Inhibition of Phosphoinositide 3-Kinase Gamma Protects Endothelial Cells via the Akt Signaling Pathway in Sepsis-Induced Acute Kidney Injury

Han Li\textsuperscript{a} Jun-Xian Xu\textsuperscript{b} Tiao-Chun Cheng\textsuperscript{a} Li-Jun Tian\textsuperscript{b} Jin-Feng Lin\textsuperscript{b}
Xi Luo\textsuperscript{c} Zhao-Lian Bian\textsuperscript{d} Xu-Dong Han\textsuperscript{b}

\textsuperscript{a}Medical School of Nantong University, Nantong, China; \textsuperscript{b}Department of Critical Care Medicine, Nantong Third People’s Hospital, Nantong University, Nantong, China; \textsuperscript{c}Nantong Institute of Liver Diseases, Nantong Third People’s Hospital, Nantong University, Nantong, China; \textsuperscript{d}Department of Gastroenterology and Hepatology, Nantong Third People’s Hospital, Nantong University, Nantong, China

\textbf{Keywords}
Sepsis · Endothelial cell · Acute kidney injury · Phosphoinositide 3-kinase gamma

\textbf{Abstract}

\textbf{Introduction:} Sepsis is a primary cause of death in critically ill patients and is characterized by multiple organ dysfunction, including sepsis-induced acute kidney injury (AKI), which contributes to high mortality in sepsis. However, its pathophysiological mechanisms remain unclear. The kidney has one of the richest and most diversified endothelial cell populations in the body. This study was designed to investigate the effects of endothelial dysfunction in sepsis-induced AKI and explore possible intervention measures to offer new insight into the pathogenesis and treatment of sepsis-induced AKI. \textbf{Methods:} The circulating levels of endothelial adhesion molecules were detected in patients with sepsis and healthy controls to observe the role of endothelial damage in sepsis and sepsis-induced AKI. A murine sepsis model induced by cecal ligation and perforation was pretreated with a phosphoinositide 3-kinase gamma (PI3K\textgamma{}) inhibitor (CZC24832), and survival, kidney damage, and renal endothelial injury were assessed by pathological examination, immunohistochemistry, quantitative polymerase chain reaction, and Western blotting. Lipopolysaccharides and CZC24832 were administered to human umbilical vein endothelial cells in vitro, and endothelial cell function and the expression of adhesion molecules were evaluated. \textbf{Results:} Endothelial damage was more serious in sepsis-induced AKI than that in non-AKI, and the inhibition of PI3K\textgamma{} alleviates renal endothelial injury in a murine sepsis model, protecting endothelial cell function and repairing endothelial cell injury through the Akt signaling pathway. \textbf{Conclusions:} In this study, endothelial cell dysfunction plays an important role in sepsis-induced AKI, and the inhibition of PI3K\textgamma{} alleviates endothelial cell injury in sepsis-induced AKI through the PI3K\textgamma{}/Akt pathway, providing novel targets for treating sepsis and related kidney injury.

\textbf{Introduction}

Sepsis is a life-threatening organ dysfunction caused by a maladaptive host response to infection [1, 2] and is the primary cause of death in critically ill patients. Sepsis often leads to multiple organ failure, including renal failure...
caused by acute kidney injury (AKI), and is the most common cause of AKI in critically ill patients, and sepsis-induced AKI accounts for 50% of AKI cases in intensive care units [3] and contributes to mortality as much as 40% [4, 5]. However, its pathophysiological mechanisms remain unclear [6]. Fluid resuscitation and vasoactive drugs for septic shock are primarily adopted in the clinical setting, as well as renal replacement therapy when necessary; however, no specific therapy for AKI is currently available.

Endothelial cell injury is considered a key factor in the progression from sepsis to organ failure [7] and contributes to sepsis outcome [8]. Vascular endothelium is a primary target of sepsis-induced events, and loss of endothelial barrier function is key to the pathogenesis of sepsis, being closely related to the systemic inflammatory and coagulopathic responses in sepsis [9]. The kidney has one of the richest and most diversified endothelial cell populations in the body [10], and renal vascular endothelium plays a critical role in several disease processes, such as glomerular nephritis, vasculitis, and lupus nephritis. During sepsis, endothelial cells, including renal vascular endothelial cells, act as the first line of defense and are activated early, increasing neutrophil activation, adhesion, and migration, increasing microvascular permeability, resulting in the rapid development of AKI [7, 11]. Yu et al. [12] have demonstrated that vascular endothelial cadherin shedding is more severe in patients with sepsis with severe AKI. Endothelial injury and endothelial barrier dysfunction contribute to the pathogenesis of sepsis and loss of organ failure [7]. Although the role of renal tubular epithelial injury in AKI has been well studied, little is known about the role of endothelial injury in AKI. Therefore, this study was designed to determine the mechanism of endothelial injury in sepsis-induced AKI to further understand its complicated pathophysiological mechanisms and to provide a scientific basis for novel treatment.

Phosphoinositide 3-kinases (PI3Ks) are a conserved family of kinases that phosphorylate phosphoinositide in the plasma membrane [13]. PI3Ks are intracellular signaling enzymes and are involved in various aspects of septic pathophysiology, including inflammatory cell recruitment and activation, apoptosis, and coagulation [14]. The PI3K family includes classes I, II, and III [15]. PI3Kγ is the only class IB member and is activated by heterotrimeric G protein-coupled receptors upon ligand binding. PI3Kγ is expressed in different cell types, such as leukocytes, platelets (PLTs), and endothelial cells, and acts as an important signaling molecule to control various immune and inflammatory responses and cardiovascular functions [16]. Martin et al. [14] have proposed that PI3Kγ contributes to sepsis and organ damage (e.g., the liver and lungs) by altering neutrophil recruitment. PI3Kγ inactivation increases endothelial recovery following arterial injury in atherosclerosis [17]. A recent study has reported that PI3Kγ is involved in the immune regulation of cardiovascular diseases by regulating cyclic adenosine monophosphate levels in cardiomyocytes [18]. Currently, PI3Kγ is thought to participate in lung and liver injuries induced by sepsis by regulating the immune response through inflammatory cells. In sepsis-induced AKI, endothelial cell injury may occur first and contribute to severe inflammation and microcirculation dysfunction; however, the role of PI3Kγ in endothelial injury during sepsis-induced AKI remains unknown. In this study, we investigated the role of PI3Kγ in endothelial cell injury in sepsis-induced AKI. Our data indicated that PI3Kγ inhibition protects endothelial cells from sepsis-induced AKI by regulating the Akt pathway.

### Materials and Methods

**Patients**

We enrolled 150 participants including 40 healthy controls (HCs) and 110 sepsis patients from September 2017 to December 2020. Sepsis was diagnosed according to the updated Sepsis-3 definition [1]. Patients with sepsis who were younger than 18 years, had long-term regular dialysis, had undergone kidney transplantation, or had incomplete data were excluded from the study. Moreover, we divided sepsis patients into the AKI (n = 54) and non-AKI

| Characteristic | HC (n = 40) | Sepsis (n = 110) | p value |
|---------------|------------|-----------------|--------|
| Age (years)   | 70.00±9.95 | 72.79±14.23     | 0.225  |
| Male, n (%)   | 20 (50)    | 72 (65.5)       | 0.086  |
| WBC (10^9/L)  | 6.21±1.39  | 17.58±9.49      | <0.001 |
| NEUT (10^9/L) | 3.86±1.04  | 15.92±9.17      | <0.001 |
| Lymph (10^9/L)| 1.81±0.54  | 0.55±0.44       | <0.001 |
| HCT (L)       | 39.40±4.44 | 29.81±7.10      | <0.001 |
| HGB (g/L)     | 136.38±15.45 | 98.19±23.40   | <0.001 |
| PLT (10^9/L)  | 216.98±59.92 | 130.75±91.72 | <0.001 |
| Scr (μmol/L)  | 64.83±13.14 | 145.92±130.62  | <0.001 |
| BUN (mmol/L)  | 5.49±1.23  | 14.67±9.20      | <0.001 |
| ALT (U/L)     | 18.10±7.49 | 245.12±1,015.51 | 0.021  |
| AST (U/L)     | 23.14±5.83 | 570.01±2,718.86 | 0.037  |

HC, healthy control; WBC, white blood count; NEUT, neutrophil count; Lymph, lymphocyte count; HCT, hematocrit; HGB, hemoglobin; PLT, platelet; Scr, serum creatinine; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
(n = 56) groups based on the KDIGO criteria: an increase in serum creatinine (SCr) level (≥26.4 μmol/L within 48 h or ≥1.5 times the baseline within 7 days) or a reduction in urine output (documented oliguria of less than 0.5 mL/kg per hour for more than 6 h). The demographic and clinical data of sepsis patients (n = 110) and HCs (n = 40) are presented in Table 1, and the data of the patients with sepsis with AKI (n = 54) and without AKI (n = 56) are shown in Table 2. This study was approved by the Ethics Committee of Nantong Third People’s Hospital, Nantong University, and the subjects provided signed informed consent.

**Plasma Endothelial Cell Adhesion Molecule Detection**

The circulating levels of intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were measured using the Human Magnetic Luminex Assay Kit (R&D Systems, MN, USA) in human plasma samples. The results were read and analyzed on Luminex MAGPIX Instrument System (Luminex, Austin, TX, USA).

**Mice and Cecal Ligation and Puncture Model**

Cecal ligation and puncture (CLP) surgery was performed to induce sepsis as in a previous study [19]. In brief, male C57BL/6 mice (8–10 weeks old) were obtained from Shanghai Laboratory Animal Central (Shanghai, China) and housed in specific pathogen-free conditions at Nantong University. The cecum was ligated using silk sutures, then perforated using a 19-gauge needle and gently squeezed to extrude a small amount of feces. Then, the cecum was returned to the peritoneal cavity, and the peritoneum was closed. The mice were fluid resuscitated using 1 mL of prewarmed normal saline.

(III) Normal control (NC) group: mice in the NC group (n = 10) underwent the same surgery but without CLP [20] and received normal saline.

The mice were monitored every 1 h for survival for 24 h. All mice were sacrificed 24 h after surgery; the blood and kidneys of the animals were harvested for further study. All animal experiments in this manuscript were performed according to the Animal Research: Reporting of In Vivo Experiments guidelines for reporting in vivo experiments, and all procedures were approved by the Animal Care and Use Committee of Nantong University.

**Renal Function and Histological Analysis**

SCr (Determiner L CRE, Hitachi Chemical Diagnostics Systems Co., Ltd, Tokyo, Japan) and blood urea nitrogen (BUN) (Urea Nitrogen Determination Kit, Shanghai Kehua Bio-Engineering co., Ltd, Shanghai, China) levels were determined using commercial kit reagents. Formalin-fixed and paraffin-embedded kidney tissues were cut into 4-μm sections, stained with hematoxylin and eosin, and visualized using a light microscope by a pathologist in a blinded manner. Pathology scores were determined using the scoring system described in a previous study [21]. The criteria of kidney injury scores included glomerular Bowman’s space dilation, tubular cell necrosis, loss of brush border, vacuolization, tubule dilation, and inflammatory cell infiltration, which were scored as follows: 0, no injury; 1, less than 25%; 2, less than 50%; 3, less than 75%; and 4, more than 75%.

**Immunohistochemistry**

Routine immunohistochemistry (IHC) staining was performed to detect the expression of PI3Kγ, phosphorylation of Akt (pAkt), ICAM-1, and VCAM-1. Paraffin-embedded sections were dehydrated and rehydrated in graded alcohols, and Tris-ethylenediaminetetraacetic acid buffer was used for antigen retrieval followed by incubation with rabbit anti-PI3Kγ (abs148249, 1:100; Abcam, Cambridge, MA, USA), and anti-VCAM-1 (ab134047, 1:800; Abcam, Cambridge, MA, USA). The sections were then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (PV-9001; Zhongshan Jinqiao Biotechnology Co., Ltd, Beijing, China) for 20 min. All sections were stained with diaminobenzidine (DAB-0031; MXB Biotechnology, Danvers, MA, USA), and anti-ICAM-1 (ab179707, 1:2,000; Abcam, Cambridge, MA, USA), and anti-VCAM-1 (ab134047, 1:800; Abcam) antibodies at 4°C overnight. Then, sections were washed and rehydrated in Tris-ethylenediaminetetraacetic acid buffer followed by incubation with rabbit anti-PI3Kγ (abs148249, 1:100; Absin, Shanghai, China), anti-pAkt (4060T, 1:100; Cell Signaling Technology, Danvers, MA, USA), anti-ICAM-1, and VCAM-1. Immunohistochemical staining was performed using the scoring system described in a previous study [21]. The percentage of areas positive for IHC was measured using ImageJ (version 1.52; National Institutes of Health, Bethesda, MD, USA) using the IHC profiler plugin [22].

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in Dulbecco’s modified Eagle medium (C12430500BT, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco), 100-U/mL penicillin, and 100-mg/mL streptomycin (Gibco) in an incubator at 37°C with 5% CO2. HUVECs were treated with indicated concentrations of lipopolysaccharide (LPS) (L4391; Sigma-Aldrich Co. Ltd, MO, USA) for different times. To examine the role of PI3Kγ in LPS-treated endothelial cells, 2-μmol/L CZC24832 (S7018; Selleck Chemicals) was added to culture medium 2 h before the administration of LPS.
Inhibition of PI3Kγ Protects Sepsis-Induced Acute Kidney Injury

Cell Proliferation
Cell proliferation was determined using the Cell-Light EdU DNA Imaging Kit (C10310-3; RiboBio, Guangzhou, China) according to the manufacturer’s instructions. In brief, HUVECs were cultured in 96-well plates, and labeling medium was added to each well for 2 h at 37°C with 5% CO₂, and 4% paraformaldehyde were added for 30 min at room temperature. Cells were then permeabilized in 0.5% Triton X-100 at 4°C for 10 min, and the samples were washed with PBS. Then, cells were stained with Hoechst 33342 for 30 min. Finally, images were obtained using a microscope (Olympus, Tokyo, Japan) and were analyzed using ImageJ software (version 1.52; National Institutes of Health), referring to previous recommendations [23].

Cellular Migration by Wound Healing Assay
Migration of HUVECs was measured using the scratch wound assay. When cell confluence reached approximately 100%, the media was removed. A 10-μL tip was used to make a vertical wound, and cell fragments were washed off with PBS and cultured continuously. Images of cells migrating to the wound were obtained at 0, 24, and 48 h using a microscope (Olympus). The wound area was analyzed using ImageJ software [24] (version 1.52; National Institutes of Health).

Tube Formation Analysis
Tube formation analysis was performed using Matrigel Matrix (354234; BD Biosciences, Franklin Lakes, NJ, USA) in a 96-well plate. The plate was coated with Matrigel under chilling conditions and incubated for solidification at 37°C for 30 min. Cells were seeded on Matrigel-coated wells and incubated for 24 h at 37°C. Tube-like structures were imaged intermittently during the incubation, and images were analyzed using ImageJ software [25] (version 1.52; National Institutes of Health).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction
Total RNA from kidney tissues and HUVECs was extracted using TRIzol reagent (9109; TaKaRa Bio, Dalian, China). RNA purity and concentration were determined using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using PrimeScript RT Reagent Kit (RR036A; TaKaRa Bio). Polymerase chain reaction primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). β-Actin was used as an internal control. Primer sequences are presented in Table 3. Reactions were performed in a Bio-Rad real-time polymerase chain reaction detection system using SYBR Green Master Mix (RR820; TaKaRa Bio).

Western Blotting
Total protein was extracted from HUVECs and kidney tissues using radioimmunoprecipitation assay lysis buffer (P0013B; Beyotime Institute of Biotechnology, Shanghai, China) containing phosphatase inhibitor cocktail (P0145; Beyotime), and protein concentrations were determined using BCA Protein Assay Kit (P0010; Beyotime). Protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrochemically transferred onto nitrocellulose membranes (Pall Corporation, Cortland, NY, USA) at 100 V for 90 min. After blocking with 5% nonfat milk, membranes were incubated with anti-PI3Kγ (5405, 1:1,000; Cell Signaling Technology), pAkt (4060, 1:2,000, Cell Signaling Technology), Akt (4691, 1:1,000; Cell Signaling Technology), VCAM-1 (ab134047, 1:2,000; Abcam), and ICAM-1 (ab179707, 1:1,000; Abcam). Target proteins were examined using an electrochemiluminescence system (Thermo Fisher Scientific) and visualized using X-ray films (Carestream, Xiamen, China). β-Actin was used as a control. The results were measured using ImageJ [26] (version 1.52; National Institutes of Health).

Statistical Analysis
Numerical variables were analyzed using the unpaired Student’s t test or Mann-Whitney U test, whereas categorical variables were compared using the χ² test or Fisher’s exact test. For survival studies, the Kaplan-Meier analysis method, followed by log-rank tests, was performed. All data were expressed as mean ± standard deviation. IBM SPSS Statistics 21.0 and GraphPad Prism 8.0 software were used for data analysis. Differences with p values of less than 0.05 were considered statistically significant. All experiments were repeated at least three times.

Results

Characteristics of the Participants
The baseline characteristics of HCs (n = 40) and sepsis patients (n = 110) are shown in Table 1. No differences in age and gender were observed between HCs and sepsis

### Table 3. Primer sequences for real-time polymerase chain reaction (PCR)

| Gene name | Primers | Sequences (5′-3′) |
|-----------|---------|-----------------|
| β-Actin   | Forward | CTACCTCATGAGATCCTGACC |
|           | Reverse | CACAGCTTCTTGTGATGTCAC |
| IL-1β     | Forward | TGGCAGAGGAGCCTACATCAACAGAG |
|           | Reverse | TGCTCTAGTCTTCATCTGGAAGG |
| IL-6      | Forward | CTCGAGACAGGTCTGCTTACCA |
|           | Reverse | CCATGTCACATTGTAGCTCT |
| MCP-1     | Forward | CCAATGGAAGCTTCCTTGAGA |
|           | Reverse | TCTGGACCCATCCCTCTCTG |
| TNF-α     | Forward | ATGTCTACGCTTCCTTCT |
|           | Reverse | GCTGTCACTGGAATTTTGAGA |
| ICAM-1    | Forward | CGCAGAGGCCCTTAACAGTCTACAC |
|           | Reverse | GACGGCGCTGACAAAGACCAC |
| VCAM-1    | Forward | TGAATTGGAAGAGACAAAGACAGAGT |
|           | Reverse | AGCAGACACGCAGAAACCAAC |
| NGAL      | Forward | CGACTACTGAGTCAGAACATTGG |
|           | Reverse | CTTGCAACATTGTAGCTGTAC |
| KIM-1     | Forward | CCTGCTGCTACTGCTTCTTG |
|           | Reverse | CCACGTTAGAGATGACTCC |

DOI: 10.1159/000526916

Kidney Blood Press Res 2022:47:616–630
patients. Compared with those in HCs, the white blood count, neutrophil count, and the levels of SCr, BUN, alanine aminotransferase, and aspartate aminotransferase were obviously increased in sepsis patients, whereas the levels of lymphocyte count, hematocrit, hemoglobin, and PLT were significantly decreased in the sepsis group (Table 1). Table 2 shows the characteristics of patients with sepsis with AKI \((n=54)\) and those without AKI \((n=56)\), which presented remarkable differences in PLT, SCr, BUN, Sequential Organ Failure Assessment score, and Acute Physiologic Assessment and Chronic Health Evaluation II score, but there were no differences in age, gender, white blood count, neutrophil count, lymphocyte count, and hemoglobin (Table 2).

**Endothelial Cell Damage Plays an Important Role in the Pathogenesis of Sepsis and Sepsis-Induced AKI**

The plasma levels of ICAM-1 and VCAM-1 were significantly higher in sepsis patients than those in HCs (ICAM-1: 357.46 ± 273.92 ng/mL vs. 223.39 ± 174.99 ng/mL \(p<0.01\); VCAM-1: 3,675.83 ± 1,378.28 ng/mL vs. 897.67 ± 491.05 ng/mL \(p<0.0001\)) (Fig. 1). Importantly, we also found significant increases in ICAM-1 and VCAM-1 in patients with sepsis with AKI compared with those in patients without AKI (ICAM-1: 400.56 ± 308.12 ng/mL vs. 305.89 ± 235.38 ng/mL \(p<0.05\); VCAM-1: 4,002.58 ± 1,218.23 ng/mL vs. 3,360.75 ± 1,459.03 ng/mL \(p<0.05\)) (Fig. 1).

**Renal Endothelial Damage Plays an Important Role in Kidney Injury in a Murine Sepsis Model**

After establishing a murine sepsis model, we found increases in BUN (NC vs. sepsis: 12.78 ± 1.11 mmol/L vs. 39.71 ± 7.38 mmol/L; \(p<0.01\)) and SCr (NC vs. sepsis: 11.40 ± 4.54 μmol/L vs. 21.84 ± 5.45 μmol/L; \(p<0.05\)) in mice with sepsis (Fig. 2a, b). The mRNA levels of the kidney injury markers KIM-1 and NGAL were also increased in sepsis mouse model (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000526916 for all online suppl. material). The histopathological manifestations and mRNA expression of inflammatory cytokines in the kidney, including interleukin (IL)-1β, IL-6, TNF (tumor necrosis factor)-α, and MCP (monocyte chemoattractant protein)-1, were more severe in mice with sepsis than those in the NC group (Fig. 2c–g).

To assess endothelial injury in AKI induced by sepsis, we further examined the expression of ICAM-1 and VCAM-1 in the kidneys. The results showed that mRNA and protein levels of ICAM-1 \((p<0.01)\) and VCAM-1 \((p<0.05)\) were significantly higher in mice with sepsis than those in the NC group (Fig. 2h), indicating aggravated kidney endothelial injury in sepsis.
Inhibition of PI3Kγ Protects Sepsis-Induced Acute Kidney Injury

Fig. 2. Renal endothelial damage plays an important role in kidney injury in a murine sepsis model. a, b BUN and SCr levels in normal control (NC, n = 10) and mice with sepsis (n = 12). c–f The mRNA expression of cytokines in mouse kidney tissues in the NC (n = 10) and sepsis (n = 12) groups. g Representative images of kidney from NC (n = 10) and sepsis (n = 12) mice stained with hematoxylin and eosin with corresponding pathology scores. h The expression of ICAM-1 and VCAM-1 in the kidneys by qRT-PCR and Western blotting. *p < 0.05; **p < 0.01; ***p < 0.001.
Fig. 3. PI3Kγ/Akt signaling pathway plays an important role in septic kidney injury. a Representative Western blotting of PI3Kγ, pAkt, and total Akt of the kidneys in the NC (n = 10) and sepsis (n = 12) groups. b Representative IHC analysis of PI3Kγ and pAkt expression in murine kidneys in the NC (n = 10), sepsis (n = 12), and CZC (n = 12) groups. c The protein levels of PI3Kγ, pAkt, and total Akt by Western blotting in the sepsis (n = 12) and CZC (n = 12) groups. *p < 0.05; **p < 0.01; ***p < 0.001.
Inhibition of PI3Kγ Protects Sepsis-Induced Acute Kidney Injury

Inhibition of PI3Kγ Protects Sepsis-Induced Acute Kidney Injury

Kidney Blood Press Res 2022;47:616–630
DOI: 10.1159/000526916

(For legend see next page.)
PI3Kγ/Akt Signaling Is Activated in a Murine Sepsis Model

To explore the underlying mechanisms, we found that the protein levels of PI3Kγ were increased in mice with sepsis ($p < 0.001$). As Akt is a critical downstream target of PI3K and pAkt is an active conformation of Akt, we assessed and discovered that pAkt was also markedly elevated in kidney tissues in mice with sepsis ($p < 0.01$) (Fig. 3a, b), and after blocking PI3Kγ, the activation of pAkt in the kidneys of mice with sepsis was significantly inhibited ($p < 0.05$) (Fig. 3b, c), indicating that the PI3Kγ/Akt signaling pathway was activated and might contribute to the pathogenesis of sepsis-induced AKI. Furthermore, we observed that the activation of PI3Kγ and pAkt was mainly concentrated in the glomerulus, including abundant capillaries, as well as some other vascular-rich regions such as the peritubular capillaries (Fig. 3b).

PI3Kγ Inhibition Alleviates Sepsis-Induced AKI

To confirm the role of PI3Kγ in sepsis and sepsis-induced AKI, the Kaplan-Meier curve showed a significant improvement in survival in mice pretreated with a PI3Kγ inhibitor (CZC group) ($p < 0.05$) (Fig. 4a). Compared with those in the sepsis group, the levels of BUN (sepsis vs. CZC: 39.71 ± 7.38 mmol/L vs. 10.40 ± 8.41 mmol/L; $p < 0.01$) and SCr (sepsis vs. CZC: 21.84 ± 5.45 μmol/L vs. 14.50 ± 4.43 μmol/L; $p < 0.05$) were significantly decreased in the CZC group compared with those in mice with sepsis without CZC (Fig. 5b left); these results were statistically significant (Fig. 5b right). Next, IHC was performed to confirm these results; from the data, we observed that VCAM-1 and ICAM-1 were increased in the kidneys of mice with sepsis compared with those in NC. More importantly, PI3Kγ inhibition with CZC decreased the expression of VCAM-1 and ICAM-1 in the kidneys of mice with sepsis (Fig. 5c).

The PI3Kγ/Akt Signal Pathway Is Involved in Endothelial Cell Injury Induced by LPS in vitro

Our data revealed that the expression of pAkt in LPS-treated HUVECs increased in a time- and concentration-dependent manner (Fig. 6a). In addition, the protein levels of ICAM-1 and VCAM-1 were higher in HUVECs treated with LPS (10 μg/mL). More importantly, HUVECs in the CZC group treated with LPS and PI3Kγ inhibitor presented a significant reduction in the expression of ICAM-1 and VCAM-1 (Fig. 6b). To further verify the role of the PI3Kγ/Akt signaling pathway in sepsis-induced endothelial cell injury, we demonstrated that the protein levels of PI3Kγ and pAkt were markedly increased in HUVECs in the LPS group compared with those in controls and obviously decreased in HUVECs in the CZC group compared with those in HUVECs in the LPS group (Fig. 6c).

PI3Kγ Inhibition Promotes Endothelial Cell Proliferation, Migration and Tube Formation

In the proliferation analysis, LPS treatment markedly inhibited HUVEC proliferation ($p < 0.001$), whereas PI3Kγ inhibition effectively promoted LPS-treated HUVEC proliferation ($p < 0.05$) (Fig. 7a). Furthermore, PI3Kγ inhibition promoted the migration of LPS-treated HUVECs ($p < 0.01$), as shown by the area of migrated cells in the wound assay (Fig. 7b). In addition, LPS treatment weakened the vascular formation ability of HUVECs, and PI3Kγ inhibition increased the number of tube structures compared with that in HUVECs treated with LPS only (Fig. 7c).
Inhibition of PI3Kγ Protects Sepsis-Induced Acute Kidney Injury

(a) Relative ICAM-1 expression of mRNA

(b) Vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and β-actin bands. The intensity ratios of VCAM-1 and ICAM-1 were calculated.

(c) Immunohistochemistry analysis of ICAM-1 and VCAM-1 in kidney tissues.
Discussion

The unique anatomical structure of the kidney conveys the importance of endothelial cell injury in the mechanisms of kidney injury, as it consists of two specialized capillary beds (glomerular and peritubular beds), and specialized endothelial cells line each capillary bed and are the first line of defense against microbial infection [7]. It has been reported that endothelial cell injury and dysfunction lead to the destruction of the microvascular barrier, increasing microvascular permeability, resulting in leakage in both the glomerular and peritubular capillary beds [27, 28], causing interstitial edema and increasing oxygen delivery distance to renal tubular epithelial cells, resulting in the rapid development of AKI [6]. Sepsis is the most common cause of AKI in critically ill patients. Endothelial activation accounts for most of the pathology observed in sepsis, and endothelial dysfunction is an early indicator of sepsis [29, 30]. Under pathological conditions, endothelial adherens junctions are destroyed and endothelial cell adhesion molecules leak into the circulation, especially after the vascular endothelial injury that occurs during sepsis, making them measurable. Studies have demonstrated that sepsis patients have high levels of serum ICAM-1 and VCAM-1, which were associated with the development of multiple organ failure and in-hospital mortality [31]. In this study, we observed higher expression of endothelial cell adhesion molecules in patients with sepsis and sepsis-induced AKI, and we also found significant kidney damage in mice with sepsis with obvious endothelial cell injury, suggesting that endothelial cell dysfunction and injury are involved in the pathophysiological processes of sepsis and contribute to sepsis-induced AKI.

In this study, we found PI3Kγ signaling was activated in kidney tissues of a sepsis mouse model. We observed a significant increase in PI3Kγ levels in the kidneys of sepsis mice, with aggravated AKI and renal endothelial injury, whereas PI3Kγ inhibition alleviated AKI and renal endothelial injury. Moreover, we observed significantly increased PI3Kγ in LPS-treated HUVECs, and inhibition of PI3Kγ kinase activity protected endothelial cell function and repaired endothelial cell injury by promoting cell proliferation and migration and tube formation. In summary, PI3Kγ signaling is activated in a sepsis mouse model and contributes to AKI by regulating endothelial cell function and mediating endothelial cell injury.

Martin et al. [14] have reported that PI3Kγ is involved in sepsis and organ damage (liver and lung) by altering neutrophil recruitment; however, they did not mention the role of PI3Kγ in the pathogenesis of kidney injury induced by sepsis. Our data revealed that the inhibition of PI3Kγ attenuated kidney injury in sepsis, and distinct from their proposed mechanism in the liver and lung, we found that PI3Kγ plays an important role in endothelial injury in sepsis-induced AKI. The inflammation and endothelial injury mechanisms are both critical during sepsis, and the abnormal interactions between endothelial and inflammatory cells and the resulting microvascular injury may underlie the pathological processes of sepsis and related organ dysfunction, which can be further studied.

As a downstream target of PI3Kγ signaling, Akt signaling was also reported to participate in the pathogenesis of sepsis. Zheng et al. [32] have demonstrated that Akt signaling was positively correlated with LPS-induced inflammatory production of alveolar macrophages, thereby regulating sepsis-induced acute lung injury, as well as cardiac inflammation [33, 34] and cardiomyocyte apoptosis [35] or modulating PLT activity [36] in sepsis. During AKI, Akt signaling was proved to be involved in regulating energy metabolism of tubular epithelial cells [37] and renal tubular cell apoptosis [38], inflammation, and mitochondrial damage [39], among others. In addition, other scholars have confirmed that the PI3Kγ expression plays a crucial role in angiogenesis during myocardial infarction [40] and the microvascular protection by inhibition of PI3Kγ after embolic stroke [16], proving the expression and important role of PI3Kγ in vascular in other diseases. Our results showed that the expression levels of PI3Kγ and pAkt were markedly increased in the kidneys of mice with sepsis and in LPS-treated HUVECs, and after the inhibition of PI3Kγ, pAkt was obviously suppressed in mouse kidneys and in HUVECs in the CZC group, indicating that PI3Kγ/Akt signaling plays an important role in sepsis and sepsis-induced AKI. Moreover, this study demonstrated that the inhibition of PI3Kγ preserved endothelial cell function and alleviated its injury in AKI induced by sepsis, including promoting endothelial cell proliferation and migration and tube formation through Akt signaling.
Inhibition of PI3Kγ Protects Sepsis-Induced Acute Kidney Injury

(For legend see next page.)
Fig. 7. Inhibition of PI3Kγ promotes endothelial cell proliferation and migration and tube formation. 

a Proliferation in HUVECs measured by EdU. 

The scratch wound assay was used to assess endothelial cell migration and analyzed by the ratio of nonmigrated area divided by the baseline wound area. c The tube formation assay was used to analyze the vascular formation ability of HUVECs. Values are expressed as means ± standard deviations, and p values were determined using the unpaired Student’s t test. *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 6. PI3Kγ/Akt signaling is involved in endothelial cell injury induced by LPS in vitro. 

a Umbilical vein endothelial cells (HUVECs) were stimulated with LPS for indicated times and concentrations for assessment of the protein levels of pAkt and total Akt by Western blotting. 

b Western blotting of the protein levels of ICAM-1 and VCAM-1 in HUVECs from the three groups. c The protein levels of PI3Kγ, pAkt, and total Akt in HUVECs by Western blotting. *p < 0.05; **p < 0.01; ***p < 0.001.
In conclusion, we first focused on the pathogenesis of endothelial injury in sepsis-induced AKI and demonstrated its aggravation of endothelial injury. The inhibition of PI3Kγ alleviated endothelial cell injury in the kidneys, preserved endothelial cell function, and repaired endothelial cell injury via the Akt signaling pathway, indicating that PI3Kγ/Akt signaling is pivotal for regulating endothelial cell function in AKI in response to sepsis, which may represent a potential novel therapeutic treatment for sepsis-induced AKI. Determining more detailed mechanisms will require further research.

Statement of Ethics

The study was conducted according to the guidelines of the Declaration of Helsinki. Our research was approved by the Ethics Committee of Nantong Third People’s Hospital, Nantong University (Approval ID: EK2021016), and all subjects provided signed informed consent. All animal experiments were performed in accordance with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals. All procedures were approved by the Animal Care and Use Committee of Nantong University (Approval ID: 220191546).

Conflict of Interest Statement

The authors declare no conflicts of interest.

Funding Sources

The research was supported by Nantong Key Discipline (wx2017002); Nantong Health Bureau Scientific Research Fund Project (MB2020036, MSZZ21030); and Scientific Research Project of Nantong Municipal Health Commission (QA2019033).

Author Contributions

Han Li and Jun-Xian Xu contributed equally to this work. Methodology: Han Li, Jun-Xian Xu, and Xu-Dong Han; software and writing – original draft: Han Li; validation: Jun-Xian Xu, Tao-Chun Cheng, and Li-Jun Tian; formal analysis: Han Li and Jin-Feng Lin; investigation, Jun-Xian Xu; resources: Xu-Dong Han; writing – review and editing and supervision: Zhao-Lian Bian and Xu-Dong Han; visualization: Zhao-Lian Bian and Xi Luo; funding acquisition: Jun-Xian Xu, Jin-Feng Lin, and Xu-Dong Han. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

References

1 Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The third international consensus definitions for sepsis and septic shock (Sepsis-3). JAMA. 2016;315(8):801–10.
2 Rhodes A, Evans LE, Alhazzani W, Levy MM, Antonelli M, Ferrer R, et al. Surviving sepsis campaign: international guidelines for management of sepsis and septic shock: 2016. Intensive Care Med. 2017;43(3):304–77.
3 Bellomo R, Kellum JA, Ronco C, Wald R, Martensson J, Maiden M, et al. Acute kidney injury in sepsis. Intensive Care Med. 2017;43(6):816–28.
4 Poston JT, Koyner JL. Sepsis associated acute kidney injury. BMJ. 2019;364:k4891.
5 Zhi DY, Lin J, Zhuang HZ, Dong L, Ji XI, Guo DC, et al. Acute kidney injury in critically ill patients with sepsis: clinical characteristics and outcomes. J Invest Surg. 2019;32(8):689–96.
6 Peerapornratana S, Manrique-Caballero CL, Gómez H, Kellum JA. Acute kidney injury from sepsis: current concepts, epidemiology, pathophysiology, prevention and treatment. Kidney Int. 2019;96(5):1083–99.
7 Ince C, Mayeux PR, Nguyen T, Gomez H, Kellum JA, Osypina-Tascón GA, et al. The endothelium in sepsis. Shock. 2016;45(3):259–70.
8 McGarrity S, Anuforo Ó, Halldórsson H, Antonelli M, Ferrer R, et al. Surviving sepsis campaign: international guidelines for management of sepsis and septic shock: 2016. Intensive Care Med. 2017;43(3):304–77.
9 Kellum JA, Gómez H, Gómez A, Murray P, Ronco C; ADQI XIV Workgroup. Acute dialysis quality initiative (ADQI) XIV sepsis phenotypes and targets for blood purification in sepsis; the BogotÁ Consensus. Shock. 2016;45(3):242–8.
10 Verma SK, Mollitoris BA. Renal endothelial injury and microvascular dysfunction in acute kidney injury. Semin Nephrol. 2015;35(1):96–107.
11 Xu C, Chang A, Hack BK, Eadon MT, Alper SL, Cunningham PN. TNF-mediated damage to glomerular endothelium is an important determinant of acute kidney injury in sepsis. Kidney Int. 2014;85(1):72–81.
12 Yu WK, McNeil JB, Wickersham NE, Shaver CM, Bastarache JA, Ware LB. Vascular endothelial cadherin shedding is more severe in sepsis patients with severe acute kidney injury. Crit Care. 2019;23(1):18.
13 An C, Wen J, Hu Z, Mitch WE, Wang Y. Phosphoinositide 3-kinase deficiency attenuates kidney injury and fibrosis in angiotensin II-induced hypertension. Nephrol Dial Transplant. 2020 Sep;1;35(9):1491–500.
14 Martin EL, Souza DG, Fagundes CT, Amaral FA, Assenzio B, Pantorieri V, et al. Phosphoinositide-3 kinase gamma activity contributes to sepsis and organ damage by altering neutrophil recruitment. Am J Respir Crit Care Med. 2010;182(6):762–73.
15 Guo H, German P, Bai S, Barnes S, Guo W, Qi X, et al. The PI3K/AKT pathway and renal cell carcinoma. J Genet Genom. 2015;42(7):343–53.
16 Jin R, Xiao AY, Li J, Wang M, Li G. PI3Kγ (phosphoinositide 3-Kinase-γ) inhibition attenuates tissue-type plasminogen activator-induced brain hemorrhage and improves microvascular patency after embolic stroke. Hypertension. 2019;73(1):206–16.
17 Lupieri A, Smirnova NF, Solinhasc R, Malet N, Benamar M, Saoudi A, et al. Smooth muscle cells-derived CXCL10 prevents endothelial healing through PI3Kγ-dependent T cells response. Cardiovasc Res. 2020;116(2):438–49.

18 Lupieri A, Blaise R, Ghigo A, Smirnova N, Sarthou MK, Malet N, et al. A non-catalytic function of PI3Kγ drives smooth muscle cell proliferation after arterial damage. J Cell Sci. 2020;133(13):jcsc245969.

19 MG Toscano, D Ganea, AM Gamero. Cecal ligation puncture procedure. J Vis Exp. 2011; (51):e2860.

20 Ferré S, Deng Y, Huen SC, Lu CY, Scherer PE, Sherman AB, Gilger BC, Berglund AK, O’Hara RE, Arsenault MG, Esparza Gonzalez MG, Toscano, D Ganea, AM Gamero. Cecal ligation puncture procedure: an immunohistochemical quantitative analysis. J Oral Maxillofac Pathol. 2017;21(2):211–7.

21 Jia P, Teng J, Zou J, Fang Y, Wu X, Liang M, Ferrè S, Deng Y, Huen SC, Lu CY, Scherer PE, Sherman AB, Gilger BC, Berglund AK, O’Hara RE, Arsenault MG, Esparza Gonzalez MG, Toscano, D Ganea, AM Gamero. Cecal ligation puncture procedure: an immunohistochemical quantitative analysis. J Oral Maxillofac Pathol. 2017;21(2):211–7.

22 Mane DR, Kale AD, Belaldavar C. Validation of immunoexpression of tenascin-C in oral precancerous and cancerous tissues using Image analysis with novel immunohistochemistry profiler plugin: an immunohistochemical quantitative analysis. J Oral Maxillofac Pathol. 2017;21(2):211–7.

23 Mane DR, Kale AD, Belaldavar C. Validation of immunoexpression of tenascin-C in oral precancerous and cancerous tissues using Image analysis with novel immunohistochemistry profiler plugin: an immunohistochemical quantitative analysis. J Oral Maxillofac Pathol. 2017;21(2):211–7.

24 Sherman AB, Gilger BC, Berglund AK, Schnabel LV. Effect of bone marrow-derived mesenchymal stem cells and stem cell supernatant on equine corneal wound healing in vitro. Stem Cell Res Ther. 2017;8(1):120.

25 Carpenter G, Berndt S, Ferratge S, Rasband W, CUendet M, Uzan G, et al. Angiogenesis analyzer for ImageJ: a comparative morphometric analysis of “endothelial tube formation assay” and “laminin bead assay”. Sci Rep. 2020;10(1):11568.

26 Carvajal-Vergara X, Sevilla A, D’Souza SL, Ang YS, Schaniel C, Lee DF, et al. Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. Nature. 2010;465(7299):808–12.

27 Adembri C, Smabati E, Vitali L, Selmi V, Margheri M, Tani A, et al. Sepsis induces albuminuria and alterations in the glomerular filtration barrier: a morphofunctional study in the rat. Crit Care. 2011;15(6):R277.

28 Wang Z, Holthoff JH, Seely KA, Pathak E, Spencer HJ, Golden N, et al. Development of oxidative stress in the peritubular capillary microenvironment mediates sepsis-induced renal microcirculatory failure and acute kidney injury. Am J Pathol. 2012;180(2):505–16.

29 Lelubre C, Vincent JL. Mechanisms and treatment of organ failure in sepsis. Nat Rev Nephrol. 2018;14(7):417–27.

30 Martin-Fernandez M, Vaquero-Roncero LM, Almansa R, Gomez-Sanchez E, Martin S, Tamayo E, et al. Endothelial dysfunction is an early indicator of sepsis and neutrophil degranulation of septic shock in surgical patients. BJ Open. 2020;4(3):524–34.

31 Amalakuan B, Habib SA, Mangat M, Reyes LF, Rodriguez AH, Hinojosa CA, et al. Endothelial adhesion molecules and multiple organ failure in patients with severe sepsis. Cytokine. 2016;88:267–73.

32 Zheng H, Liang W, Hu RH, Zhu L, Hu CC, Cheng F, et al. Annexin A1 mimetic peptide AC2-26 inhibits sepsis-induced cardiomyocyte apoptosis through LXA4/PI3K/akt signaling pathway. Curr Med Sci. 2018;38(6):997–1004.

33 Zhang L, Zheng YL, Hu RH, Zhu L, Hu CC, Cheng F, et al. Annexin A1 mimetic peptide AC2-26 inhibits sepsis-induced cardiomyocyte apoptosis through LXA4/PI3K/akt signaling pathway. Curr Med Sci. 2018;38(6):997–1004.