USF2 Knockdown Downregulates THBS1 to Inhibit the TGF- β Signaling Pathway and Reduce Pyroptosis in Sepsis-Induced Acute Kidney Injury

Jian Sun

Shanghai Jiaotong University School of Medicine Xinhua Hospital

Yang Wang

Shanghai Jiaotong University School of Medicine Xinhua Hospital

Lei Niu

Shanghai Jiaotong University School of Medicine Xinhua Hospital

Lujia Tang

Shanghai Jiaotong University School of Medicine Xinhua Hospital

Shuming Pan (✉ drpanshuming0331@163.com)

Shanghai Jiaotong University School of Medicine Xinhua Hospital <https://orcid.org/0000-0003-2518-0290>

Research

Keywords: USF2, THBS1, TGF- β , Pyroptosis, Sepsis-induced acute kidney injury

Posted Date: December 16th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-126763/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Pharmacological Research on October 1st, 2021. See the published version at <https://doi.org/10.1016/j.phrs.2021.105962>.

Abstract

Background

Acute kidney injury (AKI) is a serious complication of sepsis. This study was performed to explore the mechanism that THBS1 mediated pyroptosis by regulating TGF- β signaling pathway in sepsis-induced AKI.

Methods

Gene expression microarray related to sepsis-induced AKI was obtained from the GEO database, and the mechanism in sepsis-induced AKI was predicted by bioinformatics analysis. qRT-PCR and ELISA were performed to detect expressions of THBS1, TNF- α , IL-1 β , and IL-18 in sepsis-induced AKI patients and healthy volunteers. Mouse model of sepsis-induced AKI was established, with serum creatinine, urea nitrogen, 24-h urine output measured, and renal tissue lesions observed by HE staining. Cell model of sepsis-induced AKI was cultured *in vitro*, with expressions of TNF- α , IL-1 β , and IL-18, pyroptosis, Caspase-1 and GSDMD-N, and activation of TGF- β pathway detected. The upstream transcription factor USF2 was knocked down in cells to explore its effect on sepsis-induced AKI.

Results

THBS1 was highly expressed in patients with sepsis-induced AKI. Silencing THBS1 protected mice against sepsis-induced AKI, and significantly decreased the expressions of NLRP3, Caspase-1, GSDMD-N, IL-1 β , and IL-18, increased cell viability, and decreased LDH activity, partially reversing changes in cell morphology. Mechanistically, USF2 promoted oxidative stress response by transcriptionally activating THBS1 to activate TGF- β /NLRP3/Caspase-1 signaling pathway and stimulate pyroptosis, and finally exacerbated sepsis-induced AKI.

Conclusions

USF2 knockdown downregulates THBS1 to inhibit TGF- β signaling pathway and reduce pyroptosis and further to ameliorate sepsis-induced AKI.

Background

As a common complication of critically ill patient, sepsis-induced acute kidney injury (sepsis-induced AKI) is related to high risk of chronic complication, drastic physiologic changes and high mortality [1, 2]. About 35% of sepsis patients develop renal failure, and nearly half of critically ill patients with AKI also developed sepsis [1, 3]. In the past, sepsis-induced AKI was considered to be caused by renal hypoperfusion, but studies have found inflammatory response and apoptosis may also be involved in the pathogenesis of sepsis-induced AKI [1, 4]. Oxidative stress has been reported to be related to sepsis and may contribute to AKI by generating reactive oxygen species (ROS) [5]. Existing evidence also reports that pyroptosis of renal tubular epithelial cells induced by Caspase-11 is a crucial event in the development of

sepsis-induced AKI [3]. Current treatment of sepsis-induced AKI mainly focuses on renal replacement therapy, fluid resuscitation, vasoactive drugs and antibiotics [5]. Since the pathogenesis of sepsis-induced AKI involves multiple factors such as apoptosis, immune and inflammatory processes, molecular mechanism targeting signaling pathways may have potential therapeutic value for sepsis-induced AKI.

Thrombospondin1 (THBS1), also called TSP1, is a homotrimeric glycoprotein released by activated platelets and involved in fibrin clots reacting to injury [6]. Previous study demonstrates that THBS1 bound to CD47 to promote AKI and blocking of THBS1 signaling through CD47 improved renal interstitial fibrosis [7]. Pyroptosis, a new programmed cell death mode discovered in recent years, is characterized by dependence on cysteine-aspartic proteases 1 (caspase-1), NLRP3 inflammasome activation and the release of a large number of proinflammatory factors [8]. In human cells, TSP1 is an upstream inhibitor of IL-1 β and caspase-1 mRNA, but not NLRP3 induced in the presence of LPS [9]. The involvement of THBS1 binding to the CD36 receptor in apoptosis has been reported in endothelial cells [10]. However, no study concerns the correlation between THBS1 and pyroptosis at present.

Previous study reports that activation of TGF- β can be attenuated by suppressing THBS1 with the LSKL peptide [6]. TGF- β can induce the activation of NLRP3 inflammasome and the cleavage of Gasdermin D (GSDMD) in rat with chronic kidney disease [11]. Those evidences support the association among THBS1, inflammatory response, pyroptosis and AKI. We further searched the upstream transcriptional regulators of THBS1 in the TTRUST database, including E2F1, NR1H2, SNAI1, TP53, USF2 and WT1. Overexpression of USF2 is found in previous study to result in renal injury [12]. Hence, in this study, we aim to explore the mechanism that transcriptional regulator USF2 regulates THBS1 and pyroptosis via the TGF- β signaling pathway in sepsis-induced AKI.

Methods

Ethics statement

This study was performed with the approval of the Clinical Ethical Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. Written informed consents were obtained from all patients and healthy volunteers. The animal experiments were conducted based on minimized animal number and the least pains.

Bioinformatics analysis

GSE60088 chip (loading in GPL1261 platform file) related to sepsis-induced AKI was obtained from GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), including 5 renal tissue control samples (control) and 5 sepsis-induced AKI samples (disease). Differential expression analysis of genes was performed by R language “limma” package (<http://www.bioconductor.org/packages/release/bioc/html/limma.html>), with $|\log_2FC| > 1$ and $P < 0.05$ as the screening criteria. Related genes were obtained by “searching sepsis-induced kidney injury” in GeneCards database (<https://www.genecards.org/>). Intersection between differentially expressed genes and the genes related to sepsis-induced AKI was performed by jvenn

(<http://jvenn.toulouse.inra.fr/app/example.html>). The heatmap of candidate gene expressions was plotted by using R language “pheatmap” package (<https://cran.r-project.org/web/packages/pheatmap/index.html>). Enrichment analysis on candidate genes was performed by WebGestalt (<http://www.webgestalt.org/>). Upstream transcriptional regulators of genes were found by TTRUST database (<https://www.grnpedia.org/trust/>).

Clinical samples

Fifteen patients with sepsis-induced AKI were enrolled in this study. Inclusion criteria were: aged 18 ~ 80 years, male or female, and the diagnosis of all patients was in accordance with the International Sepsis Guidelines Diagnostic Criteria [13] and Acute Kidney Injury Diagnostic Criteria [14]. Exclusion criteria were: chronic kidney disease or kidney transplantation or AKI caused by non-sepsis, or the presence of autoimmune diseases. Fifteen healthy volunteers (confirmed absence of renal impairment, autoimmune diseases, or malignant tumors) were also enrolled in this study. Peripheral blood was collected from all subjects, and the samples were centrifuged within 2 h and stored at -80 °C.

Establishment of animal model

A total of 120 C57BL/6J mice (aged 6 ~ 8 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China with animal license number (SYXK (Jing) 2016-0011). Mice were housed in separate cages at 20–26 °C, with free access to food and water and a 12-hlight/dark cycle. After adaptive feeding for 6 days, mice were assigned into 5 groups randomly, with 24 mice in each group: sham group, AKI group, AKI + sh-NC group, AKI + sh-THBS1 group and AKI + sh-USF2 group. Mice in all groups (except the sham group) were injected intraperitoneally with lipopolysaccharide (LPS) 10 mg/kg. The mice in AKI + sh-THBS1 group and AKI + sh-NC group were injected with adenovirus low expression vector sh-THBS1 and its control adenovirus in the tail vein, respectively, and the mice in AKI + sh-USF2 group and AKI + sh-NC group were injected with adenovirus low expression vector sh-USF2 and its control adenovirus in the tail vein, respectively, with a dose of 0.1 mL. Adenovirus and control vectors were synthesized and packaged by Hanheng Biological Company (Shanghai, China), and the adenovirus virus was 10^{10} /mL. After injection of lipopolysaccharide or phosphate buffered saline (PBS) for 24 h, 6 mice in each group were placed in sealed metabolic cages and fasted. The 24-h natural urine output of mice was collected and accurately weighed by an electronic balance. The remaining mice in each group were sacrificed after injection of LPS or PBS for 48 h, and serum samples were separated. Contents of serum creatinine and urea nitrogen were measured using an Automated Chemistry Analyzer (Shenzhen Rayto Life Science Co., Ltd, Shenzhen, Guangdong, China).

Hematoxylin-eosin (HE) staining

Mouse kidney tissues were collected and fixed with 4% formaldehyde for 6 h and embedded in paraffin. Paraffin-embedded tissue sections were deparaffinized in xylene and successively soaked in 100%, 95%, 80%, and 75% ethanol, and then washed with distilled water. Then sections were stained with hematoxylin staining solution for 10 min, rinsed with distilled water, differentiated tissues in differentiation solution for 30 s, soaked in distilled water for 15 min, stained with eosin staining solution

for 2 min and then rinsed with distilled water, successively soaked in 95%, 95%, 100%, and 100% ethanol for 1 min. Next, sections were placed in xylene carbonic acid (3:1) for 1 min, soaked in xylene (I) for 1 min and soaked in xylene (II) for 1 min. Finally, sections were mounted with neutral resin and observed under a microscope (Olympus, Japan) with images randomly collected.

Establishment of cell model of sepsis-induced AKI

Human umbilical vein endothelial cell (HUVEC) line was purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were incubated in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum, penicillin (1×10^5 U/L), and streptomycin (100 mg/L) in a cell incubator at 37 °C with 5% CO₂. When the cell density reached 90%, HUVECs were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) solution, then seeded in six-well plates, cultured again to reach a cell density of 90%, and continued to be cultured in serum-free medium for 24 h. Cells were randomly assigned into control group, LPS group, LPS + si-NC group, LPS + si-THBS1 group, LPS + si-USF2 + pc-NC, and LPS + si-USF2 + pc-THBS1 group. Cells in all groups (except control group) were added with LPS (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at a concentration of 1 µg/mL and treated for 24 h. Control cells were treated with the same amount of PBS for 24 h.

Cell transfection

pcDNA-THBS1, pcDNA-NC, siRNA-THBS1, siRNA-NC, and siRNA-USF2 were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). After cell transfection was performed as per the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), the cells in each group were cultured in an incubator for subsequent detection. pcDNA-THBS1 and its controls were transfected into LPS-induced cells which were transfected with si-USF2 for further assays. The TGF-β activator SRI-011381 hydrochloride (HY-100347A, 10 µM, MCE, Monmouth Junction, NJ, USA) was added into LPS-induced cells transfected with si-THBS1 for 24 h, namely LPS + si-THBS1 + SRI group. The control cells were treated with the same amount of dimethyl sulfoxide (DMSO) and named LPS + si-THBS1 + DMSO group.

Enzyme-linked immunosorbent assay (ELISA)

HUVECs and the supernatant after being centrifuged were collected to detect the expressions of TNF-α, IL-18, and IL-1β by ELISA. ELISA kits were purchased from Shanghai Bogoo Biotechnology Co., Ltd. (Shanghai, China). Kidney tissues were taken from mice and weighed, with cold PBS added (1:9 v/w) and tissues thoroughly ground. The homogenate was centrifuged at 300 g for 10 min at 4 °C and its supernatant was taken. The contents of TNF-α, IL-18, and IL-1β were detected using Elisa kits (Shanghai, China).

FAM-FLICA caspase assay

FAM-YVAD-FMK and propidium iodide (PI) double staining was performed using FAM-FLICA Caspase-1 Detection Kit (ImmunoChemistry, Bloomington, MN, USA) to assess changes in the number of pyroptotic cells (FAM-YVAD-FMK, green; PI, red; Hoechst 33343, blue).

Transmission electron microscope

HUVECs were detached using trypsin containing disodium EDTA, centrifuged at 100 g for 5 min, with supernatant discarded, and the cells were fixed by adding fixative for electron microscopic. The fixed cells were dehydrated through graded ethanol, stained with 70% ethanol and uranyl acetate, embedded, cut in section and observed under a transmission electron microscope (BX53, Olympus, Tokyo, Japan).

Co-immunoprecipitation (Co-IP)

The procedure was performed in strict accordance with the Co-IP Pierce® Direct IP Kit 26148. Amino Link Plus Resin (20 µL) and Pierce Control Agarose Resin (20 µL) (the negative control, without reacting with the antibody to remove non-specific binding protein) were incubated with 4 µg of THBS1 monoclonal antibody for 90 min, followed by the addition of 500 µL of protein samples overnight at 4 °C and washed four times with IP lysis/wash buffer and once with solution buffer. USF2 protein complex was eluted with solution buffer. The expressions of THBS1 and USF2 in the above samples were detected by Western blot after electrophoresis with 10% SDS-PAGE. The above antibodies were purchased from Abcam (Cambridge, MA, USA).

Cell viability test

The viability of HUVECs in each group was detected using the CellTiter-Glo® Luminescent Cell Viability Assay kit (G7570, Promega, Beijing, China). HUVEC cells were adjusted to a density of 1×10^5 cells/mL, seeded into 6-well plates at 1 mL per well, and cells were collected for experiments when the cell confluence reached 80%~90%. Reagents were prepared per the manufacturer's instructions, and then each group of cells (50 µL) was seeded into a 96-well plate. After mixing and being incubated for 10 min, detection was performed on a plate-reading luminometer (Varioskan™; Thermo Scientific, Rockford, IL, USA).

Lactate dehydrogenase (LDH) activity test

HUVECs were seeded into 96-well plates (5×10^3 cells/well). After appropriate treatment, LDH activity released into the culture medium was measured using the Cytotoxicity Assay Kit (Roche Applied Science, Mannheim, Germany). The release of LDH from cells was obtained by measuring the absorbance at 490 nm with a microplate reader.

Oxidative stress-related test

HUVECs in logarithmic growth phase were taken with the density adjusted to 2×10^5 cells/mL, and seeded to 6-well plates with 2 mL per well; after the supernatant was discarded, cells were washed with PBS twice, added with serum-free RPMI-1640 culture medium containing 10 µmol/L DCFH-DA, and incubated in an incubator containing 5% CO₂ at 37 °C for 30 min. The cells were shaken well to make the probe in full contact with the cells. Next, culture medium was discarded and cells were washed with PBS 3 times. Intracellular ROS content was measured by microplate reader (excitation wavelength 493 nm, emission wavelength 525 nm).

HUVECs were taken with the density adjust to 2×10^5 cells/mL and seeded into 6-well plates at 2 mL per well. At the end of the culture, cells were detached with trypsin, washed twice with PBS, and centrifuged. The supernatant was discharged before the cells were collected. Total glutathione (GSH) content was calculated by measuring the absorbance at 410 nm with a microplate reader (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The absorbance at 560 nm was measured to calculate superoxide dismutase (SOD) activity and the absorbance at 532 nm was measured to calculate malondialdehyde (MDA) content.

Western blot

HUVECs were lysed with RIPA lysate, and total cellular protein was extracted to detect protein concentration using a bicinchoninic acid (BCA) kit. Protein (50 ~ 100 µg) was taken for electrophoresis. After protein separation, protein was transferred to poly(vinylidene fluoride) (PVDF) membrane, incubated with 5% non-fat dry milk for 2 h to block non-specific sites, and incubated with primary antibodies THBS1 (1:1000, ab226383, Abcam), TGF-β (1/1000, ab215715, Abcam), Caspase-1 (1 µg/mL, ab138483, Abcam), GSDMD-N (1:1000, ab219800, Abcam), and β-actin (1:1500, ab8227, Abcam) overnight at 4 °C. Next, protein was washed and incubated with IgG H&L secondary antibody (1:2000; ab205718, Abcam) for 1 h and washed three times with TBST for 10 min each time. The results were observed after development and fixation. Western blot images were analyzed with ImageJ2x V2.1.4.7 (Rawak Software, Inc. Germany) software.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from HUVECs by TRIzol one-step method, and cDNA was synthesized per the instructions of the reverse transcription kit and placed in a fluorescence quantitative PCR instrument for amplification with an amplification system of 20 µL. qRT-PCR was performed on an ABI7900HT fast PCR real-time system (Applied Biosystems, Foster city, CA, USA) using cDNA as a template and GAPDH as an internal reference per the instructions of SYBR Premix ExTaqII (Takara, Dalian, China), and relative expression was calculated by the $2^{-\Delta\Delta C_t}$ method. All primers (Table 1) were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Table 1
Primers Sequence of qRT-PCR

Gene	Primer
THBS1	F: 5'-ATGGGGCTGGCCTGGGGACTAG-3'
	R: 5'-TTAGGGATCTCTACATTCTGATTT-3'
TNF- α	F: 5'-ATGGAGGGGTATGCGATGACAC-3'
	R: 5'-TCATAGTGCAAACACACCAAAG-3'
IL-1 β	F: 5'-ATGGCAACTGTTCTGAACTCA-3'
	R: 5'-TTAGGAAGACACGGATTCCATG-3'
IL-18	F: 5'-ATGGCTGCTGAACCAGTAGAAGAC-3'
	R: 5'-CTAGTCTTCGTTTTGAACAGTGAA-3'
USF2	F: 5'-ATGGACATGCTGGACCCGGGTC-3'
	R: 5'-TCACTGCCGGGTGCCCTCGCCC-3'
GAPDH	F: 5'-GGGAGCCAAAAGGGTCAT-3'
	R: 5'-GAGTCCTTCCACGATACCAA-3'

Statistical analysis

Data analysis was performed with SPSS 21.0 (IBM Corp. Armonk, NY, USA) software. The data were normally distributed as tested by Kolmogorov-Smirnov, and the data were expressed as mean $\pm$ standard deviation (SD). Comparisons between two groups were analyzed by independent sample *t*-test. Comparisons among multiple groups were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA. Post-hoc tests were performed by Tukey multiple comparison test. All tests of significance were 2-tailed, and $P < 0.05$ ($P < 0.01$) was considered statistically significant.

Results

THBS1 was highly expressed in patients with sepsis-induced AKI

Totals of 213 differentially expressed genes were obtained by differential expression analysis on microarray GSE60088 (related to sepsis-induced AKI), containing 154 significantly upregulated genes and 59 downregulated genes (Fig. 1A). Totals of 241 genes related to sepsis-induced AKI were obtained from the GeneCards database. The intersection between differentially expressed genes and the genes related to sepsis-induced AKI were performed and 23 candidate genes obtained (Fig. 1B). The expression heatmap of candidate genes was plotted (Fig. 1C). We focused on the 4 genes BMP6, MYC, TGIF1 and

THBS1. Considering the difference value and innovation and the searched literature, we found few reports on the study about the relationship between THBS1 and sepsis-induced AKI. THBS1 was highly expressed in diseased samples in the microarray GSE60088 (Fig. 1D). THBS1 expression was detected by qRT-PCR in patients with sepsis-induced AKI, and the results showed that the mRNA expression of THBS1 was significantly up-regulated ($P < 0.01$) (Fig. 1E). Previous study reported that excessive release of inflammatory mediators was a major trigger for sepsis-induced AKI [15]. ELISA was performed to detect the expressions of TNF- α , IL-1 β , and IL-18 in the serum of patients with sepsis-induced AKI and normal subjects, and the results showed that the expressions of TNF- α , IL-1 β , and IL-18 in patients with sepsis-induced AKI were significantly higher than those in healthy subjects ($P < 0.01$) (Fig. 1F). Correlation analysis between THBS1 and inflammatory factors showed that the expression of THBS1 was positively correlated with the expression of inflammatory factors (all $P < 0.05$) (Fig. 1G).

Protective effect of silencing THBS1 on sepsis-induced AKI mice

Mice in each group were intraperitoneally injected with 10 mg/kg LPS to construct a mouse model of sepsis-induced AKI, and qRT-PCR showed that the expression of THBS1 in mice was significantly increased ($P < 0.01$) (Fig. 2A). sh-NC and sh-THBS1 were transfected into the mouse model and the results of qRT-PCR indicated successful transfection ($P < 0.01$) (Fig. 2A). Compared with the sham-operated mice, serum creatinine and urea nitrogen were significantly elevated and 24-h urine output was reduced in mice in the AKI group ($P < 0.01$) (Fig. 2B-D), while the opposite results were presented in the AKI + sh-THBS1 group. HE staining showed that the renal sections of sham-operated mice had normal morphology, clear histological structure, no degeneration, atrophy, swelling and necrosis or inflammatory infiltration of renal tubular cells, and no dilatation of lumen of renal tubulars (Fig. 2E). Renal tubular epithelial cell edema, vacuolar degeneration, disappeared brush border and dilatation of lumen of renal tubulars were observed in the renal tissue in mice of AKI group and AKI + sh-NC group, while silencing THBS1 could significantly improve the renal histopathological damage in AKI mice (Fig. 2E). ELISA was shown that the expressions of TNF- α , IL-1 β , and IL-18 in serum in the AKI mice were significantly increased, while silencing THBS1 could significantly reduce the expression of inflammatory factors in AKI mice (all $P < 0.01$) (Fig. 2F).

Silencing THBS1 protected mice against sepsis-induced AKI by inhibiting pyroptosis

Pyroptosis might result in cell death, renal inflammation and renal injury, while inhibition of pyroptosis ameliorates pathological injury [3, 16]. Hence, we speculated that the protective effect of si-THBS1 on mice with sepsis-induced AKI might be achieved by regulating pyroptosis. FAM-FLICA Caspase-1 Detection Kit showed that the number of pyroptotic cells was significantly increased in the AKI group, but decreased in the AKI + sh-THBS1 group (all $P < 0.05$) (Fig. 3A). Western blot showed that the levels of caspase-1 and GSDMD-N were significantly increased in the AKI group, while the levels were decreased in

the AKI + sh-THBS1 group (all $P < 0.01$) (Fig. 3B). The above results confirmed that the protective effect of si-THBS1 on sepsis-induced AKI might be achieved by inhibiting pyroptosis.

THBS1 knockdown inhibited the activation of NLRP3 inflammasome and reduced inflammation

The above *in vivo* experiments demonstrated that silencing THBS1 significantly reduced the expressions of inflammatory factors in a mouse model of sepsis-induced AKI. *In vitro* cell experiment was performed to further confirm those findings. LPS at a concentration of 1 $\mu\text{g/mL}$ was used to induce HUVECs for 24 h for establishing a sepsis-induced AKI cell model. qRT-PCR results showed that THBS1 in the LPS group was significantly elevated ($P < 0.01$) (Fig. 4A). si-NC and si-THBS1 were transfected to HUVECs to reduce THBS1 expression ($P < 0.01$) (Fig. 4A). qRT-PCR and ELISA showed that TNF- α , IL-1 β , and IL-18 in the LPS group were significantly increased, and THBS1 knockdown significantly reduced the expression of inflammatory factors in cells (all $P < 0.01$) (Fig. 4B-C). Western blot demonstrated that the expression of NLRP3 was significantly increased in the LPS group, while clearly decreased after down-regulating THBS1 expression (all $P < 0.01$) (Fig. 4D). Briefly, THBS1 knockdown inhibited the activation of NLRP3 inflammasome and decreased inflammatory factors.

THBS1 knockdown inhibited pyroptosis and attenuated cell injury

FAM-FLICA Caspase-1 Detection Kit showed that the number of pyroptotic cells was significantly reduced in the LPS + si-THBS1 group compared with the LPS group (Fig. 5A). Western blot revealed that THBS1 knockdown significantly decreased the levels of caspase-1 and GSDMD-N (all $P < 0.01$) (Fig. 5B). Cell viability and LDH activity in each group were detected by different kits. The results showed that cell viability was significantly increased and LDH activity was significantly decreased after down-regulating THBS1 expression (all $P < 0.01$) (Fig. 5C-D). Transmission electron microscopy showed that cells in the LPS group were characterized with cell membrane rupture, release of cytoplasmic contents, and chromatin condensation; while THBS1 knockdown partially reversed the morphological changes of the cells in the LPS group (Fig. 5E).

THBS1 mediated pyroptosis and aggravated cell injury by activating the TGF- β /NLRP3/Caspase-1 pathway

Enrichment analysis of candidate genes was performed by WebGestalt tool (Fig. 6A) and the results indicated that THBS1 gene was mainly enriched in the AGE-RAGE and TGF- β pathways. Previous study reported that THBS1 could activate the TGF- β pathway [6]. TGF- β could induce the activation of NLRP3 inflammasome and the cleavage of GSDMD in rat with chronic kidney disease [11]. Thus we speculated that THBS1 could regulate pyroptosis through the TGF- β /NLRP3/Caspase-1 pathway. Western blot revealed that THBS1 was able to regulate TGF- β expression (Fig. 6B). LPS-induced cells were treated with

the TGF- β activator SRI-011381 hydrochloride and THBS1 knockdown. Western blot showed that the levels of TGF- β , NLRP3, caspase-1, and GSDMD-N were significantly increased in the LPS + si-THBS1 + SRI group compared with the LPS + si-THBS1 + DMSO group (all $P < 0.01$) (Fig. 6C), suggesting that THBS1 was able to activate the NLRP3/caspase-1 pathway through TGF- β . Results of ELISA showed that the expressions of TNF- α , IL-1 β , and IL-18 were significantly increased in the LPS + si-THBS1 + SRI group (all $P < 0.01$) (Fig. 6D). Cell viability was notably decreased in the LPS + si-THBS1 + SRI group compared with the LPS + si-THBS1 + DMSO group, while LDH activity was significantly increased (all $P < 0.01$) (Fig. 6E,F). All those findings suggested that THBS1 accelerated pyroptosis and cell injury by activating the TGF- β /NLRP3/caspase-1 pathway.

Transcription factor USF2 exacerbated cellular oxidative stress response by up-regulating THBS1 to activate the TGF- β /NLRP3/Caspase-1 axis

The upstream transcriptional regulators of THBS1 were searched through the TTRUST database (Table 2), and overexpression of USF2 was found in previous study to result in renal injury [12]. Oxidative stress is involved in the pathogenesis of sepsis-induced AKI [17]. We speculated that the transcription factor USF2 might exacerbate the cellular oxidative stress response by activating the TGF- β pathway through up-regulation of THBS1. The presence of USF2 and THBS1 binding was revealed by Co-IP (Fig. 7A). USF2 was elevated in LPS-induced cells, and si-USF2 successfully decreased USF2 expression (Fig. 7B) and significantly reduced THBS1 in LPS-induced cells (Fig. 7B) (all $P < 0.01$). PcDNA-THBS1 and its control were transfected to LPS-induced cells that were previously transfected with si-USF2 (Fig. 7C). The ROS content in the cells was detected using fluorescent probe DCFH-DA, and the results showed that the fluorescence was significantly increased in the LPS + si-USF2 + pc-THBS1 group compared with the LPS + si-USF2 + pc-NC group ($P < 0.01$) (Fig. 7D). Compared with the LPS + si-USF2 + pc-NC group, the contents of GSH and SOD in the LPS + si-USF2 + pc-THBS1 group were reduced, while the content of MDA was significantly increased (all $P < 0.01$) (Fig. 7E-G). Western blot showed that the protein levels of THBS1, TGF- β , NLRP3, Caspase-1, and GSDMD-N were significantly elevated in the LPS + si-USF2 + pc-THBS1 group ($P < 0.01$) (Fig. 7H). The results of ELISA also confirmed that the expressions of TNF- α , IL-1 β , and IL-18 were significantly increased in the LPS + si-USF2 + pc-THBS1 group (all $P < 0.01$) (Fig. 7I). In summary, transcription factor USF2 upregulated THBS1 to activate the TGF- β /NLRP3/caspase-1 pathway and finally exacerbate the cellular oxidative stress response.

Table 2
Transcription factors that regulate THBS1

TF	Target	Mode of Regulation	References (PMID)
E2F1	THBS1	Activation	20976175
NR1H2	THBS1	Activation	21072196
SNAI1	THBS1	Repression	19874710
TP53	THBS1	Activation	10029407;10101800
TP53	THBS1	Unknown	19826414
USF2	THBS1	Activation	15184388
WT1	THBS1	Activation	15542786
WT1	THBS1	Repression	15618543
Note: TF: Transcription factor.			

USF2 downregulation inhibited THBS1 to block the TGF- β /NLRP3/caspase-1 pathway and ameliorate sepsis-induced AKI

In vivo experiments in mice were performed to confirm the finding that USF2 downregulation inhibited the TGF- β pathway by down-regulating THBS1 to ameliorate sepsis-induced AKI. qRT-PCR revealed that USF2 was significantly increased in AKI mice ($P < 0.01$) (Fig. 8A). sh-NC and sh-USF2 were transfected into the AKI mouse model ($P < 0.01$). The effect of USF2 downregulation on the levels of THBS1, TGF- β , NLRP3 and Caspase-1 in mice was detected. Western blot showed that the levels of THBS1, TGF- β , NLRP3 and Caspase-1 in the AKI + sh-USF2 group were significantly decreased (all $P < 0.01$) (Fig. 8B). Compared with the sham group, mice in the AKI group had significantly enhanced serum creatinine, urea nitrogen, and lowered 24-h urine output (all $P < 0.01$) (Fig. 8C-E), while the AKI + sh-USF2 group showed the opposite trend. ELISA showed that the expressions of TNF- α , IL-1 β , and IL-18 were upregulated in the AKI group, while USF2 downregulation could significantly reduce the expression of inflammatory factors in the AKI mice (all $P < 0.01$) (Fig. 8F). GSDMD-N was significantly increased in the AKI group, but decreased in the AKI + sh-USF2 (all $P < 0.01$) (Fig. 8B). HE staining also demonstrated that USF2 downregulation had protective effects on mice with sepsis-induced AKI (Fig. 8G). The above results suggested that the protective effect of USF2 downregulation on mice with sepsis-induced AKI.

Discussion

AKI is a serious complication of sepsis with high morbidity and mortality [3]. Current studies and reviews mainly concern the pathogenesis, prevention and supportive treatment of sepsis-induced AKI [1, 2].

THBS1 is significantly involved in the progression of renal diseases [7]. By bioinformatics analysis, THBS1 is found as one of the candidate genes related to sepsis-induced AKI. In our study, USF2 knockdown downregulated THBS1 and inhibited TGF- β pathway and reduced pyroptosis, thus ameliorating sepsis-induced AKI.

After bioinformatics analysis and detection in clinical samples, we found that THBS1 was highly expressed in patients and mouse model with sepsis-induced AKI. Additionally, expression of THBS1 was positively related with the expressions of inflammatory factors. Previous study reports that THBS1 is elevated in human renal fibrotic conditions and affects renal fibrosis through regulating inflammation [18]. After transfecting sh-THBS1 into the AKI mice, we found that silencing THBS1 using sh-THBS1 significantly reduced the expression of inflammatory factors. A review reports that THBS1 is involved in the inflammatory response after hemorrhagic stroke [19]. Interestingly, Maimaitiyiming H et al. previously reported that THBS1 was a significant contributor to the progression of renal disease and THBS1 deficiency reduced renal macrophage infiltration and inflammation [20]. The findings in the previous studies are consistent with our results.

Pyroptosis might result in cell death, renal inflammation and renal injury, while inhibition of pyroptosis ameliorates pathological injury [3, 16]. Central to pyroptosis is the activation of the NLRP3 inflammasome and mediation of GSDMD, rapidly causing cell membrane rupture and release of cellular contents and finally resulting in an inflammatory response [16]. In our study, the pyroptotic cells and Caspase-1 and GSDMD-N levels were clearly increased in AKI mice, but decreased after sh-THBS1 treatment. Cell experiment also showed that THBS1 knockdown inhibited pyroptosis, the activation of NLRP3 inflammasome and inflammatory factors, and attenuated cell injury. THBS1 is involved in the pathophysiology of kidney ischemia and considered as a new mediator of cell injury [21]. However, no study has been performed on the correlation between THBS1 and pyroptosis. All those results initially confirmed that the association between of THBS1 and pyroptosis and concluded that the protective effect of THBS1 knockdown on sepsis-induced AKI might be achieved by inhibiting pyroptosis.

Enrichment analysis of candidate genes indicated that THBS1 was mainly enriched in the AGE-RAGE and TGF- β pathways. Suppression of THBS1 can attenuate the activation of TGF- β [6]. Additionally, TGF- β can induce the cleavage of GSDMD and the activation of NLRP3 inflammasome in rat with chronic kidney disease [11]. Our study showed that the levels of TGF- β , NLRP3, Caspase-1, and GSDMD-N were significantly increased in the LPS + si-THBS1 + SRI group, suggesting that THBS1 was able to activate the NLRP3/caspase-1 pathway through TGF- β . It's noted that the constitutive activation of NLRP3 inflammasome leads to serious liver inflammation and pyroptotic cell death in hepatocytes [22]. Dai et al. found that interaction of C/EBP β and TFAM promoted pyroptosis through the activation of NLRP3/caspase-1, and further boosted the progression of AKI [23]. Taken together, all those evidences support our findings that THBS1 knockdown ameliorated sepsis-induced AKI by inhibiting pyroptosis and alleviating cell injury by inactivating the TGF- β /NLRP3/Caspase-1 pathway.

USF2 is an upstream transcriptional regulator of THBS1 searched in TTRUST database and the presence of USF2 and THBS1 binding was confirmed by Co-IP. Elevated expression of USF2 suppresses the transcriptional activity of the Smurf and results in the enhanced activity of TGF- β [24]. Transgenic USF-2 mice overexpress TGF- β 1 to modulate the progression of kidney disease and up-regulated TGF- β 1 level is related to the activation of profibrotic pathways triggered by oxidative stress [25]. Oxidative stress has been proven to be related to AKI development [17]. High expression of THBS1-CD47 signaling is responsible for the enhanced oxidative stress in diabetes [26]. Consistently, our finding revealed that USF2 upregulated THBS1 to activate the TGF- β /NLRP3/caspase-1 pathway and finally exacerbate the cellular oxidative stress response. Serum creatinine concentration and urine output are two early indicators for the diagnosis of AKI [5]. Our study found that silencing USF2 decreased serum creatinine, urea nitrogen and increased urine output. USF2 downregulation could significantly reduce inflammatory factors in AKI mouse model by inhibiting THBS1. Overexpression of USF2 is found in a previous report to result in renal injury [12]. Overexpression of USF2 contributes to the elevation of THBS1 and activation of TGF- β , leading to the development of diabetic nephropathy [27]. Taken together, USF2 downregulation inhibited THBS1 to inactivate the TGF- β /NLRP3/caspase-1 pathway and ameliorated sepsis-induced AKI.

Conclusion

In conclusion, USF2 activates the TGF- β pathway by up-regulating THBS1 to promote pyroptosis and aggravate sepsis-induced AKI, which may provide therapeutic strategy for patients with sepsis-induced AKI. However, our study only simply revealed that USF2 had a protective mechanism against sepsis-induced AKI by regulating THBS1 to affect the TGF- β pathway, the direct role in the treatment of clinical septic patients has not yet been deeply identified, which requires further study in future.

Abbreviations

AKI : Acute kidney injury ;

ROS : reactive oxygen species ;

THBS1:Thrombospondin1 ;

GSDMD : Gasdermin D ;

LPS : lipopolysaccharide ;

PBS : phosphate buffered saline ;

HE : Hematoxylin-eosin ;

HUVEC : Human umbilical vein endothelial cell ;

EDTA : ethylenediaminetetraacetic acid ;

DMSO : dimethyl sulfoxide ;

ELISA : Enzyme-linked immunosorbent assay ;

PI : propidium iodide ;

Co-IP : Co-immunoprecipitation ;

LDH : Lactate dehydrogenase ;

GSH : glutathione ;

SOD : superoxide dismutase ;

MDA : malondialdehyde ;

PVDF : poly(vinylidene fluoride) ;

qRT-PCR : Quantitative reverse transcription polymerase chain reaction ;

SD : standard deviation ;

ANOVA : analysis of variance

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Conceptualization: JS and YW; validation, research, resources, data reviewing, and writing: JS, LN, LT; review and editing: JS and SP. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

The data that support this study are available from the corresponding author upon reasonable request.

Authors' contributions

Conceptualization: JS and YW; validation, research, resources, data reviewing, and writing: JS, LN, LT; review and editing: JS and SP. All authors read and approved the final manuscript.

Consent for publication

All patients agreed and signed the informed consent.

Competing interests

The authors declare that they have no competing interests.

Author details

Department of Emergency, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Yangpu District, Shanghai, China

References

1. Skube SJ, Katz SA, Chipman JG, Tignanelli CJ. Acute Kidney Injury and Sepsis. *Surg Infect (Larchmt)*. 2018; 19:216-224.
2. Peerapornratana S, Manrique-Caballero CL, Gomez H, Kellum JA. Acute kidney injury from sepsis: current concepts, epidemiology, pathophysiology, prevention and treatment. *Kidney Int*. 2019; 96:1083-1099.
3. Ye Z, Zhang L, Li R, Dong W, Liu S, Li Z, et al. Caspase-11 Mediates Pyroptosis of Tubular Epithelial Cells and Septic Acute Kidney Injury. *Kidney Blood Press Res*. 2019; 44:465-478.
4. Lerolle N, Nochy D, Guerot E, Bruneval P, Fagon JY, Diehl JL, Hill G. Histopathology of septic shock induced acute kidney injury: apoptosis and leukocytic infiltration. *Intensive Care Med*. 2010; 36:471-478.
5. Keir I, Kellum JA. Acute kidney injury in severe sepsis: pathophysiology, diagnosis, and treatment recommendations. *J Vet Emerg Crit Care (San Antonio)*. 2015; 25:200-209.
6. Atanasova VS, Russell RJ, Webster TG, Cao Q, Agarwal P, Lim YZ, et al. Thrombospondin-1 Is a Major Activator of TGF-beta Signaling in Recessive Dystrophic Epidermolysis Bullosa Fibroblasts. *J Invest Dermatol*. 2019; 139:1497-1505 e1495.
7. Julovi SM, Sanganerla B, Minhas N, Ghimire K, Nankivell B, Rogers NM. Blocking thrombospondin-1 signaling via CD47 mitigates renal interstitial fibrosis. *Lab Invest*. 2020; 100:1184-1196.
8. Yu ZW, Zhang J, Li X, Wang Y, Fu YH, Gao XY. A new research hot spot: The role of NLRP3 inflammasome activation, a key step in pyroptosis, in diabetes and diabetic complications. *Life Sci*. 2020; 240:117138.
9. Stein EV, Miller TW, Ivins-O'Keefe K, Kaur S, Roberts DD. Secreted Thrombospondin-1 Regulates Macrophage Interleukin-1beta Production and Activation through CD47. *Sci Rep*. 2016; 6:19684.
10. Wu Q, Finley SD. Predictive model identifies strategies to enhance TSP1-mediated apoptosis signaling. *Cell Commun Signal*. 2017; 15:53.

11. Zhang K, Fan C, Cai D, Zhang Y, Zuo R, Zhu L, et al. Contribution of TGF-Beta-Mediated NLRP3-HMGB1 Activation to Tubulointerstitial Fibrosis in Rat With Angiotensin II-Induced Chronic Kidney Disease. *Front Cell Dev Biol.* 2020; 8:1.
12. Shi L, Nikolic D, Liu S, Lu H, Wang S. Activation of renal renin-angiotensin system in upstream stimulatory factor 2 transgenic mice. *Am J Physiol Renal Physiol.* 2009; 296:F257-265.
13. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock, 2012. *Intensive Care Med.* 2013; 39:165-228.
14. Stevens PE, Levin A, Kidney Disease: Improving Global Outcomes Chronic Kidney Disease Guideline Development Work Group M. Evaluation and management of chronic kidney disease: synopsis of the kidney disease: improving global outcomes 2012 clinical practice guideline. *Ann Intern Med.* 2013; 158:825-830.
15. Rabb H, Griffin MD, McKay DB, Swaminathan S, Pickkers P, Rosner MH, et al. Inflammation in AKI: Current Understanding, Key Questions, and Knowledge Gaps. *J Am Soc Nephrol.* 2016; 27:371-379.
16. Lin J, Cheng A, Cheng K, Deng Q, Zhang S, Lan Z, Wang W, Chen J. New Insights into the Mechanisms of Pyroptosis and Implications for Diabetic Kidney Disease. *Int J Mol Sci.* 2020; 21.
17. Liao W, Wang Z, Fu Z, Ma H, Jiang M, Xu A, Zhang W. p62/SQSTM1 protects against cisplatin-induced oxidative stress in kidneys by mediating the cross talk between autophagy and the Keap1-Nrf2 signalling pathway. *Free Radic Res.* 2019; 53:800-814.
18. Murphy-Ullrich JE. Thrombospondin 1 and Its Diverse Roles as a Regulator of Extracellular Matrix in Fibrotic Disease. *J Histochem Cytochem.* 2019; 67:683-699.
19. Wu X, Luo X, Zhu Q, Zhang J, Liu Y, Luo H, Cheng Y, Xie Z. The Roles of Thrombospondins in Hemorrhagic Stroke. *Biomed Res Int.* 2017; 2017:8403184.
20. Maimaitiyiming H, Zhou Q, Wang S. Thrombospondin 1 Deficiency Ameliorates the Development of Adriamycin-Induced Proteinuric Kidney Disease. *PLoS One.* 2016; 11:e0156144.
21. Thakar CV, Zahedi K, Revelo MP, Wang Z, Burnham CE, Barone S, et al. Identification of thrombospondin 1 (TSP-1) as a novel mediator of cell injury in kidney ischemia. *J Clin Invest.* 2005; 115:3451-3459.
22. Wree A, Eguchi A, McGeough MD, Pena CA, Johnson CD, Canbay A, Hoffman HM, Feldstein AE. NLRP3 inflammasome activation results in hepatocyte pyroptosis, liver inflammation, and fibrosis in mice. *Hepatology.* 2014; 59:898-910.
23. Dai XG, Li Q, Li T, Huang WB, Zeng ZH, Yang Y, et al. The interaction between C/EBPbeta and TFAM promotes acute kidney injury via regulating NLRP3 inflammasome-mediated pyroptosis. *Mol Immunol.* 2020; 127:136-145.
24. Tan Y, Chen Y, Du M, Peng Z, Xie P. USF2 inhibits the transcriptional activity of Smurf1 and Smurf2 to promote breast cancer tumorigenesis. *Cell Signal.* 2019; 53:49-58.
25. Samarakoon R, Overstreet JM, Higgins SP, Higgins PJ. TGF-beta1 -> SMAD/p53/USF2 -> PAI-1 transcriptional axis in ureteral obstruction-induced renal fibrosis. *Cell Tissue Res.* 2012; 347:117-128.

26. Bitar MS. Diabetes Impairs Angiogenesis and Induces Endothelial Cell Senescence by Up-Regulating Thrombospondin-CD47-Dependent Signaling. *Int J Mol Sci.* 2019; 20.

27. Liu S, Shi L, Wang S. Overexpression of upstream stimulatory factor 2 accelerates diabetic kidney injury. *Am J Physiol Renal Physiol.* 2007; 293:F1727-1735.

Figures

Figure 1

THBS1 was highly expressed in patients with sepsis-induced AKI. A: Volcano plot differentially expressed genes from GSE60088 (Dataset related to sepsis-induced AKI); abscissa represents $-\log_{10}(P \text{ value})$ and ordinate represents $\log_2FC$, with green dot indicating significantly differentially expressed genes with low expression and red dot indicating significantly differentially expressed genes with high expression; B: Venn diagram of intersection of differentially expressed genes and genes related to sepsis-induced AKI; C: Heatmap of candidate gene expression, with color scales blue to red indicating expression from low to high; D: THBS1 was significantly highly expressed in diseased samples in GSE60088; E: THBS1 expression in patients with sepsis-induced AKI and healthy subjects detected by qRT-PCR; F: Expressions of TNF- α , IL-1 β , and IL-18 in serum of patients with sepsis-induced AKI and healthy subjects measured by ELISA; G: Correlation between THBS1 and inflammatory factors analyzed by Pearson analysis. Results were obtained from three independent replicates and data were presented as mean $\pm$ standard deviation. The panel of E was analyzed by t test and the panel of F was analyzed by two-way ANOVA; Post-hoc tests were performed using Tukey's Multiple Comparisons Test; ** $P < 0.01$.

Figure 1

THBS1 was highly expressed in patients with sepsis-induced AKI. A: Volcano plot differentially expressed genes from GSE60088 (Dataset related to sepsis-induced AKI); abscissa represents $-\log_{10}(P \text{ value})$ and ordinate represents $\log_2 \text{FC}$, with green dot indicating significantly differentially expressed genes with low expression and red dot indicating significantly differentially expressed genes with high expression; B: Venn diagram of intersection of differentially expressed genes and genes related to sepsis-induced AKI; C: Heatmap of candidate gene expression, with color scales blue to red indicating expression from low to high; D: THBS1 was significantly highly expressed in diseased samples in GSE60088; E: THBS1 expression in patients with sepsis-induced AKI and healthy subjects detected by qRT-PCR; F: Expressions of TNF- α , IL-1 β , and IL-18 in serum of patients with sepsis-induced AKI and healthy subjects measured by ELISA; G: Correlation between THBS1 and inflammatory factors analyzed by Pearson analysis. Results were obtained from three independent replicates and data were presented as mean $\pm$ standard deviation. The panel of E was analyzed by t test and the panel of F was analyzed by two-way ANOVA; Post-hoc tests were performed using Tukey's Multiple Comparisons Test; ** $P < 0.01$.

Figure 2

Protective effect of silencing THBS1 on mice with sepsis-induced AKI. sh-NC and sh-THBS1 were transfected into mice model of sepsis-induced AKI. A: Expressions of THBS1 in each group of mice detected by qRT-PCR; B-D: Detection of serum creatinine, urea nitrogen and 24-h urine output in each group; E: Renal tissue of mice in each group stained by HE staining; F: Expression of TNF- α , IL-1 β , and IL-18 in serum of mice in each group detected by ELISA. N = 24 and data were presented as mean $\pm$ standard deviation. Data between multiple groups were compared by one-way ANOVA or two-way ANOVA, and post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** P < 0.01.

Figure 2

Protective effect of silencing THBS1 on mice with sepsis-induced AKI. sh-NC and sh-THBS1 were transfected into mice model of sepsis-induced AKI. A: Expressions of THBS1 in each group of mice detected by qRT-PCR; B-D: Detection of serum creatinine, urea nitrogen and 24-h urine output in each group; E: Renal tissue of mice in each group stained by HE staining; F: Expression of TNF- α , IL-1 β , and IL-18 in serum of mice in each group detected by ELISA. N = 24 and data were presented as mean $\pm$ standard deviation. Data between multiple groups were compared by one-way ANOVA or two-way ANOVA, and post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** P < 0.01.

Figure 3

Silencing THBS1 ameliorated sepsis-induced AKI mice model by inhibiting pyroptosis. sh-NC and sh-THBS1 were transfected into mice model with sepsis-induced AKI. A: Changes in the number of pyroptotic cells in tissues detected by FAM-FLICA Caspase-1 Detection Kit; B: Levels of pyroptosis-related proteins Caspase-1 and GSDMD-N measured by Western blot. N = 24 and data were presented as mean $\pm$ standard deviation. Data between multiple groups were compared by two-way ANOVA, and post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** P < 0.01.

Figure 3

Silencing THBS1 ameliorated sepsis-induced AKI mice model by inhibiting pyroptosis. sh-NC and sh-THBS1 were transfected into mice model with sepsis-induced AKI. A: Changes in the number of pyroptotic cells in tissues detected by FAM-FLICA Caspase-1 Detection Kit; B: Levels of pyroptosis-related proteins Caspase-1 and GSDMD-N measured by Western blot. N = 24 and data were presented as mean $\pm$

standard deviation. Data between multiple groups were compared by two-way ANOVA, and post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** $P < 0.01$.

Figure 4

THBS1 knockdown inhibited the activation of NLRP3 inflammasome and reduced inflammatory factors expressions. si-NC and si-THBS1 were transfected into LPS-induced HUVECs. A: Expression of THBS1 in cells of each group detected by qRT-PCR; B-C: Expressions of TNF- α , IL-1 β , and IL-18 detected by qRT-PCR and ELISA; D: Expression of NLRP3 measured by Western blot. Cell experiments were repeated three times and data were presented as mean $\pm$ standard deviation. Data between multiple groups were compared by one-way ANOVA or two-way ANOVA, and post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** $P < 0.01$.

Figure 4

THBS1 knockdown inhibited the activation of NLRP3 inflammasome and reduced inflammatory factors expressions. si-NC and si-THBS1 were transfected into LPS-induced HUVECs. A: Expression of THBS1 in cells of each group detected by qRT-PCR; B-C: Expressions of TNF-α, IL-1β, and IL-18 detected by qRT-PCR and ELISA; D: Expression of NLRP3 measured by Western blot. Cell experiments were repeated three times and data were presented as mean ± standard deviation. Data between multiple groups were compared by one-way ANOVA or two-way ANOVA, and post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** P < 0.01.

Figure 5

THBS1 knockdown inhibited pyroptosis and attenuated cell injury. si-NC and si-THBS1 were transfected into LPS-induced HUVEC cells. A: Change of number of pyroptotic cells in each group detected by AM-FLICA Caspase-1 Detection Kit; B: Levels of pyroptosis-related proteins Caspase-1 and GSDMD-N measured by Western blot; C: Cell viability detected by Cell Titer Glo Luminescent Cell Viability Assay kit; D: LDH activity detected by the kit to verify cell membrane integrity; E: Morphological changes of cells in each group observed by transmission electron microscopy. Cell experiments were repeated three times and data were presented as mean $\pm$ standard deviation. Data between multiple groups were compared by one-way ANOVA or two-way ANOVA, and post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** $P < 0.01$.

Figure 5

THBS1 knockdown inhibited pyroptosis and attenuated cell injury. si-NC and si-THBS1 were transfected into LPS-induced HUVEC cells. A: Change of number of pyroptotic cells in each group detected by AM-FLICA Caspase-1 Detection Kit; B: Levels of pyroptosis-related proteins Caspase-1 and GSDMD-N measured by Western blot; C: Cell viability detected by Cell Titer Glo Luminescent Cell Viability Assay kit; D: LDH activity detected by the kit to verify cell membrane integrity; E: Morphological changes of cells in each group observed by transmission electron microscopy. Cell experiments were repeated three times and data were presented as mean $\pm$ standard deviation. Data between multiple groups were compared by one-way ANOVA or two-way ANOVA, and post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** $P < 0.01$.

Figure 6

THBS1 mediated pyroptosis and aggravated cell injury by activating the TGF-β/NLRP3/Caspase-1 pathway. A: Enrichment analysis results for candidate genes, with abscissa indicating Enrichment ratio; B: Expression of TGF-β measured by Western blot; C: Levels of TGF-β, NLRP3, Caspase-1 and GSDMD-N measured by Western blot; D: Expressions of TNF-α, IL-1β, and IL-18 detected by ELISA; E: Cell viability detected by Cell Titer Glo Luminescent Cell Viability Assay kit; D: LDH activity detected by the kit to verify cell membrane integrity; Cell experiments were repeated three times and data were presented as mean ± standard deviation. The panel of B was analyzed by one-way ANOVA; the panel of C-D was analyzed by two-way ANOVA; the panel of E-F was analyzed by t test; Post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** P < 0.01.

Figure 6

THBS1 mediated pyroptosis and aggravated cell injury by activating the TGF-β/NLRP3/Caspase-1 pathway. A: Enrichment analysis results for candidate genes, with abscissa indicating Enrichment ratio; B: Expression of TGF-β measured by Western blot; C: Levels of TGF-β, NLRP3, Caspase-1 and GSDMD-N measured by Western blot; D: Expressions of TNF-α, IL-1β, and IL-18 detected by ELISA; E: Cell viability detected by Cell Titer Glo Luminescent Cell Viability Assay kit; D: LDH activity detected by the kit to verify cell membrane integrity; Cell experiments were repeated three times and data were presented as mean ± standard deviation. The panel of B was analyzed by one-way ANOVA; the panel of C-D was analyzed by two-way ANOVA; the panel of E-F was analyzed by t test; Post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** P < 0.01.

Figure 7

Transcription factor USF2 exacerbated cellular oxidative stress response by activating THBS1. A: Binding relationship of USF2 and THBS1 detected by Co-IP; B: mRNA expression of USF2 detected by qRT-PCR; C: mRNA level of THBS1 in each group detected by qRT-PCR; D: ROS content in cells detected by fluorescent probe DCFH-DA; E-G: Contents of GSH, SOD and MDA detected by kits; H: Protein expressions of THBS1, TGF- β , NLRP3, Caspase-1, and GSDMD-N in each group measured by Western blot; I: Expressions of TNF- α , IL-1 β , and IL-18 detected by ELISA. Cell experiments were repeated three times and data were presented as mean $\pm$ standard deviation. The panel of B-C was analyzed by one-way ANOVA; the panel of D-G was analyzed by t test; the panel of H-I was analyzed by two-way ANOVA; Post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** $P < 0.01$.

Figure 7

Transcription factor USF2 exacerbated cellular oxidative stress response by activating THBS1. A: Binding relationship of USF2 and THBS1 detected by Co-IP; B: mRNA expression of USF2 detected by qRT-PCR; C: mRNA level of THBS1 in each group detected by qRT-PCR; D: ROS content in cells detected by fluorescent probe DCFH-DA; E-G: Contents of GSH, SOD and MDA detected by kits; H: Protein expressions of THBS1, TGF- β , NLRP3, Caspase-1, and GSDMD-N in each group measured by Western blot; I: Expressions of TNF- α , IL-1 β , and IL-18 detected by ELISA. Cell experiments were repeated three times and data were presented as mean $\pm$ standard deviation. The panel of B-C was analyzed by one-way ANOVA; the panel of D-G was analyzed by t test; the panel of H-I was analyzed by two-way ANOVA; Post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** $P < 0.01$.

Figure 8

USF2 transcriptionally activated THBS1 to regulate the TGF-β/NLRP3/caspase-1 signaling pathway and exaggerate sepsis-induced AKI. sh-NC and sh-USF2 were transfected into mice model of sepsis-induced AKI. A: Expression of USF2 in mice in each group detected by qRT-PCR; B: TGF-β, NLRP3, Caspase-1, and GSDMD-N in each group measured by Western blot; C-E: Detection of serum creatinine, urea nitrogen and 24-h urine output in each group; F: Expressions of TNF-α, IL-1β, and IL-18 detected by ELISA; G: Renal tissue of mice in each group stained by HE staining. N = 24 and data were presented as mean ± standard

deviation. Data between multiple groups were compared by one-way ANOVA or two-way ANOVA, and post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** P < 0.01.

Figure 8

USF2 transcriptionally activated THBS1 to regulate the TGF-β/NLRP3/caspase-1 signaling pathway and exaggerate sepsis-induced AKI. sh-NC and sh-USF2 were transfected into mice model of sepsis-induced AKI. A: Expression of USF2 in mice in each group detected by qRT-PCR; B: TGF-β, NLRP3, Caspase-1, and GSDMD-N in each group measured by Western blot; C-E: Detection of serum creatinine, urea nitrogen and

24-h urine output in each group; F: Expressions of TNF- α , IL-1 β , and IL-18 detected by ELISA; G: Renal tissue of mice in each group stained by HE staining. N = 24 and data were presented as mean $\pm$ standard deviation. Data between multiple groups were compared by one-way ANOVA or two-way ANOVA, and post-hoc tests were performed by Tukey's Multiple Comparisons Test; ** P < 0.01.