BBA - Molecular Basis of Disease 1865 (2019) 558–569

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Downregulation of TIMP2 attenuates sepsis-induced AKI through the NF-κb pathway

Yi-Ming Li[a], Jing Zhang[a], Lian-Jiu Su[a], John A. Kellum[b], Zhi-Yong Peng[a,b,*]

[a] Dept. of Critical Care Medicine, Zhongnan Hospital of Wuhan University, Wuhan 430071, Hubei, China
[b] Center of Critical Care Nephrology, Department of Critical Care Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

ARTICLE INFO

Keywords:
Tissue inhibitor of metalloproteinases 2
Sepsis-induced acute kidney injury
NF-κb pathway
Apoptosis

ABSTRACT

Acute kidney injury (AKI) is a frequent complication of sepsis and contributes to increased morbidity and mortality. Urinary tissue inhibitor of metalloproteinases-2 (TIMP2) has been recently recognized as an early biomarker to predict AKI in critically ill patients. However, the biological functions of TIMP2 remain largely unknown. In this study, we investigated the role of TIMP2 in mediating inflammation and tubular cell apoptosis in AKI. In kidney tissue taken from mice exposed to cecal ligation and puncture (CLP) and in human kidney 2 (HK-2) cells exposed to lipopolysaccharide (LPS) in culture, TIMP2 expression was significantly upregulated. The expression of TIMP2 in the kidney tissue correlated with the severity of AKI *in vivo*. In cultured HK-2 cells, LPS challenge markedly induced cytokine release, and recombinant cytokines promoted TIMP2 expression and apoptosis. However, TIMP2 silencing ameliorated LPS-induced cytokine release, apoptosis, and cell injury. We further found that the effects of downregulation of TIMP2 on a suppression of release of inflammatory cytokines were mediated by p-P65. Stable, kidney-specific TIMP2 knockdown mice were transduced by injecting the TIMP2 knockdown lentiviral vector into kidney parenchyma. TIMP2 silencing ameliorated CLP-induced proinflammatory cytokines, kidney dysfunction as measured by serum creatinine level, and histopathological changes. Downregulation of TIMP2 showed renoprotective effects on endotoxin-induced AKI, which was associated with the anti-inflammatory activity through inhibition of the nuclear factor (NF)-κB pathway. Collectively, our results indicate that TIMP2 plays an important role in mediating sepsis-induced AKI through regulation of NF-κB. These findings reveal the pathogenic role of TIMP2 in AKI and suggest a novel target for the treatment of AKI.

1. Introduction

Acute kidney injury (AKI) is a systemic disease occurring in 10–15% of hospital admissions [1] and in > 50% of patients who are critically ill [2]. AKI is a life-threatening condition closely associated with prolonged ICU stay and multiple complications. Like the BEST Kidney study [3] from more than a decade ago, the recent AKI-EPI study [2] demonstrated that the most frequent etiology of AKI in patients with critical illness is sepsis, which contributes to 40–50% of AKI. Septic AKI is a syndrome of acute impairment of function and organ damage linked with long-term adverse outcomes depending on the extent of acute injury of the underlying organ reserve. Pathogens causing sepsis and their toxins affect the whole body as well as specific organs such as the kidneys. Kidney injury involves morphological and functional changes in endothelial cells that trigger the infiltration of neutrophils, macrophages, natural killer cells, and lymphocytes into the injured kidneys and the release of inflammatory mediators by tubular and endothelial cells [4]. Toxic molecules can reach the proximal renal tubular cells in high concentrations and trigger kidney injury, followed by inflammation and oxidative stress and, finally, cell damage [5,6].

The NF-kappaB family of transcription factors regulates the induction and resolution of inflammation. NF-κB activation regulates neutrophil, macrophage, lymphocyte, and dendritic cell biology. In addition, NF-κB activation has been documented *in vivo* and *in vitro* in intrinsic glomerular cells such as podocytes and mesangial, tubular, and endothelial cells in renal injury or after exposure to inflammatory stimuli [7]. Many stimuli activate canonical NF-κB in cultured renal cells to regulate the transcription of multiple proinflammatory molecules and leading kidney injury [8]. Despite standardization of the definition and staging of AKI, its early recognition remains the most important method of ameliorating renal injury and reducing mortality [9]. In recent decades, numerous clinical investigations have been performed to evaluate the utility of several biomarkers in the early diagnosis and risk stratification of AKI. In 2014, the US Food and Drug Administration

* Corresponding author at: Dept. of Critical Care Medicine, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, China.
E-mail address: zn001590@whu.edu.cn (Z.-Y. Peng).

https://doi.org/10.1016/j.bbadis.2018.10.041
Received 14 July 2018; Received in revised form 3 October 2018; Accepted 31 October 2018
Available online 15 December 2018
0925-4439/

approved the marketing of a test based on the combination of urine concentrations of tissue inhibitor of metalloproteinase-2 and insulin-like growth factor binding protein 7 ([TIMP2] × [IGFBP7]) to determine whether critically ill patients are at an imminent risk of developing moderate to severe AKI [10]. In addition, [TIMP2] × [IGFBP7] levels are associated with adverse long-term outcomes in patients with AKI [11]. TIMP2, a 21-kDa protein, is a member of the tissue inhibitor of metalloproteinase (TIMP) family, which includes endogenous inhibitors of metalloproteinase activity. TIMP2 is recognized as an early biomarker to predict AKI in critically ill patients, especially in septic patients [5,12–14] and septic animals [15], thus indicating a preinjury or stress status that often leads to AKI [16]. It is unknown whether TIMP2 participates in the pathophysiological process of AKI or is simply a marker of damage or stress [17]. Given the promising performance of these markers in some clinical studies, research on their pathophysiology should be prioritized. Recently, Castellano et al. reported that TIMP2 is necessary for the cognitive benefits conferred by cord plasma during depletion experiments in aged mice [18]. However, most prior studies have focused on the potential diagnostic value of TIMP2, particularly the diagnostic value of urinary TIMP2 in AKI. We hypothesize that TIMP2 also plays important roles in the development of septic AKI. The present study therefore sought to identify the detailed functions of TIMP2 in lipopolysaccharide (LPS)-injured human kidney 2 (HK-2) cells, an *in vitro* model of septic AKI, and in a septic mice model by using cecal ligation and puncture (CLP). In addition, we tested whether underexpression of TIMP2 would benefit or harm in the setting of sepsis.

2. Materials and methods

2.1. Cell culture

HK-2 cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences. The cells were cultured in Minimum Essential Medium (MEM, HyClone) with 5 ng/mL of human recombinant epidermal growth factor (EGF, Novus), 10% fetal bovine serum (FBS, GIBCO), and a penicillin–streptomycin supplement. HK-2 cells were treated with 10 μg/mL LPS (Sigma, L3129) for stimulation. To study the role of cytokines in tubular cell injury, HK-2 cells were treated with IL-1β (20 ng/mL), IL-6 (200 ng/mL), TNF-α (10 ng/mL), or IFN-γ (100 ng/mL) for 24 h with serum-free MEM medium. Cytomix is the mixture of the abovementioned cytokines. The doses of cytokines were based on previous reports [19].

2.2. Stable cell lines

HK-2 cells were stably transduced with commercially available lentiviral particles, which express TIMP2 siRNA (termed as TIMP2-S) or control lentivirus particles (termed as TIMP2-C) (Suzhou Gene Pharma, China). TIMP2 siRNA sequences are listed as follows: GGAAAGAAGG AATATCTCA. The plasmid is based on the pSMART vector with a puromycin selection and RFP cassette. HK-2 was transduced with the lentiviral vector by using a multiplicity of infection (MOI) of 50 and 6 μg/mL polybrene for 24 h. HK-2 cells were then maintained in 6-well plates and purified with 5 μg/mL puromycin for 1 week prior to experiments.

2.3. RNA extraction and qRT-PCR

Total RNA was extracted from kidney tissues and HK-2 cells by YPH EASY spin tissue/cell RNA quick extraction kit (YPH, Beijing China). In addition, mRNA reverse transcription (RT) was performed by the ReverTra Ace Kit (Toyobo, Osaka, Japan). The cDNA then served as the template for SYBR real-time polymerase chain reaction (PCR). Primer sequences are listed as Table 1. All reactions were run in triplicate on the Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

2.4. Western blot analysis

Total protein was extracted from HK-2 cells and mice kidney tissues. Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Millipore). After blocking with 5% non-fat milk, the membrane was incubated with anti-TIMP2 (1:1000, Abcam, ab180630), t-P65(1:2000, Proteintech, 10745-1-AP), p-P65(1:1000, Cell Signaling Technology, #3033), t-IκBα (1:2000, Proteintech, 15649-1-AP), p-IκBα (1:1000, Cell Signaling Technology, #2859), Bcl2 (1:1500, Proteintech, 12789-1-AP), Bcl-XL(1:10,000, Proteintech, 66020-1-lg), BAX (1:1000, Proteintech, 50599-2-lg), or Caspase 3 (1:600, Proteintech, 19677-1-AP) antibody or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000, Proteintech, 10494-1-AP). All western blot data were repeated 3 times independently.

2.5. Co-immunoprecipitation assays

HK-2 cells (5×10^6) seeded in 10 cm dishes were exposed with LPS or not. The cells were harvested and lysed by lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM NaF, 2 mM EGTA, 1 mM Na3VO4, 0.5% Triton-X-100, and 2 mM DTT) containing cocktail for 30 min on ice. After centrifugation at 12000*g* for 20 min at 4 °C, the supernatants were collected and incubated with the Protein A/G PLUS-Agarose beads and appropriate primary antibody or IgG isotype control antibody at 4 °C overnight. After washing four times, these compounds were mixed with 2× loading buffer and boiled for 5 min at 100 °C. The immune complexes were resolved on 10% SDS–PAGE gels and subjected to western blotting that was performed according to the instructions.

2.6. Immunofluorescence

For immunofluorescence (IF), 2-μm acetone-fixed cryostat sections and 4-μm paraffin sections were cut from the polyoxymethylene-fixed mice kidneys. The sections were incubated with TIMP2 antibody (1:100). To detect the primary antibodies, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:100, Abcam) for TIMP2. The nuclei were stained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI). The TIMP2 protein on HK-2 cell slides was detected in the same way. The sections were visualized using a laser-scanning confocal microscope (Olympus FluoView™ FV1000, Tokyo, Japan).

2.7. ELISA

Kidney tissues were homogenized, and the protein concentration was determined using the Coomassie blue method. TIMP2, IL-1β, IL-6, TNF-α, and IGFBP-7 levels in tissue and cell culture supernatant were measured with commercially available standard sandwich enzyme-linked kit (Bio-Swamp Life Science, Shanghai, China) in accordance with the manufacturer's instructions. Each sample was measured in triplicate.

2.8. Cell apoptosis

Stable cell lines were seeded at a density of 4.0×10^5 cells/well in 6-well tissue culture plates. After treatment with LPS, the cells were washed three times with ice-cold phosphate-buffered saline (PBS). The cells were then resuspended in binding buffer (100 μL) containing 5 μL of FITC stock (Annexin V-FITC kit, Becton-Dickinson, San Jose, CA, USA). Then, 400 μL of binding buffer was added and mixed gently after incubation at room temperature for 15 min in a dark room. CytExpert software (Beckman Coulter) was used to analyze the apoptotic cells (annexin V-positive cells).

Table 1
Primer sequences for real-time polymerase chain reaction (PCR).

Gene name (source)	Forward (from 5′ to 3′)	Reverse (from 5′ to 3′)
GAPDH (human)	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
GAPDH (mouse)	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
TIMP2 (human)	CTGGACGTTGGAGGAAAGAA	GTCGAGAAACTCCTGCTTGG
TIMP2 (mouse)	GCAACCCCATCAAGAGGATTC	GGGGCCGTGTAGATAAACTCG
IL-1β (human)	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL-1β (mouse)	TTCAGGCAGGCAGTATCACTC	GAAGGTCCACGGGAAAGACAC
IL-6 (human)	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
IL-6 (mouse)	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF-α (human)	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
TNF-α (mouse)	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG

2.9. TUNEL assay

Cells (2×10^6) were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were treated with 0.2% Triton X-100, washed with PBS, and incubated with FITC-labeled dUTP and terminal deoxynucleotidyl transferase. Following a series of washes with PBS and nuclear staining with DAPI, the cells were analyzed with an epifluorescence microscope in 5 random visual fields at ×400 magnification.

2.10. Cell viability

TIMP2-S and TIMP2-C HK-2 cells were seeded in 96-well plates at a density of 5×10^3 cells/well with LPS for 4 to 60 h. Cell viability was detected by the Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan). The plates were incubated for an additional 2 h at 37 °C. The absorbance at 450 nm was measured using a multimode plate reader (PerkinElmer).

2.11. Animal model of AKI

CLP surgery was performed to induce sepsis as previously described [20]. Briefly, C57BL/6 mice (male, 10–15 weeks old) were anesthetized with isoflurane, and a midline incision (2 cm) was made below the diaphragm to expose the cecum. The cecum was ligated at the colon juncture with a 5–0 silk ligature suture, punctured twice with a 22-gauge needle, and laced back in the abdomen, and the incision was closed in 2 layers with a 5–0 silk ligature suture. Sham operation was performed in the same way as CLP but without ligation and puncture of the cecum. Mice were fluid-resuscitated with 0.7 ml normal saline injected subcutaneously. Twenty-four hours after CLP surgery, blood was collected by an intracardiac puncture. Heparinized blood was centrifuged at 10,000*g* for 10 min to separate the plasma, which was collected and stored at −80 °C for further analysis. For tissue analyses, kidneys were harvested, and one half of the harvested kidneys was fixed in 4% paraformaldehyde and processed for H&E staining analysis, while the other half was snap-frozen in liquid nitrogen and stored at −80 °C for mRNA and protein analysis. All experiments were performed in accordance with Chinese legislation on the use and care of laboratory animals and were approved by the Animal Care and Use Committee of Wuhan University.

2.12. In-vivo intraparenchymal lentiviral vector injection

C57BL/6 mice were anesthetized with isoflurane and positioned supine on the operating table. The left kidney was exposed through flank incision, and 50 μL of viral solution was microinjected into the left renal vein through a 33-G needle and a 100 μL glass Hamilton syringe into 1 to 4 sites; care was taken not exceed the 100-μL volume. Approximately 1 to 3×10^7 viral particles were delivered as described above.

2.13. In vivo bioluminescence imaging

Mice were anesthetized with isoflurane. As the lentivirus carried RFP cassette, the bioluminescence image analyzer (BRUKER, Bruker Xtreme BI) could detect fluorescence emitted from the kidney. Images were photographed using the bioluminescence imaging system.

2.14. Immunohistochemistry

Formalin-fixed, paraffin-embedded archival mice kidney tissues were stained with TIMP2 (1:200) antibodies. All stained tissues were visualized by incubation with secondary peroxidase-conjugated antibodies. After immunostaining, representative photos were taken at ×400 magnifications.

2.15. Evaluation of renal function and histology

The concentration of creatinine in serum was measured using commercial kit reagents (Institute of Jiancheng Bioengineering, Nanjing, China). The absorbance at 546 nm was detected by a multimode plate reader (PerkinElmer). Formalin-fixed and paraffin-embedded kidney tissues were cut into 3-μm sections, stained with H&E, and visualized under an optical microscope (Olympus Optical, Tokyo, Japan).

2.16. Statistical analysis

Data are presented as mean ± SEM or median with 25th to 75th percentile from at least 3 independent experiments. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (San Diego, California, USA) were used for statistical analysis. Two groups were compared by Student's *t*-test. Associations between groups were determined by Spearman rank correlation. P value of 0.05 or less was considered as statistically significant difference.

3. Results

3.1. TIMP2 expression was increased in renal tubular cells exposed to LPS and in the kidney after CLP in mice; the latter correlated with changes in the serum creatinine level

We first confirmed the upregulation of mRNA and increased protein expression of TIMP2 in the kidney cortex of mice beginning 12 h after CLP (Fig. 1, A and B). The serum creatinine level was also significantly increased in the CLP group compared to that in the sham group at 24 h (2.74 times higher in CLP *vs* that in sham, Fig. 1C). To explore the association between serum creatinine and TIMP2, we performed correlation analysis and showed that the protein content of TIMP2 in renal tissues was correlated with the levels of serum creatinine ($r = 0.62$, $P = 0.003$) in mice after CLP (Fig. 1D); this finding indicated an association between TIMP2 and the severity of kidney dysfunction. Through

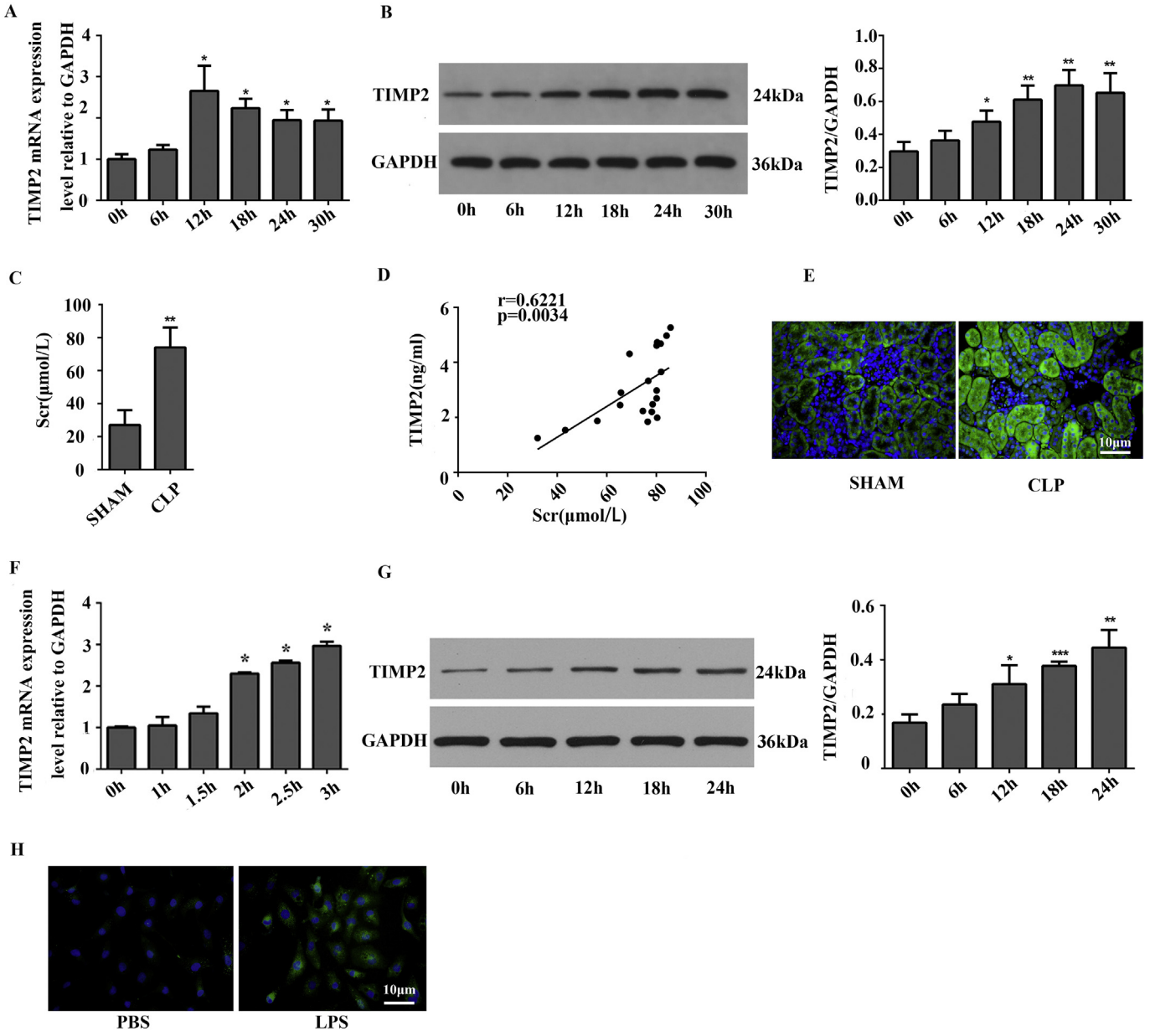


Fig. 1. TIMP2 and inflammatory cytokine levels were increased in renal tubular cells exposed to LPS and kidney challenged by CLP in mice
(A) TIMP2 mRNA expression relative to GAPDH analyzed by quantitative real-time PCR (qPCR) in a CLP-induced AKI mouse model.
(B) TIMP2 protein expression relative to GAPDH analyzed by western blot in CLP-induced AKI mice.
(C) Serum creatinine was measured in the CLP and SHAM groups to confirm the severity of AKI.
(D) The correlation between renal tubular expression of TIMP2 and serum creatinine in CLP mice.
(E) Immunofluorescence of TIMP2 in kidney tissue from CLP mice and SHAM mice. Mice kidney tissues were double-labeled with TIMP2 (green, FITC), and 4′,6-diamidino-2-phenylindole (blue). Green indicates the location of increased TIMP2 expression in renal tubular cells. Scale bar, 10 μm.
(F) TIMP2 mRNA expression relative to GAPDH analyzed by qPCR in HK-2 cells after LPS treatment.
(G) TIMP2 protein expression relative to GAPDH analyzed by western blot in HK-2 cells after LPS treatment.
(H) Immunofluorescence of TIMP2 in HK-2 cells after 24 h of LPS insult. Data were expressed as mean ± SEM. Scale bar, 10 μm.
Fig. 1A–C, E, n = 4 animals per group. D, 20 mice were received CLP. Each cell experiment was repeated 3 times independently. For each experiment, differences between mean values were considered statistically significant at $P < 0.05$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

immunofluorescence staining, upregulation of TIMP2 was detected primarily in the renal tubular cells (Fig. 1E).

To further explore the mechanisms of TIMP2 in AKI, we investigated the roles of TIMP2 in human kidney cells. Cultured human proximal tubular epithelial cells (HK-2) treated with LPS showed significantly increased expression of TIMP-2 in a time-dependent manner (Fig. 1F and G). The expression and intracellular localization of TIMP2 in HK-2 cells were determined by fluorescent immunocytochemistry assay (Fig. 1H).

3.2. Cytokines were increased together with increased TIMP2 expression, and increased cytokines further boosted TIMP2 secretion

To explore the relationship between the inflammatory response and the expression of TIMP2, we also evaluated the mRNA expression of interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNFα) after LPS treatment. These cytokines were significantly increased (Fig. 2A) accompanied with the increase in TIMP2 expression. After exposure to recombinant cytokines, including IL-1β, IL-6, TNFα, and interferon

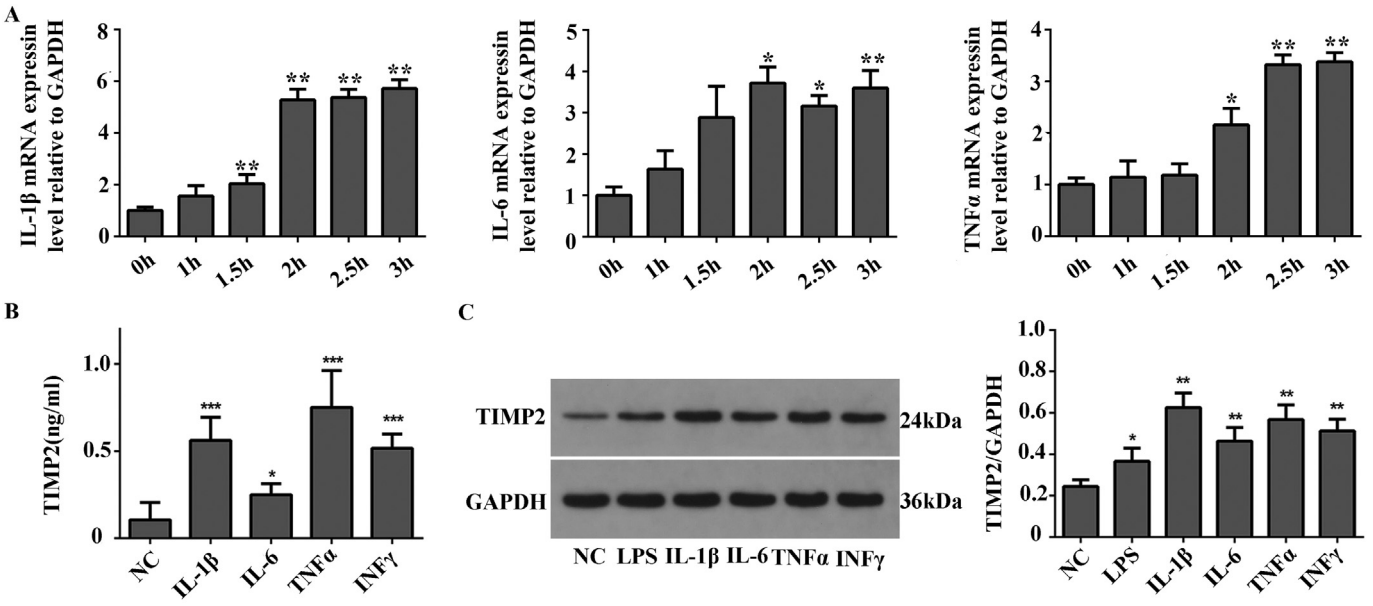


Fig. 2. TIMP2 expression was increased in the status of inflammation
(A) The mRNA of cytokines IL-1β, IL-6 and INF-γ induced by LPS in HK-2 cells were determined by qRT-PCR.
(B) The concentration of TIMP2 in the HK-2 cell culture medium was detected by ELISA in the presence of cytokines for 24 h.
(C) TIMP2 protein in HK-2 cells was quantified by western blot after administration of PBS or LPS for 24 h. *P < 0.05, **P < 0.01, ***P < 0.001.

gamma (IFN-γ) for 24 h, TIMP2 expression was increased in HK-2 cells intracellularly and in the supernatant of the culture medium, which were detected by enzyme-linked immunosorbent assay (ELISA) and western blot ($p < 0.05$) (Fig. 2B and C). These findings indicated that LPS and cytokines could boost TIMP2 secretion.

3.3. Inflammatory response promoted apoptosis and injury of HK-2 cells exposed to LPS, and silencing of TIMP2 antagonized these effects

To evaluate the apoptotic effect of cytokines on cells, we measured the apoptosis rate of HK-2 cell in the presence of IL-1β, IL-6, TNFα, and IFNγ alone and in combination (Cytomix) and found that the cellular apoptosis was induced by multiple cytokines, corresponding with caspase-3 activation and Bax upregulation and Bcl-2 and Bcl-XL downregulation (Fig. 3A and B). Cytomix induced a higher apoptosis rate compared to IL-1β and TNFα group ($P = 0.042$ and 0.014, respectively) (Fig. 3A). Apoptosis was markedly increased in TIMP2-C cells with exposure to LPS, and this response was attenuated in the TIMP2-S cells ($P < 0.05$).

To further understand the relationship between TIMP2 expression and cytokine release in renal tubular cells, we first constructed stable TIMP2-silenced cell lines from HK-2 cells. HK-2 cells were infected with lentiviral vectors delivering short hairpin RNAs (shRNAs) targeting TIMP2 to generate the TIMP2-silenced cell lines (TIMP2-S). As a control, the cell line TIMP2-C was generated using lentiviral vectors delivering a scrambled shRNA. The mRNA level of TIMP2 in TIMP2-S was nearly 90% reduced compared to that in TIMP2-C (Fig. 3C). TIMP2 protein level in the supernatant of the culture medium of HK-2 cells was increased after LPS exposure and decreased when TIMP2 was silenced by the lentivirus ($p < 0.05$) (Fig. 3D). Because the lentivirus carried the RFP cassette, red fluorescence was emitted by TIMP2 knockdown cells and control cells (Fig. 3E). The percentage of apoptosis was detected by flow cytometry assay of annexin-V–stained cells and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (Fig. 3F and G). In parallel with the cell apoptosis, the cell viability was significantly decreased after LPS exposure, and depletion of TIMP2 increased cell viability in HK-2 cells from 24 h onward (Fig. 3H).

3.4. Knockdown of TIMP2 reduced apoptosis exposed to cytokines and attenuated cytokine secretion in HK-2 cells via the NF-κB pathway

In order to evaluate the effects of cytokines effects on apoptosis in the presence or absence of TIMP2 knockdown, flow cytometry assay was used to detect the apoptosis percentage of HK-2 cells. Knockdown of TIMP2 reduced apoptosis exposed to cytokines IL-1β, IL-6, IFNγ and cytomix (Fig. 4A). IGFBP-7 in the TIMP2-S group was much lower than that of TIMP2-C after LPS exposure. (Fig. 4B). Futhermore, IL-1β, IL-6, and TNFα levels in the supernatant of the HK-2 cell culture medium were decreased in the TIMP2-S group compared with that of the TIMP2-C group after LPS challenge($P < 0.05$) (Fig. 4C). NFκb inhibitor ammonium pyrrolidinedithiocarbamate (PDC) was used to evaluate the difference in cytokines (IL-1, IL-6 and TNF-α) expression. We found that IL-6 was much lower in TIMP2-C compared to TIMP2-S group after LPS exposure. There were no statistical differences of IL-1β and TNFα concentration between TIMP2-C and TIMP2-S group (Fig. 4C).

As NF-κB is the critical moderator of inflammatory response and the transcriptional activity of NF-κB is controlled by phosphorylation of P65 at serine 536, we hypothesized that the role TIMP2 plays in inflammatory cytokine secretion is mediated by phosphorylated P65 (p-P65). Therefore, we measured p-P65 expression and total-NF-κB P65 (t-P65), total-IκBα (t-IκBα), and phospho-IκBα (p-IκBα) levels in HK-2 cells by western blot. The increased level of phosphorylated (p-P65) indicates the high activity of the NF-κB pathway. Following LPS administration, the p-P65 level decreased in the TIMP2-S group compared to that in the TIMP2-C group. Thus, downregulation of TIMP2 expression inhibited P65 phosphorylation and subsequently decreased the NF-κB activity (Fig. 4D). To further confirm the relationship between TIMP2 and NF-κb in HK-2 cells, co-immunoprecipitation assays were performed. The results of co-immunoprecipitation assays showed that TIMP2 binding with NF-κb (Fig. 4E). These data suggest that TIMP2 silencing contributed to decreased inflammatory cytokine secretion, breaking the inflammatory response loop at the same time, and this effect was mediated by p-P65.

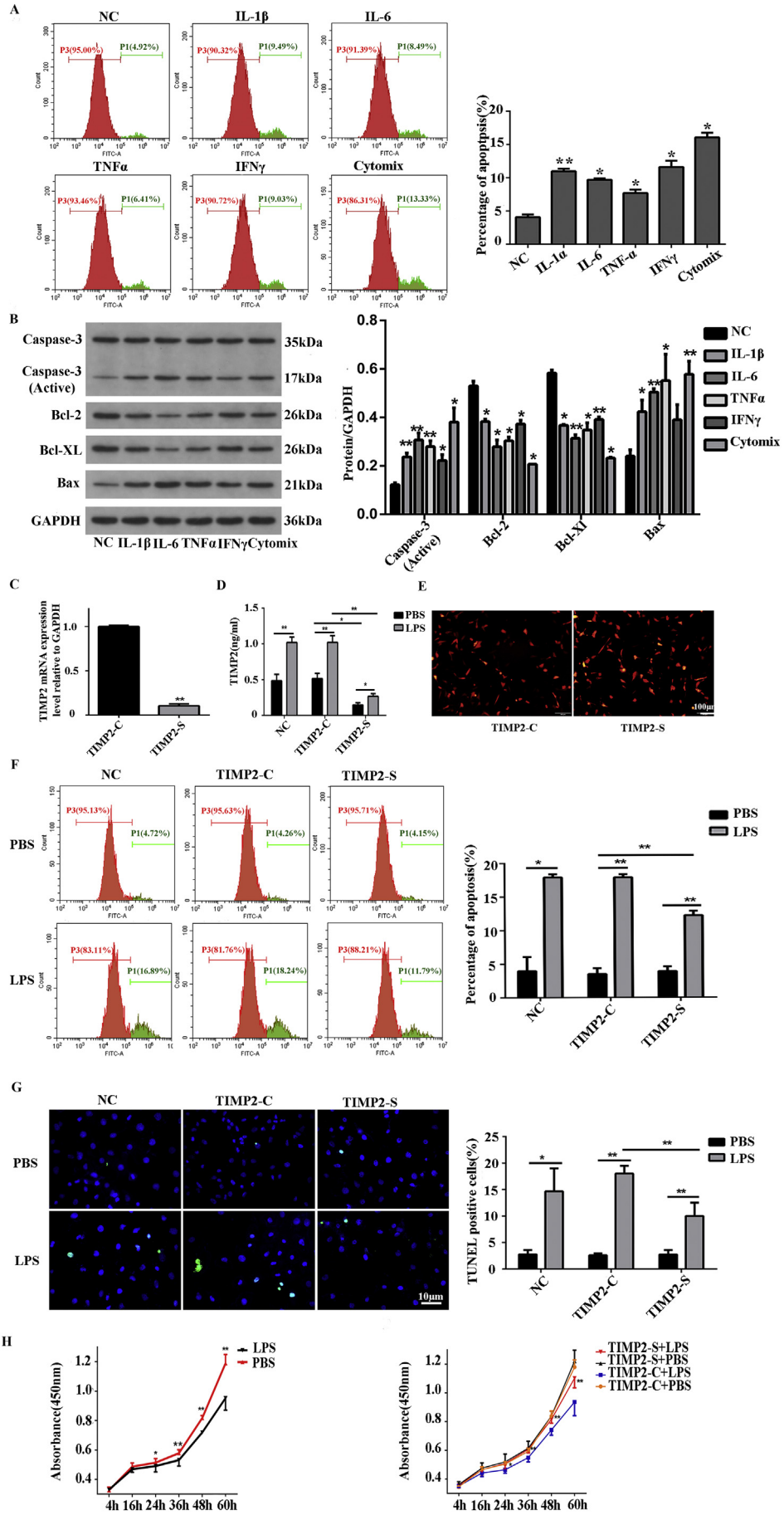


Fig. 3. Effect of TIMP2 on the apoptosis of HK-2 cells exposed to LPS.

(A) Apoptotic rate of HK-2 cells treated with IL-1β, IL6, TNFα, IFNγ, and Cytomix was measured by flow cytometry.

(B) Caspase-3(active), Bcl-2, Bcl-Xl, and Bax proteins were analyzed by western blot. HK-2 cells were subjected to IL-1β, IL6, TNFα, IFNγ, and Cytomix, respectively.

(C) TIMP2 mRNA expression level relative to GPADH was analyzed by qPCR in HK-2 cells. HK-2 cells were transduced with TIMP2 silence lentivirus or empty vector control.

(D) TIMP2 protein in the HK-2 cell culture medium was detected by ELISA.

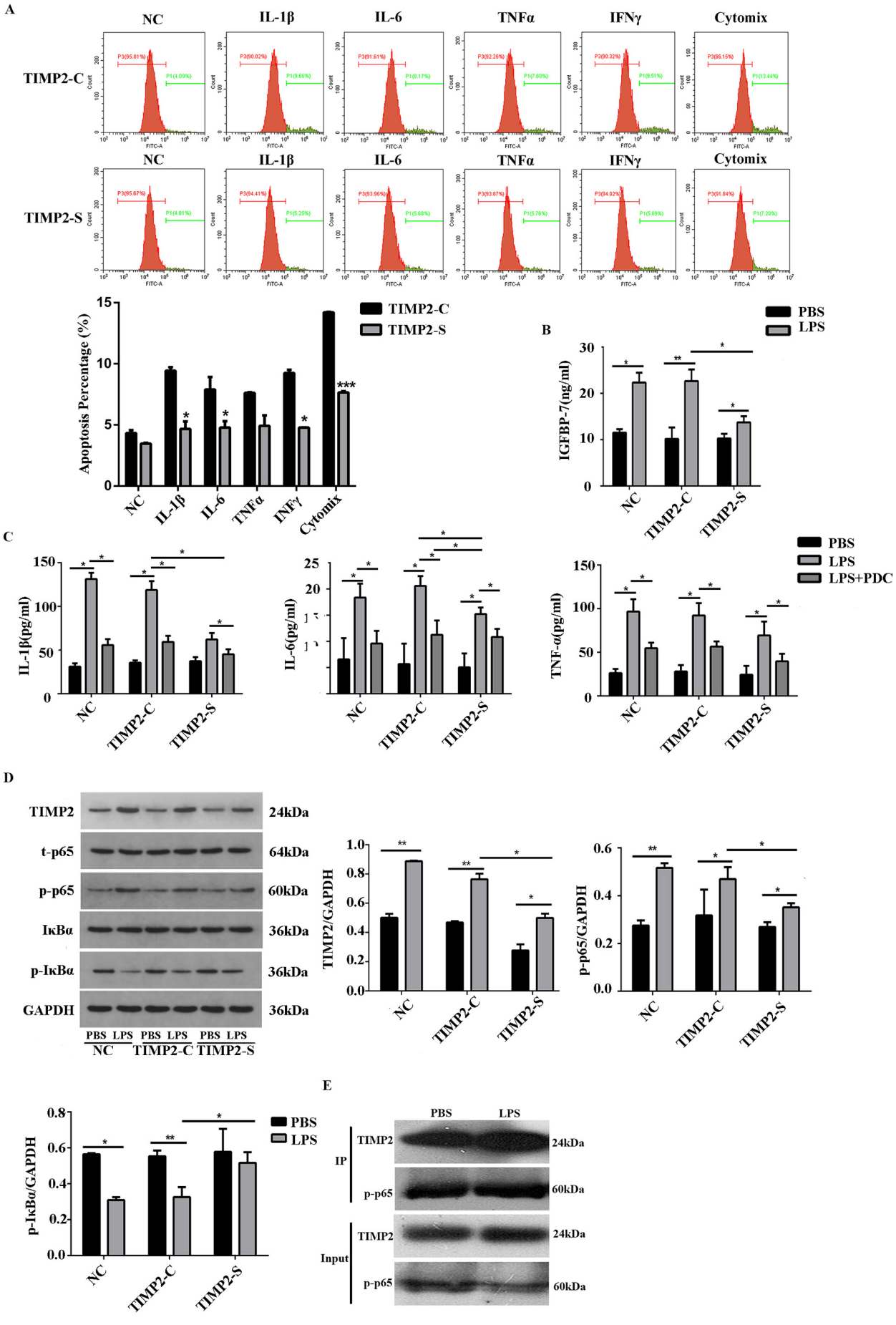
(E) Representative fluorescence microscopy images were taken; red fluorescence represents HK-2 cells were infected with TIMP2 silence lentivirus or control. Scale bar, 100 μm.

(F) Apoptotic rate of HK-2 cells subjected to PBS or LPS was measured by flow cytometry. Cells were treated with NC, TIMP2-C, and TIMP2-S HK-2. Flow cytometry assay of annexin V–stained cells.

(G) Apoptosis of HK-2 cells was measured by TUNEL assay and quantified. Scale bars: 10 μm.

(H) Cell viability of HK-2 cells was measured by CCK-8 assay after PBS or 10 μg/ml LPS treatment for 4 to 60 h.

Fig. 3 A and B HK-2 cells treated with IL-1β, IL6, TNFα, IFNγ, and Cytomix. F-H NC, TIMP2-C, and TIMP2-S HK-2 cells were subjected to PBS or LPS for 24 h. All data were expressed as the mean ± SEM. Each experiment was repeated 3 times independently. *P < 0.05, **P < 0.01, ***P < 0.001.



(caption on next page)

Fig. 4. Knockdown of TIMP2 reduced apoptosis exposed to cytokines and attenuated cytokine secretion in HK-2 cells *via* the NF-κB pathway
(A) The apoptosis percentage in TIMP2-C, and TIMP2-S HK-2 cells were detected by flow cytometry. Bar graph shows the percentage of apoptosis in different groups.
(B) IGFBP-7 protein in supernatant of HK-2 were assayed by ELISA after administration of PBS or LPS.
(C) IL-1β, IL-6, and TNFα protein in supernatant of HK-2 were assayed by ELISA after administration of PBS or LPS.
(D) TIMP2, t-P65, p-P65, t-IκBα, and p-IκBα protein were detected by western blot. Western blot data were repeated 3 times independently.
(E) HK-2 cells were exposed with LPS or PBS for 24 hours. The proteins were immunoprecipitated with anti-TIMP2 or anti-p-p65 agarose beads and then separated by SDS-PAGE. Input lanes represent 10% of cell lysate used for IP.
Fig. 4 A TIMP2-C, and TIMP2-S HK-2 cells were exposured to IL-1β, IL-6, TNFα, IFNγ and cytomix. B-D, NC, TIMP2-C and TIMP2-S HK-2 cells were subjected to PBS or LPS. E HK-2 cells were exposure with PBS or LPS. Values are presented as mean ± SEM. For each experiment, differences between means were considered statistically significant when $P < 0.05$. *$P < 0.05$, **$P < 0.01$.

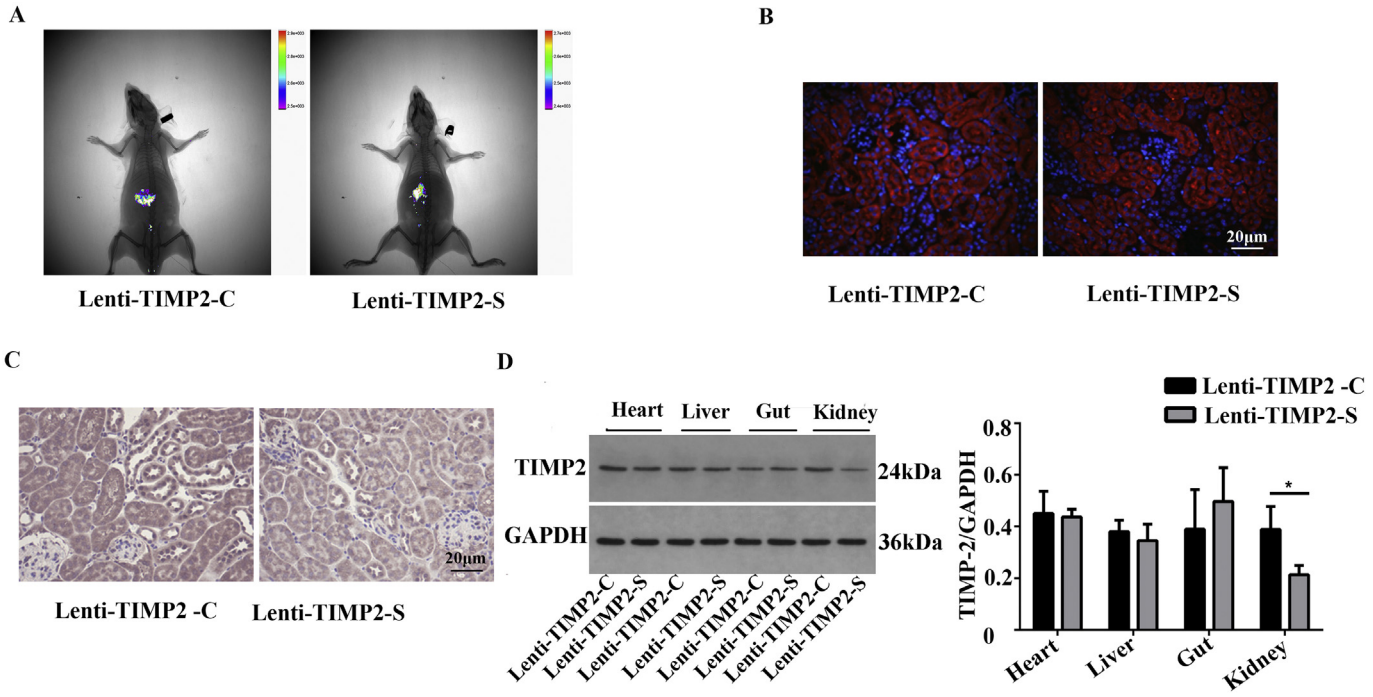


Fig. 5. Kidney TIMP2 specific knockdown mice were constructed by injection of parenchymal TIMP2-silencing lentiviral vector.
(A) *In vivo* small animal imaging techniques were used to monitor RFP cassette of lentivirus in C57BL/6 mice to determine whether the lentiviral vector was delivered into the kidney. The lentiviral vector was delivered into the kidneys of C57BL/6 mice by direct injection into the renal parenchyma.
(B) Representative fluorescence microscopy images were acquired; red fluorescence represents kidney tissues infected with TIMP2-silencing lentivirus or control. Scale bar, 20 μm.
(C) Representative renal IHC staining of TIMP2 from each group. Original magnification, ×20. Scale bar, 20 μm.
(D) TIMP2 protein expression in the heart, liver, gut, and kidney in the group of Lenti-TIMP2-C and Lenti-TIMP2-S was analyzed by western blot. n = 4 per group. *$P < 0.05$.

3.5. Downregulation of TIMP2 attenuated CLP-induced AKI by parenchymal TIMP2-silencing lentiviral vectors

To further confirm the *in vivo* role of TIMP2 in CLP-induced kidney injury, a lentiviral vector expressing shRNA targeting TIMP2 was administered to mice prior to CLP. The lentiviral vector was delivered into the kidneys of C57BL/6 mice by direct injection into the renal parenchyma. Bioluminescence imaging was used to detect whether the lentiviral vector was delivered into the kidney (Fig. 5A). Because the plasmid carried the RFP cassette, red fluorescence in the renal tubule was observed under excitation with 584 nm light (Fig. 5B). Immunohistochemistry revealed that TIMP2 expression was lower in TIMP2-knockdown lentiviral vector-injected mice (Fig. 5C). Protein expression of TIMP2 in the liver, heart, intestines, and kidney were measured by western blot. TIMP2 was knocked down in the kidney, but not in the liver, heart, and intestines (Fig. 5D). The TIMP2 lentivirus substantially reduced the TIMP2 level to 54.9% in the kidney.

The expression of IL-1β, IL-6, and TNF-α mRNAs in the kidney was much lower in the TIMP2 knockdown mice compared to that in control animals 24 h after CLP treatment (Fig. 6A). Protein content of TIMP2 and the levels of cytokines were also decreased in kidney tissues (Fig. 6B and C). In order to analyze the apoptosis of kidney, we performed TUNEL assay in kidney tissue of mice and found that the apoptosis percentage in Lenti-TIMP2-S group was much less than that in Lenti-TIMP2-S group after CLP($P < 0.05$)(Fig. 6D). Moreover, the protein levels of t-P65, p-P65, t-IκBα, and p-IκBα were also measured. (Fig. 6E). The transcriptional NF-κB activity was increased in the CLP model accompanied with the upregulated TIMP2 expression. Knockdown of TIMP2 decreased the p-P65 levels (Fig. 6E). Fig. 6F shows representative images of hematoxylin and eosin (H&E)-stained kidney tissue samples in the different groups. Notably, knocking down TIMP2 *in vivo* significantly improved renal histology and reduced the degree of renal dysfunction as assessed by the levels of serum creatinine after CLP treatment (Fig. 6G). These data provide *in vivo* evidence that TIMP2 plays an important role in mediating CLP-induced AKI and reinforces the potential of TIMP2 as a novel therapeutic target in preventing/treating AKI.

4. Discussion

AKI is a frequent complication in ICU patients, and approximately 50% of AKI cases are attributable to sepsis [21]. Sepsis and AKI

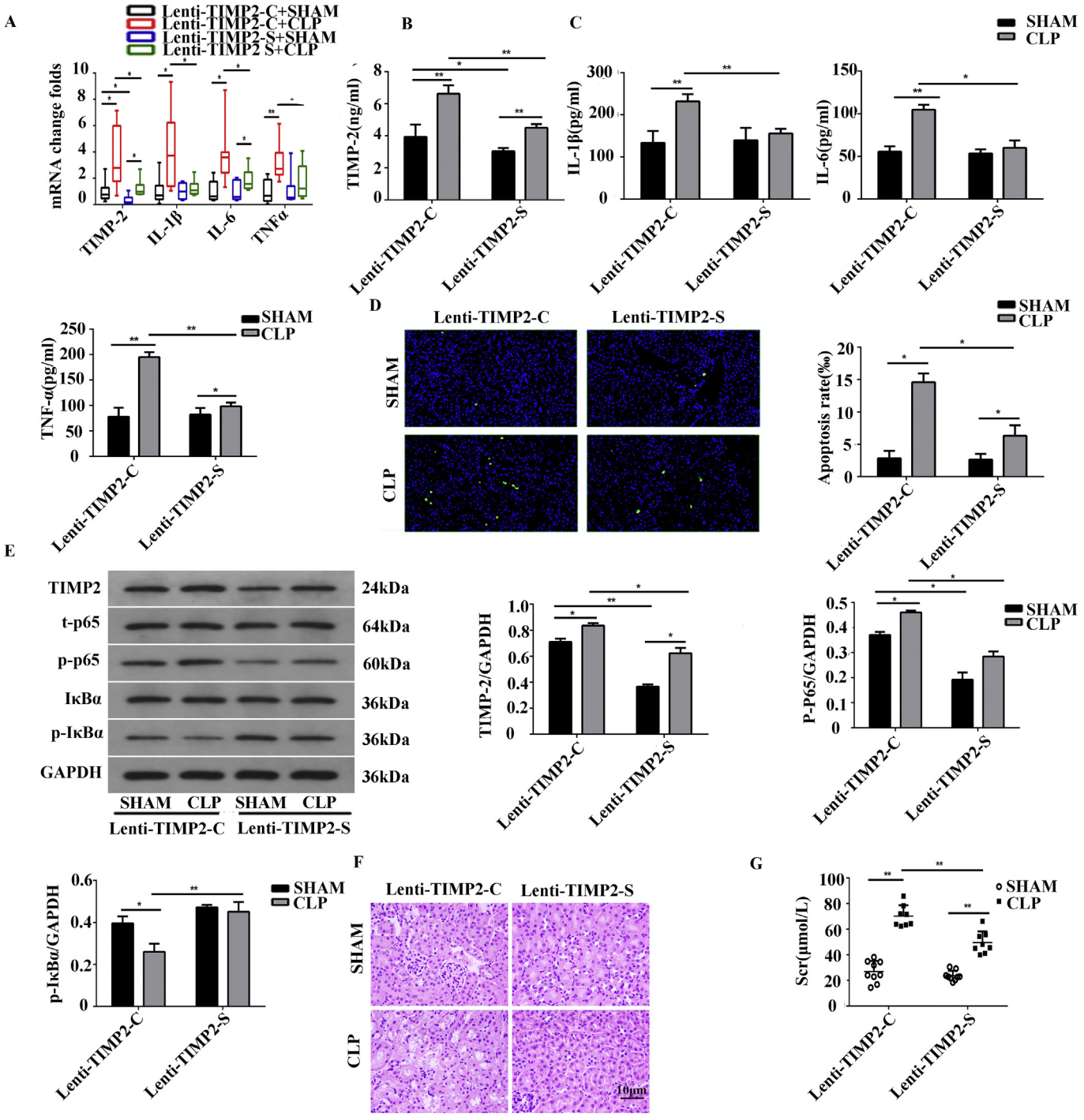


Fig. 6. Downregulation of TIMP2 attenuated CLP-induced AKI.
(A) TIMP2, IL-1β, IL-6, and TNF-α mRNA expression was measured by qPCR.
(B) The concentration of TIMP2 in the serum of mice was detected by ELISA.
(C) The cytokine concentrations of IL-1β, IL-6, and TNF-α in the serum of mice were detected by ELISA.
(D) Apoptosis rate was analysis by TUNEL in the kidney of mice.
(E) Western blot analysis of TIMP2, t-P65, p-P65, t-IκBα, and p-IκBα protein expression in mice kidney tissues. The relative protein levels were determined after normalization to GAPDH.
(F) The collected kidneys of mice were stained with H&E. Scale bars:10 μm. Kidney histology from CLP mice showing significant vacuolization in tubules.
(G) Serum creatinine level was analyzed using commercial kits in the 4 groups. n = 8 per group.
Fig. A–G. Lenti-TIMP2-C and Lenti-TIMP2-S group underwent CLP or SHAM for 24 h. *P < 0.05, **P < 0.01.

synergistically increase morbidity and mortality in ICU patients. Therefore, a more complete molecular understanding of renal tubular damage has become an area of exploration for the development of novel therapies for AKI. In the present study, we demonstrate that TIMP2 together with inflammatory cytokines are increased both *in vitro* and *in vivo* in response to LPS and sepsis, respectively. These molecules induced apoptosis, decreased cell viability *in vitro,* and compromised renal function as measured by the serum creatinine level in CLP-

induced sepsis in mice. Downregulating TIMP2 could antagonize these effects and attenuate kidney injury. We also found that the effects of TIMP2 were mediated through the NF-κB pathway.

TIMP2 is reported to be abundantly expressed in multiple human tissues such as the ovary, endometrium, gall bladder, and urinary bladder. In addition, TIMP2 acts on multiple genes, including matrix metalloproteinase (MMP), mitogen-activated protein kinase (MAPK), and β-catenin melanoma, and plays different roles in various organs, such as growth-stimulatory activity, revitalizing hippocampal function in aged mice, and promoting leukemia cell invasion [18,22–24]. Thus far, the knowledge of TIMP2 in the kidney has been limited to urinary biomarkers to predict AKI, and its exact role in kidney injury remains unknown [25]. In the present study, we used LPS-stimulated HK-2 cells and CLP-induced AKI mice and reported a prominent upregulation of TIMP2 expression primarily in renal tubular cells. This CLP model is well known to induce septic AKI [26,27]. We confirmed that TIMP2 expression increases during the early stages of AKI and shows a significant positive correlation with the severity of renal dysfunction in septic mice.

The development of AKI is related to the mechanisms of inflammation, oxidative stress, and apoptosis in cellular and molecular pathways [6,28,29]. In recent years, there has been increasing recognition that systemic inflammation can contribute to the induction of AKI [30]. Clinical studies indicate that plasma and urinary cytokine levels are correlated with degrees of tubular dysfunction, the risk of developing AKI, and subsequent patient outcomes [31,32]. In the present study, both mRNA and protein levels of IL-1β, IL-6, and TNF-α were significantly increased in the LPS-stimulated HK-2 cells and in mouse kidney tissue after CLP. Previous studies have also reported that the mRNA transcription levels of IL-1, IL-6, and TIMP2 increased significantly in renal tubular epithelial cells in septic rats [33]. Higher levels of IL-6 correlate with mortality and kidney injury [34], and transgenic knockouts of IL-6 ameliorated renal injury [35]. IL-6 deficiency attenuated neutrophil accumulation and caused mice to become relatively resistant to nephrotoxin-induced AKI and ischemic AKI as measured by the serum creatinine level and histological analysis [36]. Elevated renal inflammation was associated with increased expression of TNF-α and TIMP2 in a rat model of chronic kidney disease [37]. In our study, TIMP2 expression was increased in the presence of cytokines such as IL-6, IL-1β, TNF-α, and IFN-γ.

Our results are consistent with the studies of Lizárraga et al., which showed that the activation of NF-κB was regulated by TIMP2 in A549 lung epithelial cells [38]. In melanoma cells, overexpression of TIMP2 could upregulate NF-κB activity [39]. In our study, the activation of the NF-κB pathway induced by LPS stimulation was partially decreased by silencing TIMP2 in HK-2 cells.

We also found that in response to LPS stimulation, tubular epithelial apoptosis was induced; this finding was also reported by other researchers [40]. TIMP2 silencing resulted in decreased LPS-induced apoptosis, suggesting a pro-apoptotic role of TIMP2, perhaps through promoting cytokine synthesis.

Our animal model of CLP-induced AKI provided direct evidence of the relationship between TIMP2 and renal function. In mice, TIMP2-specific knockdown in the kidney through injections of lentiviral vectors provided a safe and efficient method for the genetic manipulation of renal tubules, thus representing a rapid and versatile alternative to genetically engineered mice for the functional characterization of disease-related genes [41]. Lentiviral vectors are particularly promising candidates because they have the ability to infect nonproliferating cells and to integrate into the genome of host cells, thereby achieving long-term transduction. Because of the slow turnover of renal cells in general, lentiviral vectors may be ideal candidates for renal gene therapy. IL-1β, IL-6, and TNF-α levels were decreased in the TIMP2 knockdown mice after CLP. The silencing of TIMP2 reduced kidney cell apoptosis percentage and decreased phosphorylation of P65. Kidney morphology and function were also ameliorated in TIMP2 knockdown mice. These results demonstrate that downregulation of TIMP2 could attenuate kidney inflammation and injury, thus suggesting that TIMP2 gene transfer into the kidney could be a novel therapeutic approach.

5. Strength and limitation

Our study has numerous strengths. Chief among these are the use of both *in vitro* and *in vivo* models involving 2 different species (human and mouse). To our knowledge the present study is the first study to show that TIMP2 is a mediator of AKI and not merely a marker. However, there were some limitations of our study. First, we could not assess survival changes with the modulation of TIMP2, as we had to sacrifice the animals early to collect tissues. Secondly, we studied only the NF-κB pathway, as it was the most important pathway to regulate the inflammatory response. It remains unknown whether other pathways also play important roles in TIMP2 signaling. Further studies are needed to understand this aspect.

6. Conclusion

In summary, we explored the possible roles of TIMP2 in septic AKI and found that TIMP2 does indeed promote kidney damage apparently by acting on NF-κb. We hypothesize a feedback loop that promotes apoptosis and leads to AKI (Fig. 7). On the contrary, suppressing TIMP2 protects against kidney damage, reduces p-P65 activity, attenuates proinflammatory cytokines, improves cell viability, and reduces apoptosis. Our results reveal a potential new therapeutic target for AKI in the setting of sepsis and possibly other conditions.

Abbreviations

AKI	acute kidney injury
HK-2	human kidney 2
TIMP-2	TIMP metallopeptidase inhibitor 2
IGFBP7	insulin like growth factor binding protein
MAPK	mitogen-activated protein kinase
CLP	cecal ligation and puncture
LPS	lipopolysaccharide
ICU	intensive care unit

Competing interests

JAK discloses grant support and consulting fees from Astute Medical. All the authors declared no conflict of interest exists.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81772046, No. 81560131); Hubei Province Technology and Innovation Project (Foreign collaboration of Science and Technology) (No. 2017AHB044); Health Commission of Hubei province (No. WJ2017Z008).

Author contributions

Conceived the project, designed the project, extract and analyzed data, drafted the manuscript and approved the final manuscript: Y.L.

Conducted the experiments: Y.L., J.Z. and J.S.

Designed the project, edited the manuscript and approved the final version: JAK, Z.P.

Transparency document

The Transparency document associated with this article can be found, in online version.

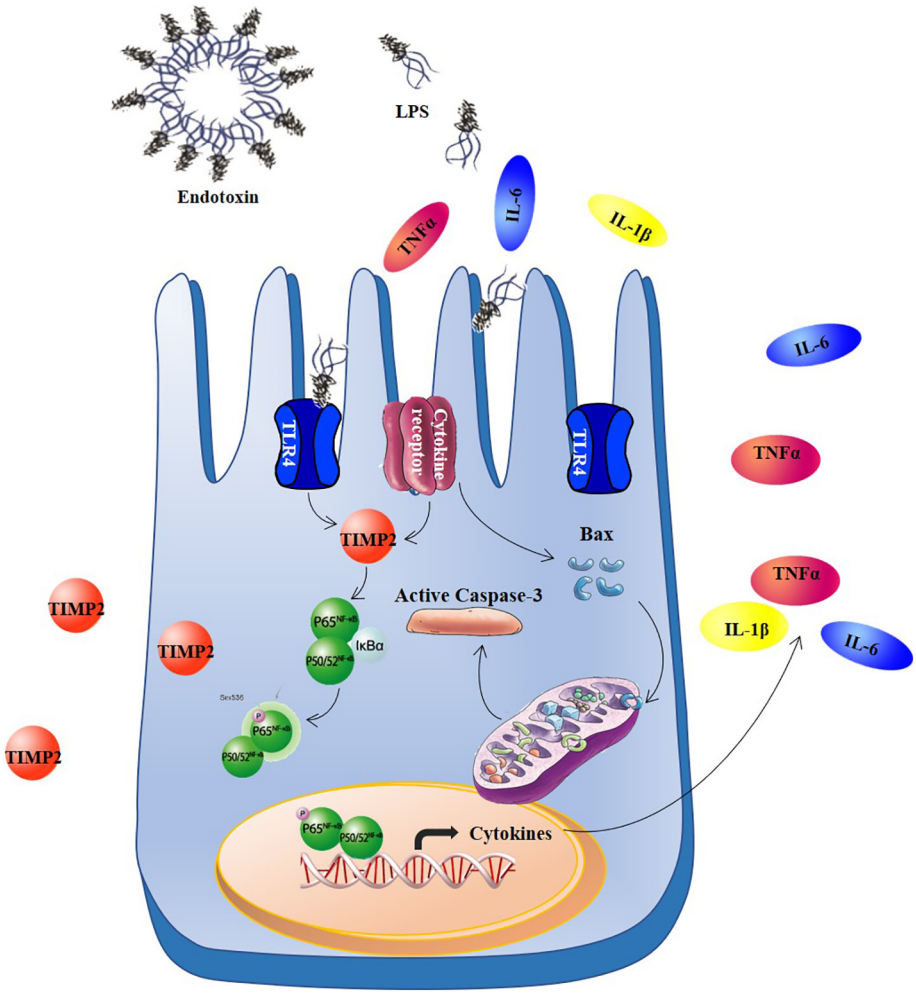


Fig. 7. Proposed mechanism for the involvement of TIMP2 in regulating sepsis-induced AKI in kidney tubular cells.
In the presence of endotoxin, high LPS levels induce the release of the TIMP2 protein, which results in the activation of the NF-κb pathway. Increased p-P65 level mediates cytokine (including IL-1β, IL-6, and TNF-α) release. The extracellular cytokines bind with their receptor and drive apoptosis. The augmented cytokine release further promotes TIMP2 synthesis and release. This, in turn, forms an inflammation loop leading to AKI.

Acknowledgments

This project was supported by grant from the National Natural Science Foundation of China (No. 81772046).

Conflict of interest

All the authors declared no competing interests.

References

[1] M. Al-Jaghbeer, D. Dealmeida, A. Bilderback, R. Ambrosino, J.A. Kellum, Clinical decision support for in-hospital AKI, J. Am. Soc. Nephrol. 29 (2018) 654–660.
[2] E.A. Hoste, S.M. Bagshaw, R. Bellomo, C.M. Cely, R. Colman, D.N. Cruz, K. Edipidis, L.G. Forni, C.D. Gomersall, D. Govil, P.M. Honore, O. Joannes-Boyau, M. Joannidis, A.M. Korhonen, A. Lavrentieva, R.L. Mehta, P. Palevsky, E. Roessler, C. Ronco, S. Uchino, J.A. Vazquez, E. Vidal Andrade, S. Webb, J.A. Kellum, Epidemiology of acute kidney injury in critically ill patients: the multinational AKI-EPI study, Intensive Care Med. 41 (2015) 1411–1423.
[3] S. Uchino, J.A. Kellum, R. Bellomo, G.S. Doig, H. Morimatsu, S. Morgera, M. Schetz, I. Tan, C. Bouman, E. Macedo, N. Gibney, A. Tolwani, C. Ronco, Beginning, I. ending supportive therapy for the kidney, acute renal failure in critically ill patients: a multinational, multicenter study, JAMA 294 (2005) 813–818.
[4] A. Akcay, Q. Nguyen, C.L. Edelstein, Mediators of inflammation in acute kidney injury, Mediat. Inflamm. 2009 (2009) 137072.
[5] M. Cuartero, J. Ballus, J. Sabater, X. Perez, N. Nin, J. Ordonez-Llanos, A.J. Betbese, Cell-cycle arrest biomarkers in urine to predict acute kidney injury in septic and non-septic critically ill patients, Ann. Intensive Care 7 (2017) 92.
[6] H. Gomez, C. Ince, D. De Backer, P. Pickkers, D. Payen, J. Hotchkiss, J.A. Kellum, A unified theory of sepsis-induced acute kidney injury: inflammation, microcirculatory dysfunction, bioenergetics, and the tubular cell adaptation to injury, Shock 41 (2014) 3–11.
[7] A.B. Sanz, M.D. Sanchez-Nino, A.M. Ramos, J.A. Moreno, B. Santamaria, M. Ruiz-Ortega, J. Egido, A. Ortiz, NF-kappaB in renal inflammation, J. Am. Soc. Nephrol. 21 (2010) 1254–1262.
[8] C. Guijarro, J. Egido, Transcription factor-kappa B (NF-kappa B) and renal disease, Kidney Int. 59 (2001) 415–424.
[9] M. Meersch, C. Schmidt, A. Hoffmeier, H. Van Aken, C. Wempe, J. Gerss, A. Zarbock, Prevention of cardiac surgery-associated AKI by implementing the KDIGO guidelines in high risk patients identified by biomarkers: the PrevAKI randomized controlled trial, Intensive Care Med. 43 (2017) 1551–1561.
[10] A. Vijayan, S. Faubel, D.J. Askenazi, J. Cerda, W.H. Fissell, M. Heung, B.D. Humphreys, J.L. Koyner, K.D. Liu, G. Mour, T.D. Nolin, A. Bihorac, American society of nephrology acute kidney injury advisory, clinical use of the urine biomarker [TIMP-2] × [IGFBP7] for acute kidney injury risk assessment, Am. J. Kidney Dis. 68 (2016) 19–28.
[11] J.L. Koyner, A.D. Shaw, L.S. Chawla, E.A. Hoste, A. Bihorac, K. Kashani, M. Haase, J. Shi, J.A. Kellum, I. Sapphire, Tissue inhibitor metalloproteinase-2 (TIMP-2)IGF-binding protein-7 (IGFBP7) levels are associated with adverse long-term outcomes in patients with AKI, J. Am. Soc. Nephrol. 26 (2015) 1747–1754.
[12] H.M. Jia, L.F. Huang, Y. Zheng, W.X. Li, Diagnostic value of urinary tissue inhibitor of metalloproteinase-2 and insulin-like growth factor binding protein 7 for acute kidney injury: a meta-analysis, Crit. Care 21 (2017) 77.
[13] M. Kimmel, J. Shi, C. Wasser, D. Biegger, M.D. Alscher, M.B. Schanz, Urinary [TIMP-2].[IGFBP7] - novel biomarkers to predict acute kidney injury, Am. J. Nephrol. 43 (2016) 375–382.
[14] K. Kashani, A. Al-Khafaji, T. Ardiles, A. Artigas, S.M. Bagshaw, M. Bell, A. Bihorac, R. Birkhahn, C.M. Cely, L.S. Chawla, D.L. Davison, T. Feldkamp, L.G. Forni, M.N. Gong, K.J. Gunnerson, M. Haase, J. Hackett, P.M. Honore, E.A. Hoste, O. Joannes-Boyau, M. Joannidis, P. Kim, J.L. Koyner, D.T. Laskowitz, M.E. Lissauer, G. Marx, P.A. McCullough, S. Mullaney, M. Ostermann, T. Rimmele, N.I. Shapiro,

A.D. Shaw, J. Shi, A.M. Sprague, J.L. Vincent, C. Vinsonneau, L. Wagner, M.G. Walker, R.G. Wilkerson, K. Zacharowski, J.A. Kellum, Discovery and validation of cell cycle arrest biomarkers in human acute kidney injury, Crit. Care 17 (2013) R25.
[15] Z.Y. Peng, F. Zhou, J.A. Kellum, Cross-species validation of cell cycle arrest markers for acute kidney injury in the rat during sepsis, Intens. Care Med. Exp. 4 (2016) 12.
[16] H. Husi, C. Human, Molecular determinants of acute kidney injury, J. Inj. Violence Res. 7 (2015) 75–86.
[17] J.A. Kellum, L.S. Chawla, Cell-cycle arrest and acute kidney injury: the light and the dark sides, Nephrol. Dial. Transplant. 31 (2016) 16–22.
[18] J.M. Castellano, K.I. Mosher, R.J. Abbey, A.A. McBride, M.L. James, D. Berdnik, J.C. Shen, B. Zou, X.S. Xie, M. Tingle, I.V. Hinkson, M.S. Angst, T. Wyss-Coray, Human umbilical cord plasma proteins revitalize hippocampal function in aged mice, Nature 544 (2017) 488–492.
[19] P.C. Baer, B. Wegner, H. Geiger, Effects of mycophenolic acid on IL-6 expression of human renal proximal and distal tubular cells in vitro, Nephrol. Dial. Transplant. 19 (2004) 47–52.
[20] T. Miyaji, X. Hu, P.S. Yuen, Y. Muramatsu, S. Iyer, S.M. Hewitt, R.A. Star, Ethyl pyruvate decreases sepsis-induced acute renal failure and multiple organ damage in aged mice, Kidney Int. 64 (2003) 1620–1631.
[21] A. Zarjou, A. Agarwal, Sepsis and acute kidney injury, J. Am. Soc. Nephrol. 22 (2011) 999–1006.
[22] H. Kim, H.S. Lee, T.H. Kim, J.S. Lee, S.T. Lee, S.J. Lee, Growth-stimulatory activity of TIMP-2 is mediated through c-Src activation followed by activation of FAK, PI3-kinase/AKT, and ERK1/2 independent of MMP inhibition in lung adenocarcinoma cells, Oncotarget 6 (2015) 42905–42922.
[23] Y. Xia, S. Wu, Tissue inhibitor of metalloproteinase 2 inhibits activation of the beta-catenin signaling in melanoma cells, Cell Cycle 14 (2015) 1666–1674.
[24] C. Wang, X. Cai, B. Chen, Z. He, Z. Chen, J. Cen, Z. Li, Up-regulation of tissue inhibitor of metalloproteinase-2 promotes SHI-1 cell invasion in nude mice, Leuk. Lymphoma 54 (2013) 2707–2711.
[25] Z. Song, Z. Ma, K. Qu, S. Liu, W. Niu, T. Lin, Diagnostic prediction of urinary [TIMP-2] × [IGFBP7] for acute kidney injury: a meta-analysis exploring detection time and cutoff levels, Oncotarget 8 (2017) 100631–100639.
[26] Z.Y. Peng, H.Z. Wang, N. Srisawat, X. Wen, T. Rimmele, J. Bishop, K. Singbartl, R. Murugan, J.A. Kellum, Bactericidal antibiotics temporarily increase inflammation and worsen acute kidney injury in experimental sepsis, Crit. Care Med. 40 (2012) 538–543.
[27] Z.Y. Peng, H.Z. Wang, M.J. Carter, M.V. Dileo, J.V. Bishop, F.H. Zhou, X.Y. Wen, T. Rimmele, K. Singbartl, W.J. Federspiel, G. Clermont, J.A. Kellum, Acute removal of common sepsis mediators does not explain the effects of extracorporeal blood purification in experimental sepsis, Kidney Int. 81 (2012) 363–369.
[28] Z. Wang, J.H. Holthoff, K.A. Seely, E. Pathak, H.J. Spencer 3rd, N. Gokden, P.R. Mayeux, Development of oxidative stress in the peritubular capillary microenvironment mediates sepsis-induced renal microcirculatory failure and acute kidney injury, Am. J. Pathol. 180 (2012) 505–516.
[29] J.A. Kellum, J.R. Prowle, Paradigms of acute kidney injury in the intensive care setting, Nat. Rev. Nephrol. 14 (4) (2018) 217–230, https://doi.org/10.1038/nrneph.2017.184 (Epub 2018 Jan 22).
[30] E.M. Simmons, J. Himmelfarb, M.T. Sezer, G.M. Chertow, R.L. Mehta, E.P. Paganini, S. Soroko, S. Freedman, K. Becker, D. Spratt, Y. Shyr, T.A. Ikizler, P.S. Group, Plasma cytokine levels predict mortality in patients with acute renal failure, Kidney Int. 65 (2004) 1357–1365.
[31] J.M. Fernandez-Real, J. Vendrell, I. Garcia, W. Ricart, M. Valles, Structural damage in diabetic nephropathy is associated with TNF-alpha system activity, Acta Diabetol. 49 (2012) 301–305.
[32] T.J. Pianta, P.W. Peake, J.W. Pickering, M. Kelleher, N.A. Buckley, Z.H. Endre, Evaluation of biomarkers of cell cycle arrest and inflammation in prediction of dialysis or recovery after kidney transplantation, Transpl. Int. 28 (2015) 1392–1404.
[33] S. Zheng, Y. Pan, C. Wang, Y. Liu, M. Shi, G. Ding, HMGB1 turns renal tubular epithelial cells into inflammatory promoters by interacting with TLR4 during sepsis, J. Interf. Cytokine Res. 36 (2016) 9–19.
[34] E. Cho, M. Kim, Y.S. Ko, H.Y. Lee, M. Song, M.G. Kim, H.K. Kim, W.Y. Cho, S.K. Jo, Role of inflammation in the pathogenesis of cardiorenal syndrome in a rat myocardial infarction model, Nephrol. Dial. Transplant. 28 (2013) 2766–2778.
[35] M.L. Kielar, R. John, M. Bennett, J.A. Richardson, J.M. Shelton, L. Chen, D.R. Jeyarajah, X.J. Zhou, H. Zhou, B. Chiquett, G.T. Nagami, C.Y. Lu, Maladaptive role of IL-6 in ischemic acute renal failure, J. Am. Soc. Nephrol. 16 (2005) 3315–3325.
[36] H. Su, C.T. Lei, C. Zhang, Interleukin-6 signaling pathway and its role in kidney disease: an update, Front. Immunol. 8 (2017) 405.
[37] Y. Liu, G. Bledsoe, M. Hagiwara, Z.R. Yang, B. Shen, L. Chao, J. Chao, Blockade of endogenous tissue kallikrein aggravates renal injury by enhancing oxidative stress and inhibiting matrix degradation, Am. J. Physiol. Renal Physiol. 298 (2010) F1033–F1040.
[38] F. Lizarraga, V. Maldonado, J. Melendez-Zajgla, Tissue inhibitor of metalloproteinases-2 growth-stimulatory activity is mediated by nuclear factor-kappa B in A549 lung epithelial cells, Int. J. Biochem. Cell Biol. 36 (2004) 1655–1663.
[39] J. Sun, W.G. Stetler-Stevenson, Overexpression of tissue inhibitors of metalloproteinase 2 up-regulates NF-kappaB activity in melanoma cells, J. Mol. Signal. 4 (2009) 4.
[40] C. Li, J. Wu, Y. Li, G. Xing, Cytoprotective effect of heat shock protein 27 against lipopolysaccharide-induced apoptosis of renal epithelial HK-2 cells, Cell. Physiol. Biochem. 41 (2017) 2211–2220.
[41] J. Espana-Agusti, D.A. Tuveson, D.J. Adams, A. Matakidou, A minimally invasive, lentiviral based method for the rapid and sustained genetic manipulation of renal tubules, Sci. Rep. 5 (2015) 11061.