Humanized anti-TLR4 monoclonal antibody ameliorates lipopolysaccharide-related acute kidney injury by inhibiting TLR4/NF-κB signaling

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Abstract. A humanized anti-Toll-like receptor 4 (TLR4) monoclonal antibody (mAb) was previously produced using phage antibody library technology, and it was found that the mAb could effectively ameliorate lipopolysaccharide (LPS)-induced damage in macrophages. The present study investigated the protective effects exerted by the humanized anti-TLR4 mAb against LPS-induced acute kidney injury (AKI), as well as the underlying mechanisms. Female C57BL/6 mice were randomly divided into four groups (n=8 per group): i) Control; ii) LPS; iii) LPS + humanized anti-TLR4 mAb (1 μg/g); and iv) LPS + humanized anti-TLR4 mAb (10 μg/g). Serum creatinine, blood urea nitrogen, IL-6, TNFα and IL-1β levels were then examined, followed by renal pathology assessment, immunohistochemical staining, reverse transcription-quantitative PCR and western blotting to assess apoptosis/survival/inflammation-related molecules and kidney injury molecule (KIM)-1. The humanized anti-TLR4 mAb successfully ameliorated LPS-induced AKI and renal pathological damage. The humanized anti-TLR4 mAb also dose-dependently suppressed LPS-induced elevations in serum IL-6, TNFα and IL-1β, and decreased the renal expression levels of myeloid differentiation primary response 88 (MyD88), IKKα/β, IκB, p65 and KIM-1. Compared with the LPS group, renal Bax and KIM-1 expression levels were significantly downregulated, and Bcl-2 expression was notably upregulated by the humanized anti-TLR4 mAb. Moreover, the humanized anti-TLR4 mAb also significantly decreased the protein expression levels of MyD88, phosphorylated (p)-IKKα/β, p-IκB and p-p65 in the renal tissues compared with the LPS group. Therefore, the present study indicated that the anti-inflammatory effects of the humanized anti-TLR4 mAb against LPS-related AKI in mice were mediated via inhibition of the TLR4/NF-κB signaling pathway.

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Abbreviations: AKI, acute kidney injury; BUN, blood urea nitrogen; KIM-1, kidney injury molecule-1; LPS, lipopolysaccharide; mAb, monoclonal antibody; SI-AKI, sepsis-induced AKI; TLR4, Toll-like receptor 4

Key words: humanized anti-TLR4 mAb, acute kidney injury, NF-κB, TLR4/LPS

Introduction

Acute kidney injury (AKI) describes acute renal function decline, which results in >60% of patients requiring intensive care (1). The etiology of AKI can be multiple, and sepsis, which is defined as the presence of any organ dysfunction that results from the deleterious response of the host to infection, is a common offender. Indeed, the kidney is one of the most commonly affected organs during sepsis and sepsis-induced (SI)-AKI substantially contributes to the morbidity and mortality of patients who suffer from sepsis (2). The most common cause of SI-AKI is gram-negative bacterial infection, and lipopolysaccharide (LPS), which is an important constituent of the outer membrane of gram-negative bacteria, can be an important contributor to risk associated with AKI (3). LPS can activate Toll-like receptor 4 (TLR4) signaling and NF-κB, leading to the generation of vital effectors, such as TNFα and IL-1β (4). Excessive inflammatory cytokine generation can be deleterious to the kidneys (5).

Pathologically, SI-AKI involves the presentation of peritubular endothelial dysfunction, tubular injury and inflammatory cell infiltration (6-8). Although the understanding of the pathophysiology of SI-AKI has improved, its incidence remains high, ≤60% of patients with sepsis have AKI (2), rendering SI-AKI a frequent complication for those suffering from critical illnesses (2). Therefore, effective treatments for SI-AKI, in which LPS-induced SI-AKI constitutes the majority cases, are urgently required.

TLR4 is a transmembrane protein with extracellular leucine-rich repeats (9). The primary ligand for binding TLR4 is LPS. Upon recognizing LPS, TLR4 triggers the IL-1 receptor-domain-containing adaptor-inducing IFN-β- and myeloid differentiation primary response 88 (MyD88)-dependent signal, causing the production of proinflammatory cytokines and type I interferon via activation of NF-κB, interferon regulatory factor-3 and MAPK signaling pathways (10). The renal expression of TLR4 is primarily located in the tubular epithelia, but it can also be found in the glomeruli and vascular endothelia (5). It is possible that TLR4 may serve as a potential therapeutic target for LPS-induced AKI and the use of a monoclonal antibody (mAb) is a promising approach (5,11). The monoclonal antibodies are frequently administered among patients with inflammation-triggered diseases (11). In prior studies, humanized anti-TLR4 Fab fragments with gene-splicing were created using phage antibody library technology and antibodies based on a prokaryotic vector were successfully constructed, followed by the collection of purified anti-TLR4 Fab antibodies (11,12). It was also revealed that the produced anti-TLR4 mAb can effectively counteract LPS-induced damage by blocking TLR4 signaling in macrophages (11,12). In light of these findings, the protective effects against LPS-related AKI exerted by the humanized anti-TLR4 mAb and the associated mechanisms in mice were further investigated in the present study.

Materials and methods

Reagents, diagnostic kits and antibodies. The humanized anti-TLR4 mAb was provided by Professor Jin Zhu (Nanjing Medical University, Nanjing, China; patent no. ZL201410765623.8). The *Escherichia coli* 0111 B4 LPS was purchased from Sigma-Aldrich (Merck KGaA). The diagnostic kit for serum creatinine and the test kit for blood urea nitrogen (BUN) were purchased from Beckman Coulter, Inc. For detecting IL-1β, IL-6 (Rockland Immunochemicals Inc. Mouse IL-1 beta AccuSignal ELISA kit; cat. no. KOA0211 and Mouse IL-6 AccuSignal ELISA kit; cat. no. KOA0226) and TNFα (Dakewe Bio-engineering Co., Ltd. Mouse TNF-α Precoated ELISA kit; cat. no. 1217202) ELISA kits were used. For western blotting, the antibodies targeted against Bax (cat. no. 14796), MyD88 (cat. no. 4283), phosphorylated (p)-IκB (cat. no. 2859), IκB (cat. no. 4812), p-IKKα/β (cat. no. 2697), p-p65 (cat. no. 3033), p65 (cat. no. 8242) and GAPDH (cat. no. 5174) were obtained from Cell Signaling Technology, Inc. The antibody targeted against kidney injury molecule-1 (KIM-1) was purchased from Novus Biologicals, LLC (cat. no. NBP1-76701) and the antibody targeted against Bcl-2 (cat. no. ab182858) and IKKα/β (cat. no. ab178870) was purchased from Abcam . The HRP-linked polymer detection system was obtained from Shanghai Changdao Biotechnology Co. Ltd.

Animal experiments: Group assignment and treatment. Female C57BL/6 mice (9 weeks old, 18-22 g) were obtained from Changzhou Cavens Lab Animal Co. Ltd. All animals were acclimated for 7 days before experiment initiation, placed in a 12-h light/dark cycle at a temperature of 22±2˚C in an air-conditioned room, and given access to food and water *ad libitum*. All procedures were carried out according to the Guidelines for Laboratory Animal Care of the US National Institutes of Health (13). The present study was approved by the Research Ethics Committee of The Affiliated Wuxi People's Hospital of Nanjing Medical University (approval no. KS202089). All applicable international, national and/or institutional guidelines for the care and use of animals were followed. A total of 32 mice were randomly divided into four groups (n=8 per group): i) Control; ii) LPS; iii) LPS + humanized anti-TLR4 antibody (1 μg/g); and iv) LPS + humanized anti-TLR4 antibody (10 μg/g). The concentration of humanized anti-TLR4 antibody was selected according to the results of pre experiments. To induce AKI, mice in the LPS group were intraperitoneally administered 10 μg/g body weight of LPS dissolved in normal saline. Mice in the LPS + humanized anti-TLR4 antibody groups received a humanized anti-TLR4 antibody injection through the tail vein at 4 h before the LPS challenge. Mice in the control group were intraperitoneally administered 10 μg/g body weight of normal saline. All mice were sacrificed at 24 h after LPS stimulation or saline injection, and the blood and kidneys were collected. Isoflurane inhalation anesthesia was used (induction concentration, 3%; maintenance concentration, 2%) and venous blood was collected from the orbital sinus, followed by the removal of the bilateral kidney. Then, mice were sacrificed by CO_2 asphyxia (50% CO_2 replacement rate).

Serum biochemical and cytokine analysis. The sera obtained from mice was isolated from total blood by centrifugation (3,000 x g for 10 min at room temperature). Subsequently, serum creatinine and blood urea nitrogen (BUN) of serum were measured using commercially available kits. The levels of serum IL-6, TNFα and IL-1β were determined using ELISA kits according to manufacturer's protocols.

Renal histopathological examination. Mice renal tissues were fixed with 10% formaldehyde, embedded in paraffin, sectioned at 4 μm thick and stained with hematoxylin and eosin (hematoxylin aqueous solution for 5 min, alcohol eosin for 1-2 min at room temperature). Pathological examination was performed under a light microscope (magnification, x200) by two pathologists, who were blinded to the treatment. The severities of renal tubuli that presented necrosis, brush border loss, interstitial edema and tubular dilation were classified into five categories: 0, none; 1, 0-20%; 2, 20-50%; 3, 50-70%; and 4, >70% (4).

For the immunohistochemical (IHC) staining, the paraffin-embedded kidney sections were deparaffinized and rehydrated in a graded ethanol series. Using a microwave, the antigen was recovered using sodium citrate solution (pH 6.0). Following antigen extraction and internal peroxidase quenching

by 3% H_2O_2, the sections were incubated overnight with Bax mAb (1:400), Bcl-2 mAb (1:500) and KIM-1 polyclonal antibody (1:100) at 4˚C. Then the sections were incubated with HRP-bonded second antibody (1:200) for 30 min and stained with hematoxylin for 3 min at room temperature, followed by the collection of four digital images of each non-overlapping microscopic field of the renal cortex and medulla using an Eclipse Ni light microscope (Nikon Corporation; magnification, x200). The positive stained areas of KIM-1, Bax and Bcl-2, based on the unit area (magnification, x200), were calculated as the percentage of all examined areas using a digital image analysis program [ImageJ V1.8.0.112 (National Institutes of Health); DS-Ri2 Special color imaging system for microscope; Nikon Corporation)].

Western blotting. RIPA buffer (CoWin Biosciences) was used to extract total protein from renal tissues. The BCA method was used to determine the protein concentration. The total protein samples (30 μg) were separated via 12% SDS-PAGE and transferred onto PVDF membranes, which were then blocked with 5% albumin from bovine serum in TBS for 60 min at room temperature. The membranes were incubated overnight at 4˚C with primary antibodies targeted against: MyD88 (1:1,000), p-p65 (1:1,000), p65 (1:1,000), p-IKKα/β (1:1,000), IKKα/β (1:1,000), p-IκB (1:1,000), IκB (1:1,000), Bax (1:1,000), KIM-1 (1:500), Bcl-2 (1:5,000) and GAPDH (1:2,000). After incubation with HRP-labeled Goat Anti-Rabbit IgG (Beyotime Institute of Biotechnology; cat. no. A0208; 1:1,000) for 60 min at 37˚C, the bands were visualized using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.). Aperio Image Analysis (Leica Microsystems GmbH; MAN-0013, revision G) was used to semi-quantify protein expression. GAPDH was used as the loading control. A total of eight samples were used for each analysis and the analysis was repeated three times each. There were three independent tests per experiment.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was performed to determine the mRNA expression levels of MyD88, IKKα/β, IκB, p65 and KIM-1 using specific primers. Total RNA was extracted from renal tissues using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Then, the RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.; cat. no. K1622) according to the manufacturer's protocol, followed by qPCR using SYBR-Green qPCR Master Mix (Thermo Fisher Scientific, Inc.) and the ABI PRISM 7300 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a 384-well PCR plate. The thermocycling conditions used for qPCR were as follows: 95˚C for 10 min; 40 cycles of 95˚C for 15 sec and 60˚C for 45 sec; followed by dissociation at 95˚C for 15 sec, 60˚C for 60 sec, 95˚C for 15 sec and 60˚C for 15 sec to detect the melting temperature. The primer sequences for each gene were: MyD88 forward, 5'-CCCCACTCGCAGTTTGTTG-3' and reverse, 5'-GATGCCTCCCAGTTCCTTTG-3'; IKKα/β forward, 5'-GAGACACGGAAGGCAACC-3' and reverse, 5'-GAGACACGGAAGGCAACC-3'; IκB forward, 5'-GACTGACATTGTGGACCTGC-3' and reverse, 5'-GACTGACATTGTGGACCTGC-3'; p65 forward, 5'-ATCTGTTTCCCCTCATCTTTCC-3' and reverse, 5'-CAGCCTCATAGTAGCCATCCC-3'; KIM-1 forward, 5'-AATGGCACTGTGACATCCTC-3' and reverse, 5'-GAGACACGGAAGGCAACC-3'; GAPDH forward, 5' CTGCCCAGAACATCATCC 3' and reverse, 5' CTCAGATGCCTGCTTCAC 3'. mRNA expression levels were normalized to the internal reference gene GAPDH. The RT-PCR data were calculated by using the $2^{-\Delta\Delta Cq}$ method (14).

Statistical analysis. SPSS (version 18; SPSS, Inc.) was used for the statistical analysis. Continuous data are presented as the mean ± SEM. The differences among multiple treatment groups were analyzed using one-way ANOVA followed by Tukey's post hoc test (parametric) or the Kruskal-Wallis test followed by Dunn's post hoc test (non-parametric). There were six samples in each group, and three multiplex RT-PCR analyses were performed on one sample. A total of eight samples for each western blot analysis run and western blotting and all other experiments were repeated three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of the humanized anti-TLR4 mAb on LPS-induced renal histopathology. The effect of the humanized anti-TLR4 mAb on LPS-related AKI was first examined based on the renal pathology. Compared with the control group, the kidneys in the LPS group displayed prominent pathologies and pretreatment with the humanized anti-TLR4 mAb (1 μg/g and 10 μg/g) significantly improved such renal injuries (Fig. 1).

Effects of the humanized anti-TLR4 mAb on the renal function of LPS-treated mice. The serum BUN and creatinine levels were further examined to evaluate the effect of the humanized anti-TLR4 mAb on renal function. Compared with the control group, mice treated with LPS displayed significantly elevated serum BUN and creatinine levels, whereas LPS-induced alterations in BUN were significantly attenuated by the administration of the humanized anti-TLR4 mAb (10 μg/g; Fig. 2). Serum creatinine (log-transformed) was also attenuated by the administration of the humanized anti-TLR4 mAb, but the difference was not significant.

Effects of the humanized anti-TLR4 mAb on LPS-induced alterations in IL-6, TNFα and IL-1 β levels based on ELISAs. Compared with the control group, LPS significantly increased the serum TNFα, IL-6 and IL-1β levels, which was attenuated by pretreatment with the humanized anti-TLR4 mAb in a dose-dependent manner (Fig. 3).

Effects of the humanized anti-TLR4 mAb on the expression of MyD88, IKKα/β, IκB, p65 and KIM-1 in renal tissues based on RT-qPCR. The mRNA expression levels of MyD88, IKKα/β, IκB, p65 and KIM-1 in the kidneys were significantly higher in the LPS group compared with the control group (Fig. 4). LPS-induced increases in the expression levels of MyD88, IKKα/β and IκB were significantly attenuated by pretreatment with the humanized anti-TLR4 mAb (10 μg/g). Pretreatment with the humanized anti-TLR4 mAb decreased

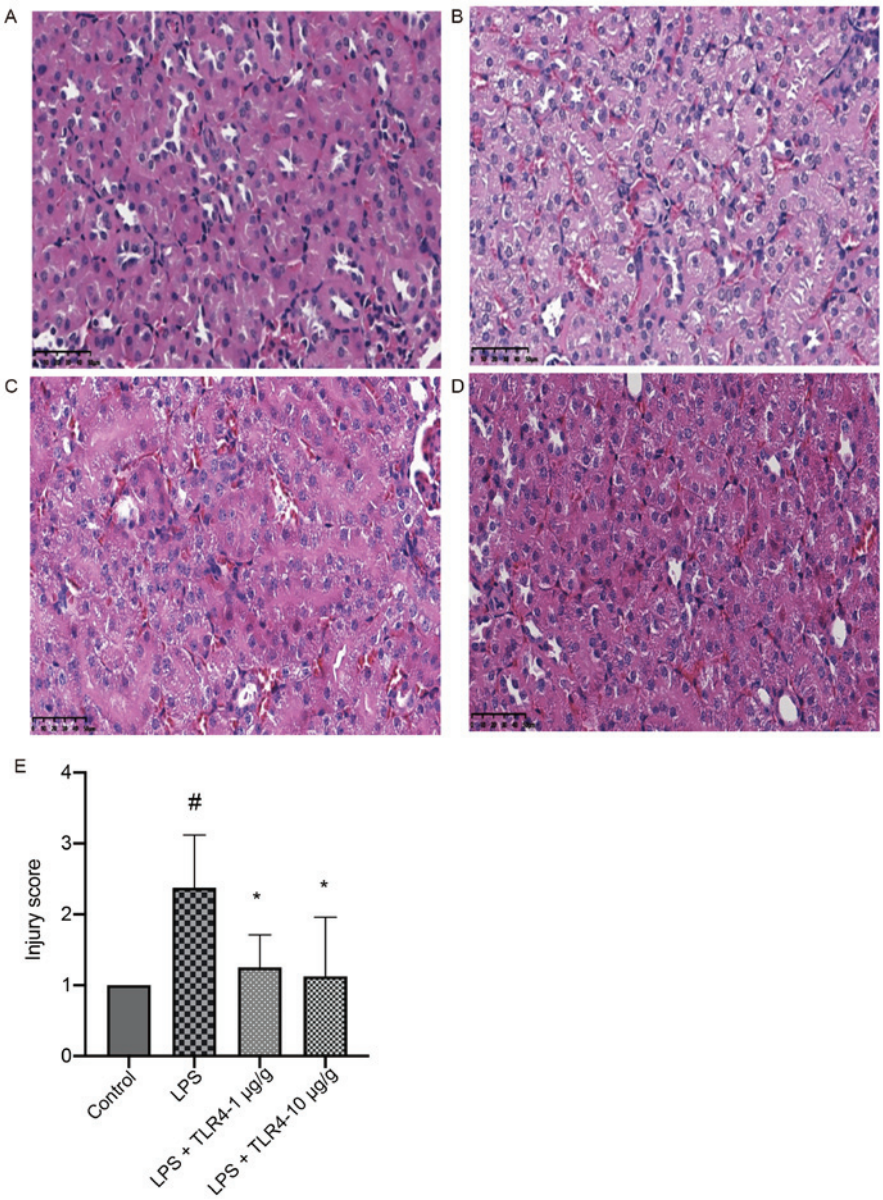

Figure 1. Effect of the humanized anti-TLR4 mAb on LPS-induced renal pathologies. The histopathological changes of renal tissues from different groups: (A) Control, (B) LPS, (C) LPS + humanized anti-TLR4 mAb (1 μg/g) and (D) LPS + humanized anti-TLR4 mAb (10 μg/g). Hematoxylin and eosin staining was performed to assess histopathological changes (magnification, x200). (E) Quantification of renal pathologies. $^{\#}$P<0.01 vs. control; *P<0.05 vs. LPS. TLR4, Toll-like receptor 4; mAb, monoclonal antibody; LPS, lipopolysaccharide.

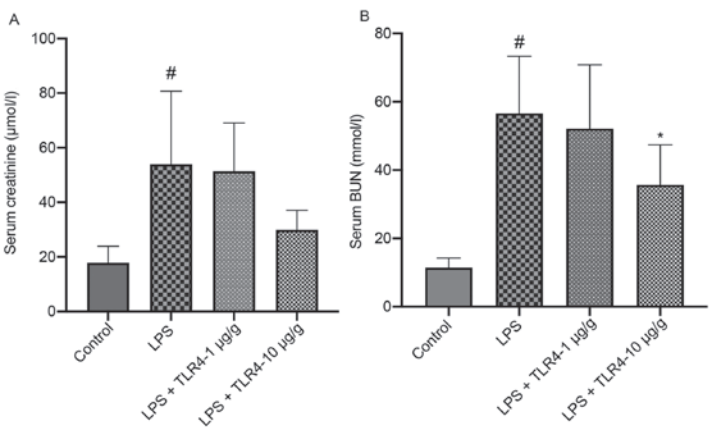

Figure 2. Creatinine and BUN levels in mice pretreated with the humanized anti-TLR4 mAb. The sera were collected at 24 h after LPS treatment and were tested for (A) creatinine and (B) BUN levels. $^{\#}$P<0.05 vs. control; *P<0.05 vs. LPS. BUN, blood urea nitrogen; TLR4, Toll-like receptor 4; mAb, monoclonal antibody; LPS, lipopolysaccharide.

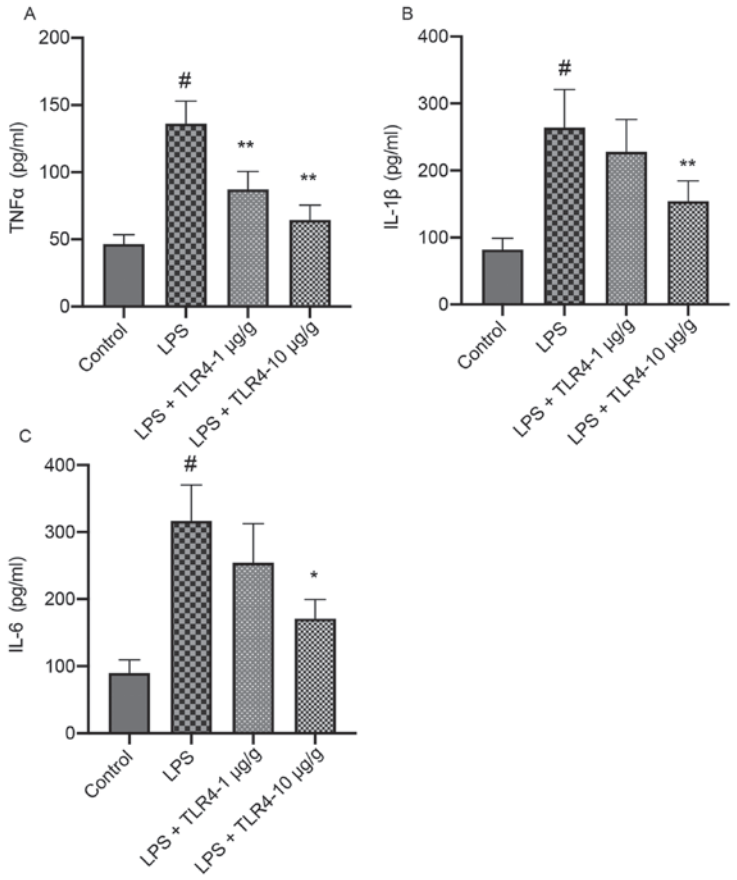

Figure 3. Effect of the humanized anti-TLR4 mAb on serum TNFα, IL-6 and IL-1β levels. The sera were collected at 24 h after LPS treatment, followed by assessment of the levels of (A) TNFα, (B) IL-1β and (C) IL-6. $^{\#}$P<0.01 vs. control; *P<0.05 and **P<0.01 vs. LPS. TLR4, Toll-like receptor 4; mAb, monoclonal antibody; LPS, lipopolysaccharide.

p65 and KIM-1 expression levels in LPS-treated mice, but the difference was not significant.

Effects of the humanized anti-TLR4 mAb on the expression of Bax, Bcl-2 and KIM-1 in renal tissues based on IHC staining. The percentage of positively stained areas of KIM-1 and Bax in the LPS group were significantly increased compared with the control group (Fig. 5). However, pretreatment with the humanized anti-TLR4 mAb significantly decreased KIM-1 and Bax expression compared with the LPS group. In the LPS group, the positively stained areas of Bcl-2 were significantly decreased compared with the control group, whereas pretreatment with the humanized anti-TLR4 mAb increased Bcl-2 positive staining, but the difference was not significant.

Effects of the humanized anti-TLR4 mAb on the expression of MyD88, p-IKKα/β, p-IκB and p-p65 in renal tissues based on western blotting. Compared with the control group, LPS treatment significantly increased the expression levels of MyD88, p-IKKα/β, p-IκB and p-p65 in renal tissues (Fig. 6). LPS-induced increases in the expression of MyD88, p-IKKα/β, p-p65 and p-IκB were significantly attenuated by pretreatment with the humanized anti-TLR4 mAb.

Effects of the humanized anti-TLR4 mAb on the expression of Bax, Bcl-2 and KIM-1 in renal tissues based on western blotting. The expression levels of KIM-1 and Bax were significantly higher in the LPS group compared with the control group. The expression of these proteins significantly declined after pretreatment with the humanized anti-TLR4 mAb (Fig. 7). Furthermore, Bcl-2 expression in the LPS group was significantly decreased compared with the control group, but pretreatment with the humanized anti-TLR4 mAb (10 μg/g) significantly increased Bcl-2 expression levels in LPS-treated mice.

Discussion

The occurrence of AKI is associated with a higher risk of developing complications that involve various organs and systems (15). Among in-patients, >5% are at risk of manifesting AKI, which increases the risk of mortality and the subsequent development of chronic kidney disease (16). Among those that require intensive care, >50% have AKI, the severity of which is closely associated with an increase in hospitalization mortality (17). At present, the management plan for patients with SI-AKI consists primarily of infection control, transfusion and optimal resuscitation, the use of vasoactive drugs and the prompt initiation of renal replacement therapy (2). However, patients with SI-AKI continue to suffer from high mortality. Therefore, novel therapeutics for effective management are urgently required. Previous studies reported that the humanized anti-TLR4 mAb can effectively ameliorate LPS-triggered inflammatory responses

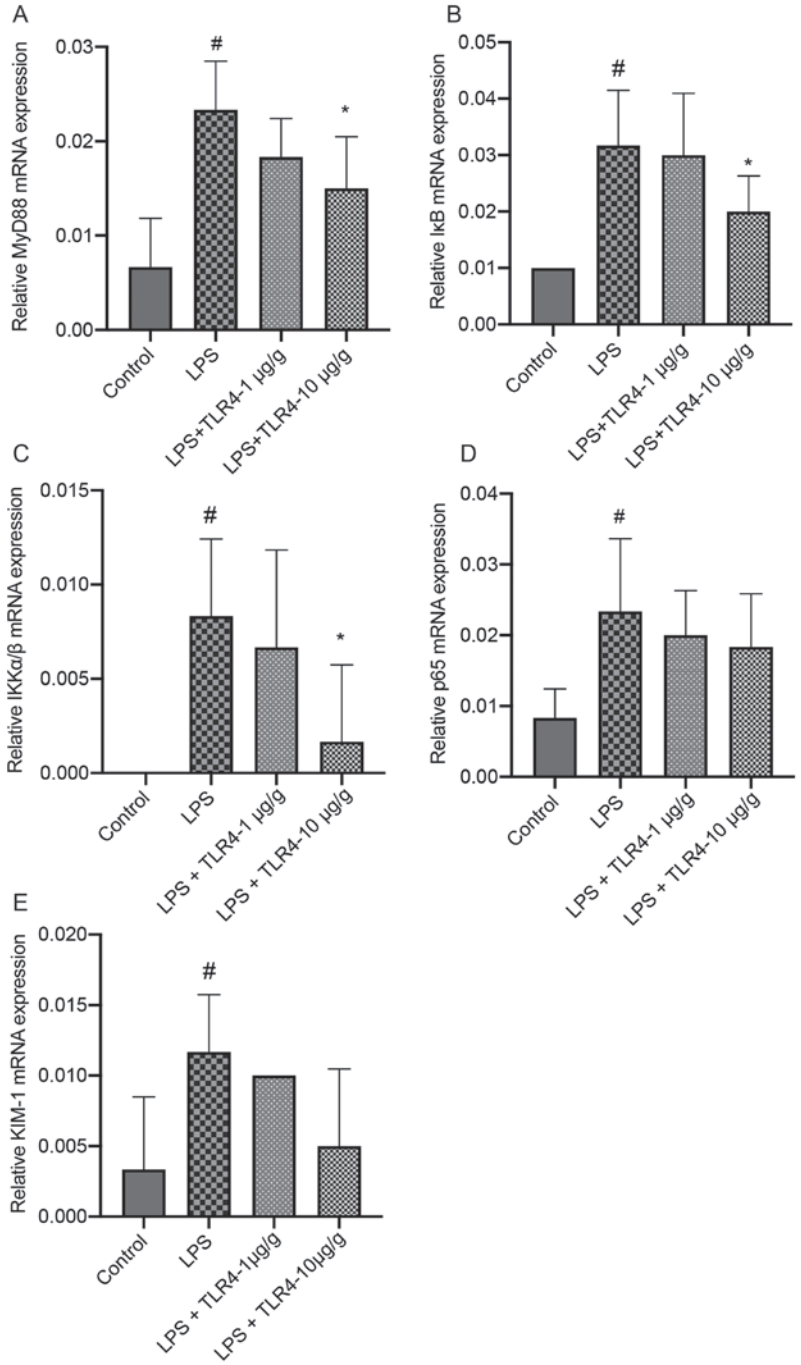

Figure 4. Effects of the humanized anti-TLR4 mAb on the expression of MyD88, IκB, IKKα/β, p65 and KIM-1 in renal tissues. The kidneys were collected at 24 h after LPS treatment. Subsequently, reverse transcription-quantitative PCR was performed to assess the expression levels of (A) MyD88, (B) IκB, (C) IKKα/β, (D) p65 and (E) KIM-1. $^{\#}$P<0.05 vs. control; *P<0.05 vs. LPS. TLR4, Toll-like receptor 4; mAb, monoclonal antibody; MyD88, myeloid differentiation primary response 88; KIM-1, kidney injury molecule-1; LPS, lipopolysaccharide.

in macrophages by blocking the TLR4 signal (11,12). In the present study, the effect of the humanized anti-TLR4 mAb on LPS-induced AKI mice was investigated and it was shown that LPS induced significant AKI in treated mice, as indicated by elevated serum creatinine, BUN and KIM-1, which is an early biomarker of AKI (18), expression levels. It was further revealed that, compared with the LPS group, the administration of the humanized anti-TLR4 mAb downregulated KIM-1 expression at the mRNA, protein and tissue level, although this was not significant at the mRNA level. Administration of the humanized anti-TLR4 mAb was accompanied by improved renal histopathological changes and downregulated serum BUN and creatinine levels in LPS-treated mice. Collectively, the results of the present study indicated that the humanized anti-TLR4 mAb may alleviate the severity of LPS-induced AKI in animal models.

Since male mice have been found to be more aggressive prior to the experiments, the differences in animal behavior might alter experimental results. Hence, female mice were selected in the present study. The periods of adolescence, middle-age and old age of mice are 60-90 days, 120-180 days and >180 days after birth, respectively. It was found that

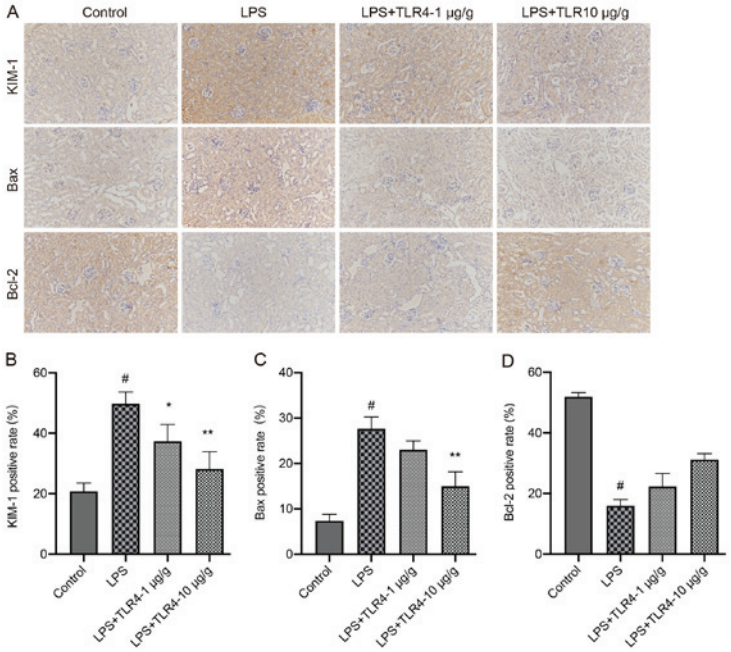

Figure 5. Effects of the humanized anti-TLR4 mAb on the expression of KIM-1, Bax and Bcl-2 in renal tissues. The renal tissues were collected at 24 h after LPS treatment. (A) Immunohistochemical staining (magnification, x200). Quantification of the immunohistochemical staining of (B) KIM-1, (C) Bax and (D) Bcl-2. #P<0.01 vs. control; *P<0.05 and **P<0.01 vs. LPS. TLR4, Toll-like receptor 4; mAb, monoclonal antibody; KIM-1, kidney injury molecule-1; LPS, lipopolysaccharide.

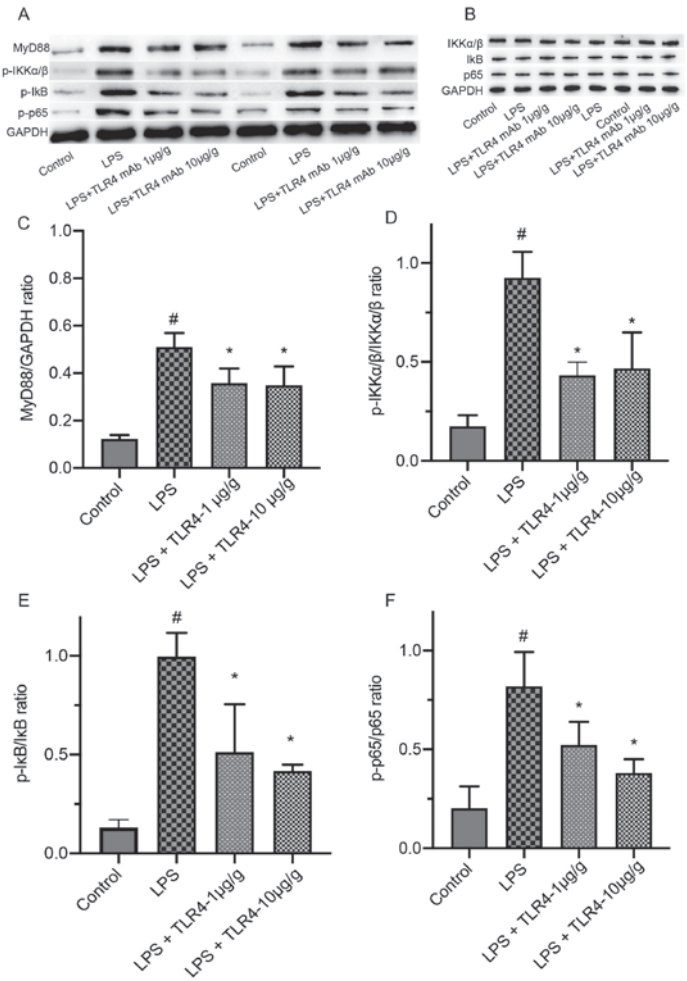

Figure 6. Effects of the humanized anti-TLR4 mAb on the expression of MyD88, p-IKKα/β, p-IκB, p-p65, IKKα/β, IκB and p65 in renal tissues by western blotting. The renal tissues were collected after LPS treatment for 24 h. (A) The expression of MyD88, p-IKKα/β, p-IκB and p-p65 and (B) IKKα/β, IκB and p65 Quantification of (C) MyD88, (D) p-IKKα/β/IKKα/β ratio (E) p-IκB/IκB ratio and (F) p-p65/p65 ratio expression levels. #P<0.01 vs. control; *P<0.01 vs. LPS. TLR4, Toll-like receptor 4; mAb, monoclonal antibody; MyD88, myeloid differentiation primary response 88; p, phosphorylated; LPS, lipopolysaccharide.

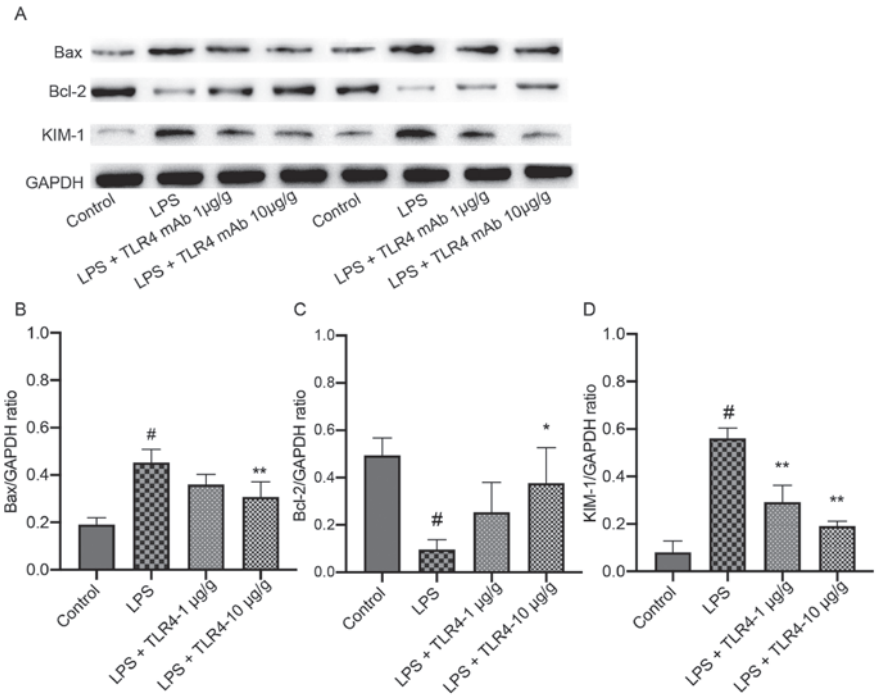

Figure 7. (A) Effects of the humanized anti-TLR4 mAb on Bax, Bcl-2 and KIM-1 expression in renal tissues by western blotting. The renal tissues were collected after LPS treatment for 24 h. Quantification of (B) Bax, (C) Bcl-2 and (D) KIM-1 expression levels. $^{\#}$P<0.01 vs. control; *P<0.05 and **P<0.01 vs. LPS. TLR4, Toll-like receptor 4; mAb, monoclonal antibody; KIM-1, kidney injury molecule-1; LPS, lipopolysaccharide.

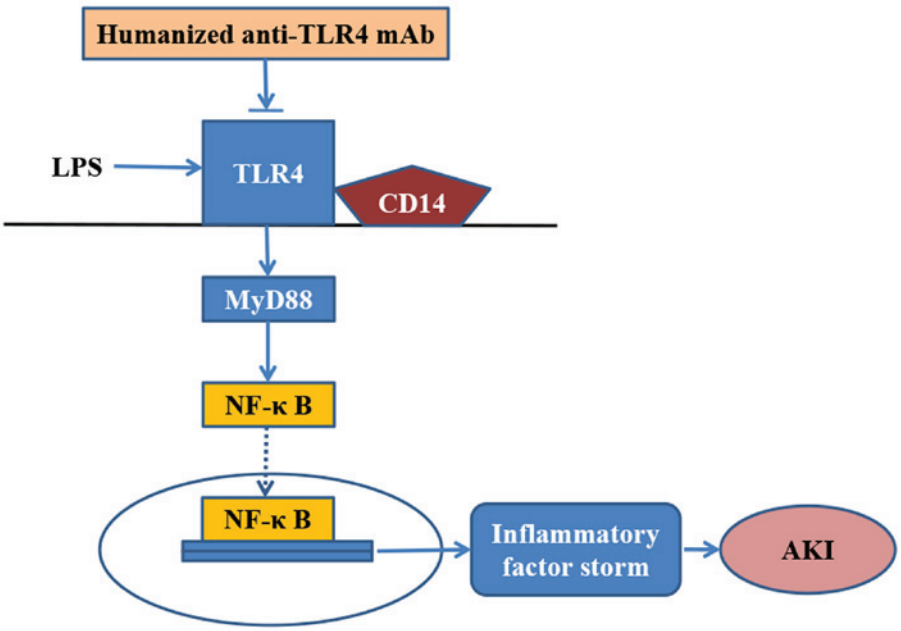

Figure 8. Humanized anti-TLR4 mAb inhibits the activation of NF-κB during LPS-related AKI. TLR4, Toll-like receptor 4; mAb, monoclonal antibody; LPS, lipopolysaccharide; AKI, acute kidney injury; MyD88, myeloid differentiation primary response 88.

6-month-old mice became old with weaker immunity, displayed a declined physical status, thinner hair and slower reactions (19), and had poorer liver and kidney function. Renal aging is associated with alterations in renal morphology and functional decline (20). The glomerular filtration rate of C57BL/6 mice also decreases with age (21). The literature suggests that most reports of LPS-induced AKI involve mice aged 8-12 weeks (15,22). Therefore, the present study used 9-week-old mice after a 7-day acclimation period.

The induction of inflammatory cytokines triggered by LPS serves an important role in the pathogenesis of AKI (23). It has been previously shown that the serum levels of TNFα, IL-1β and IL-6 significantly increase during episodes of LPS-induced AKI and that the inhibition of these cytokines may alleviate such injury (24). A previous study revealed that the humanized anti-TLR4 mAb can lower serum TNFα, IL-1 and IL-6 levels and inhibit the expression of these cytokines in macrophages stimulated by LPS (11). The present study further

showed that the humanized anti-TLR4 mAb decreased the serum TNFα, IL-1β and IL-6 levels in mice with LPS-induced AKI in a dose-dependent manner, suggesting that inhibition of these inflammatory cytokines might be partially responsible for the therapeutic effect of the humanized anti-TLR4 mAb against LPS-induced AKI.

TLR4 signaling is the primary pathway that mediates renal inflammation (25). LPS induces the release of inflammatory cytokines by activating TLR4, which accounts for the subsequent development of renal injuries (26). NF-κB is the main effector that regulates the expression of several inflammatory cytokines (27). By activating the TLR4/NF-κB signaling pathway, LPS can enhance the production of TNFα, IL-1β and IL-6 (15). It has been previously shown that the humanized anti-TLR4 mAb can inhibit LPS-induced NF-κB activation in macrophages (11,12). Previous study have shown that TLR4 may serve as a potential therapeutic target during AKI (5). The anti-inflammatory mechanism underlying the humanized anti-TLR4 mAb was examined and the results demonstrated that the humanized anti-TLR4 mAb (10 μg/g) downregulated LPS-induced elevations in the mRNA expression levels of MyD88, IKKα/β and IκB. Furthermore, the protein expression levels of MyD88, p-IKKα/β, p-IκB and p-p65 were significantly lowered by pretreatment with the humanized anti-TLR4 mAb in LPS-treated mice. The present findings suggested that the humanized anti-TLR4 mAb reduced the release of circulating cytokines during LPS-induced AKI via suppressing TLR4/NF-κB signaling.

Apoptosis also serves an important role in the pathogenesis of SI-AKI (28). Inflammatory exudates in injured kidneys induce apoptosis and promote renal epithelial loss, which is a characteristic of AKI (29,30). The primary mechanisms of renal tubular cell apoptosis are the activation of Bax and the inhibition of Bcl-2. The present study revealed that, compared with the control group, LPS exposure significantly increased Bax expression, but significantly decreased Bcl-2 expression, of which both effects were significantly reversed by pretreatment with the humanized anti-TLR4 mAb (10 μg/g). Therefore, it was hypothesized that the humanized anti-TLR4 mAb may be able to protect against AKI by reducing the expression of apoptosis-related proteins.

Overall, the present study revealed that the humanized anti-TLR4 mAb exhibited a strong anti-inflammatory effect during episodes of LPS-related AKI in mice. In addition, it demonstrated that the humanized anti-TLR4 mAb further inhibited the activation of NF-κB during LPS-related AKI (Fig. 8). Therefore, the humanized anti-TLR4 mAb may serve as a potential therapeutic for the management of SI-AKI in the future.

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Availability of data and materials

The datasets generated and analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

JZ, CX and LW conceived and designed the experiment. QZ, MW and YZ conducted the experiments. XL and LW analyzed the data. QZ and LW wrote the manuscript. JZ, CX, QZ and LW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of The Affiliated Wuxi People's Hospital of Nanjing Medical University (approval no. KS202089). All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Shu B, Feng Y, Gui Y, Lu Q, Wei W, Xue X, Sun X, He W, Yang J and Dai C: Blockade of CD38 diminishes lipopolysaccharide-induced macrophage classical activation and acute kidney injury involving NF-κB signaling suppression. Cell Signal 42: 249-258, 2018.
2. Poston JT and Koyner JL: Sepsis associated acute kidney injury. BMJ 364: k4891, 2019.
3. Kuzmich NN, Sivak KV, Chubarev VN, Porozov YB, Savateeva-Lyubimova TN and Peri F: TLR4 signaling pathway modulators as potential therapeutics in inflammation and sepsis. Vaccines (Basel) 5: 34, 2017.
4. Song J, Fan HJ, Li H, Ding H, Lv Q and Hou SK: Zingerone ameliorates lipopolysaccharide-induced acute kidney injury by inhibiting Toll-like receptor 4 signaling pathway. Eur J Pharmacol 772: 108-114, 2016.
5. Anderberg SB, Luther T and Frithiof R: Physiological aspects of Toll-like receptor 4 activation in sepsis-induced acute kidney injury. Acta Physiol (Oxf) 219: 573-588, 2017.
6. Tran M, Tam D, Bardia A, Bhasin M, Rowe GC, Kher A, Zsengeller ZK, Akhavan-Sharif MR, Khankin EV, Saintgeniez M, *et al*: PGC-1α promotes recovery after acute kidney injury during systemic inflammation in mice. J Clin Invest 121: 4003-4014, 2011.
7. Takasu O, Gaut JP, Watanabe E, To K, Fagley RE, Sato B, Jarman S, Efimov IR, Janks DL, Srivastava A, *et al*: Mechanisms of cardiac and renal dysfunction in patients dying of sepsis. Am J Respir Crit Care Med 187: 509-517, 2013.
8. Jung YJ, Lee AS, Nguyen-Thanh T, Kim D, Kang KP, Lee S, Park SK and Kim W: SIRT2 regulates LPS-induced renal tubular CXCL2 and CCL2 expression. J Am Soc Nephrol 26: 1549-1560, 2015.
9. Kim HM, Park BS, Kim J-I, Kim SE, Lee J, Oh SC, Enkhbayar P, Matsushima N, Lee H, Yoo OJ, *et al*: Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. Cell 130: 906-917, 2007.

10. Akira S, Uematsu S and Takeuchi O: Pathogen recognition and innate immunity. Cell 124: 783-801, 2006.
11. Cai B, Wang M, Zhu X, Xu J, Zheng W, Zhang Y, Zheng F, Feng Z and Zhu J: The fab fragment of a humanized anti-toll like receptor 4 (TLR4) monoclonal antibody reduces the lipopolysaccharide response via TLR4 in mouse macrophage. Int J Mol Sci 16: 25502-25515, 2015.
12. Wang M, Zheng W, Zhu X, Xu J, Cai B, Zhang Y, Zheng F, Zhou L, Yang Z, Zhang X, *et al*: A human anti-toll like receptor 4 Fab fragment inhibits lipopolysaccharide-induced pro-inflammatory cytokines production in macrophages. PLoS One 11: e0146856, 2016.
13. Care NRCU and Animals AUOL; Guide for the Care and Use of Laboratory Animals: The National Academies Collection: Reports funded by National Institutes of Health. National Academies Press, Washington, DC, 2011.
14. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
15. Ye H-Y, Jin J, Jin L-W, Chen Y, Zhou Z-H and Li Z-Y: Chlorogenic acid attenuates lipopolysaccharide-induced acute kidney injury by inhibiting TLR4/NF-κB signal pathway. Inflammation 40: 523-529, 2017.
16. Wonnacott A, Meran S, Amphlett B, Talabani B and Phillips A: Epidemiology and outcomes in community-acquired versus hospital-acquired AKI. Clin J Am Soc Nephrol 9: 1007-1014, 2014.
17. Hoste EA, Bagshaw SM, Bellomo R, Cely CM, Colman R, Cruz DN, Edipidis K, Forni LG, Gomersall CD, Govil D, *et al*: Epidemiology of acute kidney injury in critically ill patients: The multinational AKI-EPI study. Intensive Care Med 41: 1411-1423, 2015.
18. Song J, Yu J, Prayogo GW, Cao W, Wu Y, Jia Z and Zhang A: Understanding kidney injury molecule 1: A novel immune factor in kidney pathophysiology. Am J Transl Res 11: 1219-1229, 2019.
19. Qin C: Medical laboratory zoology. People's Health Publishing House: 51-57, 2015.
20. Zhou XJ, Rakheja D, Yu X, Saxena R, Vaziri ND and Silva FG: The aging kidney. Kidney Int 74: 710-720, 2008.
21. Hackbarth H and Harrison DE: Changes with age in renal function and morphology in C57BL/6, CBA/HT6, and B6CBAF1 mice. J Gerontol 37: 540-547, 1982.
22. Chen L, Yang S, Zumbrun EE, Guan H, Nagarkatti PS and Nagarkatti M: Resveratrol attenuates lipopolysaccharide-induced acute kidney injury by suppressing inflammation driven by macrophages. Mol Nutr Food Res 59: 853-864, 2015.
23. Mortensen J, Shames B, Johnson CP and Nilakantan V: MnTMPyP, a superoxide dismutase/catalase mimetic, decreases inflammatory indices in ischemic acute kidney injury. Inflamm Res 60: 299-307, 2011.
24. Xu D, Chen M, Ren X, Ren X and Wu Y: Leonurine ameliorates LPS-induced acute kidney injury via suppressing ROS-mediated NF-κB signaling pathway. Fitoterapia 97: 148-155, 2014.
25. Cunningham PN, Wang Y, Guo R, He G and Quigg RJ: Role of Toll-like receptor 4 in endotoxin-induced acute renal failure. J Immunol 172: 2629-2635, 2004.
26. Castoldi A, Braga TT, Correa-Costa M, Aguiar CF, Bassi EJ, Correa-Silva R, Elias RM, Salvador F, Moraes-Vieira PM, Cenedeze MA, *et al*: TLR2, TLR4 and the MYD88 signaling pathway are crucial for neutrophil migration in acute kidney injury induced by sepsis. PLoS One 7: e37584, 2012.
27. Blackwell TS and Christman JW: The role of nuclear factor-κ B in cytokine gene regulation. Am J Respir Cell Mol Biol 17: 3-9, 1997.
28. Lee S-Y, Lee Y-S, Choi H-M, Ko YS, Lee HY, Jo SK, Cho WY and Kim HK: Distinct pathophysiologic mechanisms of septic acute kidney injury: Role of immune suppression and renal tubular cell apoptosis in murine model of septic acute kidney injury. Crit Care Med 40: 2997-3006, 2012.
29. Peerapornratana S, Manrique-Caballero CL, Gómez H and Kellum JA: Acute kidney injury from sepsis: Current concepts, epidemiology, pathophysiology, prevention and treatment. Kidney Int 96: 1083-1099, 2019.
30. Gao L, Wu W-F, Dong L, Ren G-L, Li H-D, Yang Q, Li X-F, Xu T, Li Z, Wu B-M, *et al*: Protocatechuic aldehyde attenuates cisplatin-induced acute kidney injury by suppressing nox-mediated oxidative stress and renal inflammation. Front Pharmacol 7: 479, 2016.