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Protective effects of rapamycin induced autophagy on CLP septic mice

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ABSTRACT

Sepsis is a life-threatening condition that may develop to multiple organ failure and septic shock. Autophagy is considered to play an important role in the regulation of inflammation. The present study aims to investigate the protective role of mTORC1 inhibitor, rapamycin, on septic death using cecal ligation and puncture (CLP) mice model. Here, results showed that pretreatment with rapamycin reduced the pyroptosis of peritoneal macrophages stimulated by cecal contents and the release of inflammatory factors such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α); In septic mice, rapamycin treatment decreased the activation of inflammasome in lung, and alleviated the pathological injuries in lung, liver and spleen tissues during acute stage of sepsis. Treatment of rapamycin rescued animals from septic death significantly. Our results indicated that activation of autophagy is a potential strategy to regulate the excessive inflammation in acute stage of sepsis.

1. Introduction

According to the latest guidelines, sepsis is defined as life-threatening organ dysfunction caused by an uncontrolled host immune response to infection [1]. Although a lot of clinical trials have been conducted, the mortality rate of septic patients remains high even in developed countries [2]. It is generally accepted that there is a cytokine storm in acute sepsis. Unbalance of anti-inflammatory and pro-inflammatory reaction is the main pathogenic mechanism of sepsis [3]. Usually, the lung is the first organ damaged by sepsis, and a large number of patients die from acute respiratory distress syndrome (ARDS) in the early stage of sepsis. Recent study has confirmed that intestinal bacteria invaded into the lung during early stage of sepsis causing a disorder of the lung microbiome, mainly characterized by enrichment with the proteobacteria [4,5]. Translocation of proteobacteria in lung was significantly associated with elevated alveolar concentrations of TNF-α, which is an important mediator of pulmonary inflammation in ARDS [6].

Autophagy is a self-protection mechanism responsible for removing misfolded proteins, resisting oxidative stress and degrading microbial pathogens using lysosomal processes [7,8]. Autophagy has been suggested to be involved in the regulation of inflammatory reaction [9,10]. The mechanisms that autophagy can inhibit inflammation, is that autophagy can decrease the cell apoptosis and its secondary necrosis so that inflammation will be constrained [11–14]. Alternatively, autophagy negatively regulates inflammasome activation and inhibits the inflammation caused by pyroptosis [15,16]. Studies also found that the deficiency of autophagy-related protein in macrophages can lead to a high expression of IL-1β and IL-18 after stimulated by lipopolysaccharide (LPS) [10]. Therefore, it is of interesting to study whether autophagy is a valuable target for regulation of acute immunoreaction in early stage of sepsis. Here, mTORC1 inhibitor, rapamycin, is used in our study to activate cell autophagy. Rapamycin is a powerful anticancer agent. Studies have demonstrated that rapamycin protects septic mice through different mechanisms, such as by inhibition of Th17 cell proliferation, blocking the NF-κB pathway, promoting the expansion of Treg cells, and inhibiting the production of inflammatory mediators [17–20]. In the present study, we focused on the effect of rapamycin on regulation of the balance of autophagy and inflammasome during the acute stage of sepsis.

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2. Materials and methods

2.1. Animals

Male C57BL/6 mice (10 weeks old, SLAC, Shanghai, China) were maintained at animal experimental center of Shanghai Jiaotong University under 12-h day and night cycle with free access to food and water at least 1 week before experiments. All experimental procedures were carried out according to the guidelines of Institutional Animal Care and Use Committee of Shanghai (A2015035).

2.2. Cell culture and stimulation

Peritoneal macrophages were obtained by peritoneal lavage in C57BL/6 mice, then approximately 1 × 105 cells/well were seeded in a 96-cell plates, cultured in DMEM (Gibco) with 10% heat-inactivated fetal bovine serum (Gibco), 50 U/mL penicillin and 50 mg/mL streptomycin, and then incubated in a humidified incubator containing 5% CO_2 at 37 °C. The treatment groups was given rapamycin (Gene Operation) at a dose of 5 nmol/L, 10 nmol/L and 20 nmol/L, the control group was added the same volume of vehicle (10% DMSO, Yeasen). After 9 h, these cells were stimulated by cecal contents for 15 min, followed by washed with PBS and cultured by phenol red free DMEM medium with 100 μg/mL gentamicin. After cultured for 12 h, cells were removed by centrifugation for 5 min at 1200 rpm at room temperature and the supernatant was used for the detection of lactate dehydrogenase (LDH) and cytokines.

2.3. LDH measurement

The concentrations of LDH in the supernatant of peritoneal macrophages was measured by commercially available LDH assay (Beyotime), following the manufacturer's recommendations. Briefly, the cell-free culture supernatant samples (120 μL of each) were transferred into a 96-well plates, and mixed with 60 μL of LDH-detection assay mixture. After incubation for 30 min at room temperature, the optical densities reflecting LDH activity was determined at 560 nm using an ELISA reader (Tecan).

2.4. IL-1β and TNF-α detection

The levels of IL-1β and TNF-α were measured by using a commercially available ELISA kit (Dakewe) according to the manufacturer's instructions. The supernatant obtained from the group treated with 10 nmol/L rapamycin or the vehicle group was added to a 96-well plates with 100 μL/well, then add 50 μL diluted Biotinylated antibody to each well. After incubation at 37 °C for 90 min, the unbound materials were removed by four washing cycles with washing buffer. Then 100 μL streptavidin-HRP was added to each well, the plates were incubated at 37 °C for 30 min. The plates were washed and 100 μL of tetramethylbenzidine (TMB) was added to each well. After incubation at 37 °C for 30 min, 100 μL/well stop solution was added. The optical densities were read at a wave of 450 nm with an ELISA reader.

2.5. Sepsis model

Sepsis was induced by cecal ligation and punture (CLP) in C57BL/6 mice as described previously [21,22]. In brief, mice were anesthetized with 10% Chloral hydrate (300 mg/kg of body weight), and then the cecum was exposed by the middle abdominal incision, the cecum was ligated with a 4-0 silk tie and puntured with a 22-gauge needle. Then the cecum was returned to the anotomical position and the abdominal wall was sutured in layers. Postoperatively, 1 mL of 0.9% saline was injected subcutaneously. Sham-operated mice were treated similarly, but the cecum was not ligated or punctured. In treatment groups, rapamycin (5 mg/kg) was injected at 9 h before CLP operation or 1 h after CLP operation, intraperitoneally. The vehicle-treated group was given the same volume of vehicle (10% DMSO). Mice were killed at indicated time points and tissues were removed for analysis. Mice were observed by animal caretakers who were blinded to the treatment arms every day to evaluate the state of the mice as well as the survival.

2.6. Histological analysis

The lung, liver and spleen tissues were obtained 24 h after the establishment of the model, fixed with 4% paraformaldehyde for 24 h. The tissues were dehydrated through increasing concentrations of ethanol (30%–100%), and placed in xylene for 2 h, followed by overnight paraffin embedding. The samples were sectioned at 4 μm in thickness and mounted on slides. After placed in xylene, concentrations of ethanol (100%–50%), and the slides were stained with standard hematoxylin and eosin (HE) procedure. A blinded observer was assigned to evaluate the histopathological injuries of lung, liver and spleen in each sample. Histopathological injuries were evaluated according to the interstitial edema, infiltration of inflammatory cells, congestion, hemorrhage, hyaline membrane formation and necrosis of the tissues. Grading of the tissue sections is set from 0 to 4. "0" stands for normal; "1" for minimal injury; "2" for mild injury; "3" for moderate injury; and "4" for severe injury. Score is based on a blind test.

2.7. Western blotting analyses

The lung tissue samples from mice, either pretreatment with rapamycin or not, were removed 8 h after CLP or sham surgery, and homogenized in RIPA lysis buffer (Yeasen) containing protease inhibitor PMSF (1 mM, Yeasen) for 30 min. Protein extracts were centrifuged at 12,000 × *g* for 10 min, the supernatants were quantified by BCA protein assay (Yeasen). Equal of protein (50 μg) were loaded in SDS-polyacrylamide electrophoresis gel and transferred to polyvinylidene difluoride membrane, then blocked with 5% nonfat dry milk in TBST for 1 h at room temperature, followed by incubated overnight at 4 °C with a specific primary antibody. The following antibody were used: anti-LC3 II (Proteintech, 1:1000), anti-caspase 1 p10 (santa cruse, 1:100), anti-IL1β (R&D, 1:200), anti-p62 (Abcam, 1:10,000), anti-β-actin (Proteintech, 1:1000), anti-gapdh (Proteintech, 1:1000). The membranes was washed with TBST for 5 times and subsequently incubated with peroxidase conjugated secondary antibodies: goat anti-rabbit IgG (Signalway Antibody, 1:10,000), The membranes were washed 5 times with TBST, and the signal was then detected using enhanced chemiluminescence (ECL) reagent (Beyotime).

2.8. Statistical analysis

ANOVA test or student *t*-test was used for statistical analysis. P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Rapamycin treatment inhibited pyroptosis of macrophages and reduced the release of inflammatory cytokines

Pyroptosis is programmed cell death that can induce very deleterious inflammatory response. We are interested to investigate whether autophagy can inhibit pyroptosis of peritoneal macrophages caused by enteric bacteria infection. LDH release assay showed that cell death was significantly lower in the rapamycin-treated group compared with the control group (Fig. 1A). Protective effect of rapamycin on pyroptosis is dose dependent. Cell death in control group was 30.7 ± 3.03%, while the group treated with 5 nmol/L, 10 nmol/L, 20 nmol/L rapamycin was 12.8 ± 2.88%, 8.2 ± 0.60%, and 16.2 ± 2.51% respectively. Moreover, the release of pro-inflammatory cytokines also well decreased after autophagy stimulation. The concentrations of IL-1β, TNF-α

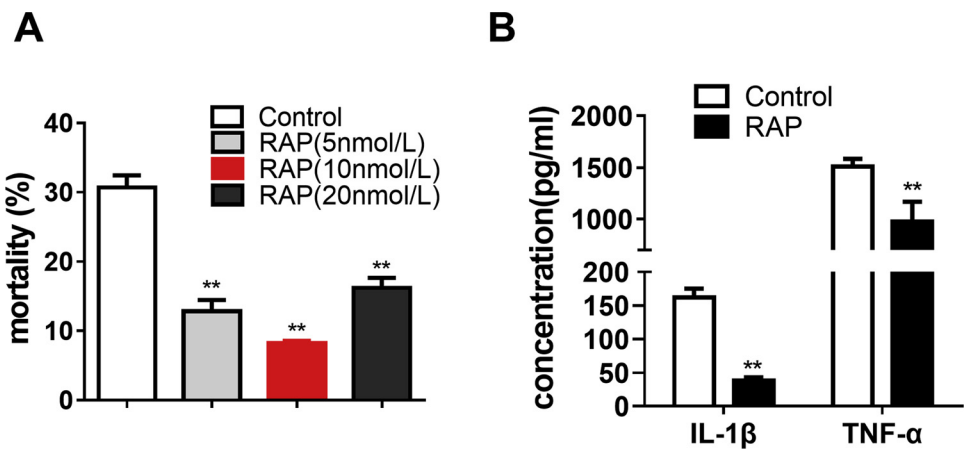

Fig. 1. Inhibition of pyroptosis and cytokines release by rapamycin treatment. (A) Pretreatment with rapamycin improved the survival of peritoneal macrophages treated with cecal contents. (B) Pretreatment with rapamycin reduced the release of inflammatory cytokines (IL-1β, TNF-α) from peritoneal macrophages stimulated by cecal contents. One of the representative experiments was presented. Significance of $p < 0.01$ was indicated as '**'.

released in the supernatant in the control group were 161.96 ± 22.83 pg/mL and 1509.88 ± 129.59 pg/mL respectively, while 37.84 ± 9.25 pg/mL and 976.87 ± 334.18 pg/mL of IL-1β and TNF-α were recorded in cells pretreated with rapamycin (Fig. 1B).

3.2. Rapamycin treatment reduced mortality in CLP mice

Here, we investigated whether rapamycin treatment protected mice from CLP-induced sepsis. Twenty mice were pretreated with rapamycin (5 mg/kg) or vehicle (same volume of 10% DMSO) 9 h before CLP or the sham surgery. The mortality of 80 h of the mice treated with rapamycin was 45%, while the group without rapamycin -treatment (control) was 70% (Fig. 2A). In viewing of the impossibility that premorbid treatment with rapamycin of septic patients, we also investigated the effect of postoperative treatment with rapamycin on septic death. Twenty animals were injected with rapamycin (5 mg/kg) or vehicle at 1 h after CLP. The mortality in 80 h of the control group was 60%, the rapamycin -treated group was 30% (Fig. 2B).

3.3. Rapamycin treatment activated autophagy and inhibited inflammasome

Autophagy activation in lung samples was determined by using western blot. The results showed that the expression of LC3-II significantly increased in rapamycin-treated group compared with the control group (Fig. 3A and B). Autophagic flux was further studied by detection of degradation of p62. Results showed that the degradation of p62 in the lung tissues of mice treated with rapamycin is most significant at 24 h post CLP (Fig. 3C and D). Then, we examined whether autophagy activated by rapamycin treatment can inhibit inflammasome assembly during early stage of sepsis. The results showed that the expression of pro-caspase-1 and pro-IL-1β in rapamycin treatment group was down regulated (Fig. 3A). Moreover, cleavage of caspase 1 and IL1β were also inhibited after rapamycin treatment (Fig. 3E and F).

3.4. Rapamycin reduces inflammatory tissue injuries

In order to investigate the effects of rapamycin on histopathological changes in septic mice, tissues were collected 36 h post CLP. In the control group (CLP without rapamycin-treatment), there were significant congestion, inflammatory cells infiltration, structure damage, interstitial cellular degeneration and necrosis in the lungs, liver, and spleens. While in the group treated with rapamycin, pathological injuries in the organ of lung, liver and spleen was attenuated significantly as evidenced by less inflammatory cell infiltration, reduced exudate blockage of capillary, lower levels of interstitial cellular degeneration and necrosis (Fig. 4A). Blind test of biopsy by three independent experiments demonstrated the protective effect of rapamycin treatment on pathological injury in septic mice (Fig. 4B).

4. Discussion

There is a storm of inflammatory factors in sepsis, the strong inflammatory reaction in the early stage leads to a long-term immune exhaustion and inhibition state in the late stage, which breaks the balance between the promotion and depression of inflammatory response. ARDS is the leading cause of death in patients with sepsis. Therefore, the control of inflammatory response in lungs during the early stage of sepsis is vital for the treatment of sepsis. Autophagy is an intracellular process of maintaining cell homeostasis. In recent years, a growing number of evidence suggests that autophagy-related proteins play an important role in inhibiting pro-inflammatory responses [23,24]. Early stage of sepsis is mostly accompanied by severe microbial infections. Usually, infection of these microorganisms activates caspase 1/11 inflammasomes and results cell pyroptosis and subsequent uncontrolled pro-inflammatory response [25]. Deletion of

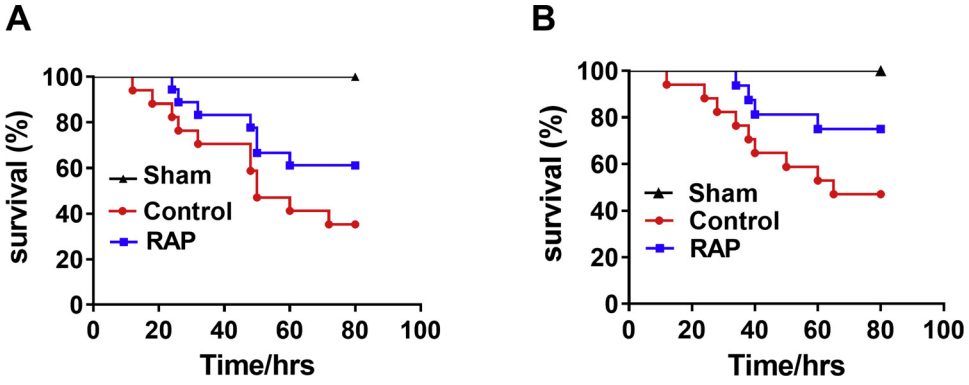

Fig. 2. Activation of autophagy protects mice from septic death. (A) Mortality of mice in groups of RAP (pretreatment with rapamycin 9 h before CLP surgery), Control (CLP without rapamycin treatment) and sham surgery was recorded. (B) Mortality of mice in groups of RPA (rapamycin treatment 1 h after CLP surgery), Control (CLP without rapamycin treatment) and sham surgery was recorded.

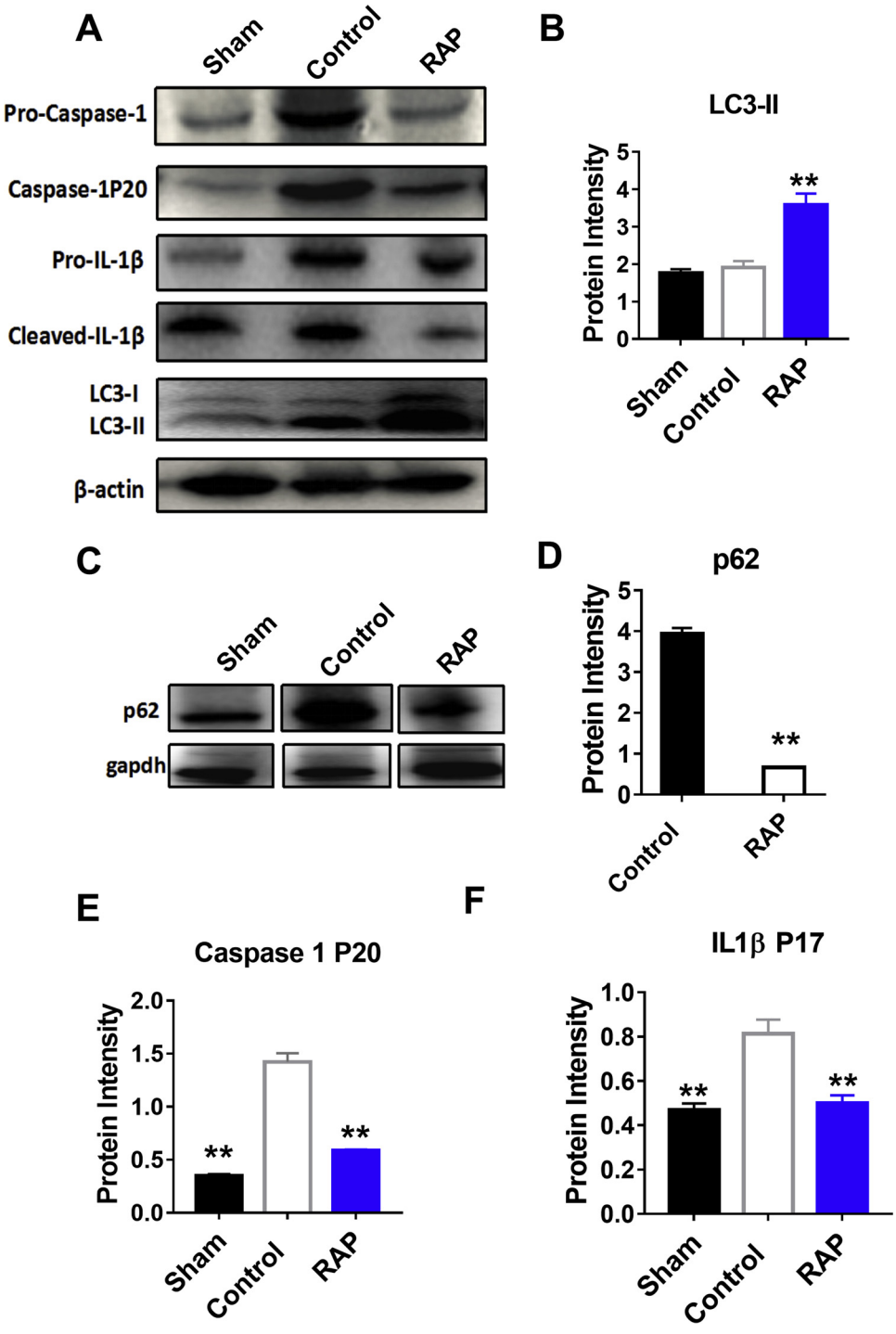

Fig. 3. Rapamycin treatment activates autophagy and inhibits inflammasome activation in lung tissues of CLP mice. (A) The expression of Pro-caspase-1, Pro-IL-1β, cleaved caspase-1 P20, cleaved IL-1β and LC3 in lung samples from sepsis mice was detected by western blot. (B) Protein intensity of LC3-II normalized with β-actin was presented. (C) Degradation of p62 in lung sample 24 h after CLP surgery was detected. (D) Protein intensity of p62 normalized with gapdh was presented. (E) Protein intensity of cleaved caspase 1 normalized with gapdh was presented. (F) Protein intensity of cleaved IL1β normalized with gapdh was presented. Experimental data from one representative replicate (of three replicates) are presented. Significance of $p < 0.01$ was indicated as '**'.

inflammsome genes well protect mice from septic shock [26–28]. Therefore, reducing the activation of inflammasomes and pyroptosis is of great significance in alleviating pathological injuries at early stage of sepsis. It has been found that autophagic proteins can negatively regulate the activation of inflammasomes by protecting mitochondria from destruction or directly targeting inflammasome for degradation [29,30]. Therefore, we are interested to investigate whether activation of autophagy by rapaymycin treatment can reduce inflammasome activation and control deleterious inflammatory response in CLP septic mice. In the present study, in vitro assays showed that after stimulated by cecum contents, the secretion of inflammatory factors (IL-1β, TNF-α) as well as the pyroptosis decreased significantly in rapamycin-treated macrophages compared with the control group, suggesting that autophagy can reduce the inflammatory response of macrophages and enhance its protective role in immune reaction. We also found that the protective effect of rapamycin is dose dependent. Due to the cytotoxicity of DMSO, treatment with high concentration of rapamycin decreases the inhibition effect on cell death. Alternatively, excessive autophagy activation can also induce cell death [31]. Subsequently, we established CLP animal model to investigate the protective effect of rapamycin on sepsis in acute inflammatory phase. The results showed that the pathological damage of affected organs was alleviated by treatment with rapamycin in comparison with untreated group. Pretreatment of rapamycin significantly reduced the mortality caused by CLP sepsis. Moreover, we identified that postoperative administration of rapamycin is more beneficial for CLP sepsis, which confirmed the

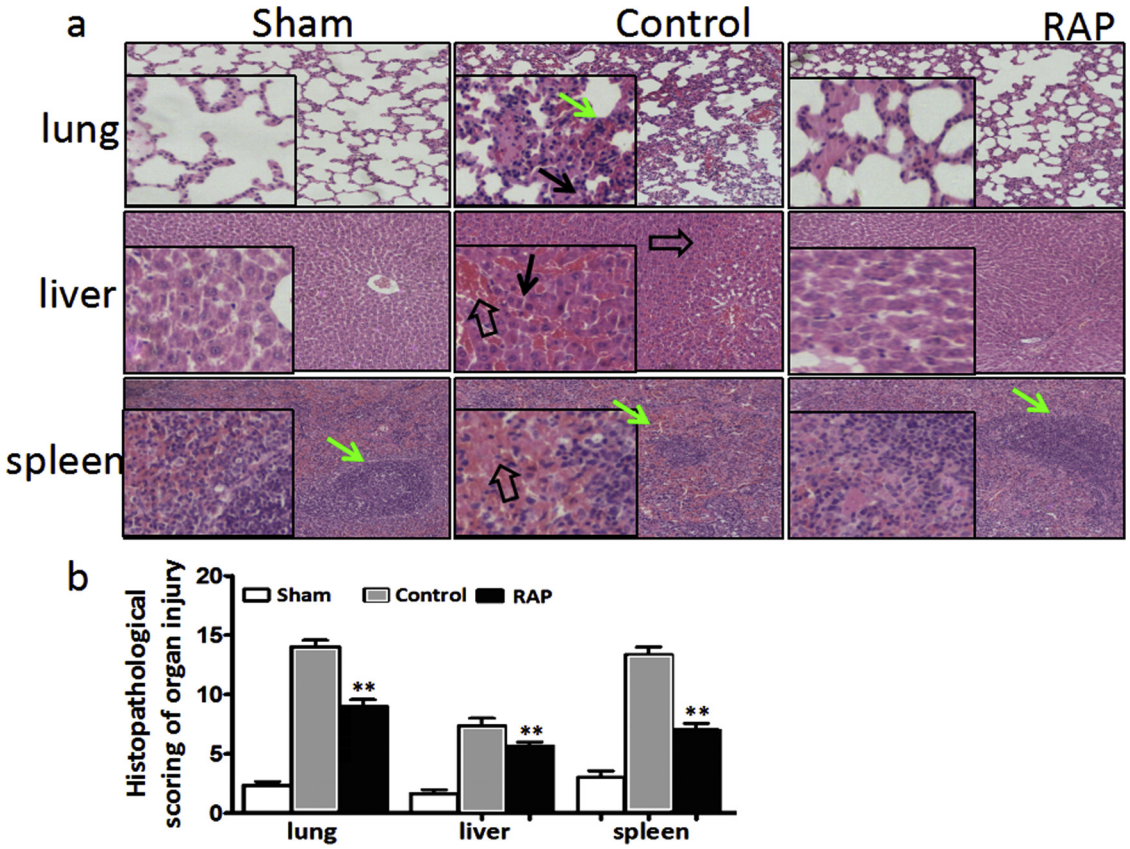

Fig. 4. Activation of autophagy reduced the pathological injuries of lung, liver and spleen. (A)The pathological injuries induced by CLP was attenuated significantly in rapamycin-treated mice, as evidenced by less inflammatory cell infiltration (black solid arrows), reduced structure damage (green solid arrows), and lower levels of congestion (black hollow arrows) in the lungs, liver, and spleens. (B) Histological score was recorded by using a blind test. Experimental data from one representative replicate (of three replicates) are presented. Significance of $p < 0.01$ was indicated as '**'.

clinical value of treatment of sepsis using rapamycin. Taken together, these data demonstrated the therapeutic potential of autophagy in the acute inflammatory phage of sepsis. It also validated that autophagy play a protective role from cell to death during the inflammation.

5. Conclusion

In summary, our study demonstrated activation of autophagy by rapamycin treatment decreases inflammsome activation and pyroptosis in lung. Treatment either pre- or post CLP sepsis, rapamycin protects mice from septic death significantly. Therefore, regulation of autophagy is a valuable target for effective therapy of sepsis in acute inflammatory phase.

Conflict of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Zhenxia Wang: Formal analysis, Validation. **Yan Li:** Conceptualization, Writing - review & editing. **Xiaowei Yang:** Formal analysis, Methodology. **Lian Zhang:** Formal analysis, Methodology. **Huiming Shen:** Funding acquisition, Resources. **Weihong Xu:** Funding acquisition, Resources. **Congli Yuan:** Investigation, Project administration, Supervision.

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