

## Original Article

# Toll like receptor 2 induces kidney inflammation via MyD88/NF- $\kappa$ B signaling pathway

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**Abstract:** Objective: Septic acute kidney injury (AKI) can be caused by an inflammatory process in the kidney. We previously have revealed that TLR2 is over expressed in podocytes of lipopolysaccharide (LPS)-induced septic AKI mice. In the current study, we aimed to investigate the significance of TLR2 overexpression in podocytes on AKI pathogenesis and underlying mechanisms. Methods: *In vitro*, podocyte cell line with or without LPS treatment was tested for the expression of TLR2 by Western blot, IL-6 and TNF- $\alpha$  by RT-PCR and ELISA, MyD88 and p65 by RT-PCR and Western blot, respectively. Subcellular location of MyD88 and p65 was determined by immunofluorescence. *In vivo*, AKI mice were induced by LPS, and the expression of TLR2, MyD88 and p65 were determined by immunohistochemistry and the expression of IL-6 and TNF- $\alpha$  was measured by ELISA. Results: *In vitro* podocyte cell line study showed that LPS treatment significantly increased the expression of TLR2, inflammatory cytokines IL-6 and TNF- $\alpha$ , and signaling pathway proteins MyD88 and p65. Moreover, a nearly full translocation of p65 from cytoplasm to nucleus was observed in LPS-treated podocytes. Of note, all the above changes induced by LPS treatment could be strongly suppressed by TLR2 knock-down. *In vivo* experiment on LPS-induced septic AKI mice confirmed that TLR2 overexpression lead to inflammatory cytokines IL-6 and TNF- $\alpha$  expression as well as the elevation of signaling pathway factors MyD88 and p65. Conclusion: Our study has revealed that TLR2 overexpression in podocytes enhances inflammatory cytokines expression through MyD88/NF- $\kappa$ B signaling pathway, consequently promoting AKI pathogenesis.

**Keywords:** Toll like receptor 2, MyD88, NF- $\kappa$ B, podocyte, inflammation, acute kidney injury

## Introduction

Up to now, acute kidney injury (AKI) still remains one of the most common entities among patients with critical illness [1]. For the past few decades, much effort has been made in the understanding of mechanisms underlying AKI pathogenesis. It is now believed that AKI is usually triggered by severe sepsis while LPS, a component of the outer membrane of Gram-negative bacteria, has been reported to be involved in sepsis development [2-4]. The establishment of this disease involves a wide range of factors including kidney hemodynamic and non-hemodynamic factors [5, 6]. However, the exact mechanisms of AKI pathology remain elusive.

Among the non-hemodynamic factors involving AKI pathogenesis, immunological factors, es-

pecially inflammatory cytokines, are believed to closely associate with AKI. Inflammatory cytokines like TNF- $\alpha$ , IL-6 and MCP1 increase significantly in the kidney as well as peripheral blood in the early phase of AKI patients and have been described to mediate AKI and sometimes maybe also injuries to distant organs [7-9].

Toll like receptors (TLRs) are major pattern recognition receptors (PPRs) and play important roles in innate immune response. Upon the engagement of pathogen-associated molecular patterns (PAMPs), TLRs could induce inflammatory responses through various signaling pathways [10]. Previous studies have documented that TLR2 is over expressed in the kidney and contributes to the development of septic AKI [11-13]. In addition, our previous study has further identified that TLR2 is over activated on

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**Table 1.** Primer pairs used for RT-PCR

Gene	Primer sequence (5'-3')
TLR2	F: CAAATGGATCATTGACAACATCATC R: TTCGTACTTGACCACTCGC
MyD88	F: TCCGGCAACTAGAACAGACAGACT R: GCGGCGACACCTTTTCTCAAT
p65	F: ACCTGGAGCAAGCCATTAGC R: GAGGCGCACTGCATTG
IL-6	F: GACAAAGCCAGAGTCCTTCAGAGAG R: CTAGGTTTGCCGAGTAGATCTC
TNF- $\alpha$	F: ATGAGCACAGAAAGCATGATC R: TACAGGCTTGCTACTCGAATT

F: forward primer ; R: reverse primer.

the podocytes in the septic AKI patients [14]. However, in what way does TLR2 contribute to AKI development has not yet been studied.

In the current study, using a lipopolysaccharide (LPS)-induced septic AKI platform, we investigated both *in vitro* and *in vivo* the signaling pathways which TLR2 triggers in the development of AKI.

### Materials and methods

#### Ethical statement

All protocols involving animals in this study were reviewed and approved by the Bioethics Committee of the First People's Hospital of Kunshan and performed in accordance with the guidelines of Laboratory Animal Science Association (IRB approval number FPHKA20-1512012).

#### Mouse podocytes culture and LPS stimulation

Mouse podocyte cell line MPC5 was a gift from professor Mundel from Mount Sinia Medical School, and propagated at 33°C in RPMI 1640 containing 10% FCS (Boehringer Mannheim, Mannheim, Germany), 100 U/ml penicillin (GIBCO BRL, Karlsruhe, Germany), 100 µg/ml streptomycin (GIBCO BRL), and 10 U/ml recombinant mouse  $\gamma$ -interferon (Sigma Chemical Co, Munich, Germany). To initiate differentiation, cells were thermoshifted to 37°C and maintained in medium without  $\gamma$ -interferon (Mundel et al, 1997). After 10 days of differentiation, podocytes were treated with LPS at a final concentration of 10 µg/mL for 24 h. For signaling pathway inhibition, inhibitors specifically targeting MyD88 (NBP2-29328), NF- $\kappa$ B (Celastrol)

and JAK1/2 (Ruxolitinib) respectively were added into cell culture 2 h before LPS treatment.

#### LPS-induced septic AKI model

LPS-induced septic AKI mice were generated as we previously described [14]. In brief, 6 to 8-week-old male BALB/c mice weighing 20 to 22 g (SLAC laboratory animal center) housed in a specific pathogen-free (SPF) environment with food and water supplied. For the generation of septic AKI mice, animals were injected with LPS (10 mg/kg, L2880, Sigma-Aldrich) intraperitoneally. For signaling pathway inhibition, inhibitors specifically targeting MyD88 (NBP2-29328), NF- $\kappa$ B (Celastrol) and JAK1/2 (Ruxolitinib) respectively were injected through the same route as LPS. Twenty-four hours post injection, mice were sacrificed and urine and serum samples were collected for renal function assessment and kidney samples were processed for analysis.

#### TLR2 knock-down

TLR2 knock-down was performed using TLR2-specific siRNA (sc-40257, Santa Cruz) according to the manufacturer's instructions. In brief, differentiated MPC5 cells were transfected with TLR2-specific siRNA or control siRNA using siRNA transfection reagent (Roche). Twenty-four hours post transfection, cells were proceeded for LPS treatment and downstream analyses.

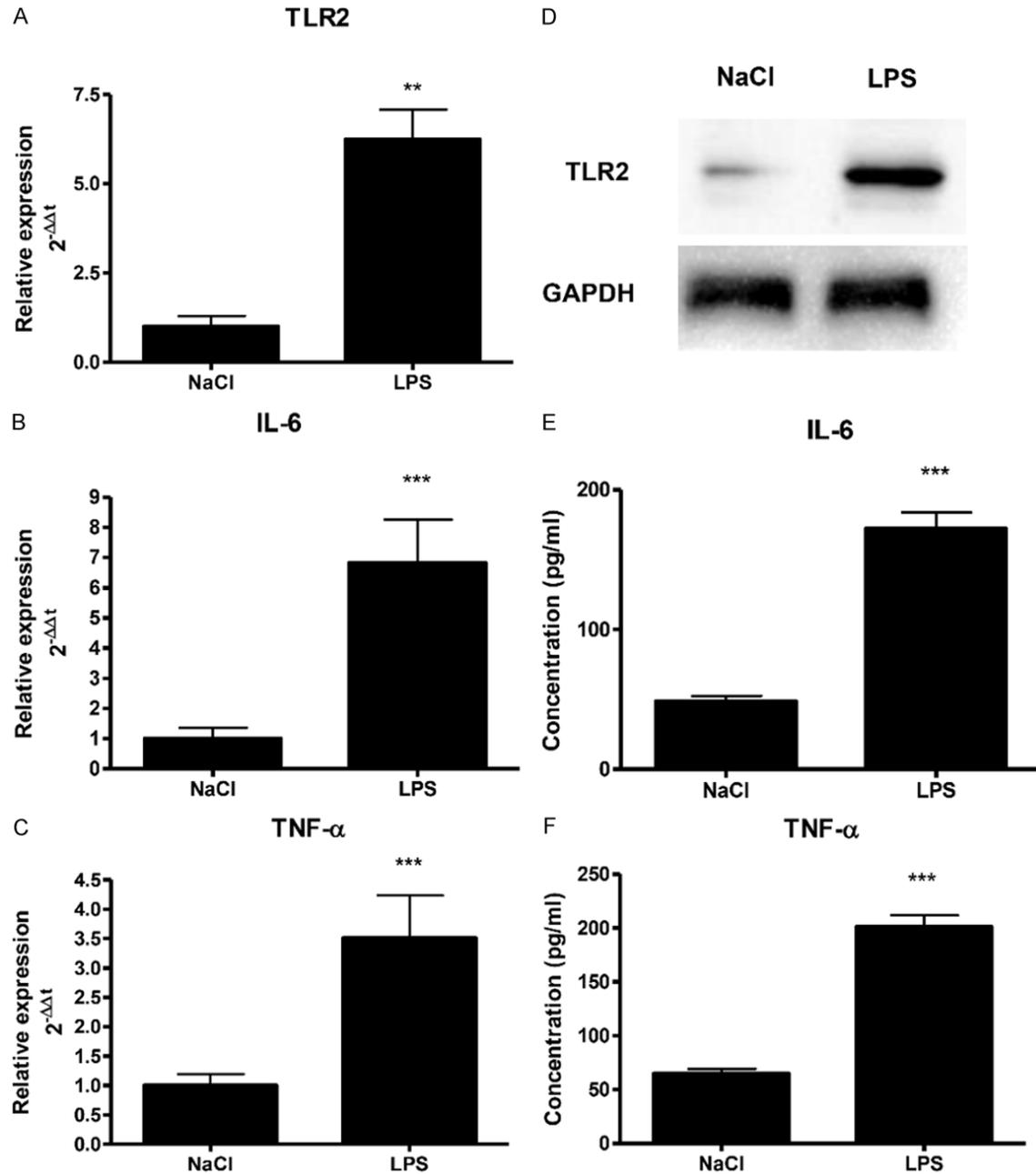
#### RNA extraction and real-time PCR

Total RNA was extracted from podocytes with or without LPS treatment using TRIzol reagent (Invitrogen, ThermoScientific) and reverse-transcribed into cDNA using M-MLV kit (Promega). Semi-quantitative real-time PCR was then performed on an ABI Prism 7300 system using SYBR Green (ThermoScientific) as reporting dye. Relative expression level of TLR2, MyD88, p65, IL-6 and TNF- $\alpha$  were calculated with the  $2^{-\Delta\Delta Ct}$  method using GAPDH as an internal control. The primer pairs used for RT-PCR in this study were listed in **Table 1**.

#### ELISA

IL-6 and TNF- $\alpha$  concentration in mouse serum samples as well as in cell culture supernatants were measured by ELISA using commercial kits according to the manufacturer's instructions

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**Figure 1.** LPS-induced TLR2 overexpression resulted in the elevation of IL-6 and TNF- $\alpha$  in LPS-treated podocytes. Podocytes were treated with LPS for 24 h and then mRNA level of TLR2 (A), IL-6 (B) and TNF- $\alpha$  (C) were measured by RT-PCR. Data shown as means  $\pm$  SD of three independent experiments. The protein level of TLR2 was determined by Western blot (D). Data shown is one out of three independent experiments. The protein level of IL-6 and TNF- $\alpha$  were measured by ELISA (E, F). Data shown as means  $\pm$  SD of three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

(IL-6 was from Biosource and TNF- $\alpha$  was from Diaclone).

### Western blot

Mice kidneys podocytes with or without LPS treatment were first lysed with RIPA buffer sup-

plemented with protease inhibitors (Roche) and then centrifuged and supernatants were collected. Protein concentration was then determined by BCA kit (ThermoScientific) and equal amount of proteins were loaded onto a 10% SDS-PAGE gel. Electrophoresis separated proteins were then transferred onto a PVDF mem-

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brane. Non-specific binding sites of the membrane was first blocked by 5% non-fat milk, and then target proteins were detected by sequential incubations of primary and corresponding HRP-conjugated secondary antibodies. Following incubations, membrane was extensively washed and immunobands were visualized using ECL substrate (GE Healthcare) under a CCD camera (Bio-Rad). The following primary antibodies were used in the current study: rabbit anti-mouse TLR2 (Sigma-Aldrich), rabbit anti-p65 (Santa Cruz), rabbit anti-mouse MyD88 (Abcam) and rabbit anti-GAPDH (Santa Cruz). The goat anti-rabbit IgG-HRP was used as secondary antibody in this study (Santa Cruz).

### *Immunohistochemistry (IHC)*

IHC was performed as previously described [14, 15]. In brief, kidney samples were fixed with paraformaldehyde and embedded in paraffin and sectioned. For immunostaining of TLR2, MyD88 and p65, slides were first dewaxed, rehydrated and then antigen retrieval was performed by immersing the slides in boiling 0.01 M citrate buffer for 15 min. Following that, endogenous peroxidase was quenched by 0.3% H<sub>2</sub>O<sub>2</sub> and non-specific binding sites were blocked by normal serum, respectively. After washes, slides were then incubated with primary antibodies targeting TLR2, MyD88 and p65 respectively overnight at 4°C, followed by sequential incubation of biotinylated secondary antibodies (R&D systems) and avidin-biotinylated horseradish peroxidase complex (Vector Laboratories), respectively. At last, slides were extensively washed with PBS and immunostaining was visualized by diaminobenzidine (Sigma-Aldrich). The following primary antibodies were used for IHC analysis in the current study: rabbit anti-TLR2 (Sigma-Aldrich), MyD88 (Abcam) and p65 (Santa Cruz).

### *Immunocytochemistry (ICC)*

ICC analysis of p65 subcellular localization was performed as previously described with modifications [16]. In brief, differentiated podocytes were treated with LPS (10 µg/mL) or NaCl for 24 h. Then cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and blocked with 5% BSA. Following that, cells were extensively washed with PBS and incubated for 1 h with rabbit anti-p65 (Santa Cruz) and FITC-conjugated goat anti-rabbit IgG, respectively. After incubations,

cells were again washed with PBS, mounted with anti-fluorescence quenching reagent (Beyotime) and visualized using Olympus IX51 microscope.

### *Statistical analysis*

The data are expressed as mean ± standard deviation (SD). For comparisons between two groups, student's t test was applied while for comparisons among three or more groups, One-way ANOVA plus SNK *post hoc* were used. A *p* value less than 0.05 was considered statistically significant. All analyses were performed with SPSS 10.0 (SPSS).

## **Results**

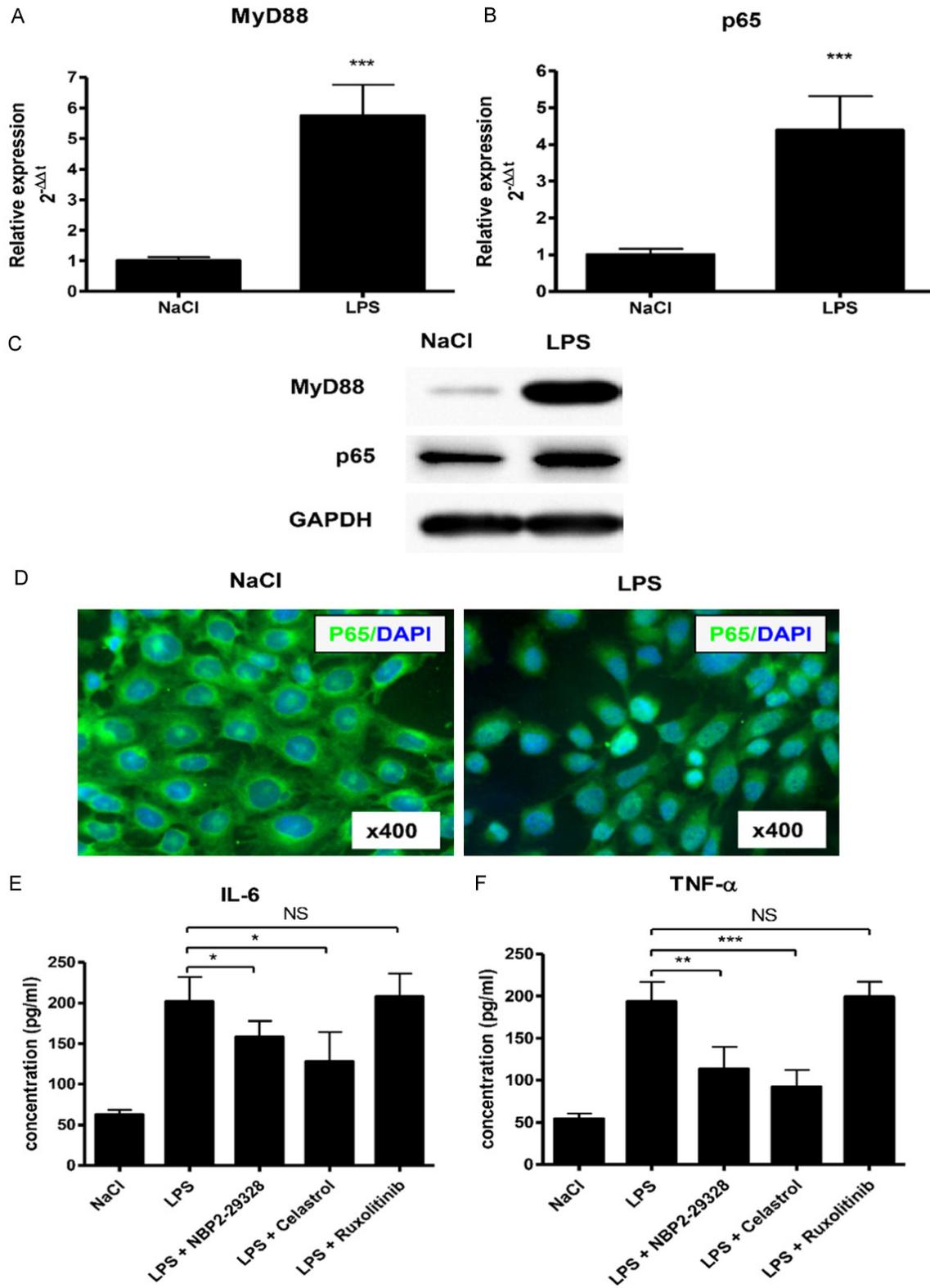
### *TLR2 overexpression in LPS-treated podocytes resulted in elevation of inflammatory cytokines*

Our previous study has revealed that TLR2 was over expressed in podocytes of septic AKI mice [14]. In the current study, we further explored the significance of this TLR2 over expression to AKI pathogenesis. Since TLR activation could usually trigger inflammatory cytokines production while increased inflammatory cytokines have been associated with AKI development, we herein first investigated *in vitro* whether TLR2 over expression could enhance the expression of inflammatory cytokines IL-6 and TNF-α. LPS-treated MPC5 podocytes were served as an *in vitro* model for septic AKI. Consistent with our previous results, podocytes with LPS treatment showed a significant increase in TLR2 expression on both mRNA and protein level (**Figure 1A** and **1D**). In accordance with the TLR2 expression, the IL-6 and TNF-α expression upon LPS treatment also showed sharp elevation on both mRNA and protein level (**Figure 1B, 1C, 1E** and **1F**). Taken together, the data herein suggested that LPS triggered TLR2 over expression lead to the expression of inflammatory cytokines including IL-6 and TNF-α.

### *Elevated inflammatory cytokines were triggered by TLR2/MyD88/NF-κB signaling pathway*

MyD88/NF-κB signaling pathway is one of the most common pathways participating in TLR regulation of inflammatory reactions. We next further investigated whether this signaling pathway also involves in TLR2 regulation of IL-6 and TNF-α expression. First, we measured the

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**Figure 2.** LPS-induced TLR2 overexpression activated MyD88/NF-κB signaling pathway. Podocytes were treated with LPS for 24 h in the presence or absence of signaling pathway inhibitors and then mRNA and protein levels of MyD88 (A, C) and p65 (B, C) were determined by RT-PCR and Western blot, respectively. (D) The subcellular location of p65 was determined by ICC. (E, F) IL-6 and TNF-α expression was quantified by ELISA. \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

expression of MyD88 and p65 on both mRNA and protein level. Our results showed that upon LPS treatment, the mRNA and protein levels of MyD88 and p65 increased significantly (**Figure 2A-C**). Since the successful NF- $\kappa$ B activation of target gene expression requires translocation of this transcription factor, we next determined the subcellular location of p65 in podocytes with or without LPS treatment. Our results demonstrated that almost a full translocation of p65 from cytoplasm to nucleus was observed in podocytes with LPS treatment (**Figure 2D**), indicating that NF- $\kappa$ B activation was triggered. To further confirm the importance of MyD88 and NF- $\kappa$ B in the expression of inflammatory cytokines IL-6 and TNF- $\alpha$ , specific signaling pathway inhibitors were introduced. As shown in **Figure 2E** and **2F**, IL-6 and TNF- $\alpha$  expression was significantly decreased when a MyD88 inhibitor (NBP2-29328) or a NF- $\kappa$ B inhibitor (Celastrol) was added. By contrast, the expression of these two cytokines was not affected by the addition of a JAK1/2 inhibitor (Ruxolitinib). Taken together, our results here suggested that LPS-induced TLR2 over expression in podocytes could result in the secretion of inflammatory cytokines via MyD88/NF- $\kappa$ B signaling pathway.

### *Knock-down of TLR2 could inhibit LPS-induced inflammatory cytokine response*

To further confirm the importance of TLR2 in LPS-induced inflammatory cytokine response in podocytes, TLR2 knock-down was performed by specific siRNA before LPS treatment. As shown in **Figure 3A**, LPS-induced expression of MyD88 and p65 was suppressed by TLR2 knock-down. In addition, nuclear translocation of p65 from cytoplasm was also significantly down-regulated when TLR2 expression was specifically knocked down (**Figure 3B**). In accordance, the elevated expression of IL-6 and TNF- $\alpha$  upon LPS treatment were also decreased by TLR2 knock-down (**Figure 3C** and **3D**). Together, these data indicate that LPS induced inflammatory cytokine response was mediated by TLR2 overactivation.

### *TLR2/MyD88/NF- $\kappa$ B signaling pathway is involved in the regulation of inflammatory cytokines expression in LPS-induced septic AKI mice*

We further confirmed our findings *in vivo* on a LPS-induced murine septic AKI model. Mice

were treated with LPS for 24 h and successful modeling was determined by renal function assessments including BUN and urinary albuminuria level and kidney histological evaluation [14]. In accordance with the results observed from *in vitro* assays, the expression of TLR2, MyD88 and p65 were significantly increased, together with the increase of inflammatory cytokines IL-6 and TNF- $\alpha$  (**Figure 4**). Furthermore, IL-6 and TNF- $\alpha$  expression could be significantly inhibited by MyD88 or NF- $\kappa$ B inhibitors, but not a control JAK1/2 inhibitor (**Figure 4B** and **4C**).

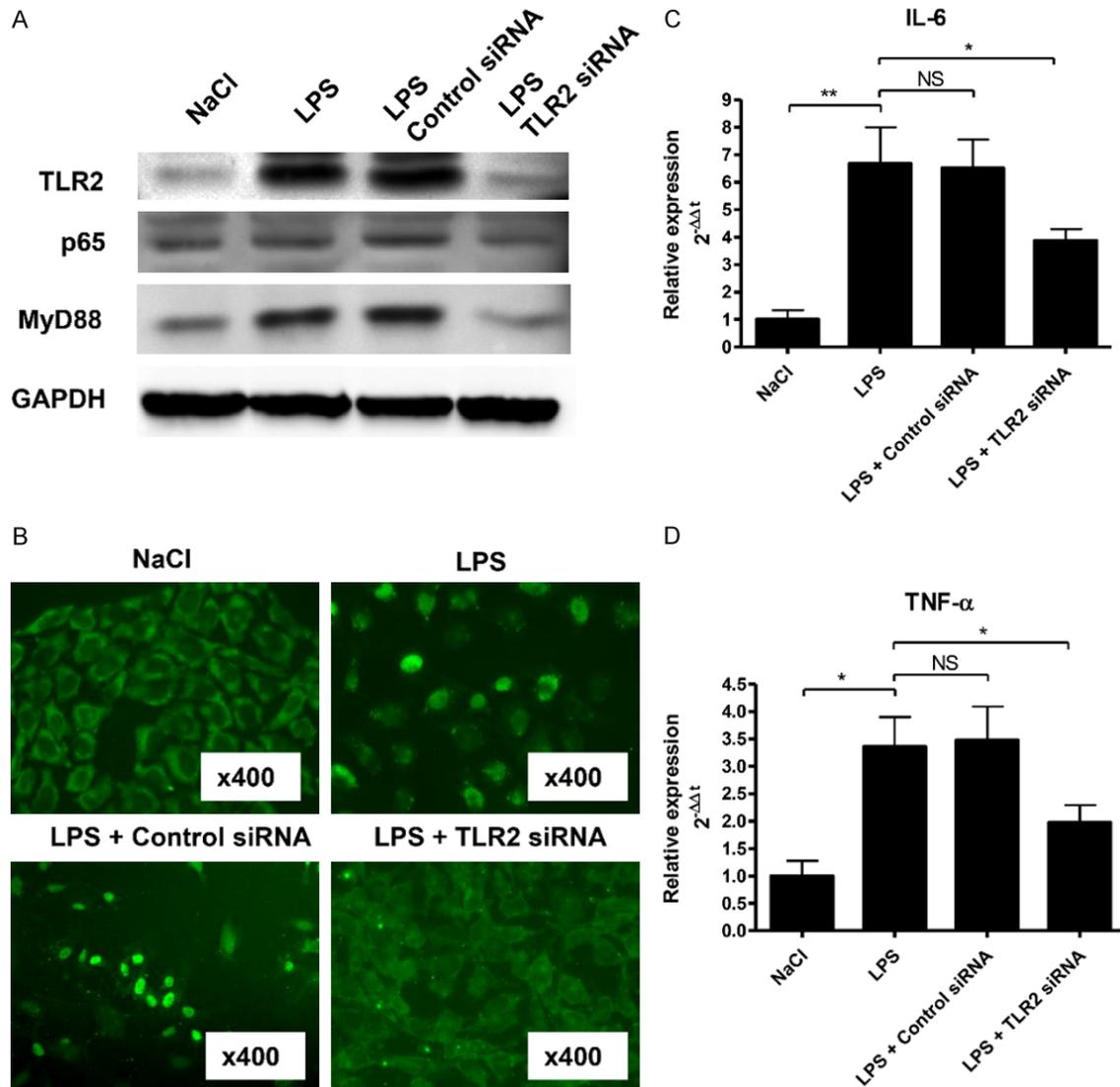
Taken together, our results in the current study revealed that septic AKI induced TLR2 over expression could enhance inflammatory cytokines production by activating MyD88/NF- $\kappa$ B signaling pathway.

## Discussions

Septic AKI is one of the most severe complications in hospitalized patients, which could be caused by an inflammatory process in the kidney. TLR2 overexpression/over activation has been reported to associate with AKI development, however, the exact mechanism has not yet been fully revealed [17, 18]. Our previous study has identified that podocytes are the main cells overexpress TLR2 in septic AKI mice. In the current study, we further investigated the mechanism underlying TLR2 overexpression in podocytes to septic AKI development. Our study revealed that TLR2 overexpression in podocytes could lead to enhanced inflammatory cytokines production, which could probably be activated through MyD88/NF- $\kappa$ B signaling pathway.

Sepsis, the main cause for septic AKI in hospitalized patients, is usually a result of gram-negative bacteria infection [2]. LPS as the main component on the membrane of gram-negative bacteria could trigger high level immunologic responses. Therefore, LPS has been usually used as a factor to induce AKI on small animal models like mouse model [19]. Our current study also adopted the well-accepted classical LPS-induced mice model. Others have also tested the possibility on the adoption of live *E. coli* to induce septic AKI on large models to generate a condition closer to clinical manifestations [20-22]. Although all the tested symptoms

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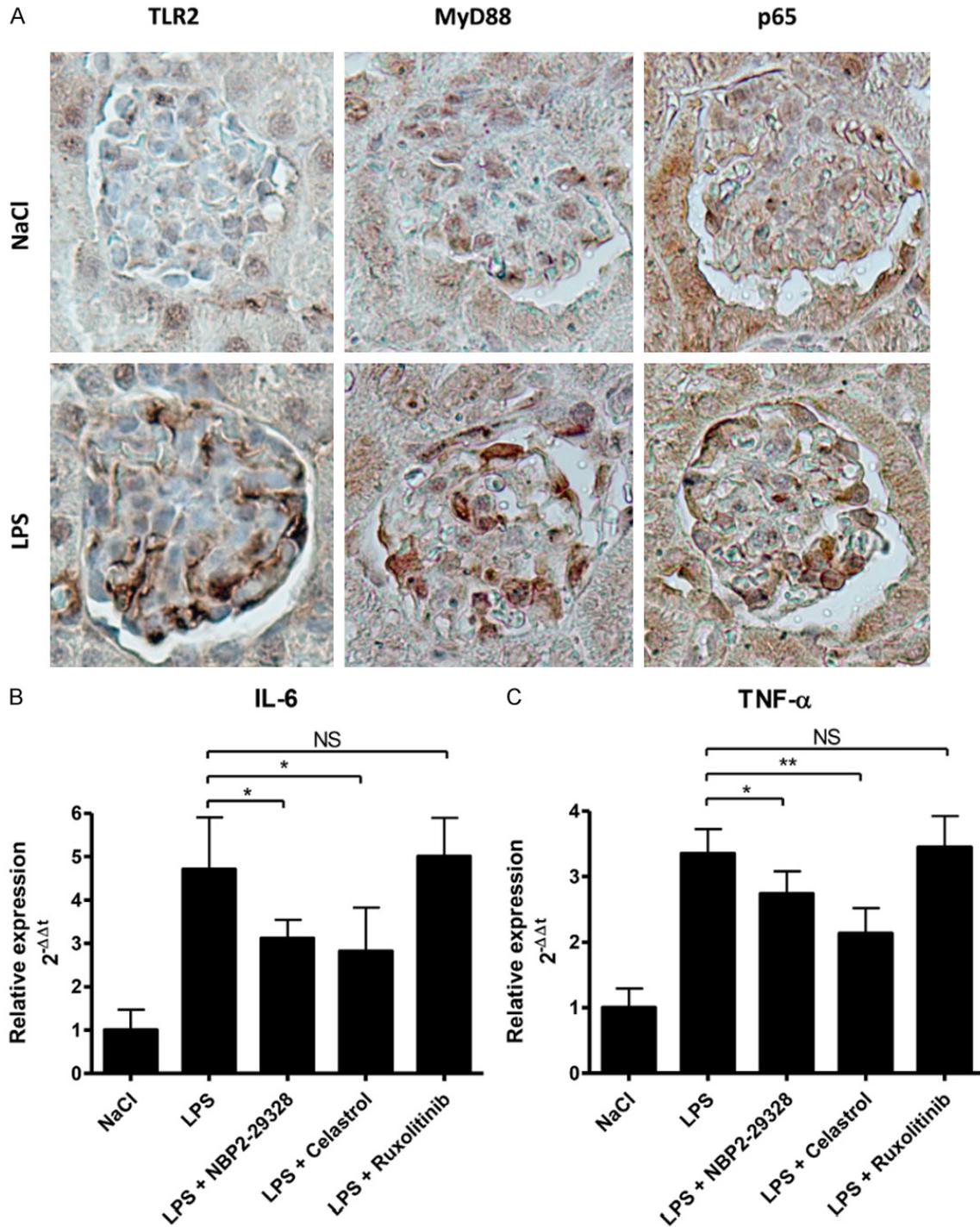


**Figure 3.** Knock-down of TLR2 could inhibit LPS-induced inflammatory cytokine response. Podocytes were treated with or without control siRNA or TLR2 specific siRNA for 24 h, and then mock-treated or treated with LPS for another 24 h. After treatment, cells were harvested and TLR2, MyD88, p65 and GAPDH expression were determined by Western blot (A), p65 subcellular location was determined by ICC (B) and IL-6 and TNF- $\alpha$  RNA levels were measured by RT-PCR (C, D). (A, B) Data shown are one representative out of three independent experiments. (C, D) Data shown as means  $\pm$  SD of three independent experiments. NS, statistically not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

mimicking a real clinical septic AKI in our study using the LPS-induced septic AKI model, a further study to confirm the findings on a live bacteria-induced septic AKI model would be benefited.

Podocytes, cells wrapping around the capillaries of the glomerulus in the kidney, constitute an important part of the Bowman's capsule filter to let through small molecules including water, salt and sugar while retaining large mol-

ecules like proteins [23, 24]. Given the importance of this cell type and in addition the TLR2 overexpression on this cell type in septic AKI, we consequently in the current study focused on the changes of this cell type to the development of septic AKI [14]. However, inflammation as well as other immunological responses to foreign antigens would usually involve cascade reactions of various cell types [25]. These may include cell proliferation, migration and altered gene expression. Therefore, further investiga-



**Figure 4.** LPS-induced TLR2 overexpression enhanced IL-6 and TNF- $\alpha$  expression in vivo via MyD88/NF- $\kappa$ B signaling pathway. Mice treated with or without signaling pathway inhibitors were induced by LPS for 24 h and then sacrificed. TLR2, MyD88 and p65 expression in the kidney were determined by IHC (A). Data shown is one out of three independent experiments. IL-6 (B) and TNF- $\alpha$  (C) expression were measured by RT-PCR. Data shown as means  $\pm$  SD of three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

tions to study the involvement of other cells within kidney and/or migrated into kidney in

septic AKI, albeit beyond the scope of the current study, would be interesting.

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For many years, AKI research has been focused mostly on acute tubular necrosis (ATN), with specifically on renal interstitial and tubular injuries, while little attention was given on the glomeruli changes. However, recent studies on AKI animal models and patient pathological analysis have revealed that ATN is not a common histopathological change for AKI, with its incidental rate only around 22% of all AKI patients [26-28]. In addition, increasingly evidence suggests that changes on glomerular hemodynamics and impairments of podocytes and endothelial cells are common AKI symptoms [29]. Moreover, studies on septic AKI models have shown that podocytes are severely impaired, resulting in elevation of inflammatory cytokines like IL-6 and TNF- $\alpha$ , as well as proteinuria, through undefined signaling pathways [30-33]. In the current study, by using in vitro and in vivo models, we have confirmed that LPS-induced septic AKI could enhance inflammatory cytokine expression, and more importantly, our data have suggested that this increased inflammatory cytokine expression is mediated by LTR2/MyD88/NF- $\kappa$ B signaling pathway.

In summary, our study here indicated that TLR2 overexpression in podocytes could enhance inflammatory cytokines expression through MyD88/NF- $\kappa$ B signaling pathway, consequently promoting AKI pathogenesis. The findings of our study not only revealed potential mechanism underlying TLR2 overexpression contributing to AKI development, but also could provide potential treatment targets for septic AKI.

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### Disclosure of conflict of interest

None.

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