Role of TLR4/MyD88/NF-κB signaling in the contrast-induced injury of renal tubular epithelial cells

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Abstract. The aim of the present study was to explore the role of toll-like receptor 4 (TLR4)/myeloid differentiation primary response 88 (MyD88)/nuclear factor (NF)-κB signaling in the contrast-induced injury of renal tubular epithelial cells, and to investigate the potential mechanisms. HK-2 cells cultured *in vitro* were randomly divided into six groups as follows: i) The blank group; ii) the iohexol group; iii) the NF-κB RNAi group (NF-κB siRNA + iohexol); iv) the TLR4 RNAi group (TLR4 siRNA + iohexol); v) the NF-κB blocker group (PDTC + iohexol); and vi) the TLR4 blocker group (CLI-095 + iohexol). The expression of the TLR4/MyD88/NF-κB signaling pathway proteins was detected by reverse transcription-quantitative (RT-q)PCR and western blot analysis, and the cellular proliferation rate was determined using the Cell Counting Kit-8 assay. The mRNA expression levels of the inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 were also detected using RT-qPCR, and apoptosis was assessed by flow cytometry and western blotting to detect apoptosis-associated proteins (caspase-3, caspase-9 and cleaved caspase-9). Compared with the blank group, the apoptotic rates and the expression levels of TLR4, MyD88, NF-κB, caspase-3, cleaved caspase-9, TNF-α, IL-1β and IL-6 were upregulated in the iohexol group ($P<0.001$). However, when TLR4 or NF-κB were blocked or silenced, these effects were reversed ($P<0.001$). Collectively, the results of the present study indicated that TLR4/MyD88/NF-κB signaling is involved in the contrast-induced injury of renal tubular epithelial cells by inducing inflammation and apoptosis.

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Introduction

Contrast-induced nephropathy (CIN) refers to acute kidney injury (AKI) that occurs following intravascular application of contrast medium (CM), in the absence of other causative factors. CIN accounts for 12% of all cases of iatrogenic AKI (1), and is the third leading cause after renal hypoperfusion and the use of nephrotoxic drugs. The morbidity rate of CIN is ~7.1% (2), and it may be higher among patients with acute coronary syndrome (3) or ST-segment elevation myocardial infarction (4). Although the risk of CIN has decreased due to technological advancements in recent years, the total number of cases remains high due to an increase in the number of patients requiring angiography. CM-induced decrease in renal perfusion and the direct toxic effect of CM on renal tubular cells are widely considered as the primary causes of CIN (5). It is also accepted that renal tubular obstruction, apoptosis, oxidative stress and immune inflammatory reactions are implicated in contrast-induced AKI (5-7). However, the molecular mechanisms of CIN are yet to be fully elucidated.

The toll-like receptors (TLRs) are important components of the immune response to pathogens, of which TLR4 is a vital regulator of the inflammatory response (8). TLR4-mediated downstream signaling pathways include the myeloid differentiation primary response 88 (MyD88)-dependent and the MyD88-independent pathways; the former primarily regulates the expression of a variety of inflammation-associated genes, which transmit intracellular signals through the TIR (Toll/IL-1 receptor) domain of MyD88. This activates transcription factors, such as nuclear factor (NF)-κB, thereby promoting the release of inflammatory factors, including interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α (9). Previous studies reported that, in a model of AKI, blocking the TLR4/NF-κB signaling pathway inhibited the expression of inflammatory factors and preserved renal function (10,11); however, the expression levels of signaling molecules upstream of NF-κB were not investigated.

Therefore, the aim of the present study was to explore the role of TLR4/MyD88/NF-κB signaling in an *in vitro* model of CIN by blocking the corresponding genetic loci of the associated signaling proteins, and to provide a new experimental basis for investigating the molecular mechanisms of CIN.

Materials and methods

Cell culture. Human renal proximal tubular cells (HK-2) were cultured in Dulbecco's modified Eagle's medium/Nutrient

Mixture F-12 (DMEM/F12; GE Healthcare Life Sciences) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere at 37˚C (5% CO_2). The medium was replaced every 2 days and the cells were passaged at ~80% confluence using trypsin (GE Healthcare Life Sciences). Between passages 4 and 6, cells in the logarithmic growth phase were transferred into 6-, 12- or 96-well plates. The cells were serum-starved by replacing the culture medium with serum-free DMEM/F12, and cultured for 12 h prior to the addition of iohexol (GE Healthcare Life Sciences).

Groupings. Serum-starved HK-2 cells were divided into the following groups: i) The blank group, cultured in serum-free DMEM/F12 only; ii) the iohexol group, where 100 mg/ml iohexol was added at 12 h post-serum starvation; iii) the NF-κB RNAi group; and iv) the TLR4 RNAi group, both of which were transfected with the corresponding siRNAs and treated with 100 mg/ml iohexol; v) the pyrrolidine dithiocarbamate (PDTC) group; and vi) the CLI-095 group, in which, 2 h prior to iohexol treatment, the cells were treated at room temperature with 100 μmol/l PDTC (Sigma; Merck KGaA) or 3 μmol/l CLI-095 (InvivoGen). The corresponding indicators mentioned below were detected in all groups after 48 h of iohexol treatment.

Small interfering (si)RNA transfection. The siRNAs were designed and synthesized by Shanghai GenePharma Co., Ltd., and subsequent experiments were conducted following confirmation of a gene-silencing efficiency of ≥80% (Fig. 1). Negative control was used for RNA interference and the sequences were as follows: Forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. The siRNA sequences were as follows: NF-κB forward, 5'-GCA CCAUCAACUAUGAUGATT-3' and reverse, 5'-UCAUCA UAGUUGAUGGUGCTT-3'; and TLR4 forward, 5'-GGAAUG AGCUAGUAAAGAATT-3' and reverse, 5'-UUCUUUACU AGCUCAUUCCTT-3'. Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) was incubated for 5 min at room temperature with moderate serum-free DMEM/F12, and was added to the diluted siRNA solution, which was diluted with an equal volume of serum-free medium (20 pmol siRNA per 2 μl Lipofectamine solution). The mixture was then co-cultured with cells seeded into 6-well plates ($1x10^5$ cells/well) overnight, to a final concentration of 40 nmol/l for both siRNAs.

Cell Counting Kit-8 (CCK-8) assay. Cells in the logarithmic growth phase were seeded into 96-well plates at a density of $5x10^3$ cells/well; each group contained six wells, including the A0 group, which was incubated with DMEM/F12 only. Following grouping, CCK-8 reagent (10 μl; Dojindo Molecular Technologies, Inc.) was added to each well, and the plates were incubated for 2 h at 37˚C in the dark. The absorbance at 450 nm was then determined by Synergy Mx (Bio Tek, Inc.).

Flow cytometry. The Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (Nanjing KeyGen Biotech Co., Ltd.) was used to detect apoptosis by flow cytometry. Cells were seeded into 12-well plates ($5x10^4$ cells/well), and the treated cells were collected, centrifuged for 5 min at 4˚C (350 x g) and washed three times with cold PBS. The cells were resuspended in 500 μl binding buffer and 5 μl Annexin V-FITC was added. After 5 min of incubation in the dark at room temperature, 5 μl PI was added and incubated under the same conditions for 10 min. Finally, apoptosis was detected by CytoFLEX (Beckman, Inc.) and analyzed using CytExpert 2.3 (Beckman, Inc.).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from treated HK-2 cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.). Reverse transcription was performed with 1 μg total RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio, Inc.) according to the manufacturer's protocol. The cDNA was then amplified using the Custom gene RT-qPCR Quantitation Kit (Shanghai GenePharma Co., Ltd.) as per the manufacturer's instructions; the primer sequences are listed in Table I. The RT-qPCR thermocycling conditions were as follows: Reverse transcription, one cycle at 95˚C for 3 min; qPCR, 40 cycles at 95˚C for 12 sec and 62˚C for 40 sec. The mRNA expression levels were quantified with the Bio-Rad CFX Manager (Bio-Rad Laboratories, Inc.) using the $2^{-\Delta\Delta Cq}$ method (12), with GAPDH as the reference gene.

Western blotting. Treated HK-2 cells were washed twice with cold PBS, and lysed on ice for 1 h with RIPA lysis buffer containing 1 mM PMSF (both from Beyotime Institute of Biotechnology). Total protein was quantified using the Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Protein samples (40 μg/lane) were separated by SDS-PAGE using a 10% gel, and transferred onto PVDF membranes (Thermo Fisher Scientific, Inc.), which were subsequently blocked with 5% skimmed milk for 2 h at room temperature. The membranes were probed with the following primary antibodies at 4˚C overnight: Anti-caspase-3 (1:1,000; cat. no. 19677-1-AP; ProteinTech Group, Inc.), anti-caspase-9 and cleaved (c)-caspase-9 (1:1,000; cat. no. 10380-1-AP; ProteinTech Group, Inc.), anti-NF-κB p65 (1:1,000; cat. no. 10745-1-AP; ProteinTech Group, Inc.), anti-MyD88 (1:1,000; cat. no. 23230-1-AP; ProteinTech Group, Inc.), anti-TLR4 (1:800; cat. no. 19811-1-AP; ProteinTech Group, Inc.) and anti-β-actin (1:5,000; cat. no. ab8227; Abcam). After washing thrice with PBS-T at room temperature, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (1:8,000; cat. no. ab 97051; Abcam) at room temperature for 1 h. Finally, the treated membranes were visualized using BeyoECL Star reagent (Beyotime Institute of Biotechnology) according to the manufacturer's instructions, and then quantified using Quantity One 1-D Analysis Software Version 4.6.8 (Bio-Rad Laboratories, Inc.) with β-actin as the loading control.

Statistical analysis. All statistical analyses were conducted using SPSS version 22.0 (IBM Corp.) and all data are presented as the mean ± standard deviation. Variations between groups were statistically assessed using a Student's t-test or one-way analysis of variance followed by Tukey's multiple comparisons test, as appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

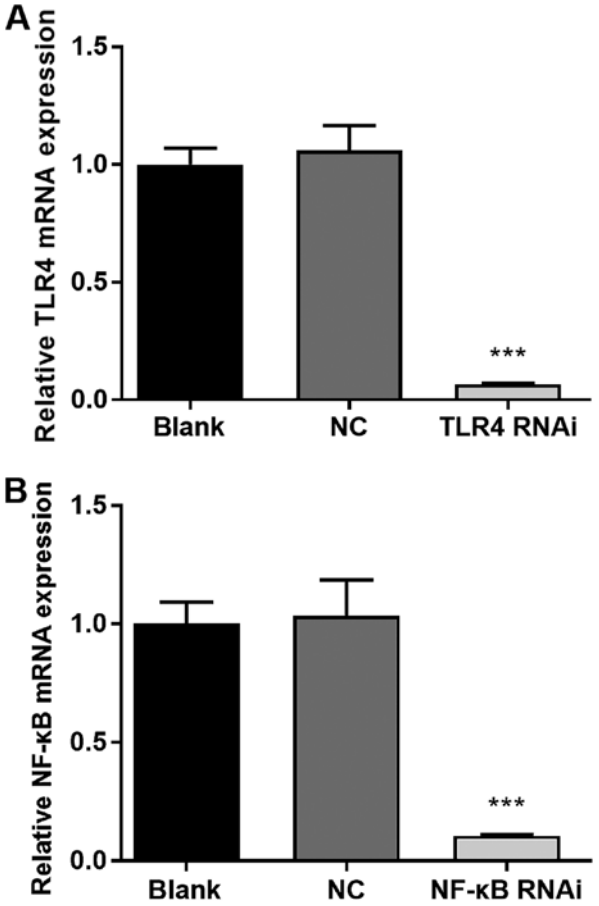

Figure 1. siRNA transfection achieves a high level of gene silencing efficiency. (A) NC group or TLR4 RNAi group: Using Lipofectamine® 3000, HK-2 cells were transfected with NC or TLR4 siRNA, respectively, to a final concentration of 40 nmol/l; blank group: Lipofectamine® 3000 alone. (B) NC group or NF-κB RNAi group: Using Lipofectamine® 3000, HK-2 cells were transfected with NC or NF-κB siRNA, respectively, to a final concentration of 40 nmol/l; blank group: Lipofectamine® 3000 alone. After 48 h, cells from each group were harvested and the relative expression levels of NF-κB or TLR4, respectively, were determined by reverse transcription-quantitative PCR. ***P<0.001 vs. the NC group. siRNA, small interfering RNA; NC, negative control; NF-κB, nuclear factor κB; TLR4, toll-like receptor 4.

Table I. Primers designed for reverse transcription-quantitative PCR analysis.

mRNA	Primer pairs (5'-3')
GAPDH	Forward: AAAATCAAGTGGGGCGATGC
	Reverse: GATGACCCTTTTGGCTCCCC
TLR4	Forward: GTCTCCTCCACATCCTCCCT
	Reverse: CTCCCAGAACCAAACGATG
MyD88	Forward: GTCTCCTCCACATCCTCCCT
	Reverse: CAGTTGCCGGATCTCCAAGT
NF-κB	Forward: TTGGGAATGGTGAGGTCACTCTAAC
	Reverse: TCTCCTGTCACCGCGTAGTCG
TNF-α	Forward: TTCTGCCTGCTGCACTTTGGAG
	Reverse: AGGGCTGATTAGAGAGAGGTCCCTG
IL-1β	Forward: AGCACCTTCTTTCCCTTCATCTTTG
	Reverse: CATAAGCCTCGTTATCCCATGTGTC
IL-6	Forward: GCCAGAGCTGTGCAGATGAGT
	Reverse: TGGCATTTGTGGTTGGGTCAG

TLR4, toll-like receptor 4; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; IL, interleukin.

Results

Iohexol stimulates the expression of TLR4, MyD88 and NF-κB. After 48 h of iohexol stimulation, the mRNA and protein expression levels of TLR4, MyD88 and NF-κB were significantly increased compared with those in the blank group (Fig. 2A and B; P<0.001). The corresponding expression levels were markedly lower following NF-κB or TLR4 silencing or blocking, respectively, compared with the iohexol group (P<0.001).

Blocking or silencing the TLR4/MyD88/NF-κB signaling pathway alleviates the inhibition of HK-2 cell proliferation induced by iohexol. The CCK-8 assay was used to evaluate HK-2 cell proliferation. As shown in Fig. 3, the proliferation rate of the blank group was considered to be 100%, and the proliferation rates of the treatment groups (%)=$(A_x-A_0)/(A_c-A_0)^*100\%$ (A_x, absorbance value of other groups; A_0, absorbance value of the A0 group; A_c, absorbance value of the blank group). After a 48-h incubation period with iohexol, the proliferation rate of the iohexol group was significantly reduced (P<0.001). Following NF-κB- or TLR4-RNAi inhibition, or treatment with PDTC or CLI-095, the proliferation rates were found to be higher compared with those of the iohexol treatment group (P<0.001), albeit lower compared with those of the blank control group (PDTC group: P<0.05; CLI-095, NF-κB RNAi and TLR4 RNAi groups: P<0.001, compared with the blank group).

Blocking or silencing the TLR4/MyD88/NF-κB signaling pathway inhibits iohexol-induced HK-2 cell apoptosis. The apoptotic rate of the iohexol group (14.71±2.55%) was significantly higher compared with that of the other groups (P<0.001), and there were no significant differences in the apoptotic rates of the PDTC (4.21±1.93%), CLI-095 (3.64±0.90%), NF-κB RNAi (5.80±0.72%) or TLR4 RNAi groups (5.42±0.54%) compared with that of the blank group (3.29±1.11%; P>0.05) (Fig. 4A). The expression levels of apoptosis-related proteins were directly associated with the corresponding apoptotic rates. Although there was no significant difference in the expression of caspase-9 amongst the groups, the expression levels of caspase-3 and c-caspase-9 were significantly increased in the iohexol group, compared with the blank group (P<0.001), and decreased in the other groups compared with the iohexol group (P<0.001; Fig. 4B).

Blocking or silencing TLR4/MyD88/NF-κB signaling attenuates iohexol-induced inflammation. As presented in Fig. 5, the mRNA expression levels of TNF-α, IL-1β and IL-6 in the iohexol group were significantly higher compared with those in the blank group (P<0.001). After blocking or silencing of the TLR4 or NF-κB loci, the expression levels were significantly decreased compared with those of the iohexol group (P<0.001).

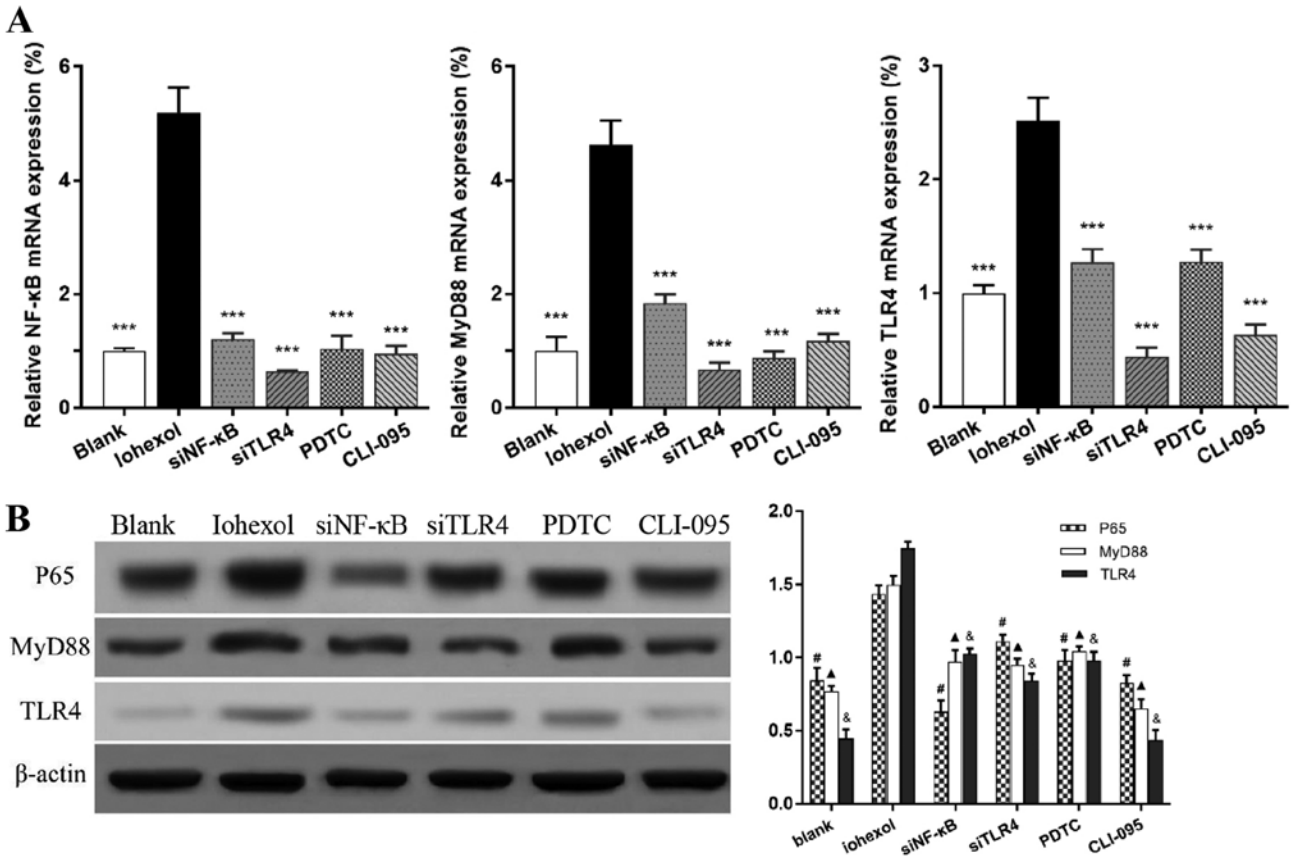

Figure 2. mRNA and protein expression levels of TLR4, MyD88 and NF-κB. (A) The mRNA expression levels of the blank group were considered as 100%. ***P<0.001 vs. the iohexol group. (B) Protein expression of TLR4, MyD88 and NF-κB in each group was quantified using Quantity One software, with β-actin as the internal control. #P<0.001, ▲P<0.001 and &P<0.001 vs. the iohexol group. TLR4, toll-like receptor 4; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor κB.

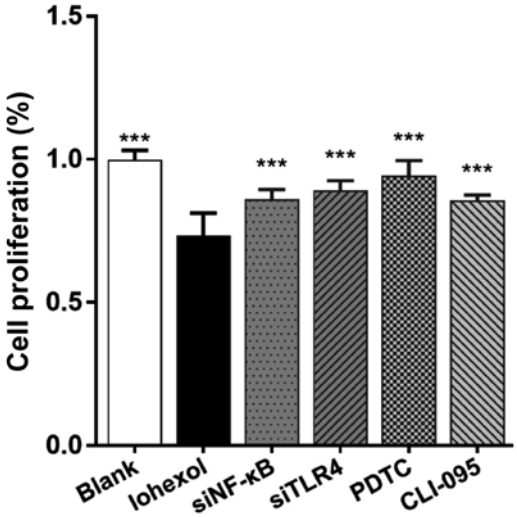

Figure 3. Change of proliferation rate in each group. Cell Counting Kit-8 assay was used to determine the proliferation rate. ***P<0.001 vs. the iohexol group.

Discussion

CIN is defined as an increase in serum creatinine of >25% or 44 μmol/l, compared with the baseline value, within 3 days of using CM and in the absence of any other causes of renal injury (13). The specific pathogenesis of CIN has not been completely elucidated, although it is widely accepted that immune inflammatory responses play an important role in the occurrence and development of CIN. Numerous studies have confirmed that the expression of specific cytokines is elevated during CIN, and it was reported that immune inflammatory responses in a CIN rat model were alleviated by NF-κB silencing (14).

TLR4, which is expressed in renal intrinsic cells (such as epithelial cells), endothelial and mesangial cells, is one of the key factors of the inflammatory response. TLR4 activation transduces transmembrane signals via the MyD88-dependent pathway, activating transcription factors such as NF-κB, and promoting the subsequent release of a variety of cytokines and inflammatory factors (15). TLR4 signaling is initiated by the recognition and binding of pathogen-associated molecular patterns and other specific ligands, including lipopolysaccharide, taxol, fusion protein, envelope proteins, heat shock proteins, oligosaccharides of hyaluronic acid, polysaccharide fragments of heparan sulfate and fibrinogen (16). A previous report verified that, in an animal model of ischemia-reperfusion injury, the gene expression and protein synthesis of TLR4 in renal tubular epithelial cells was significantly upregulated (17). Additionally, Pulskens *et al* (18) reported that, in an animal model of acute ischemic kidney injury, the inflammatory response and tubular damage were alleviated in TLR4-knockout mice compared with wild-type mice.

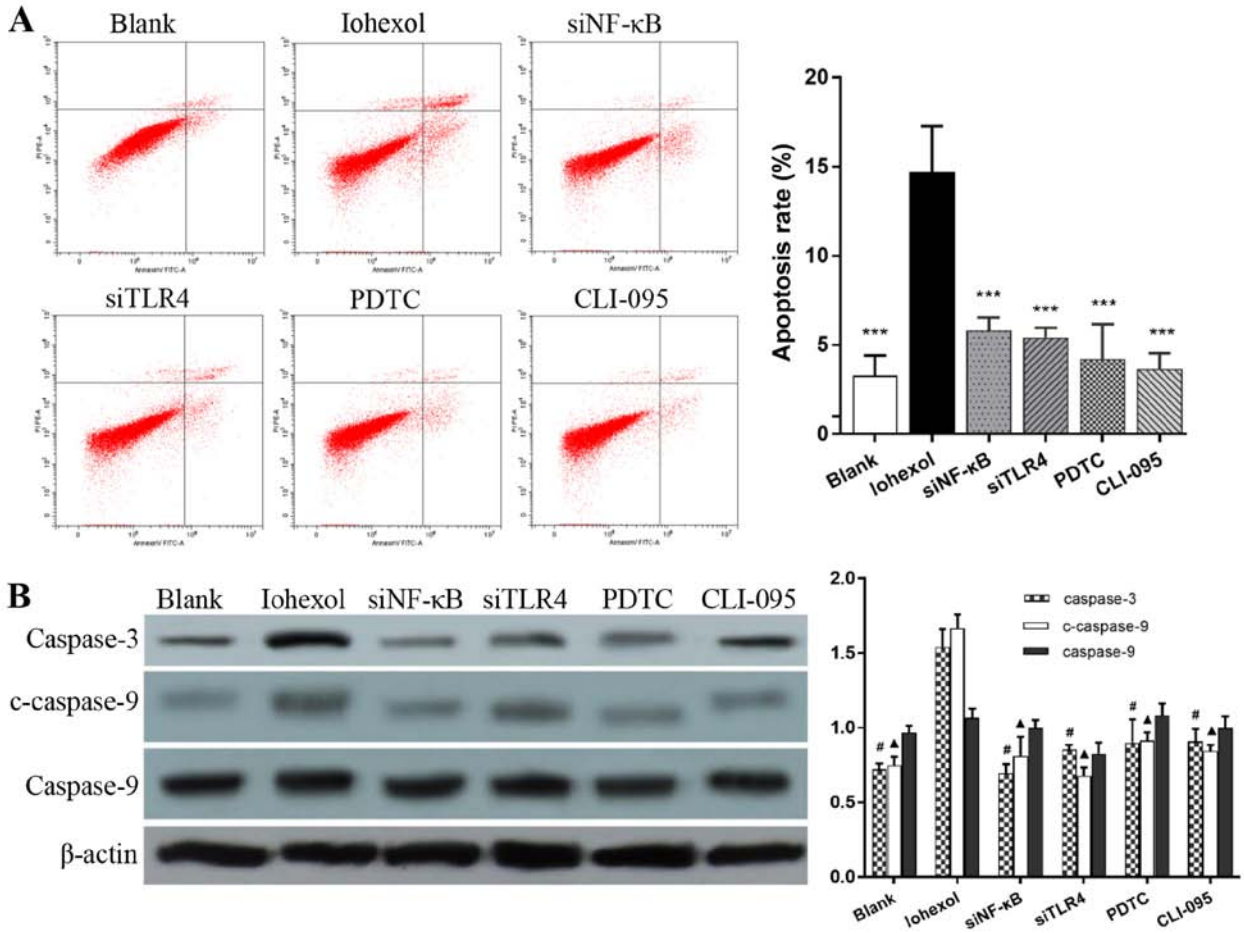

Figure 4. Apoptotic rates and expression levels of apoptosis-associated proteins. (A) HK-2 cell apoptotic rates were determined by flow cytometry using an Annexin V-FITC/PI Apoptosis Detection kit, and analyzed using CytExpert software. ***P<0.001 vs. the iohexol group. (B) The protein expressions levels of caspase-3, caspase-9 and c-caspase-9 in each group were quantified using Quantity One software, with β-actin as the internal control. #P<0.001, ▲P<0.001 vs. the iohexol group. PI, propidium iodide; c-, cleaved.

PDTC is a commonly-used NF-κB inhibitor that reduces the expression and transcriptional activity of all NF-κB subunits (19). It was previously reported that PDTC prevented the degradation of inhibitory factor B-α and inhibited the nuclear translocation of NF-κB, thus reducing the occurrence of acute and chronic inflammation (20). Borghi *et al* (21) found that PDTC reduced the levels of serum urea and creatinine, and mitigated diclofenac-induced AKI. CLI-095 (also known as TAK-242), potently inhibits TLR4 signaling and downregulates the production of nitric oxide and pro-inflammatory cytokines (22). TAK-242 binds directly to a specific amino acid, Cys747, in the intracellular domain of TLR4, which inhibits TLR4 signaling and, in turn, inhibits MyD88-dependent and -independent signaling (23). In mouse models, TAK-242 was also shown to alleviate renal inflammation, tubulointerstitial damage, fibrosis and the loss of renal function induced by cyclosporin A (24). In a randomized, double-blinded, controlled trial of patients with severe sepsis, the 28-day all-cause mortality rate was lower in patients receiving TAK-242 compared with that in the placebo treatment group (25), suggesting the potential inhibition of TLR4-mediated inflammatory responses in clinical practice.

In the present study, NF-κB and TLR4 were directly inhibited by PDTC and CLI-095, and silenced using specific siRNAs. Treated HK-2 cells were incubated with 100 mg/ml iohexol as an *in vitro* model of CIN. Notably, the mRNA and protein expression levels of TLR4, MyD88 and NF-κB in the iohexol group were significantly higher compared with those in the blank group, while even with the stimulation of iohexol, these levels were still decreased following the blocking or silencing of the TLR4/MyD88/NF-κB signaling pathway. These results indicate that iohexol is able to activate TLR4/MyD88/NF-κB signaling in CIN, and that this activation can be effectively counteracted by blocking or silencing NF-κB and TLR4. Furthermore, inhibiting TLR4/MyD88/NF-κB signaling significantly reduced the mRNA expression levels of TNF-α, IL-1β and IL-6, thereby delaying the inflammatory response. However, the changes in the expression of these inflammatory factors following blocking or silencing were not completely consistent with those in the blank group, suggesting that other pathways may be involved in the inflammation-associated injury of renal tubular epithelial cells.

In CIN, caspase-9 is activated through the mitochondrial pathway, which subsequently activates the downstream caspase-3 and induces apoptosis (26). Previous studies have demonstrated that, in hypotonic or isotonic CM-induced *in vitro* models, caspase-9 and -3 were activated, while caspase-8 and -10 were not (27). In another study, specific inhibitors of caspase-3 and -9 attenuated the CM-induced injury of LLC-PK1 cells, while specific inhibitors of caspase-8

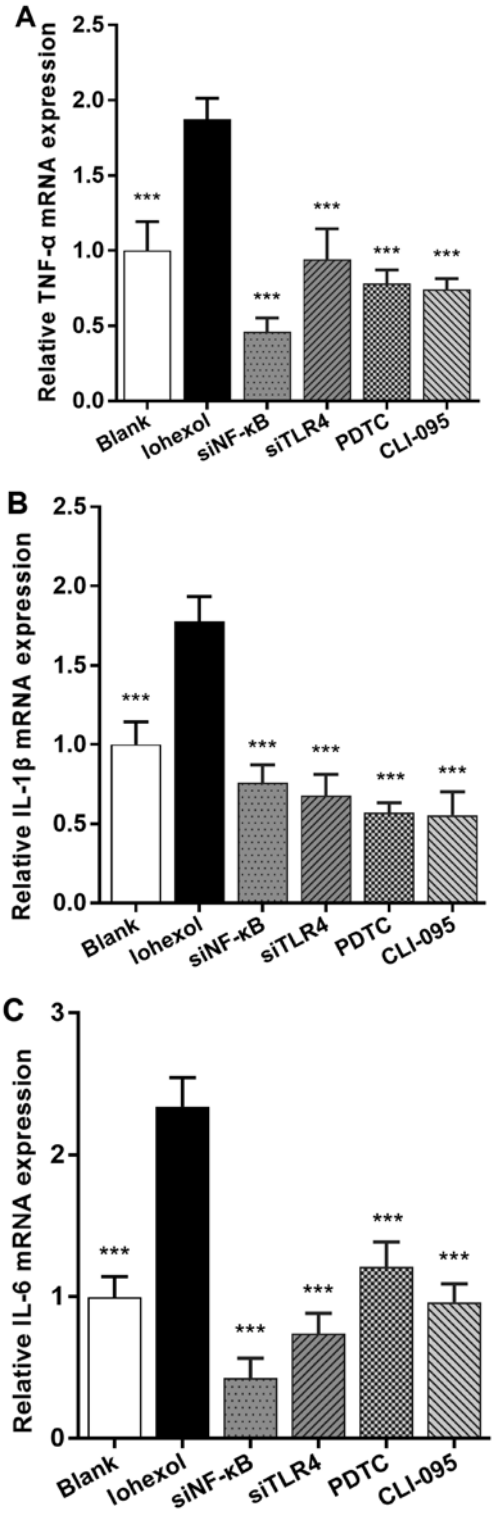

Figure 5. mRNA expression levels of (A) TNF-α, (B) IL-1β and (C) IL-6 in each group compared with the iohexol group. The mRNA expression levels were detected using reverse transcription-quantitative PCR with GAPDH as the internal control, and the expression level of the blank group was considered as 100%. ***P<0.001.

did not (28), indicating that contrast-induced apoptosis occurs primarily through the mitochondrial pathway. In the present study, the expression levels of various apoptosis-associated proteins were investigated, demonstrating that the expression of caspase-3 and c-caspase-9 were markedly higher following iohexol treatment, compared with the blank group, which supports previous findings (24-26). However, no significant differences were observed in the expression levels of caspase-9 between the two groups. After inhibiting TLR4/MyD88/NF-κB signaling, the elevated expression levels of caspase-3 and c-caspase-9 were downregulated, and no significant differences were observed when compared with the blank group. Furthermore, the apoptotic rates of HK-2 cells were assessed by flow cytometry, the results of which confirmed the elevated protein expression levels of caspase-3 and c-caspase-9. These results indicate that the TLR4/MyD88/NF-κB signaling pathway is involved in the iohexol-induced apoptosis of renal tubular epithelial cells.

There were several limitations to the present study. First, the conclusions were drawn from *in vitro* experimentation only, and have not been confirmed in *in vivo* experiments or clinical studies. Second, although its mRNA and protein levels were determined, the expression of MyD88 was not experimentally inhibited. Finally, only a 48-h time point was selected, which was insufficient to elucidate the role of TLR4/MyD88/NF-κB signaling in the entire process of CIN development. In future studies, the establishment of CIN mouse models of TLR4, MyD88 and NF-κB gene-silencing may further elucidate the role of TLR4/MyD88/NF-κB signaling in CIN.

To conclude, the results of the present study suggested that the TLR4/MyD88/NF-κB signaling pathway is involved in the development of contrast-induced nephropathy by promoting inflammatory responses and apoptosis. These findings may enable a deeper understanding of the pathogenesis of CIN, and highlight the TLR4/MyD88/NF-κB signaling pathway as a potential target for the clinical prevention of CIN.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW performed the experiments and was a major contributor to the writing of the manuscript. JZ and LY were responsible for the experimental design and statistical analysis. JY and SW participated in the experiments and data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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