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Tri-domain proteins 27 reduce inflammation and apoptosis in HK-2 cells and protect against acute kidney injury in mice

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Abstract. – OBJECTIVE: The kidney is one of the most commonly damaged organs in sepsis. Acute kidney injury (AKI) induced by sepsis is a clinically dangerous disease with a high mortality rate. Therefore, it is particularly important to find a way to prevent and treat sepsis-induced AKI.

MATERIALS AND METHODS: Human renal tubular epithelial cell line (HK-2) and 8-week-old C57BL/6 mice were used. Lipopolysaccharide (LPS) was used to induce HK-2 cell injury and mouse AKI. Lentiviruses overexpressing TRIM27 were constructed to increase TRIM27 expression in HK-2 cells. Then, the effects of TRIM27 on the inflammation and apoptosis of HK-2 cells were analyzed, and those of TRIM27 recombinant protein on AKI in mice was detected by immunohistochemical staining and Western blot.

RESULTS: It was found that TRIM27 overexpression reduced the expressions of inflammatory factors and signaling molecules in apoptosis-related pathways in HK-2 cells, but increased the ratio of Bcl-2 to Bax in HK-2 cells, indicating the anti-apoptotic effect of TRIM27. Toll-like receptor 4 (TLR4)/NF-κB signaling pathway is an important mechanism of LPS mediated renal injury, and TRIM27 overexpression in HK-2 cells significantly inhibited the activity of TLR4/NF-κB signaling pathway. In addition, AKI was significantly relieved in mice treated with TRIM27 recombinant.

CONCLUSIONS: TRIM27 exerts anti-inflammatory and anti-apoptotic effects by inhibiting the TLR4/NF-κB signaling pathway, which effectively alleviates LPS-induced HK-2 cell damage and mouse AKI.

Key Words:
TRIM27, Inflammation, Apoptosis, HK-2 cells, Acute kidney injury.

Introduction

Sepsis is a systemic inflammatory response syndrome caused by bacteria invading the blood circulation and releasing toxins, leading to direct damage to tissues and activating the body's excessive defense system[1]. Sepsis often leads to single or multiple organ dysfunction, and the kidney is one of the most susceptible organs[2]. According to statistics, about 13 million people worldwide suffer from acute kidney injury (AKI) every year, and about 1.7 million people die of AKI each year[3]. Therefore, it is especially important to find a method to treat AKI effectively.

Tri-domain proteins (TRIM) are a family of proteins with zinc-finger structure, B-box and helix-helix at the N-terminus, and a variable domain at the C-terminus. Currently, nearly 80 TRIM protein members have been discovered and identified[4,5]. TRIM27, a member of the TRIM protein family, has been reported to play important roles in various life processes such as transcriptional regulation, inflammatory response, apoptosis, cell cycle, cell differentiation, and tumor cell migration[6]. Zheng et al[7] found that TRIM27 interacts with IKB kinase ε (IKKε) through yeast two-hybrid study. *In vitro* co-immunoprecipitation studies[8] also found that there is an interaction between TRIM27 and IKKβ, IKKα and IKKε. TRIM27 inhibits the activation of IFN-stimulated response elements (ISRE) and NF-κB mediated by tumor necrosis factor (TNF) and interleukin (IL)-1. The knockdown of TRIM27 by siRNA can significantly promote the

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activation of ISRE and NF-κB mediated by TNF and IL-1. These positive and negative researches initially suggested that TRIM27 can negatively regulate the inflammatory signaling pathway. In addition, TRIM27 can also interact with Nucleic acid binding oligomerization domain 2 (NOD2). The interaction of TRIM27 with NOD2 allows for efficient K48 ubiquitination of NOD2, resulting in degradation of NOD2 *via* the proteasome pathway. It has been reported that the mutation of NOD2 is associated with Crohn's enteritis[9]. It was found that TRIM27 expression was increased significantly in patients with Crohn's enteritis, suggesting that TRIM27 may affect the development of enteritis by regulating NOD2. These findings suggest that TRIM27 can control the inflammatory response by negatively regulating the NOD2 inflammatory signaling pathway. However, whether TRIM27 has anti-inflammatory effects on AKI caused by sepsis is still unclear. Therefore, C57BL/6 mice and human renal tubular epithelial cell line (HK-2) were used to construct an AKI model to investigate the role of TRIM27 in AKI to support clinical AKI treatment through this study.

Materials and Methods

Animals

Sixty 8-week-old male C57BL/6 mice were used in this study. All mice were kept in SPF barrier facilities. Clean chow and drinking water were fed to the mice and replaced regularly. All animal experiments were conducted in accordance with the regulations on the administration of experimental animals in China. This study was approved by the Animal Ethics Committee of Yantai Affiliated Hospital of Binzhou Medical University. Lipopolysaccharide (LPS) was used to induce kidney injury in mice. Mice were intraperitoneally injected with LPS (5 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA), and urine, serum and kidney tissues were collected 24 hours later. Mice in the treatment group were subcutaneously injected with recombinant protein TRIM27 (100 μg/kg) (PrimeGene, Shanghai, China) daily for one week before modeling.

Enzyme Linked Immunosorbent Assay (ELISA)

The urine and serum of the mice were collected, and the expression of cytokines was detected by ELISA (Invitrogen, Carlsbad, CA, USA). Impurities were removed from urine and serum by centrifugation. Then, the standard substance was diluted and added to the standard well according to the instruction. Next, 50 μL of samples were added to the rest of the wells, and the horseradish peroxidase (HRP)-labeled avidin working solution was immediately added and incubated at 37°C for 1 hour. After washing in each well, the substrate solution was added for 30 min in the dark. Finally, the absorbance of each well at 450 nm was detected with the microplate reader. The detection of cell culture supernatant was similar to that of serum.

Hematoxylin-Eosin (HE) Staining

After the kidney of mice was collecting, the renal capsule was gently separated with tweezers and fixed in 4% paraformaldehyde for 24 h. Then, the kidney tissue was put in gradient alcohol and xylene and made into paraffin mass. A slicer was used to make paraffin sections of kidney tissue. Thereafter, paraffin sections were baked in a 37°C oven for 24 h, dewaxed, hydrated, and soaked in hematoxylin dye for 3 min. After rinsing in water for 3 min, the sections were soaked in alcohol hydrochloride for 3 s, rinsed in water immediately, stained with eosin for 1 min and dehydrated. Finally, neutral gum was used to seal the sections, and the staining results were observed and recorded under a microscope.

Cell Culture and Treatment

Human renal tubular epithelial cells (HK-2) were used in this study. Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) medium (Gibco, Rockville, MD, USA) was used to culture HK-2 cells and 10% complete medium was prepared using fetal bovine serum (FBS; Gibco, Rockville, MD, USA). All cell experiments were performed in a sterile ultra-clean table. LPS (0.5 mg/kg) was used to stimulate HK-2 cell injury.

Cell Transfection

HK-2 cells were transferred into the 6-well plates. After the cell growth and fusion degree reached 60%, the original culture medium was replaced with 2 mL of fresh culture medium containing 6 mg/mL polybrene (Invitrogen, Carlsbad, CA, USA), and appropriate lentivirus suspension was added. After the cells were cultured for another 24 h, fresh medium was used to replace the medium containing lentivirus.

Western Blot

Mouse kidney tissue and HK-2 cells were lysed by radioimmunoprecipitation assay (RIPA)

lysate at low temperature (Beyotime, Shanghai, China). The bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) was used to detect the protein concentration and 5×loading buffer was used to add to the lysate. An equal amount of protein is added to the running gel for electrophoresis and membrane transfer. Then, 5% skim milk was used to seal the polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). Subsequently, PVDF membrane was incubated with primary antibody solution (Abcam, Cambridge, MA, USA) at 4°C overnight, and washed by Tris-Buffered Saline and Tween-20 (TBST). After the PVDF membrane was incubated with secondary antibody solution (Abcam, Cambridge, MA, USA), the protein bands were detected with enhanced chemiluminescence (ECL).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

HK-2 cells were collected and placed in Eppendorf (EP) tubes with 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After leaving the cells on ice for 5 min, 200 μL of chloroform was added to TRIzol and mixed. After centrifugation (12000 rpm, 15 min), 400 μL of the supernatant was collected and mixed with 400 μL of isopropanol. After centrifugation (12000 rpm, 15 min), the supernatant was discarded, and 1 mL of 75% ethanol was added to wash the sediment. Finally, enzyme-free water was used to dissolve the RNA. The RNA solution was stored in a -80°C refrigerator. After measuring the RNA concentration using a spectrophotometer, the mRNA was reversely transcribed to cDNA using the Prime RT Master Mix (TaKaRa, Dalian, Liaoning, China). Then cDNA was stored in a refrigerator at -20°C and used for amplification with various primers. The reaction system was: 5 μL of SYBR Premix Ex Taq II (TaKaRa, Dalian, Liaoning, China) + 0.25 μL of PCR sense Primer + 0.25 μL of PCR anti-sense Primer + 1 μL of DNA and 3.5 μL of enzyme-free water. The primer sequences are shown in Table I. Endogenous GAPDH mRNA expressions were used as controls. $2^{-\Delta\Delta CT}$ was used to indicate the relative expression of mRNA.

Immunocytofluorescence (IF) Staining

Cell slides were put into 24-well plates and HK-2 cells were cultured in them. After different treatment of the cells, the 24-well plates were taken, and the culture medium was removed.

Table I. Primers sequences for qRT-PCR.

Name	Sense/Anti-sense	Sequence (5′-3′)
TRIM27	Sense	ACGCATTCGACGACTGAGTGTAC
	Anti-sense	CGGGCTAAATCGGACGTAGATCGT
IL-1β	Sense	GTTGACGTACTACGTACGTGATC
	Anti-sense	AACGAGCATGCGGACTCTGCTAC
TNF-α	Sense	ATCGTCGCAGCTTCAGCAGTCTGCA
	Anti-sense	ATCCTGCCGCATATCTCAGTAGCA
IL-6	Sense	ACGACGTTCTACGTACGTACGTCT
	Anti-sense	GCGTATCATGCGATCTCTGGTCA
IL-8	Sense	GTCTTTAGCAGACGGTCGTACCA
	Anti-sense	GGTAGCTTCTAGCATCGTCAGTA
caspase3	Sense	CGCATGCTCTCAGCGTACGTACAT
	Anti-sense	CTTCAGGACGTTGTGACCACGGTCA
caspase8	Sense	ACGGTCAGTCGTACGTAGCTACGT
	Anti-sense	AAGCTCGACTGACTGACGTAGT
caspase9	Sense	GTCAGCGACGTACGTATCGAT
	Anti-sense	ATCTCGTACGTCGCTCTAGCGTA
Bax	Sense	AATCTCGCGTACGTAGCTGCTA
	Anti-sense	GGCTCTAAAGCTCTCAGAGTCT
Bcl-2	Sense	GCCCTTAAGGCGTACTGACGT
	Anti-sense	GCAACTTGAGCGCGTTCGAGCT
TLR4	Sense	AACGTTTCAGGAGACTCGTACGT
	Anti-sense	CCTATCGAGACATCGTGTACTGG
p65	Sense	AAGCTCTCGGCCCGGTAGCGTA
	Anti-sense	CAGCCTTCGCACGATGCAGTCGTG
GAPDH	Anti-sense	GGTTTCGACACCGTGTGGCATCGT
	Anti-sense	AGCGCCCTTTGGGCTATCGACTA

After the cells were soaked with 4% paraformaldehyde and 0.2% Triton-100, 10% goat serum was used to seal the cells. The primary antibody solution (Abcam, Cambridge, MA, USA) was then used to incubate the cells at 4°C overnight. After washing the cells with phosphate-buffered saline (PBS), the cells were incubated with fluorescent-labeled secondary antibody solution (Abcam, Cambridge, MA, USA) at room temperature for 1 h. Finally, the slides were fixed onto the glass slide with sealing tablets containing 4',6-diamidino-2-phenylindole (DAPI), and the staining results were observed with a fluorescence microscope.

Flow Cytometry

HK-2 cells were cultured in 6-well plates. After corresponding treatment of the cells, Annexin V-FITC apoptosis detection kit (Invitrogen, Carlsbad, CA, USA) was used to determine cell apoptosis rate according to the manufacturer's instructions.

Statistical Analysis

All the statistical results were processed by the statistical analysis software Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA). All measurement units are expressed as mean ± standard deviation. Levene method was used to test homogeneity of variance of each group. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). *t*-test was used for pairwise comparison. $p<0.05$ indicated that the difference was statistically significant.

Results

TRIM27 Overexpression Reduced LPS-Induced Inflammation in HK-2 Cells

LPS (0.5 μg/mL) was used to construct AKI models at cellular level. The transfection efficiency of TRIM27 overexpressed lentivirus was detected by Western blot (Figure 1A) and qRT-PCR (Figure 1B). Changes in inflammatory factors in HK-2 cells were detected by ELISA (Figure 1C-1F) and the results showed that the expressions of IL-1β, TNF-α, IL-6 and IL-8 in LPS-induced HK-2 cells were increased in LPS group while the overexpression of TRIM27 decreased the expression of these inflammatory factors. The results of qRT-PCR (Figure 1G-1J) were similar to those of ELISA. These results indicate that TRIM27 has a good anti-inflammatory effect on HK-2 cells.

TRIM27 Overexpression Reduced LPS-Induced Apoptosis of HK-2 Cells

To determine the effect of TRIM27 on the apoptosis level of HK-2 cells, the expression of apoptotic pathway related molecules in HK-2 cells was detected. IF staining results (Figure 2A, 2B) indicated that Bax expression was increased and Bcl-2 expression was decreased in LPS-induced HK-2 cells. However, overexpression of TRIM27 attenuated LPS-induced apoptosis, which was manifested as an increase in Bcl-2/Bax. qRT-PCR (Figure 2C-2G) also detected the mRNA expressions of caspase3/8/9, Bax and Bcl-2, and the results showed that TRIM27 significantly reduced the expressions of caspase3/8/9 and Bax and increased the expression of Bcl-2.The apoptosis rate of HK-2 cells was determined by flow cytometry (Figure 2H), which also indicated the anti-apoptotic effect of TRIM27.

TRIM27 Inhibits the Activity of Toll-Like Receptor 4 (TLR4)/NF-κB Signaling Pathway

TLR4/NF-κB signaling pathway is an important pathway mediating LPS-induced injury. IF staining (Figure 3A, 3B) and qRT-PCR (Figure 3C, 3D) detected the expressions of TLR4 and p65 in HK-2 cells, and the results showed that LPS stimulation increased the expressions of TLR4 and p65 in HK-2 cells while TRIM27 decreased their expressions. The phosphorylation level of p65 was detected by Western blot (Figure 3E), and the results showed that TRIM27 inhibited the phosphorylation of p65, indicating that TRIM27 can inhibit the nuclear translocation of p65.

Recombinant TRIM27 Protein Attenuates LPS-Induced Mouse AKI

To verify the renal protective effect of TRIM27, injected TRIM27 recombinant protein was subcutaneously into LPS-induced mice. HE staining (Figure 4A) detected changes in the renal morphology of mice treated with LPS and TRIM27. The results showed that the renal tubules of mice treated with LPS were swollen and the glomeruli were deformed, while the glomeruli and renal tubules of mice treated with TRIM27 were significantly improved. In addition, urine from mice was collected to detect changes in renal damage-related signaling molecules Kim-1, NGAL,

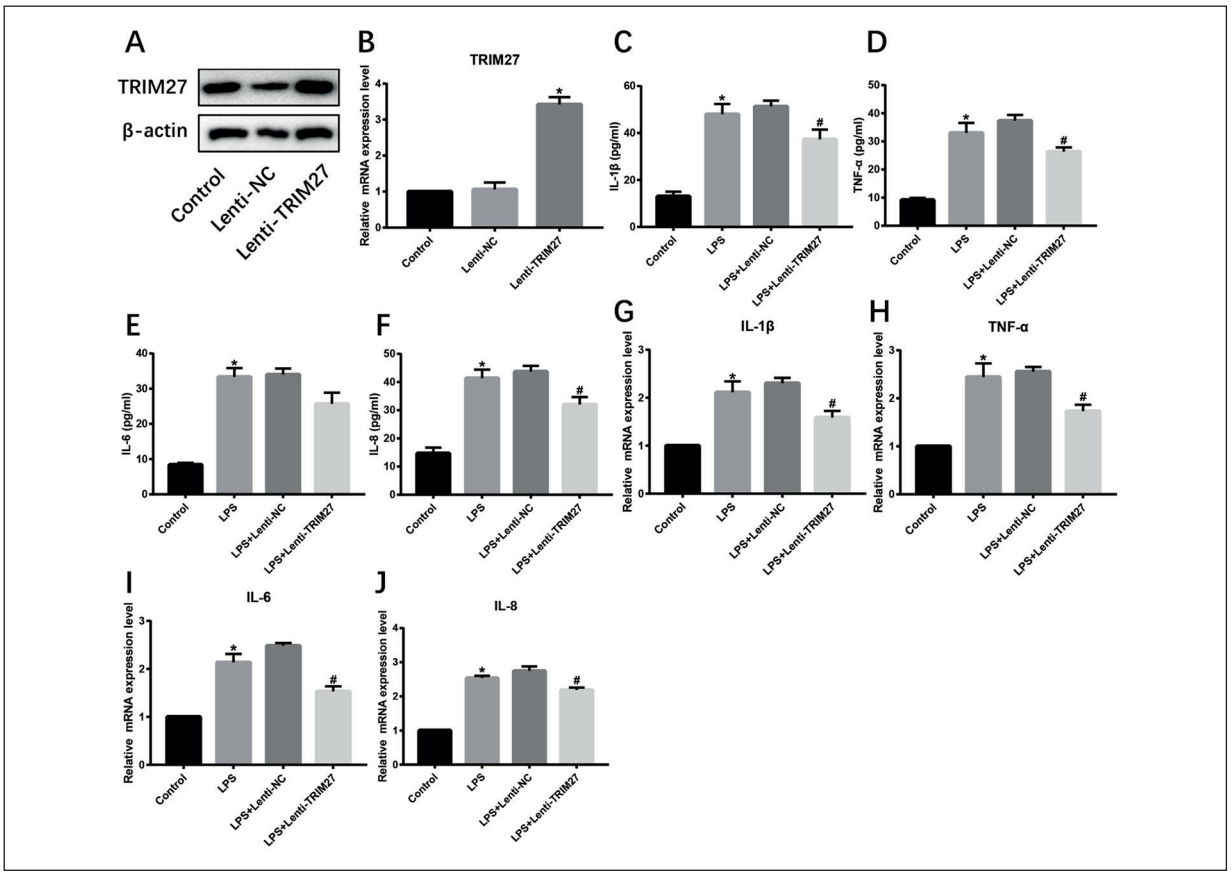

Figure 1. TRIM27 overexpression reduces LPS-induced inflammation in HK-2 cells. **A, B,** Protein expression and mRNA expression of TRIM27 were determined by Western blot and qRT-PCR. **C-F,** ELISA results of IL-1*β*, TNF-*α*, IL-6 and IL-8. **G-J,** mRNA expressions of IL-1β, TNF-α, IL-6 and IL-8. ("*" means $p<0.05$ *vs.* the control group and "#" means $p<0.05$ *vs.* the LPS+Lenti-NC group).

L-FAPB, and netrin-1 (Figure 4B-4E), and the results showed that urine expressions of kidney damage-related molecules in the AKI group were increased while TRIM27 decreased their expressions. Scr and BUN in mouse serum were also detected and TRIM27 was shown to reduce Scr and BUN (Figure 4F, 4G).

Discussion

Sepsis is often caused by a host's dysregulation of the inflammatory response to infection. Due to its high mortality and high economic burden, it has become a research hotspot in the medical field[10-12]. AKI is one of the most common complications of sepsis and is an independent risk factor for increased risk of sepsis death. As a potential therapeutic target, TRIM27 has shown superior anti-inflammatory and anti-apoptotic effects, which has greatly helped the treatment of AKI.

The anti-inflammatory effect of TRIM27 on HK-2 cells significantly reduced LPS-induced HK-2 cell damage. In sepsis, pathogen invades the body, and harmful molecules released are called pathogen associated molecular pattern (PAMP), such as LPS, DNA and lipoteichoic acid, which are related to the pattern recognition receptor (PRR) of the body[13]. The combination of PRR initiates the body's immune response and subsequent pro-inflammatory or anti-inflammatory responses. In addition, after this process is initiated, it will inevitably cause damage to cells of the body, which in turn will release the contents of the cells, namely the damage associated molecular pattern (DAMP). DAMP can also be combined with PRR, which in turn expands the body's immune response and inflammatory response[14]. PRR includes retinoic acid inducible gene-I like receptors, Toll like receptors (TLR) and NOD-like receptors, which are not only present in immune cells, but also present in ep-

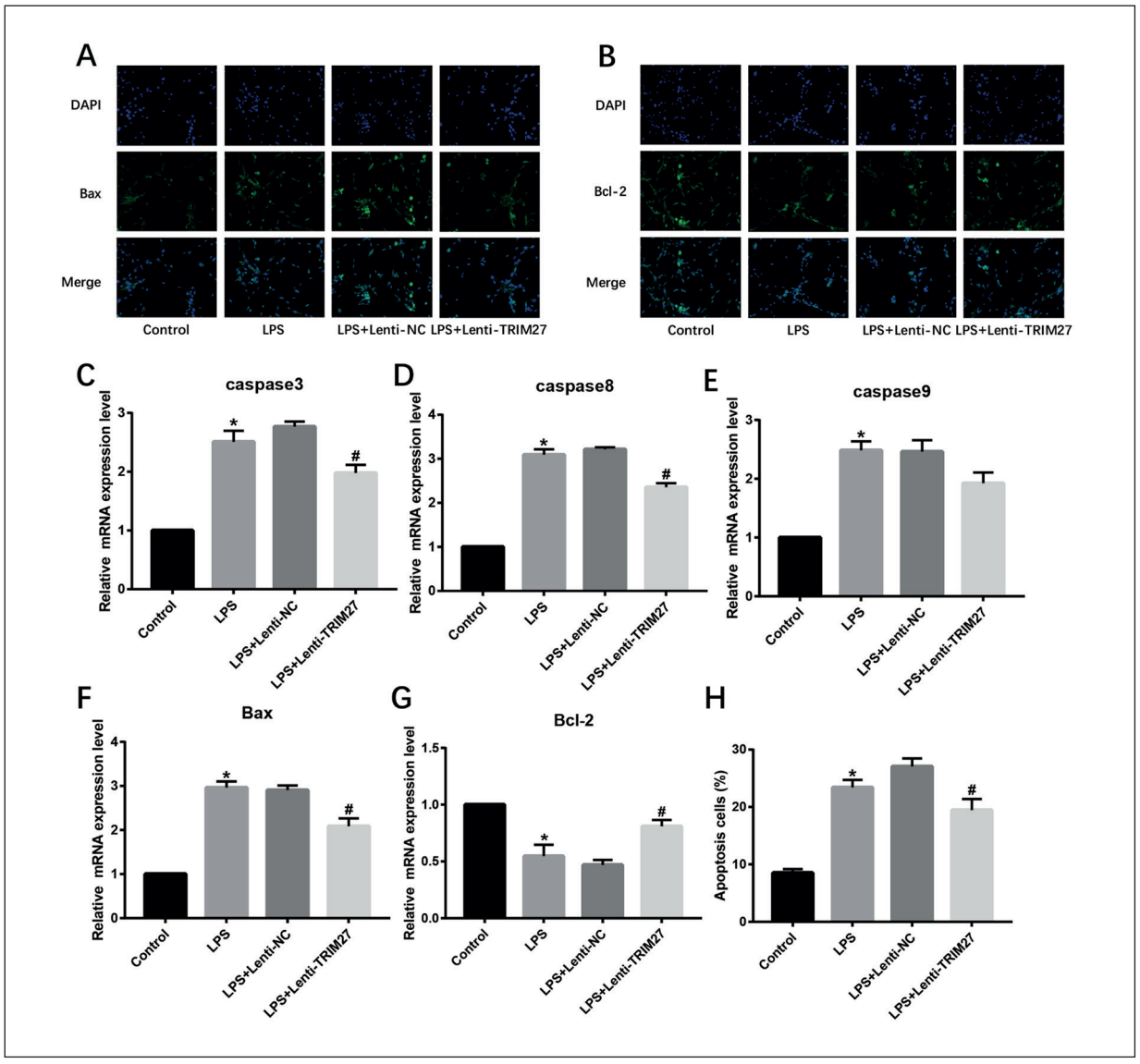

Figure 2. TRIM27 overexpression reduces LPS-induced apoptosis of HK-2 cells. **A, B,** IF staining results of Bax and Bcl-2 in HK-2 cells (magnification: 200×). **C-G,** mRNA expressions of caspase3, caspase8, caspase9, Bax and Bcl-2. **H,** Cell apoptosis rate of HK-2 cells. ("*" means $p<0.05$ *vs.* the control group and "#" means $p<0.05$ *vs.* the LPS+Lenti-NC group).

ithelial cells and endothelial cell surfaces. After pathogen invasion, renal tubular epithelial cells, endothelial cells, immune cells, etc. recognize PAMP and DAMP through PRR, initiate complex immune network responses, and release a large number of cytokines (including TNF-α, IL-6, IL- 8, INF-γ, etc.), which activates the cytokine cascade and forms a "waterfall effect"[15]. The outbreak of inflammatory factors leads to the continuous activation of the immune system, stimulates the massive release of other inflammatory factors (such as reactive oxygen species, thromboxane, endothelin, etc.), aggravates microcirculatory dysfunction, and ultimately causes damage to renal tissue cells. After HK-2 cell injury was induced with LPS, the inflammatory factors in HK-2 cells continued to rise, showing severe inflammatory response. Similarly, studies have found that the pathology of patients with septic shock and AKI is characterized by massive leukocyte infiltration, apoptosis and rare thrombosis[16]. Ren et al[17] found that long-chain non-coding RNA PVT1 aggravated LPS-induced HK-2 cell injury by stimulating TNF-α and JNK/NF-κB signaling pathways. Other researchers believe that renal dendritic cells can take up antigen-me-

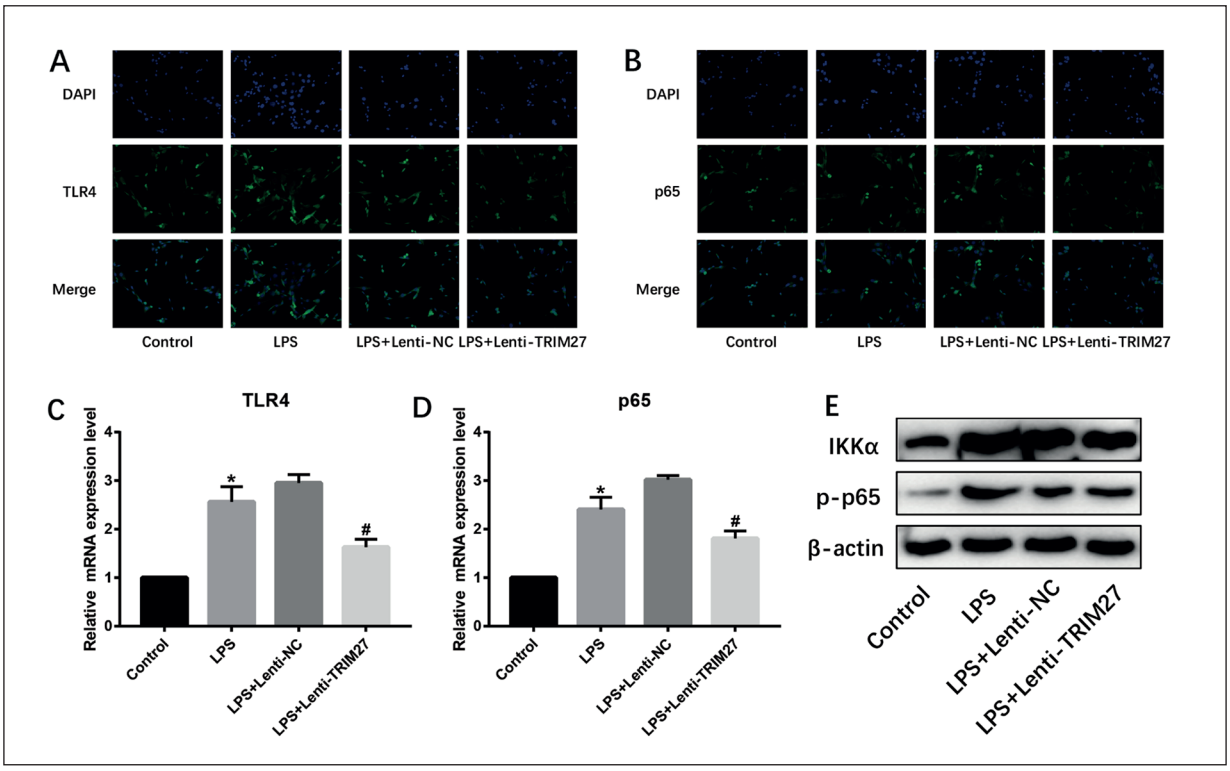

Figure 3. TRIM27 inhibits the activity of Toll-like receptor 4 (TLR4)/NF-κB signaling pathway. **A, B,** IF staining results of TLR4 and p65 in HK-2 cells (magnification: 200×). **C, D,** mRNA expressions of TLR4 and p65. **E,** Western blot results of IKKα and p-p65 in HK-2 cells. ("*" means $p<0.05$ *vs.* the control group and "#" means $p<0.05$ *vs.* the LPS+Lenti-NC group).

diated T cells in the capillaries surrounding the renal tubules to infiltrate the kidneys, thereby initiating inflammatory response[18]. Similarly, in sepsis, DAMP produced by damaged cells or blood circulation in the kidney can also expand local inflammation of the kidney through inflammatory factor release, leukocyte activation, increased adhesion molecules, platelet aggregation, microthrombus formation, and proinflammatory response[19]. TRIM27 showed significant anti-inflammatory effects in LPS-induced HK-2 cell injury and mouse kidney injury and reduced the expression of inflammatory factors in HK-2 cells.

NF-κB is a transcription factor of eukaryotic cells that is present in almost all cells. It mainly functions as a p50-p60 heterodimer[20]. In normal cases, NF-κB is present in the cytosol in combination with inhibitory proteins (IκBα) in the form of inactive complexes. When TNF acts on the corresponding receptor, NF-κB can be activated by the second messenger system[21]. Viral infection, lipopolysaccharide reactive oxygen species intermediates, double-stranded RNA, and activated protein kinase C, protein kinase A, etc. can directly activate NF-Kb[22]. The system changes the conformation of the inhibitory protein and separates it from NF-κB. The inhibition of NF-κB was removed and activated to transfer into the nucleus, leading to the overexpression of inflammatory factors[23]. Among the IκB proteins, the p65-p50 dimer is a heterodimer that is most frequently activated by various stimuli associated with kidney damage. Of note, the use of NF-κB inhibitors to block the activation of NF-κB can significantly reduce the expression of inflammatory factors in the kidney and improve the inflammatory response of the kidney[24]. TLR4 is a PRR that acts as an LPS sensor that activates recruitment of inflammatory factors and causes kidney injury. Moreover, the activation of TLR4 sensitizes the NF-κB pathway, which is linked to the initiation of the pro-inflammatory cytokines[25]. Activation of the NF-κB pathway is thought to be a major cause of renal inflammation, and inhibition of the TLR4/NF-κB has been shown to have a renal protective effect on LPS-induced AKI. In this study, lentivirus expressing TRIM27 was used to transfect HK-2 cells, and it was found that

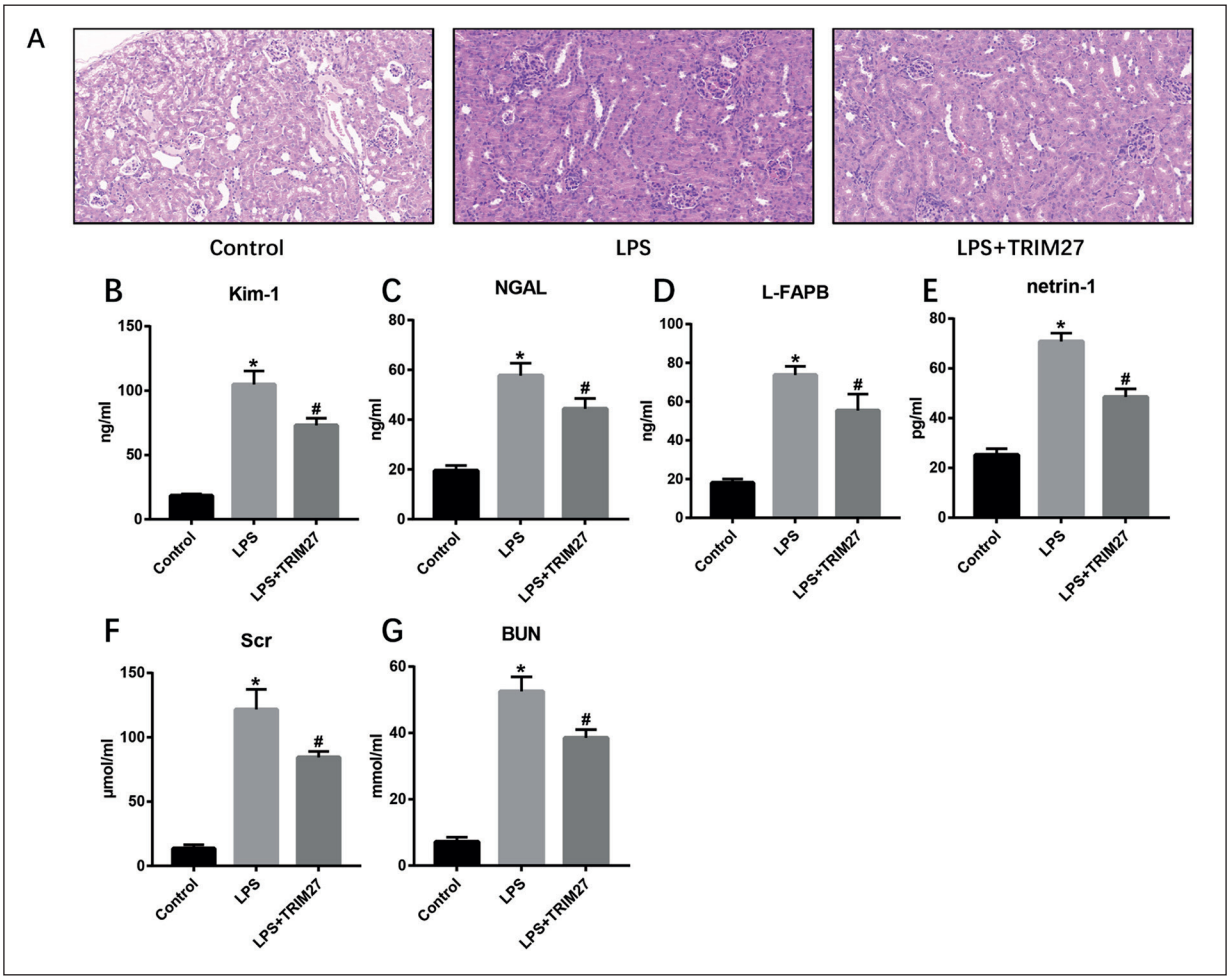

Figure 4. Recombinant TRIM27 protein attenuates LPS-induced mouse AKI. **A,** HE staining of mice kidney tissue (magnification: 200×). **B-E,** Content of Kim-1, NGAL, L-FAPB and netrin-1 in urine. **F, G,** Content of Scr and BUN in serum. ("*" means $p<0.05$ *vs.* the control group and "#" means $p<0.05$ *vs.* the LPS+TRIM27 group).

overexpression of TRIM27 significantly reduced the activity of the TLR4/NF-κB signaling pathway, suggesting a mechanism by which TRIM27 protects HK-2 cells from LPS-induced damage.

Conclusions

These results demontrated that overexpression of TRIM27 significantly reduced LPS-induced inflammation levels in HK-2 cells. After LPS stimulated HK-2 cells, the apoptosis level of HK-2 cells was increased, while TRIM27 decreased the apoptosis level of HK-2 cells. In addition, TRIM27 also inhibited the activity of the TLR4/NF-κB signaling pathway in HK-2 cells. In animal experiments, TRIM27 recombinant protein-treated mice had significantly lower kidney injury than the LPS group, which also demonstrated the protective effect of TRIM27 on the kidney.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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