Macrophage-derived exosomal miRNA-155 promotes tubular injury in ischemia-induced acute kidney injury

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Abstract. Tubule injury is a characteristic pathological feature of acute kidney injury (AKI) and determines the prognosis of kidney disease. However, the exact mechanism of tubule injury remains largely unclear. In the present study, the exact mechanism of tubule injury was investigated. Bilateral renal ischemia/reperfusion (I/R) injury (I/RI) was induced in mice and exosome secretion inhibitor GW4869 and miRNA-155 inhibitor were used. In addition, the exosomal microRNA (miR)-155-mediated cross-talk between macrophage and tubular cells was also investigated. It was determined that tubular injury was observed in an I/R-induced AKI model, which was closely associated with macrophage infiltration. Interestingly, blocking exosome production using GW4869 ameliorated tubular injury in I/R-induced AKI. Mechanistically, once released, activated macrophage-derived exosomal miR-155 was internalized by tubular cells, resulting in increased tubule injury through targeting of suppressor of cytokine signaling-1 (SOCS-1), a negative regulator of NF-κB signaling. In addition, a dual-luciferase reporter assay confirmed that SOCS-1 was the direct target of miR-155 in tubular cells. Notably, injection of these miR-155-enriched exosomes into renal parenchyma resulted in increased tubule injury *in vivo*. Thus, the present study demonstrated that exosomal miR-155 mediated the communication between activated macrophages and injured tubules, leading to progression of AKI, which not only provide novel insights into the pathophysiology of AKI but also offer a new therapeutic strategy for kidney diseases.

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Introduction

Acute kidney injury (AKI) is a serious syndrome characterized by a rapid decline in kidney function, with high mortality and no effective therapy currently. Renal ischemia-reperfusion (I/R) injury is the most common cause of AKI, where the proximal tubule is the mainstay of injury (1). Increasing evidence indicates that severity of proximal tubule injury determines renal prognosis (2). More importantly, promoting tubular repair protects from the ischemic AKI (3). However, the exact mechanism of tubule injury remains poorly understood.

Histologically, tubule injury and infiltration of immune cells are the characteristic pathological changes of I/R injury. Among immune cell populations, the macrophage is the major contributor to renal injury. For example, pharmacologic strategies with deletion of macrophages effectively protects the kidney from injury induced by ischemic, obstructive or toxic insults (4). Interestingly, in addition to the change in quantity, phenotype switching of macrophages also plays a crucial role in the prognosis of kidney diseases (5). However, the molecular signals through which macrophages induce tubule cell injury during AKI remains to be elucidated.

Exosomes are nanometer-scale, membrane-enclosed extracellular vesicles, which are released by almost all cell types under physiological and pathological conditions. Over the past decades, increasing studies have determined that exosomes can mediate intracellular communication by transferring cell-specific cargos, including proteins, lipids and genetic information (such as DNA, mRNA, and microRNA (miR), to target cells, even at a distance from the parent cells (6,7). Recently, convincing studies demonstrated that under some specific conditions, increased production of exosomes is induced and content of exosomes is also modulated to regulate the key biological functions of recipient cells (8,9). Interestingly, exosomes are also found to mediate the cross-talk between tubular epithelial cells (TECs) and fibroblasts in kidney fibrosis (10). In addition, exosomes derived from injured TECs can transfer specific mRNAs into macrophages to alter the

biological functions of macrophages in both AKI and chronic kidney disease (11). However, whether exosomes derived from macrophages influence the function of TECs has not been reported.

miRNAs are a class of epigenetic regulators with the capability of modulating gene expression at the post-transcriptional level, which plays important roles in kidney diseases. A previous study revealed that miRNAs are commonly enriched in exosomes (12). Interestingly, the miRNA class was observed to be the largest and most consistent proportional change in exosomes using total RNA-sequencing in a model of I/R (12), suggesting that miRNAs are emerging as crucial regulators of cellular function through exosome-mediated cellular communication. Notably, Li *et al* found that miR-23a which transfers from tubules to immune cells through exosomes could promote kidney injury (13). In addition, tubular cells could also take up exosomal miRNAs to regulate acute tubular injury as previously reported (14). However, the role of macrophage-derived exosomal miRNAs in tubular injury remains largely unclear.

Recently, increasing evidence demonstrated that miR-155 expression in the M1 polarized macrophage was significantly upregulated (GSE33453). Notably, the level of miR-155 was significantly enhanced in the exosomes derived from M1 macrophages as well. Furthermore, transfer of miR-155 via exosomes could modulate various pathophysiological functions (15,16). In the present study, it is suggested that exosomal miR-155 released by macrophages could promote tubular injury by conveying the injury signals. Elucidating the exact mechanism underlying tubular injury in AKI not only provides novel insights into the pathophysiology of tubular injury but also offers a new therapeutic strategy for kidney diseases.

Materials and methods

Reagents. Lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (product no. L3012) were purchased from Sigma-Aldrich; Merck KGaA. Recombinant mouse interferon-γ (IFN-γ; cat. no. 485-MI-100; R&D Systems, Inc.) was used for macrophage intervention. The levels of plasma blood urea nitrogen (BUN; cat. no. C013-2-1) and serum creatinine (SCr; cat. no. C011-2-1) were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Animals. The experimental animal procedures were approved (approval no. N2017-078) by the Ethics Committee of Soochow University (Suzhou, China). All the mice were housed under pathogen-free conditions in a standard laboratory with a controlled room temperature (22±1˚C) and humidity (65-70%), and a 12:12-h light-dark cycle, with free access to food and water. For the ischemic AKI model (n=6), bilateral I/R injury was induced in mice by clamping the renal pedicle for 30 min as previously described (17). Mice were sacrificed by cervical dislocation at 24 h after I/R injury, and the renal cortex was harvested. Mice in the GW4869 group received intraperitoneal injection of GW4869 (2.5 mg/kg; Sigma-Aldrich; Merck KGaA; n=6) 2 h before I/R surgery. The intraparenchymal injection (n=6) was performed as previously described (18). Briefly, the inferior pole of the left kidney was exposed. Exosomes derived from M1 or M0 macrophages (20 μg in 60 μl of PBS) were injected into 2 sites (the left and right sides of the inferior pole of the kidney) via a 50-G needle. The *in vivo* miRNA-155 inhibitor (Suzhou GenePharma Co., Ltd.) was transfected into the mouse kidneys through tail vein injections using the *in vivo*-jetPEI (Polyplus-transfection SA). Briefly, the miR-155 inhibitor (50 μg) or negative control (NC; 50 μg) dissolved in 5% glucose solution was injected into each mouse at 24 h before surgery as instructed by the manufacturer's protocols. Mice were sacrificed at 24 h after I/R, and their serum and renal cortex were harvested. To ameliorate the suffering of mice throughout the experimental period, the mice were euthanized with isoflurane inhalation followed by the dislocation of the cervical vertebra, where isoflurane was used at 4% for induction and 2% for maintenance of anesthesia. Animal sacrifice was confirmed by respiratory and cardiac arrest, and no righting reflex.

Kidney histology and tubular injury scoring. Periodic Acid-Schiff (PAS) staining was performed. Kidneys were fixed in 10% buffered formalin at room temperature for 24 h, embedded in paraffin, cut into 3-μm sections, stained with Periodic Acid for 20 min and Schiff for 20 min at room temperature, and visualized at a magnification of x400 under an optical microscope (Olympus Optical Co., Ltd.). To evaluate the tubular injury score, 10 random tissue section images per animal were assessed on PAS-stained sections in a blinded manner by nephropathologists, and semi-quantitatively scored as previously described (19).

Immunohistochemical staining. For immunohistochemical staining, 10% buffered formalin-fixed at room temperature for 24 h, and paraffin-embedded tissue sections (4 μm) were blocked with 10% goat serum (Wuhan Servicebio Technology Co., Ltd.) for 2 h at room temperature and were incubated with primary antibodies against F4/80 (1:200; product code ab6640; Abcam) and kidney injury molecule-1 (KIM-1) (1:200; cat. no. MA5-28211; Invitrogen; Thermo Fisher Scientific, Inc.) for 12 h at 4˚C and then analyzed using a streptavidin peroxidase detection system (50 μl; cat. no. KIT-9720; Fuzhou Maixin Biotech Co., Ltd.) at room temperature according to the manufacturer's protocol. DAB (Fuzhou Maixin Biotech Co., Ltd.) was used as an HRP-specific substrate. The images were visualized under an optical microscope (Olympus Corporation).

Cell culture. TECs were isolated for primary culture using an established method (20) and then were cultured in DMEM-Ham's-F12 medium (Hyclone; Cytiva) supplemented with 10% fetal bovine serum (FBS; ScienceCell Research Laboratories, Inc.), and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Ischemia/reperfusion injury (I/RI) conditions of TECs were modeled *in vitro* (1% oxygen, 94% N_2 and 5% CO_2 with glucose-free and FBS-free for 12 h and then regular culture medium with 21% oxygen for 2 h of reoxygenation). The mouse RAW264.7 macrophage cell line (American Type Culture Collection; ATCC no. TIB-71) was used for this study. RAW264.7 cells were cultured in RPMI-1640 (Hyclone; Cytiva) supplemented

Table I. Primers used for quantitative-PCR.

Genes	Forward (5'-3')	Reverse (5'-3')
Mouse IL-1β	TGCCACCTTTTGACAGTGATG	AAGGTCCACGGGAAAGACAC
Mouse TNF-α	TCTTCTCATTCCTGCTTGTGG	GGTCTGGGCCATAGAACTGA
Mouse MCP-1	CATCCACGTGTTGGCTCA	GATCATCTTGCTGGTGAATGAGT
Mouse SOCS-1	CACTCACTTCCGCACCTTCC	CAGCCGGTCAGATCTGGAAG
Mouse KIM-1	CGGTACAACTTAAAGGGGCA	GACGTGTGGGAATCTCTGGT
Mouse β-actin	GAGACCTTCAACACCCCAGC	ATGTCACGCACGATTTCCC
Mouse miR-155	GGGGGTTAATGCTAATTGTGAT	AGTGCGTGTCGTGG
Mouse U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; SOCS-1, suppressor of cytokine signaling-1; KIM-1, kidney injury molecule-1; miR-155, microRNA-155.

with 10% FBS and 1% penicillin-streptomycin. Both cell lines (TECs and RAW264.7 macrophages) were cultured in an atmosphere of 5% CO_2 and 95% air at 37˚C.

Isolation and characterization of exosomes. For kidney exosome extraction, 100 mg of the kidney cortex derived from the ischemic kidney was collected. Renal exosomes were isolated using ultracentrifugation as previously reported (21). For *in vitro* experiments, RAW264.7 macrophages were cultured with the presence or absence of LPS (100 ng/ml) plus IFN-γ (20 ng/ml) in serum-free RPMI-1640 medium. The medium was then used for exosome purification using differential ultracentrifugation (Type 70 Ti Rotor; Beckman Coulter Optima L-80 XP) as previously described (21). Briefly, the medium was centrifuged at 2,000 x g for 20 min at 4˚C to eliminate the cells and debris and at 13,500 x g for 20 min at 4˚C to eliminate the microvesicles, followed by ultracentrifugation at 200,000 x g for 120 min at 4˚C. The exosome pellet was washed in 20 ml of PBS and collected by subsequent ultracentrifugation at 200,000 x g for 120 min at 4˚C.

The size and morphology were detected by electron microscopy and the specific surface markers (Alix, CD63, CD9) of the isolated exosomes were also detected by western blotting for characterization of the exosomes.

Transmission electron microscope (TEM). The exosome sample was diluted 10 times with PBS and then stained with 2% phosphotungstic acid for 10 min at room temperature. The samples were detected using a TEM (Hitachi HT 7700; Hitachi, Ltd.) at 80 kV.

Transwell study. To study the process of macrophage-derived exosomes communicating with TECs, a Transwell Permeable Support system (Corning, Inc.) with a 0.4-μm pore-size filter was used according to the manufacturer's protocol. Macrophages ($2x10^6$) were seeded into the upper chamber containing RPMI-1640 medium supplemented with 10% FBS. Subsequently, recipient TECs were then seeded in 12-well plates (lower chamber) in DMEM-Ham's-F12 medium supplemented with 10% FBS. Both cell lines were cultured for 12 h at an atmosphere of 5% CO_2 and 95% air at 37˚C. Macrophages with LPS plus IFN-γ treatment were then assessed in the upper chamber followed by DiO-labeling for 6 h at 37˚C. All co-cultured experiments were then conducted in hypoxia or normoxia for 12 h. Uptake of DiO-labeled exosomes by TECs was visualized by the confocal microscope (FV1000; Olympus Corporation).

Bioinformatics analysis. miRNA targets were predicted using 5 online databases: TargetScan (Human 8.0/Mouse 8.0; https://www.targetscan.org/mmu_80/), miRDB (http://www.mirdb.org/), miRanda (http://www.microrna.org/microrna/home.do), DIANA-TarBase (v7.0; http://www.microrna.gr/tarbase), and PicTar (http://www.pictar.org/).

miRNA inhibitor interference studies. RAW264.7 cells were transfected with NC inhibitor (forward, 5'-CCC CCCCCCCCCCCCCCC-3' and reverse, 5'-CCCCCC CCCCCCCCCCCCC-3') or miR-155 inhibitor (forward, 5'-ACCCCUAUCACGAUUAGCAUUAA-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3') at a concentration of 100 nM using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following transfection for 6 h at 37˚C, the medium was removed and fresh medium was added. The subsequent experiments were performed after 18 h of transfection.

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted using TRIzol following the manufacturer's instructions (Takara Bio, Inc.). mRNA was reverse-transcribed using PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions and PCR was performed using SYBR Premix Ex Taq and 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The amplification level was programmed with a denaturation step at 95˚C for 30 sec, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec. miRNA was reverse-transcribed and detected with All-in-One miRNA First-Strand cDNA Synthesis kit and All-in-One miRNA qPCR kit (GeneCopoeia, Inc.) according to the manufacturer's instructions. The housekeeping genes β-actin and U6 were used as controls. The $2^{-\Delta\Delta Cq}$ method was used as previously

reported (22). All the primers for RT-qPCR are listed in Table I.

Western blot analysis. Samples of cells and cortical tissues were lysed in cold RIPA lysis buffer (Thermo Fisher Scientific, Inc.) supplemented with protease inhibitor cocktail and the protein concentration was determined using a BCA protein assay kit (Nanjing KeyGen Biotech Co., Ltd.). Proteins (20-40 μg) were subjected to 10% SDS-PAGE (Thermo Fisher Scientific, Inc.) and transferred to PVDF membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk for 2 h at room temperature. The primary antibodies used were anti-Alix (1:500; cat. no. sc-53540; Santa Cruz Biotechnology, Inc.), anti-CD63 (1:1,000; product code ab213090; Abcam), anti-suppressor of cytokine signaling-1 (SOCS-1; 1:1,000; product code ab62584; Abcam), and anti-β-actin (1:3,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) and the membranes were incubated with the primary antibodies for 12 h at 4˚C. Goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:3,000; product nos. 7076 and 7074, respectively; Cell Signaling Technology, Inc.) were used for detection for 2 h at room temperature. The signals were then detected using an enhanced chemiluminescent kit (GE Healthcare; Cytiva). Finally, ImageJ software (v1.8.0; National Institutes of Health) was used for densitometry.

Luciferase reporter assay. The plasmids were all obtained from Shanghai Genechem Co., Ltd. TECs were transfected with 3'UTR luciferase reporter constructs (3'UTR-NC and 3'UTR-SOCS-1), miRNA (miR-155 mimic, 5'-TTAAUG CTAATCGTGATAGGGGT-3'; and miR-NC, 5'-CCCCCC CCCCCCCCCCCCC-3') and *Renilla* luciferase using Lipofectamine 3000, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection for 6 h at 37˚C, the medium was removed and fresh medium was added. To assess the binding specificity, the sequences in the 3'UTR-SOCS-1 (3'UTR-SOCS-1-mut) that interact with the miR-155 seed sequence were mutated. After 48 h of transfection, the luciferase activity of cells was detected using a Dual Luciferase Assay kit (cat. no. E1910; Promega Corporation). *Renilla* luciferase activity was normalized to Firefly luciferase activity.

Statistical analyses. Data were obtained from at least three independent experiments and expressed as the means ± SEM. Statistical analysis was performed using unpaired Student's t-tests or one-way analysis of variance (ANOVA) followed by Bonferroni correction. Statistical analyses were performed using SPSS version 20.0 (IBM Corp.). A 2-sided P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Tubular injury and macrophage infiltration are observed in I/R-induced AKI. In the present study, markedly elevated levels of SCr and BUN were observed in the I/R-treated mice (Fig. 1A). Histologically, TEC injury (necrosis, detachment, cellular debris, and cast formation) and increased inflammatory cell infiltration were observed in the I/R-treated kidneys (Fig. 1B). In addition, KIM-1, a marker of tubular injury, was detected to reveal tubular injury (Fig. 1C and D). Concomitantly, there were significant increases in the mRNA expression of renal inflammatory cytokines [monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β)] (Fig. 1E). Notably, the number of $F4/80^+$ macrophages was also revealed to be significantly increased (Fig. 1F). Thus, these results suggested that macrophage infiltration was associated with tubular injury in I/R-induced AKI.

Blocking exosome production ameliorates tubular injury in I/R-induced AKI. In this experiment, GW4869 was used to block exosome production (23). Notably, it was determined that the levels of SCr and BUN were markedly decreased in GW4869-treated mice (Fig. 2A). Histologically, ameliorated TEC injury was observed in GW4869-treated kidneys (Fig. 2B). In addition, the results of PCR and immunohistochemical analysis of KIM-1 also confirmed similar results (Fig. 2C and D). Concomitantly, the renal mRNA expression of inflammatory factors (MCP-1, TNF-α, and IL-1β) was significantly decreased in the GW4869-treated group compared with the vehicle (Fig. 2E). Thus, the findings suggested that exosome secretion is an important mechanism of tubular injury in I/R-induced AKI.

Exosomes derived from activated macrophages promote tubular cell injury. To explore the potential mechanism for the effect of exosomes on tubular injury, exosomes from I/R-injured kidneys were first isolated and characterized via western blotting (using Alix and CD63, as exosome markers) and TEM (Fig. 3A and B). To mimic the microenvironment in which exosomes are released from the activated macrophages to promote tubular injury, a schematic diagram depicting the process of the experiment is provided in Fig. 3C. As anticipated, it was determined that exosomes released from macrophages with LPS and IFN-γ treatment (activated macrophages, namely M1 macrophages) transferred to TECs (Fig. 3D). To study the effect of exosomes derived from M1 macrophages on tubular cells, TECs with exosomes derived from M1 macrophages were cultured under hypoxia (exo-M1). It was determined that exo-M1 promoted tubular injury compared with the exosomes derived from non-activated macrophages (exo-M0) (Fig. 3E and F). Concomitantly, the mRNA expression of the inflammatory factors was significantly increased in the exo-M1-treated group (Fig. 3G). These findings indicated that exosomes secreted from activated macrophages promote tubular injury.

Exosomal miR-155 level is associated with tubular injury. For this experiment, the role of exosomal miR-155 in ischemia-induced kidney injury was explored. Interestingly, the kidneys with I/R injury secreted exosomes that were highly enriched in miR-155 (Fig. 4A). M1 macrophage exosomes were then isolated for miR-155 detection. As expected, exosomes derived from M1 macrophages were highly enriched in miR-155 (Fig. 4B). To examine the effects of exosomal miRNA-155, miR-155 was silenced in M1 macrophages, and exosomes derived from M1 macrophages were isolated. The miR-155-silenced exosomes (Exo-miR-155 inhibitor) were then

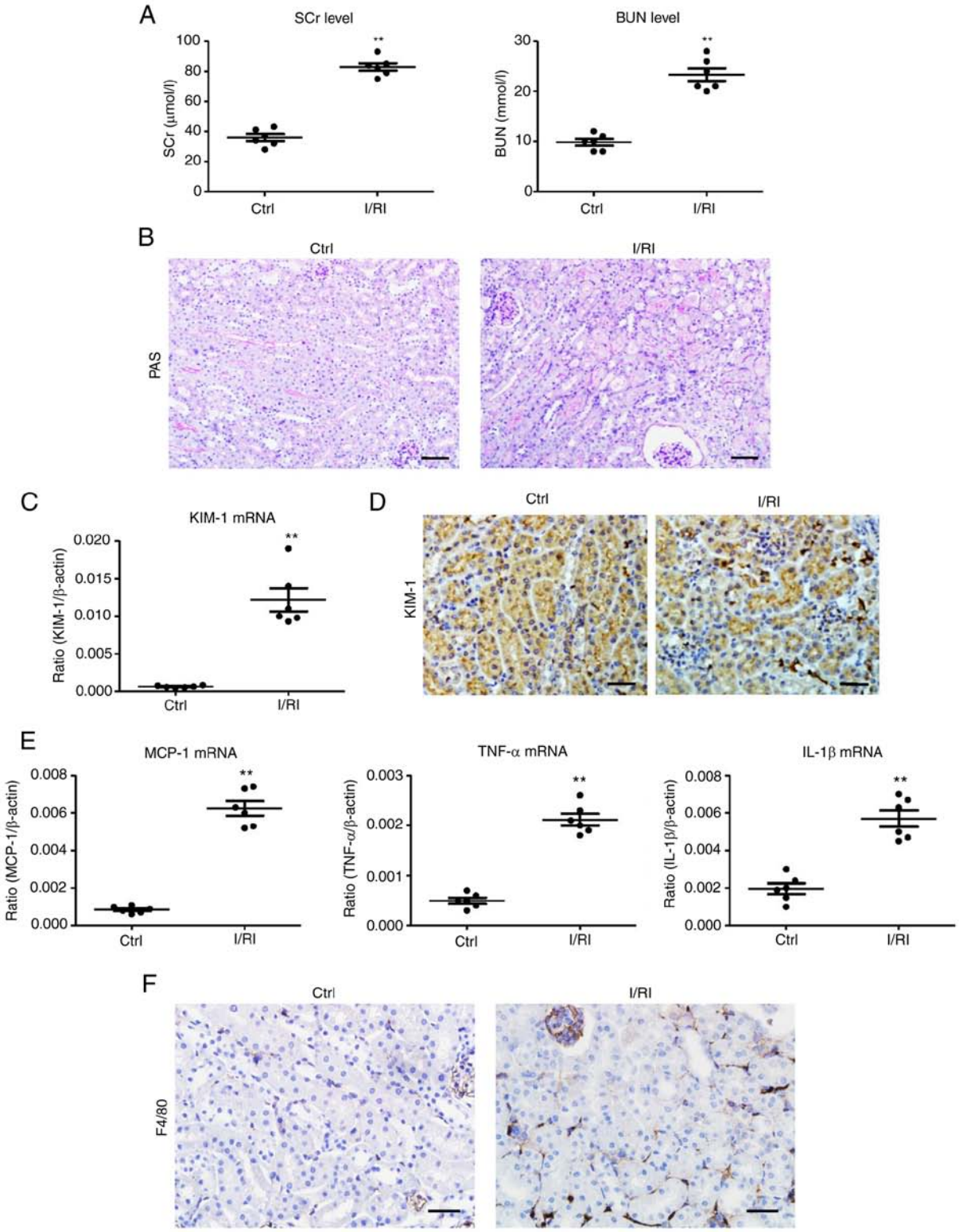

Figure 1. Tubule injury and macrophage infiltration are observed during I/RI of the kidney. (A) The levels of SCr and BUN in I/R-induced renal injury. (B) Histological changes (PAS staining; scale bar, 100 μm). (C) RT-qPCR analysis of the mRNA level of KIM-1 in kidney tissues. (D) Representative images of KIM-1 expression in kidney tissues from I/R-injured mice assessed by immunohistochemistry (scale bar, 50 μm). (E) Representative images of F4/80 expression in kidney tissues from I/R-injured mice assessed by immunohistochemistry (scale bar, 50 μm). (F) RT-qPCR analysis of inflammatory cytokines, MCP-1, TNF-α, and IL-1β mRNA levels in kidney tissues (n=6). Data are presented as the means ± SEM. **P<0.01 vs. the Ctrl group. I/RI, ischemia/reperfusion injury; SCr, serum creatinine; BUN, blood urea nitrogen; I/R, ischemia/reperfusion; PAS, periodic acid-Schiff; RT-qPCR, reverse transcription quantitative-PCR; KIM-1, kidney injury molecule-1; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; Ctrl, control.

used to treat TECs. Notably, compared with the Exo-miR-NC group, Exo-miR-155 inhibitor administration significantly ameliorated tubular injury (Fig. 4C and D). In addition, the mRNA expression of inflammatory factors (MCP-1, TNF-α, and IL-1β) was decreased in the Exo-miR-155 inhibitor-treated group (Fig. 4E). Therefore, the level of miR-155 in exosomes

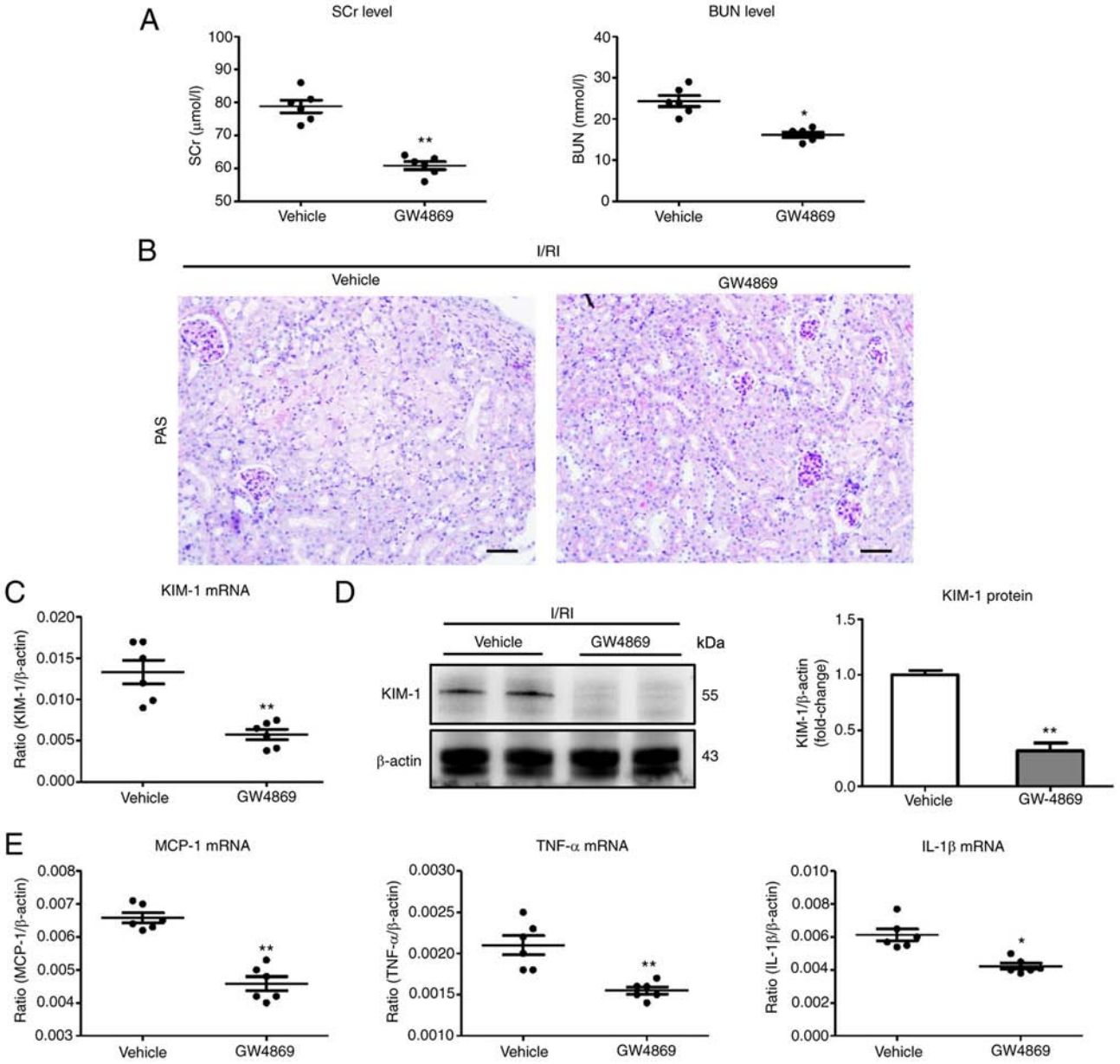

Figure 2. Blocking exosome production using GW-4869 suppresses tubule injury in I/R-induced kidney injury. (A) The SCr and BUN levels in I/R-treated mice with GW-4869 administration. (B) Histological changes (PAS staining; scale bar 100 μm). (C and D) RT-qPCR and western blot analysis of KIM-1 expression in I/R-induced kidney injury with GW-4869 administration. (E) RT-qPCR analysis of inflammatory cytokine mRNA levels in kidney tissues (n=6). Data are presented as the means ± SEM. *P<0.05 and **P<0.01 vs. the vehicle group. I/R, ischemia/reperfusion; SCr, serum creatinine; BUN, blood urea nitrogen; PAS, periodic acid-Schiff; RT-qPCR, reverse transcription quantitative-PCR; KIM-1, kidney injury molecule-1; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; I/RI, ischemia/reperfusion injury.

parallels tubular injury, suggesting that the secretion of miR-155-laden exosomes by M1 macrophages may be associated with the pathological mechanism of tubular injury.

miR-155 inhibition alleviates tubular injury in I/R-induced kidney injury. To determine the effect of miR-155 on I/R-induced kidney injury, miR-155 inhibitor or scrambled NC was administered before I/R injury to the kidney. Interestingly, compared with the scrambled NC-treated mice, the miR-155 inhibitor efficiently reversed the upregulation of SCr and BUN levels (Fig. 5A). Notably, miR-155 inhibitor could ameliorate TEC injury and significantly reduce protein casts (Fig. 5B). The results of PCR and immunohistochemical analysis of KIM-1 also confirmed similar results (Fig. 5C and D). In addition, the mRNA expression of inflammatory factors was similarly attenuated in the I/RI + miR-155 inhibitor group (Fig. 5E). Thus, these data indicated that miR-155 inhibition could attenuate I/R-induced tubular injury.

Exosomal miR-155 promotes tubular injury by targeting SOCS-1. To further investigate the exact molecular mechanism of exosomal miR-155 on tubular cells, possible miR-155 targets that contribute to tubular injury were predicted using 5 online databases: TargetScan (Human 8.0/Mouse 8.0), miRDB, miRanda, DIANA-TarBase (v7.0), and PicTar. Overlap analysis revealed that the possible target of miR-155 was SOCS-1 (Fig. 6A), a negative regulatory factor of NF-κB. It was determined that exosomes released from

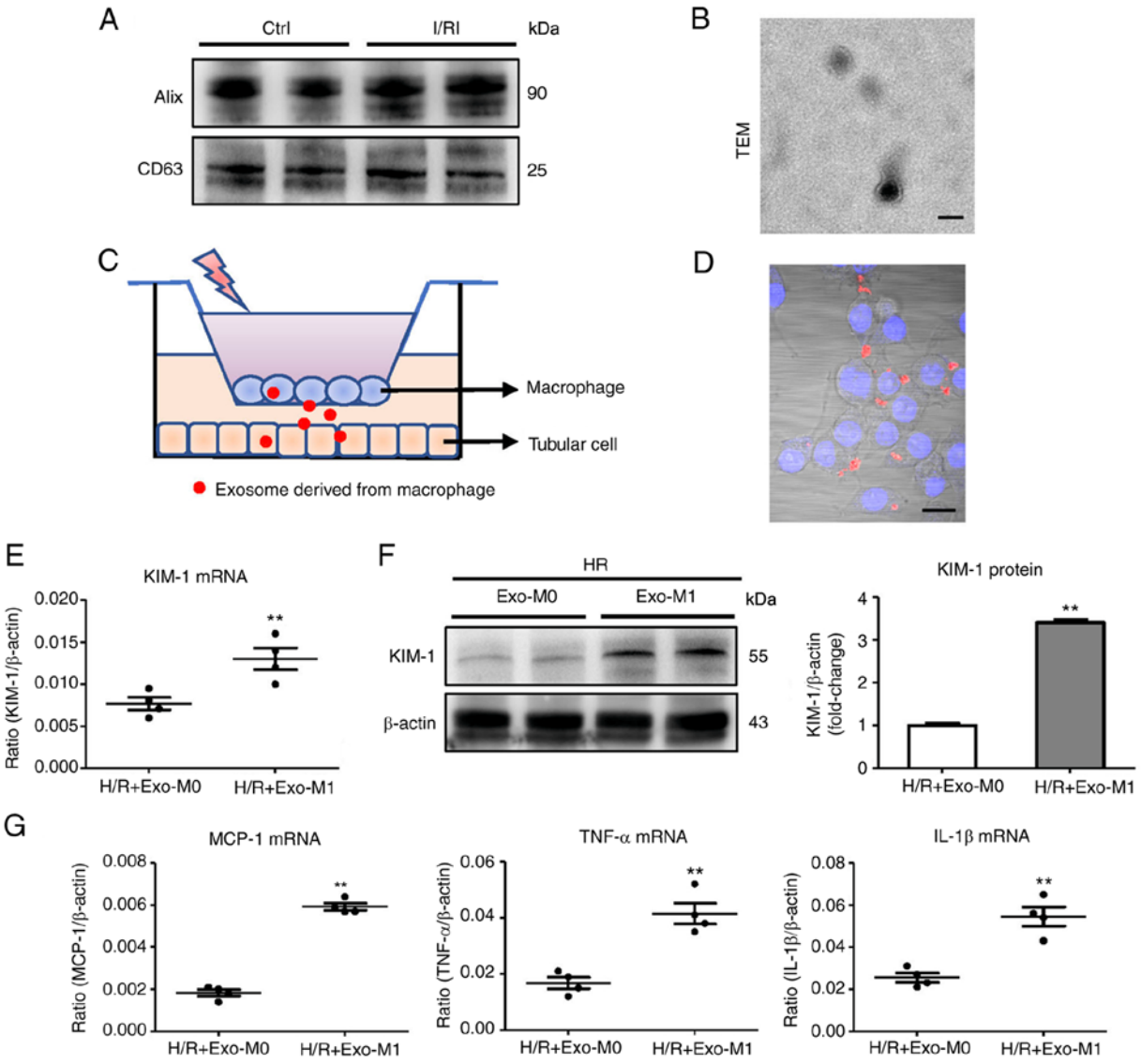

Figure 3. Activated macrophage-derived exosomes promote TEC injury. (A) Immunoblot analysis of Alix, and CD63 in exosome kidney samples. (B) Representative electron micrograph of exosomes purified from the kidneys. Scale bar, 100 nm. (C) Schematic diagram of the experimental design. (D) Representative images of exosomes released from Dio-labeled activated macrophages and internalized by TECs (scale bar, 10 μm). (E and F) RT-qPCR and western blot analysis of KIM-1 expression in H/R-treated TECs treated with exosomes derived from activated macrophages (exo-M1). (G) RT-qPCR analysis of inflammatory cytokine mRNA levels in H/R-treated TECs treated with exo-M1 (n=4). Data are presented as the means ± SEM. **P<0.01 vs. the H/R + Exo-M0 group. TEC, tubular epithelial cell; RT-qPCR, reverse transcription quantitative-PCR; H/R, hypoxia/reoxygenation; TEM, transmission electron microscope; Exo, exosome; I/RI, ischemia/reperfusion injury.

M1 macrophages decreased the levels of SOCS-1 protein in tubule cells (Fig. 6B and C). In addition, the *in vitro* study revealed that the miR-155-silenced exosomes (Exo-miR-155 inhibitor) were used to treat TECs. As expected, SOCS-1 expression was increased in the Exo-miR-155 inhibitor group (Fig. 6D and E), indicating that SOCS-1 was modulated by exosome-containing miR-155. To examine whether miR-155 could regulate the expression of SOCS-1 in I/R-induced kidney injury, as revealed in Fig. 6F, miR-155 inhibitor significantly increased SOCS-1 protein expression. Notably, luciferase reporter assay (Fig. 6G) showed that the activity of luciferase reporters was markedly reduced by miR-155 overexpression as compared with the control. Furthermore, the activity of SOCS-1-3'-UTR-mut luciferase reporter was not affected by the miR-155 overexpressed vector compared with the control, demonstrating that miR-155 could directly interact with the 3'-UTR of SOCS-1. These results indicated that increased miR-155 in macrophage-derived exosomes contributed to tubular injury via targeting SOCS-1.

Exosomes derived from M1 macrophages promote tubular injury in mice. To determine the role of exosomes derived from M1 macrophages *in vivo*, exosomes derived from activated macrophages (exo-M1) were transferred into the kidneys by direct renal parenchyma injection. As anticipated, it was determined that exo-M1 treatment promoted the damage of kidney function (Fig. 7A). Histologically, increased TEC injury was observed in the exo-M1-treated kidneys (Fig. 7B). In addition, the results of PCR and immunohistochemical analysis for KIM-1 also confirmed the effect of tubular injury promotion (Fig. 7C and D). Furthermore, the renal mRNA expression of inflammatory factors (MCP-1, TNF-α, and IL-1β) was

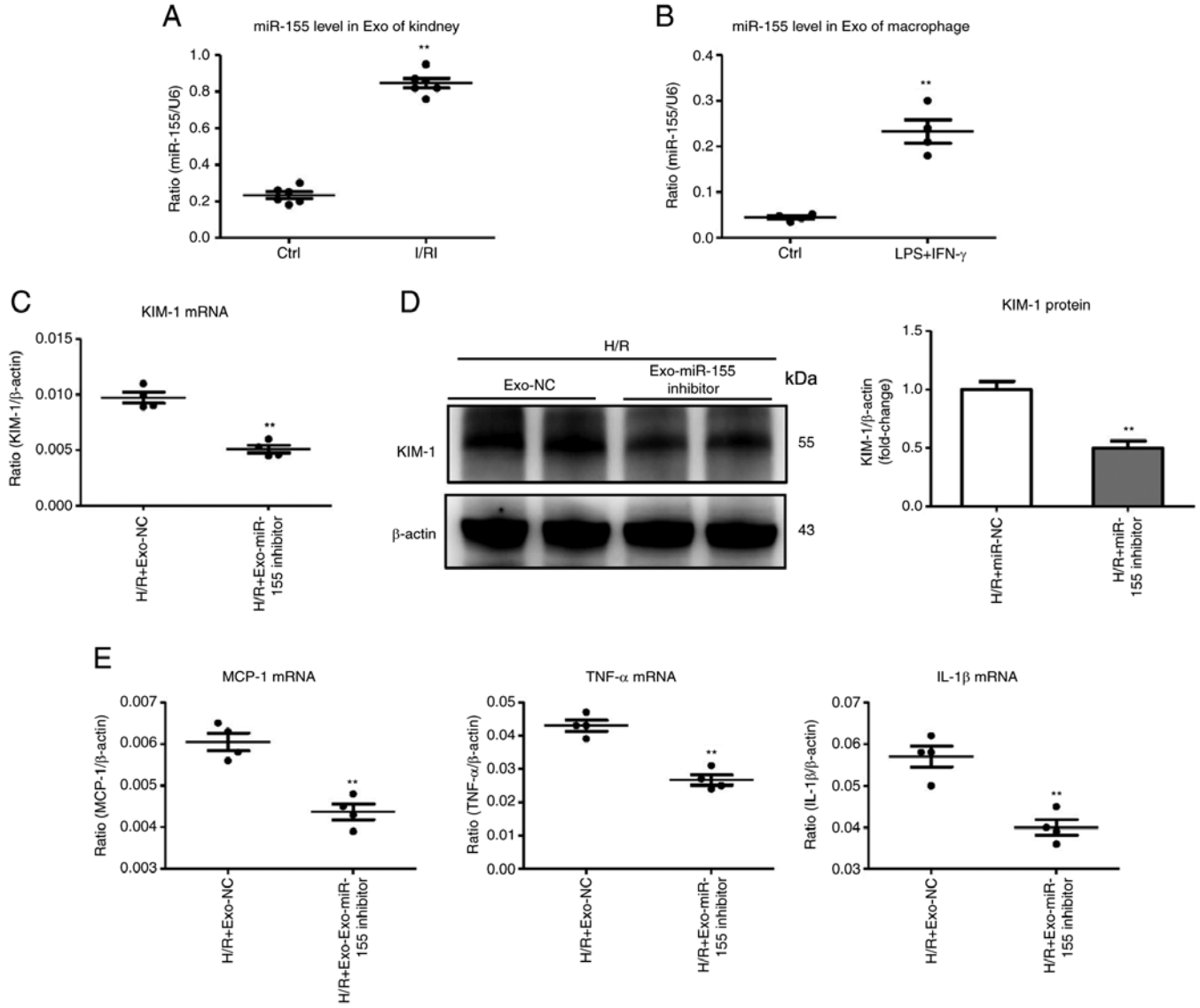

Figure 4. Exosomal miR-155 is associated with TEC injury. (A) miR-155 expression in exosomes derived from the I/R-injured kidneys was assessed by RT-qPCR. Data are expressed as the mean ± SEM for groups of 6 mice. (B) miR-155 in exosomes derived from the activated macrophages was detected by RT-qPCR. (C and D) mRNA and protein expression of KIM-1 in TECs after treatment with Exo-miR-155-inhibitor/Exo-NC (the exosomes derived from activated macrophages transfected with miR-155 inhibitor/miR-NC). (E) RT-qPCR analysis of inflammatory cytokine mRNA levels in TECs treated with Exo-miR-155-inhibitor/Exo-NC. **P<0.01 vs. the H/R + Exo-NC. miR-155, microRNA-155; TEC, tubular epithelial cell; I/R, ischemia/reperfusion; RT-qPCR, reverse transcription quantitative-PCR; KIM-1, kidney injury molecule-1; Exo, exosome; NC, negative control; H/R, hypoxia/reoxygenation; LPS, lipopolysaccharides; IFN-γ, interferon-γ; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

similarly elevated in the exo-M1-treated group (Fig. 7E). Additionally, immunohistochemical analysis revealed a reduction of SOCS-1 expression in the exo-M1 recipients (Fig. 7D). Thus, the findings demonstrated that exosomes derived from M1 macrophages contribute to tubular injury.

Discussion

Renal tubule injury is a characteristic pathological feature of AKI and determines the outcome of kidney disease. However, the mechanism of TEC injury remains unclear. In the present study, a novel mechanism through which the macrophage-derived exosomal miR-155/SOCS-1 axis mediated renal tubule injury was demonstrated. The findings not only provide novel insights into the pathophysiology of AKI but also offer a new therapeutic strategy for kidney diseases.

Renal tubules, which are packed with mitochondria and dependent on oxidative phosphorylation, are particularly vulnerable to a variety of injuries including obstructive, ischemic, hypoxic, toxins, and metabolic, and determine the prognosis of kidney diseases (24). There are several studies that revealed that TECs could undergo changes and function as inflammatory cells, with the consequent production of various inflammatory molecules (MCP-1, TNF-α, and IL-1β) that drive progression of kidney diseases (25,26). Thus, elucidating the exact mechanism underlying tubular injury is urgent.

As a type of principal innate immune cell, the macrophage plays a central role in the maintenance of tissue homeostasis (27). Increasing studies have demonstrated that macrophages exhibit high diversity and plasticity in response to different microenvironments, thereby exerting diverse functions. For example, classically activated macrophages (M1)

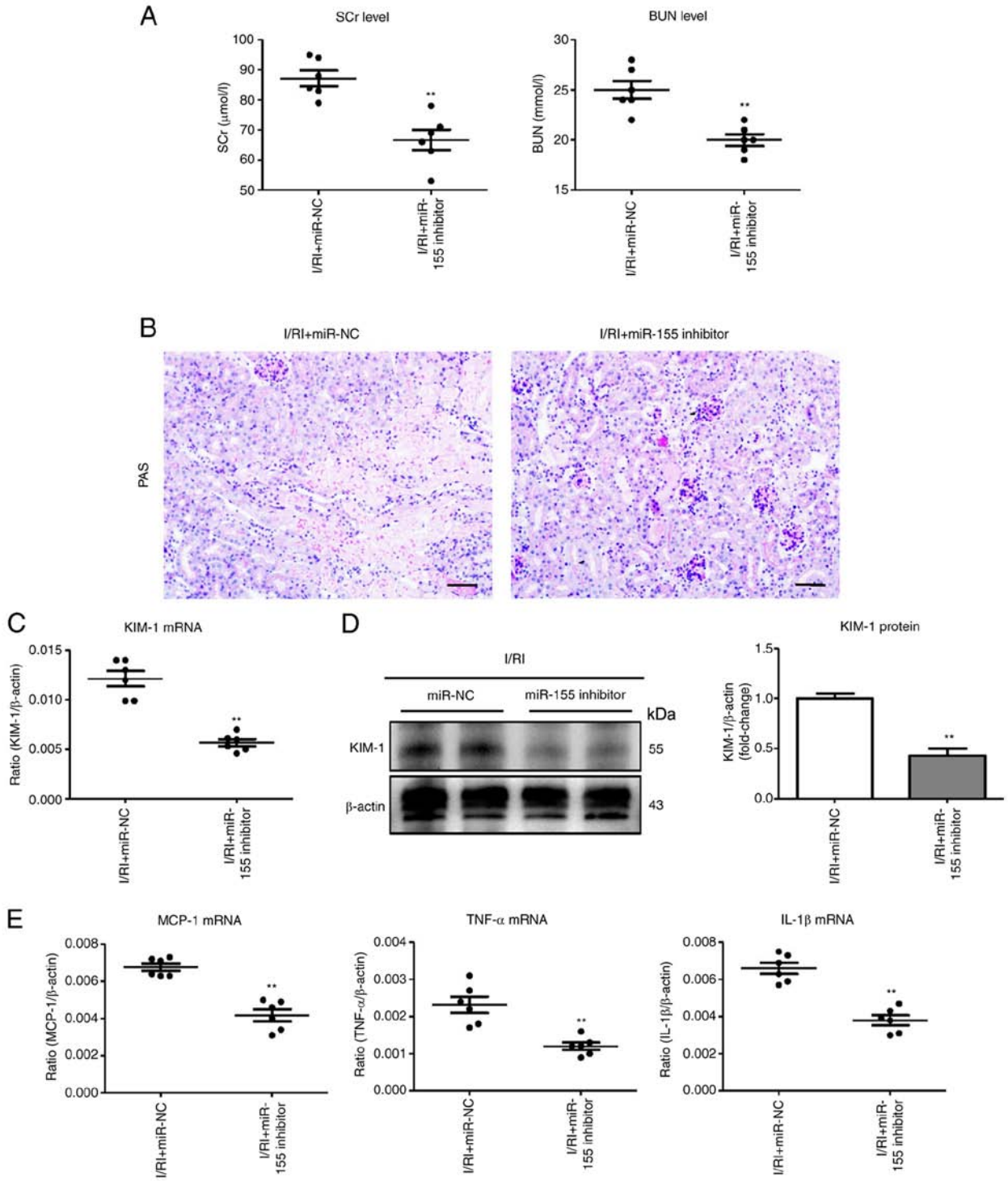

Figure 5. miR-155 inhibition ameliorates tubule injury in I/R-induced kidney injury. (A) The SCr and BUN levels in I/R-injured mice following miR-155 inhibitor administration. (B) Histological changes (PAS staining; scale bar, 100 μm). (C and D) RT-qPCR and western blot analysis of KIM-1 expression in I/R-treated kidneys with miR-155 inhibitor administration. (E) mRNA expression of inflammatory cytokines in I/R-treated kidneys with miR-155 inhibitor administration. n=6. Data are presented as the means ± SEM. **P<0.01 vs. the I/RI + miR-NC group. miR-155, microRNA-155; I/R, ischemia/reperfusion; SCr, serum creatinine; BUN, blood urea nitrogen; PAS, periodic acid-Schiff; RT-qPCR, reverse transcription quantitative-PCR; KIM-1, kidney injury molecule-1; I/RI, ischemia/reperfusion injury; NC, negative control; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

and alternatively activated macrophages (M2) could perform a variety of biological functions via the production of large amounts of cytokines (28). Recently, convincing evidence revealed that macrophages also play a critical role in innate immunity and adaptive immune response through the secretion of exosomes (29). More interestingly, the function of exosomes derived from macrophages is gaining increasing attention in the course of various diseases (30,31). With regard to kidney diseases, Huang *et al* (32) recently reported that macrophage-derived exosomes improved high glucose-induced podocyte injury by transferring specific miRNA. However, the roles of exosomes derived from macrophages in AKI remains

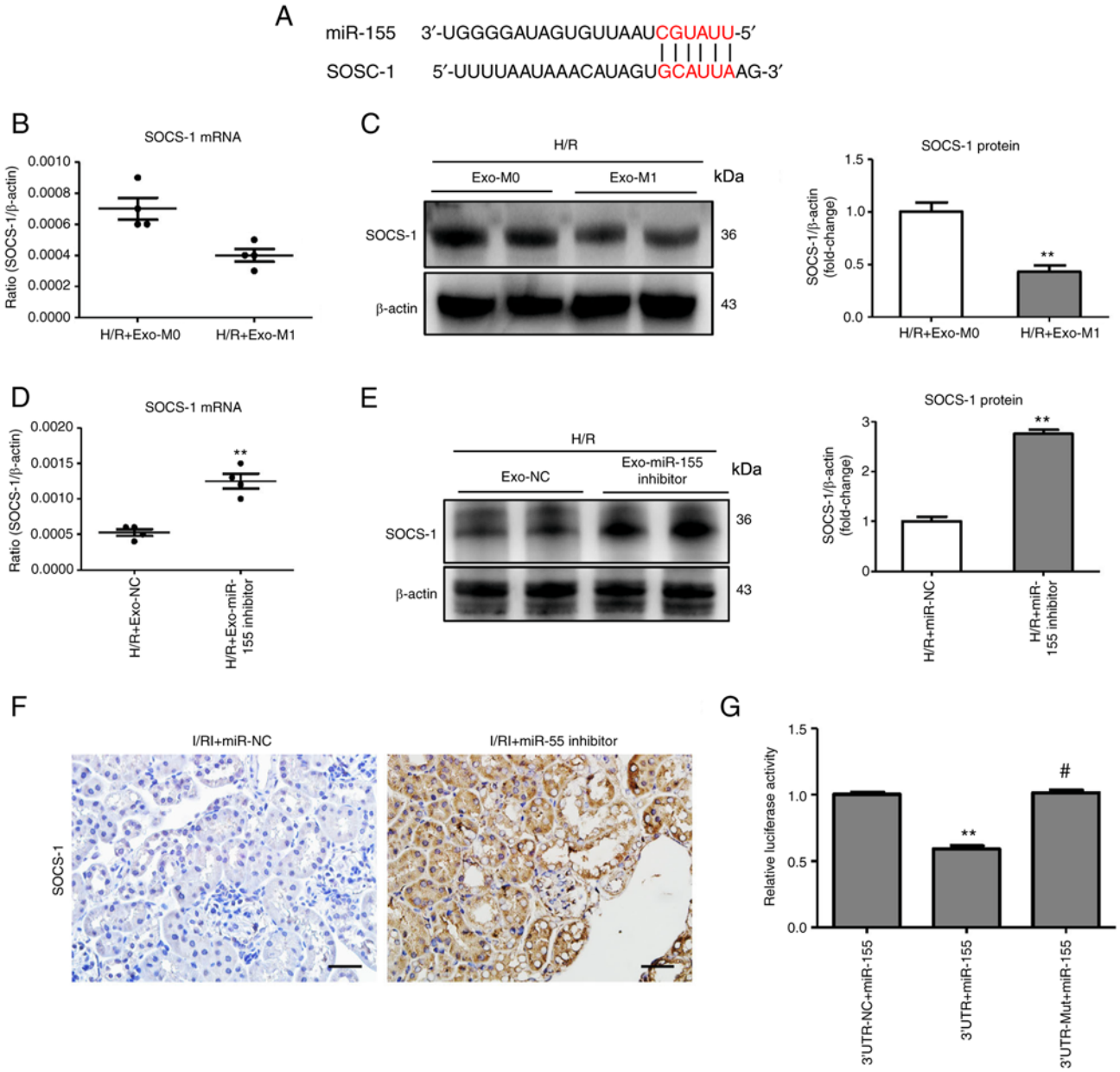

Figure 6. Exosomal miR-155 promotes tubule injury by targeting SOCS-1. (A) Schematic diagram depicting the predicted mmu-miR-155 targets using TargetScan, release 7.1 (http://www.targetscan.org/mmu_71/). (B and C) RT-qPCR and western blot analysis of SOCS-1 expression in H/R-treated TECs treated with exo-M1. Data are presented as the means ± SEM. **P<0.01 vs. H/R + Exo-M0. (D and E) mRNA and protein expression of SOCS-1 in TECs after treatment with Exo-miR-155-inhibitor/Exo-NC. Data are presented as the means ± SEM. **P<0.01 vs. the H/R + Exo-NC group. (F) Representative images of SOCS-1 expression in I/R-treated kidney with miR-155 inhibitor administration (scale bar, 50 μm). (G) A luciferase reporter assay was performed using constructs with SOCS-1 3'-UTR or SOCS-1 3'-UTR-mut. TECs were co-transfected with these constructs along with the miR-155 overexpressed plasmid. **P<0.01 vs. the 3'-UTR-NC + miR-155 group; $^{\#}$P<0.01 vs. the 3'-UTR + miR-155 group. miR-155, microRNA-155; SOCS-1, suppressor of cytokine signaling-1; RT-qPCR, reverse transcription quantitative-PCR; H/R, hypoxia/reoxygenation; Exo, exosomes; TECs, tubular epithelial cells; NC, negative control; UTR, untranslated region; mut, mutated; I/RI, ischemia/reperfusion injury.

poorly understood. In the present study, it was determined that exosomes derived from M1 macrophages mediated tubule injury during AKI. Notably, blocking exosome production ameliorated tubular injury in I/R-induced AKI, suggesting that exosomes play a crucial role in the pathogenesis of tubule injury. To the best of our knowledge, this is the first time the function of exosomes derived from macrophages during the AKI was explored. Therefore, the present study plays a vital role in understanding ischemia-induced kidney injury.

To further investigate the exact mechanism of exosomes derived from M1 macrophages on renal tubule injury, the specific cargos of exosomes was analyzed. Given that miRNAs are commonly enriched in exosomes and the miRNA class was observed to be the largest and most consistent proportional change in exosomes using total RNA-sequencing in a model of I/R (12), it was hypothesized that exosomes could regulate tubular cells by transferring cell-specific miRNA. Notably, it was determined that it was miR-155 that was specifically assembled into the exosomes and miR-155 inhibition alleviated tubular injury *in vitro* and *in vivo*. In a previous study by Ying *et al*, it was reported that miR-155 was loaded into macrophage exosomes (33). In addition,

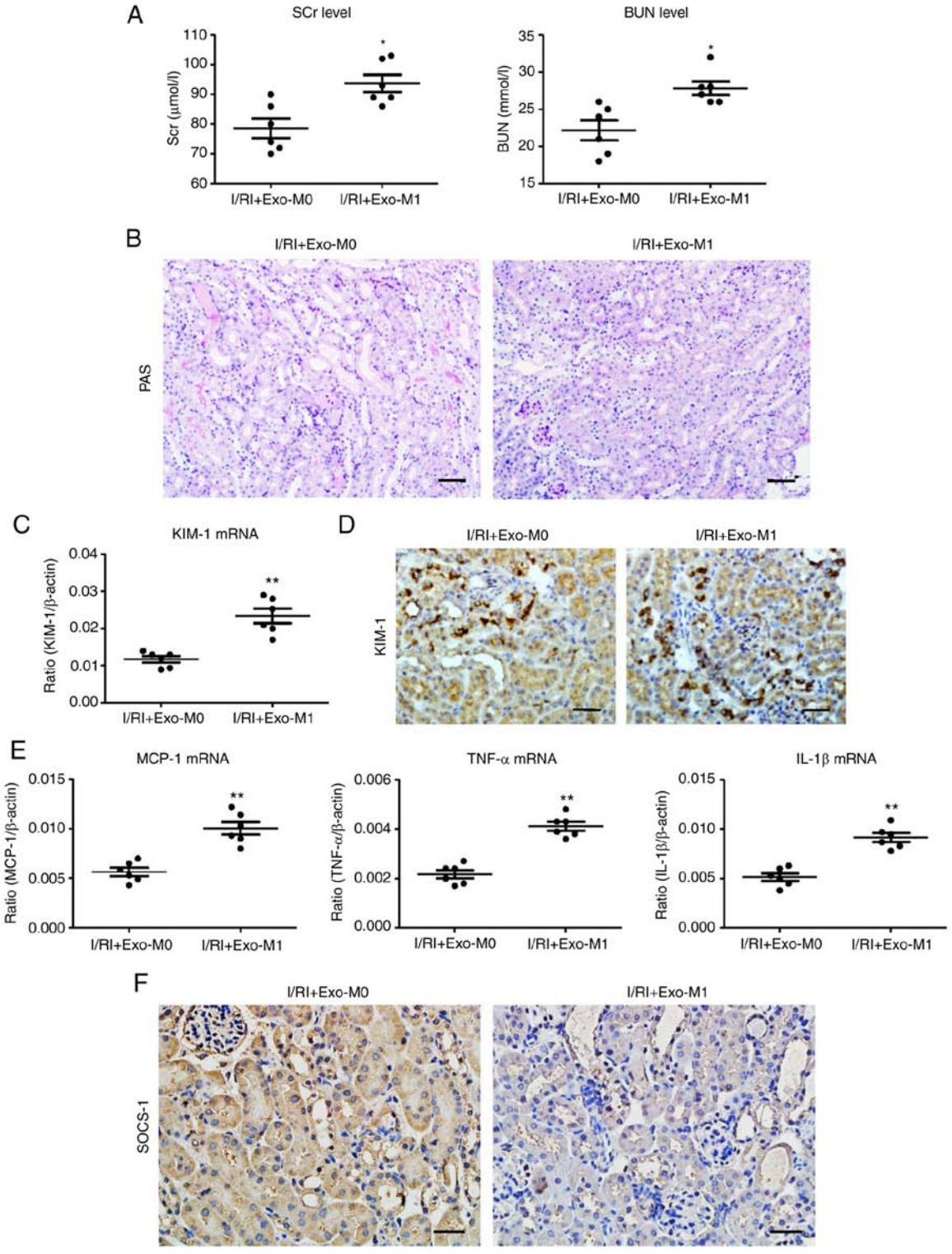

Figure 7. Exosomes secreted from M1 macrophages promote tubule injury in I/R-induced kidney injury. (A) The SCr and BUN levels in I/R-injured mice after exo-M1 administration. (B) Representative PAS-stained kidney sections after exo-M1 injection (scale bar, 100 μm). (C) RT-qPCR analysis of KIM-1 expression in I/R-treated kidney with Exo-M1 injection. (D) Representative images of KIM-1 expression assessed by immunohistochemistry (scale bar, 50 μm). (E) mRNA expression of inflammatory cytokines in the exosome-injected kidney. (F) Representative images of SOCS-1 expression in the exosome-injected kidney, assessed by immunohistochemistry (scale bar, 50 μm). Data are expressed as the mean ± SEM. n=6. *P<0.05 and **P<0.01 vs. I/RI + Exo-M0 (Ctrl). I/R, ischemia/reperfusion; SCr, serum creatinine; BUN, blood urea nitrogen; PAS, periodic acid-Schiff; RT-qPCR, reverse transcription quantitative-PCR; KIM-1, kidney injury molecule-1; Exo, exosome; SOCS-1, suppressor of cytokine signaling-1; I/RI, ischemia/reperfusion injury; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

convincing evidence has shown that macrophage-derived miR-155-containing exosomes promoted cardiac hypertrophy and fibrosis by targeting FoxO3a in uremic mice (34). Therefore, these results indicated that exosomes derived from M1 macrophages are implicated in tubule injury via transferring of miR-155.

Finally, the exact mechanism through which macrophage-derived exosomal miR-155 regulated tubule injury was further investigated. Notably, it was determined that exosomal miR-155 was implicated in the pathogenesis of tubule injury by suppression of SOCS-1, a dual guanine nucleotide exchange factor that plays an important role in regulating cellular hemostasis (35). Silencing of miR-155 markedly increased the levels of SOCS-1 expression *in vitro* and *in vivo* and resulted in reduced tubule injury. SOCS-1 has previously been demonstrated as an inflammation suppressor that normally functions as a negative regulator of NF-κB signaling pathways (36), and which aggravates tubular injury during ischemic AKI (37). More interestingly, a previous study indicated that SOSC-1 is a target of miR-155 involved in the regulation of immune response (38). Thus, the findings of the present study, demonstrated that SOCS-1, a target of miR-155, played a new molecular role in regulating the function of tubular cells.

In summary, it was demonstrated that exosomal miR-155 mediated the cross-talk between tubules and macrophages and contributed to tubule injury in ischemia-induced AKI. The exosome/miR-155/SOCS-1 axis played a critical role in renal tubule injury. The findings suggested that macrophage-derived exosomal miR-155 provides a novel understanding of the molecular mechanisms of renal tubule injury and represents a new therapeutic target for the treatment of AKI.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

ZZ, HC, LZ, GL, and LW designed the study, carried out experiments, analyzed the data, and wrote the paper. CL carried out the experiments and analyzed the data. ZZ, GL, and LW prepared the figures and edited the manuscript. ZZ and HC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experimental animal procedures were approved (approval no. N2017-078) by the Ethics Committee of Soochow University (Suzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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