

## Original Article

# Toll-like receptor 4 affects the expression of matrix metalloproteinase 13 in human chondrocytes

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**Abstract:** Background: Currently, toll-like receptor 4 (TLR-4) was implied to participate in multiple biological processes. This study was aimed to investigate the effects of TLR-4 knockdown on osteoarthritis (OA). Methods: Human chondrocytes collected from patients suffering OA were transfected with small interfering RNA targets TLR-4 (siTLR4) or its negative control (siNC). The expression of TLR-4 was determined by quantitative reverse transcription PCR (qRT-PCR). Then, the expression of matrix metalloproteinases 13 (MMP-13) was detected by enzyme-linked immunosorbent assay (ELISA). After that, cell proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Finally, the expression of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon  $\gamma$  (IFN- $\gamma$ ) and interleukin 10 (IL-10) were all measured by ELISA. Results: The mRNA expression level of TLR-4 in chondrocytes transfected with siTLR4 was significantly reduced in comparison with chondrocytes transfected with siNC ( $P < 0.001$ ). Knockdown of TLR-4 remarkably decreased the expression of MMP-13 compared with negative control ( $P < 0.001$ ). Then, the TLR-4-knockdown showed prominent elevation on cell viability at 3 d and 7 d after transfection in comparison with negative control ( $P < 0.001$ ). Finally, the expression of inflammatory cytokines (TNF- $\alpha$ , IFN- $\gamma$  and IL-10) were all down-regulated in TLR-4 knockdown chondrocytes in comparison with negative control ( $P < 0.001$ ). Conclusion: Knockdown of TLR-4 in OA chondrocytes reduced expression of MMP-13 and promoted cell proliferation accompanied by down-regulation of inflammatory cytokines.

**Keywords:** Human chondrocytes, toll-like receptor 4, matrix metalloproteinases 13, inflammatory cytokines

## Introduction

As a chronic joint disease, osteoarthritis (OA) causes long-term disability and significant pain [1]. The World Health Organization (WHO) declared that the prevalence of OA reached 9.6% in men and 18% in women amongst people aged over 60 years globally. With an increasing number of aging populations, the prevalence of OA was predicted to double by the year 2020 [2]. Concerning the treatment of OA is still limited, it is imperative to investigate novel therapeutic target for OA.

In common, OA is mainly characterized by the degradation and loss of cartilage [3]. There is sufficient extracellular matrix (ECM) in articular cartilage; 90-95% of the ECM was type II collagen. The degradation and loss of ECM were observed in early OA [4, 5]. Matrix metalloproteinases (MMPs) which cleave cartilage matrix

proteins act as the key feature for OA joint destruction [6]. Studies focus on human OA have demonstrated that many MMPs are observed to express in articular cartilage [7]. Among these MMPs, the MMP-13 (collagenase 3) is proved to be the most essential for the processing of ECM degradation on account of the preference on type II collagen, which donates to tensile strength to cartilage and abundantly presents in ECM [8]. There is strong evidence to support that MMP-13 acts substantial role in OA pathogenesis [9, 10].

Toll-like receptors (TLRs) are a kind of transmembrane proteins involved with numerous biological processes, including innate immunity, the host defense response, autoimmune and inflammatory disease [11]. Kim *et al.* claimed that the expressions of TLR-2 and TLR-4 were both elevated in OA cartilage [12]. Meanwhile, multiple lines of evidence suggest that

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TLRs are concerned with MMP-13 expression. Ru *et al.* proposed that combined deficiency of TLR-2/TLR-4 attenuated induction of MMP-13 in mouse [13]. Another study found that selective TLR-9 agonist remarkably increased the gene expression of MMP-13 in both LS174 and SW620 cells [14]. Recent study also implied that the expression of TLR-1, TLR-2 and TLR-6 were all improved in septic chondrocytes accompanied by the up-regulation of MMP-13 [15].

With a view to the interaction of TLRs and MMPs as well as the relationship between MMPs and OA, we employed human chondrocyte to construct TLR-4 knockdown cell lines for the first time. After that we investigated the changes of MMP-13 and inflammatory cytokines expression to confirm the effects of TLR-4 on OA as a potential therapeutic target.

### Materials and methods

#### *Patients*

This investigation was approved by our local institutional ethics committee. Human OA cartilage was acquired from five patients (two males and three females, average age  $63.60 \pm 2.70$  years) underwent total knee replacement from February 2014 to November 2014. OA was diagnosed according to the American College of Rheumatology criteria. Patients were stopped taking non-steroidal anti-inflammatory drugs or steroids at least 2 weeks before surgery as well as intra-articular injection at least 1 month before surgery. All the samples were collected after receiving the written informed consents. All the operations in our study were conducted following to the approved guide line and regulations.

#### *Cell culture*

Cartilage cultures were performed according to the method described previously [3]. In brief, cartilage was cut into pieces and digested by 0.2% collagenase II (Sigma, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) at 37°C for 24 h. After resuspension, the chondrocytes were filtrated through a 0.075 mm cell strainer. Accordingly, the chondrocytes were centrifuged at  $132 \times g$  for 10 min and the pellets were gently resuspended in DMEM supplemented

with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA). Chondrocytes were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. After 2 weeks culturing with exchange of cell medium every 2 days, the first passage chondrocytes were harvested. All the experiments were performed within 3 days of passage 1 cells.

#### *Cell transfection*

Human chondrocytes were transfected with small interfering RNA (siRNA) utilizing Lipofectamine 3000 (Invitrogen, CA, USA) according to the manufacturer's instructions. The siRNA against TLR-4 (siTLR4, sc-40260) and none-silencing siRNA (siNC, sc-37007) were both purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The transfected cells were used after transfection for 48-96 h. The siNC was transfected as a negative control. Cells without transfection were acted as control.

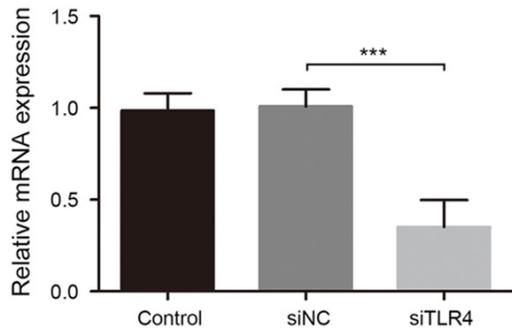
#### *Matrix metalloproteinase 13 (MMP-13) enzyme-linked immunosorbent assay (ELISA)*

The culture supernatants of human chondrocytes transfected with siTLR4 or siNC as well as of chondrocytes without transfection were collected after culturing for 24 h. The protein levels of MMP-13 were estimated by ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocols. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Richmond, CA, USA).

#### *Cell viability assay*

Cell viability assay was conducted with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the protocol described previously [16]. In brief, transfected and wild chondrocytes were respectively seeded into the 96-well plates with a density of  $1.5 \times 10^3$  cells/well and maintained for 0 d, 3 d and 7 d. Subsequently, 10  $\mu$ L of MTT solution (5 mg/mL, Sigma, St. Louis, MO, USA) was added to each well and incubated at 37°C for 4 h. Following addition of 150  $\mu$ L dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), cell viability was determined by measurement of the absorbance at 570 nm utilizing a microplate reader (Bio-Rad, Richmond, CA, USA).

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**Figure 1.** The difference of toll-like receptor 4 (TLR-4) expression in transfected chondrocytes. Chondrocytes were transfected with either specific small interfering RNA against TLR-4 (siTLR) or non-silencing small interfering RNA (siNC). After transfection, cells were collected for quantitative reverse transcription PCR (qRT-PCR). Data presented were the mean of five independent experiments. Error bars indicated standard deviation (SD). \*\*\*,  $P < 0.001$ .

### Quantitative reverse transcription PCR (qRT-PCR)

Total RNAs were isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and RNase-free DNase I (Promega, Madison, WI, USA) following the manufacturer's instructions. After that, cDNAs were synthesized using M-MLV First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The mRNA expression levels of TLR-4 were measured by qRT-PCR using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Primers were designed as follows: TLR-4-forward 5'-TGG ATA CGT TTC CTT ATA AG-3', reverse 5'-GAA ATG GAG GCA CCC CTT C-3'; GAPDH-forward 5'-CGC CCG CAG CCG ACA CAT C-3', reverse 5'-CGC CCA ATA CGA CCA AAT CCG-3'. Synthesis of primers was performed by Sangon Biotech Co., Ltd. (Shanghai, China). TLR-4 mRNA expression was estimated by the comparative threshold (Ct) using  $2^{-\Delta\Delta Ct}$  method [17]. GAPDH acted as an internal normalized reference.

### ELISA of inflammatory cytokines

The concentrations of various inflammatory cytokines in chondrocytes were detected using Multi-analyte ELISArray kits (SABiosciences Fredrick, MD, USA) followed the manufacturer's instructions. The relevant cytokines were tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon  $\gamma$  (IFN- $\gamma$ ) and interleukin 10 (IL-10). The absorbance was

measured at 450 nm by a microplate reader (Bio-Rad, Richmond, CA, USA).

### Statistical analysis

The results were presented as the mean  $\pm$  standard deviation (SD) except box-whisker plot. Measurement data were tested by one-way analysis of variance (ANOVA). Statistical calculations were processed by Graphpad Prism 5.0 software (GraphPad, San Diego, CA, USA). A statistical significance was defined when  $P < 0.05$ .

## Results

### SiTLR-4 down-regulated the expression of TLR-4

The mRNA expression level of TLR-4 in transfected chondrocytes were estimated by qRT-PCR. As shown in **Figure 1**, the mRNA expression of TLR-4 in chondrocytes transfected with siTLR4 were significantly reduced compared with chondrocytes transfected with siNC ( $P < 0.001$ ). There was no significant difference between control and chondrocytes transfected with siNC; thus, we concluded that TLR-4 knockdown chondrocytes were successfully obtained.

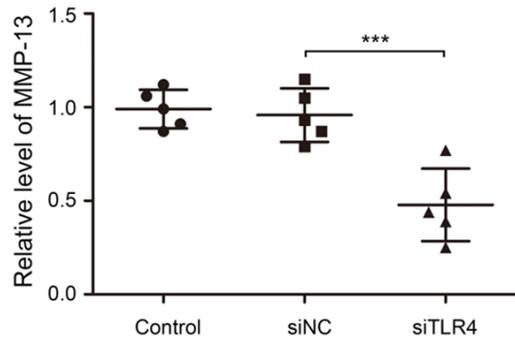
### Knockdown of TLR-4 decreased the level of MMP-13

MMP-13 production in culture supernatants was determined by ELISA. In comparison with chondrocytes transfected with siNC, the expression level of MMP-13 was remarkably decreased in TLR-4 knockdown chondrocytes ( $P < 0.001$ ) (**Figure 2**). Based on the difference at MMP-13 expression between control and chondrocytes transfected with siNC was not significant, we demonstrated that the knockdown of TLR-4 decreased the expression of MMP-13.

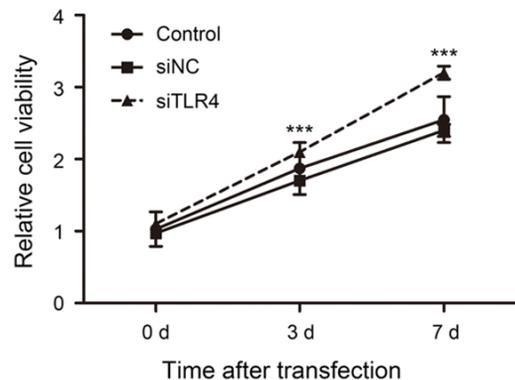
### Knockdown of TLR-4 promoted chondrocyte viability

Zero day, three days and seven days after transfection, the chondrocyte viability was determined by MTT assay. The **Figure 3** showed that TLR-4 knockdown markedly improved the cell viability compared with chondrocytes transfected with siNC ( $P < 0.001$ ). As comparison between control and chondrocytes transfected with siNC was not significant, we implied that knockdown of TLR-4 promoted chondrocyte viability.

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**Figure 2.** Influence of toll-like receptor 4 (TLR-4) on expression of matrix metalloproteinases 13 (MMP-13) in chondrocytes. Chondrocytes were transfected with either specific small interfering RNA against TLR-4 (siTLR) or non-silencing small interfering RNA (siNC). After transfection, cells were collected for determination of MMP-13 expression by enzyme-linked immunosorbent assay (ELISA). Data presented were the mean of five independent experiments. Error bars indicated standard deviation (SD). \*\*\*,  $P < 0.001$ .



**Figure 3.** Effect of toll-like receptor 4 (TLR-4) knockdown on chondrocyte proliferation. Chondrocytes were transfected with either specific small interfering RNA against TLR-4 (siTLR) or non-silencing small interfering RNA (siNC). At 0 d, 3 d and 7 d after transfection, cells were collected for the measurement of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data presented were the mean of five independent experiments. Error bars indicated standard deviation (SD). \*\*\*,  $P < 0.001$ .

### Knockdown of TLR-4 reduced the levels of inflammatory cytokines

Further studies on inflammatory cytokines were also conducted by ELISA. In **Figure 4A-C**, the concentration of TNF- $\alpha$ , IFN- $\gamma$  and IL-10 were all down-regulated in TLR-4 knockdown chondrocytes, in comparison with cells transfected with siNC ( $P < 0.05$  or  $P < 0.01$ ). The expression differ-

ence of these cytokines between control and chondrocytes transfected with siNC was not significant; therefore, we proposed that TLR-4 knockdown reduced the secretion of inflammatory cytokines.

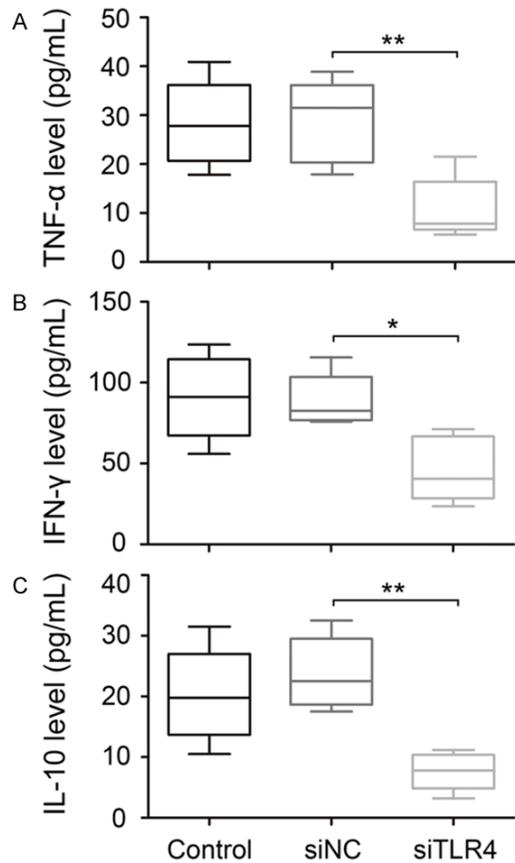
### Discussion

With a prevalence continues to rise, OA becomes a substantial public health burden. As the single cell type in the cartilage, chondrocyte plays essential role in the process of OA. Meantime, TLR-4 was proved to be expressed both at mRNA and protein levels in human chondrocytes [11]. Therefore, we utilized chondrocyte to construct TLR-4 knockdown cell lines via transfection with siTLR4. Subsequent ELISA assay suggested that the expression of MMP-13 was markedly suppressed in TLR-4 deficiency chondrocytes. Besides, detection of cell viability implied that TLR-4 knockdown significantly elevated the cell viability of chondrocytes. Finally, the ELISA assays focused on protein levels of TNF- $\alpha$ , IFN- $\gamma$  and IL-10 suggested that the expressions of these inflammatory cytokines were all down-regulated in TLR-4 deficient chondrocytes.

A large numbers of evidences supported that MMP-13 played a crucial role in OA. Previous studies proved that knockdown of histone deacetylase (HDAC) whose inhibitor acted as a depressor of cartilage degradation strongly suppressed MMP-13 expression in human chondrosarcoma cells. While the expression of HDAC and MMP-13 were both elevated in OA patients, the correlation between MMP-13 and OA was suggested to be existed [18]. Selective MMP-13 inhibitors were proved to completely block type II collagen degradation in bovine explants and 80% inhibition in human OA cartilage [19]. The OA cartilage erosions in MMP-13 deficient mice were markedly reduced [20]. Together, human articular chondrocytes, which ablated MMP-13 expression, were able to stabilize their ECM and impede their differentiation [21]. All the findings narrated above draw a conclusion that down-regulation of MMP-13 alleviates OA. The results in our study indicated that the expression of MMP-13 was down-regulated which emphasized the important effects of TLR-4 deficiency for OA.

OA is implicated with signaling mechanisms involved with stress- and inflammation-induced

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**Figure 4.** Influence of toll-like receptor 4 (TLR-4) knockdown on expressions of inflammatory cytokines in chondrocytes. Chondrocytes were transfected with either specific small interfering RNA against TLR-4 (siTLR) or non-silencing small interfering RNA (siNC). After transfection, cells were collected for the measurement of inflammatory cytokines including tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon  $\gamma$  (IFN- $\gamma$ ) and interleukin 10 (IL-10). Data presented were the median of five independent experiments. Error bars indicated minimum and maximum. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

factors. When suffered with extrinsic stress, the damaged cartilage matrix releases molecules into synovial fluid and implicated the release of proteolytic enzymes by synovial cells accompanied by recruitment of inflammatory cells [22, 23]. These released molecules act as damage-associated molecular patterns (DAMPs) and then activate TLRs, whose activation is followed by induction of inflammatory gene expression [24]. In allusion to TLR-4, the interaction between TLR-4 and its adaptors activate both myeloid differentiation primary response gene 88 (MyD88)-dependent and toll-receptor-associated activator of interferon (TRIF)-de-

pendent pathways [25, 26]. Nuclear Factor- $\kappa$ B (NF- $\kappa$ B) is located downstream of these two pathways. Once the NF- $\kappa$ B is activated, numerous pro-inflammatory cytokines are produced such as TNF- $\alpha$  [27]. Additionally, the NF- $\kappa$ B induces the chondrocyte differentiation towards hypertrophy [28]. The hypertrophic chondrocytes were suggested to express multiple phenotypic markers including MMP-13 and reduce the expression of type II collagen [29]. In our study, the TLR-knockdown “chopped off” the effects of DAMPS, thus suppressed the secretion of inflammatory cytokines, which were followed with reduced expression of MMP-13. Besides, previous study suggested that heat shock protein 70 (Hsp70) was released in response to TNF- $\alpha$  and then acted as a paracrine/autocrine inducer of IL-10 production in fibroblast-like synoviocytes (FLSs) [30]. This might be an explanation for the decrease of IL-10 along with decrease of TNF- $\alpha$ . Furthermore, the treatment options for OA were mainly focused on management of pain and swelling, and minimum the functional impairment for maintaining the quality of life [3]. If the TLR-4 was knockdown, the interactions between stimuli and inflammatory response were interrupted resulting alleviation of pain. Thus, the TLR-4 might be an effective therapeutic target for OA.

As the single cell type in the cartilage, death of chondrocyte has been proved to be involved with the initiation and severity of cartilage degradation [31]. Previous studies had lent support on the effects of inflammatory cytokines for the cell proliferation. Schuerwegh *et al.* studied various cytokines and concluded that TNF- $\alpha$  and IFN- $\gamma$  induced bovine chondrocytes proliferation [32]. Wang *et al.* showed that the apoptosis was facilitated in human chondrocytes with TNF- $\alpha$  stimulation [33]. Simultaneously, Kim *et al.* found that TLR4  $-/-$  mice showed low IFN- $\gamma$  along with attenuated arthritis [34]. Likewise, the secretions of TNF- $\alpha$  and IFN- $\gamma$  in our study were observed to down-regulated which might affect the proliferation of chondrocytes and thereby relieve the symptom of OA.

In conclusion, the knockdown of TLR-4 in chondrocytes improved the cell proliferation and reduced the expression of MMP-13 accompanied by decrease of inflammatory cytokines expressions. The results suggested that the

TLR-4 deficiency might be a potential therapeutic target on treatment of OA. Exact connection between vast types of inflammatory cytokines induced during OA and TLR-4 deficiency need to be further studied.

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### Disclosure of conflict of interest

None.

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