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Toll-like receptor 4 promotes high glucose-induced catabolic and inflammatory responses in chondrocytes in an NF-κB-dependent manner

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ABSTRACT

Diabetes is an independent risk factor for knee osteoarthritis (OA), and hyperglycaemia-induced inflammation is considered to play an important role in their connection. The Toll-like receptor 4 (TLR4) regulates inflammatory responses in several pathological conditions including diabetes and OA. However, its role in diabetes-associated OA is poorly understood. In this study, we found that TLR4 expression was higher in OA cartilage from patients with type 2 diabetes mellitus (T2DM) than that from non-T2DM patients. Similarly, its expression was induced in primary mouse chondrocytes treated with high glucose, which suggests that TLR4 upregulation in T2DM-associated OA cartilage may originate from hyperglycaemia stimulation. We further discovered that TLR4 promoted high glucose-induced catabolic and inflammatory responses in chondrocytes, and mechanistically, these effects could be explained by the exacerbated activation of the transcription factor nuclear factor kappa B (NF-κB) pathway, since its inhibition by Bay 11-7082 abrogated TLR4 effects on high glucose-treated chondrocytes. Taken together, these findings may reveal a promotive role of TLR4 in regulating hyperglycaemia-induced catabolism and inflammation in T2DM-associated OA, and also implicate that TLR4 inhibition might be of therapeutic significance in treating T2DM-associated OA.

1. Introduction

Osteoarthritis (OA) is the most common type of joint arthritis disease and also one of the leading causes of pain and disability worldwide [1]. OA is characterized by progressive degeneration of articular cartilage and signs and symptoms of inflammation, leading to structural and functional impairments of the joint [2]. The heritability, ageing, female gender and previous knee trauma have long been recognized as the major risk factors of OA [3]. Several recent studies have pointed out that the metabolic factors might also be involved in the pathophysiology of OA, including obesity, diabetes, hypertension and dyslipidemia [4]. The type 2 diabetes mellitus (T2DM) is a chronic metabolic disease associated with many complications, and accumulating epidemiological and experimental observations support that T2DM is an independent risk factor for OA progression [5–7].

However, the molecular mechanisms underlying the connection between OA and T2DM are less known. It's proposed that the local inflammation may be an important mechanistic link, since it plays a key role in both OA and T2DM diseases [8]. For example, the synovitis occurring in OA may be exacerbated by the increased level of inflammatory cytokines, adipokines and prostaglandins, which can also be observed in T2DM tissues [9]. Additionally, hyperglycaemia is the main trigger of joint degradation in OA and it also has tight connection with inflammation [10]. Local hyperglycaemia can disorganize the cartilage matrix by increasing the level of advanced glycation end products (AGEs), that in turn activate the production of catabolic and proinflammatory mediators, thereby leading to tissue remodeling, matrix stiffness and subchondral bone destruction [11,12]. Moreover, hyperglycaemia causes a low-grade systemic inflammation that may contribute to OA progression [13,14]. Thus, the hyperglycaemia-induced inflammation may underlie the OA phenotypes associated with T2DM.

The Toll like receptor 4 (TLR4) belongs to the family of pattern recognition receptors (PRRs) that are essential for the innate immune response in the host cells [15]. One study has reported that TLR4 mediates hyperglycemia-induced inflammation in macrovascular aortic endothelial cells [16]. Further, TLR4 has been associated with the regulation of inflammation in both OA and T2DM [17–19]. However, to

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date, its role in T2DM-associated OA is not investigated. In this study, we show the upregulation of TLR4 in knee OA cartilage from T2DM patients, and we demonstrate that TLR4 promotes high glucose-induced catabolic and inflammatory responses in chondrocytes through exacerbating the activation of the transcription factor nuclear factor kappa B (NF-κB) pathway, thus implicating TLR4 as a novel regulator of T2DM-associated OA.

2. Materials and methods

2.1. Antibodies and reagents

The antibodies and reagents utilized in this study were obtained from the following sources: anti-TLR4 (abcam, ab22048), anti-β-Actin (Proteintech, 66009-1-Ig), anti-COX-2 (Novus, NB100-868), anti-MMP13 (abcam, ab39012), anti-COL2 (abcam, ab34712), anti-p-IκBα (Cell Signaling, 2859), anti-IκBα (Cell Signaling, 9242), anti-p65 (abcam, ab16502), anti-H3 (Millipore, 06-755), goat anti-rabbit IgG-HRP (Santa Cruz, sc-2004), goat anti-mouse IgG-HRP (Santa Cruz, sc-2005), glucose (Sigma, G8270), CLI-095 (InvivoGen, tlrl-cli95), Bay 11-7082 (Sigma, B5556).

2.2. Clinical cartilage samples

The specimens of human knee articular cartilage were isolated from OA patients who received the total knee replacement at The First Affiliated Hospital of Jinan University. The knee OA diagnosis conformed to the American College of Rheumatology classification criteria [20]. A total number of 13 OA combined with T2DM patients were recruited in this study according to their medical files and drug prescriptions. In addition, 15 non-T2DM patients with matched age and Body Mass Index (BMI) were included in the study as controls. There was no significant difference in OA degree between these two groups according to the Osteoarthritis Research Society International (OARSI) assessment system [21]. The informed consent was obtained from each patient. The homogeneous isolated cartilage samples included all remaining cartilage zones and were resected as previously described [22]. The sampling procedures were performed in accordance with the terms of the Medical Ethical Committee of The First Affiliated Hospital of Jinan University.

2.3. Animals and chondrocyte isolation

Male C57BL/6J mice were used for primary chondrocyte isolation. Mice were maintained under specific pathogen-free conditions throughout. All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committees of The First Affiliated Hospital of Jinan University. The isolation of chondrocytes was processed as described previously [23]. Briefly, knee articular cartilage tissues were dissected from the joint surfaces and rinsed in sterile phosphate buffered saline (PBS). The cartilage tissues were cut into small pieces with a surgical blade, and then incubated with 1.5 mg/ml pronase (Sigma) for 2 h and digested in 2 mg/ml collagenase II (Sigma) with oscillation for 8 h at 37 °C. The cell suspensions were filtered by a 70 μm size strainer (BD). After cell counting, chondrocyte monolayers with a density of 20,000 cells/cm^2 were cultured in DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% L-glutamine. Culture plates were maintained in a humidified incubator at 37 °C with 5% CO_2 atmosphere.

2.4. Chondrocyte culture and treatment

For high glucose treatment, freshly isolated mouse primary chondrocytes were cultured in complete DMEM/F12 supplemented with different concentrations of glucose for 24 h or treated with 45 mM glucose for different time periods. The normal medium containing 5 mM glucose was used as control treatment. For the treatment of CLI-095 or Bay 11-7082, chondrocytes were treated with 1 μg/ml CLI-095 or 1 μM Bay 11-7082 for 24 h in culture medium containing varied concentrations of glucose, and equal volume of DMSO was added to serve as a control.

2.5. Quantitative reverse transcription PCR analysis

The total RNA was extracted from the homogenized cartilage tissues and chondrocytes with TRIzol reagent (Thermo Fisher Scientific, 15596026), and 3 μg total RNA were reverted into complimentary cNDA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1621) following the manufacturer's instructions. The quantitative reverse transcription PCR (qRT-PCR) analysis of the transcript levels of target genes was performed by SYBR green real-time PCR kit (TakaRa, RR420A) and 7500 Real-Time PCR System (Applied Biosystems). The transcript level of *Actb* gene was used as a normalization. Data were analyzed using the comparative Ct method. The primer pairs used for amplifying human and mouse genes are listed as follows: Human *TLR4* forward 5′-TGTACCCTTCTCACTGCCAG-3′, reverse 5′-CTGTTTCTGAGGAGGCTGGA-3′; Mouse *Tlr4* forward 5′-AGG CAGCAGGTGGAATTGTA-3′, reverse 5′-GGTCCAAGTTGCCGTTT CTT-3′; Human ACTB forward 5′-GGGAAATCGTGCGTGACATT-3′, reverse 5′-AGGTAGTTTCGTGGATGCCA-3′; Mouse Actb forward 5′-TAG GCGGACTGTTACTGAGC-3′, reverse 5′-GCCTTCACCGTTCCAGTTTT-3′.

2.6. Immunoblot analysis

For protein extraction, the cartilage tissues and chondrocytes were homogenized in RIPA lysis buffer supplemented with 1 tablet of protease inhibitors Cocktails (Sigma) for 20 min on ice. The whole lysates were centrifuged at 12,000*g* for 10 min at 4 °C. The supernatants were collected and denatured in 1 × SDS sample buffer for 10 min at 100 °C. Immunoblot was performed as described previously [24]. In brief, equal amount of total proteins for each sample were loaded and resolved by SDS-PAGE. Proteins were then transferred onto nitrocellulose (NC) membrane (Millipore), followed by 1 h block with 5% skimmed milk soluted in TBS supplemented with 0.1% Tween (TBST). NC membrane was probed overnight with primary antibodies at 4 °C. After wash with TBST for 4 times, NC membrane was incubated with secondary antibodies for 1 h at room temperature. After repeated wash with TBST, the enhanced chemiluminescence reagent (GE Healthcare, RPN2209) was added onto the NC membrane for visualizing protein bands with the ImageQuant LAS 4010 imaging system (GE). The images of protein bands were analyzed using ImageJ software.

2.7. Immunohistochemistry

The immunohistochemistry (IHC) staining was performed as previously documented [25]. Briefly, the cartilage sections were fixed with 4% formalin and embedded in paraffin. Cartilage sections (5 μm thickness) were prepared and stained with anti-TLR4 (1:200 dilution) or control nonimmune IgG antibody (1:200). After the reaction with diaminobenzidine (DAB) and counterstaining with hematoxylin, cartilage sections were visualized under a light microscopy. The IHC score was calculated with the following formula: [(value of weak-intensity pixels) (value of moderate-intensity pixels) (value of strong-intensity pixels)] / total value of pixels. The mean IHC score across 5 stochastic regions on each section was calculated and results relative to control were shown.

2.8. Adenovirus-mediated overexpression

The adenovirus expressing mouse *Tlr4* was constructed as described previously [26]. Mouse *Tlr4* cDNA was cloned into the pShuttle-CMV

vector. The empty vector was used a control. The constructed plasmids were transfected into human embryonic kidney (HEK) 293 cells using Lipofectamine 2000 (ThermoFisher Scientific). At 48 h after transfection, the generated adenovirus particles in supernatants were collected and purified using Adenovirus Purification Kits (Takara). The cultured chondrocytes were infected overnight with adenovirus solution in the presence of 4 μg/ml polybrene. The overexpression of TLR4 was confirmed by Immunoblot.

2.9. Cell viability

After treatment, chondrocytes were collected and washed with PBS for 3 times. The viability of chondrocytes was measured by trypan blue exclusion following the manufacturer's instructions (ThermoFisher Scientific, 15250061). Cell number was counted by a Countess Automated Cell Counter (Invitrogen). The trypan blue negative cells were considered as viable cells. The cell viability was calculated as the number of viable cells divided by the total number of cells. The results were expressed as relative to control (%). Each treatment was performed with 5 replicates.

2.10. ELISA assay

After treatment, the supernatants of chondrocyte culture medium were collected. The levels of IL-1β, IL-6, or PGE_2 in supernatants were quantified by using the commercially available PGE2, IL-6, and MMP-13 specific ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's instructions.

2.11. Luciferase reporter assay

The reporter plasmid pNF-κB-luc was used to evaluate the NF-κB activity, which includes the NF-κB enhancer consensus sequences [5 × (TGGGGACTTTCCGC)] as well as NF-κB-dependent firefly luciferase gene. One day before transfection, the mouse chondrocytes were seeded in 24-well plates, and at the next day, the chondrocytes were transiently transfected with pNF-κB-luc plasmids using the Lipofectamine 2000. In the meantime, 50 ng Renilla luciferase (pRL-null) plasmid were co-transfected for normalizing transfection efficiency. After treatment, cells were lysed, and the luciferase activity was measured using the Dual-Luciferase Assay System kit (Promega, E1960) according to the manufactures' instructions.

2.12. Statistics

All data are presented as means ± standard deviation (SD) from at least 3 independent replicates. Statistical comparisons were performed using unpaired Student's *t*-test or one-way ANOVA followed by Dunnett's test if more than two groups were analyzed simultaneously, with $P < 0.05$ being considered statistically significant.

3. Results

3.1. TLR4 is elevated in OA cartilage from patients with T2DM

To explore the possible role of TLR4 involved in T2DM-associated knee OA, we first measured the mRNA level of TLR4 in OA cartilage from patients with (OA-T2DM, $n = 13$) or without T2DM (OA, $n = 15$) by qRT-PCR analysis. The result showed that compared with that of OA group, the mRNA level of TLR4 was significantly higher in OA cartilage from OA-T2DM (Fig. 1A, $P = 0.0219$). In accordance with the expression change of its mRNA, the protein level of TLR4 was also found to be upregulated in OA cartilage from OA-T2DM, as determined by immunoblot analysis (Fig. 1B). Moreover, the upregulation of TLR4 was confirmed by immunohistochemistry staining on sections of OA cartilage from OA-T2DM, which predominantly displayed membrane-cytoplasmic distribution (Fig. 1C). Collectively, these results indicate that compared with that in OA cartilage from OA patients, TLR4 expression level is higher in OA cartilage from OA-T2DM patients, and also imply that TLR4 may play a role in T2DM-associated OA.

3.2. High glucose induces TLR4 expression in chondrocytes

Hyperglycaemia, the main pathophysiological feature of T2DM, induces pathological changes in cartilage [11]. We suspected that the TLR4 upregulation in OA cartilage from OA-T2DM patients may be associated with the stimulation of hyperglycaemia. To test this possibility, we utilized primary mouse chondrocytes cultured in vitro and treated them with high concentrations of glucose for imitating hyperglycaemia in diabetic patients. The treatment of high concentrations of glucose for 24 h, ranging from 15 mM to 45 mM, did not obviously affect the cell viability of chondrocytes (Fig. 2A), suggesting that these high concentrations of glucose have little toxicity on cultured chondrocytes. However, under these treatments, the mRNA level (Fig. 2B) and protein level (Fig. 2C) of TLR4 were both upregulated in chondrocytes in a dose-dependent manner. To know more about the inducible effect of high glucose on TLR4 expression, we treated chondrocytes with high glucose for up to 72 h. Likewise, longer exposure of chondrocytes to high glucose did not affect cell viability (Fig. 2D). Interestingly, the upregulation of TLR4 peaked at 24 h exposure, which to some extent declined thereafter until 72 h, but still remained higher than control treatment, at both mRNA and protein levels (Fig. 2E–F). These observations suggest that a negative feedback may exist to restrain the excessive upregulation of TLR4 when exposed to high glucose with a longer time. In any rate, these results together indicate that high glucose induces TLR4 expression in chondrocytes, at least in vitro, and hint that the TLR4 upregulation in OA cartilage from OA-T2DM patients may be associated with hyperglycaemia.

3.3. TLR4 promotes high glucose-induced catabolic and inflammatory responses in chondrocytes

High glucose has been shown to favor the catabolic program and inflammatory responses in chondrocytes [27,28]. Indeed, we found that high glucose induced catabolic program of chondrocytes, as shown by increased expression of COX-2 and MMP13 and decreased expression of COL2 (Fig. 3A). More importantly, TLR4 overexpression aggravated these changes, which vanished when chondrocytes were treated with its specific inhibitor CLI-095 [29], however TLR4 overexpression alone did not have similar effect in normal glucose-treated chondrocytes (Fig. 3A), suggesting that TLR4 promotes high glucose-induced catabolic program of chondrocytes. What's more, similarly, TLR4 overexpression also enhanced high glucose-induced production of pro-inflammatory cytokines, such as IL-1β (Fig. 3B), IL-6 (Fig. 3C), and PGE_2 (Fig. 3D), and these effects were abrogated by further treatment of CLI-095. Thus, in addition to promoting high glucose-induced catabolic program, TLR4 also displays inducible effect on inflammatory responses in chondrocytes exposed to high glucose.

3.4. TLR4 promotes NF-κB pathway activation in high glucose-treated chondrocytes

In order to understand how TLR4 exerts its role, we focused on interrogating the NF-κB pathway, since it mediates a plenty of biochemical responses to high glucose stimulation and is also a central regulator of catabolic and inflammatory responses in chondrocytes [30–33]. We found that high glucose treatment activated NF-κB pathway in chondrocytes, as evidenced by increased level of p-IκBα in the cytosol and elevated level of p65 in the nucleus (Fig. 4A). The activation of NF-κB pathway in high glucose-treated chondrocytes was further enhanced when TLR4 was overexpressed, however, which was completely abrogated in the presence of CLI-095 (Fig. 4A), suggesting

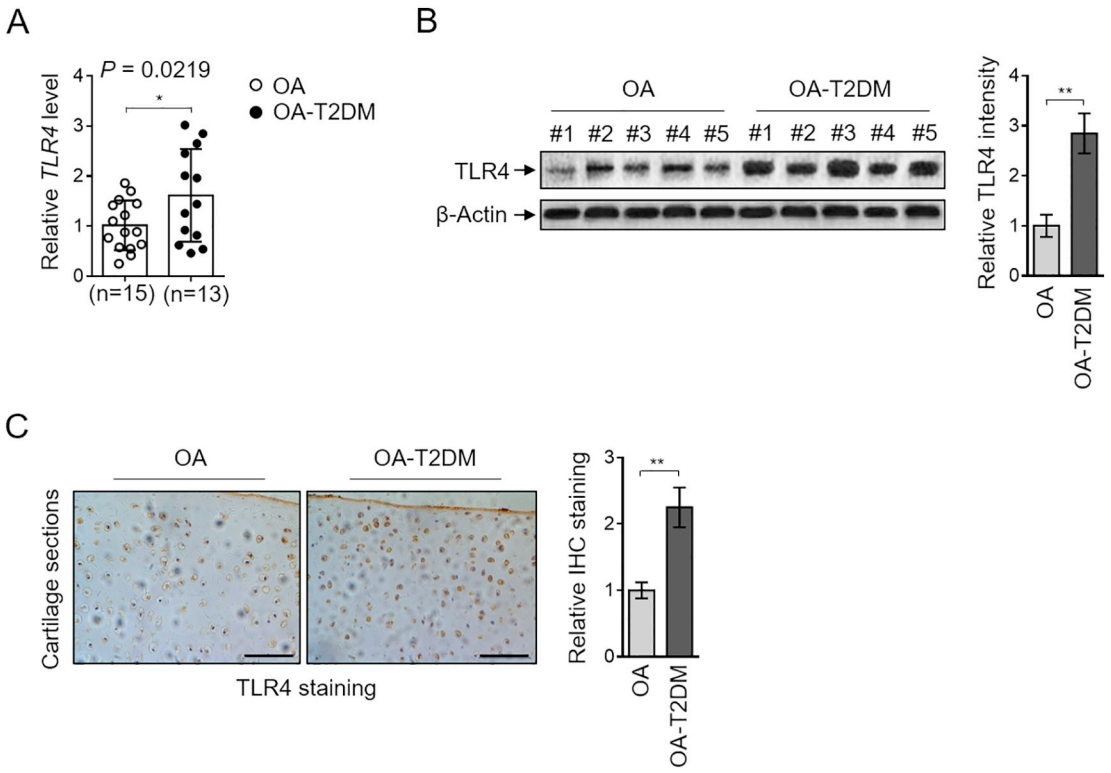

Fig. 1. TLR4 is upregulated in OA cartilage from T2DM patients.
(**A**) The mRNA level of *TLR4* in OA cartilage from patients with (OA-T2DM, $n = 15$) or without (OA, $n = 13$) T2DM was determined by qRT-PCR. The results relative to OA are shown. (**B**) The protein level of TLR4 in 5 representative OA cartilage samples from OA group and OA-T2DM group was determined by immunoblot. β-Actin was used as a loading control. The representative images (left) and band intensity of TLR4 relative to OA group (right) are shown. (**C**) The protein expression of TLR4 in cartilage sections prepared from OA group and OA-T2DM group was detected by immunohistochemistry (IHC). Scale bar, 50 μM. The quantification of IHC staining relative to OA group is also shown at right. Data are mean ± SD. Unpaired student's *t*-test. **, $P < 0.01$; *, $P < 0.05$.

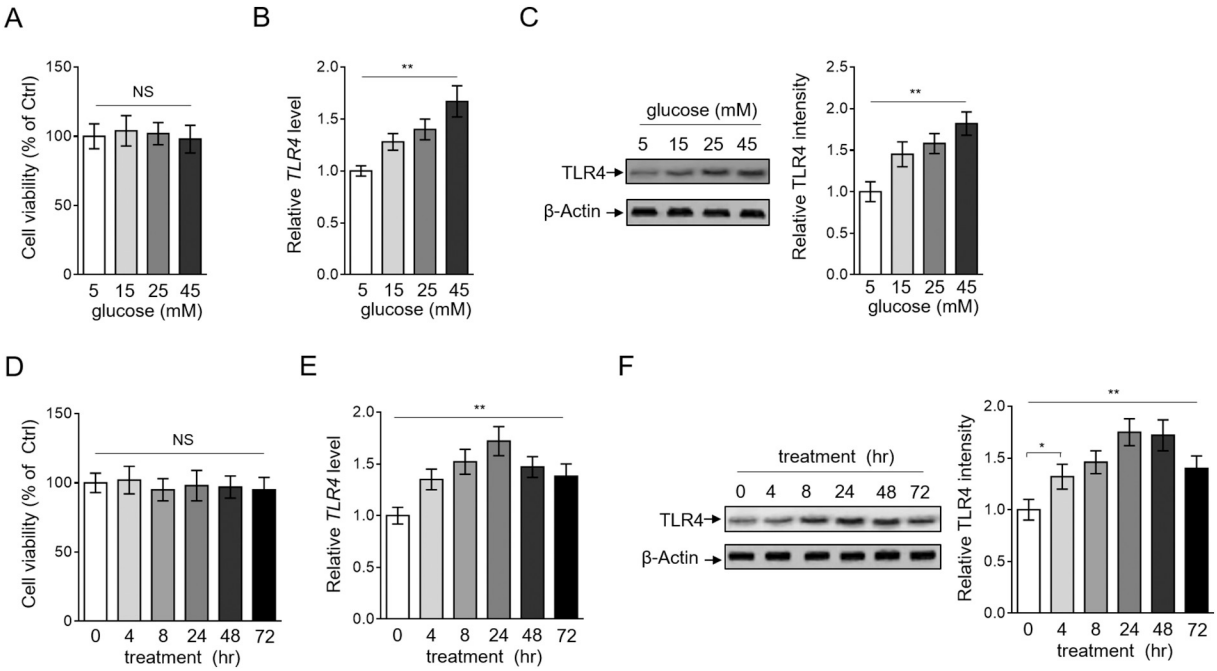

Fig. 2. TLR4 expression is induced in high glucose-treated chondrocytes.
(**A–C**) The primary mouse chondrocytes were cultured with different concentrations of glucose as indicated for 24 h. (**A**) The cell viability was measured by MTT assay. The results are expressed as relative to 5 mM glucose group (%). (**B**) The mRNA of *TLR4* in each group was determined by qRT-PCR. The results relative to 5 mM glucose group are shown. (**C**) The protein expression of TLR4 was determined by immunoblot. β-Actin was used as a loading control. The representative images (left) and band intensity of TLR4 relative to 5 mM glucose group (right) are shown. (**D–F**) The primary mouse chondrocytes were cultured with 45 mM glucose for different time periods as indicated. The cell viability (**D**), the mRNA level of *TLR4* (**E**), and protein level of TLR4 (**F**) were determined and analyzed as in (A-C). All data are mean ± SD. One-way ANOVA followed by Dunnett's test. **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

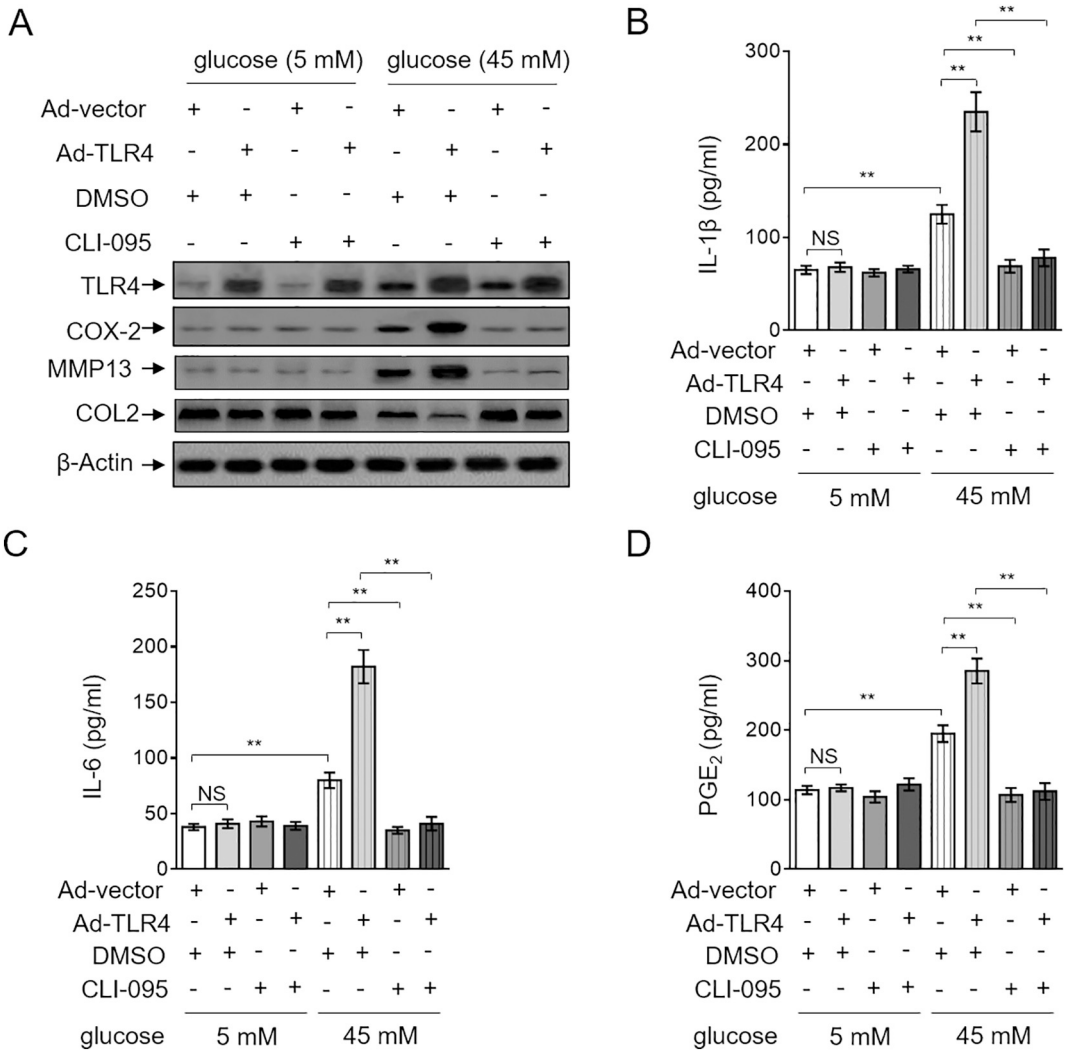

Fig. 3. TLR4 promotes high glucose-induced catabolic and inflammatory responses in chondrocytes.
(**A–D**) The primary mouse chondrocytes overexpressing vector control (Ad-vector) or *TLR4* (Ad-*TLR4*) were treated with DMSO or CLI-095 and cultured with 5 mM or 45 mM glucose for 24 h as indicated. (**A**) The protein expression of TLR4, COX-2, MMP13 and COL2 was determined by immunoblot. β-Actin was used as a loading control. The production of IL-1β (**B**), IL-6 (**C**) and PGE_2 (**D**) in the culture supernatants was measured by ELISA. All data are mean ± SD. Unpaired student's *t*-test. **, $P < 0.01$; NS, not significant.

that TLR4 promotes the activation of NF-κB pathway in high glucose-treated chondrocytes. This notion was further confirmed by luciferase reporter assay when transfected with pNF-κB-Luc (Fig. 4B). In sum, high glucose-induced NF-κB pathway activation can be amplified when chondrocytes were overexpressed with TLR4.

3.5. NF-κB inhibition abrogates TLR4-promoted catabolic and inflammatory responses in high glucose-treated chondrocytes

To elucidate the role of NF-κB pathway involved in TLR4-regulated catabolic and inflammatory responses in chondrocytes exposed to high glucose, the activation of this pathway was blocked by exploiting its specific inhibitor Bay 11-7082 [34]. As shown in Fig. 5A, the promoted activation of NF-κB pathway by TLR4 overexpression in high glucose-treated chondrocytes was efficiently inhibited by Bay 11-7082 treatment. Moreover, along with the suppressed NF-κB pathway activation, the TLR4 overexpression-induced catabolic program was correspondingly attenuated, as seen by the reversal of expression of COX-2, MMP13 and COL2 (Fig. 5A). Likewise, similar tendency of production of proinflammatory cytokines, including IL-1β, IL-6 and PGE_2, was observed when NF-κB pathway activation in high glucose-treated chondrocytes was inhibited by Bay 11-7082 (Fig. 5B–D). Taken together, these findings indicate that the TLR4-promoted catabolic and inflammatory responses in high glucose-treated chondrocytes is dependent on the activation of NF-κB pathway.

4. Discussion

Accumulating evidence indicates that diabetes is an independent risk factor of OA, and several models have been proposed to explain their enigmatic connection [35–37]. However, it has to be admitted that the complicated pathophysiology of these two related diseases hinders the discovery of molecular mechanisms which could account for the diabetes-associated OA phenotypes. To date, the effects engendered by hyperglycaemia toxicity are paid much attention, and a general paradigm based on the latest findings is described [11]. It's suggested that due to its local toxicity and systemic toxicity, hyperglycaemia could induce a plenty of biochemical events, such as formation of advanced glycation end products (AGEs), lo-grade inflammation, neuromuscular impairment and oxidative stress, thus causing matrix stiffness, abnormal subchondral bone, and chondrocytes/synoviocytes activation, thereby hyperglycaemia represents the main trigger of joint degradation in OA patients [11,38]. Nevertheless, much molecular evidence from mechanistic studies is needed to

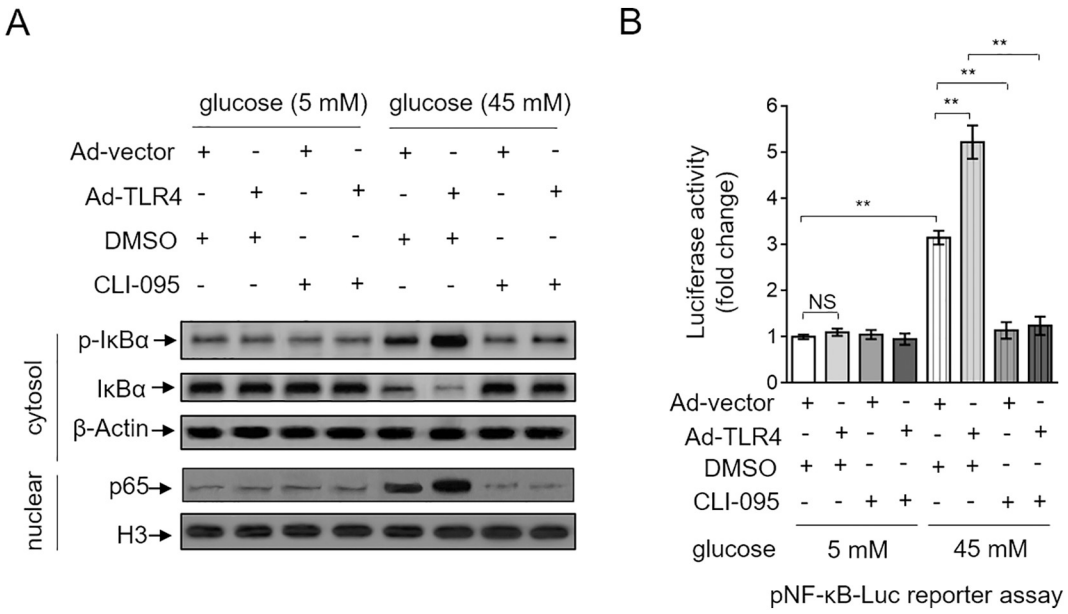

Fig. 4. TLR4 promotes NF-κB pathway activation in chondrocytes exposed to high glucose.
(**A**) The primary mouse chondrocytes overexpressing vector control (Ad-vector) or *TLR4* (Ad-*TLR4*) were treated with DMSO or CLI-095 and cultured with 5 mM or 45 mM glucose for 24 h as indicated. The protein expression of p-IκBα and IκBα in the cytosol (upper) and p65 level in the nuclear (lower) was determined by immunoblot. β-Actin and H3 were used as loading controls, respectively. (**B**) The primary mouse chondrocytes overexpressing vector control (Ad-vector) or *TLR4* (Ad-*TLR4*) were transfected with 5 × NF-κB-Luc plasmid, along with 50 ng Renilla luciferase (pRL-null) plasmid for normalizing transfection efficiency. At 24 h after transfection, cells were further treated with DMSO or CLI-095 and cultured with 5 mM or 45 mM glucose for 24 h as indicated. The firefly luciferase activity was measured and normalized to the Renilla luciferase activity. The results relative to Ad-vector in 5 mM glucose group are shown. All data are mean ± SD. Unpaired student's *t*-test. **, $P < 0.01$; NS, not significant.

support the role of hyperglycaemia in diabetes-associated OA. More importantly, elucidating the mechanisms of their association could help us to develop the disease-modifying drugs in the treatment of diabetes-associated OA [39]. In our present study, we uncover TLR4 as a novel regulator of high glucose-induced catabolic and inflammatory responses in chondrocytes, and mechanistically, we show that this regulation is executed in an NF-κB-dependent manner. Moreover, together with the finding that the higher TLR4 expression level was observed in OA-T2DM, these results implicate that TLR4 may be a regulator involved in the formation of diabetes-associated OA phenotypes associated with hyperglycaemia.

By examining clinical samples, we found that both the mRNA level and protein level of TLR4 were higher in cartilage tissues from OA-T2DM patients than those from OA patients without T2DM. However, caution should be mentioned here, as the recruited size of objects is limited. Preferably, future studies with larger sample size are warranted to consolidate this finding. Besides, whether this tendency could also be extended to patients with type 1 diabetes mellitus merits further investigations. We subsequently observed that similar to the findings obtained from clinical samples, high glucose induced the TLR4 expression in a dose-dependent manner. The pattern of the upregulation of TLR4 suggests a transcriptional induction in response to stimulation of high glucose. It has been reported that high glucose induces TLR4 in human monocytes via PKC-δ by stimulating NADPH [40]. The PKC-δ kinase appears to be involved in mediating the reactive oxygen species-dependent chondrocyte cell death [41,42]. Therefore, we suspect that oxidative stress aroused by high glucose is probably associated with the regulation of TLR4 expression in chondrocytes [22,43]. Nonetheless, whether the induction of TLR4 by high glucose in chondrocytes is also mediated by PKC-δ and NADPH stimulation is unknown at present. Interestingly, we also noticed that the extent of TLR4 induction declined when exposed to high glucose for a relatively longer time. We guess that a negative feed-back loop might exist that restrains the appearance of excessive expression of TLR4 under that condition. Addressing this issue may advance our understanding of the regulation of TLR4 under the exposure of high glucose.

We demonstrate that TLR4 exaggerates high glucose-induced catabolic and inflammatory responses in chondrocytes via promoting NF-κB pathway activation. With certain coincidence, TLR4 was found to mediate inflammatory responses in endothelial cells exposed to high glucose [44]. Although the activation of TLR4 in human and mouse chondrocytes has been demonstrated to induce inflammatory and catabolic responses in chondrocytes [45,46], we for the first time show the positive loop formed between high glucose and TLR4 that functions to aggravate high glucose-induced inflammatory and catabolic responses in chondrocytes. Notably, under normal culture conditions, TLR4 overexpression has no similar effects on inflammatory and catabolic responses in chondrocytes. We guess it is possible that there are no inflammatory responses initiated or no inflammatory mediators and ligands are excreted outside the extracellular space, where they are bound to activate TLR4. In this case, overexpression of TLR4 or not could engender no obvious effects. Instead, upon high glucose treatment, TLR4 overexpression could serve to aggravate the pre-existing inflammation and catabolism in chondrocytes.

The signals transduced by TLR4 and NF-κB pathway appear essential for these effects, since their blockade by specific inhibitors abrogates them. As known, the ligand-triggered TLR4 dimerization activates two major downstream signaling pathways, the MyD88-dependent and the MyD88-independent pathways, and then activates TNF-receptor associated factor 6 (TRAF6), which in turn propagates the central proinflammatory transcription factor NF-κB [47,48], thereby exerting pro-catabolic and pro-inflammatory effects. Future studies are needed to clarify which intracellular pathway downstream of TLR4 is connected to NF-κB activation under the treatment of high glucose.

In summary, this study suggests a TLR4-exaggerated and NF-κB-dependent promotive effect of high glucose on catabolic program and inflammatory responses in chondrocytes, which may underlie the pathophysiology of T2DM-associated OA (Fig. 5E). Based on the provided results, we propose that as a potential molecular regulator of T2DM-associated OA, TLR4 deserves further investigations, and it might

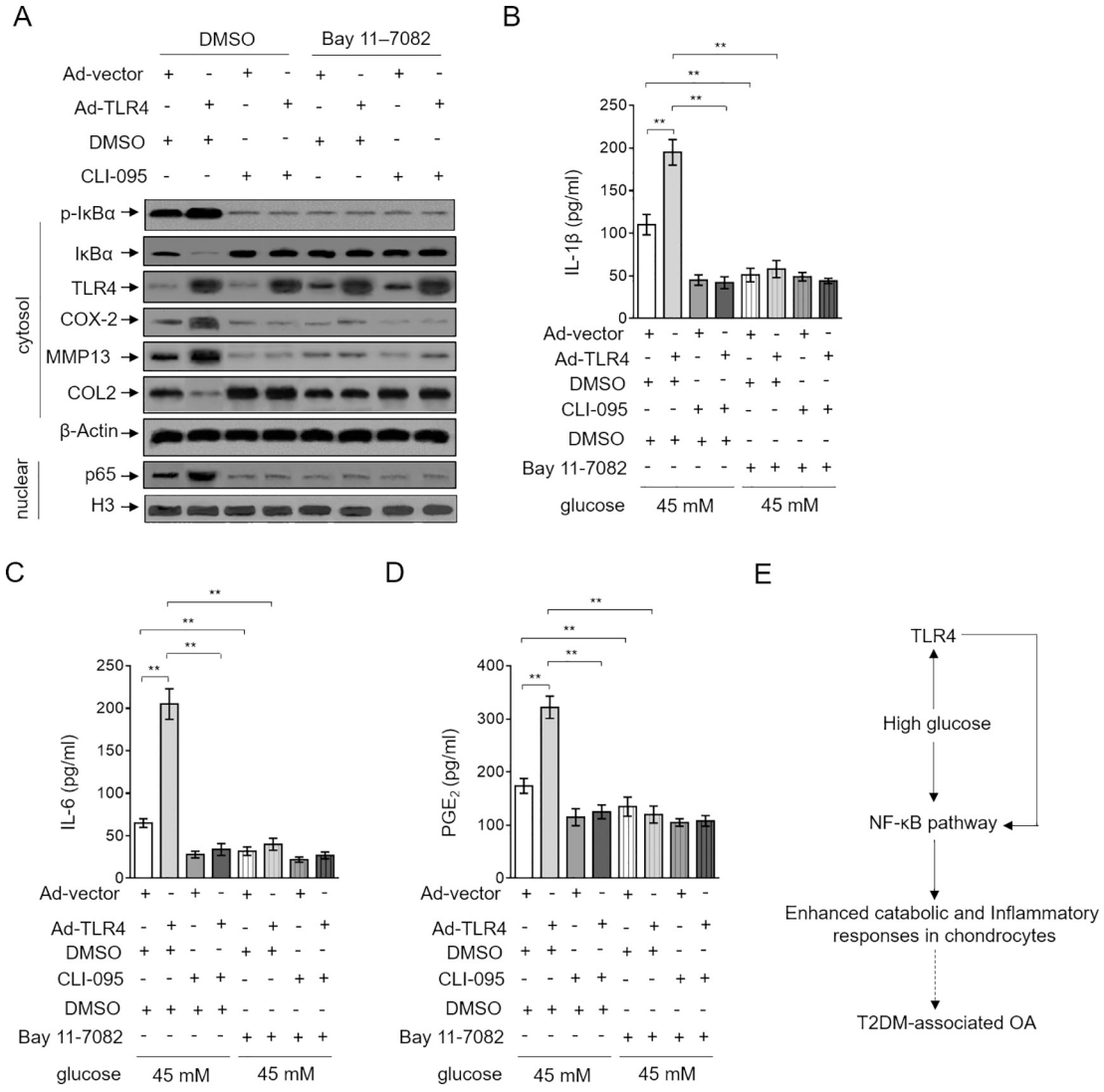

Fig. 5. TLR4-promoted catabolic and inflammatory responses are abrogated by NF-κB inhibition in high glucose-treated chondrocytes.
(**A–D**) The primary mouse chondrocytes overexpressing vector control (Ad-vector) or *TLR4* (Ad-*TLR4*) were treated with DMSO or CLI-095 and cultured with 45 mM glucose in the presence or absence of Bay 11-7082 for 24 h as indicated. (A) The protein expression of p-IκBα and IκBα in the cytosol (upper) and p65 level in the nuclear (lower) was determined by immunoblot. β-Actin and H3 were used as loading controls, respectively. The production of IL-1β (B), IL-6 (C) and PGE_2 (D) in the culture supernatants was measured by ELISA. (E) Proposed model of this study. All data are mean ± SD. Unpaired student's *t*-test. **, $P < 0.01$.

present a promising therapeutic target for interfering the progression of this disease.

Disclosure of conflict of interest

None.

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