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Research Article

Exosomes Derived from *Schistosoma japonicum* Cystatin-Treated Macrophages Attenuated CLP-Induced Sepsis in Mice

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Sepsis is a disease caused by multiple microbial infections resulting in multiple organ failure. *Schistosoma japonicum* secreted cystatin (*Sj*-Cys) is a strong immunomodulator that stimulates M2 macrophages and alleviates inflammatory damage caused by sepsis. To determine whether the therapeutic effect of *Sj*-Cys on sepsis can be conveyed by the exosomes released by *Sj*-Cys-stimulated macrophages, RAW264.7 macrophages were stimulated with r*Sj*-Cys *in vitro*, the exosomes were obtained from the cell culture supernatant by ultracentrifugation. Sepsis was induced in BALB/c mice by cecal ligation and puncture (CLP). The septic mice were treated with exosomes derived from *Sj*-Cys-treated macrophages. The treatment effect of exosomes on sepsis was assessed by examining the survival rate of mice up to 72 hr and measuring serum levels of inflammatory cytokines, liver/kidney damage biomarkers, and observing pathological changes in tissue sections. The tissue levels of M1, M2 macrophage surface markers, and TRL2/MyD88 were measured to explore possible mechanisms. *Results*. Exosomes derived from *Sj*-Cys-treated macrophages exhibited significant therapeutic effect on CLP-induced sepsis in mice with prolonged survival rate and less damage of critical organs by downregulating the proinflammatory factors TNF-α and IL-6 and upregulating the anti-inflammatory factor TGF-β. The therapeutic effect of exosomes is associated with macrophage polarization from M1 to M2 in the infected tissues via downregulating TRL2/MyD88 inflammatory pathway. *Conclusions*. Exosomes derived from *Sj*-Cys-treated macrophages attenuated sepsis in mice through promoting macrophage polarization from M1 to M2 and reducing inflammatory responses, possibly via downregulating TLR2/MyD88 inflammatory signaling pathway. This offers new approaches for immunotherapy of sepsis.

1. Introduction

Sepsis is a systemic inflammatory syndrome attributed to the dysregulated host immune responses to bacterial infections and a life-threatening medical emergency [1]. The variability of sepsis between patients makes treatment challenging, despite the level of care and medical instruments being updated [2, 3] the mortality rate remains as high as 40% [4]. The evolution of sepsis is broadly categorized into two phases, systemic inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response syndrome (CARS) [5]. SIRS is originated by various innate immune cells, which are activated by pathogen signaling when a pathogen invades, stimulating tumor necrosis factor-α (TNF-α) production, and speeding up the activation of other inflammatory cytokines and exacerbating tissue damage. This active phase of inflammatory factors is the main cause of organ damage in sepsis [6, 7]. Another aspect, CARS is a mechanism of systemic immune system by secreting anti-inflammatory factors such as IL-4, IL-10, and TGF-β in order to balance the

inflammatory state and reduce inflammation [8]. Therefore, the timing and balancing of the SIRS and CARS have become crucial for the treatment of prognosis of sepsis.

Macrophages are innate immune cells that play a key role in initiating, amplifying, and clearing inflammation and are major target cells in the treatment of sepsis [9–11]. Macrophages exhibit different phenotypes and functions depending on the surrounding microenvironment [12]. Macrophages are distinguished into two types according to phenotype: the classical activation (M1) type inspired by type-1T helper cytokine (Th1), which secretes proinflammatory factors such as TNF-α and IL-6 and is engaged in the development of inflammation; and the alternative activation (M2) antigen induced by T helper type-2 (Th2) cytokine, which secretes transforming growth factor (TGF-β) and stimulates tissue repair [12]. All studies indicated that the proportion of M1 to M2 macrophages affects the development and consequence of inflammatory diseases.

Exosomes are nanoscale vesicles produced by various cells and play an important role in mediating intercellular signaling and communicating [13, 14]. Under the inflammatory response, the donor cells release active substances such as proteins, genes, and lipids into exosomes to the recipient cells resulting in the receptor phenotypic variation [15, 16]. In recent years, the function and possible application of exosomes in sepsis has attracted growing attention. It has been shown that exosomes are released from diverse effector cells and associated with various tissue or organ damage or repair in sepsis. For example, exosomes released by macrophages from TLR-9 knockout mouse ameliorated sepsis-induced mitochondrial oxidative stress and apoptosis in cardiomyocytes [17]. Adipose-derived stem cells released exosomes inhibited inflammation and oxidative stress in mice with lipopolysaccharide (LPS) induced acute kidney injury [18]. Mouse bone marrow mesenchymal stem cells released serum amyloid A1 (SAA1) protein in exosomes that reduced sepsis-induced lung injury in mice [19]. On the contrary, exosomal miR-155 stimulated macrophage proliferation and inflammation in lung tissue and mediated septic lung injury [20].

A large body of experimental evidence demonstrates that helminth infections modulate host immune responses by releasing a number of functional proteins to diminish immune attack on parasites as a survival strategy during infection, on the other hand, host may acquire the beneficial effect of the helminth-mediated immunomodulation to reduce inflammatory responses to some exogenous allergens or indigenous autoantigens [21, 22]; therefore, these helminth-derived immunomodulatory proteins have been experimentally tested as therapy agents for the treatment of allergic or inflammatory diseases [23]. In particular, cysteine protease inhibitors (cystatin) from various parasitic helminths are strong immunomodulatory proteins to reduce host inflammatory immune responses [24–26]. *Schistosoma japonicum* secreted cystatin (*Sj*-Cys) has been identified as a strong immunomodulatory protein [27]. It has been determined that proinflammatory factors released from LPS-stimulated macrophages can be inhibited by *Sj*-Cys. The recombinant protein of *Sj*-Cys (r*Sj*-Cys) reduced the seriousness of collagen-induced arthritis [28] and cecal ligation and puncture (CLP)-induced bacterial sepsis in mice [29]. Further investigation revealed that the therapeutic effect of r*Sj*-Cys on sepsis was taken placed through activating regulatory macrophages [29]. It is still unknown the therapeutic mechanism of r*Sj*-Cys on sepsis. In this study, the exosomes extracted from *Sj*-Cys-treated macrophages (*Sj*-Cys-EXO) were used to treat CLP-induced bacterial sepsis in mice. We found that *Sj*-Cys-EXO conducted similar therapeutic effect on CLP-induced bacterial sepsis with reduced organ injury as our previous study with treatment of r*Sj*-Cys *in vivo* in septic mice [29], demonstrating at the first time that *Sj*-Cys induced M2 polarization can be transferred to macrophages in septic tissue through exosomes to mitigate sepsis caused tissue or organ damage.

2. Materials and Methods

2.1. Expression of Recombinant Sj-Cys Protein (rSj-Cys). The coding DNA for *Sj*-Cys (GenBank: FJ617450) was amplified from cDNA of *S. japonicum* adult worms and cloned into pPIC9k yeast expression vector, as described previously. The expression of r*Sj*-Cys was induced with 0.5% methanol and the expressed r*Sj*-Cys was purified using an immobilized metal affinity chromatography (IMAC) (Thermo, USA) [30]. The contaminated endotoxin in the purified r*Sj*-Cys was removed using a Endotoxin Removal Kit (BioVision, USA) and confirmed by a LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, USA). The concentration of r*Sj*-Cys was determined with bicinchoninic acid (BCA) protein quantification kit (Biosharp, Hefei, China). Purity and molecular weight of r*Sj*-Cys were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2. Exosomes Isolation from Macrophages Treated with rSj-Cys. The RAW264.7 cells were cultivated in DMEM media supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China) and 1x penicillin/streptomycin (Sigma–Aldrich, Steinheim, Germany). When the cell growth density reached about 80%, the culture media was changed to media with exosome-free serum (System Biosciences, UK). Then r*Sj*-Cys was added into the culture up to 2 μg/ml and the culture was continued for additional 48 hr. The culture supernatant was harvested, the cells and debris were removed by centrifugation at 300×*g*; 2,000×*g* and 10,000×*g*, respectively and then filtrated through 0.22 μm filter. The filtered supernatant was centrifuged at 120,000×*g* for 90 min, the pellet was washed once with 1x phosphate-buffered saline, pH 7.4, and recovered by centrifugation at 120,000×*g* for another 90 min to obtain exosomes (EXO). The protein concentration of obtained EXO was quantified by BCA and the exosome-positive proteins (CD9, TSG101) in EXO were measured by Western blot with specific antibodies (Abcam, UK) [30].

Negative-staining transmission electron microscopy (TEM) was conducted to analyze the shape and size of exosomes. The exosomes were suspended in 2% glutaraldehyde, loaded on a copper grid and negatively stained with 1% uranyl acetate, then imaged with a HT-7700 TEM (Hitachi, Japan) at 100 kV [31]. The collected exosomes were also analyzed using a nanoflow cytometer (nFCM), as described in Tian et al.'s [32] study. Briefly, the concentration of each

extracellular vesicle (EV) sample was determined by employing 100 nm orange FluoSpheres of known particle concentration to calibrate the sample flow rate. Two single-photon counting avalanche photodiodes were used for the simultaneous detection of the side scatter and orange fluorescence of individual EVs. The sample nFCM data were collected for 1 min at a sample pressure of 1.0 kPa. The particle concentrations and particle size distributions were calculated using NanoFCM software (Xiamen, China) [32].

2.3. Animals and Ethics. Male BALB/c mice at 6 weeks old and weight of 18–22 g were purchased from the Animal Centre of Henan Province. All animal experiments were conducted with protocol approved by Ethics Review Committee of Bengbu Medical College (approval number: LAEC-2022-413).

2.4. Murine Model of Sepsis. Sepsis model was established in mice through CLP that causes polymicrobial infection to mimic the pathophysiological characteristics of peritonitis and sepsis in patients in a clinical setting [33, 34]. As described previously, mice were kept fasted for 12 hr with access to water only. Under anesthesia with isoflurane inhalation, mice were stabilized and the abdominal cavity was open, the cecum was located and tied at the top 1.0 cm of the cecum. The cecum was punctured with an 18-gauge needle, a small amount of faeces was squeezed out. The cecum was placed back into the cavity and the abdomen was closed by suturing level by level. The control mice were subjected to incision without any manipulation of the cecum (sham surgery). The survival rate of the mice was observed for 72 hr.

2.5. Therapeutic Effect of Sj-Cys-EXO on Septic Mice. Total 75 BALB/c mice were randomly assigned to five groups with 15 each. Three groups of mice have performed CLP surgery, 30 min after surgery mice in each group were injected intraperitoneally with 10 μg of EXO derived from r*Sj*-Cys-treated macrophages (CLP + *Sj*-Cys-EXO) or 10 μg EXO from non-treated macrophages (CLP + EXO) or PBS only (CLP + PBS) in the total volume of 100 μl, respectively. Another two groups performed sham surgery and treated with the same amount of *Sj*-Cys-EXO (Sham + *Sj*-Cys-EXO) or PBS only as control (Sham + PBS). Twelve hours after treatment, five mice in each group were sacrificed. Blood was drawn and sera were separated for testing the levels of cytokines and some biochemical makers for liver and kidney functions described below, liver, lung, and kidney were collected from each mouse for histopathological examination. The survival rate of the remaining 10 mice was monitored for 72 hr. The experiment was repeated for three times.

2.6. Serological Test. Sera from five mice euthanized 12-hr posttreatment were tested for the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the liver function biomarkers, blood urea nitrogen (BUN) and creatinine (Cr) (kidney function markers), using an automatic chemistry analyzer (Beckman, USA). The proinflammatory IL-6, TNF-α, and anti-inflammatory TGF-β cytokine were detected in the sera by ELISA with specific antibodies (Dakewe, China). The standard range of IL-6 was 0–1,000 pg/ml with a serum dilution of 1 : 40, and the standard range of TGF-β was 0–500 pg/ml with a serum dilution of 1 : 100.

Table 1: Liver injury score parameters.

Hepatocellular edema, congestion, and inflammatory cell infiltration	Pathological score
No	0 (normal)
<25%	1 (moderate)
25%–50%	2 (severe)
50%–70%	3 (extremely severe)
>75%	4 (critical)

Table 2: Lung injury score parameters.

Alveolar congestion and wall thickness, inflammatory cell infiltration	Pathological score
No	0 (normal)
<25%	1 (moderate)
25%–50%	2 (severe)
50%–70%	3 (extremely severe)
>75%	4 (critical)

Table 3: Kidney injury score parameters.

Kidney tube injury and glomerular reduction	Pathological score
No	0 (normal)
<25%	1 (moderate)
25%–50%	2 (severe)
50%–70%	3 (extremely severe)
>75%	4 (critical)

2.7. Histopathological Examination in Tissues of Liver, Lung, and Kidney. The livers, lungs, and kidneys were obtained from five mice euthanized 12-hr posttreatment and fixed with formalin. Tissue sections were cut and stained with hematoxylin and eosin (H&E) and examined for histopathological changes under the microscope (Olympus, Tokyo, Japan). Haemorrhage, hepatocyte necrosis, inflammatory cell infiltration, cytoplasmic vacuolation, and nuclear condensation were assessed in liver injury and pathological score was evaluated, as shown in Table 1 [35]. Alveolar wall thickening, vascular congestion, interstitial, and alveolar leukocyte infiltration were evaluated for lung injury, as shown in Table 2 [36]. Injured renal tubules and shrunk glomerulus were assessed in kidney injury, as shown in Table 3, based on the previous study [37].

2.8. Detection of Macrophage Phenotypic Markers. The levels of inducible nitric oxide synthase (iNOS), the macrophage M1 marker, and arginase-1 (Arg-1), M2 marker, were measured in the homogenate of liver, lung, and kidney tissues of treated mice using the corresponding ELISA kits (Elabscience, China).

Total RNA was extracted from liver, lung, and kidney tissues of each mouse using TRIzol-up (TransGen Biotech,

Table 4: The related primers of target genes in qPCR.

Target gene	Primer sequences (5′→3′)
iNOS (NM_001313922.1)	Forward: CAAGCACCTTGGAAGAGGAG
	Reverse: AAGGCCAAACACAGCATACC
Arg-1 (XM_021173919.1)	Forward: CTCCAAGCCAAAGTCCTTAGAG
	Reverse: AGGAGCTGTCATTAGGGACATC
GAPDH (XM_036165840.1)	Forward: ACCCAGAAGACTGTGGATGG
	Reverse: CACATTGGGGGTAGGAACAC

China) and quantified by measuring A260/280. Total 2 μg of total RNA from each sample was used to reverse transcribed into cDNA using a reverse transcription kit (TransGen, China) on S1000 Thermal Cycler (Bio-Rad) in a total volume of 50 μl. The relative mRNA expression of macrophage polarization-related markers (iNOS and Arg-1) against housekeeper control GAPDH in these tissues was determined by a reverse transcription quantitative polymerase chain reaction (RT-qPCR) by using SYBR® Green I fluorescent dye to quantitatively detect amplified double-strand DNA in LightCycler® 96 Real-Time PCR System, Switzerland. The primers for each target gene used for the real-time PCR (Table 4) were designed and optimized to achieve similar efficiency for the targets and the internal control gene (GAPDH) [38]. Total 2 μl of total cDNA was used in the qPCR reaction for each sample, the reaction condition is 95°C for 5 s, 55°C for 15 s, and 72°C for 10 s with total 45 cycles. The final value was determined by the $2^{-\Delta\Delta Ct}$ formula.

2.9. Detection of TLR2 and MyD88 in the Liver, Kidney, and Lung Tissues of Mice by Western Blotting. The expression levels of TLR2 and MyD88 in the tissues of liver, kidney, and lung were determined by Western blot using specific antibodies. Briefly, tissues obtained from treated mice were lysed with lysis solution and the tissue lysate supernatant was collected by centrifugation. The protein concentration in the lysate supernatant was measured using the BCA method. The same amount of tissue lysate (20 μg) was separated on a 10% acrylamide gel and transferred onto polyvinylidene difluoride membranes. The membrane was then blocked with skimmed milk for 3 hr and incubated overnight at 4°C with rabbit anti-TLR2 antibody (1 : 2,000) (Abcam, USA), or rabbit anti-MyD88 antibody (1 : 1,000) (Cell Signaling Technology, USA), or rabbit anti-β-actin antibody (1 : 5,000) (Cell Signaling Technology, USA). The washed membrane was followed by reincubation with HRP-conjugated goat antirabbit IgG secondary antibody (Biosharp, Hefei, China) (1 : 7,000). The bands were visualized and density of the target bands was scanned by Image Lab (Bio-Rad). Results are expressed as a ratio of TLR2 and Myd88 to β-actin control.

2.10. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, USA) for one-way or two-way analysis of variance for multiple group comparisons. All data are expressed as mean and standard deviation. Kaplan–Meier survival analysis was used to compare differences in survival between groups. $P < 0.05$ was considered to be statistically significant.

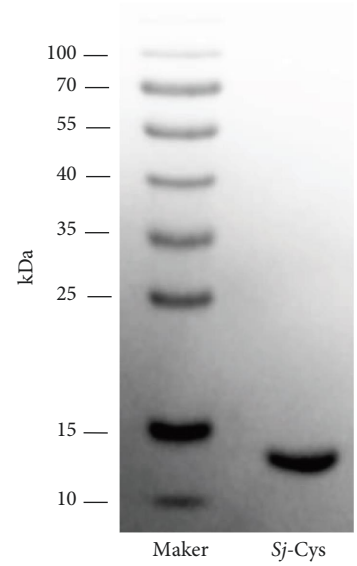

Figure 1: SDS-PAGE analysis of r*Sj*-Cys. Total 2 μg of r*Sj*-Cys was separated by 12% polyacrylamide gel electrophoresis.

3. Results

3.1. Expression, Purification, and Identification of rSj-Cys. The *Sj*-Cys was expressed successfully as a soluble recombinant protein. SDS-PAGE revealed that the purified r*Sj*-Cys had a size of approximately 11 kDa as expected based on the sequence (Figure 1). The endotoxin level in the purified r*Sj*-Cys protein was less than 0.06 EU/ml.

3.2. Successful Isolation of Exosomes from rSj-Cys Treated or Nontreated Macrophages. RAW264.7 macrophage cell line was incubated with 2 μg/ml r*Sj*-Cys for 48 hr. The exosomes released by the r*Sj*-Cys-treated or untreated macrophages were obtained by ultracentrifuging the culture supernatants. The obtained exosomes showed vesicles of 40–150 nm in diameter with a typical bilayer structure measured by TEM (Figure 2(a)). Flow cytometry analysis confirmed the size of 40–150 nm for those vesicles in the exosomes (Figure 2(b)). The exosome-specific markers, TSG101 and CD9, were detected in the obtained EXO via Western blotting using corresponding specific antibodies. There is no significant difference between r*Sj*-Cys stimulated macrophage exosomes (*Sj*-Cys-EXO) or exosomes derived from untreated macrophages (EXO) in terms of morphology and intensity of TSG101 or CD9 (Figure 2(c)).

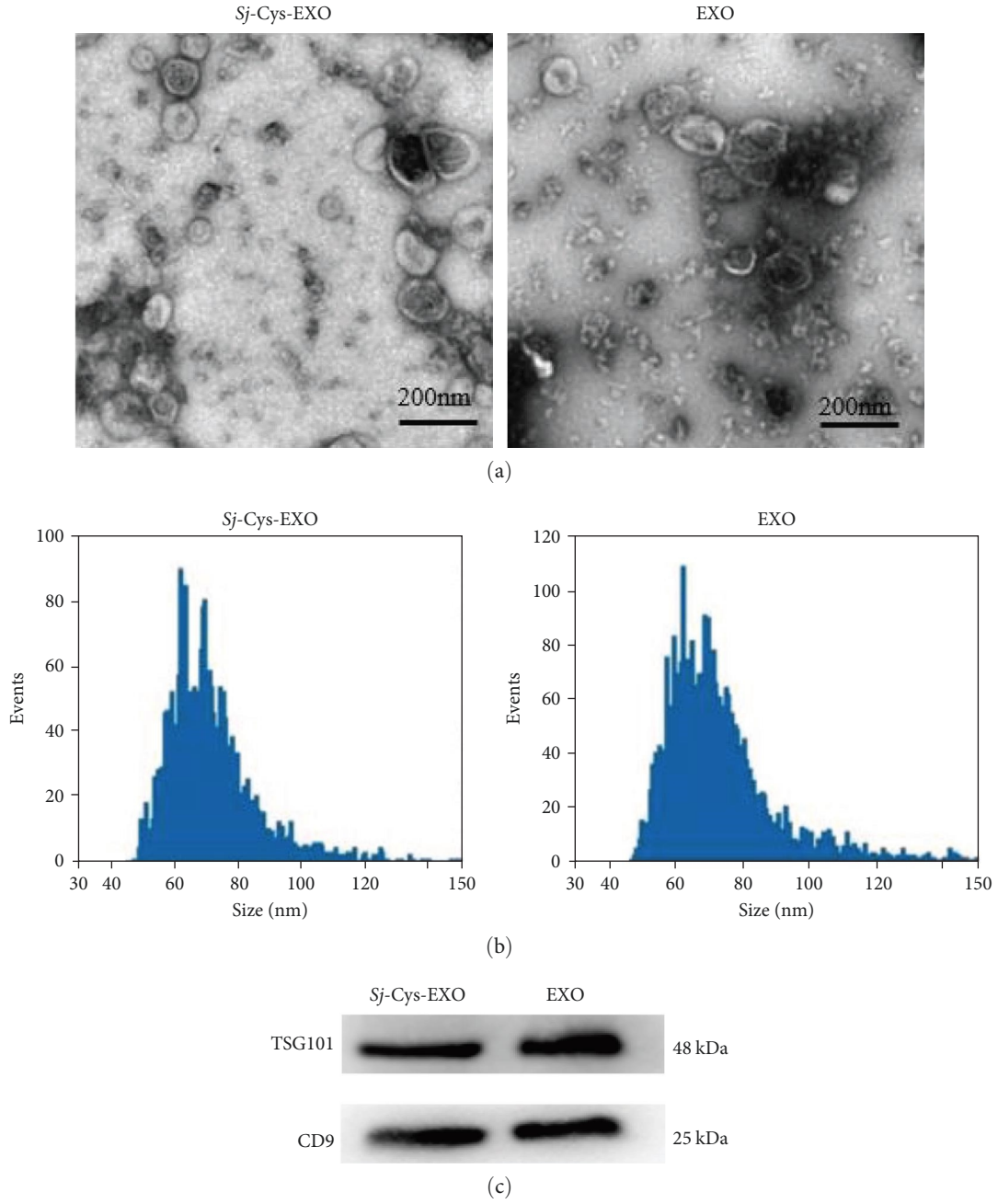

Figure 2: Identification of exosomes released by r*Sj*-Cys-treated (*Sj*-Cys-EXO) or untreated macrophages (EXO). (a) Exosome ultrastructure was measured by transmission electron microscopy (TEM). (b) The size distribution profile of the exosomes was measured using flow cytometry. (c) The exosome-specific markers TSG101 and CD9 were determined in exosomes via Western blotting with specific antibodies.

3.3. Treatment with Sj-Cys-EXO Improved the Survival Rate of Mice with CLP-Induced Sepsis. To test the therapeutic effect of exosomes derived from r*Sj*-Cys-treated macrophages on sepsis, septic mice were each injected intraperitoneally with 10 μg of *Sj*-Cys-EXO or the same amount of EXO from untreated macrophage. The survival rate of mice up to 72 hr in each group was observed. As seen in Figure 3, all mice receiving PBS in the CLP group (CLP + PBS) died within 48 hr. However, the 72 hr survival rate in CLP + *Sj*-Cys-EXO group increased to 30% that is significantly improved compared with CLP + PBS group ($P < 0.05$). The 72 hr survival rate for mice in the CLP + EXO group was only 10%, without statistic difference compared to control group of CLP + PBS. All mice in the sham-operated group with or without *Sj*-Cys-EXO treatment survived up to 72 hr.

3.4. Sj-Cys-EXO Improved Visceral Organs Function of Septic Mice. The sera collected from different groups of mice were analyzed using an automatic chemistry analyzer to measure the levels of ALT, AST (liver function), BUN, and Cr (kidney

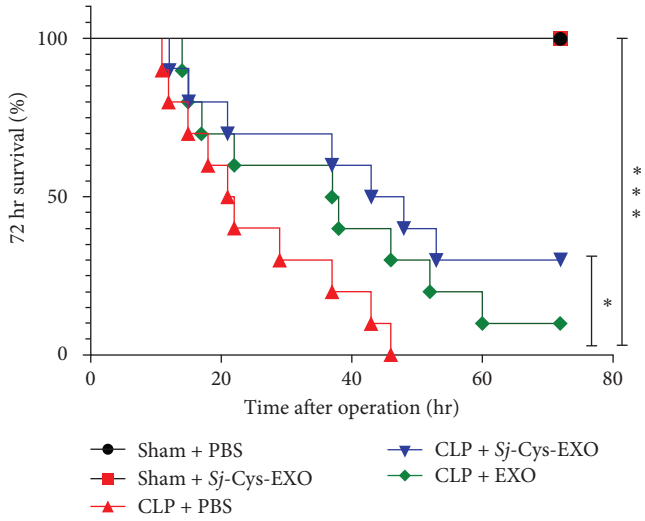

FIGURE 3: Treatment with *Sj*-Cys-EXO improved the survival rate of mice with CLP-induced sepsis. After CLP or sham surgery, mice were treated intraperitoneally with 10 μg of *Sj*-Cys-EXO, EXO, or PBS. The survival rate was determined using Kaplan–Meier method and compared by log-rank test. $n = 10$. $^{*}P < 0.05$, $^{***}P < 0.001$.

function). The findings showed that serological ALT, AST, BUN, and Cr levels were significantly elevated in the CLP group compared to the Sham group (Figure 4), indicating that sepsis caused severe damage to the liver and kidney. However, treatment with *Sj*-Cys-EXO in mice with sepsis (CLP + *Sj*-Cys-EXO) significantly reduced the levels of ALT, AST, BUN, and Cr in their sera compared to mice receiving PBS only. Mice treated with normal macrophages-derived EXO (CLP + EXO) also showed some extent of reduced levels of these liver or kidney injury markers, but the reduced level is not as significant as *Sj*-Cys-EXO. These results indicate that exosomes from macrophages, especially from *Sj*-Cys-treated macrophages, significantly improve the liver and kidney functions of septic mice. *Sj*-Cys-EXO has no effect on mice with sham surgery.

3.5. Sj-Cys-EXO Attenuates the Pathological Injury Caused by Sepsis. The liver, lung, and kidney tissue showed significant inflammation and damage in mice with CLP-induced sepsis without treatment (CLP + PBS), exhibiting inflammatory cell infiltration, edema, hemorrhage, tissue structure damage, and cell death. After being treated with exosomes from *Sj*-Cys-treated macrophages (CLP + *Sj*-Cys-EXO), the tissue damage in liver, lung, and kidney was significantly improved with less inflammation cell infiltration and edema and less hemorrhage and tissue structure distortion (Figure 5(a)). The organ tissue damage was also improved in septic mice treated with normal macrophage exosome (CLP + EXO); however, the improvement was not as significant as mice treated with *Sj*-Cys-EXO. The pathological injury scores in liver, lung, and kidney showed the similar level of improvement in septic mice treated with *Sj*-Cys-EXO or normal macrophage EXO, but with more significance with the former (Figure 5(b)). The above results showed that treatment with *Sj*-Cys-EXO greatly reduced the damage and pathology of important organs including liver, lung, and kidney in mice with CLP-induced sepsis.

3.6. Sj-Cys-EXO Regulated Macrophage Polarization in Liver, Kidney, and Lung Tissues of Septic Mice. To further investigate whether therapeutic effect of *Sj*-Cys-EXO on sepsis is taken placed through modulating macrophage polarization, the protein and mRNA expression levels of M1 macrophage marker iNOS and M2 macrophage marker Arg-1 were measured in liver, kidney, and lung tissues using ELISA and RT-qPCR, respectively. The results showed iNOS was significantly increased in those tissues of mice with CLP-induce sepsis in both protein and transcriptional levels compared to mice with sham surgery. However, the protein level of Arg-1 was significantly reduced, although the mRNA level was slightly increased. These results suggest that the serious polymicrobial infection significantly induces M1 macrophage polarization in infected organs. Treatment with *Sj*-Cys-EXO significantly reduced iNOS but increased Arg-1 in both protein and mRNA levels in liver, lung, and kidney of mice with CLP-induced sepsis (CLP + *Sj*-Cys-EXO), indicating that *Sj*-Cys-EXO induces the macrophage polarization from M1 to M2 in these organs (Figure 6). Treatment with normal macrophage-derived exosome (EXO) also promoted the M2 polarization in these organs but not so significant as *Sj*-Cys-EXO.

3.7. Sj-Cys-Induced Macrophage-Derived Exosome Reduced Proinflammatory Cytokines and Induced TGF-β in Mice with CLP-Induced Sepsis. Inflammatory cytokines TNF-α, IL-6, and regulatory cytokine TGF-β have a major impact on the pathogenesis of sepsis. To measure whether exosomes from r*Sj*-Cys-treated macrophages regulate cytokine production in mice with sepsis, the concentrations of inflammatory cytokins TNF-α and IL-6 and anti-inflammatory cytokine TGF-β were examined in sera by ELISA. The results demonstrated that both TNF-α and IL-6 levels were highly elevated in CLP-induced septic mice (CLP + PBS) relative to sham-operated mice. The regulatory cytokine TGF-β was also reduced in septic mice. However, septic mice treated with *Sj*-Cys-EXO had significantly lower levels of these proinflammatory cytokines compared to septic mice receiving PBS. These proinflammatory cytokines were also reduced in mice treated with EXO from normal macrophages; however, the decreased level was not as significant as mice treated with *Sj*-Cys-EXO (Figure 7(a)). In contrast, the serological level of TGF-β was significantly increased in septic mice treated with *Sj*-Cys-EXO (CLP + *Sj*-Cys-EXO) compared with mice received with PBS. Septic mice received with normal macrophage-derived EXO showed slightly increased TGF-β level (CLP + EXO) compared to nontreated CLP + PBS (Figure 7(b)). These outcomes indicate that exosomes from r*Sj*-Cys-stimulated macrophages have ability to downregulate Th1 inflammatory response and upregulate regulatory response.

3.8. Sj-Cys-EXO Inhibits the Expression of TLR2 and MyD88. To verify whether *Sj*-Cys-EXO regulates macrophage polarization in septic mice through TLR2/MyD88 signaling

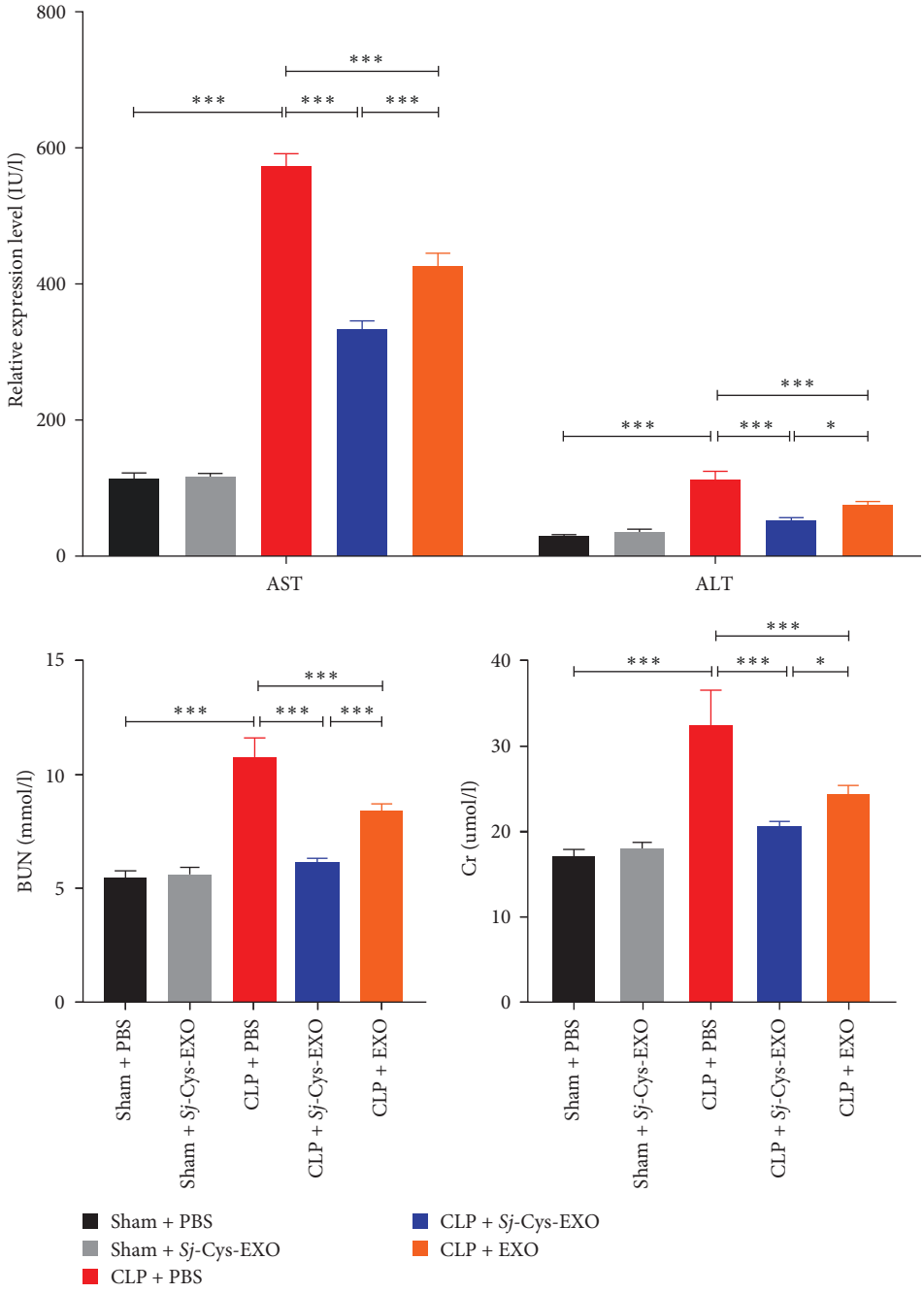

FIGURE 4: The serological levels of ALT, AST, BUN, and Cr were reduced in mice treated with *Sj*-Cys-EXO. $n = 5$ per group. The results are presented as mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$.

pathway, TLR2 and MyD88 expression level was measured in liver, lung, and kidney of septic mice. As shown in Figure 8, CLP-induced sepsis resulted in consistent activation of the TLR2/MyD88 signaling pathway in liver, lung, and kidney tissues. However, while the acute damage to these vital organs caused by sepsis was alleviated by the treatment with *Sj*-Cys-EXO, the TLR2 and MyD88 protein expression levels were also reduced in these tissues of treated mice. The reduced TLR2 and MyD88 level in EXO treated group was not as significant as those in *Sj*-Cys-EXO treated group (Figure 8).

4. Discussion

Sepsis is a life-threatening disease induced by an overwhelming immune responses to bacterial infection, including overreactive cellular immune response and proinflammatory cytokine storm that cause injury and disfunctions in key organs including liver, lung, and kidney, the major cause of death in sepsis [39]. Therefore, there is an acute need to develop new approach targeting the inflammatory drivers of sepsis. Although the mechanism of overwhelming immune responses has not been fully understood, macrophages are

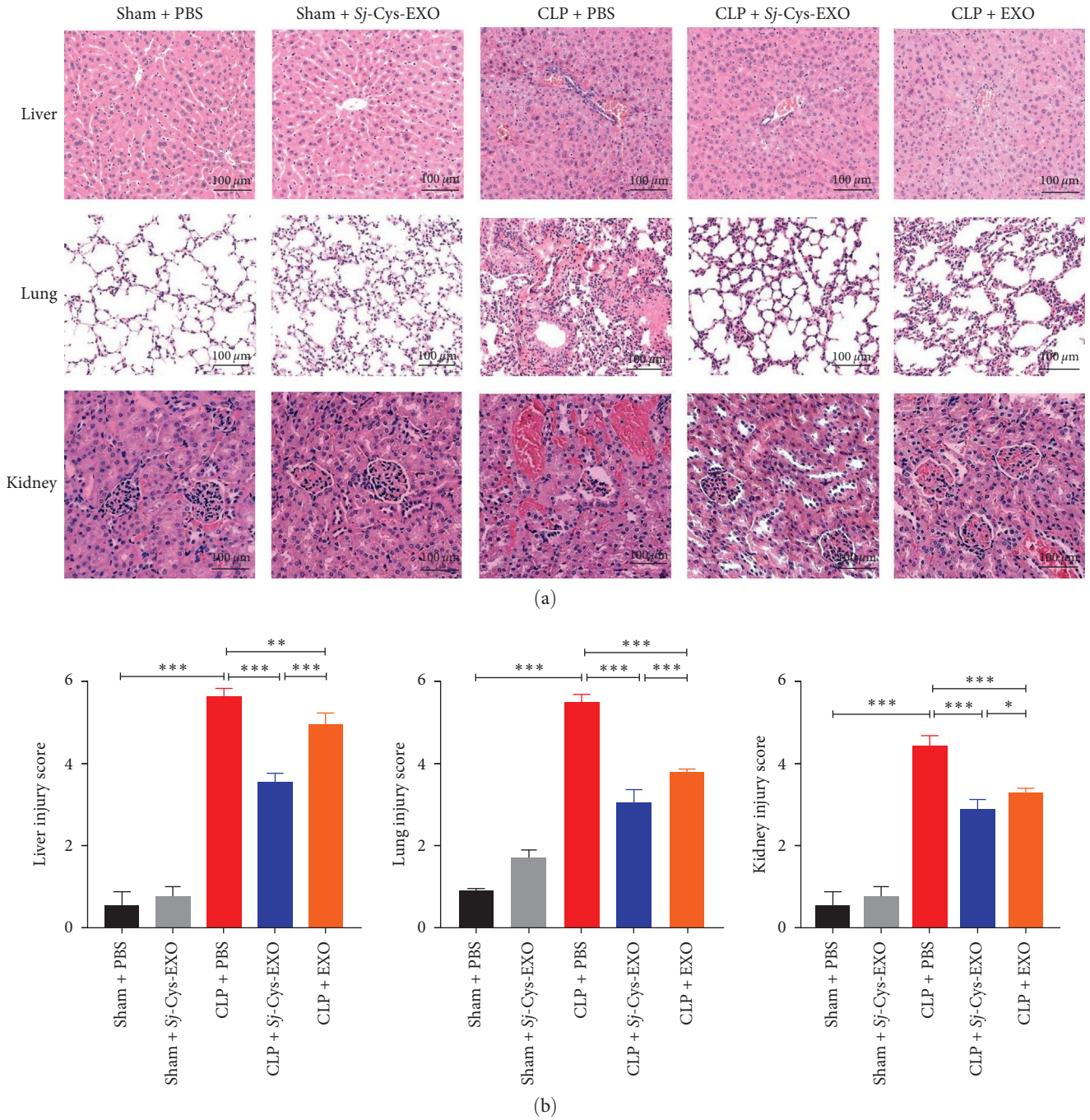

FIGURE 5: Treatment with *Sj*-Cys-EXO reduced liver, kidney, and lung tissue damage caused by CLP-induced sepsis. (a) The representative histopathology results of liver, kidney, and lung stained with H&E from mice 12 hr after CLP or sham surgery treated with *Sj*-Cys-EXO, EXO, or PBS. (b) The pathological score in liver, lung, and kidney from different groups of mice. The magnification ×200, scale bar = 100 μm. For (b), $n = 5$ per group. The results are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

major producers of inflammatory mediators in sepsis and other inflammatory diseases [40]. Our previous study has determined that *Sj*-Cys was a strong immunomodulator that reduces Th1 proinflammatory responses and mitigates the severity of sepsis-induced organ injury and damage mostly through polarizing M1 to M2 macrophages in a mouse model [29]. In this study, we used r*Sj*-Cys to stimulate RAW264.7 macrophage cell line *in vitro* to obtain *Sj*-Cys stimulated macrophage-derived exosomes (*Sj*-Cys-EXO). These exosomes were used to intraperitoneally treat mice with CLP-induced sepsis to determine whether exosomes derived from *Sj*-Cys-treated macrophages can convey the therapeutic efficacy of r*Sj*-Cys to mice with sepsis. Indeed, treating with *Sj*-Cys-EXO significantly decreased the severity of sepsis and enhanced the survival rate of septic mice up to 30% within 72 hr observation period compared to mice

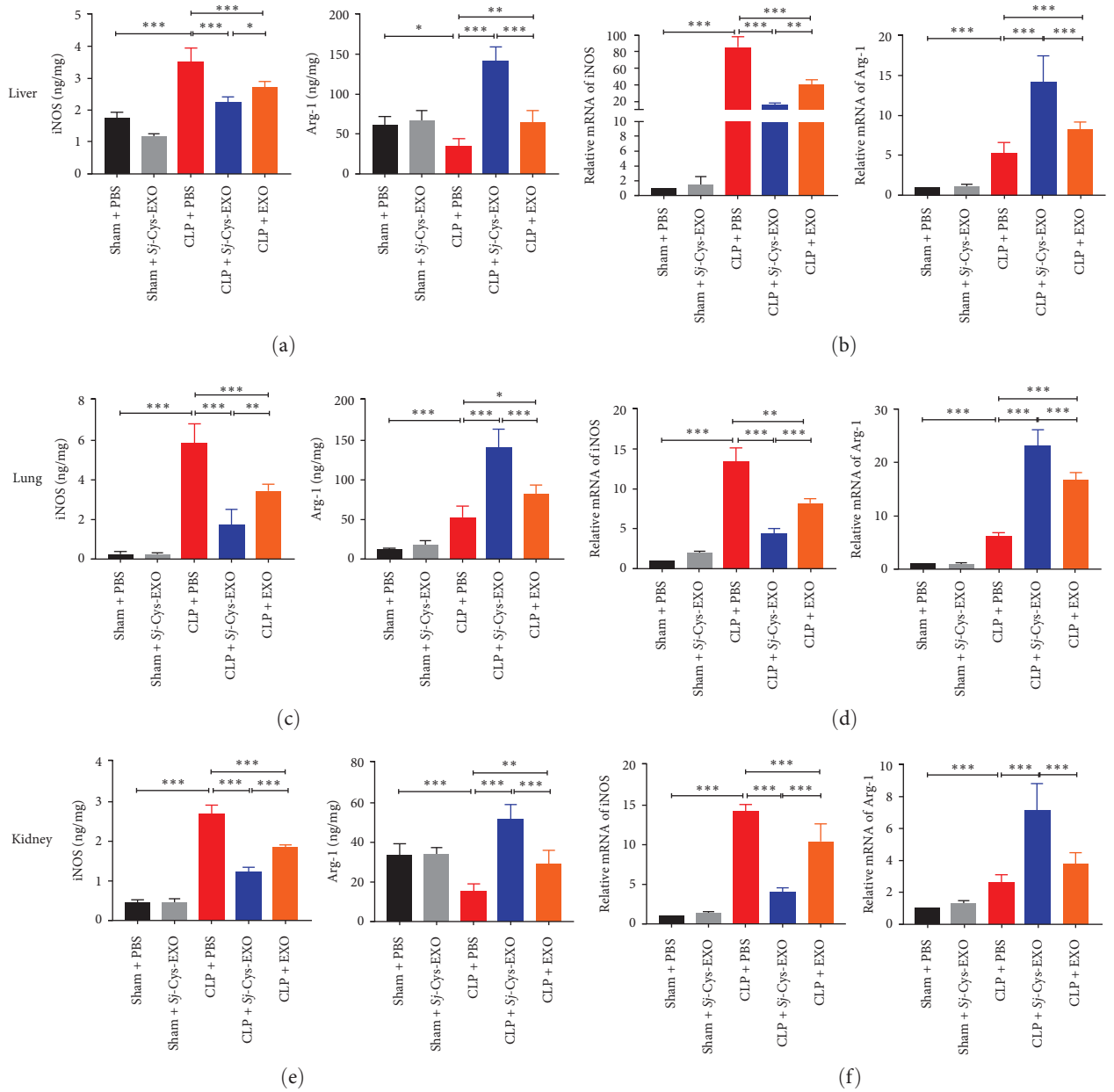

FIGURE 6: Treatment with *Sj*-Cys-EXO significantly induced macrophage polarization from M1 to M2 in liver, lung, and kidney of mice with CLP-induced sepsis identified by measuring protein expression of M1 marker iNOS and M2 marker Arg-1 in liver (a), lung (c), and kidney (e) tissues using ELISA, or measuring mRNA expression levels of these markers in liver (b), lung (d), and kidney (f) tissues by RT-qPCR. $n = 5$ per group. Results are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

without treatment that all died within 48 hr after CLP surgery (Figure 3), which is similar to the results from the direct treatment with *Sj*-Cys recombinant protein itself in our previous study [29]. The increased survival rate seems to be *Sj*-Cys-EXO specific since exosomes derived from naïve RAW264.7 macrophage displaced less therapeutic effect in terms of survival rate. The improved survival rate was also reflected with reduced pathology damage of key organs such as liver, lung, and kidney, with less inflammatory cell infiltration, less congestion and edema, less dead cell, and structural disruption (Figure 5). The reduced organ injury caused by the sepsis was also confirmed by reduced levels of ALT and AST (biomarkers of liver cell damage) and reduced levels of BUN and Cr (biomarkers for kidney function damage) in sera (Figure 4). All results suggest that exosomes derived from r*Sj*-Cys treated macrophage can transfer the anti-inflammatory and therapeutic effect of r*Sj*-Cys to mice with sepsis. We also observe some therapeutic effects of exosomes derived from naïve macrophages on sepsis, possibly because the exosomes from normal macrophage cell line may contain some components directly from natural M2 cells that may partially reduce inflammation.

Further investigation in this study revealed that the immunological mechanism behind the reduced inflammation and tissue damage was related to the polarization of

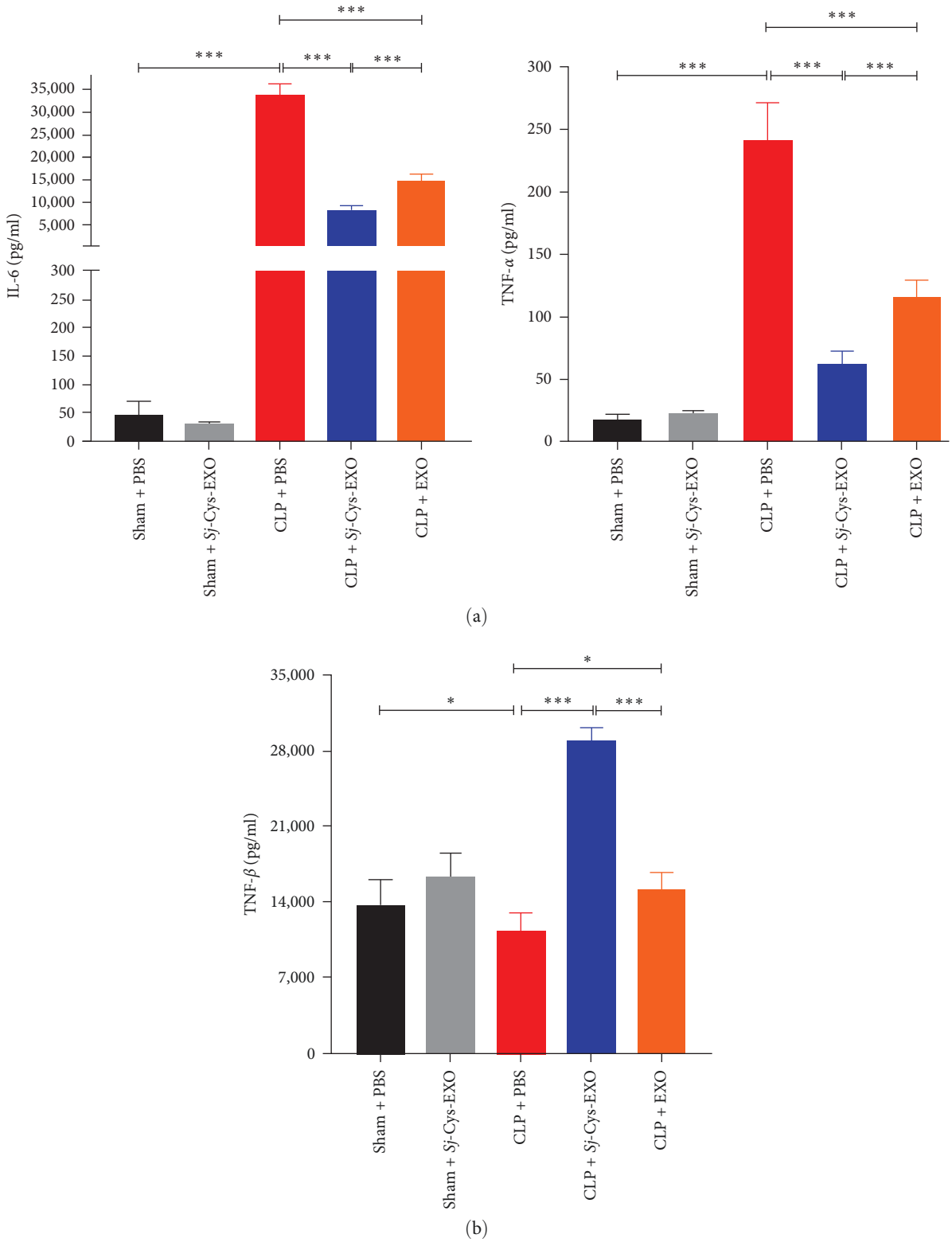

Figure 7: Treatment with *Sj*-Cys-EXO reduced the levels of inflammatory cytokines (IL-6 and TNF-α) and increased the level of TGF-β in sera of mice with CLP-induced sepsis. (a) The levels of inflammatory cytokine IL-6 and TNF-α in sera of each group of mice measured by ELISA with specific antibodies. (b) Level of TGF-β in sera of each group of mice measured by ELISA. $n = 5$ per group. The results are presented as mean $\pm$ SD. $^{*}P < 0.05$, $^{***}P < 0.001$.

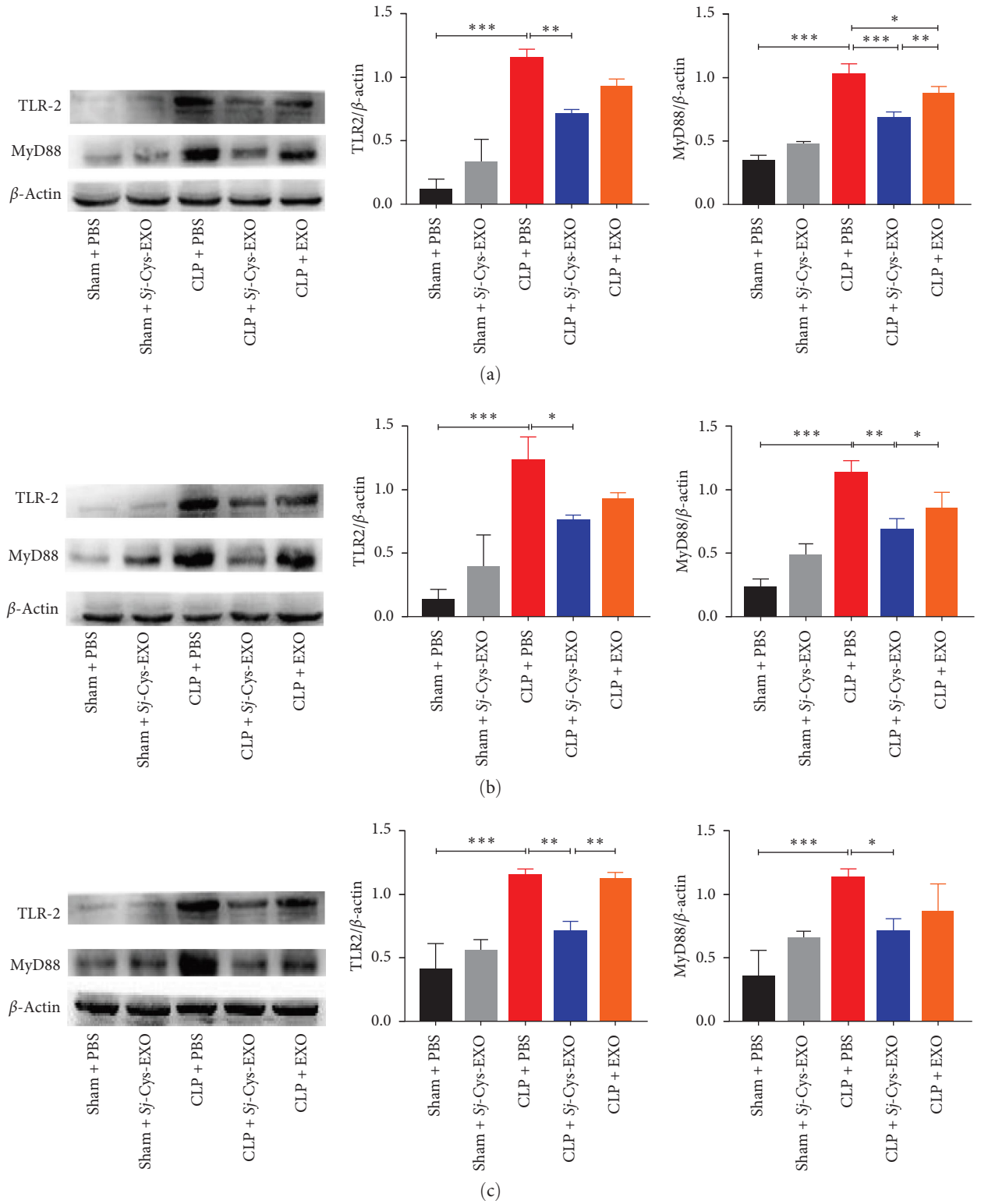

Figure 8: Treatment with *Sj*-Cys-EXO suppressed the expression of TLR-2 and MyD88 in liver (a), lung (b), and kidney (c) of mice with CLP-induced sepsis detected by Western blot. The β-actin was detected as control. The density ratio of TLR-2/β-actin and MyD88/β-actin are shown on the right. The results are shown as the density mean $\pm$ SEM for each group. $n = 3$ per group. The results are presented as mean $\pm$ SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

macrophage from M1 to M2 induced by the *Sj*-Cys-EXO, evidenced by the reduced expression levels (protein and mRNA) of macrophage M1-related markers iNOS and increased expression levels of M2 marker Arg-1 in liver, lung, and kidney tissues. The cytokine profile in sera was consistent with the M2 macrophage polarization in mice treated with *Sj*-Cys-EXO, exhibiting higher level of regulatory cytokine TGF-β and reduced levels of proinflammatory cytokines TNF-α and IL-6 (Figure 7). Therefore, the results clearly show that exosomes derived from *Sj*-Cys treated macrophages transfer the signal to macrophages in mice with CLP-induced sepsis to induce macrophage polarization from M1 to M2 resulting in the inhibition of proinflammatory reaction caused by the bacterial sepsis.

It has been identified that exosomes released into the extracellular environment by different types of immune cells play important roles in intercellular communication by transferring biological information between cells [41–43]. LPS-stimulated M1-type macrophage-secreted exosomes that promote the occurrence of inflammation and cause acute lung injury in mice [44]. However, M2-type macrophage-derived exosomes (M2EXO) played important roles in vascular tissue repair and skin wound healing possibly through reprograming M1-type macrophages to M2-type macrophages [45, 46]. Our findings are in agreement with above investigations and demonstrated that *Sj*-Cys-induced M2-type macrophage polarization that can transfer signals to the local macrophages by releasing exosomes to amplify the anti-inflammatory response and reduce immunopathology in septic mice.

In pathogenesis of sepsis, TLRs are critical in maintaining the delicate equilibrium between immune tolerance and activation [47, 48]. TLR2 is a lipoprotein-specific receptor for host defence against bacterial infection. During bacterial infection, TLR2 is activated that triggers downstream MyD88 signaling pathway and the activation of inflammatory cells. This activation results in the greater synthesis and release of cytokines causing SIRS or multiorgan dysfunction syndrome, the major complication and consequence of bacterial sepsis [49, 50]. There was evidence that *Staphylococcus aureus* released lipoprotein via EV to stimulate TLR2 on innate immune cells to expand inflammatory responses [51]. It was also found that macrophages infected with *Mycobacterium* could release exosomes to stimulate resting macrophages and activate the TLR2/TLR4/MyD88 signaling pathway, thereby promoting the expression of proinflammatory cytokines [52]. Exosomes were also involved in the transcellular transcytosis of peptidoglycan between intestinal epithelium cells to regulate intestinal immune response and homeostasis [53]. To investigate whether exosomes derived from r*Sj*-Cys-treated macrophage alleviate sepsis in mice by inhibiting TLR2/MyD88-dependent signaling pathway, we measured the protein expression levels of TLR2 and MyD88 in liver, lung, and kidney tissues of septic mice after being treated with *Sj*-Cys-EXO. As expected, the TLR2 and MyD88 expressed were significantly elevated in mice with CLP-induced sepsis, indicating the TLR2/MyD88 signaling pathway is activated in septic mice. However, after being treated with *Sj*-Cys-EXO, the protein expression levels of TLR2 and MyD88 in these tissues were significantly reduced, suggesting *Sj*-Cys-EXO regulates macrophage polarization and reduces inflammatory immune responses by inhibiting TLR2/MyD88 signaling pathway.

The results identified in this study clearly demonstrate that the therapeutic effect of *Sj*-Cys on inflammatory sepsis can be conducted by the exosomes released by *Sj*-Cys-activated M2 macrophages. The specific components in the exosomes that conduct immunomodulatory efficacy are under investigation.

5. Conclusions

The findings of this study demonstrated that exosomes derived from *Sj*-Cys treated macrophage (*Sj*-Cys-EXO) attenuate the severity of CLP-induced sepsis in mice by promoting macrophages polarization from M1 to M2 and inhibiting TLR2/MyD88 signaling pathway.

Data Availability

All research data involved in this article were obtained from the article and the submitting authors.

Disclosure

A previous version of this manuscript was published as a preprint [54].

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Xiaodi Yang, Rui Zhou, and Feifei Huang conceived and designed the study. Feifei Huang, Yayun Qian, Qianqian Li, Xiuxiu Li, Xinyue Wu, and Qili Shen performed the experiments. Huihui Li, Liang Chu, and Chen Wan analyzed the data. Feifei Huang and Yayun Qian wrote the manuscript. Bin Zhan and Xiaodi Yang critically revised the manuscript. Feifei Huang and Yayun Qian contributed equally to this work and shared first authorship.

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