

TNF- α increases inflammatory factor expression in synovial fibroblasts through the toll-like receptor-3-mediated ERK/AKT signaling pathway in a mouse model of rheumatoid arthritis

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Received November 16, 2017; Accepted March 9, 2018

DOI: 10.3892/mmr.2018.8897

Abstract. Osteoarthritis is a type of joint disease that may lead to other joint diseases. Previous research has demonstrated that tumor necrosis factor (TNF)- α is associated with osteoarthritis activity and pathology. The possible mechanisms of the TNF- α -mediated signaling pathway have not been clearly elaborated in synovial fibroblasts. The present study aimed to investigate the potential mechanisms of TNF- α in a mouse model of iodoacetate-induced osteoarthritis. Reverse transcription-quantitative polymerase chain reaction, ELISA, western blotting and immunohistochemistry were performed to evaluate the role of TNF- α in the progression of osteoarthritis. The results revealed that the serum levels of TNF- α , interleukin (IL)-1 β , IL-4 and IL-6 were significantly upregulated in a mouse model of iodoacetate-induced osteoarthritis compared with healthy mice ($P < 0.01$). TNF- α , IL-1 β , IL-4 and IL-6 mRNA and protein levels were also significantly upregulated in synovial fibroblasts in the experimental mice ($P < 0.01$). It was demonstrated that TNF- α increased pro-inflammation factors matrix metalloproteinase (MMP)-3, MMP-9, nuclear factor (NF)- κ B and receptor activator of NF- κ B ligand (RANKL) in synovial fibroblasts. It was also observed that the toll-like receptor (TLR)-3 was significantly upregulated and extracellular signal-regulated kinase (ERK) and protein kinase B (AKT) were significantly downregulated in synovial fibroblasts in osteoarthritis mice ($P < 0.01$). An *in vitro* assay demonstrated that TNF- α inhibitor decreased mRNA and protein levels of IL-1 β , IL-4 and IL-6 in synovial fibroblasts. The knockdown of TLR-3 abolished the TNF- α upregulated mRNA and protein levels of IL-1 β , IL-4 and IL-6 in synovial fibroblasts. In addition, the knockdown of TLR-3 also reversed TNF- α -upregulated ERK and AKT expression in synovial fibroblasts. *In vivo* assays demonstrated that TNF- α inhibitor

significantly decreased the deposition of IL-1 β , IL-4 and IL-6 as well as bone destruction and significantly increased the body weight and osteoarthritis score for osteoarthritic mice ($P < 0.01$). TNF- α inhibitor decreased TLR-3 and significantly increased the expression and phosphorylation of ERK and AKT in articular cartilage ($P < 0.01$). In conclusion the results of the present study indicate that TNF- α serves an essential role in synovial fibroblasts in osteoarthritis, suggesting that inhibition of TNF- α may decrease inflammation via the TLR-3-mediated ERK/AKT signaling pathway in a mouse model of monosodium iodoacetate-induced osteoarthritis.

Introduction

Knee osteoarthritis is a disease with the degenerative changes in the articular hyaline cartilage (1). The incidence of knee osteoarthritis is increasing due to prevalence in aging societies worldwide, which is characterized by degenerative disease that mainly caused by inflammation and dysfunction of synovial cells (2-4). A systematic review has given the overview of factors (age, body mass index, level of physical function and level of physical activity) related to patients with hip or knee osteoarthritis (5). The pathological reasons of osteoarthritis are complex in classification system manner and the cellular pathogenesis in joints (6). Previous study has indicated that inflammation is a variable feature of knee osteoarthritis, and is associated with joint symptoms and progression of this disease (7). A report has showed that decreasing of tumor necrosis factor (TNF)- α , interleukin (IL)-1 and IL-6 contents in joint fluid markedly improved symptoms in the rabbit with knee osteoarthritis (8).

A previous study has investigated the association between systemic and local inflammation and incident and progressive radiographic secondary osteoarthritis and results found that TNF- α inhibitor infliximab therapy is effective against hand osteoarthritis (9). Qin *et al* reported that TNF/TNFR signal transduction pathway-mediated anti-apoptosis and anti-inflammatory effects of sodium ferulate on IL-1 β -induced rat osteoarthritis chondrocytes (10). Study also identified that decreasing of circulating levels of TNF- α inhibited the development of osteoarthritis (11). Systemic blockade of STAT-3 can alleviate medial meniscus-induced osteoarthritis in mice (12). However, the associations between TNF- α and STAT3 pathway has not reported.

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Key words: osteoarthritis, inflammation, tumor necrosis factor- α , toll-like receptor-3, extracellular signal-regulated kinase/protein kinase B

Currently, toll-like receptor-3 (TLR-3) can regulate the STAT3 pathway in rheumatoid arthritis fibroblast-like synoviocytes (13). Evidences have demonstrated that TLR-3 is overexpressed in synovial tissue in patients with early rheumatoid, suggesting that TLR-3 signal pathway may associate with the persistent inflammation and joint destruction in this disease process (14). Zhu *et al* have found that pristane-induced arthritis rats presented higher TLR3 expression levels in the synovium, and increased the activity of the TLR3 signal pathway (15). Domagala *et al* have reported that inhibition of IL-1 β -induced activation of extracellular signal-regulated kinase (ERK) signal pathway showed benefits for the treatment of osteoarthritis (16). Klosowska *et al* have found that targeting of ERK/ protein kinase B (AKT) signal pathway reduced Fractalkine-induced osteoarthritis fibroblast migration via alterations of cytoskeletal structure in the pathologic processes of osteoarthritis (17). However, the relationships between ERK/AKT signal pathway and osteoarthritis have not well investigated, yet.

In the present study, we investigated the role of TNF- α -mediated TLR-3/ERK/AKT pathways in synovial fibroblasts in osteoarthritis model. We also analyzed the *in vivo* effects of inhibition of TNF- α activity in monosodium iodoacetate-induced osteoarthritis mice model.

Materials and methods

Ethics statement. This study was approved by Ethics Committee of the 309th Hospital of People's Liberation Army. All surgeries were performed under intravenous injection of sodium pentobarbital anesthesia (35 mg/kg).

Animals study. A total of 40 male C57BL/6 mice (25-32 g, 5-6 months of age) were purchased from Shanghai SLAC Laboratory Animals Co., Ltd (Shanghai, China). All mice were housed under controlled temperatures in a 12 h light/dark cycle with easy access to food and water. All mice were identified by ear punching and used to establish osteoarthritis mice model induced by monosodium iodoacetate (0.2 mg per mice; Sigma-Aldrich Co., St. Louis, MO, USA) as described previously (18). On day 10 after monosodium iodoacetate administration, mice were randomly assigned to two groups vehicle group (n=20) and TNF- α inhibitor group (n=20). Mice were received subcutaneous injection of vehicle (0.2 mg/kg/day) or TNF- α inhibitor (0.2 mg/kg/day; Lenalidomide, Sigma-Aldrich) for a total of 32 days. All mice were sacrificed on day 40 for histological analysis. Body weights of experimental mice were measured prior and post treatments on day 40. The osteonecrosis mice were anesthetized under IV pentobarbital anesthesia (35 mg/kg) and sacrificed by cervical dislocation on day 40 for further analysis.

Cells isolation and culture. Synovial fibroblasts were isolated from experimental mice as described (19) and cultured in DMEM medium with 10% fetal bovine serum (FBS; both Sigma-Aldrich) at 37°C and 5% CO₂ humidified atmosphere. Synovial fibroblasts (1x10⁶ cells/well) were seeded into six-well plates and treated by TNF- α (2 mg/ml), TNF- α inhibitor (2 mg/ml) or PBS (all Sigma-Aldrich) for 24 h at 37°C for RT-qPCR and western blot analysis.

Enzyme-linked immunosorbent assay (ELISA). Concentrations of TNF- α (MTA00B, Bio-Rad), IL-1 β (MLB00C), IL-4 (M4000B) and IL-6 (DY406; all Bio-Rad, Berkeley, CA, USA) concentrations in serum from experimental mice were analyzed using ELISA. TNF- α , IL-1 β , IL-4 and IL-6 were measured by using ELISA kits (Bio-Rad) according to the manufacturer's instructions. The results were performed by ELISA reader system (1775xMark™; Bio-rad).

Knockdown of TLR-3. Small interference RNAs (siRNA) for TLR-3 (siRTLRL-3) were synthesized by Ribobio Co., Ltd. (Guangzhou, China). siRTLRL-3 sense, 5'-CCUGAGCUGUCA AGCCACUACCUUU-3' and antisense, 5'-AAAGGUAGU GGCUGACAGCUCAGG-3'; siRcontrol sense, 5'-CCUGUC GAACUACCGCAUCCAGUUU-3' and antisense, 5'-AAA CUGGAUGCGGUAGUUCGACAGG-3'. Synovial fibroblasts (1x10⁶ cells/well) were seeded onto 6-well plates and transiently transfected with 120 nmol siRTLRL-3 with negative control (NC) siRNA as control using RNAi MAX (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturers' instructions. All experiments were performed in triplicate and further analysis was performed after a 48-h transfection. After a 48-h transfection, cells were then treated with TNF- α inhibitor (2 mg/ml), or PBS for 24 h at 37°C for further analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays. Total RNA was extracted from synovial fibroblasts using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturers' instructions. Extracted mRNA (1 μ g) was transcribed into cDNA at 42°C for 2 h using a reverse transcription kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. The cDNA (10 ng) was used for qPCR using the SYBR-Green Master Mix system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instrument. All primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) (Table I). After 120 sec incubation at 95°C, PCR was performed using 40 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and elongation at 72°C for 30 sec. Relative gene expression levels were calculated using the 2^{- $\Delta\Delta$ C_q} method (20). The results were presented as the n-fold change compared with β -actin using Quantiscan 2.1 (software Demo of AB QuantStudio™ 12K Flex System; Thermo Fisher Scientific, Inc.).

Western blot analysis. Western blotting was used to evaluate protein levels in this study as previously described (21). Briefly, a total of 1x10⁷ synovial fibroblasts were lysed in a lysis buffer containing 1% phenylmethanesulfonyl fluoride (Sigma-Aldrich) for 3 times of freezing-thawing and centrifuged at 8,000 x g for 10 min at 4°C. Concentration of proteins was measured by BCA kit (no. 23225; Thermo Fisher Scientific, Inc.). Proteins (20 μ g) were separated using the 12% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE). Then proteins were transferred onto proteins to polyvinylidene fluoride (PVDF) membrane (Millipore, Massachusetts, MA, USA). Then the nitrocellulose membrane was blocked with 5% (w/v) nonfat dry milk dissolved in tris-buffered saline

Table I. Sequences of primers for reverse transcription-quantitative polymerase chain reaction.

Gene	Sequence (5'-3')	
	Reverse	Forward
TNF α	CCTATGTCTCAGCCTCTTCT	CCTGGTATGAGATAGCAAAT
IL-1 β	GGCTGCTTCCAAACCTTTGA	GAAGACACGGATTCCATGGT
IL-6	GTGAGGAACAAGCCAGAG	TGACCAGAAGAAGGAATGC
IL-4	TACAGCCACCATGAGAAGGAC	TGATCGTCTTTAGCCTTTCCA
MMP-3	GCCCTGGAACCTCACACGACA	TTGGAACTCACACGCCAGAAG
RANKL	AAGGCGAGAGATTCTTTCCCTG'	ACTGGGGACAATTCACTAGAGC
MMP-9	CGGAGCACGGAGACGGGTAT	TGAAGGGGAAGACGCACAGC
NF- κ B	CCGAAGAACCATCCGA	CGGGAAGGACTTTATGTA
β -actin	CAAGAGATGGCCACGGCTGCT	TCCTTCTGCATCCTGTTCGGCA

TNF, tumor necrosis factor; IL, interleukin; MMP, matrix metalloproteinase; NF, nuclear factor; RANKL, receptor activator of NF- κ B ligand.

plus Tween-20 (TBST) solution for 2 h at 37°C and followed by incubation with primary rabbit anti-mouse antibodies: TNF- α (1:1,000; ab6671), IL-1 β (1:1,000; ab200478), IL-4 (1:1,000; ab9728), IL-6 (1:500; ab7737), PI3K (1:2,000; ab1678), ERK (1:2,000; ab196883), pERK (1:2,000; ab214362), AKT (1:1,000; ab8805), pAKT (1:1,000; ab133458), matrix metalloproteinase (MMP)-3 (1:1,000; ab53015) and MMP-9 (1:1,000; ab38898), receptor activator of NF- κ B ligand (RANKL; 1:1,000; ab216484), NF- κ Bp65 (1:1,000; ab32536) and β -actin (1:2,000; ab8226; all Abcam, Cambridge, UK) for 12 h at 4°C. HRP-conjugated goat anti-rabbit IgG mAb (1:5,000; PV-6001; ZSGB-BIO, Beijing, China) was added for 24 h at 4°C. A Ventana Benchmark automated staining system was used for analyzing protein expression (Olympus BX51; Olympus, Tokyo, Japan). Protein expression signal was analyzed by scanning densitometry using a Microtek ScanMaker 8700 (Zhongjing Technology Co. Ltd, Beijing, China) with ScanWizard 5 software (Informer Technologies, Inc., Walnut, CA, USA).

Tissue preparation and histopathologic analysis. The osteonecrosis mice were sacrificed on day 40 and the joints and articular cartilages were separated and fixed in 10% formalin. Paraffin-embedded joints and articular cartilages were cut into at a 4- μ m thickness sections. Tissue sections were stained with 5% hematoxylin and eosin (H&E) for histological evaluation. Safranin O-fast green and Toluidine blue staining was used to evaluate proteoglycans in the cartilage matrix. Tissue sections were also fixed in 10% formalin at 37°C for 24 h and decalcified using Gooding and Stewart's fluid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and subsequently embedded in paraffin. 4- μ m thick sections were stained with 0.05% toluidine blue (pH 4.1) and the severity of the degree of osteonecrosis was evaluated by the modified Mankin scoring system (22). The Mankin scoring system was scored as follows: 0, normal; 1, irregular surface; 2, pannus; 3, absence of superficial cartilage layers; 4, slight disorganization; 5, fissure into the calcified cartilage layer, and 6, disorganization. Histopathological evaluation was performed by two independent blinded observers.

Immunohistochemistry. The paraffinized joint tissue sections were heated in an oven at 65°C for 24 h, dewaxed to water and rinsed with PBS three times. The washed sections were placed in EDTA buffer (Beinuo Bioscience Inc., Shanghai, China), and then boiled at a low heat following an interval of 10 min at 65°C for a total of three intervals. Following natural cooling, the sections were washed with PBS three times, and were placed into 3% hydrogen peroxide solution (Beina Bioscience Inc.), for incubation at 37°C for 10 min, to block endogenous peroxidase. Free-floating sections were rinsed with PBS and placed in a solution containing primary mouse monoclonal antibodies directed against TNF- α (1:1,000; ab6671, Abcam), IL-1 β (1:1,000, ab200478), IL-4 (1:1,000, ab9728), IL-6 (1: 500; ab7737), PI3K (1:2,000; ab1678), ERK (1:2,000; ab196883), pERK (1:2,000; ab214362), AKT (1:1,000; ab8805), pAKT (1:1,000; ab133458; Abcam) at 4°C for 12 h. After rinsing, sections were incubated for 1 h at 37°C with the avidin-biotin-peroxidase complex (1:5,000 dilution; PV-6001; ZSGB-BIO). The sections were then washed with PBS and observed by fluorescent video microscopy (BZ-9000; Keyence Corp., Osaka, Japan).

Statistical analysis. Statistical analysis was completed using SPSS 19.0 statistical software (IBM SPSS, Armonk, NY, USA) with the assistance of Microsoft Excel (Windows 2010; Microsoft Corporation, Redmond, WA, USA). All data are expressed as the mean \pm standard deviation and experiments were performed three times. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison post hoc tests using Graph Pad Prism 5 software. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Inflammatory cytokines expression in iodoacetate-induced osteoarthritis mice. Inflammatory cytokines were investigated in osteoarthritis mice model. As shown in Fig. 1A, serum levels of TNF- α , IL-1 β , IL-4 and IL-6 were significantly upregulated in osteoarthritis mice compared to healthy mice ($P < 0.01$). Results demonstrated that TNF- α , IL-1 β , IL-4 and

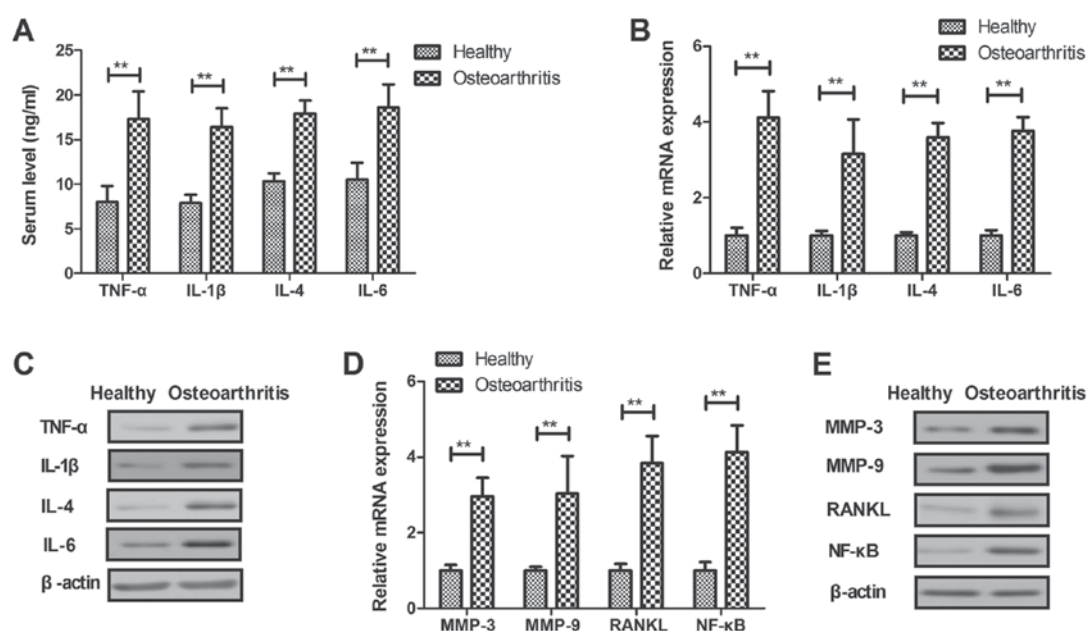


Figure 1. Inflammatory cytokines expression in osteoarthritis and healthy mice. (A) Serum levels of TNF- α , IL-1 β , IL-4 and IL-6 in osteoarthritic and healthy mice. (B) mRNA and (C) protein levels of TNF- α , IL-1 β , IL-4 and IL-6 in synovial fibroblasts in osteoarthritic and healthy mice. (D) mRNA and (E) protein levels of MMP-3, MMP-9, RANKL and NF- κ B in synovial fibroblasts in osteoarthritic and healthy mice. ** $P < 0.01$. TNF, tumor necrosis factor; IL, interleukin; MMP, matrix metalloproteinase; NF, nuclear factor; RANKL, receptor activator of NF- κ B ligand.

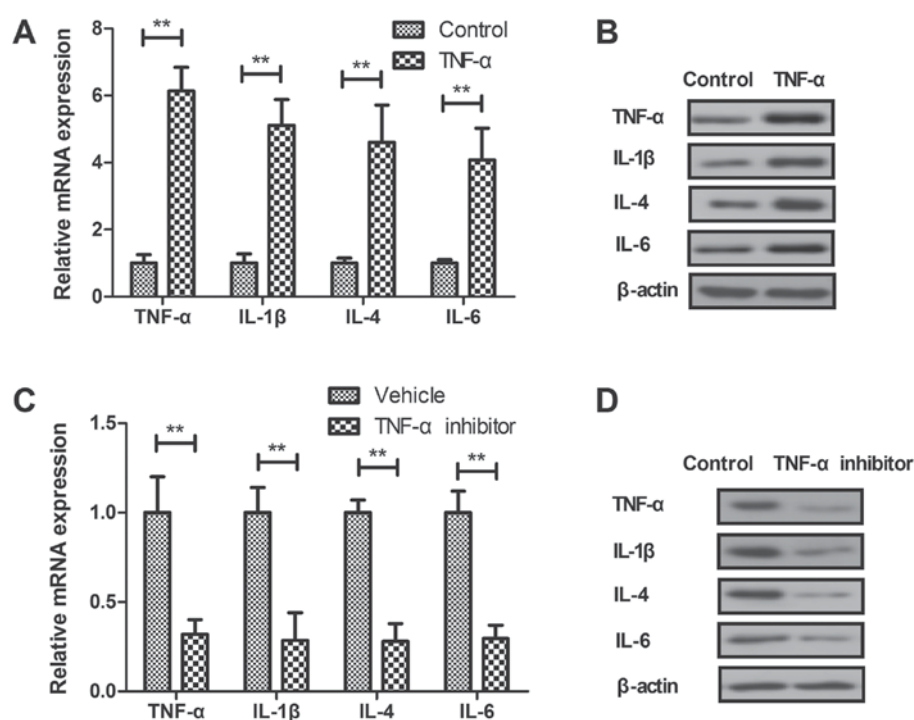


Figure 2. Effect of TNF- α on inflammatory cytokines expression levels in synovial fibroblasts. TNF- α increases TNF- α , IL-1 β , IL-4 and IL-6 (A) mRNA levels and (B) protein expression in synovial fibroblasts. TNF- α inhibitor decreases TNF- α , IL-1, IL-4 and IL-6 (C) mRNA levels and (D) protein expression in synovial fibroblasts. ** $P < 0.01$. TNF, tumor necrosis factor; IL, interleukin.

IL-6 mRNA and protein levels were significantly upregulated in synovial fibroblasts in osteoarthritis mice compared to healthy mice ($P < 0.01$, Fig. 1B and C). We observed that MMP-3, MMP-9, RANKL and NF- κ B mRNA and protein levels were significantly upregulated in synovial fibroblasts in osteoarthritis mice compared to healthy mice ($P < 0.01$, Fig. 1D and E). These results indicate that inflammatory

cytokines are markedly upregulated in serum and synovial fibroblasts in osteoarthritis mice model.

Effect of TNF- α on inflammatory cytokines expression levels in synovial fibroblasts. *In vitro* assay showed that TNF- α increased TNF- α , IL-1 β , IL-4 and IL-6 mRNA and protein levels were significantly upregulated in synovial fibroblasts

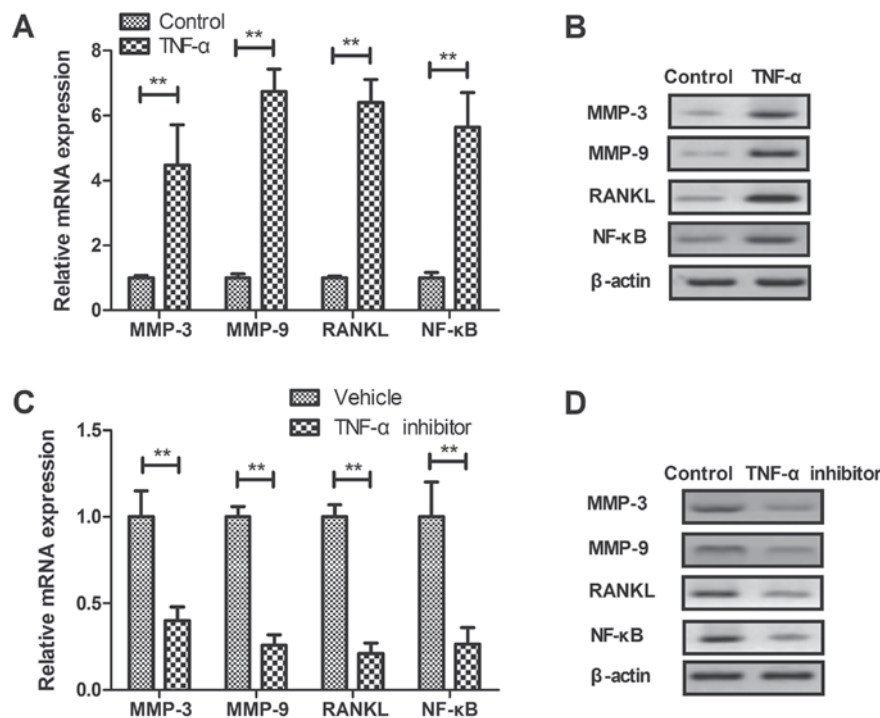


Figure 3. Effect of TNF- α on the expression of pro-inflammation factors in synovial fibroblasts. TNF- α treatment stimulates the (A) mRNA levels and (B) protein expression of MMP-3, MMP-9, RANKL and NF- κ B in synovial fibroblasts. TNF- α inhibitor decreases the (C) mRNA levels and (D) protein expression of MMP-3, MMP-9, RANKL and NF- κ B in synovial fibroblasts. ** $P < 0.01$. TNF, tumor necrosis factor; MMP, matrix metalloproteinase; NF, nuclear factor; RANKL, receptor activator of NF- κ B ligand.

(Fig. 2A and B). TNF- α inhibitor treatment downregulated TNF- α , IL-1, IL-4 and IL-6 mRNA and protein levels in synovial fibroblasts (Fig. 2C and D). These results indicate that TNF- α inhibitor can lead to downregulation of inflammatory cytokines expression levels in synovial fibroblasts *in vitro*.

Effect of TNF- α on expression of pro-inflammation factors in synovial fibroblasts. We investigated the effects of TNF- α on pro-inflammation factors in synovial fibroblasts. We showed that TNF- α treatment stimulated mRNA and protein expression levels of MMP-3, MMP-9, RANKL and NF- κ B in synovial fibroblasts ($P < 0.01$, Fig. 3A and B). TNF- α inhibitor treatment significantly decreased mRNA and protein expression levels of MMP-3, MMP-9, RANKL and NF- κ B in synovial fibroblasts ($P < 0.01$, Fig. 3C and D). These results suggest that TNF- α is a target for inhibition of pro-inflammation factors in synovial fibroblasts.

Effect of TNF- α on TLR-3-mediated ERK/AKT signal pathway. We investigated the regulatory effects of TNF- α on TLR-3-mediated ERK/AKT signal pathway in synovial fibroblasts. Results demonstrated that TLR-3, ERK and AKT expression levels were significantly upregulated in synovial fibroblasts in osteoarthritis mice compared to healthy mice ($P < 0.01$, Fig. 4A). TNF- α treatment increased TLR-3, ERK and AKT expression levels in synovial fibroblasts compared to control ($P < 0.01$, Fig. 4B). Conversely, TNF- α inhibitor significantly decreased TLR-3, ERK and AKT expression levels in synovial fibroblasts compared to control ($P < 0.01$, Fig. 4C). These results suggest that TNF- α can regulate TLR-3-mediated ERK/AKT signal pathway in synovial fibroblasts.

TNF- α regulates inflammatory factors expression via TLR-3-mediated ERK/AKT signal pathway. We further analyzed the possible mechanism mediated by TNF- α in synovial fibroblasts. Results revealed that knockdown of TLR-3 (siTLR-3) canceled TNF- α inhibitor-decreased expression and phosphorylation levels of ERK and AKT in synovial fibroblasts (Fig. 5A). TNF- α inhibitor-decreased TNF- α , IL-1 β , IL-4 and IL-6 expression levels were also abolished by siTLR-3 in synovial fibroblasts (Fig. 5B). Also, TNF- α inhibitor-decreased MMP-3, MMP-9, RANKL and NF- κ B expression levels were canceled by TLR-3 knockdown in synovial fibroblasts (Fig. 5C). These results indicate that TNF- α regulates inflammatory factors expression via TLR-3-mediated ERK/AKT signal pathway in synovial fibroblasts.

TNF- α inhibitor improves bone destruction in iodoacetate-induced osteoarthritis mice model. Finally, we further investigated the *in vivo* role of TNF- α inhibitor in the iodoacetate-induced osteoarthritis mice model. As shown in Fig. 6A, TNF- α inhibitor improved the osteoarthritis determined by osteoarthritis score. Results demonstrated that TNF- α inhibitor markedly downregulated bone destruction compared to control (Fig. 6B). TNF- α inhibitor also improved body weight for osteoarthritis mice compared to control (Fig. 6C). Inflammatory factors of TNF- α , IL-1, IL-4 and IL-6 expression levels were downregulated in cartilago articularis (Fig. 6D). TNF- α inhibitor decreased TLR-3 and increased expression and phosphorylation levels of ERK and AKT in cartilago articularis (Fig. 6E). These results suggest that TNF- α inhibitor presents many benefits for the treatment of osteoarthritis mice model.

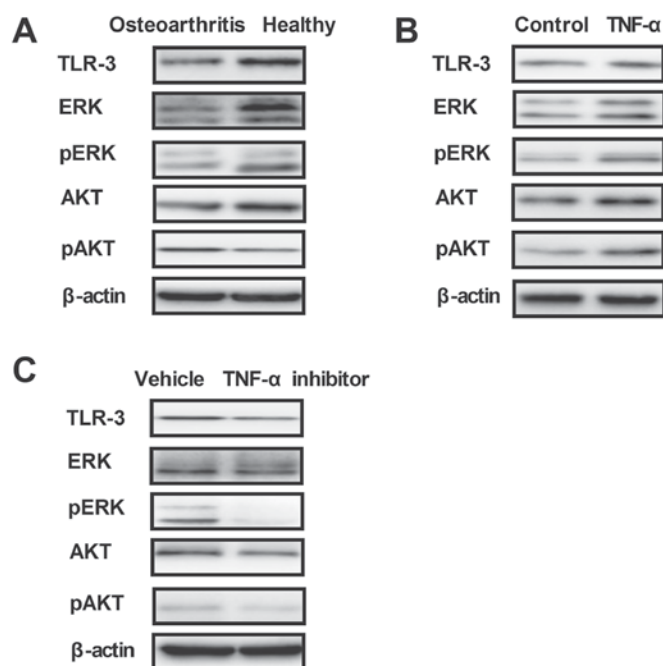


Figure 4. Effect of TNF- α on the TLR-3-mediated ERK/AKT signaling pathway. (A) Protein expression of TLR-3, ERK and AKT in synovial fibroblasts in osteoarthritic and healthy mice. (B) TNF- α treatment increases the TLR-3, ERK and AKT protein expression in synovial fibroblasts. (C) TNF- α inhibitor decreases the TLR-3, ERK and AKT protein expression in synovial fibroblasts. TNF, tumor necrosis factor; TRL, toll-like receptor; ERK, extracellular signal-regulated kinase; AKT, protein kinase B; p, phosphorylated.

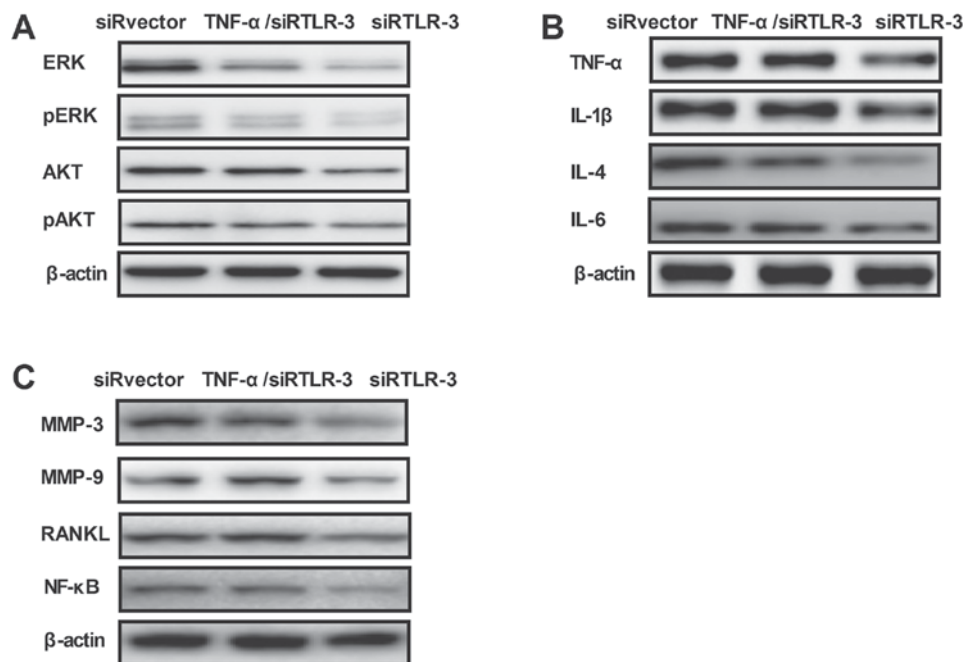


Figure 5. TNF- α regulates inflammatory factor expression via the TLR-3-mediated ERK/AKT signaling pathway. (A) Knockdown of TLR-3 reverses TNF- α inhibitor-decreased protein expression and phosphorylation of ERK and AKT in synovial fibroblasts. (B) Knockdown of TLR-3 abolishes TNF- α inhibitor-decreased TNF- α , IL-1 β , IL-4 and IL-6 protein expression in synovial fibroblasts. (C) Knockdown of TLR-3 abolishes TNF- α inhibitor-decreased protein expression of MMP-3, MMP-9, RANKL and NF- κ B in synovial fibroblasts. siRTLR-3, TLR-3 knockdown; TNF, tumor necrosis factor; MMP, matrix metalloproteinase; NF, nuclear factor; RANKL, receptor activator of NF- κ B ligand; TLR, toll-like receptor; ERK, extracellular signal-regulated kinase; AKT, protein kinase B; p, phosphorylated; IL, interleukin.

Discussion

In recent years, many reports have found that anti-TNF- α therapy has achieved the therapeutic effects for patients with osteoarthritis by targeting TNF- α in synovial fluid (9,23-25).

In this study, we analyzed the relationships between TNF- α and TLR-3-mediated ERK/AKT signal pathway in synovial fibroblasts in mice with osteoarthritis. Güler-Yüksel *et al* have indicated that treatment with TNF- α inhibitor infliximab could reduce hand osteoarthritis (9). Findings in this study indicate

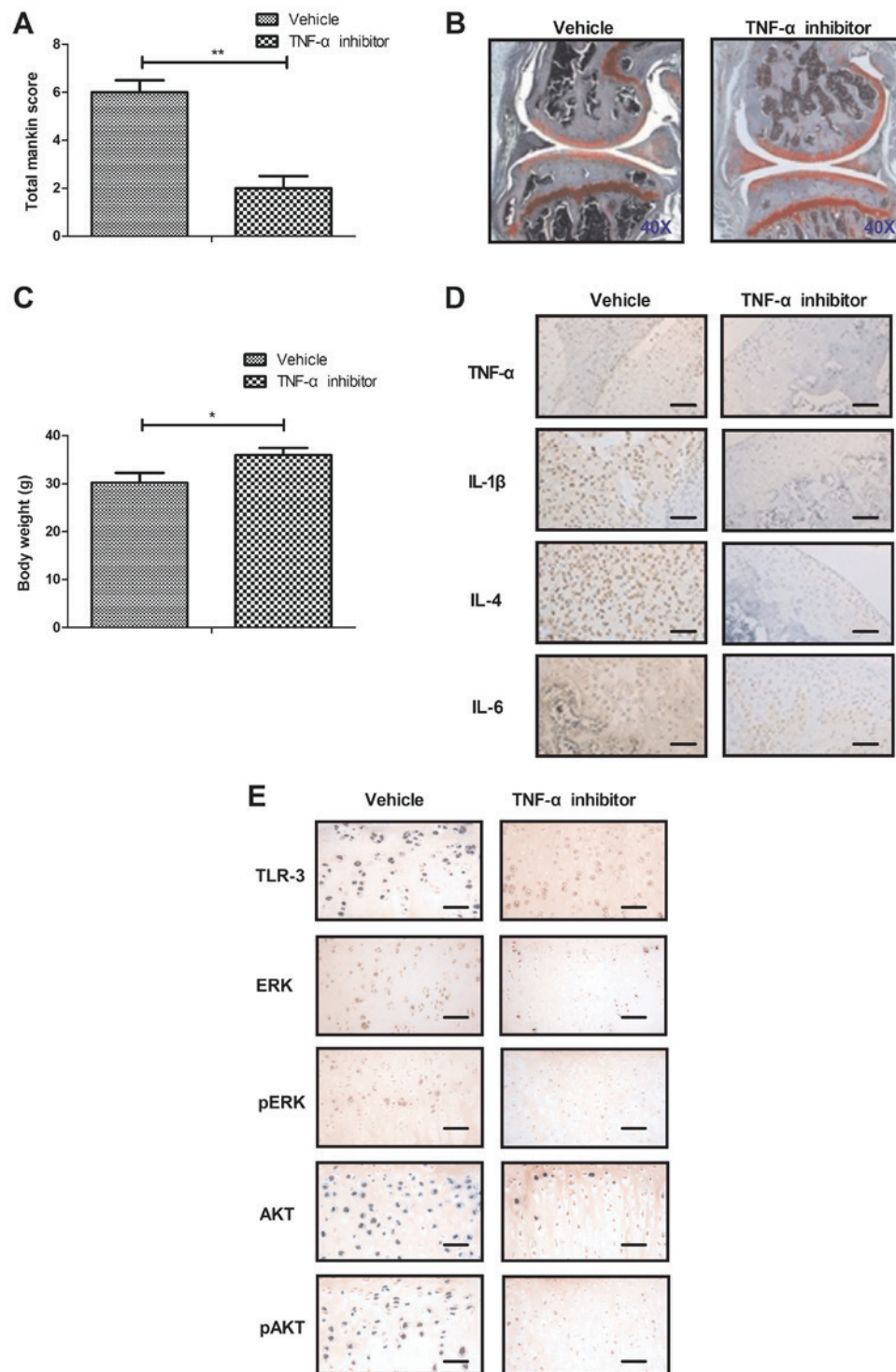


Figure 6. TNF- α inhibitor improves bone destruction in a mouse model of iodoacetate-induced osteoarthritis. (A) TNF- α inhibitor improves osteoarthritis as determined by the osteoarthritis score. (B) Representative articular tissue in vehicle and TNF- α inhibitor treated mice. TNF- α inhibitor downregulates bone destruction compared with the control. Magnification, x40. (C) TNF- α inhibitor improves body weight of osteoarthritis mice compared with the control. (D) TNF- α inhibitor decreases TNF- α , IL-1, IL-4 and IL-6 expression in articular cartilage. Scale bars, 20 μ m. (E) TNF- α inhibitor decreases TLR-3 and increases the expression and phosphorylation of ERK and AKT in articular cartilage. Scale bars, 20 μ m. *P<0.05 and **P<0.01. TNF, tumor necrosis factor; TLR, toll-like receptor; ERK, extracellular signal-regulated kinase; AKT, protein kinase B; p, phosphorylated; IL, interleukin.

that TNF- α upregulates the inflammatory cytokines and decreases ERK/AKT signal pathway in synovial fibroblasts in mice with osteoarthritis. Notably, we found that TNF- α increased TLR-3 expression in synovial fibroblasts in mice with osteoarthritis. Here, we reported that TNF- α can increase regulate inflammation in synovial fibroblasts via regulation

of TLR-3-mediated ERK/AKT signal pathway in mice with osteoarthritis both *in vitro* and *in vivo*.

Study has observed that osteoarthritis Marker YKL-39 is stimulated by IL-4 in differentiating macrophages (26). We reported that TNF- α increased IL-4 expression in synovial fibroblasts. Treatment with TNF- α inhibitor infliximab

treatment significantly reduced hand osteoarthritis in patients with rheumatoid arthritis (9). In addition, suppressing the release of synovial IL-1 β and TNF- α in knee osteoarthritis of rabbits may contribute to the treatment of osteoarthritis (27). Our results showed that TNF- α treatment upregulated IL-1 and IL-6 expression levels in synovial fibroblasts and in cartilage articularis in osteoarthritis mice model. Study has showed that TNF- α and MMP-3 correlated significantly with the swollen joint count (28). MMP-3 and MMP-9 expression levels were upregulated in osteoarthritis mice, which can be downregulated by TNF- α inhibitor (29). Evidences have showed that RANKL and NF- κ B expression levels were increased in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathy and osteoarthritis (30). We reported that TNF- α inhibitor significantly downregulated RANKL and NF- κ B in synovial fibroblasts and in synovial tissue in osteoarthritis mice. These results indicate that TNF- α is a mediator for inflammation in the processes of osteoarthritis mice. We will evaluate the effects TNF- α on P-38, P-65 and IRF-3 expression in synovial fibroblasts in our future work.

Currently, TLR-3 SNP is associated with knee osteoarthritis and increasing of TLR-3 levels has been observed in a Chinese Han population (31). We found that TLR-3 was upregulated and TNF- α inhibitor decreased TLR-3 expression levels in synovial fibroblasts. Domagala *et al* have suggested that inhibition of IL-1 β -induced activation of MEK/ERK pathway provided a potential mechanism for the treatment of osteoarthritis (16). Fu *et al* have showed that regulation of the PI3K/AKT signaling pathway could inhibit inflammation and chondrocyte apoptosis in a rat model of osteoarthritis (32). In this study, we found that TNF- α inhibitor downregulated TLR-3, ERK and AKT expression levels in synovial fibroblasts in mice model of osteoarthritis. Ballak *et al* have found that TLR-3 appears to play a redundant role in obesity-induced inflammation and insulin resistance (33). Additionally, TLR-3 mediated the synovial inflammation in rheumatoid arthritis (34). We reported that TNF- α regulates inflammation via TLR-3-mediated ERK/AKT signal pathway in synovial fibroblasts.

In conclusion, this study found that TNF- α inhibitor treatment not only decreased inflammatory factors, but also associated with osteoarthritis score in mice model. The pro-inflammatory cytokines in osteoarthritis, such as MMP-3, MMP-9, RANKL and NF- κ B, which participate in the pathogenesis of osteoarthritis, are downregulated by treatment of TNF- α inhibitor. Importantly, findings have indicated that TNF- α can regulate inflammation expression via TLR-3-mediated ERK/AKT signal pathway in synovial fibroblasts both *in vitro* and *in vivo*. Therefore, ERK/AKT may provide a novel potential target for osteoarthritis therapy. However, further reports need to elucidate the possible mechanisms mediated by TNF- α in the pathogenesis of osteoarthritis.

Acknowledgements

Not applicable.

Funding

The present study was supported by National Science Foundation (grant no. 30801159), the 12th five-year plan of the

military (grant no. 39770714) and Beijing municipal starting special (grant no. 2016-3-5071).

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

FYY, CQX, CLJ and JTS performed the experiments and analyzed the experimental data. XWH designed the study.

Ethics approval and consent to participate

The present study was approved by Ethics Committee of the 309th Hospital of People's Liberation Army.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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