Treatment with toll-like receptor 2 inhibitor ortho-vanillin alleviates lipopolysaccharide-induced acute kidney injury in mice

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Abstract. Reducing inflammation is a promising approach for the prevention and treatment of septic acute kidney injury (AKI), since AKI is characterized by excessive inflammation in the kidney. Previous studies have demonstrated that toll-like receptor 2 (TLR2) is overstimulated, which promotes inflammation by activating the NF-κB signaling pathway, in a lipopolysaccharide (LPS)-induced model of AKI mice. For the present study, it was hypothesized that TLR2 inhibition could reduce inflammation and consequently prevent septic AKI. Therefore, the potential renal protective effects of ortho-vanillin (OV), an inhibitor of TLR2, were investigated in the present study *in vitro* and *in vivo*. *In vitro* treatment with OV on LPS-stimulated mouse podocyte cell line MPC5 did not affect TLR2 expression but interrupted the interaction between TLR2 and its downstream adaptor MyD88, resulting in the reduction of inflammatory cytokines IL-6 and TNF-α expression. *In vivo* OV treatment in an LPS-challenged mouse model effectively alleviated LPS-induced kidney injury as indicated by histology analysis and the significantly reduced blood urea nitrogen and serum creatinine levels. Additionally, inflammatory cytokines TNF-α, IL-6 and IL-1β expression were also significantly reduced in mice with OV treatment. Signaling pathway analysis further demonstrated that OV treatment did not affect the expression of TLR2 and p65 but suppressed p65 phosphorylation. Taken together, data from the present study demonstrated that OV was effective in protecting renal function against LPS-induced AKI through the inhibition of TLR2/NF-κB signaling and subsequent inflammatory cytokine production. These findings indicated that OV or targeting TLR2 signaling in general, represents a novel therapeutic approach for use in the prevention and treatment of AKI.

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Introduction

Despite clinical progress in the past few decades, acute kidney injury (AKI) remains to be major medical concern. Each year, ~13.3 million are afflicted with AKI worldwide (1). In general, AKI is temporary disease and does not usually result in mortality directly; however, this injury is associated with high morbidity and mortality, with ~1.7 million deaths each year worldwide (1,2). In developed countries, AKI is mainly observed in elderly patients within the intensive care unit, whereas in lower- and middle-income countries young adults are likely to be affected by AKI and be at risk of mortality (3-5). One-third of individuals that experience AKI may develop chronic kidney disease and end-stage renal disease, drastically reducing the quality of life and incurring high long-term medical costs (6).

Despite the health problems that are caused by AKI, effective therapeutic strategies other than dialysis are not yet available to increase the survival rate, reduce injury or accelerate recovery (7). Potential tubular, vascular and inflammatory processes have been reported to be associated with AKI pathogenesis (8). Among these factors, inflammation is generally believed to serve a predominant role in the pathophysiology of AKI, especially in septic AKI (7-9). Therefore, developing novel strategies to reduce inflammation at injured sites may represent a feasible and practical way to treat or prevent septic AKI, and has previously been investigated on murine models with promising results (9,10). By using a lipopolysaccharide (LPS)-induced septic AKI mouse as a model, Hu *et al* (11) demonstrated that the systemic delivery of a plasmid expressing an immunosuppressive cytokine interleukin-35 (IL-35) effectively prevented LPS-induced AKI by inhibiting NF-κB activation. Additionally, another study has revealed that the inhibition of leukocyte infiltration into the kidneys could reduce renal injury and protect renal function (12), whilst the activation of the cholinergic anti-inflammatory pathway, which is mediated by α7-nicotinic acetylcholine receptor on $CD4^+$ T cells, has also been demonstrated to exhibit renal protection (13).

As a key component of the innate immune response, Toll-like receptors (TLRs) serve to recognize pathogen-associated molecular patterns (PAMPs) that are present on pathogens, and initiate the innate immune response by producing inflammatory cytokines (14,15). In particular, previous studies have shown that TLR2 could be activated in mice using LPS stimulation, which contribute to the development of septic AKI by enhancing inflammatory cytokine production via the NF-κB signaling pathway (14-19). Histological evaluation has demonstrated that TLR2 overactivation in AKI is mainly identified in podocytes, which may be indicative of the important roles podocytes serve in septic AKI pathogenesis (17).

Since preventing inflammation has been demonstrated to be an effective approach for the treatment and prevention of septic AKI, it was hypothesized that TLR2 inhibition may serve as a potentially valuable target for inhibiting inflammation, consequently reducing the risk of AKI. To assess this, the potential protective effects of ortho-vanillin (OV), a small molecule inhibitor against TLR2, were investigated on LPS-induced septic AKI *in vitro* and *in vivo*. OV is an organic solid that is present in a number of plants and has been reported to inhibit TLR2 signaling without inducing cytotoxicity (20,21). Therefore, the results of the present study revealed that treatment with OV effectively alleviated LPS-induced septic AKI in a podocyte cell line and in a mouse AKI model.

Materials and methods

Cell line culture, LPS and OV treatment. The mouse podocyte cell line MPC5 was a kind gift from Professor Mundel, Department of Neuro-Ophthalmology, Mount Sinai School of Medicine and was cultured at 33˚C with 5% CO_2 in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA), 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA) and 10 U/ml recombinant mouse interferon-γ (Sigma-Aldrich; Merck KGaA). To initiate differentiation, cells were transferred to RPMI-1640 medium without interferon-γ and cultured at 37˚C with 5% CO_2 for 10 days (18). After differentiation, podocytes were treated at 37˚C, 5% CO_2 with LPS to a final concentration of 10 μg/ml for 24 h. For OV treatment at 37˚C, 5% CO_2, OV was added to a final concentration of 50 μM 2 h prior to LPS treatment and remained for the duration of the LPS treatment. Podocytes treated with vehicle (saline solution for LPS and H_2O for OV) were used as negative controls.

Cell viability. Differentiated MPC5 cells were treated with 0, 10, 50 or 150 μM OV at 37˚C and 5% CO_2 for 24 h before cell viability was assessed using a MTT assay kit (Abcam) according to the manufacturer's protocol. Briefly, following OV treatment, medium was removed and cells were resuspended in serum-free RPMI-1640 medium at $1x10^6$ cells/ml. A total of 50 μl of the cell suspension was mixed with 50 μl MTT reagent and incubated at 37˚C for 3 h. Following incubation, MTT solvent was added and incubated at room temperature for an additional 15 min on a shaker. Finally, optical absorbance was read at OD_{590} nm and cell viability was calculated with cells without OV treatment considered to be 100%.

Animals and ethical statement. A total of 60 male BALB/c mice (age, 6-8-weeks; weight, 20-22 g; Shanghai SLAC Laboratory Animal Co., Ltd.) were used in the current study. All mice were housed in a specific pathogen-free environment (18˚C, 50% humidity and light from 04.00 to 17.00) with freely available food and water supplied. All protocols involving animals in the present study were reviewed and approved by the Bioethics Committee of the First People's Hospital of Kunshan (Kunshan, China) and performed in accordance with the guidelines of the Laboratory Animal Science Association (IRB approval no. FPHKA201512012).

Mouse OV treatment and AKI induction. OV treatment was performed as previously described (21). Briefly, mice were administered with two intraperitoneal (i.p.) doses of OV 1 h apart. At 1 h after the second OV injection, LPS was injected to induce septic AKI as previously described (17). Animals were injected with LPS (10 mg/kg; cat. no. L2880; Sigma-Aldrich; Merck KGaA) i.p. The mice were sacrificed 24 h post-injection, after which serum samples were collected for renal function assessment by centrifugation at 10,000 x g for 10 min at 4˚C and kidney samples were processed for subsequent analysis. For ELISA, kidney tissue samples were homogenized in RIPA buffer (Thermo Fisher Scientific, Inc.) supplemented with protease inhibitor cocktail (Roche Diagnostics) on ice, and then centrifuged at 10,000 x g for 10 min at 4˚C to pellet cell debris. The resulting supernatants were collected and adjusted to a concentration of 1 mg/ml [concentrations were determined using a NanoDrop™ One (Thermo Fisher Scientific, Inc.)], and stored at -80˚C until use. For histological analysis, kidney samples were fixed with 4% paraformaldehyde for 16 h at room temperature, embedded in paraffin and cut into 4 μm sections and stored at room temperature until use.

ELISA. IL-6, TNF-α and IL-1β levels in cell culture supernatants and murine kidney tissue samples were measured using ELISA. For cell culture supernatants, cell debris was cleared by centrifugation (400 x g, 5 min at 4˚C). Tissue samples were homogenized as described above. ELISA kits for IL-6 (cat. no. 5017218), TNF-α (cat. no. 5017331) and IL-1β (cat. no. 501129749) were purchased from eBioscience; Thermo Fisher Scientific, Inc. and used according to the manufacturer's protocols.

Co-immunoprecipitation (co-IP) and western blot analysis. MPC5 cells or mouse kidney tissue samples were lysed or homogenized in RIPA buffer (Thermo Fisher Scientific, Inc.) supplemented with protease inhibitor cocktail (Roche Diagnostics) and then centrifuged at 10,000 x g for 10 min at 4˚C to remove cell debris and tissue clumps. Cleared supernatants were either used for co-IP or directly for western blot analysis. Co-IP was performed using a Dynabeads™ co-immunoprecipitation kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. In brief, rabbit anti-mouse MyD88 antibody (5 μg; cat. no. 4283; Cell Signaling Technology, Inc.) or anti-TLR2 antibody (5 μg; cat. no. AF1530; R&D systems, Inc.)-coupled Dynabeads were incubated with cleared cell lysis supernatants overnight at 4˚C. After washes with wash buffer, proteins bound to the beads were eluted using elution buffer. Vehicle treated cells were used as control.

Protein concentrations were determined using a Bicinchoninic Acid assay (Thermo Fisher Scientific, Inc.). For western blot analysis, lysed cell supernatants, homogenized tissue supernatants or Co-IP samples were first resolved using 12% SDS-PAGE at 20 μg per lane and then transferred to PVDF membranes (Millipore; Merck KGaA). After blocking with 5% non-fat milk for 1 h at room temperature, the membranes were then sequentially incubated with primary antibodies overnight at 4˚C and with HRP-conjugated secondary antibodies for 1 h at room temperature. The membranes were then extensively washed with PBST and the immune-bands were visualized using an ECL substrate (Millipore; Merck KGaA) under a CCD camera (Bio-Rad Laboratories, Inc.). Gray-scale analysis of the western blot bands was performed using Image J (version 2; National Institutes of Health) (22). The following primary antibodies were used in the current study (all at 1:1,000 dilution): Goat anti-mouse TLR2 (cat. no. AF1530; R&D systems, Inc.), goat anti-mouse GAPDH (cat. no. sc-48166; Santa Cruz Biotechnology, Inc.), rabbit anti-mouse MyD88 (cat. no. 4283; Cell Signaling Technology, Inc.), rabbit anti-mouse p65 (cat. no. 8242; Cell Signaling Technology, Inc.) and rabbit anti-mouse phosphorylated (p)-p65 (cat. no. 3033; Cell Signaling Technology, Inc.). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (H+L) (cat. no. SA00001-4) and HRP-conjugated goat anti-rabbit IgG (H+L) (cat. no. SA00001-2) secondary antibodies were purchased from Proteintech Group, Inc.

Histology evaluation. Histological evaluation of kidney samples was performed as previously described (17,18). Briefly, kidney samples were fixed with 4% paraformaldehyde for 16 h at room temperature, embedded in paraffin and cut into 4 μm sections. Slides were then de-waxed, rehydrated in descending alcohol series (100, 95, 70 and 50%) and stained with periodic acid Schiff's reagent (PAS). For PAS staining, slides were sequentially stained with periodic acid solution and Schiff's reagent for 5 and 15 min respectively, at room temperature. Stained slides were visualized under a light microscope and images were captured at x200 magnification using an NiU upright microscope coupled with a DS-Fi3 camera (Nikon Corporation). A total of 10 kidney sections per mouse were analyzed and two independent experienced investigators randomly evaluated 20-30 glomeruli per kidney section in a blinded manner. NIS-Element D imaging software (version 4.30.00; Nikon Corporation) was used for image capture. Kidney injury was assessed using a previously described criteria (23), which classifies the kidney injury severity into a 0-4 scoring system: No injury, Score 0; <10% injury, score 1; 10-25% injury, score 2; 25-75% injury, score 3; and >75% injury, score 4.

Measurement of Blood Urea Nitrogen (BUN) and Serum Creatinine (SCr). At the time of mice sacrifice, blood samples were collected and kept at room temperature undisturbed for 30 min to enable blood clotting. Subsequently, blood samples were centrifuged at 2,000 x g for 10 min at 4˚C and sera (supernatants) were subsequently collected. BUN and SCr concentrations in murine sera were determined using a Hitachi 7060 automated Chemistry Analyzer (Diamond Diagnostics, Inc.) according to the manufacturer's protocols.

Statistical analysis. The data are expressed as mean ± standard deviation. Student's t-test was applied for statistical comparisons between two groups while a One-way ANOVA followed by Student-Newman-Keuls post hoc test was used for comparisons between three or more groups. $P<0.05$ was considered to indicate a statistically significant difference. All analyses were performed using Prism 7.03 (GraphPad Software, Inc.).

Results

OV inhibits TLR2 signaling and pro-inflammatory cytokine production in murine podocytes. Since TLR2 was previously found to be overexpressed and activated in podocytes in septic AKI mouse (18), the potential anti-inflammation effects of OV were first investigated on the murine podocyte cell line MPC5. Cell cytotoxicity of OV was first measured by treating MPC5 cells with increasing doses (0, 10, 50 and 100 μM) of OV for 24 h. No apparent cell cytotoxicity was observed within the dose range tested (10-150 μM; Fig. 1A). As OV at all tested doses showed similar results, a moderate dose within the tested range was selected (final concentration of 50 μM) for the subsequent experiments on cell lines.

The impact of OV treatment on TLR2 expression was subsequently determined. MPC5 cells pretreated with or without OV were stimulated with LPS for 24 h, and then TLR2 expression was determined using western blot analysis. In accordance with previous findings, LPS treatment markedly increased TLR2 expression, but treatment with OV did not result in any changes to LPS-induced TLR2 expression (Fig. 1B) (18).

TLR2 is activated upon recognition of PAMPs, which bind to the intracellular adaptor protein MyD88 to activate a number of signaling pathways that lead to the expression of pro-inflammatory cytokines (24). To investigate if OV could inhibit TLR2 signaling by interfering with TLR2-MyD88 interaction, a Co-IP assay was performed with lysates from MPC5 cells with or without OV treatment and LPS stimulation using an anti-MyD88 antibody. TLR2 was not associated with MyD88 in the absence of LPS stimulation, but TLR2-My88 complexes were detected upon LPS stimulation (Fig. 1C). Of note, when cells were pretreated with OV, the TLR2-MyD88 association was considerably reduced, suggesting an inhibition of TLR2-MyD88 interaction following OV treatment (Fig. 1C). To further confirm the findings, an additional Co-IP with anti-TLR2 antibody was performed, and the results were consistent with the anti-MyD88 Co-IP. Namely, LPS stimulation induced TLR2-MyD88 interaction, while such interaction was inhibited by OV treatment (Fig. 1C).

To determine whether the interrupted TLR2-MyD88 interaction induced by OV treatment resulted in reduced inflammation downstream, the supernatants of MPC5 cells with or without OV treatment and LPS stimulation were tested for IL-6, TNF-α and IL-1β expression, which are pro-inflammatory cytokines that have been previously reported to serve important roles in AKI pathogenesis (25-27). The ELISA results revealed that when compared with vehicle control, LPS stimulation significantly increased the release of IL-6, TNF-α and IL-1β, which was inhibited by OV treatment (Fig. 1D-F).

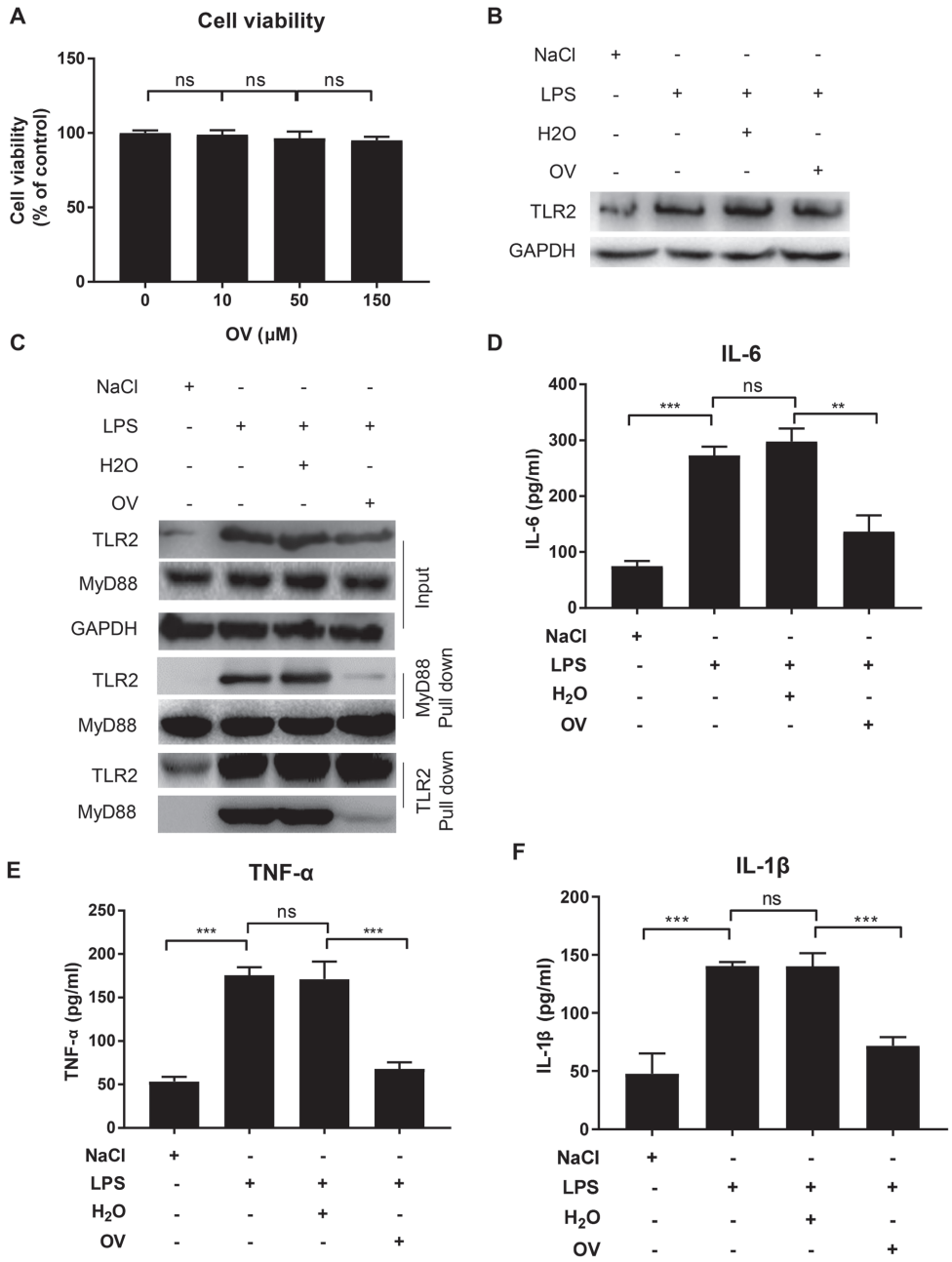

Figure 1. OV inhibits TLR2 signaling in the murine podocyte cell line MPC5. (A) OV cytotoxicity assay. MPC5 cells were treated with increasing doses of OV for 24 h, following which an MTT assay was performed to assess the cytotoxicity of OV. Data are presented as the mean ± SD from three independent experiments. MPC5 cells were pretreated with or without OV, and then treated with vehicle control or stimulated with LPS for 24 h. After stimulation, (B) TLR2 expression was determined using western blotting. Data shown are representative of three independent experiments. (C) TLR2-MyD88 interaction was assessed using co-immunoprecipitation assay. Data shown are one representative of three independent experiments. (D) IL-6, (E) TNF-α and (F) IL-1β expression in cell culture supernatant as measured using ELISA. Data are presented as the mean ± SD from three independent experiments. ns, not statistically significant; **P<0.01 and ***P<0.001. OV, ortho-vanillin; LPS, lipopolysaccharide; TLR2, toll-like receptor 2; MyD88, myeloid differentiation primary response 88; IL, interleukin; TNF-α, tumour necrosis factor-α; SD, standard deviation.

In conclusion, these results demonstrated that OV exhibited no cell toxicity and treatment of MPC5 cells with OV reduced inflammatory cytokine production by inhibiting the TLR2-MyD88 interaction in podocytes.

OV treatment reduces LPS-induced AKI in mice. The renal protective effects of OV were determined using an LPS-induced AKI mouse model. BALB/c mice were first pretreated with OV before being challenged with LPS, and renal function was subsequently assessed using staining. A period of 24 h after LPS challenge, severe renal pathological lesions, including glomeruli abnormality in morphology, loss of brush border with notable inflammatory cell infiltration were observed in renal tissues (Fig. 2A and B). In contrast, significantly attenuated injury score was identified in kidney tissues isolated from mice pre-treated with OV. Measurements of BUN and SCr, which are two biomarkers for septic AKI, were also consistent with the histological evaluation, in that LPS challenge increased BUN and SCr levels in the blood when compared with vehicle control, an effect that was significantly reduced by OV treatment

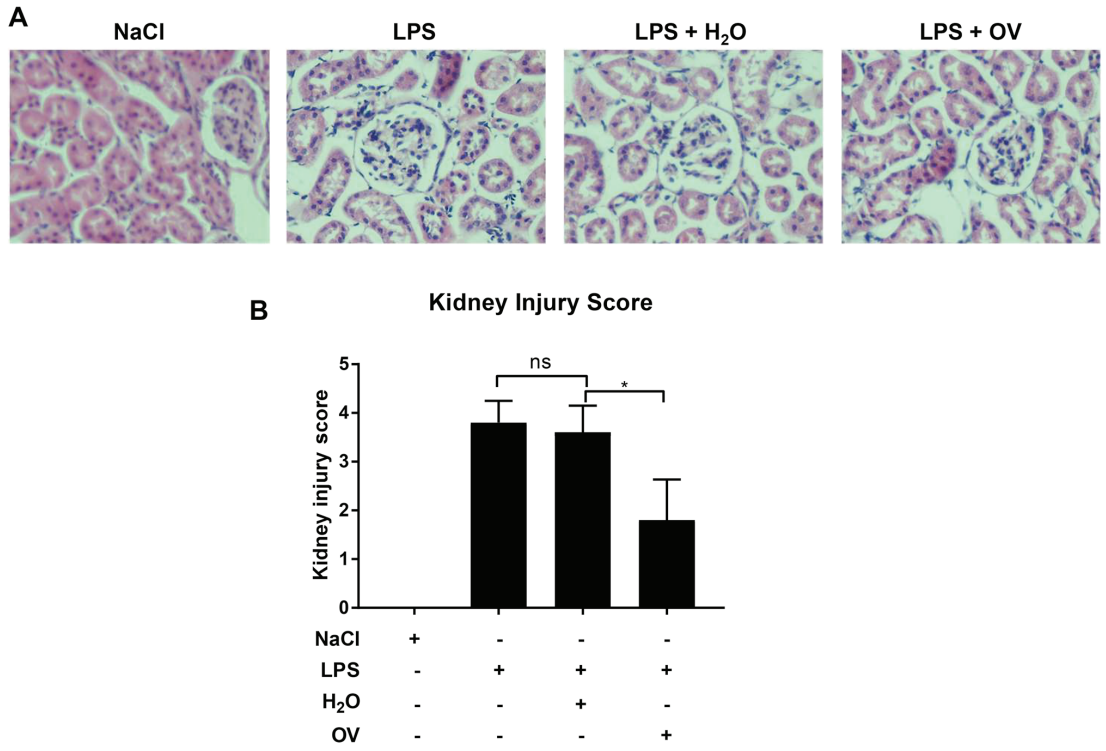

Figure 2. OV treatment reduces pathological injury in an LPS-induced AKI mouse model. Mice were pretreated with OV for 2 h and then challenged with LPS for a further 24 h. Mice were then sacrificed and kidney samples were collected for preparation of paraffin sections. Tissue sections were subsequently dewaxed, rehydrated and stained with periodic acid solution and Schiff's reagent. Images were captured at magnification x200. (A) One representative image of three is shown per condition. (B) Kidney injury was scored according to scales 0-4. Data are presented as the mean ± standard deviation (n=5 mice/group) from three independent experiments. Asin the vehicle control group no apparent kidney injury was observed, the injury score was consequently designated 0. *P<0.05. AKI, acute kidney injury; ns, not statistically significant; OV, ortho-vanillin; LPS, lipopolysaccharide.

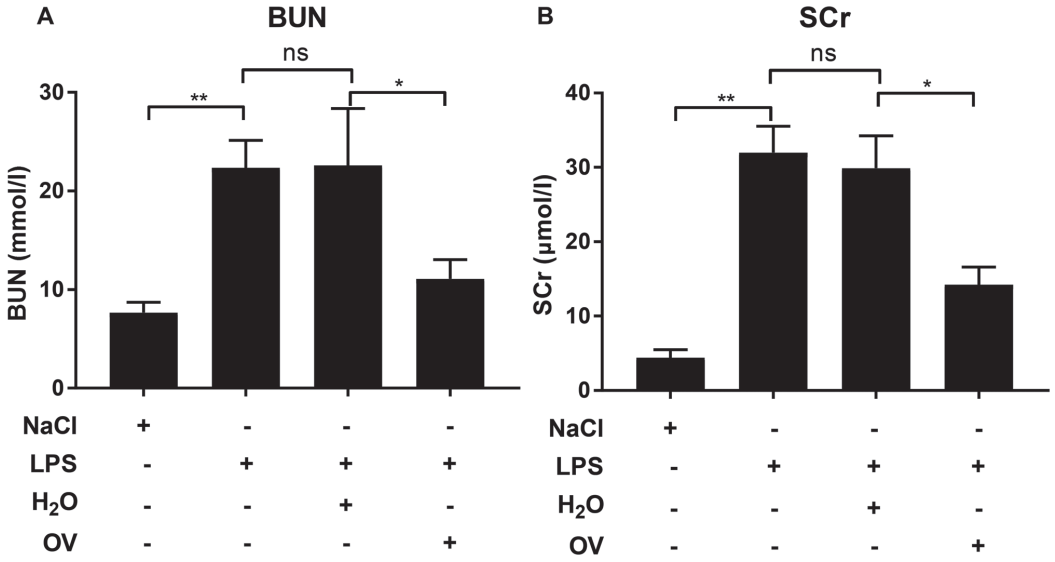

Figure 3. OV treatment improves serum BUN and SCr levels in LPS-induced AKI model mice. Mice were pretreated with OV and then challenged with LPS. (A) BUN and (B) SCr were measured in the sera collected 24 h after challenge. Data are presented as mean ± standard deviation (n=5 mice/group) from three independent experiments. *P<0.05 and **P<0.01. AKI, acute kidney injury; ns, not statistically significant; OV, ortho-vanillin; LPS, lipopolysaccharide; BUN, blood urea nitrogen; SCr, serum creatinine.

(Fig. 3A and B). Taken together, these data demonstrated that OV conferred protective properties against LPS-induced AKI.

OV treatment reduces proinflammatory cytokine production in LPS-induced AKI mice. The hypothesis that OV treatment also inhibited inflammatory responses that are induced by LPS challenge was subsequently investigated using ELISA. The levels of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in kidney tissue extracts were significantly increased following LPS challenge, which was prevented by OV

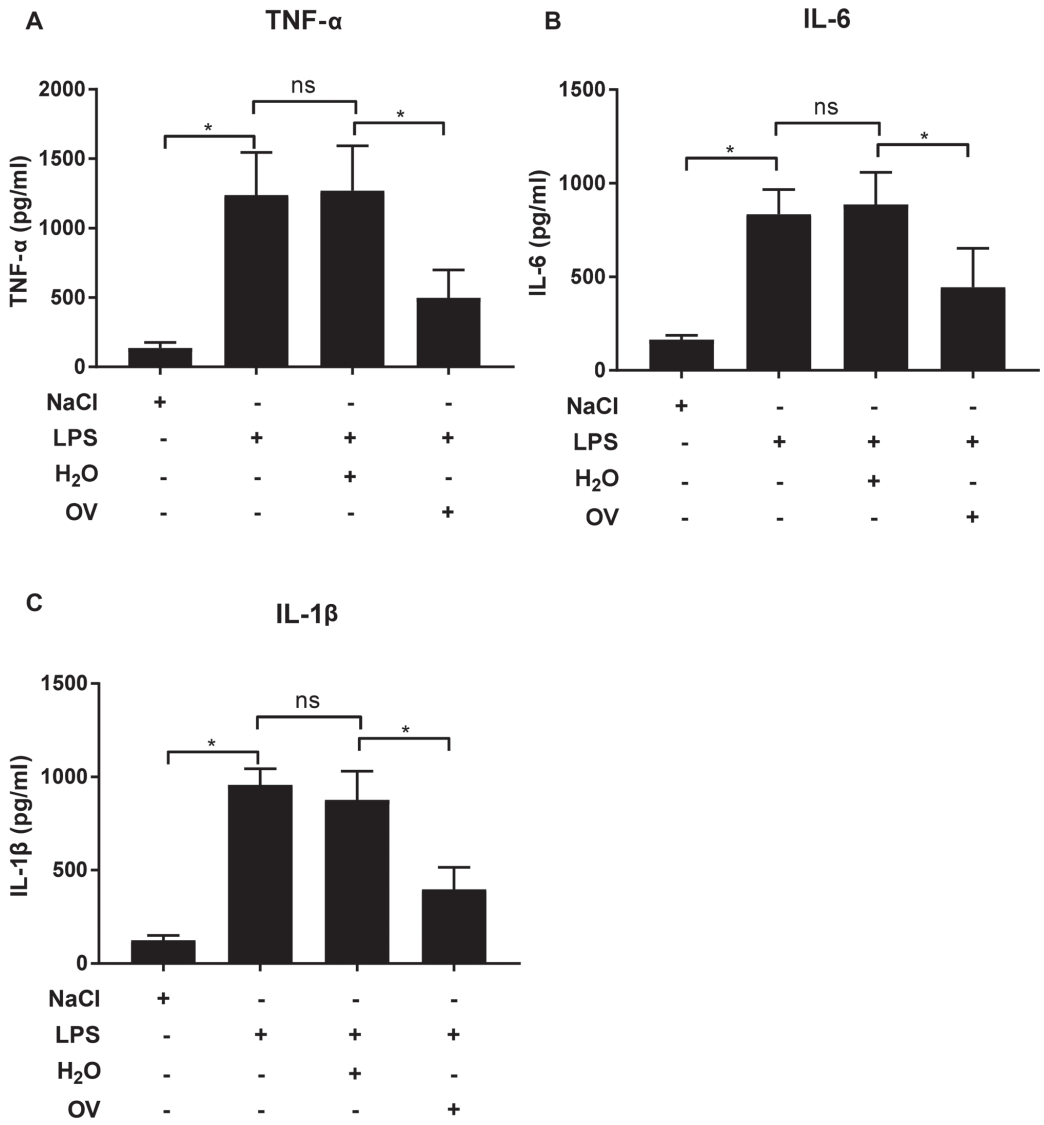

Figure 4. OV treatment reduces renal pro-inflammatory cytokine production in LPS-induced AKI model mice. Mice were pretreated with OV, challenged with LPS and were then sacrificed 24 h after challenge and kidney samples were harvested and homogenized. (A) TNF-α, (B) IL-6 and (C) IL-1β were quantified in kidney tissue lysates using ELISA. Data are presented as the mean ± standard deviation (n=5 mice/group) from three independent experiments. *P<0.05. ns, not statistically significant; AKI, acute kidney injury; OV, ortho-vanillin; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumour necrosis factor-α.

treatment (Fig. 4A-C). These data indicated that OV treatment could alleviate renal damage by inhibiting the expression of pro-inflammatory cytokines.

OV treatment inhibits the NF-kB signaling pathway in LPS-induced AKI mice. Previous studies have revealed that TLR2 induces inflammation by activating the NF-κB signaling pathway in LPS-treated AKI mice (17,18). Since OV is a small molecule inhibitor against TLR2 and treatment with this inhibitor has been previously demonstrated to reduce TLR2 activation and downstream inflammatory cytokine production, it was hypothesized that the NF-κB signaling pathway may also be inhibited by OV. To test this, TLR2 expression was measured in kidney tissues. In similarity with the data obtained from MPC5 cells, LPS challenge significantly increased TLR2 expression, compared with that in vehicle control, but this was not alleviated by treatment with OV (Fig. 5A and C). The expression of p65 and p-p65 were then measured in kidney tissues. In comparison with vehicle control, LPS challenge significantly upregulated the expression of p65 and p-p65 (Fig. 5B and D). OV treatment did not induce the expression of p65 but significantly inhibited the phosphorylation of this protein, suggesting that OV inhibited the activation of the NF-κB signaling pathway (Fig. 5B and D). Total p65 levels were unchanged by treatment with OV. Under some circumstances, the upregulation of p65 expression together with phosphorylation have been previously observed (28,29). These findings support the results of the current study, which determines the expression of p65 was upregulated in the kidney upon LPS stimulation.

Taken together, the results of the present study revealed that OV inhibited the activation of the TLR2 signaling pathway and subsequent inflammatory cytokine production both *in vitro* and *in vivo*. Furthermore, treatment with OV can effectively protect against LPS-induced AKI via inhibiting TLR2/NF-κB signaling pathway, representing a novel therapeutic approach for the treatment and possible prevention of AKI.

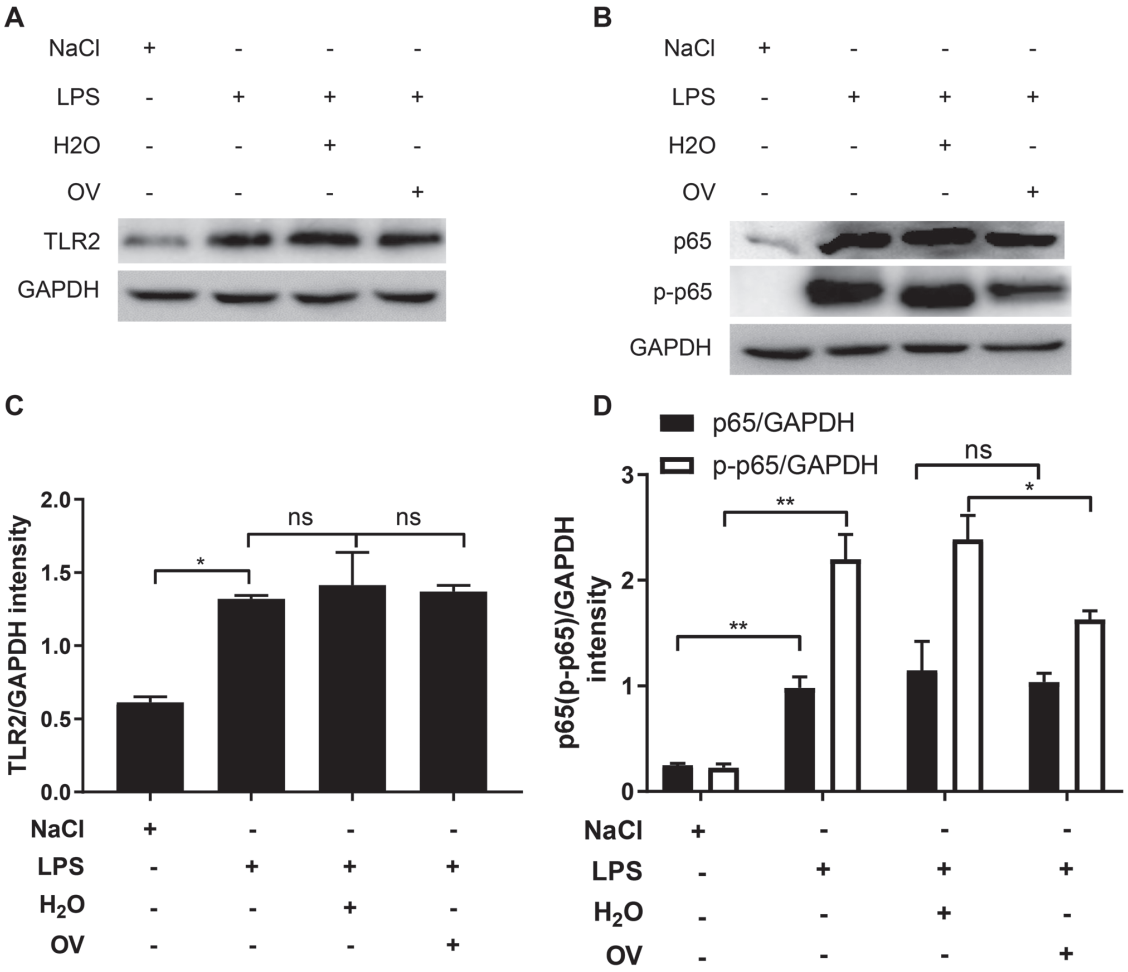

Figure 5. OV treatment inhibits NF-κB signaling activation in LPS-induced AKI mice. Mice were pretreated with OV and then challenged with LPS and were sacrificed 24 h later where kidney samples were harvested and homogenized. (A) TLR2, (B) p65 and p-p65 in the kidney tissue lysates were quantified using western blot analysis. One representative image from three is shown. (C) Quantified densitometric values of TLR2, (D) p65 and p-p65 performed using ImageJ. Data are presented as the mean ± standard deviation (n=5 mice/group) of three independent experiments. *P<0.05 and **P<0.01. ns, not statistically significant; AKI, acute kidney injury; OV, ortho-vanillin; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumour necrosis factor-α; TLR2, toll-like receptor 2; p65, NF-κB p65 subunit; p-p65, phosphorylated p65.

Discussion

As one of the most severe complications in hospitalized patients, AKI is a multi-factorial disease that is associated with a rapid loss of renal function. AKIs are categorized into 3 groups: Pre-renal (reduced blood supply into the kidney), renal (kidney tissue damage, including sepsis and accident) and post-renal (urine retention in kidney). Among these different AKIs, ~50% of the cases are septic AKI (30). Excessive inflammation is generally considered to be a key factor in triggering and aggravating AKI, whilst AKI can in turn aggravate inflammation further, resulting in exacerbated renal dysfunction and tissue damage (31). Given the roles of inflammation in AKI initiation and progression, preventing inflammation represents a promising therapeutic strategy for use in AKI (32).

As a general mechanism to combat harmful pathogens and remove damaged cells, inflammation can be triggered by a number of factors, and distinctive pathways can lead to the expression of effectors such as pro-inflammatory cytokines (33). The initiation of inflammation is usually mediated by immune cells that reside in the tissue, which can recognize PAMPs and damage-associated molecular patterns (DAMPs) through their surface pattern recognition receptors (PPRs). Upon recognizing PAMPs or DAMPs, PPRs are activated and release inflammatory mediators through highly regulated signaling pathways. Since inflammation is a multi-factorial and highly regulated cascade response, anti-inflammation strategies can be developed to target each of these factors and/or steps, including the elimination of harmful stimuli, inhibition of pro-inflammation pathways, neutralization of pro-inflammatory mediators and enhancement of anti-inflammatory mediators. LPS is a substance that can cause septic AKI, where the infusion of alkaline phosphatase dephosphorylates LPS to the nontoxic, monophosphorylated form of LPS has prevented renal damage in patients with AKI (34,35). Elevated levels of pro-inflammatory cytokines, including TNF-α and IL-1β, are potent mediators of immunopathological responses during LPS-induced septic AKI, such that the neutralization of TNF-α has been previously demonstrated to reduce both mortality and renal failure in animal studies (36-38). In another study, injection of an immunosuppressive cytokine IL-35 has been demonstrated to reduce renal damage in a LPS-induced AKI mouse model (11). Previous studies performed in the

Laboratory of the Intensive Care Unit, The First People's Hospital of Kunshan, Jiangsu, China have demonstrated that TLR2 is excessively activated in LPS-induced AKI, and triggers the production of pro-inflammatory cytokines, subsequently leading to aggravated renal damage. This finding gave rise to the hypothesis that targeting TLR2 may be a promising approach for septic AKI prevention or treatment (17,18). Therefore, in the present study, the potential protective effects of a TLR2 small molecule inhibitor OV was investigated in a LPS-induced AKI mouse model. OV treatment significantly alleviated renal damage in LPS-induced AKI mice, and this protective effect of OV appeared to be the result of an inhibition of TLR2/NF-κB signaling that was induced by OV. Of note, despite the apparent success achieved by the various anti-inflammation strategies, a single approach does not appear to be sufficient to fully prevent or treat AKI (5,7). Although it is beyond the scope of the present study, it would potentially be useful to assess the protective efficacies of combined therapies in AKI.

In the present study, OV was used as the TLR2 inhibitor to block TLR2-mediated inflammation in LPS-induced AKI mice for a number of reasons. OV is an organic compound present in many plants and has been demonstrated to exhibit no cytotoxicity in numerous studies so far, making this inhibitor a suitable candidate with great potential for drug development (21,39). In particular, OV has been proven to specifically inhibit mouse and human TLR2 signaling, which can facilitate the translation of research from animal to a more clinical setting (21). The mechanism in which OV inhibits TLR2 activity has been elucidated. TLR2 signaling is initiated by dimerization of intracellular Toll/IL-1 receptor resistance (TIR) domains, where the resultant downstream signaling relies on the binding of MyD88 to TIR domains. OV can bind in the BB loop pocket of TIR domains, which inhibits TLR2 trimerization and MyD88 binding (21). However, other measures to inhibit TLR2 activity may still need further investigation to optimize strategies for septic AKI prevention and/or treatment. A number of studies have been previously performed that investigate a number of different TLR2 inhibition strategies for the treatment of diseases that may also be applicable to septic AKI. In the treatment of acute gut inflammation, Shmuel-Galia *et al* (40) indicated that inhibiting TLR2 dimerization by a TLR2 transmembrane peptide significantly reduced monocyte activation and pro-inflammatory cytokine production. In human cytomegalovirus infection, the virus-derived microRNA-UL112-3p could effectively inhibit the activation of the TLR2/NF-κB signaling pathway by targeting TLR2 (41). One structural study has also revealed that staphylococcal superantigen-like protein 3, which is secreted by *staphylococcus aureus*, can antagonize TLR2 by inhibiting its ligand binding and subsequent receptor dimerization (42). Although each disease has its own characteristics and requires unique treatment formulations, it would still be warranted to investigate if the aforementioned TLR2 inhibition strategies could also apply to the prevention and/or treatment of septic AKI.

In terms of the dose used, a previous study has determined that OV administrated at a dose of 1.314 mM/g twice i.p. exhibited good TLR2 inhibition in mice (21). As a proof-of-concept study, this administration route and dose for OV was adopted in septic AKI prevention in the present study. However, to determine the protective effects of OV, a systematic evaluation of different administration routes and doses is required. In addition, as with many other studies in septic AKI treatment, only the preventative effects of OV and not effects on AKI post-injury were examined. Although this is beyond the scope of the present study, it would be interesting to investigate whether OV could also have exhibit effects following AKI injury.

In summary, the present study revealed that targeting TLR2 signaling by treatment with OV could effectively alleviate LPS-induced AKI *in vitro* and *in vivo*, by inhibiting the TLR2/NF-κB signaling pathway. Although further characterization is required, OV, and the general targeting of TLR2, represents a promising target for the prevention of septic AKI.

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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

YP and SL designed the study. YP, LL, YW, JY, FJ, TT, HY performed the experiments. YP, LL, HY and LS analyzed the data. YP and SL wrote the manuscript. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

All protocols involving animals in the present study were reviewed and approved by the Bioethics Committee of the First People's Hospital of Kunshan (Kunshan, China) and performed in accordance with the guidelines of the Laboratory Animal Science Association (IRB approval no. FPHKA201512012).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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