

Macrophage-derived exosome piR-50971 exacerbates sepsis-induced myocardial injury by inhibiting autophagy through the upregulation of N6-Methyladenosine modification of mTOR

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Abstract

Background: Sepsis-induced myocardial injury (SIMI) is recognized as a severe complication of sepsis which characterized by a high mortality rate. Notably, the pathophysiology of SIMI involves complex mechanisms, including dysregulation of autophagy. Exosomes contribute to crucial biological processes during sepsis, modulating autophagy processes and facilitating communication between cells. PIWI-interacting RNAs (piRNAs) are highly expressed in myocardial tissue and exert cardiovascular regulation properties. Therefore, we investigated the role of macrophage-derived exosome piRNAs, specifically piR-50971, in SIMI and their impact on autophagy through N6-Methyladenosine (m⁶A) modification of mTOR.

Methods: A cecal ligation and puncture model was established to mimic the pathophysiological features of SIMI. Plasma exosomes were isolated and sequenced to characterize the expression of sepsis-related piRNAs. Bioinformatics analysis was employed to predict the potential regulatory mechanisms involving piR-50971. To investigate the direct interaction between piR-50971 and mTOR, a dual-luciferase reporter assay was conducted. Moreover, a methylated RNA immunoprecipitation assay was conducted to verify the involvement of piR-50971 in the m⁶A methylation modification of mTOR transcripts. Additionally, the m⁶A methylation level was assessed using dot blotting. Left ventricular ejection fraction and left ventricular fractional shortening of rats were detected by animal echocardiography. Transmission electron microscopy was used to detect autophagy flux in the myocardial tissue of rats *in vivo*. Cardiac enzymes were detected using a biochemical analyzer.

Results: piR-50971 was identified as a key piRNA upregulated in plasma exosomes during SIMI, which was correlated with the inhibition of autophagy. Increased macrophage infiltration was observed in the myocardium of rats with SIMI. Additionally, cardiomyocytes treated with macrophage-derived exosomes exhibited impaired autophagy. RNA binding protein immunoprecipitation assay demonstrated an interaction between Wilms' tumor 1-associated protein (WTAP) protein and mTOR mRNA. piR-50971 interacted with mTOR, leading to increased m⁶A modification through the regulation of WTAP and subsequent suppression of autophagy. Notably, this regulation upregulated mTOR translation, thereby inhibiting autophagy and exacerbating myocardial injury under septic conditions. *In vivo* experiments demonstrated that piR-50971 inhibition ameliorated myocardial injury and improved autophagy in rats with SIMI.

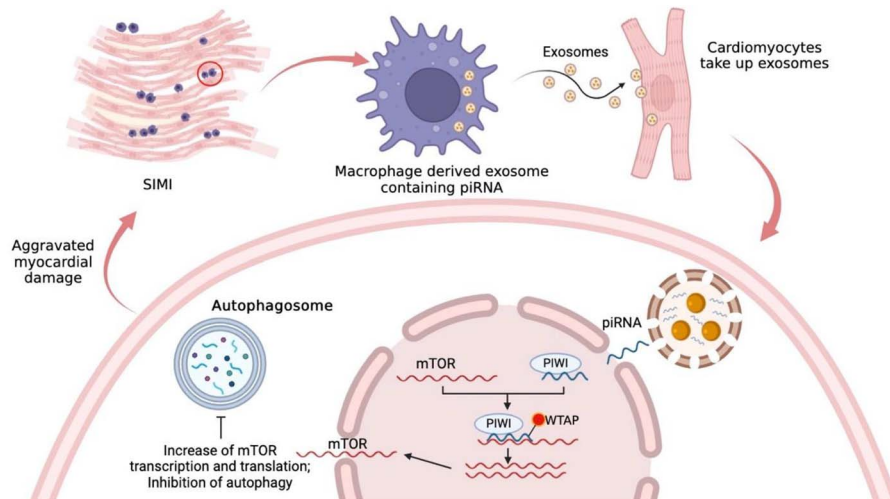
Conclusions: Our findings reveal a novel mechanism by which macrophage-derived exosome piR-50971 contributes to SIMI by suppressing autophagy via m⁶A modification of mTOR. Overall, our results implicate piR-50971 as a potential target for therapeutic intervention in sepsis-related myocardial dysfunction.

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Graphical Abstract



Keywords: Autophagy; mTOR; m⁶A modification; piRNAs; piR-50971; Sepsis-induced myocardial injury; WTAP

Highlights

- This study reveals a novel mechanism by which macrophage-derived exosome piR-50971 contributes to sepsis-induced myocardial injury (SIMI).
- Exosome piR-50971 suppresses autophagy via m⁶A modification of mTOR.
- piR-50971 inhibition ameliorates myocardial injury and improves autophagy in rats with SIMI.

Background

Sepsis is a multifaceted pathological condition triggered by a dysregulated host response to infection, frequently resulting in a cascade of life-threatening complications [1–4]. Sepsis-induced myocardial injury (SIMI) is an important determinant of sepsis regression, which increases the mortality rate from 20% to nearly 70%–90% [5–9]. SIMI has a multifactorial pathophysiology involving inflammatory responses, mitochondrial dysfunction, and immune dysregulation, etc. [10–12]. Autophagy is a process by which cells self-digest and recycle the components. Notably, dysfunctional autophagy has been observed under septic conditions in preclinical studies under septic condition [13, 14]. Therefore, maintaining normal autophagy has been recognized as a critical mechanism mitigating SIMI [15, 16]. The mammalian target of rapamycin (mTOR) plays a critical regulatory role upstream of autophagy signaling, mediating the inhibition of the autophagy initiation complex and consequently suppressing autophagic activity. However, the precise molecular mechanisms underlying autophagy regulation in SIMI remain incompletely unclear.

Exosomes are cup-shaped bilayer membrane extracellular vesicles carrying nucleic acids, lipids and proteins [17], serving as ‘signal messengers’ between cells and the microenvironment. Circulating mononuclear macrophages are the main immune cells that infiltrate the myocardium during SIMI, with an observed increase in exosome production accompanied by a reduction in uptake [18]. PIWI-interacting RNAs (piRNAs) are single-stranded non-coding RNAs (ncRNAs) initially discovered in testicular tissues [19, 20], and are predominantly abundant in the reproductive system, brain and cardiac tissues [21]. Under septic conditions, classical ncRNAs—including

miRNAs, circRNAs, and lncRNAs—are involved in the modulation of autophagy [22, 23]. However, the involvement of piRNAs in the autophagy regulation in SIMI and the potential mechanisms remain undiscovered.

N⁶-methyladenosine (m⁶A) methylation, the predominant modification occurring post-transcriptionally on RNA, is characterized by the methylation of the amino group at the sixth carbon of adenosine and serves essential functions in the regulation of RNA biology [24, 25]. The dynamic and reversible nature of m⁶A modification is governed by three classes of proteins: methyltransferases, demethylases, and readers that bind specifically to m⁶A-modified transcripts. m⁶A regulates programmed cell deaths in SIMI, including apoptosis, autophagy, pyroptosis, and ferroptosis [26]. Moreover, alterations in methylation proteins have been detected in clinical samples, including enhanced expression of Wilms’ tumor 1-associated protein (WTAP) and methyltransferase-like 3 (METTL3) [27]. Autophagy plays a critical role in determining the clinical outcome of sepsis, and this effect may be partly attributed to m⁶A-dependent regulation of autophagy-related gene expression [28]. mTOR signaling plays a role in m⁶A modification [29–33], mostly in cancer field. However, the relationship between m⁶A regulation and mTOR expression in sepsis remains unclear.

piRNAs have been reported to recruit methyltransferases, and facilitate epigenetic modifications and post-transcriptional regulation of RNA, DNA and histones [34]. In the cardiovascular system, the piRNA CHAPIR can recruit METTL3 and enhance m⁶A methylation of PARP10, resulting in cardiac hypertrophy and remodeling [35]. Additionally, piRNAs also exert autophagy-regulating effects, an important area of cancer research [36–38]. However, it

remains unclear whether piRNAs exert a regulatory effect on autophagy signaling via m⁶A modification of target gene mTOR in SIMI. piRNAs have been implicated in modulating mTOR signaling in various clinical diseases, including cancer and the cardiovascular system [39–41]. Although SIMI involves complex and multifactorial mechanisms, emerging evidence suggests that autophagy dysregulation may serve as a promising therapeutic target. Considering the critical involvement of exosomes in intercellular communication during sepsis and the potential regulatory functions of piRNAs in cardiovascular processes, we hypothesized that macrophage-derived exosome piRNAs may contribute to SIMI by inhibiting autophagy. Therefore, we investigated the role of macrophage-derived exosome piRNAs in SIMI and their impact on autophagy through m⁶A modification of mTOR. Overall, the study offers new perspectives on the mechanistic link between exosomal piRNAs and autophagy regulation in SIMI, potentially paving the way for future therapeutic investigations.

Methods

Sequencing and bioinformatic workflow for small RNA analysis

Small RNA sequencing of the rat serum was performed by RiboBIO Guangzhou, China). Briefly, exosomes were extracted from the rat serum by ultrafiltration. Total RNA quality, including concentration and purity, was determined using the Nano 6000 and the Agilent Bioanalyzer System (Agilent Technologies, USA). For sequencing, 500 ng of RNA was used as input material, followed by library quality assessment. Thereafter, high-quality reads were sequenced using a HiSeq 2500 V4 (Illumina, USA).

Cell culture

H9C2 and AC16 cell lines were obtained from Fuheng Biology (China). H9C2 cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM), while AC16 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS). All cells were incubated at 37°C in a humidified incubator with 5% CO₂. Cells were collected for subsequent experiments when they reached ~80% confluency.

Animals

Male Sprague–Dawley rats (8 weeks old, ~300 g) were purchased from GemPharmatech Co., Ltd (Nanjing, China). All animals were housed and maintained under specific pathogen-free conditions at the Animal Science Centre of Ruijin Hospital, affiliated with Shanghai Jiao Tong University School of Medicine (Shanghai, China). All animal procedures were conducted in strict accordance with the ethical guidelines established by the Animal Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. The experimental protocol was reviewed and approved by the committee, ensuring ethical justification of all procedures and minimization of animal suffering.

Surgical procedure

A rat model of SIMI was generated using the cecal ligation and puncture (CLP) method. Rats were randomly allocated to either the Sham or CLP group. To establish a sublethal sepsis

model, rats were anesthetized via intraperitoneal injection of 0.3% pentobarbital sodium at a dose of 30 mg/kg. A midline laparotomy was then performed to expose the cecum, which was ligated at ~75% of its length and punctured once with a 7-gauge needle. A small amount of fecal content was gently extruded to ensure patency before the cecum was returned to the abdominal cavity. Small-animal echocardiography was performed 24-hour post-CLP model establishment. Blood samples were collected from the abdominal aorta, while left ventricular myocardial tissues were harvested and immediately snap-frozen in liquid nitrogen for subsequent analysis. Bupivacaine and buprenorphine were subcutaneously administered to alleviate postoperative pain. Following injury, all rats received intraperitoneal fluid resuscitation with lactated Ringer's solution at a dose of 30 ml/kg body weight and were subsequently housed individually. All surgical procedures were performed under aseptic conditions by experienced personnel. Notably, the CLP procedure was carefully controlled to induce sublethal sepsis and avoid excessive trauma. The animals were maintained under controlled environmental conditions with free access to food and water throughout the duration of the experiment. Additionally, the rats were monitored daily for signs of distress, and animals showing severe discomfort were euthanized.

Western blotting

Protein samples extracted from cellular or cardiac tissues were quantified using a BCA kit (CST, USA). Proteins were mixed with loading buffer and denatured at 100°C for 5 minutes prior to separation by SDS-PAGE. Subsequently, the proteins were transferred onto PVDF membranes. The membranes were then incubated overnight at 4°C with primary antibodies against mTOR, PIWIL2, and WTAP (all from Proteintech, USA), as well as p62 and LC3 I/II (both from Cell Signaling Technology, USA). Following five washes with TBST, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection kit.

Total RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from myocardial tissues of mice in the LPS (n=4) and control (n=4) groups, as well as from AC16 cells, using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Complementary DNA was synthesized via reverse transcription using a reverse transcription kit (Vazyme, China). Quantitative real-time PCR (qRT-PCR) was performed on a QuantStudio 3 system (Thermo Fisher Scientific, USA) using SYBR Green Premix (Vazyme, China). Relative gene expression levels were calculated using the 2^{-ΔΔCt} method, with GAPDH or U6 serving as internal controls. Primer sequences for piRNAs and mRNAs are listed in Tables S1 and S2, respectively.

piR-50971 mimic and inhibitor transfection

piR-50971 mimic and inhibitors were procured from Calmbio Technology (Shanghai, China). Briefly, AC16 cells were seeded into 6-well plates at a density of 1 × 10⁶ cells per well and cultured until reaching 70–90% confluency. For transfection,

3.75 μ l or 7.5 μ l of Lipofectamine 3000 reagent was diluted in 125 μ l of Opti-MEM (a reduced-serum medium) within a sterile Eppendorf tube. Additionally, piR-50971 mimic (final concentration 30 nM) or inhibitor (final concentration 50 nM) and 5 μ l of P3000 reagent was diluted in 125 μ l of Opti-MEM medium. Thereafter, the solution containing the diluted piR-50971 mimic or inhibitor and P3000 reagent was combined with the diluted Lipofectamine 3000 reagent, and the mixture was gently mixed, followed by incubation at room temperature for 15 min. The incubated complex was introduced into the cell culture wells, followed by gentle vortexing. After ensuring that the complex was evenly distributed throughout the wells, the cell culture plates were maintained at 37°C under a 5% CO₂ condition for 2 days, after which the transfected cells were analyzed.

RNA binding protein immunoprecipitation assay

RNA binding protein immunoprecipitation (RIP) assays were conducted using the Magna RIP Kit (Millipore, USA) according to the manufacturer's instructions. Briefly, protein A/G magnetic beads conjugated with either control IgG or WTAP antibodies (Proteintech, USA) were incubated with cell lysates at 4°C overnight. Following six washes to remove non-specific bindings, the RNA-protein complexes were treated with proteinase K buffer for subsequent RNA isolation. Thereafter, RNA was extracted for quantitative PCR (qPCR) to assess the relative interactions.

Methylated RNA immunoprecipitation (MeRIP)

After RNA extraction, the total m⁶A level was determined using the m⁶A RNA methylation quantitative kit (Epigen-tek, USA), according to the manufacturer's protocol. Briefly, 1000 ng of RNA was transferred to the assay wells, followed by the separate addition of appropriate concentrations of capture and detection antibodies. qPCR was performed to quantify the m⁶A levels of mTOR.

Confocal microscopy

Myocardial cells were cultured on glass-bottom dishes and subsequently infected with RFP-GFP-LC3 lentivirus for a duration of 48 hours. Thereafter, the cells were fixed with paraformaldehyde and stained with DAPI to facilitate nuclear examination. Autophagic flux was assessed using confocal laser scanning microscopy (Leica, USA).

Immunofluorescence assay

Briefly, the cells were harvested, fixed in 4% paraformaldehyde for 30 minutes, deparaffinized, and treated with sodium citrate for antigen-retrieval. After placing on slides, the cells were treated with 3% hydrogen peroxide, permeabilized with 0.1% Triton X-100, followed by blocking with 10% bovine serum albumin (BSA) at room temperature. Subsequently, the cells were incubated overnight at 4°C with the appropriate primary antibodies. After five washes with PBS, the cells were incubated with corresponding fluorophore-conjugated secondary antibodies for 1 hour at room temperature, followed by nuclear staining with DAPI.

Immunohistochemical assay

Briefly, the slides were preprocessed according to the methodology outlined in the immunofluorescence assay section. Thereafter, the slides were incubated with primary antibodies overnight at 4°C, followed by incubation with

appropriate secondary antibodies. Subsequently, the slides were developed using 3,3'-diaminobenzidine tetrahydrochloride as the chromogenic substrate and visualized under an immunofluorescence microscope.

Dot blot assay

Dot blot assay was performed to identify global m⁶A levels in left ventricle tissues as well as in H9C2 and AC16 cells treated with or without exosomes. Total RNA was extracted, denatured, and transferred to a Hybond N+ membrane (Amersham, UK). Thereafter, the membrane was crosslinked under ultraviolet light for 30 minutes, blocked with 5% BSA, and incubated with an anti-m⁶A antibody (Proteintech, USA) and a secondary antibody. Finally, m⁶A dots were assessed using ECL chemiluminescence.

m⁶A modification sites prediction

The prediction of m⁶A modification sites on the mTOR mRNA was conducted using SRAMP (<http://www.cuilab.cn/sramp/>), an online tool that integrates sequence features and machine learning algorithms to identify putative m⁶A sites.

Dual-luciferase reporter assay

The binding sites of the piR-50971 target gene mTOR were predicted using the miRanda algorithm. Specifically, the mTOR coding sequence (CDS) reporter vectors were constructed using Calmbio (Shanghai, China). 293 T cells were cultured in 12-well plates to 80% confluence and subsequently co-transfected with either wild-type (mTOR-WT) or mutated (mTOR-Mut) reporter vectors, along with piR-50971 mimic or negative control (NC) using Lipofectamine 3000. Luciferase reporter assays were conducted 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega, USA), following the manufacturer's instructions. Luminescence was measured with a microplate luminometer, and firefly luciferase activity was normalized to Renilla luciferase activity to control for transfection efficiency.

In vivo transfection of piR-50971 inhibitor

In vivo transfection experiment was performed to investigate the effects of piRNA inhibitors. Briefly, 12 rats were randomly allocated to three groups: Sham, CLP, and CLP+piR-50971 inhibitor. Mice in the CLP+piR-50971 inhibitor group were transfected with the transfection reagent (Entranster™) and piR-50971 inhibitor on the first day via the caudal vein. Rats in the CLP and CLP+piR-50971 inhibitor groups underwent cecal ligation and perforation on the second day, whereas those in the sham group underwent laparotomy, separation of the distal cecum, and subsequent abdominal closure. A subcutaneous injection of 30 ml·kg⁻¹ saline solution was administered immediately after the surgical procedures for resuscitation purposes.

Small animal cardiac ultrasound

A day before cardiac ultrasound, the rats were depilated in the chest region. Anaesthesia was administered using 3% isoflurane for induction and 2% isoflurane for maintenance throughout the procedure. A conductive gel was applied to the chest skin and an ultrasound probe was placed on the gel-covered area. M-mode echocardiographic recordings were obtained from the parasternal long-axis view to assess left ventricular systolic function. Parameters including ejection

fraction (EF) and fractional shortening (FS) were calculated using standard formulas.

Transmission electron microscopy

Transmission electron microscopy (TEM) was employed to examine autophagosomes in cardiac tissue. Briefly, myocardial samples were fixed overnight at 4°C in a solution containing paraformaldehyde and glutaraldehyde, followed by graded dehydration and embedding in epoxy resin. Ultrathin sections were then prepared and subjected to double staining with uranyl acetate and lead citrate prior to imaging, followed by examination under a ZEISS Xradia 520 Versa electron microscope (ZEISS, Germany).

Acquisition of exosome

Human monocytic leukemia Tohoku Hospital Paediatrics-1 (THP-1) cells were obtained from the Chinese Academy of Sciences (Shanghai, China) and used for exosome collection. Briefly, 1×10^7 THP-1 cells were seeded into a 75 cm² culture flask containing 10 ml of complete DMEM supplemented with 100 µg/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, USA) to induce cell adherence. After 24 hours of induction, the medium was removed, and the cells were washed three times with PBS. The medium was then replaced with 10 ml of serum-free RPMI 1640 medium for subsequent exosome isolation. The cells were then randomly assigned to either a control group (CTL-Exo, receiving no treatment) or an LPS-treated group (LPS-Exo, with the medium adjusted to a final LPS concentration of 1 µg/ml).

To collect serum samples, rats in the sham operation ($n = 3$) and CLP surgery ($n = 3$) were anesthetized with 3% pentobarbital sodium 48-hour post-surgery. Thereafter, a needle attached to a blood collection tube containing an anticoagulant was inserted into the abdominal cavity of the rats for blood sample collection. After blood collection, the anticoagulant tube was inverted and centrifuged at 2000 rpm for 15 minutes to collect the plasma, which was used for further exosome extraction.

Exosomes extraction

Exosomes were purified from the collected supernatants using an ultrafiltration method, which involved sequential centrifugation and membrane filtration to concentrate extracellular vesicles of appropriate size. Briefly, the supernatant of the culture medium or plasma supernatant was transferred to an AU-15 ultrafiltration tube (10 kDa MWCO, Amicon Ultra) and centrifuged at 4000 g for 30 minutes at 4°C. Thereafter, the ultrafiltration device was removed from the centrifuge, and the liquid in the collection tube was discarded. PBS (14 ml) was added to the filtration device and gently aspirated multiple times with a pipette, followed by centrifugation at 4000 g for 30 minutes at 4°C to recover concentrated exosomes from the filtration device. Notably, the concentration of exosome used in the study was 100 µg/ml.

WTAP plasmid transfection

WTAP overexpression and knockdown plasmids were purchased from OBiO Technology (Shanghai, China) and Beyotime Biotechnology (China), respectively. AC16 cells were seeded in 6-well plates at a density of 1×10^6 cells per well and cultured until reaching 70–90% confluency. For transfection, 3.75 µl or 7.5 µl of Lipofectamine™ 3000 (Invitrogen, USA) was diluted in 125 µl of Opti-MEM™ reduced-serum medium

(Gibco, USA) in a sterile Eppendorf tube. In a separate sterile tube, 2500 ng of plasmid DNA and 5 µl of P3000™ reagent were diluted in another 125 µl of Opti-MEM. The DNA-P3000 solution was then gently added to the diluted Lipofectamine 3000 reagent and incubated at room temperature for 15 minutes to allow complex formation. The transfection mixture was subsequently added to the cells, and the plate was gently rocked to ensure even distribution. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 48 hours prior to downstream analysis.

Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 9. Comparisons between two groups were conducted using Student's t-test, while differences among multiple groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A P -value < 0.05 was considered statistically significant. Significance levels are indicated as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Results

RNA-seq demonstrated the connection between piRNAs and autophagy signaling in rats with SIMI

To explore the potential roles of piRNAs in autophagy regulation, exosomes were isolated from the serum of rats in the sham-operated group ($n = 3$) and the CLP-induced sepsis group ($n = 3$) using an ultrafiltration-based protocol. TEM showed that the extracted exosomes displayed a disk-like morphology, with diameters ranging from 40 to 100 nm and expressing specific markers. Small RNA transcriptome sequencing was conducted, followed by differential expression analysis to identify miRNAs and piRNAs exhibiting significant expression changes in plasma-derived exosomes. Notably, the numbers of miRNAs and piRNAs with log₂ fold change ≥ 1 in the CLP model vs. control groups were 279 and 1190, respectively. Additionally, the numbers of miRNAs and piRNA with P < 0.05 in the CLP model vs. control groups were 173 and 232, respectively (Figure 1a). Importantly, we identified a larger number of differentially expressed piRNAs than miRNAs in the sepsis model, suggesting that piRNAs significantly contribute to the regulatory landscape of SIMI. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed to investigate the functional enrichment of identified piRNAs. The results demonstrated significant enrichment of piRNAs in autophagy-related pathways and the mTOR signaling pathway (P < 0.05), suggesting their potential involvement in regulating these critical biological processes (Figure 1b and c). Additionally, the mTOR signaling pathway and autophagy emerged as key terms within the environmental information processing category in the KEGG database (Figure 1d).

Macrophage derived exosomes inhibit myocardial autophagy

HE staining revealed prominent pathological features of SIMI in rats subjected to CLP, including increased macrophage infiltration and elevated expression of the macrophage marker CD68 compared to the sham-operated group (Figure 2a). Furthermore, western blot analysis was conducted to evaluate autophagy-related protein levels in septic myocardial tissue.

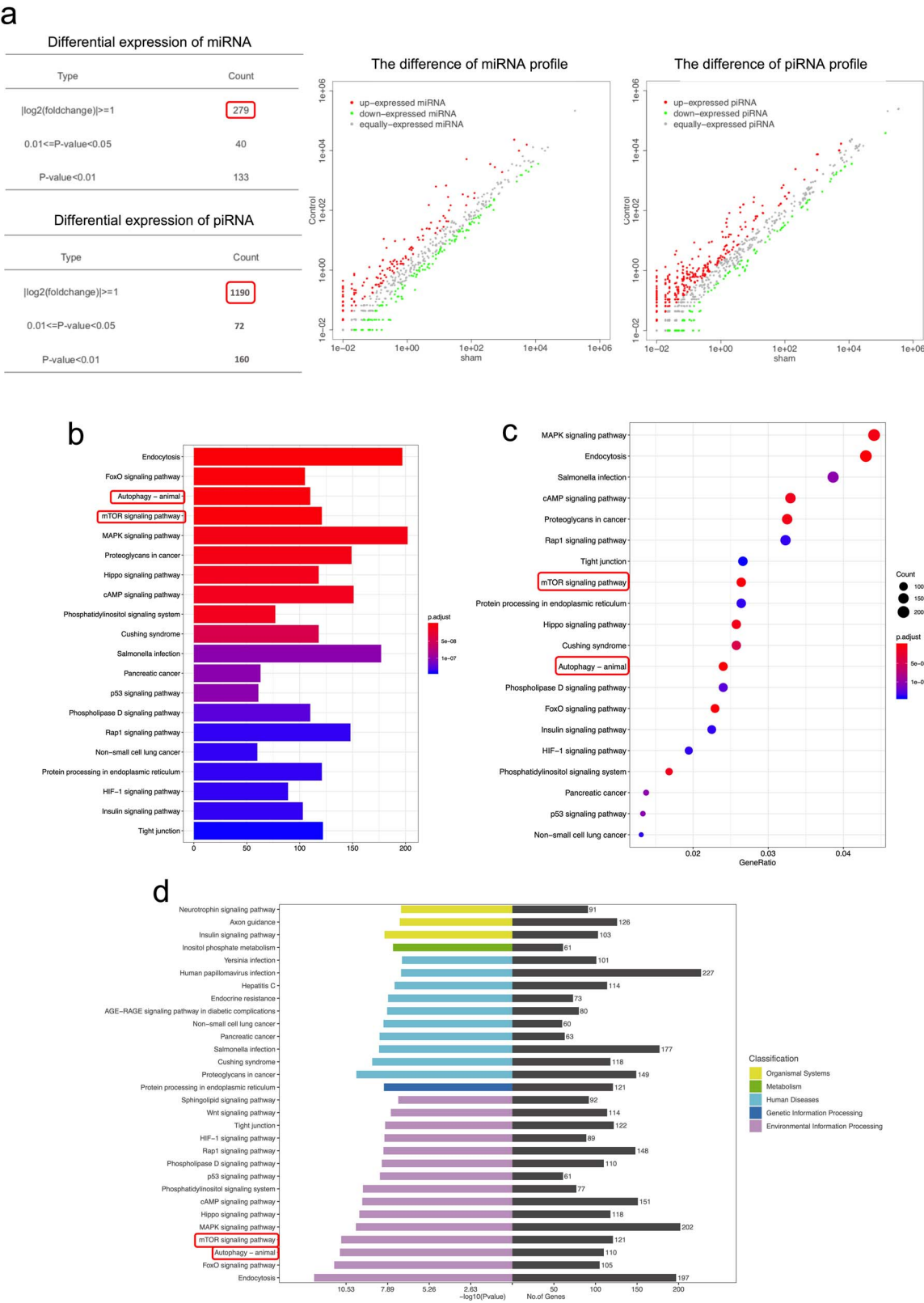


Figure 1. Small RNA-seq of piRNA and miRNA expressions in rats with CLP-induced sepsis. **(a)** Differential expression of piRNAs and miRNAs in plasma exosomes from the CLP and sham groups. **(b)** KEGG bar plot showed gene count in enriched autophagy and mTOR signalling pathways. **(c)** KEGG dot plot highlighted gene ratio in key pathways of autophagy and mTOR. **(d)** KEGG analysis of environmental information progress shows that mTOR signalling pathway and autophagy are among the cardinal terms. *piRNA* piwi-interacting RNA, *miRNA* micro RNA, *CLP* cecal ligation and puncture, *mTOR* mammalian target of rapamycin

Relative to the sham group, myocardial tissues from CLP rats exhibited upregulated mTOR and p62 expression alongside a decreased LC3 II/I ratio, indicative of autophagy inhibition (Figure 2b).

To investigate the effects of macrophage-derived exosomes on myocardial cells, the rat myocardial cell line H9C2 and human myocardial cell line AC16 were separately co-cultured with exosomes derived from PMA-stimulated THP-1 cells (CTL-Exo) or PMA- and LPS-stimulated THP-1 cells (LPS-Exo). Western blotting revealed that there was a significant increase in the expression of the autophagy-related proteins mTOR and p62 and a decrease in LC3 II/I expression compared with those in the CTL (no exosomes) and CTL-Exo groups (Figure 2b).

Additionally, we investigated the autophagic flux in the presence of exosomes derived from LPS-stimulated macrophages. PKH26-labeled exosomes derived from macrophages were effectively taken up by AC16 cells (Figure 2c). Additionally, AC16 cells were transfected with a lentivirus encoding the RFP-GFP-LC3 fusion protein to monitor autophagy. In the CTL-Exo group, the formation of autophagic lysosomes resulted in the quenching of GFP fluorescence owing to the acidic environment within the lysosomes, leading to a predominance of red spots in the merged images. Conversely, the formation of autophagic lysosomes was inhibited in the LPS-Exo group, resulting in an increased number of yellow spots in the merged images (Figure 2d). Collectively, our findings indicate that cardiomyocytes can take up macrophage-derived exosomes, which inhibits autophagic flux.

PIWIL2 is highly expressed in the myocardium of rats with SIMI

piRNAs exert their biological functions through interactions with PIWI proteins. Recent studies have demonstrated that PIWI family proteins, including PIWIL1, PIWIL2, and PIWIL4, are expressed not only in testicular tissues but also in various other tissues. Our analysis demonstrated a significant upregulation of PIWIL2 mRNA expression in cardiac tissue (Figure 3a). Western blotting indicated a marked increase in PIWIL2 expression in myocardial tissue in the CLP-induced sepsis group (Figure 3b and 3c). Additionally, immunofluorescence assay was performed to assess PIWIL2 expression in myocardial tissue and AC16 cells. PIWIL2 expression was significantly elevated in the myocardial tissue of rats subjected to CLP compared to the sham group (Figure 3d). Likewise, co-culture with LPS-derived exosomes (LPS-Exos) markedly upregulated PIWIL2 expression in AC16 cells relative to the CTL-Exos group (Figure 3e).

Macrophage derived exosome piR-50971 inhibits autophagy via mTOR signaling

Based on small RNA sequencing data, we constructed a Venn diagram to identify the intersection of piRNAs associated with autophagy, the mTOR signalling pathway, and the top 10 piRNAs. Notably, six piRNAs were identified: piR-50971, piR-53 694, piR-44 502, piR-62 717, piR-56 067, and piR-56 326 (Figure 4a). Additionally, the expression of piR-50971 in cardiac tissue was assessed in both the sham and CLP groups using quantitative PCR. The results revealed a significant upregulation of piR-50971 in the CLP group compared to the sham group (Figure 4b). In contrast, no

significant differences were observed in the expression levels of the other five candidate piRNAs between the two groups (Figure S1). Based on the small RNA sequence data, piR-50971 was highly expressed in serum exosomes in the CLP group (Figure 4c). To verify whether the difference in piR-50971 expression was due to macrophage-derived exosomes under septic conditions, we compared piR-50971 expression levels in the CTL-Exos and LPS-Exo groups. qPCR analysis demonstrated that piR-50971 expression was significantly elevated in the LPS-Exo group compared to the CTL-Exo group (Figure 4d). Consistent with these findings, piR-50971 expression was markedly increased in AC16 cells following treatment with LPS-derived exosomes (Figure 4e).

Based on the exosome sRNA sequence results, we constructed a Venn diagram to identify the intersection between piR-50971 and autophagy-related genes. Importantly, the intersection revealed four target genes: mTOR, RPTOR, MAPK9, and MAPK10 (Figure 4f). Therefore, we hypothesized that the macrophage-derived exosome piR-50971 inhibits autophagy by interacting with its target gene mTOR. To further investigate the role of piR-50971 in mTOR signaling and its effect on autophagy, we designed and synthesized piR-50971 mimic and an piR-50971 inhibitor. Notably, the transfection efficiency of the mimic and inhibitor was confirmed 24-hour post-transfection (Figure 4g). Additionally, both qPCR and western blot analyses revealed that mTOR mRNA and protein expression levels were upregulated following transfection with the piR-50971 mimic, and downregulated upon treatment with the piR-50971 inhibitor (Figure 4h). Consistent with the findings in the LPS-Exo group, transfection with piR-50971 mimic inhibited autophagy, as evidenced by p62 and LC3 II/I proteins expression levels (Figure 4i). Immunofluorescence assay revealed that transfection with piR-50971 mimic impaired autophagic flux, whereas transfection with piR-50971 inhibitors improved it (Figure 4j). Collectively, these findings suggest that the piR-50971 mimic inhibits autophagy by regulating the mTOR pathway.

Additionally, we predicted the presence and location of binding sites between piR-50971 and mTOR using miRanda software. Importantly, a single binding site was identified within the mTOR CDS (Figure 4k). Moreover, we conducted a luciferase assay to confirm the interaction between piR-50971 and mTOR in 293 T cells. The fluorescence activity in the piR-50971 transfection group was significantly diminished, suggesting a targeted binding interaction between piR-50971 and mTOR (Figure 4l).

piR-50971 inhibiting autophagy level via upregulation of methyrase WTAP

Recent studies have indicated that autophagy plays a significant role in determining the clinical outcomes of patients with sepsis, with enhanced autophagic activity being closely associated with improved prognosis. Additionally, detailed transcriptome map analyses suggest a potential correlation between the regulation of m⁶A methylation and autophagy-associated genes [42]. In this study, both mRNA and protein levels of WTAP were significantly upregulated in AC16 cells treated with LPS-derived exosomes compared to controls (Figure 5a and b). Additionally, transfection with piR-50971 mimic upregulated WTAP protein expression in AC16 cells (Figure 5b). To investigate the role of WTAP in

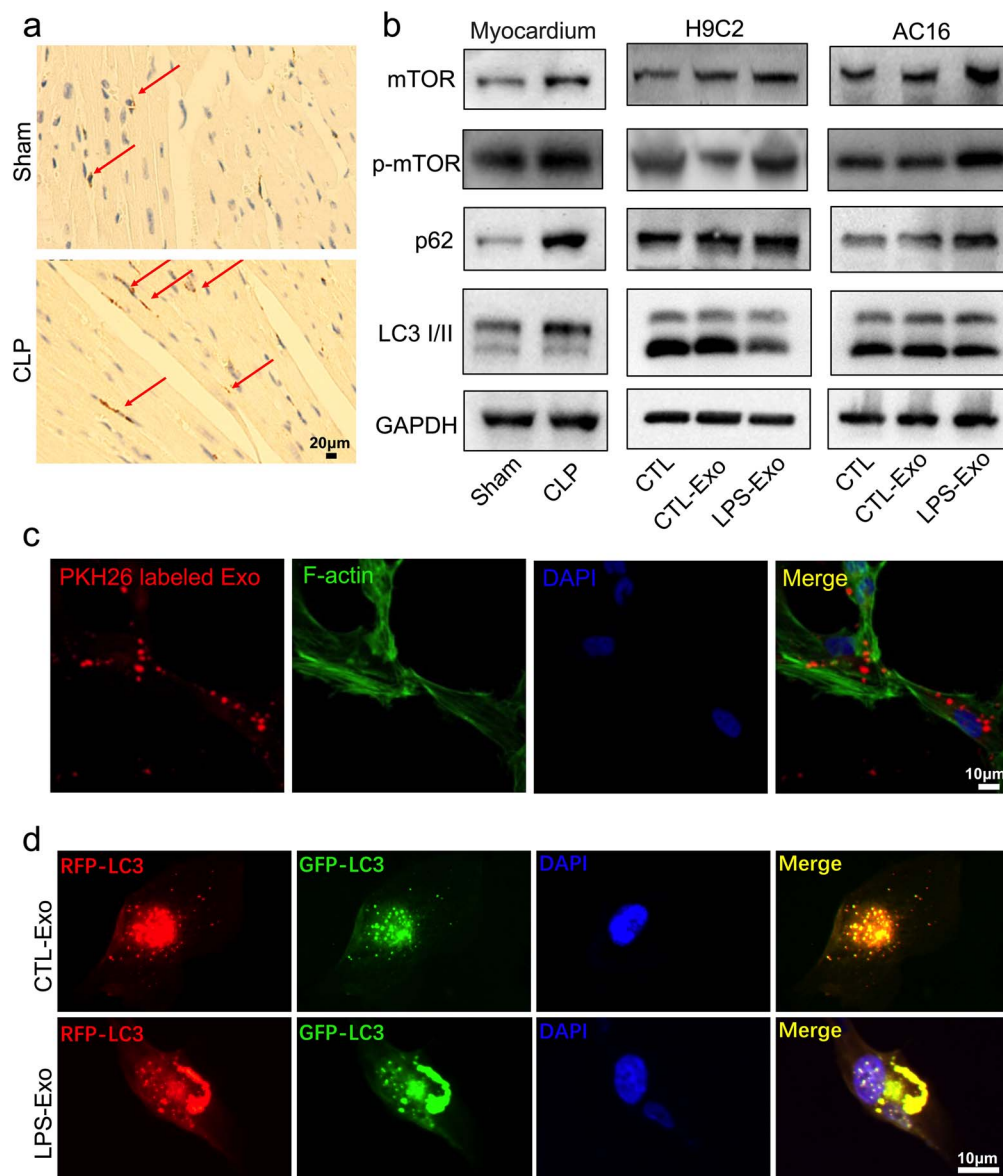


Figure 2. Macrophage derived exosomes inhibits autophagy in myocardial cells. **(a)** Immunohistochemical staining showed that the infiltration of macrophage marker CD68 increased in the myocardial tissue of rats with SIMI. **(b)** Western blot analysis showed autophagy inhibition in the myocardial tissue of rats with SIMI, as well as in H9C2 and AC16 cells (20X). **(c)** Macrophage derived exosomes labelled with PKH26 can be taken up by AC16 cells. **(d)** LPS-Exo stimulated myocardial AC16 cells showed inhibited autophagy flux (40X). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). *SIMI* sepsis-induced myocardial injury, *LPS* Lipopolysaccharide, *Exo* exosome

the regulation of autophagy, we conducted rescue studies of either overexpressing and knocking down WTAP in LC3 lentivirus transfected AC16 cells. WTAP overexpression significantly elevated mTOR and autophagy-related protein expression, increased P62 and decreased LC3II/I ratio, whereas WTAP knockdown reversed the properties and ameliorated autophagy (Figure 5c) in AC16 cells. Although WTAP overexpression significantly inhibited autophagy flux in AC16 cells, WTAP knockdown restored the autophagic flux (Figure 5d).

To further investigate piR-50971-mediated regulation of autophagy via WTAP, we downregulated WTAP in piR-50971 mimic-transfected AC16 cells or upregulated WTAP in piR-50971 inhibitor-transfected AC16 cells. WTAP knockdown significantly decreased mTOR expression and activation, downregulated autophagy-related proteins and

increased LC3II/I ratio in AC16 cells transfected with piR-50971 mimic. WTAP overexpression significantly increased mTOR expression and activation, upregulated autophagy-related proteins and decreased LC3II/I ratio in AC16 cells transfected with piR-50971 inhibitor (Figure 5e). Autophagy flux assay showed that piR-50971 mimic significantly inhibited autophagic flux in AC16 cells, whereas WTAP downregulation markedly restored autophagic flux. Although the piR-50971 inhibitor restored autophagic flux in AC16 cells, upregulation of WTAP significantly inhibited autophagic flux (Figure 5f).

piRNA-50971 enhances m⁶A methylation level of mTOR via WTAP

Since piR-50971 inhibited autophagy via upregulation of WTAP, we consequently investigated whether WTAP altered

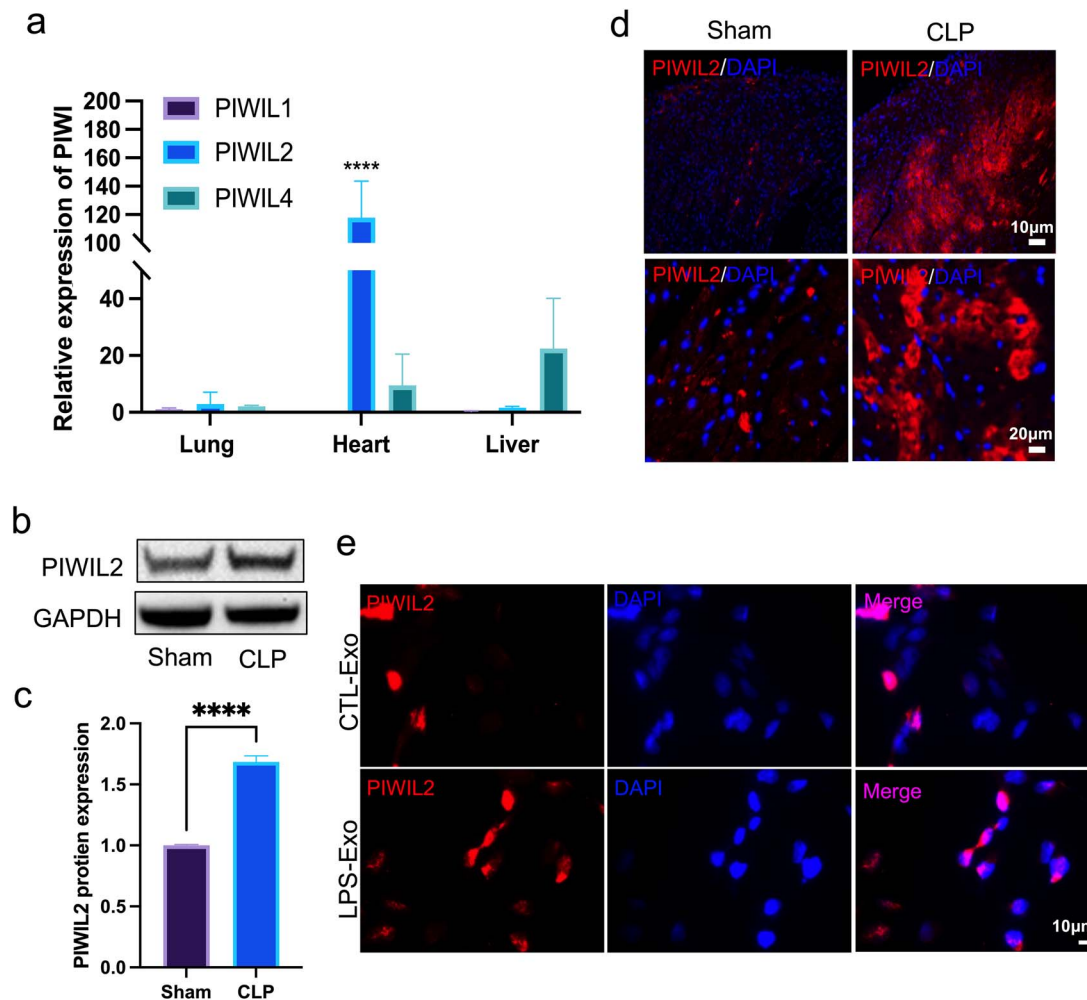


Figure 3. PIWIL2 is highly expressed in myocardial cells and tissue in mice with SIMI. (a) qPCR results indicated that PIWIL2 expression was significantly elevated in the myocardial tissue under septic conditions. (b–c) Western blot analysis verified increased PIWIL2 expression in myocardial tissue of rats with SIMI. (d) Immunofluorescence assay showed enhanced expression of PIWIL2 in the myocardial tissue of rats with SIMI. (e) Immunofluorescence assay showed that incubation with LPS-Exo markedly increased PIWIL2 expression in AC16 cells compared with that in the CTL-Exo incubation. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). SIMI sepsis-induced myocardial injury, PIWIL2 piwi-like protein 2, LPS lipopolysaccharide, Exo exosome, qPCR quantitative polymerase chain reaction

m⁶A modification of the target gene mTOR. Subsequently, we first conducted a dot blot assay to assess total m⁶A levels. The results demonstrated a significant elevation in total m⁶A methylation levels in the myocardial tissue of rats subjected to CLP, as well as in LPS-Exo-treated and piR-50971 mimic-transfected AC16 cells (Figure 6a). Furthermore, we predicted the m⁶A sites of mTOR and associated confidence scores using SRAMP. Notably, we identified several m⁶A sites within mTOR, including five sites with very high confidence scores (8249, 8240, 8104, 8020, and 7927) as shown in Figure 6b, with the specific structures of the five high-confidence sites displayed in Figure 6c. Consequently, we designed a pair of primers encompassing these five high-confidence sites to further investigate the alteration of m⁶A levels in mTOR using MeRIP-qPCR assay following an established protocol. LPS-Exo treatment and piR-50971 mimic transfection elevated m⁶A modification level in mTOR (Figure 6d and 6e). Additionally, RIP-qPCR assay showed that transfection with piR-50971 mimic enhanced the binding of WTAP protein to mTOR mRNA, thereby facilitating m⁶A methylation of mTOR (Figure 6f). To validate WTAP-mediated regulation of

m⁶A modification of mTOR, we measured m⁶A level in piR-50971 mimic-transfected AC16 cells following WTAP downregulation and in piR-50971 inhibitor-transfected AC16 cells following WTAP upregulation. WTAP downregulation in piR-50971 mimic-transfected AC16 cells significantly decreased m⁶A level of mTOR. In contrast, WTAP upregulation in piR-50971 inhibitor-transfected AC16 cells significantly increased m⁶A level of mTOR (Figure 6g).

piR-50971 inhibition alleviated SIMI in *in vivo*

In this study, we investigated whether piR-50971 silencing could mitigate myocardial damage and autophagy inhibition in rats with SIMI. Cardiac ultrasound examination revealed that rats with CLP-induced sepsis exhibited compromised systolic and diastolic cardiac functions, indicative of ventricular dysfunction. However, treatment with piR-50971 inhibitor ameliorated these adverse effects, suggesting that piR-50971 may act as a potential therapeutic target that contributes to the exacerbation of myocardial injury under septic conditions (Figure 7a). Histological examination indicated that rats with SIMI exhibited pronounced myocardial oedema, disruption of

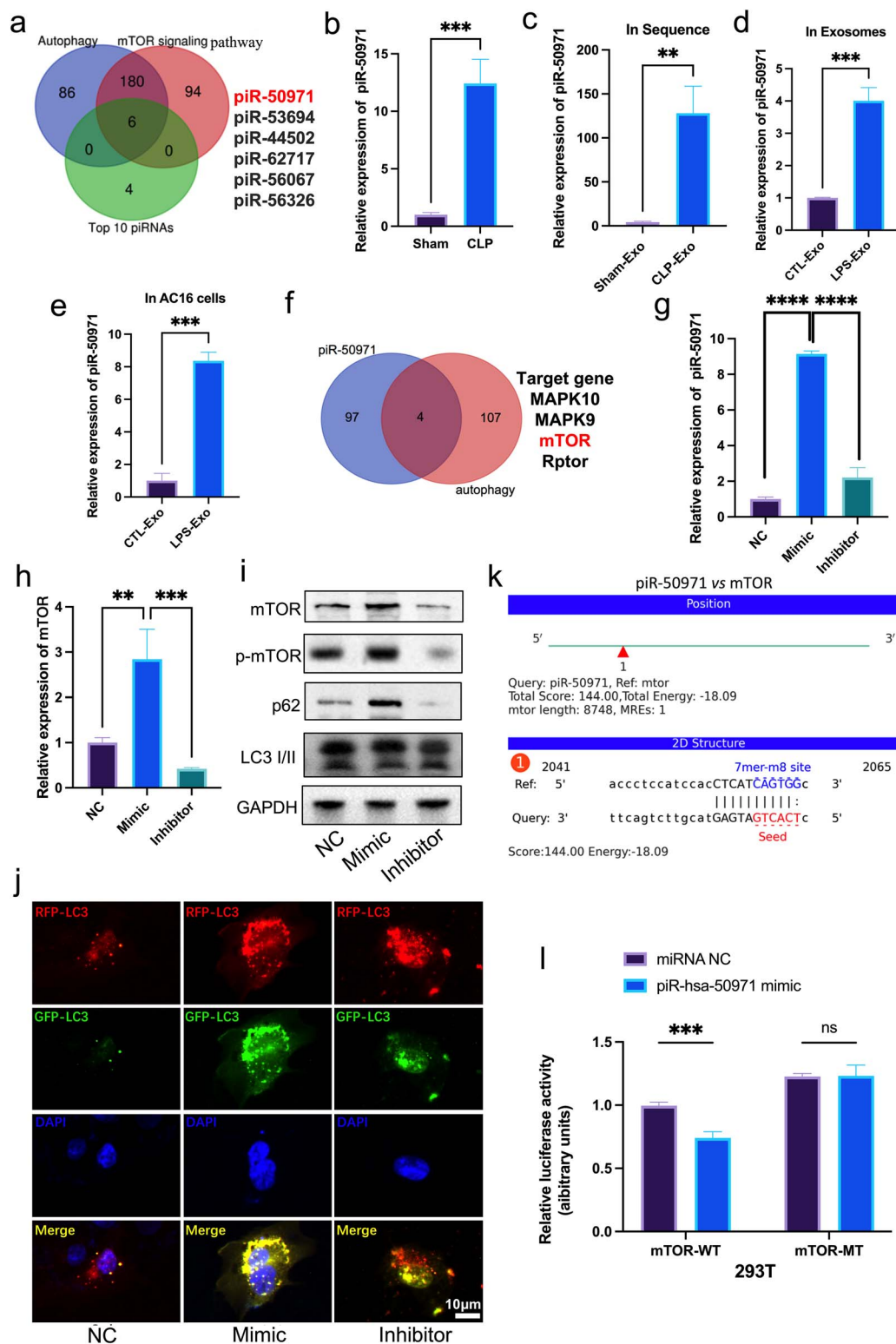


Figure 4. Macrophage derived exosome piR-50971 inhibits autophagy via mTOR signalling. **(a)** Venn diagram of autophagy related piRNAs, mTOR signalling pathway-related piRNAs, and top 10 piRNAs. **(b)** qPCR showed high expression of piR-50971 in the myocardial tissue of rats with SIMI. **(c)** qPCR showed the expression piR50971 increased significantly in serum exosomes in the CLP group. **(d)** qPCR showed that the expression of piR-50971 was significantly upregulated in the LPS-Exo group. **(e)** qPCR demonstrated that piR-50971 expression was remarkably elevated in LPS-Exo-stimulated AC16 cells. **(f)** Venn diagram of piR-50971-related autophagy genes showed that mTOR was one of the four intersected genes. **(g)** qPCR verification of piR-50971 mimic and inhibitor transfection efficacies in AC16 cells. **(h)** qPCR revealed that the mRNA level of mTOR was upregulated by piR-50971 mimic and downregulated by piR-50971 inhibitor. **(i)** Western blotting showed that piR-50971 mimic transfection inhibited autophagy via regulation of autophagy-related proteins. **(j)** Confocal microscopy showed that autophagy flux was inhibited by piR-50971 mimic and restored by piR50971 inhibitor in AC16 cells. **(k)** miRanda software predicted the binding site of piR-50971 and mTOR (40X). **(l)** Dual luciferase reporter assay verified the targeted binding site between piR-50971 and mTOR. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). piRNA piwi-interacting RNA, mTOR mammalian target of rapamycin, LPS lipopolysaccharide, Exo exosome, qPCR quantitative polymerase chain reaction

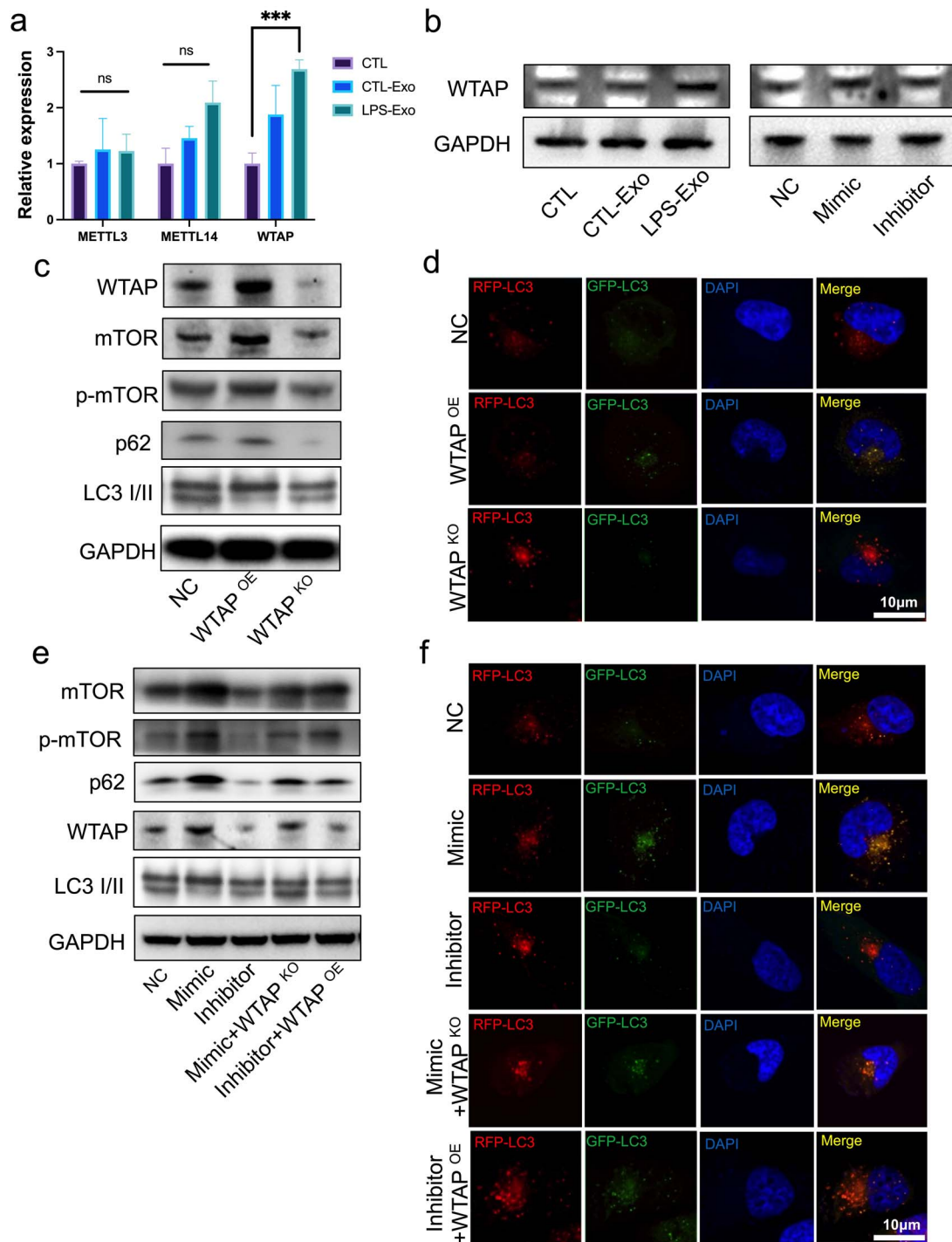


Figure 5. Macrophage-derived exosome piR-50971 inhibited autophagy via the upregulation of WTAP. **(a)** qPCR showed that LPS-Exo stimulation increased methylase WTAP expression in AC16 cells. **(b)** Co-culturing of LPS-Exo or piR-50971 mimic increased WTAP protein expression in AC16 cells. **(c)** WTAP overexpression inhibited autophagy level, while WTAP knockdown improved autophagy level in cardiomyocyte AC16 cells. **(d)** WTAP upregulation inhibited autophagic flux, whereas WTAP downregulation restored autophagy flux in AC16 cells (40X). **(e)** WTAP downregulation following transfection with piR-50971 mimic significantly ameliorated autophagy, whereas WTAP upregulation following transfection with piR-50971 inhibitor inhibited autophagy in AC16 cells. **(f)** WTAP downregulation restored autophagic flux inhibited by piR-50971 mimic transfection, whereas WTAP upregulation inhibited autophagic flux restored by piR-50971 inhibitor transfection (40X). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). WTAP wilms tumor 1 associated protein, LPS lipopolysaccharide, Exo exosome, qPCR quantitative polymerase chain reaction

myocardial fibres, increased interstitial spaces, and enhanced infiltration of inflammatory cells. Echocardiographic analysis demonstrated that SIMI significantly compromised cardiac function, as evidenced by reduced left ventricular ejection fraction (LVEF) and shortening fractions (LVFS). However,

treatment with piR-50971 inhibitor prior to CLP ameliorated these effects (Figure 7b and 7c). Myocardial injury biomarkers, including lactate dehydrogenase (LDH), creatine kinase (CK), and creatine kinase-MB (CK-MB), exhibited consistent trends following CLP induction and administration

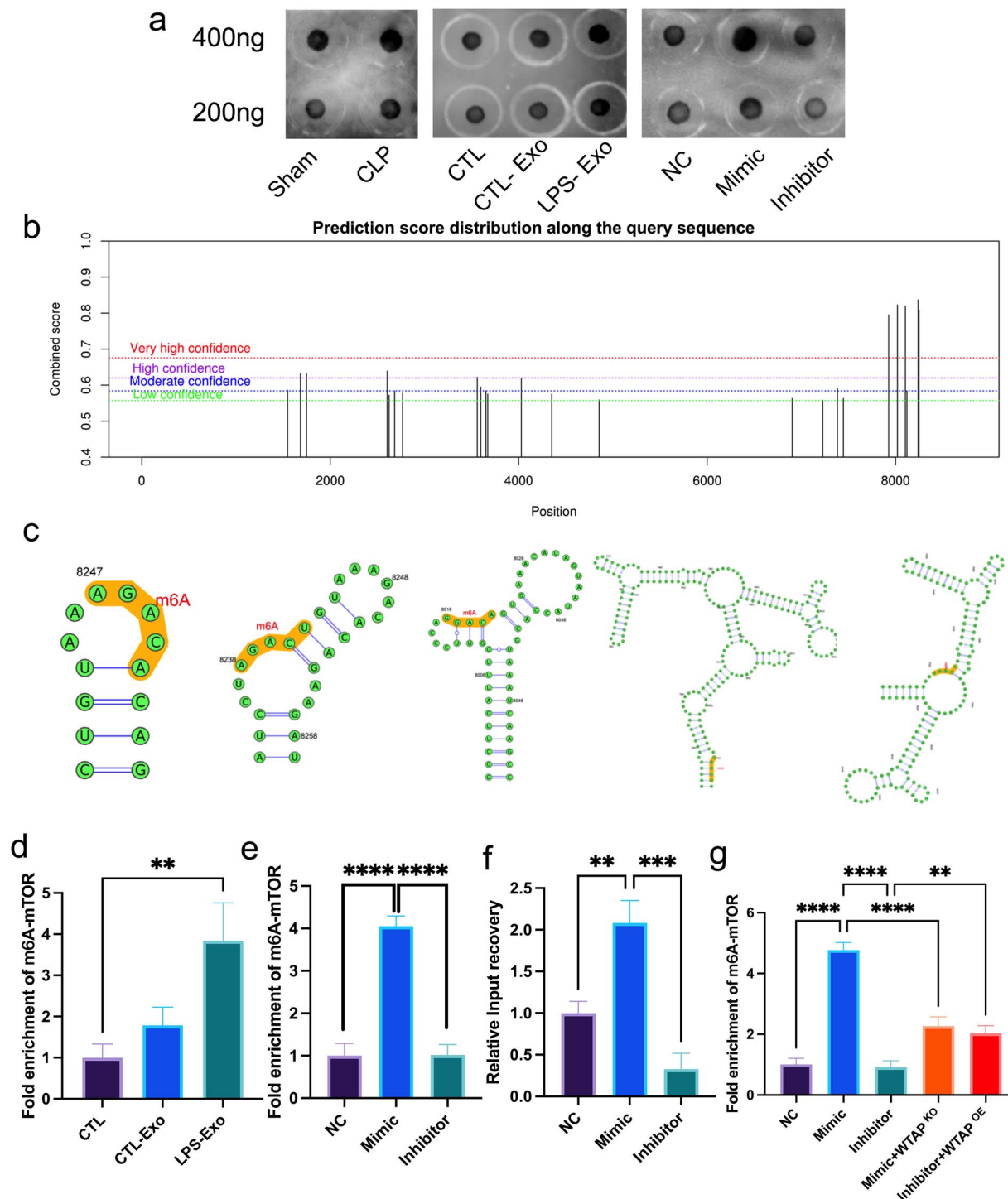


Figure 6. piR-50971-mediated WTAP upregulation on m6A modification of mTOR in AC16 cells. **(a)** Dot blot assay showed total methylation level was upregulated in the CLP, LPS-Exo, and piRNA-50971 mimic groups both *in vivo* and *in vitro*. **(b)** Prediction of m6A sites of mTOR by SRAMP. **(c)** The structures of 5 m6A sites of mTOR with extremely high confidence scores. **(d)** MeRIP-qPCR assay showed that LPS-Exo treatment increased m6A level of mTOR in AC16 cells. **(e)** MeRIP-qPCR assay demonstrated that piR-50971 mimic transfection significantly increased m6A level of mTOR in AC16 cells. **(f)** RIP-qPCR assay indicated the interaction between WTAP protein and mTOR mRNA. **(g)** MeRIP showed that the upregulation of m6A methylation levels of mTOR by piR-50971 mimics was reversed by WTAP knockdown in AC16 cells, whereas the downregulation of m6A methylation of mTOR by piR-50971 inhibitor was reversed by WTAP overexpression in AC16 cells. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). WTAP Wilms tumor 1 associated protein, CLP cecal ligation and puncture, qPCR quantitative polymerase chain reaction, mTOR mammalian target of rapamycin, MeRIP methylated RNA immunoprecipitation, RIP RNA immunoprecipitation, LPS lipopolysaccharide, Exo exosome, SRAMP sequence-based RNA adenosine methylation site predictor

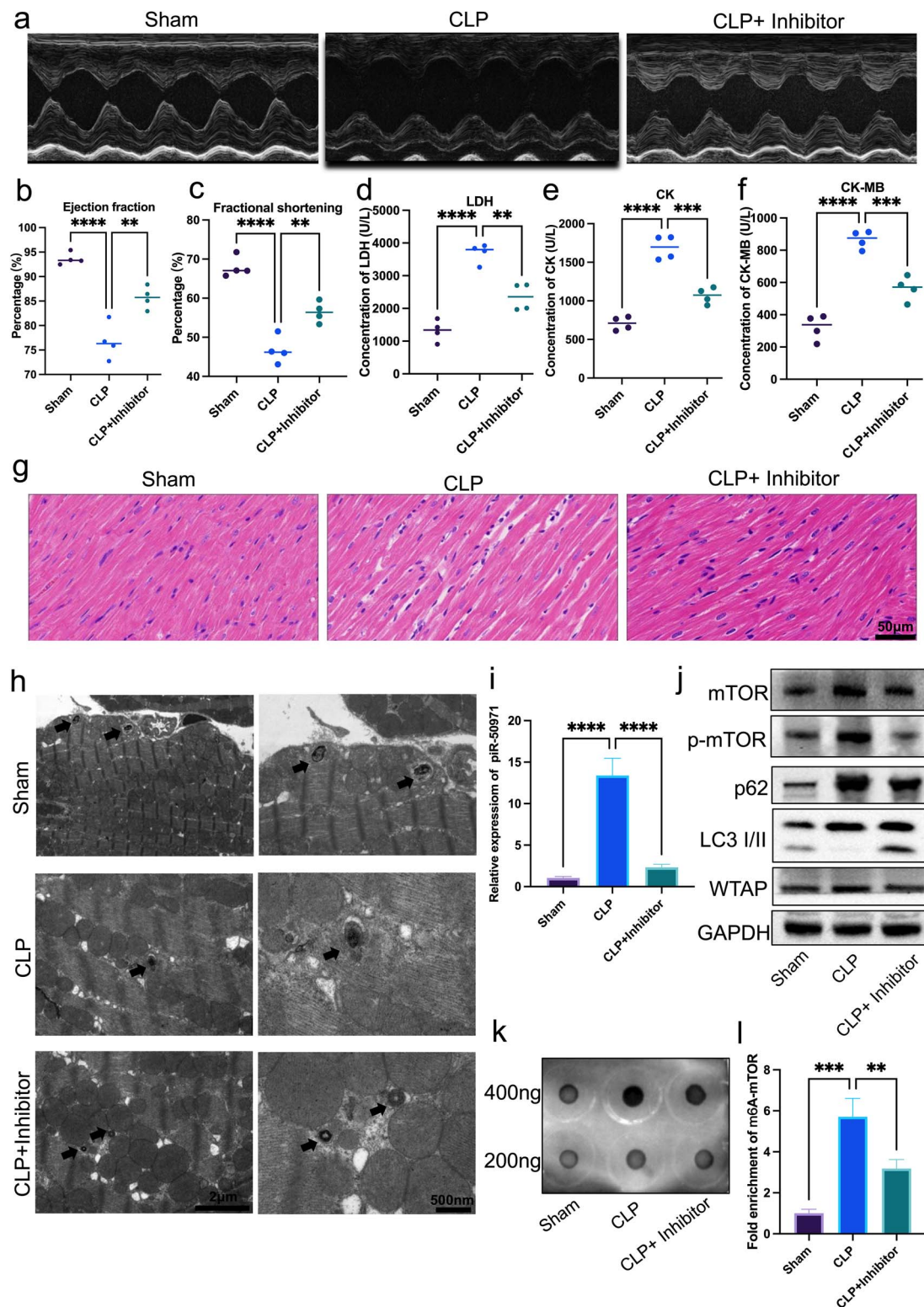


Figure 7. piR-50971 inhibition reversed cardiac dysfunction in mice with SIMI. **(a)** Echocardiography showed that piR-50971 inhibitor ameliorated SIMI-associated cardiac dysfunction *in vivo*. **(b–c)** piR-50971 inhibitor ameliorated SIMI-induced impairment in LVEF and LVFS. **(d–f)** piR-50971 inhibitor downregulated the expression of the myocardial injury marker of LDH, CK, and CK-MB *in vivo*. **(g)** H&E staining indicated that piR-50971 inhibitor improved cardiac function in mice with SIMI. **(h)** TEM showed that piR-50971 inhibitor ameliorated SIMI-induced inhibition of autophagy flux in the myocardial tissue of rats *in vivo*. **(i)** qPCR validated the transfection efficacy of piR-50971 inhibitor in myocardial tissue *in vivo*. **(j)** Western blot showed that piR-50971 inhibitor reversed sepsis induced myocardial autophagy inhibition *in vivo*. **(k)** Dot blot assay showed that piR-50971 inhibitor downregulated total m6A level in the myocardial tissue of rats with SIMI. **(l)** MeRIP assay showed that piR-50971 inhibitor downregulated m6A level of mTOR in the myocardial tissue of rats with SIMI. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). SIMI sepsis-induced myocardial injury, LVEF left ventricular ejection fraction, LVFS left ventricular shortening fractions, H&E haematoxylin and eosin, TEM transmission electron microscopy, qPCR quantitative polymerase chain reaction, mTOR mammalian target of rapamycin, MeRIP methylated RNA immunoprecipitation, LDH lactate dehydrogenase, CK creatine kinase, CK-MB creatine kinase-mb

of the piR-50971 inhibitor (Figure 7d and 7f). Treatment with piR-50971 inhibitor ameliorated CLP-induced myocardial injury, as evidenced by the restoration of myocardial fibres and horizontal stripes and the reversal of inflammatory infiltration and interstitial oedema (Figure 7g). TEM of rat myocardial tissue showed a reduced number of autophagosomes and obstructed autophagy flux in the CLP group compared to the sham group. However, transfection with piR-50971 inhibitor ameliorated CLP-induced disruption of autophagic flux (Figure 7h). Additionally, qPCR confirmed successful *in vivo* transfection of the piR-50971 inhibitor (Figure 7i). Western blotting showed that treatment with piR-50971 inhibitor via the tail vein significantly enhanced myocardial autophagy levels, which were suppressed under septic conditions (Figure 7j). Additionally, dot blot assay indicated that the piR-50971 inhibitor reduced the overall methylation levels in myocardial tissue compared with those in the CLP group (Figure 7k). Moreover, MeRIP assays confirmed a reduction in m⁶A methylation levels of mTOR in the myocardial tissue of rats with CLP-induced sepsis following injection with piR-50971 inhibitor (Figure 7l).

Discussion

Sepsis is a complex and life-threatening condition characterized by organ dysfunction resulting from a dysregulated host immune response to infection [43, 44]. SIMI, a major complication of sepsis, is characterized by impaired cardiac function and structural damage to myocardial tissue. Preclinical and clinical evidences indicates that substantial improvements in the prognosis of patients with sepsis are largely dependent on autophagy [41]. The pathogenesis of SIMI is complex and remains incompletely understood, with dysregulated autophagy emerging as a central contributing mechanism. Normal autophagy may exacerbate sepsis-induced cardiac dysfunction [6]. During the early stages of sepsis, mitochondrial dysfunction triggers autophagy, which may contribute to the restoration of both mitochondrial integrity and myocardial function [45]. Beclin-1 overexpression in cardiomyocytes during sepsis can mitigate inflammation through PINK1/Parkin-mediated autophagy [16], thereby exerting cardioprotective effects.

Under septic conditions, mTOR signaling serves as a central regulator of multiple cellular processes, including metabolism, inflammation, and autophagy. In sepsis-associated immunosuppression, the mTOR pathway mediates autophagy-lysosomal disorders [46]. In addition, lysosomal membrane permeabilization plays a critical role in the regulation of programmed cell death pathways such as necroptosis and pyroptosis [47, 48]. Moreover, the mTOR signaling pathway has been implicated in the metabolic reprogramming of cholesterol, a key process during the differentiation of naïve CD4⁺ T cells into Tregs [49]. Notably, the administration of rapamycin, a specific mTOR inhibitor, has been suggested to confer cardioprotective effects in SIMI by facilitating autophagy activation [13]. Epigenetic reprogramming dependent on the AKT/mTOR/HIF1 α pathway is reported to modulate immune response to sepsis. Bioinformatics analysis of small RNA sequences revealed that mTOR and autophagy signaling were enriched in the plasma exosomes of piRNAs in rats with SIMI. Our findings provide novel insights into the involvement of piRNAs in mTOR-regulated autophagy,

a previously unexplored mechanism contributing to the pathogenesis of SIMI.

Exosomes are critical in sepsis pathogenesis in animals. Circulating mononuclear macrophages have been identified as the main immune cells infiltrating the myocardium in the acute stage of SIMI. Emerging evidence from preclinical *in vitro* and *in vivo* studies indicates that sepsis is associated with enhanced exosome secretion and a concomitant impairment in their cellular uptake [50–52]. Additionally, administration of exosome generation inhibitors markedly enhanced the survival rates of mice with septic insults [53]. piRNAs and PIWI proteins have recently been reported to play regulatory roles in autophagy [54]. Specifically, piR-004800 knockdown inhibited the activation of the AKT/mTOR pathway and induced autophagy in patients with multiple myeloma [39]. In contrast, PIWIL1 upregulation facilitated autophagosome formation and modulated the PINK1/Parkin pathway of mitochondrial autophagy [55]. Our study revealed that the macrophage-derived exosome piR-50971 inhibited autophagy through the upregulation of mTOR under septic conditions, which further aggravated myocardial damage. Moreover, PIWIL2 was highly expressed in the cardiac tissue of rats with CLP-induced sepsis, implying a regulatory role for PIWIL2 in SIMI. Overall, our study elucidates a novel aspect of exosome-mediated intercellular communication and provides significant insights into the functions of piRNAs in SIMI.

m⁶A modification is the most abundant internal modification of eukaryotic mRNA and plays a critical role in regulating complex biological processes. Transcriptomic analysis suggests a potential link between sepsis prognosis and the m⁶A-mediated regulation of autophagy-related genes [42]. A clinical database revealed that METTL3 and WTAP expressions was significantly elevated in patients with SIMI [56]. Notably, the increase in WTAP was ~100-fold greater than that of METTL3, suggesting that WTAP may play a more prominent biological role in SIMI. In macrophages, METTL14 acetylation upregulated m⁶A methylation of Spi2a, which subsequently inhibited NF- κ B activation, reduced inflammatory response, and ameliorated SIMI [57]. Moreover, m⁶A methylation contributes to the regulation of immune responses during sepsis, in part by limiting the formation of neutrophil extracellular traps (NETs) and attenuating macrophage-mediated phagocytosis [58]. piRNAs have been implicated in key biological processes, including epigenetic regulation, genome rearrangement, and the post-transcriptional regulation of gene expression [34]. piRNAs recruit methyltransferases and facilitate post-transcriptional regulation of RNA, DNA, and histones [20, 50]. Our findings of our study indicated that piR-50971 recruited and interacted with WTAP, leading to upregulated m⁶A level of mTOR and enhanced mTOR expression subsequently, which unimpeded autophagy flux and aggravated myocardial damage. Increased autophagic flux has been reported to inhibit cellular oxidative stress, thereby attenuates apoptosis and pyroptosis [59]. This suggests a potential mechanism by which piRNAs modulate m⁶A modification in target genes and regulates autophagy signaling.

Traditionally, miRNAs have been recognized for their role in gene silencing via binding to the 3' UTR of target mRNAs [20]. However, recent studies have revealed that miRNAs also interact with CDS to exert their biological functions [60]. In this study, we used miRanda to predict the binding sites

of piR-50971 and mTOR and found that the binding sites were located at 7 mer-m8 sites within the CDS region of piR-50971. Dual-luciferase reporter assay was performed to validate the binding of piR-50971 to mTOR. Previous studies have demonstrated that PIWI/piRNA complexes can activate translation [61], challenging existed paradigms within the field and highlighting the necessity for continued investigation of miRNA dynamics.

Our research demonstrates that macrophage-derived exosome piR-50971 exacerbates sepsis-induced myocardial damage by inhibiting autophagy via the upregulation of m⁶A level in mTOR. *In vivo* experiments further conformed the therapeutic potential of targeting piR-50971, as its inhibitor mitigated myocardial damage and improved autophagy levels in SIMI. These findings emphasized the potential target for developing targeted therapies in SIMI. While we have identified a specific piRNA and its role in myocardial damage, the sepsis syndrome is multifactorial, involving numerous cellular and molecular pathways. Future research should aim to identify other potential piRNAs and other targets that may be involved in the pathogenesis of SIMI. Additionally, the long-term effects of piRNA modulation on cardiac function and the potential systemic effects of such interventions need to be thoroughly investigated.

Our study demonstrated that macrophage-derived exosome piR-50971 exacerbated SIMI by inhibiting autophagy via the upregulation of m⁶A levels in mTOR. *In vivo* experiments further confirmed the therapeutic potential of targeting piR-50971, as its inhibition mitigated myocardial damage and improved autophagy in SIMI. Although we identified a specific piRNA and its role in myocardial damage, the sepsis syndrome is multifactorial and involves numerous cellular and molecular pathways. Future studies should aim to identify other potential piRNAs and targets that may be involved in SIMI pathogenesis. Additionally, the long-term effects of piRNA modulation on cardiac function and the potential systemic effects of such interventions need to be thoroughly investigated.

Despite the promising findings, the study had some limitations. For instance, the rat model employed in this study may not fully capture the complexity of human SIMI. Moreover, inherent physiological and genetic differences between rats and humans may constrain the direct translatability of these findings to clinical applications. Although we demonstrated the interaction between piR-50971 and mTOR mRNA, the upstream mechanisms regulating piR-50971 expression and potential off-target effects remain to be explored. Moreover, our *in vivo* studies lacked genetic manipulation experiments, such as CRISPER-Cas9 or gene-edited rats, to directly validate the role of piR-50971 in SIMI, and long-term outcomes were not assessed. Future studies should focus on validating our findings in human samples to facilitate clinical translation.

Our findings suggest several promising hotspots for future research and potential clinical applications. Specifically, the identification of piR-50971 as a key regulator of SIMI opens the possibility of developing targeted therapies aimed at inhibiting piRNAs to mitigate cardiac damage and enhance autophagy. Additionally, the role of m⁶A methylation in modulating autophagy through mTOR presents an opportunity to explore treatments that target the methyltransferases or demethylases involved in this process. piR-50971 may represent a promising diagnostic biomarker for the early detection and prognostic evaluation

of SIMI. Future research should aim to further elucidate the molecular mechanisms underlying the interaction between piR-50971 and WTAP, with particular emphasis on its role in modulating m⁶A RNA methylation. Additionally, it is crucial to assess the long-term implications of targeting piR-50971 for cardiac function and patient outcomes. Overall, these approaches may hold significant potential for improving the management and prognosis of sepsis-induced cardiac complications.

Conclusions

Our study provides robust evidence that piRNAs, specifically piR-50971, play crucial roles in SIMI pathogenesis. piR-50971 upregulates m⁶A levels in mTOR by recruiting WTAP, consequently inhibiting autophagy and aggravating myocardial dysfunction in sepsis. In conclusion, these findings provide valuable insights into the molecular mechanisms driving SIMI and highlight components of the piRNA pathway as potential therapeutic targets for the prevention and treatment of septic myocardial dysfunction.

List of abbreviations

Abbreviations	English full name
SIMI	sepsis-induced myocardial injury
MODS	multiple organ dysfunction syndrome
CLP	cecal ligation and puncture
ncRNA	non-coding RNA
mTOR	mammalian target of rapamycin
LPS	lipopolysaccharide
miRNA	microRNA
lnc RNA	long non-coding RNA
circRNA	circular RNA
piRNA	PIWI (P-element induced wimpy testis)-interacting RNA
m ⁶ A	N6-methyladenosine
METTL3	methyltransferase like 3
WTAP	Wilms tumor 1 associated protein
THP-1	Tohoku Hospital Pediatrics-1
Exo	exosome
H&E	haematoxylin and eosin
LVEF	left ventricular ejection fraction
LVFS	left ventricular fractional shortening
TEM	transmission electron microscopy
qPCR	quantitative polymerase chain reaction
MeRIP	methylated RNA immunoprecipitation
RIP	RNA immunoprecipitation
LDH	lactate dehydrogenase
CK	creatine kinase
CK-MB	creatine kinase-mb

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Author contributions

Jiaqiang Wang (Data curation [equal], Formal Analysis [equal], Funding acquisition [supporting], Methodology [equal], Writing—original draft [equal], Writing—review & editing [equal]), Jie Zhang (Data curation [equal], Formal Analysis [equal], Methodology [equal], Writing—original draft [equal], Writing—review & editing [equal]), Shan

Zhong, Xuelian Chen and Hsin-Ying Liu (Formal Analysis [supporting], Methodology [supporting]), Chenghao Lu, Hanting Zhu, Yunsheng Chen, Jizhuang Wang and Jiarong Yu (Methodology [supporting], Project administration [supporting], Visualization [supporting]), Xiong Zhang (Conceptualization [equal], Project administration [equal]), Yan Liu (Conceptualization [equal], Project administration [equal]) and Min Gao (Funding acquisition [lead], Conceptualization [lead], Project administration [equal])

Supplementary data

Supplementary data are available at *Burns & Trauma Journal* online.

Ethics approval and consent to participate

Animal ethics has been approved by Animal Ethics Committee of Ruijin Hospital Shanghai Jiao Tong University School of Medicine (No. RJ2024065).

Consent for publication

Not applicable.

Conflicts of interest

The authors declare that they have no competing interests.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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