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Original Article

Silencing of TLR7 protects against lipopolysaccharide-induced chondrocyte apoptosis and injury by blocking the p21-mediated JAK2/STAT3 pathway

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Abstract: Involvement of toll-like receptor 7 (TLR7) in the immune response has been reported in diverse inflammatory diseases. However, the role of TLR7 in the pathogenesis of osteoarthritis (OA) is poorly understood. In this study, we sought to investigate the contribution of TLR7 in regulating chondrocyte apoptosis, inflammation, and degradation of the extracellula matrix (ECM), and its underlying mechanisms. We found that TLR7 expression was increased in cartilage tissues of OA patients and in lipopolysaccharide (LPS)-induced chondrocytes. Silencing of TLR7 alleviated LPS-induced chondrocyte apoptosis, inflammation, and ECM degradation. Mechanistically, TLR7 silencing inhibited the JAK2/STAT3 signaling pathway by inducing p21 expression. Moreover, p21 knockdown and colivein (an activator of JAK2/STAT3 signaling) partially rescued the suppressive role of TLR7 silencing on chondrocyte apoptosis, the inflammatory response, and ECM underproduction. Taken together, our data revealed that knockdown of TLR7 attenuated chondrocyte apoptosis and injury by blocking the p21-mediated JAK2/STAT3 pathway, suggesting that TLR7 may be a therapeutic target in OA.

Keywords: Toll receptor 7, apoptosis, injury, chondrocyte, osteoarthritis

Introduction

Osteoarthritis (OA) is a common chronic disease that affects the joint spaces and muscle strength, and is a leading cause of disability [1]. OA is characterized by degeneration of articular cartilage, and its pathogenic mechanisms include a disorder of anabolic and catabolic gene expression and chondrocyte apoptosis, which results in irreversible joint damage, pain, and synovial inflammation [2]. Chondrocytes, as the only cell type present in cartilage, play a vital role in OA initiation and progression. Synthesis of structural components of the extracellular matrix (ECM) and matrix-degrading proteases occurs in chondrocytes. Damage of the ECM, increased expression of matrix-degrading proteases, and chondrocyte apoptosis are the main causes of degeneration of articular cartilage [3, 4]. Furthermore, accumulation of pro-inflammatory cytokines, including interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and IL-6, has been found in the synovial tissues and cartilage of OA patients. These cytokines affect cartilage matrix degradation by regulating the production of matrix metalloproteinases (MMPs) in OA [5]. Therefore, it is important to investigate the mechanism of chondrocyte dysfunction for OA therapy.

Toll-like receptor 7 (TLR7), an important member of the TLR family, plays a vital role in immune-mediated inflammatory disorders by triggering the production of downstream signaling cascades [6, 7]. Studies show that TLR7 signaling triggers the secretion of chemokines and pro-inflammatory cytokines by immune cell activation, thereby promoting the development of inflammatory disorders, such as systemic lupus erythematosus [8], glomerulonephritis [9], and rheumatoid arthritis [10]. Moreover, TLR7 ligation can affect the expression of cytokines, such as p21 and p-STAT3, and induces pancreatic tumorigenesis progression by regulating

stromal inflammation [11]. In acute kidney injury, TLR7 also promotes apoptosis and inflammatory responses [12]. On the other hand, TLRs have been reported to regulate anabolic and catabolic pathways and chondrocyte apoptosis of OA by activating innate immune responses [13], namely TLR4 [14], and TLR2 [15]. Notably, TLR7 expression is upregulated in the lipopolysaccharide (LPS)-induced inflammatory response of post-traumatic OA [16]. Higher expression of TLR7 has been found in synovial membrane samples and blood samples of OA [17]. Furthermore, TLR7 signaling activation by miR-21 results in OA knee pain [18]. However, the molecular mechanism by which TLR7 regulates apoptosis and chondrocyte injury in OA is poorly understood.

JAK2 signaling pathways, especially the classical JAK2/STAT3 pathway are involved in orthopaedic diseases, such as OA of the knee [19], osteosarcoma [20], and rheumatoid arthritis [21]. The related genes of the JAK2/STAT3 signaling pathway (JAK2 and STAT3) are usually activated in chondrocytes of various orthopaedic diseases [22, 23]. Studies suggested that the JAK2/STAT3 pathway can regulate apoptosis and the production of MMPs and pro-inflammatory cytokines in chondrocytes to mediate OA progression [24, 25]. JAK2/STAT3 signaling activated by IL-1 induces chondrocyte apoptosis and MMP expression in OA [26]. In addition, the JAK2/STAT3 pathway induced by angiotensinogen regulates IL-6-mediated inflammatory responses in OA [27]. Therefore, targeted inhibition of the JAK2/STAT3 pathway is expected to maintain normal physiologic function of chondrocytes and to improve OA.

In our research, we first studied the function of TLR7 in LPS-induced chondrocyte apoptosis, inflammation and ECM degradation, and then investigated the underlying mechanisms. Our results showed that TLR7 was involved in LPS-induced chondrocyte apoptosis, inflammation, and ECM degradation through regulating the p21-mediated JAK2/STAT3 signaling pathway. Our findings may provide a new solution for OA treatment.

Materials and methods

Ethics statement

All procedures were strictly performed accordance with the Animal Care and Use Committee of Yantaishan Hospital (ethic code: wydw20140129). The collection of human articular cartilage tissue was approved by the Ethical Committee of Yantaishan Hospital and followed the guidelines of the Declaration of Helsinki.

Cartilage tissues of patients

OA human articular cartilages were collected from 24 patients who underwent knee arthroplasty surgery (15 women and 9 men aged 64-72 years old). The normal human articular cartilages from 24 donors with no significant clinical and imaging features of OA were obtained from femoral condyles and tibial plateaus at autopsy (10 women and 14 men aged 56-68 years old). Informed consent was obtained from all participants and the study was approved by the Ethic Committee of Yantaishan Hospital.

Cell culture

Human chondrocytes (C28/I2) were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China) and incubated in DMEM/F-12 culture medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS: Gibco, Rockville, MD) using a humidified incubator (37°C, 5% CO_2). When cells reached 70-80% confluence they were treated with 1, 5 and 10 μg/ml of LPS (Sigma, St. Louis, MO, USA) for 6, 12 and 24 h.

Transfection

TLR7 siRNA plasmid, p21 siRNA plasmid and control siRNA plasmid were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc, California, USA).

TLR7 siRNA sequence: 5'-AACTTCTTGGGCTTAGAACA-3'; p21 siRNA sequence: 5'-GGGTCGAAAACGGCGGCAGACC-3'; siRNA negative control sequence: 5'-UCUUCCGAACGUGUCACGUTT-3'. C28/I2 cells (1×10^6 cells/well) were cultured in a six-well plate for 24 h at 37°C, and then 2 μg of plasmids transcribing siRNA were transiently transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Besides, cells were transfected with the TLR7 overexpression plasmid (Addgene plasmid #13022) according to the method of Chamberlain *et al.* [28].

RNA extraction and RT-qPCR

Total RNA was extracted from cartilage tissues and chondrocytes using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, USA). The cDNA was synthesized by reverse transcription reaction and the quantitative reverse transcription PCR (RT-qPCR) was performed using PrimeScriptTM RT-PCR Kit (TaKaRa; Dalian) in QuantStudio™ 12K Flex system (Life Tech, USA). The cycling conditions were as follows: 95°C for 10 min for 1 cycle and 95°C for 15 s and 60°C for 1 min repeated for 40 cycles, followed by a dissociation stage. Relative gene expression was quantified by the cycle threshold (Ct) $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Each sample was analyzed in triplicate. Primer sequences are listed in Table S1.

Western blot analysis

The chondrocytes were lysed in RIPA lysis buffer (Sigma, St. Louis, MO, USA) for 15 min. Total protein concentration in chondrocytes was detected using a BCA protein assay kit (Solarbio, Beijing, China). The total protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and blocked with 5% skimmed milk at room temperature for 1 h. The membranes were incubated with the primary antibody overnight at 4°C and then incubated with goat anti-rabbit horseradish peroxidase antibody (Sigma, St. Louis, MO, USA). The protein complexes were detected by enhanced chemiluminescence (Luminata Forte, Millipore, USA). Chemiluminescent signal was quantified by BandScan 5.0 software. The primary antibodies human anti-TLR7 (1:100, #sc-57463), MMP-3 (1:1000, #sc-21732), MMP-13 (1:1000, #sc-515284), aggrecan (1:1000, #sc-33695) and GAPDH (1:1000, #sc-47724) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p21 (1:1000, #2947), iNOS (1:1000, #39898), STAT3 (1:2000, #4904), phospho-STAT3 (1:2000, #9131), JAK2 (1:1000, #3230), phospho-JAK2 (1:1000, #3771), Bcl-2 (1:1000, #3498), Bax (1:1000, #2772) and cleaved caspase-3 (1:1000, #9661) were purchased from Cell Signaling Technology (Beverly, MA, USA). GAPDH was used as an internal control.

MTT assay

Human chondrocyte proliferation was measured using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Dojindo Laboratories, Kumamoto, Japan). Chondrocytes (5×10^3/ml) were inoculated in 96-well plates, cultivated to logarithmic growth phase and then added to the DMEM/F-12 medium supplemented with 5 mg/ml of MTT reagent. MTT formazan crystals were mixed with dimethyl sulfoxide (Sangon, Shanghai, China) and oscillated for 10 min at a low speed. Cell viability was measured using a microplate reader (Thermo Fisher Scientific, Massachusetts, USA) by spectrophotometry at 570 nm.

Apoptosis assay

Human chondrocyte apoptosis was detected using the Cell Apoptosis Detection Kit (Qiagen, Valencia, CA, USA). Briefly, after 24 h cultured with LPS, chondrocytes transfected with plasmids were incubated with 10 μl of annexin V-FITC and propidium iodide (Becton, Heidelberg, Germany) at room temperature for 15 min and the annexin V-FITC staining protocol was followed to measure apoptosis.

ELISA assay

The corresponding ELISA kits were used to measure the contents of IL-6, TNF-α and IL-1β (BioSite, Paris, France). The experimental steps were performed in accordance with the manufacturer's instructions.

Measurement of nitrite oxide production

The content of nitrite oxide (NO) in human chondrocytes was detected using the Nitric Oxide assay kit (Thermo Fisher Scientific, Massachusetts, USA). The experimental steps were carried out according to the manufacturer's instructions. The absorbance of NO at 550 nm was determined.

Statistical analysis

Statistical analyses of all data were achieved by using the GraphPad Prism 6 (GraphPad Software, Inc.). Statistical significance was performed using one-way or two-way ANOVA, with Tukey's post hoc test used for comparisons between the two groups. The data were repre-

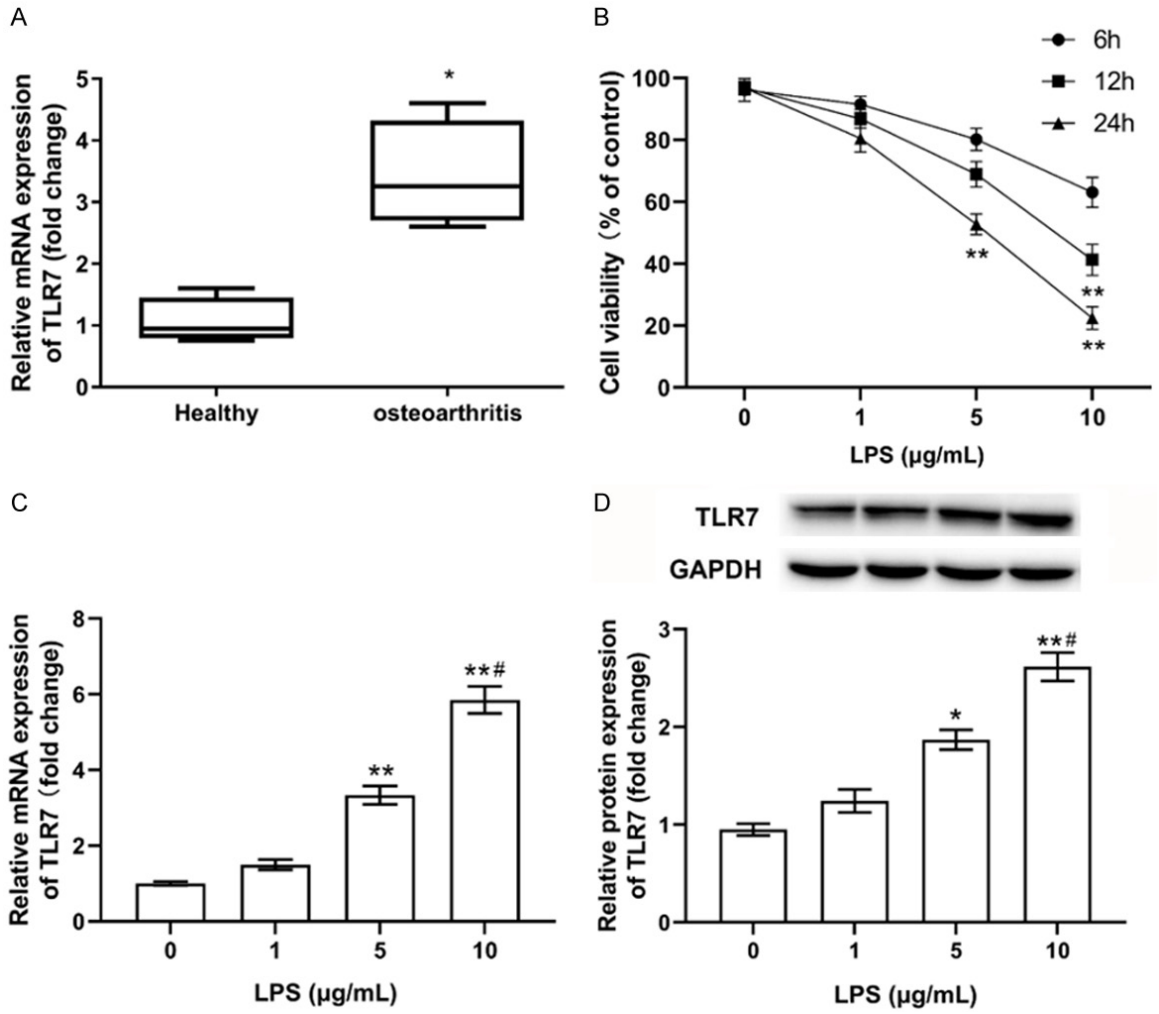

Figure 1. TLR7 expression is upregulated in cartilage tissues of OA-patients and LPS-stimulated chondrocytes. Chondrocytes were treated with different concentrations of LPS (0, 1, 5, and 10 μg/ml) for 6, 12, and 24 h and cell supernatants were collected after culturing for 48 h. A. TLR7 expression in cartilage tissues of OA patients was detected by RT-qPCR. $^{*}P<0.05$ compared with healthy controls. B. The viability of chondrocytes after LPS treatment. C. mRNA expression of TLR7 in LPS-induced chondrocytes was detected by RT-qPCR. D. Expression of TLR7 in LPS-induced chondrocytes was detected by western blot assay. C28/I2 chondrocytes were treated with different concentrations of LPS for 12 h; $^{*}P<0.05$ and $^{**}P<0.01$ compared with 0 μg/ml LPS treatment; $^{\#}P<0.05$ compared with 5 μg/ml LPS treatment. Columns present the mean ± SEM ($n\geq3$).

sented as means ± SEM and each experiment was performed in triplicate in this study. $P<0.05$ was considered significant.

Results

TLR7 was upregulated in cartilage tissues of OA patients and LPS-induced chondrocytes

Expression of TLR7 was detected in cartilage tissues of OA patients and healthy participants. The results showed that mRNA expression of TLR7 was dramatically higher in cartilage tissues of OA patients than that in healthy participants (**Figure 1A**). Chondrocytes were treated with different concentrations of LPS (0, 1, 5, and 10 μg/ml) for 6, 12, and 24 h. The results indicated that LPS significantly suppressed the cell viability of chondrocytes in a dose- and time-dependent manner (**Figure 1B**). Moreover, LPS treatment significantly increased mRNA and protein expression of TLR7 in chondrocytes (**Figure 1C**, **1D**).

Silencing of TLR7 inhibited LPS-induced chondrocyte apoptosis and ECM degradation

To investigate the effect of TLR7 on LPS-induced chondrocytes, cells were transfected with TLR7 siRNA (si-TLR7) or control siRNA (si-

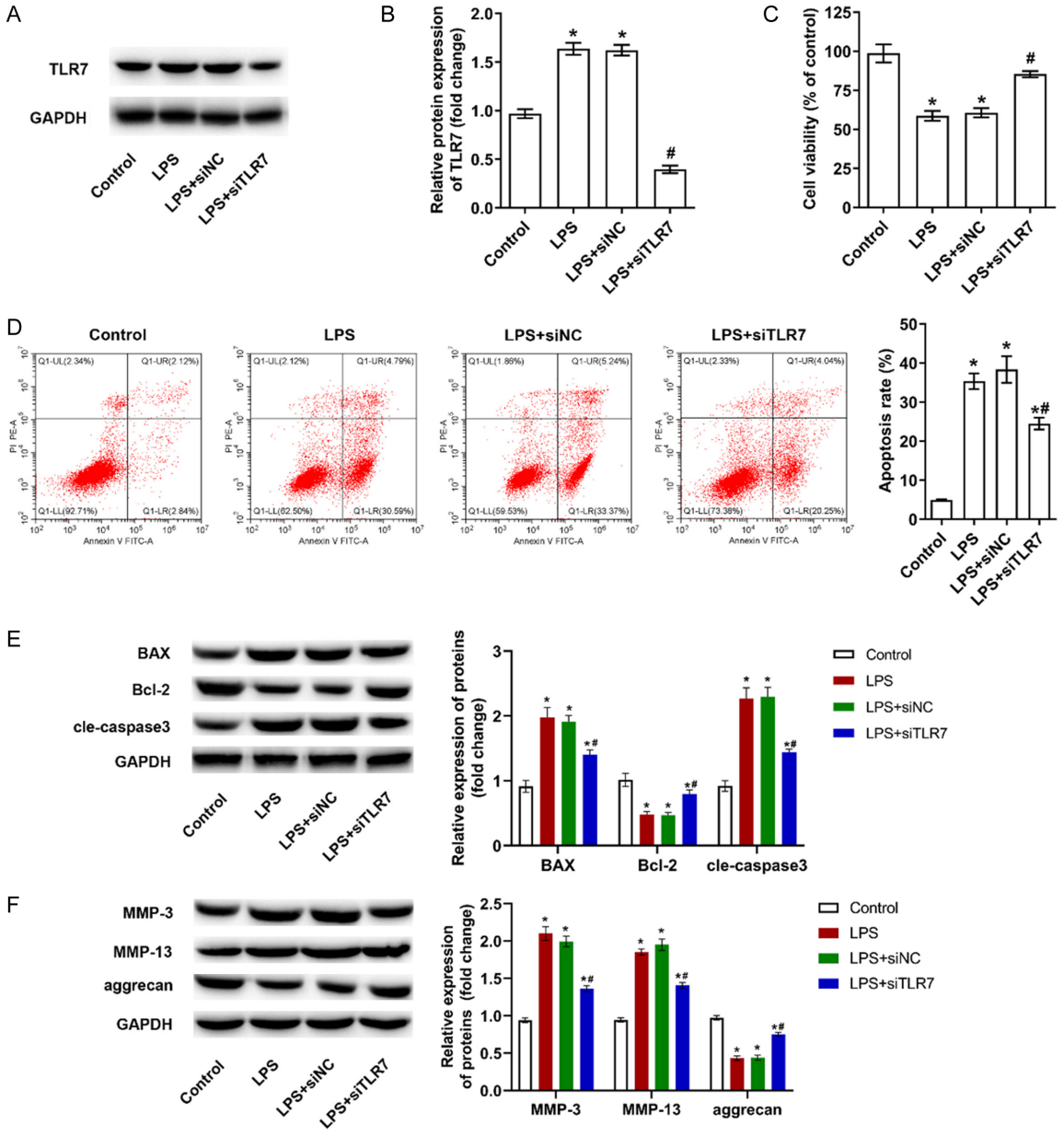

Figure 2. TLR7 silencing inhibited LPS-induced cell apoptosis and ECM degradation. Chondrocytes were transfected with control siRNA (si-NC, 1 μg/ml) and TLR7 siRNA (si-TLR7, 1 μg/ml) for 48 h and then cells were treated with 5 μg/ml LPS for 12 h. A, B. TLR7 expression in chondrocytes analyzed by western blot assay. C. The viability of chondrocytes evaluated by MTT assay. D. Apoptosis rate detected by flow cytometry analysis. E. Expression of cle-caspase3, BAX and Bcl-2 assayed by western blot analysis. F. Expression of MMP-3, MMP-13 and aggrecan assayed by western blot analysis. GAPDH was used as an internal control. Columns present the mean ± SEM ($n \geq 3$); $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus LPS group.

NC) for 48 h and then treated with 5 μg/ml LPS for 12 h. The results showed that protein expression of TLR7 was increased significantly after LPS treatment, whereas silencing of TLR7 suppressed the increase of TLR7 expression (**Figure 2A**, **2B**). As expected, the downregulation of TLR7 significantly increased cell viability (**Figure 2C**). Flow cytometry showed that transfection of si-TLR7 inhibited apoptosis of chondrocytes compared with the si-NC group (**Figure 2D**). TLR7 knockdown significantly reduced the protein expressions of cleaved caspase-3 (cle-caspase3) and BAX, and enhanced Bcl-2 expression in LPS-induced chon-

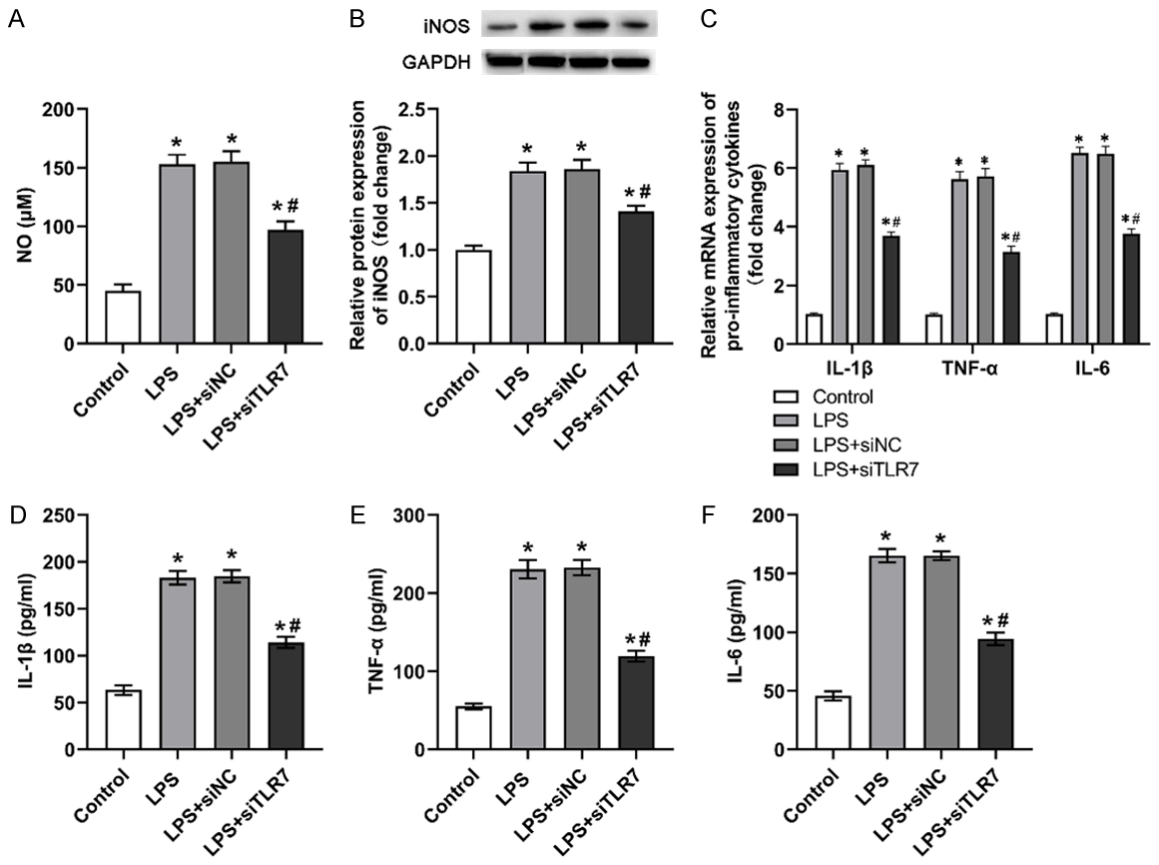

Figure 3. Production of inflammatory mediators and cytokines in LPS-stimulated chondrocytes were reduced by TLR7 knockdown. Chondrocytes were transfected with si-NC and si-TLR7 for 48 h and then cells were treated with 5 μg/ml LPS for 12 h. A. The content of NO was detected in cells. B. The protein level of iNOS in cells was analyzed by western blot assay. C. mRNA expressions of IL-1β, TNF-α, and IL-6 in chondrocytes were assessed by RT-qPCR. D-F. The contents of IL-1β, TNF-α, and IL-6 in chondrocytes were detected through ELISA assay. Columns present the mean ± SEM ($n \geq 3$); $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus LPS group.

drocytes (**Figure 2E**). In addition, we further investigated whether TLR7 regulates the ECM degradation in LPS-induced chondrocytes. The results showed that the expressions of MMP-3 and MMP-13 were upregulated by LPS, which were significantly inhibited by TLR7 silencing. Moreover, the inhibiting effect of LPS on aggrecan expression was compensated by TLR7 knockdown (**Figure 2F**).

Cells were transfected with TLR7 overexpression plasmids (Figure S1A). Overexpression of TLR7 further reduced the LPS-inhibited cell viability (Figure S1B). The upregulation of TLR7 significantly elevated the apoptosis rate in LPS-treated chondrocytes, together with an increase of apoptosis-related protein expression (Figure S1C-E). Furthermore, TLR7 overexpression upregulated the expressions of MMPs proteins and decreased aggrecan expression in LPS-induced cells (Figure S1F).

Silencing of TLR7 inhibited LPS-induced inflammatory response in chondrocytes

Inflammation plays an essential role in the pathogenesis of OA [31]. Thus, we further verified the effect of TLR7 on inflammatory factors in chondrocytes. As shown in **Figure 3A** and **3B**, the results confirmed that the NO content and the protein level of iNOS were elevated in LPS-induced cells; however, it was markedly reduced by TLR7 knockdown. Moreover, the downregulation of TLR7 significantly suppressed the mRNA expressions of IL-1β, TNF-α, and IL-6 in LPS-stimulated chondrocytes (**Figure 3C**). the increased levels of IL-1β, TNF-α and IL-6 in LPS-treated chondrocytes were dramatically inhibited by TLR7 silencing (**Figure 3D-F**). In contrast, overexpression of TLR7 further increased the production of these inflammatory mediators and cytokines induced by LPS in cells (Figure S2).

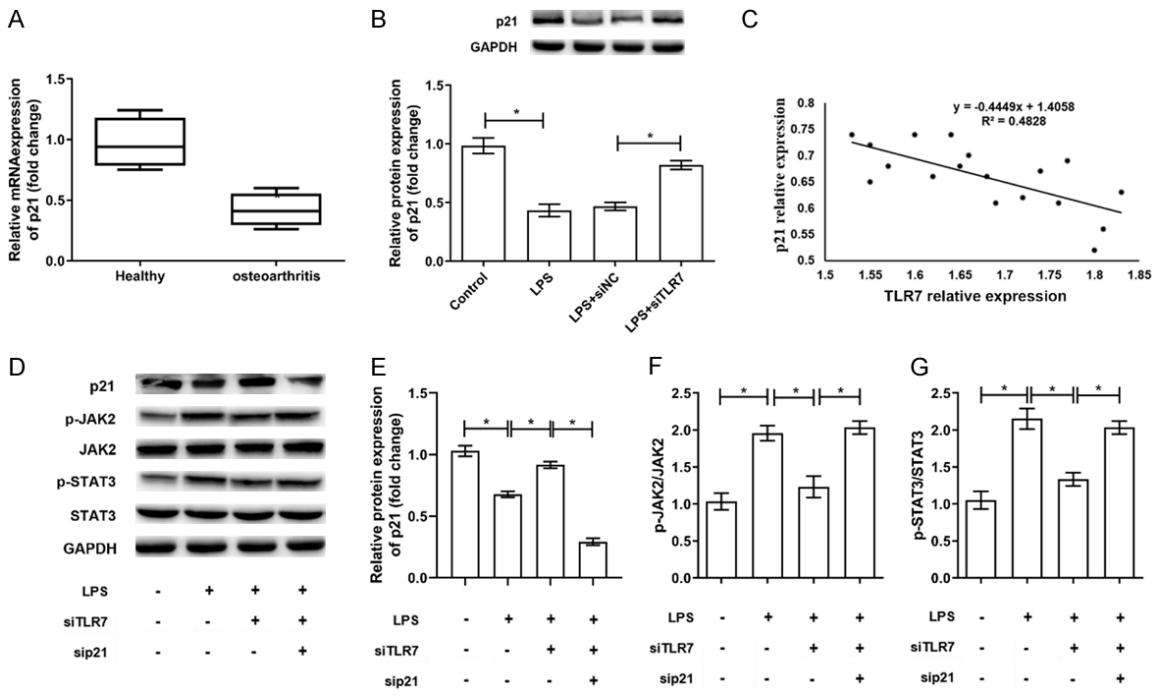

Figure 4. TLR7 modulated the JAK2-STAT3 pathway by regulating p21 expression. Chondrocytes were transfected with si-NC, si-TLR7, and p21 siRNA (si-p21). Samples were collected after transfection for 48 h. A. mRNA expression of p21 in cartilage tissues of OA patients detected by RT-qPCR. B. p21 expression in LPS-induced chondrocytes detected by western blot assay. C. Relation between TLR7 and p21 evaluated by Pearson analysis. D-G. Protein expressions of p21, p-JAK2/JAK2 and p-STAT3/STAT3 assayed by western blot. GAPDH was used as an internal control. *, stands for $P<0.05$. Columns present the mean ± SEM ($n\geq3$).

TLR7 modulated the JAK2/STAT3 pathway by suppressing p21 expression.

Accumulating evidence demonstrated that p21 and the JAK2/STAT3 pathway were closely associated with OA progression [19, 32]. Our results showed that mRNA expression of p21 in cartilage tissues of OA patients was lower than in healthy participants (**Figure 4A**). LPS treatment decreased p21 expression, whereas TLR7 silence reversed the inhibition effect of LPS on p21 expression (**Figure 4B**). Pearson correlation analysis indicated that a negative correlation existed between TLR7 expression and p21 expression (**Figure 4C**). Furthermore, expressions of p-JAK2 and p-STAT3 were upregulated by LPS treatment, but they was suppressed significantly by TLR7 knockdown (**Figure 4D**, **4F**, **4G**). These results suggested that TLR7 knockdown elevated the phosphorylation levels of JAK2 and STAT3 and reduced p21 expression in LPS-treated chondrocytes.

To investigate the relationship between TLR7, p21 and JAK2/STAT3 signaling, p21 siRNA and TLR7 siRNA were co-transfected into LPS-treated cells. The results showed that p21 expression was upregulated in LPS-induced cells transfected with si-TLR7, which was significantly inhibited by p21 knockdown (**Figure 4D**, **4E**). Importantly, the suppressive effect of TLR7 knockdown on the phosphorylation levels of JAK2 and STAT3 was reversed by p21 silencing (**Figure 4F**, **4G**).

P21-mediated JAK2-STAT3 signaling is involved in TLR7-knockdown alleviation of chondrocyte apoptosis and injury

To investigate the role of p21-mediated JAK2-STAT3 signaling in TLR7-knockdown induced alleviation of chondrocyte apoptosis, ECM degradation and inflammation, the cells were transfected with si-p21 and treated with colivelin (an activator of JAK2/STAT3 signaling). The results indicated that cell viability increased by downregulation of TLR7 was significantly reduced by p21 silencing or colivelin (**Figure 5A**), and that p21 silencing or colivelin significantly increased the apoptosis rate compared

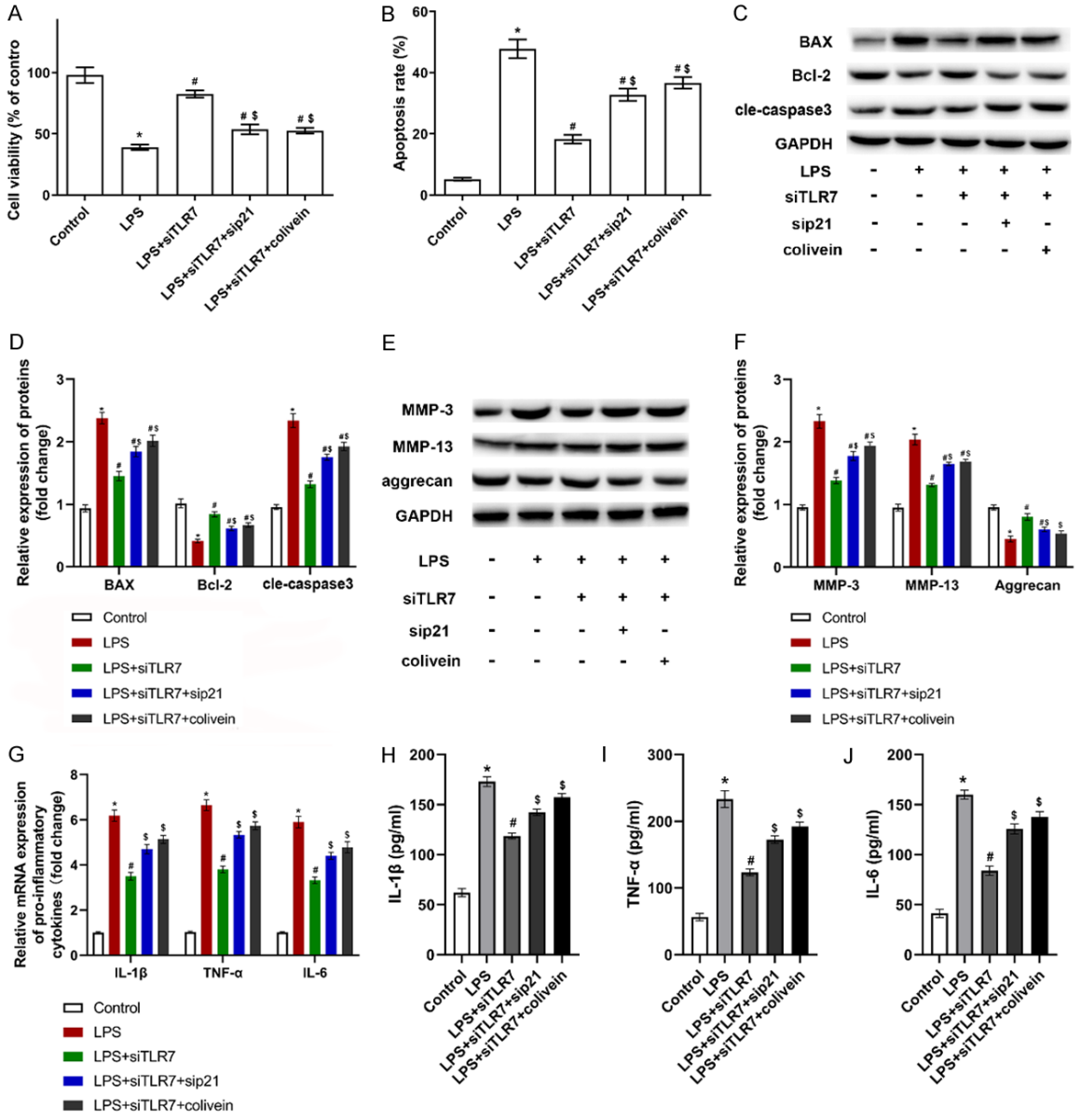

Figure 5. TLR7 silencing protected against chondrocyte apoptosis and injury by suppressing the p21-mediated JAK2/STAT3 pathway. Chondrocytes were transfected with si-NC, si-TLR7 and si-p21 and pre-treated with 0.25 μmol l^{-1} colivelin for 2 h. Samples were collected after transfection for 48 h. A. The viability of chondrocytes evaluated by MTT assay. B. Apoptosis rate of chondrocytes detected by apoptosis assay. C, D. Expressions of cle-caspase3, BAX and Bcl-2 assayed by western blot. E, F. Expressions of MMP-3, MMP-13 and aggrecan assayed by western blot analysis. G. mRNA expressions of IL-1β, TNF-α, and IL-6 in chondrocytes assessed by RT-qPCR. H-J. The levels of IL-1β, TNF-α, and IL-6 in chondrocytes were detected by ELISA. GAPDH was used as an internal control. Columns present the mean ± SEM ($n \geq 3$); $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus LPS; $^{\$}P<0.05$ versus LPS+siTLR7.

with TLR7 silence in LPS-treated cells (**Figure 5B**). Expressions of BAX and caspase-3 in cells transfected with si-TLR7 were increased by p21 knockdown and colivelin, respectively. Bcl-2 expression was upregulated by TLR7 knockdown in LPS-stimulated cells, but this was reversed by si-p21 or colivelin (**Figure 5C**, **5D**). Expressions of MMP-3 and MMP-13 were reduced notably by TLR7 silencing, whereas p21 silencing or colivelin reversed the suppressive roles of TLR7 knockdown in MMP expression. Furthermore, the expression of aggrecan was decreased by downregulation of p21 and colivelin, (**Figure 5E**, **5F**) respectively. In addi-

tion, mRNA expression and the levels of IL-1β, TNF-α, and IL-6 were decreased by silencing of TLR7 in LPS-treated cells, which were recovered by p21 knockdown or colivelin (**Figure 5G-J**).

Discussion

Degeneration of articular cartilage is the main cause of OA pathogenesis. The only cells present in cartilage are chondrocytes, which play a vital role in the development of cartilage damage and joint dysfunction [33, 34]. In normal conditions, anabolic and catabolic functions of chondrocytes exist in a state of equilibrium. The loss of phenotypic stability of chondrocytes contributes to chondrocyte hypertrophy, ultimately resulting in the appearance of OA [35]. Therefore, investigating the function of chondrocytes in OA pathogenesis has received much attention. LPS-stimulated chondrocytes have been widely used as an *in vitro* model for OA [36, 37]. In our study, we also constructed an LPS-stimulated chondrocyte model as an *in vitro* method for investigating OA pathogenesis. The results showed that LPS treatment caused chondrocyte apoptosis, an inflammatory response, and ECM underproduction.

TLR activation results in the production of chemokines and increased pro-inflammatory cytokines in synovium or cartilage, thereby promoting OA progression [38, 39]. Moreover, TLRs can cause articular cartilage dysfunction by involving chondrocyte ECM degradation or apoptosis [40, 41]. In this study, we investigated the role of TLR7 in chondrocyte apoptosis, inflammation, and ECM degradation. We found that TLR7 expression was upregulated significantly in cartilage tissues of OA patients and in LPS-induced chondrocyte model. Similarly, it was reported that expression of TLR7 was upregulated in synovial membrane samples of OA patients and LPS-treated joint tissue of mice [16, 17]. Furthermore, previous studies confirmed that TLR7 induced the production of TNF-αand type 1 interferons, thereby promoting an inflammatory response and apoptosis of regulatory T cells [42]. TLR7 knockout attenuated cell apoptosis in enterovirus 71-infected mice [43]. Our results demonstrated that LPS-induced inflammation was alleviated by TLR7 knockdown by decreasing the production of pro-inflammatory factors IL-1β, TNF-α and IL-6. TLR7 silencing rescued the LPS-induced chondrocyte apoptosis by regulating apoptosis-related genes. Besides, it has been reported that TLR7 induced the expression of MMPs, such as MMP-9 [44]. In our study, TLR7 aggravated LPS-induced chondrocyte catabolic events by inducing the expression of ECM-related genes. Therefore, we speculated that TLR7 is involved in the OA progression by regulating chondrocyte apoptosis, inflammation, and ECM degradation.

The role of p21 in OA has been reported widely. p21 is not only involved in chondrocyte regeneration and differentiation [45], but also regulates apoptosis, inflammation, and catabolic response in chondrocytes [32, 46, 47]. In this study, we found that p21 expression was decreased in cartilage tissues of OA patients and in LPS-induced chondrocytes. Importantly, p21 expression was inhibited by TLR7. A study shows that p21 expression is regulated by TLR7 ligation in pancreatic ductal adenocarcinoma [11]. Moreover, our results further indicated that the downregulation of p21 expression reversed the effect of TLR7 knockdown on LPS-induced apoptosis, the inflammatory response, as well as ECM degradation. Thus, we believed that silencing of TLR7 alleviated chondrocyte apoptosis and injury by regulating p21 expression.

The JAK2/STAT3 pathway plays an important role in the processes of OA [24] and is associated with p21 or TLR7 in various diseases [11, 32]. In OA patients and rat model, expression of JAK family members JAK1 and JAK3 was significantly upregulated [48]. Inhibition of JAK2/STAT3 effectively protects against OA in mice [49]. Our results indicated that LPS activated the JAK2/STAT3 pathway in chondrocytes. On the other hand, JAK2/STAT3 signaling is activated by TLR7 or p21 to mediate catabolic and inflammatory responses and apoptosis. Evidence suggested that TLR7 ligation regulated the expressions of p21, STAT3, and p-STAT3, and accelerated inflammatory response and apoptosis of pancreatic cancer [11]. p21 silencing caused the increased STAT3 phosphorylation and thereby upregulated the expression MMPs and accelerated the chondrocyte catabolic response [32]. In this study, silencing of TLR7 inhibited LPS-induced JAK2/STAT3 signaling by regulating p21 expression. Impor-

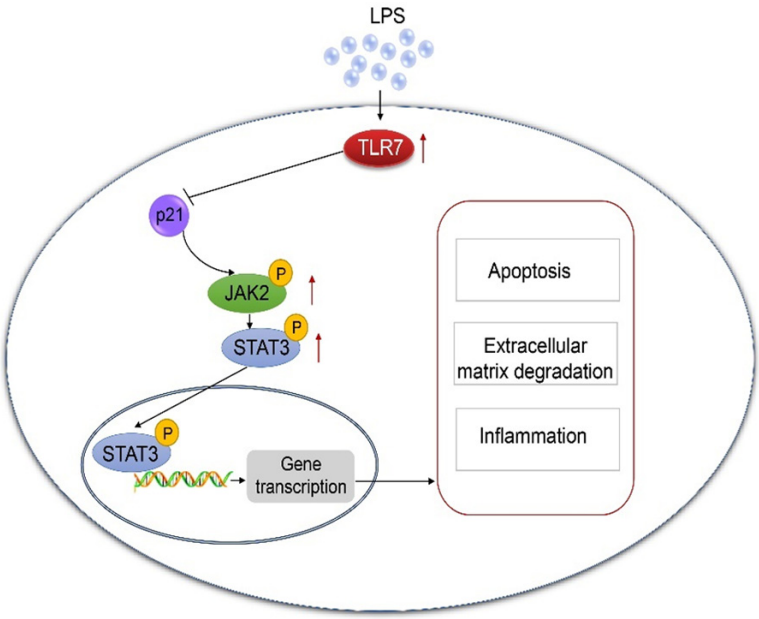

Figure 6. The molecular mechanism of TLR7 regulating apoptosis, ECM degradation, and inflammation in the LPS-induced chondrocytes.

tantly, colivelin, an activator of JAK2/STAT3 signaling, reversed the effect of TLR7 knockdown on apoptosis, the inflammatory response and ECM underproduction. Based on the above analysis, we uncovered novel findings that silencing of TLR7 protected against LPS-induced chondrocyte apoptosis, inflammation and ECM degradation by the p21-mediated JAK2/STAT3 pathway (**Figure 6**). Although our results confirmed the effect of TLR7 on LPS-induced chondrocytes, limitations still existed in this study. How TLR7 reduced the p21 expression requires further study. Besides, the regulation mechanism of TLR7 in *in vivo* models needs to be investigated to support the results from *in vitro* experiments. These limitations will be solved in our further research.

Conclusion

We identified that TLR7 expression was increased in cartilage tissues of OA patients and in LPS-induced chondrocytes. Silencing of TLR7 attenuated LPS-induced chondrocyte apoptosis, the inflammatory response, and ECM degradation. Additionally, p21 was involved in TLR7 regulation of the JAK2/STAT3 signaling pathway. Taken together, these results suggested that silencing of TLR7 protected against LPS-induced chondrocyte apoptosis, inflammation, and ECM degradation by blocking the p21-mediated JAK2/STAT3 pathway. This finding provides new insights into mechanisms of OA.

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Wei Liu, Limin Jiang and Shengjie Dong: Conceptualization, Software, Dan Liu and Chao Wan: Data curation, Methodology, Writing-Original draft preparation. Shijun Wang: Investigation. Weihua Ma: Software.

Disclosure of conflict of interest

None.

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References

[1] Andriacchi TP and Favre J. The nature of in vivo mechanical signals that influence cartilage health and progression to knee osteoarthritis. Curr Rheumatol Rep 2014; 16: 463.

[2] Wei Y and Bai L. Recent advances in the understanding of molecular mechanisms of cartilage degeneration, synovitis and subchondral bone changes in osteoarthritis. Connect Tissue Res 2016; 57: 245-261.

[3] Aigner T, Soeder S, Gebhard PM, McAlinden A and Haag J. Mechanisms of disease: role of chondrocytes in the pathogenesis of osteoarthritis-structure, chaos and senescence. Nat Clin Pract Rheum 2007; 3: 391-399.

[4] Galiniak S, Krawczyk-Marc I, Wawrzyniak A and Orkisz S. Chondrocytes apoptosis in osteoarthritis. Postep Hig Med Dosw 2018; 72: 875-883.

[5] Wang T and He C. Pro-inflammatory cytokines: the link between obesity and osteoarthritis. Cytokine Growth Factor Rev 2018; 44: 38-50.

[6] Petes C, Odoardi N and Gee K. The toll for trafficking: toll-like receptor 7 delivery to the endosome. Front Immunol 2017; 8: 1075.

[7] Luo X, Cui J, Long X and Chen Z. TLRs play crucial roles in regulating RA synoviocyte. Endocr Metab Immue 2020; 20: 1156-1165.

[8] Chodisetti SB, Fike AJ, Domeier PP, Singh H, Choi NM, Corradetti C, Kawasawa YI, Cooper

TK, Caricchio R and Rahman ZSM. Type II but not type I IFN signaling is indispensable for TLR7-promoted development of autoreactive B cells and systemic autoimmunity. J Immunol 2020; 204: 796-809.
[9] Zheng N, Xie K, Ye H, Dong Y, Wang B, Luo N, Fan J, Tan J, Chen W and Yu X. TLR7 in B cells promotes renal inflammation and Gd-IgA1 synthesis in IgA nephropathy. JCI insight 2020; 5: e136965.
[10] Hahnlein JS, Ramwadhdoebe TH, Semmelink JF, Choi IY, Berger FH, Maas M, Gerlag DM, Tak PP, Geijtenbeek TBH and van Baarsen LGM. Distinctive expression of T cell guiding molecules in human autoimmune lymph node stromal cells upon TLR3 triggering. Sci Rep 2018; 8: 1736.
[11] Ochi A, Graffeo CS, Zambirinis CP, Rehman A, Hackman M, Fallon N, Barilla RM, Henning JR, Jamal M, Rao R, Greco S, Deutsch M, Medina-Zea MV, Bin Saeed U, Ego-Osuala MO, Hajdu C and Miller G. Toll-like receptor 7 regulates pancreatic carcinogenesis in mice and humans. J Clin Invest 2012; 122: 4118-4129.
[12] Huang Y, Zhou F, Xiao Y, Shen C, Liu K and Zhao B. TLR7 mediates increased vulnerability to ischemic acute kidney injury in diabetes. Rev Assoc Med Bras (1992) 2019; 65: 1067-1073.
[13] Barreto G, Manninen M and Eklund KK. Osteoarthritis and toll-like receptors: when innate immunity meets chondrocyte apoptosis. Biology (Basel) 2020; 9: 65.
[14] Ding Y, Wang L, Zhao Q, Wu Z and Kong L. MicroRNA-93 inhibits chondrocyte apoptosis and inflammation in osteoarthritis by targeting the TLR4/NF-B signaling pathway. Int J Mol Med 2019; 43: 779-790.
[15] Hwang HS, Park SJ, Cheon EJ, Lee MH and Kim HA. Fibronectin fragment-induced expression of matrix metalloproteinases is mediated by MyD88-dependent TLR-2 signaling pathway in human chondrocytes. Arthritis Res Ther 2015; 17: 320.
[16] Mendez ME, Sebastian A, Murugesh DK, Hum NR, McCool JL, Hsia AW, Christiansen BA and Loots GG. LPS-induced inflammation prior to injury exacerbates the development of post-traumatic osteoarthritis in mice. J Bone Miner Res 2020; 35: 2229-2241.
[17] Wang X, Yu Y, Huang Y, Zhu M, Chen R, Liao Z and Yang S. Identification of potential diagnostic gene biomarkers in patients with osteoarthritis. Sci Rep 2020; 10: 13591.
[18] Hoshikawa N, Sakai A, Takai S and Suzuki H. Targeting extracellular miR-21-TLR7 signaling provides long-lasting analgesia in osteoarthritis. Mol Ther Nucleic Acids 2020; 19: 199-207.
[19] Jiang M, He J, Gu H, Yang Y, Huang Y, Xu X and Liu L. Protective effect of resveratrol on obesity-related osteoarthritis via alleviating JAK2/STAT3 signaling pathway is independent of SOCS3. Toxicol Appl Pharm 2020; 388: 114871.
[20] Ge X, Liu W, Zhao W, Feng S, Duan A, Ji C, Shen K, Liu W, Zhou J, Jiang D, Rong Y, Gong F, Wang J, Xu Z, Li X, Fan J, Wei Y, Bai J and Cai W. Exosomal transfer of LCP1 promotes osteosarcoma cell tumorigenesis and metastasis by activating the JAK2/STAT3 signaling pathway. Mol Ther Nucleic Acids 2020; 21: 900-915.
[21] El-Ghafar OAMA, Helal GK and Abo-Youssef AM. Apixaban exhibits anti-arthritic effects by inhibiting activated factor X-mediated JAK2/STAT3 and MAPK phosphorylation pathways. Inflammopharmacology 2020; 28: 1253-1267.
[22] Huang CY, Lai KY, Hung LF, Wu WL, Liu FC and Ho LJ. Advanced glycation end products cause collagen II reduction by activating Janus kinase/signal transducer and activator of transcription 3 pathway in porcine chondrocytes. Rheumatology (Oxford) 2011; 50: 1379-1389.
[23] Xu X, Lv H, Li X, Su H, Zhang X and Yang J. Danshen attenuates cartilage injuries in osteoarthritis in vivo and in vitro by activating JAK2/STAT3 and AKT pathways. Exp Anim Tokyo 2018; 67: 127-137.
[24] Teng Y, Ni G, Zhang W, Hua J, Sun L, Zheng M, Dong Q and Huang W. TRIM59 attenuates IL-1 beta-driven cartilage matrix degradation in osteoarthritis via direct suppression of NF-kappa B and JAK2/STAT3 signaling pathway. Biochem Bioph Res Commun 2020; 529: 28-34.
[25] Lu W, Ding Z, Liu F, Shan W, Cheng C, Xu J, He W, Huang W, Ma J and Yin Z. Dopamine delays articular cartilage degradation in osteoarthritis by negative regulation of the NF-kappa B and JAK2/STAT3 signaling pathways. Biomed Pharmacother 2019; 119: 109419.
[26] Yao ZZ, Hu AX and Liu XS. DUSP19 regulates IL-1 ss-induced apoptosis and MMPs expression in rat chondrocytes through JAK2/STAT3 signaling pathway. Biomed Pharmacother 2017; 96: 1209-1215.
[27] Wang W, Han X, Zhao T, Zhang X, Qu P and Zhao H. AGT, targeted by miR-149-5p, promotes IL-6-induced inflammatory responses of chondrocytes in osteoarthritis via activating JAK2/STAT3 pathway. Clin Exp Rheumatol 2020; 38: 1088-1095.
[28] Chamberlain ND, Kim SJ, Vila OM, Volin MV, Volkov S, Pope RM, Arami S, Mandelin AM 2nd and Shahrara S. Ligation of TLR7 by rheumatoid arthritis synovial fluid single strand RNA induces transcription of TNFα in monocytes. Ann Rheum Dis 2013; 72: 418-426.
[29] Zhang X, Zhu Y, Chen X, Zhang Y, Zhang Y, Jia Y, Wang H, Liu Y and Xiao L. Baicalein ameliorates inflammatory-related apoptotic and cata-

bolic phenotypes in human chondrocytes. Int Immunopharmacol 2014; 21: 301-308.
[30] Won Y, Shin Y, Chun CH, Cho Y, Ha CW, Kim JH and Chun JS. Pleiotropic roles of metallothioneins as regulators of chondrocyte apoptosis and catabolic and anabolic pathways during osteoarthritis pathogenesis. Ann Rheum Dis 2016; 75: 2045-2052.
[31] Woodell-May JE and Sommerfeld SD. Role of inflammation and the immune system in the progression of osteoarthritis. J Orthop Res 2020; 38: 253-257.
[32] Hayashi S, Fujishiro T, Hashimoto S, Kanzaki N, Chinzei N, Kihara S, Takayama K, Matsumoto T, Nishida K, Kurosaka M and Kuroda R. p21 deficiency is susceptible to osteoarthritis through STAT3 phosphorylation. Arthritis Res Ther 2015; 17: 314.
[33] Kim J, Xu M, Xo R, Mates A, Wilson GL, Pearsall AW and Grishko V. Mitochondrial DNA damage is involved in apoptosis caused by pro-inflammatory cytokines in human OA chondrocytes. Osteoarthr Cartilage 2010; 18: 424-432.
[34] Akkiraju H and Nohe A. Role of chondrocytes in cartilage formation, progression of osteoarthritis and cartilage regeneration. J Dev Biol 2015; 3: 177-192.
[35] Singh P, Marcu KB, Goldring MB and Otero M. Phenotypic instability of chondrocytes in osteoarthritis: on a path to hypertrophy. Ann N Y Acad Sci 2019; 1442: 17-34.
[36] Lei J, Fu Y, Zhuang Y and Zhang K. Sema4D aggravated LPS-induced injury via activation of the MAPK signaling pathway in ATDC5 chondrocytes. Biomed Res Int 2020; 2020: 8691534.
[37] Mawatari T, Nakamichi I, Suenaga E, Maloney WJ and Smith RL. Effects of heme oxygenase-1 on bacterial antigen-induced articular chondrocyte catabolism in vitro. J Orthop Res 2013; 31: 1943-1949.
[38] Nie F, Ding F, Chen B, Huang S, Liu Q and Xu C. Dendritic cells aggregate inflammation in experimental osteoarthritis through a toll-like receptor (TLR)-dependent machinery response to challenges. Life Sci 2019; 238: 116920.
[39] Herrero-Beaumont G, Perez-Baos S, Sanchez-Pernaute O, Roman-Blas JA, Lamuedra A and Largo R. Targeting chronic innate inflammatory pathways, the main road to prevention of osteoarthritis progression. Biochem Pharmacol 2019; 165: 24-32.
[40] Liu YX, Wang GD, Wang X, Zhang YL and Zhang TL. Effects of TLR-2/NF-kappa B signaling pathway on the occurrence of degenerative knee osteoarthritis: an in vivo and in vitro study. Oncotarget 2017; 8: 38602-38617.
[41] Zhang Q, Hui W, Litherland GJ, Barter MJ, Davidson R, Darrah C, Donell ST, Clark IM, Cawston TE, Robinson JH, Rowan AD and Young DA. Differential toll-like receptor-dependent collagenase expression in chondrocytes. Ann Rheum Dis 2008; 67: 1633-1641.
[42] Roh YS, Kim JW, Park S, Shon C, Kim S, Eo SK, Kwon JK, Lim CW and Kim B. Toll-like receptor-7 signaling promotes nonalcoholic steatohepatitis by inhibiting regulatory T cells in mice. Am J Pathol 2018; 188: 2574-2588.
[43] Luo Z, Su R, Wang W, Liang Y, Zeng X, Shereen MA, Bashir N, Zhang Q, Zhao L, Wu K, Liu Y and Wu J. EV71 infection induces neurodegeneration via activating TLR7 signaling and IL-6 production. PLoS Pathog 2019; 15: e1008142.
[44] Lee YH, Huang JH, Chang TH, Yang HC and Wu-Hsieh BA. Mucosa-associated lymphoid tissue lymphoma translocation protein 1 positively modulates matrix metalloproteinase-9 production in alveolar macrophages upon toll-like receptor 7 signaling and influenza virus infection. Front Immunol 2017; 8: 1177.
[45] Ibaraki K, Hayashi S, Kanzaki N, Hashimoto S, Kihara S, Haneda M, Takeuchi K, Niikura T and Kuroda R. Deletion of p21 expression accelerates cartilage tissue repair via chondrocyte proliferation. Mol Med Rep 2020; 21: 2236-2242.
[46] Kihara S, Hayashi S, Hashimoto S, Kanzaki N, Takayama K, Matsumoto T, Chinzei N, Iwasa K, Haneda M, Takeuchi K, Nishida K and Kuroda R. Cyclin-dependent kinase inhibitor-1-deficient mice are susceptible to osteoarthritis associated with enhanced inflammation. J Bone Miner Res 2017; 32: 991-1001.
[47] Xu M, Feng M, Peng H, Qian Z, Zhao L and Wu S. Epigenetic regulation of chondrocyte hypertrophy and apoptosis through Sirt1/P53/P21 pathway in surgery-induced osteoarthritis. Biochem Biophys Res Commun 2020; 528: 179-185.
[48] Qiao Z, Tang J, Wu W, Tang J and Liu M. Acteoside inhibits inflammatory response via JAK/STAT signaling pathway in osteoarthritic rats. BMC Complem Altern Med 2019; 19: 264.
[49] Zou L, Yu L, Zhao X, Liu J, Lu H, Liu G and Guo W. MiR-375 mediates chondrocyte metabolism and oxidative stress in osteoarthritis mouse models through the JAK2/STAT3 signaling pathway. Cells Tissues Organs 2019; 208: 13-24.

Table S1. List of primers used for qPCR in this study

Primer title	Orientation
Human TLR7 F	5'-TGGAAATTGCCCTCGTTGTT-3'
Human TLR7 R	5'-GTCAGCGCATCAAAAGCATT-3'
Human IL-1β F	5'-AACCTGCTGGTGTGTGACGTTC-3'
Human IL-1β R	5'-AGCACGAGGCTTTTTTGTTGT-3'
Human IL-6 F	5'-GTACATCCTCGACGGCATCTCA-3'
Human IL-6 R	5'-GCACAGCTCTGGCTTGTTCCTC-3'
Human TNFα F	5'-TCTCGAACCCCGAGTGACA-3'
Human TNFα R	5'-GGCCGGCGGTTCA-3'
Human GAPDH F	5'-AAGGCTGTGGGCAAGG-3'
Human GAPDH R	5'-TGGAGGAGTGGGTGTCG-3'
Human p21 F	5'-TGGACCTGTCACTGTCTTGT-3'
Human p21 R	5'-TCCTGTGGGCGGATTAG-3'

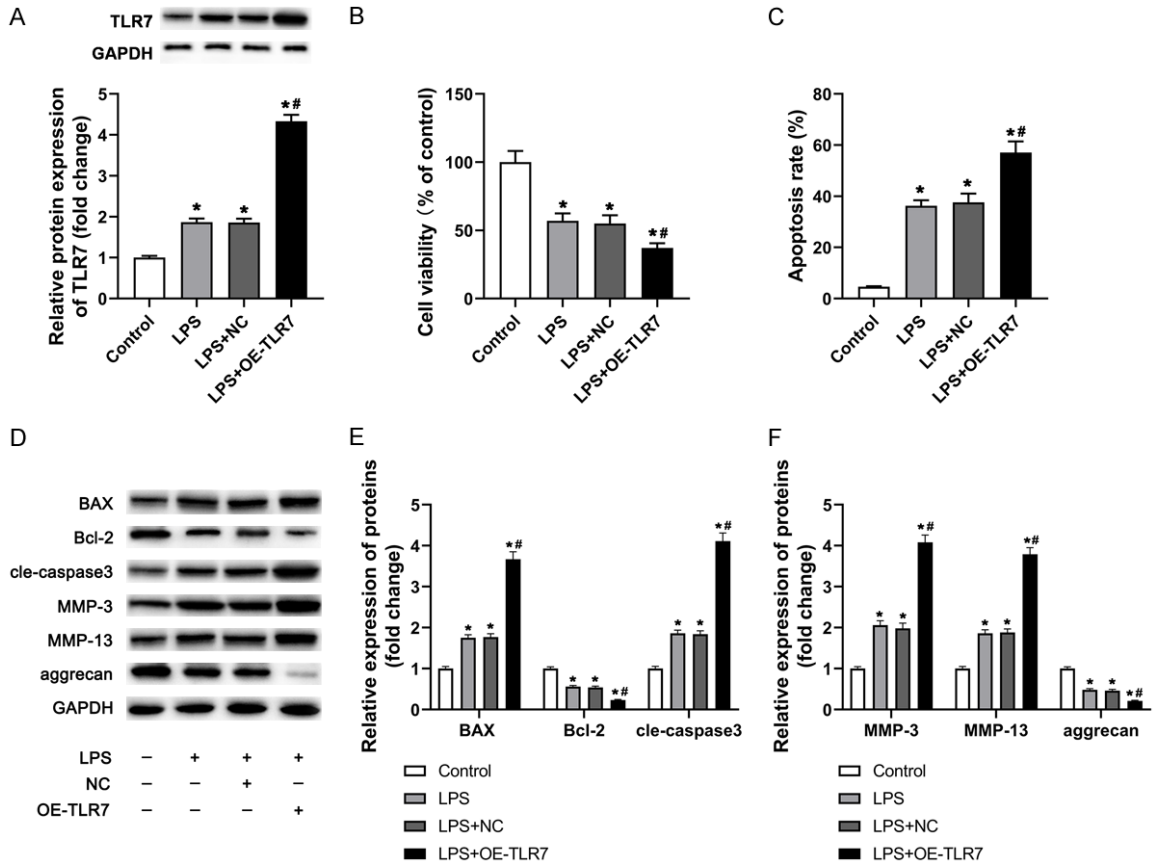

Figure S1. TLR7 overexpression promoted LPS-induced cell apoptosis and ECM degradation. Chondrocytes were transfected with NC vector and OE-TLR7 for 48 h and then cells were treated with 5 μg/ml LPS for 12 h. A. TLR7 expression in chondrocytes was analyzed by Western blot assay. B. The viability of chondrocytes evaluated by MTT assay. C. Apoptosis rate detected by flow cytometry analysis. D-F. Expressions of cle-caspase3, BAX, Bcl-2, MMP-3, MMP-13 and aggrecan were assayed by Western blot analysis. GAPDH was used as an internal control. Columns presented the mean ± SEM of result data ($n \geq 3$); $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus LPS+NC group.

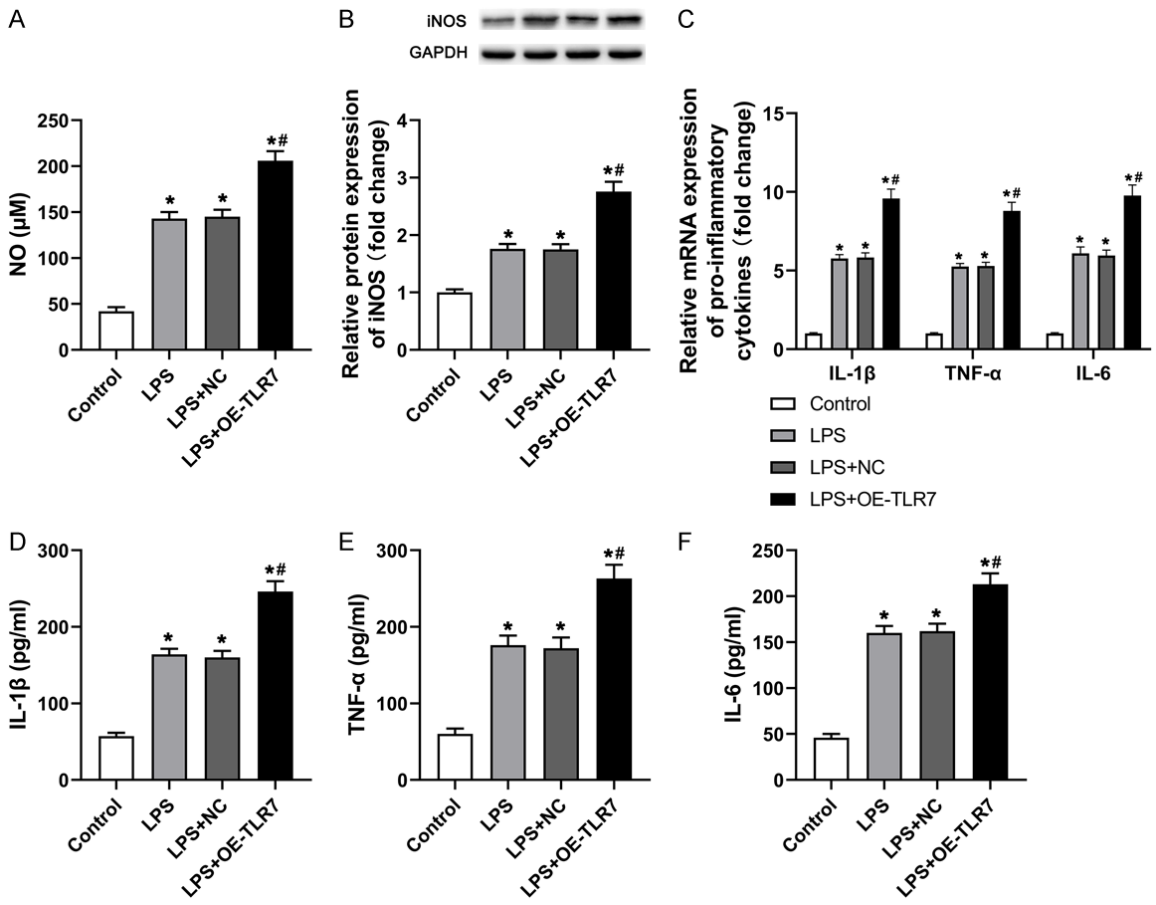

Figure S2. Production of inflammatory mediators and cytokines in LPS-stimulated chondrocytes were promoted by TLR7 overexpression. Chondrocytes were transfected with NC vector and OE-TLR7 for 48 h and then cells were treated with 5 μg/ml LPS for 12 h. A. The content of NO was detected in cells. B. The protein level of iNOS in cells was analyzed by western blot assay. C. mRNA expressions of IL-1β, TNF-α and IL-6 in chondrocytes were assessed by RT-qPCR. D-F. The contents of IL-1β, TNF-α and IL-6 in chondrocytes were detected through ELISA assay. Columns present the mean ± SEM of data ($n \geq 3$); $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus LPS+NC group.