invitrogen, CA, USA) according to the product manual. The purity and concentration of the extracted total RNA was examined by Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific, Grand Island, USA). The Cham QTM SYBR® qPCR Master Mix (Vazyme, Nanjing, China) was applied to amplify the FPR1, tumor necrosis factor-α (TNF-α), Interleukin 6 (IL-6), and IL-1β complementary DNAs (cDNAs) using the QuantStudio 6 Flex RT-PCR system (Life Technologies, Carlsbad, CA) according to the product manual. The expression values of target gene were normalized to U6 expression. The PCR primers were designed and chemically synthesized by Tsingke Technology (Beijing, China). Relative expressions of FPR1, TNF- α , IL-6, and IL-1B in each experimental group were analyzed using the $2^{\triangle\triangle Ct}$ method.^{16,17} Primer sequences are exhibited in Table 1. All reactions were executed in triplicate.

Cell transfection

The FPR1 stable knockdown cell lines were constructed by lentivirus, which contained pFU-GW-009 vector (Gene Chem, China). All lentiviral vectors expressed enhanced green fluorescent protein (GFP), which was used for measuring their infection efficiency. The 293T cells were used to package the lentivirus. We found that the infection efficiency of ATDC5 cell lines was highest if the multiplicity of infection (MOI) was 200. The medium containing

Table 1 Primers for *FPR1*, *TNF-a*, *IL-6*, *IL-1B*, and reference genes.

Forward Reverse	CATGGGAGGACATTGGCCTT
Doverse	
Keverse	CACGGATTCTGACTGTGGCT
Forward	ATGAGCACAGAA AGCATGATC
Reverse	TACAGGCTTGTCACTCGAATT
Forward	TTCCCTACTTCACAAGTC
Reverse	ACTAGGTTTGCCGAGTAG
Forward	ACAGATGAAGTGCTCCTTCCA
Reverse	GTCGGAGATTCGTAGCTGGAT
Forward	GTGACGTTGACATCCGTAAAGA
Reverse	GCCGGACTCATCGTACTCC
	Reverse Forward Reverse Forward Reverse Forward

0.3-mg/mL puromycin (Thermo Fisher Scientific) was used to select stably transfected cells. Finally, the infected cells were divided into two groups: (i) Infection of control virus (shNC); and (ii) infection of FPR1 knockdown virus (shFPR1).

MTT assay

MTT assay was employed to assess the extent of cell viability. Briefly, the cells $(2.5 \times 10^3 \text{ cells/well})$ were plated into 96-well plates in triplicate and treated with different conditions as indicated in each experiment. Following treatment, a final concentration of 0.5-mg/mL MTT solution (Beyotime, Shanghai, China) was added into each well, and the cells were incubated for another 4 h at 37°C. Subsequently, the culture medium was discarded and 100-µL dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added for visualization. Optical density (OD) of each sample was detected at 490 nm through a microplate reader (BioTek, Winooski, VT, USA).

Enzyme-linked immunosorbent assay (ELISA)

TNF- α , IL-6, and IL-1B protein levels in LPS-ATDC5 cell lines were examined by ELISA using kits from eBioscience (Cat. No. 88-7013-88; San Diego, CA, USA) following manufacturer's instructions.

Apoptosis assay

Annexin V-PE-Cy5 Apoptosis Staining/detection kit (ab14159; Abcam, Cambridge, MA, USA) was applied to analyze cell apoptosis. Flow cytometry results were obtained from BD Accuri™ C6 (CA, USA). Briefly, the cells were digested, washed, and resuspended in binding buffer and cultured in dark in Annexin V-PE-Cy5-FITC. Again, the cells were kept in dark for at least 30 min at 37°C and quantified through flow cytometry.

Western blot

Briefly, cells were washed thrice in pre-cooled phosphate buffer solution (PBS), and the total protein was extracted by radioimmunoprecipitation assay (RIPA) buffer (Beyotime). Protein concentration was measured by using bicinchoninic acid (BCA) protein assay kits (CoWin Biotechnology, Jiangsu, China). Equal amounts of total proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). They were then transferred to the polyvinylidene difluoride membranes (PVDF; Millipore) and blocked by 5% non-fat milk at room temperature for 1 h. The protein was identified through overnight incubation at 4°C with the following specific primary antibodies: FPR1 (rabbit anti-FPR1 antibody, PA1-41398, 1:2000; Invitrogen), Bax (rabbit anti-Bax antibody, ab32053, 1:5000; Abcam), Bcl-2 (rabbit anti-Bcl-2 antibody, ab182858, 1:2000; Abcam), cleaved Caspase-3 (rabbit anti-cleaved Caspase-3 antibody, ab32042, 1:500; Abcam), p-ERK (rabbit anti-p-ERK antibody, ab201015, 1:1000; Abcam), ERK (rabbit anti-ERK antibody, ab32537, 1:1000; Abcam), p-p38 (rabbit anti-p-p38 antibody, 44-684G, 1:1000; Invitrogen), p38 (rabbit anti-p38 antibody, ab170099, 1:3,000; Abcam), p-JNK (rabbit anti-p-JNK antibody, ab76572, 1:5000; Abcam), JNK (rabbit anti-JNK antibody, ab199380, 1:2500; Abcam), B-actin (rabbit anti-B-actin antibody, ab8227, 1:3000; Abcam). The membranes were further incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (ab205718, 1:1500; Abcam) and the bands on the membranes were visualized by the ECL chemiluminescence reagent (Beyotime). The B-actin was used to normalize the amount of analyzed samples, and protein bands were quantified by gray scale analysis through ImageJ software (National Institutes of Health).

Statistical analysis

All data are presented as mean \pm standard deviation obtained from three independent assays. Student's t-test was employed to calculate comparisons between two groups. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was employed for analysis. P < 0.05 indicates statistically significant differences.

Results

Expression of FPR1 was increased in LPS-ATDC5

The FPR1 expression level was examined in chondrogenic cell line ATDC5, which was stimulated with 0-, 2.5-, 5-, and 10-µg/mL LPS concentrations for 12 h. RT-qPCR result showed that the FPR1 mRNA expression level increased in an LPS dose-dependent manner (Figure 1A). Western blot assay result revealed that LPS treatment upregulated the protein expression level of FPR1 in a dose-dependent manner, which was consistent with RT-qPCR results (Figure 1B). These two results suggested that the expression of FPR1 was upregulated in LPS-ATDC5.

Downregulation of FPR1 improved the survival rate of LPS-ATDC5

The role of FPR1 in ATDC5 cell lines induced by 5-µg/mL LPS concentration was explored. It was observed while making a toxicity curve of LPS (see Supplementary Material, Figure S1) that the optimal concentration of 5-µg/ mL LPS was required, and FPR1 was highly expressed after induction by 5-µg/mL LPS in our results as shown above. The results of Western blot (Figure 2A) and RT-qPCR (Figure 2B) assays displayed that LPS treatment notably increased the protein and mRNA expression level of FPR1 compared to control group in ATDC5 cells, and the FPR1 protein and mRNA expression level were downregulated in LPS+shFPR1 group compare to LPS+shNC group. Then MTT assay was employed to analyze cell viability in ATDC5 cells of control, LPS, LPS+shNC, and LPS+shFPR1 groups. The result demonstrated that viability of ATDC5 cells was enhanced in LPS+shFPR1 group compared to LPS+shNC group (Figure 2C). These results revealed that downregulation of FPR1 improved the survival rate of LPS-ATDC5.

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Downregulation of FPR1 reduced inflammatory response of LPS-ATDC5

In order to explore whether FPR1 mediates LPS-ATDC5 inflammatory response, the expression levels of TNF- α , IL-6, and IL-1B were examined in LPS-ATDC5. We found that

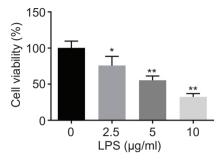


Figure S1 Toxicity of LPS on ADTC5 cells. CCK8 assay showed the cell viability of ADTC5 cells treated with LPS at 0-, 2.5-, 5-, and 10-ug/mL concentrations. 'P < 0.05. "P < 0.01.

the concentration and mRNA expression levels of TNF- α , IL-6, and IL-1B increased notably in the LPS group compared to the control group. The ELISA and RT-qPCR results showed that both concentration of inflammatory factors and levels of genes were upregulated by the induction of LPS. The results of ELISA presented that FPR1 knockdown decreased the concentration of TNF- α , IL-6, and IL-1B (Figure 3A). Results of RT-qPCR assay revealed that the expression levels of TNF- α , IL-6, and IL-1B were markedly decreased in LPS-ATDC5 after knocking down of FPR1 (Figure 3B). Hence, LPS-ATDC5 inflammatory response was reduced with down-regulation of FPR1.

Downregulation of FPR1 decreased apoptosis of LPS-ATDC5

The apoptosis rate of LPS-ATDC5 cells was analyzed. The flow cytometry assay results revealed that the apoptosis rate increased prominently in the LPS group compared to the control group. Knockdown of FPR1 decreased the

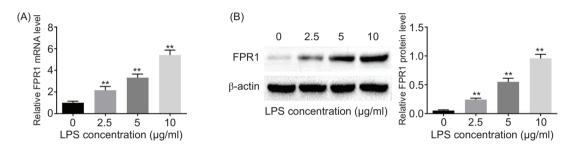


Figure 1 The expression of FPR1 was increased in LPS-ATDC5. (A) The mRNA expression level of FPR1 at 0-, 2.5-, 5-, 10-μg/mL LPS concentrations; **P < 0.01. (B) The protein expression level of FPR1 at 0-, 2.5-, 5-, 10-μg/mL LPS concentrations; **P < 0.01. β-actin was used as an internal control.

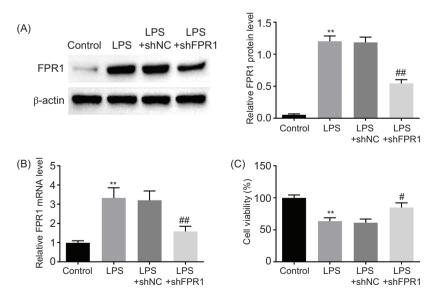


Figure 2 Downregulation of FPR1 improved the survival rate of LPS-ATDC5. (A) The protein expression level of FPR1 in ATDC5 cell of control, LPS, LPS+shNC, and LPS+shFPR1 groups; "P < 0.01. ß-actin was used as an internal control. (B) The mRNA expression level of FPR1 in ATDC5 cells of control, LPS, LPS+shNC, and LPS+shFPR1 groups; **P < 0.01. (C) MTT analysis of ATDC5 cell viability in control, LPS, LPS+shNC, and LPS+shFPR1 groups; *P < 0.01. *shNC versus shFPR1.

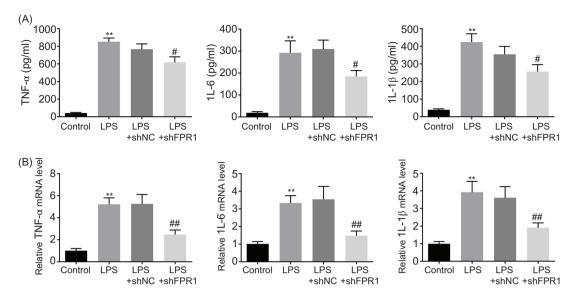


Figure 3 Downregulation of FPR1 reduced inflammatory response of LPS-ATDC5. ELISA was used to measure the concentrations of TNF- α , IL-6, and IL-1B in LPS-ATDC5 of control, LPS, LPS+shNC, and LPS+shFPR1 groups; *P < 0.05, **P < 0.01. The (A) protein and (B) mRNA expression levels of TNF- α , IL-6, and IL-1B in LPS-ATDC5 of control, LPS, LPS+shNC, and LPS+shFPR1 groups. **P < 0.01. *shNC versus shFPR1.

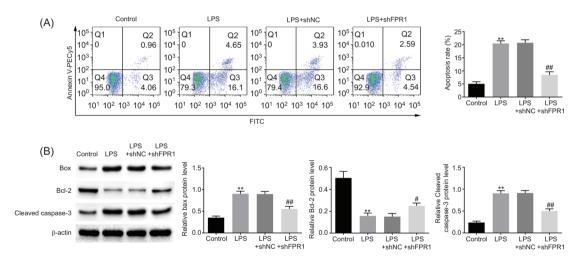


Figure 4 Downregulation of FPR1 reduced apoptosis of LPS-ATDC5. (A) The flow cytometry assay analysis of apoptosis rate. (B) Western blot analysis of Bax, cleaved Caspase-3, and Bcl-2 in Ishikawa and HEC-1-B cells after treatment of GA at 0-, 20-, 50-, 100 μM concentrations. **P < 0.01. GAPDH was used as an internal control. #shNC versus shFPR1.

apoptosis rates of LPS-ATDC5 cell lines compared to the control group (Figure 4A). Further, Western blot assay was employed to examine the expression levels of apoptosis-related proteins. The results showed that after knocking down FPR1, the expression levels of Bax and cleaved Caspase-3 were decreased whereas the expression level of Bcl-2 was upregulated in LPS-ATDC5 (Figure 4B). Collectively, these results illustrated that FPR1 knockdown decreased LPS-ATDC-5 apoptosis.

Downregulation of FPR1 inhibited the MAPK signaling pathway

Finally, the expression levels of related proteins of MAPK signaling pathway were examined by Western blot assay.

The result depicted that the activity of MAPK signaling pathway was remarkably enhanced in the LPS group compared to the control group. The expression levels of ERK, p38, and JNK all phosphorylated, were downregulated in FPR1 stable knockdown LPS-ATDC5 cells compared to the control group, while the expression levels of ERK, p38, and JNK did not change. Collectively, these results revealed that downregulation of FPR1 suppressed the MAPK signaling pathway (Figure 5).

Discussion

Osteoarthritis is the most common chronic disease of joints, affecting majority of population over the age of 65 years. It is a leading musculoskeletal cause of impaired mobility in

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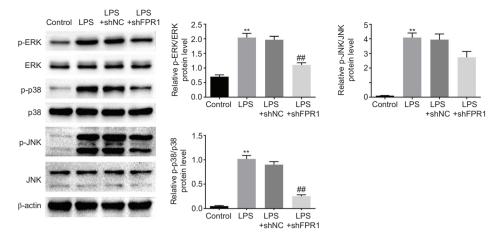


Figure 5 Downregulation of FPR1 inhibited the MAPK signaling pathway. Western blot analysis of phosphorylated ERK, ERK, phosphorylated p38, p38, phosphorylated JNK, and JNK in ATDC5 cell lines of control, LPS, LPS+shNC, and LPS+shFPR1 groups. *P < 0.05, **P < 0.01. β-actin was used as an internal control. #shNC versus shFPR1.

the elderly.¹⁸ Previous studies have proved that local inflammatory response leads to the pathogenesis of osteoarthritis. For example, tripterine upregulates miR-223 to relieve LPS-induced injury in osteoarthritis;19 tan IIA protected ATDC5 cells from LPS-induced injury by reducing miR-203a expression level and inhibiting JAK/STAT and JNK pathways;20 piperine relieves LPS-induced inflammatory injury by decreasing the expression level of miR-127 in osteoarthritis;²¹ and pretreatment with green tea polyphenols (GTP) reduces LPS-induced inflammatory response in osteoarthritis by inhibiting the MAPK and NF-kB pathways through regulating miR-9 positively.²² Therefore, exploring effective molecules that could reduce the inflammatory response of osteoarthritis is necessary. Our study for the first time validated that FPR1 promotes the injury of osteoarthritis, suggesting that FPR1 could be involved in its pathogenesis.

FPR1 has been found to play multiple roles in many animal diseases. These roles are ambivalent, and could be beneficial or harmful.²³ FPR1 has a function in the tumorigenicity of human cervical cancer.⁹ Suppressing FPR1 could be a potential intervention strategy to manage triple-negative breast cancer (TNBC) displaying the characteristics of breast cancer.²⁴ Blocking FPR1 suppresses activation of neutrophil and protects against acute lung injury by targeting dipeptide HCH6-1.²⁵ It is found in this study that the expression level of FPR1 was increased in LPS-ATDC5, and the downregulation of FPR1 improved the survival rate, and reduced inflammatory response and apoptosis of LPS-ATDC5. Thus, FPR1 might serve as clinical therapeutic targets in many diseases.

FPR1 regulates multiple signaling pathways. FPR1 can decrease NF-κB nuclear translocation, and can regulate NLRP3 inflammasome signaling and MAPK signaling pathways. FPR1-knocked out mice displayed decreased acute inflammation (MAPK and NF-κB signaling pathway activation and NRLP3 inflammasome pathway induction). FPR1 on intestinal epithelial cells to produce reactive oxygen species, causing extracellular signal-regulated kinase MAPK, and rapid phosphorylation of focal adhesion kinase (FAK). Consistent with the studies that established FPR1 interact with multiple signal pathways, the present study revealed that FPR1 promotes LPS-induced inflammatory injury and

apoptosis through activating MAPK signaling pathway in murine chondrogenic ATDC5 cell lines. In future, it will be investigated whether FPR1 plays a role in osteoarthritis by influencing other signaling pathways.

Conclusion

It was discovered in the present study that the expression of FPR1 was increased in LPS-ATDC5. On the other hand, downregulation of FPR1 improved the survival rate of LPS-ATDC5, decreased the inflammatory response and apoptosis of LPS-ATDC5, and, finally, inhibited the MAPK signaling pathway. These findings suggested that downregulation of FPR1 abated LPS-induced inflammatory injury and apoptosis by upregulating the activity of MAPK signaling pathway in murine chondrogenic ATDC5 cells. However, as this study was restricted to explore the functioning of FPR1 in MAPK signaling pathway, the future research would investigate whether it works on other signal pathway as well.

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Competing Interests

The authors stated that there were no conflicts of interest to disclose.

Authors' Contributions

Hongtao Chen and Li Zhang designed the study and supervised data collection. Hongtao Chen analyzed and interpreted the data. Li Zhang prepared and reviewed the draft of the manuscript for publication. Both authors read and approved the final manuscript.

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