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Original Article

HMGB1 mediates acute liver injury in sepsis through pyroptosis of liver macrophages

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Abstract: Sepsis is a systemic inflammatory response syndrome caused by infection. Septic patients often show an acute liver dysfunction during the onset of sepsis in ICU. We found the levels of ALT, AST, TBIL increased significantly in septic patients and returned after recovery from sepsis in our ICU ($P<0.05$), and had a similar trend for HMGB1. To explore the role of hepatic macrophage in acute liver injury, we simulated the process of acute liver injury by cecal ligation and puncture (CLP) in mice. We assessed the inflammatory infiltration of the liver by HE, and examined the levels of ALT and AST in serum and the expression of HMGB1, IL-1β in the serum and the relative expression of mRNA in the liver at the different time of CLP model. Also we found the rate of pyroptosis cells in liver was about 18.19%, while 16.29% in macrophages by Flow cytometry. So our study has demonstrated that HMGB1 may promote the pyroptosis of liver macrophages to mediate acute liver injury in sepsis.

Keywords: HMGB1, Sepsis, acute liver injury, macrophage, pyroptosis

Introduction

Sepsis is still an acute syndrome that endangers human health. It is one of the main causes of death in the intensive care unit (ICU) for critically ill patients. The essence of sepsis is the systemic inflammatory response syndrome that occurs after the body encounters infection, accompanied by multiple organ dysfunction (MODS) [1]. Despite declining age-standardized incidence and mortality, sepsis remains a major cause of health loss worldwide (Acar, Atalan et al. 2018) [2]. In addition to high incidence rate and high mortality rate, sepsis also causes huge medical costs. The cost of sepsis treatment in the United States and Europe is 16 billion 700 million US dollars and 58 Euro 7 billion 600 million per year [3].

In sepsis, the most commonly damaged target organ is the liver, which is both the center of energy metabolism and the most important immune organ in the body [4]. In sepsis, macrophages in the liver have a huge clearance effect on pathogens, and lymphocytes in the liver also play an important role in the development of sepsis, such as controlling bacteremia, regulating the production, releasing various inflammatory factors, and synthesizing acute-phase proteins [5]. Changes in the structure, function, and metabolism of the liver affect the development and outcome of sepsis. Failure of liver function can also induce the occurrence of MODS. Therefore, the damage of liver function in sepsis is an important factor in determining the prognosis of sepsis. Studies have suggested that most of the organ damage caused by sepsis is functional damage rather than structural damage, and the damage is potentially reversible [6, 7]. Therefore, an effective intervention treatment for acute liver injury caused by sepsis is of great clinical significance to prevent secondary multifunctional damage and improve the prognosis of sepsis.

High mobility group box 1 protein (HMGB1) is an important inflammatory factor of sepsis discovered in recent years. Some experiments have shown that after injecting recombinant HMGB1 intraperitoneally into mice that are insensitive to sensitive LPS and sensitive mice, they can both cause the death. This shows that LPS is not a direct cause of the death of mice, but is caused by other lethal factors. This advanced

Table 1. Primers used for detection of gene expression

Genes	Primer sequences
Mouse-β-actin-S	5'-GTGACGTTGACATCCGTAAAGA-3'
Mouse-β-actin-A	5'-GTAACAGTCCGCCTAGAAGCAC-3'
Mouse-IL-1β-S	5'-GCAACTGTTCCTGAACTCAACT-3'
Mouse-IL-1β-A	5'-ATCTTTTGGGGTCCGTCAACT-3'
Mouse-HMGB1-S	5'-CGGATGCTTCTGTCAACTTCTC-3'
Mouse-HMGB1-A	5'-GTTTCTTCGCAACATCACCAAT-3'

IL, interleukin; HMGB1, High mobility group box 1 protein

proinflammatory and lethal factor is HMGB1 [8]. Animal experiments show that in the CLP model experiment, the concentration of mouse serum HMGB1 is also significantly increased. It is also confirmed that immune cells stimulated by HMGB1 can release TNF-α, IL-6, IL-1β, MCP-1 and many other inflammatory factors in vitro experiments [9]. Pyroptosis is a new form of programmed cell death. It is an inflammatory death form dependent on caspase-1, with morphological features of inflammation and necrosis. When associated with infection, it is present in various cell types, as well as monocytes, macrophages and dendritic cells. Pyroptosis may cause the release of proinflammatory cytokines (including IL-1β and IL-18) outside the cell. As we all know, IL-1β is a key inflammatory cytokine of the host against pathogens, and plays as a role of gatekeeper [10]. Some studies have confirmed that HMGB1 endocytosis of mononuclear macrophages leads to caspase-1 dependent special programmed death, as pyroptosis, and the large number of immune cell deaths is just a important part of sepsis patients with organ damage Important reasons [11]. Therefore, studying the pyroptosis of macrophage has an important role in the process and treatment of acute liver injury in sepsis.

Materials and methods

Materials

FAM-FLICA® Caspase-1 Assay Kit was purchased from ImmunoChemistry Technologies (FAM-YVAD-FMK 655), and 7AAD from BD Pharmingen™.

Experimental animal

C57BL/6 mice (6-8 weeks old, 18-22 g) were purchased from the Animal Lab Center of the Central Laboratory. All animal experiments were performed according to the guidelines of animals approved by The Animal Care and Use Committee of the Nanjing Medical University.

Sepsis model

Sepsis was induced in mice by CLP. The mice were completely anesthetized with 0.75% Pentobarbital solution (10 μg/g), and the midline abdominal incision was made. The cecum was ligated at 1/2 of the distal end and punctured with No. 7 sterile needle to induce polymicrobial peritonitis. The abdominal wall was divided into two layers, and 1 ml 0.9% sodium chloride solution was injected subcutaneously for fluid resuscitation. The animals in the sham operation group underwent laparotomy and intestinal operation without ligation and perforation. After recovery from anesthesia, all mice were free to obtain food and water.

Hematoxylin-eosin staining (HE)

The liver tissue was fixed in 4% formaldehyde for at least 24 hours. Paraffin sections were made using standard techniques. Section (4 μm) was stained with hematoxylin-eosin, and observed and photographed under the microscope.

Enzyme linked immunosorbent assay (ELISA)

Serum samples were collected. The levels of IL-1β, AST, ALT, TBIL and HMGB1 in serum were determined by ELISA kits (R&D Systems) according to the instructions.

Real-time quantitative polymerase chain reaction (qRT-PCR)

Total-RNA from tissue was extracted using TRIzol reagent. RNA was converted to cDNA with Superscript II reverse transcriptase by following the manufacturer's instructions. Primers used in this study as **Table 1**. Each sample was tested for analysis of relative gene expression and transcript levels of β-actin were monitored as internal control.

Flow cytometry

The liver tissues were homogenized to obtain single cell suspension. The cells were washed twice and 1×10^6 cells were suspended in 50 μL PBS supplemented with 1% FBS. Then stained with FLICA (FAM-VAD-FMK655) at 37°C for 30 minutes. 7AAD (BD Pharmingen™) was added

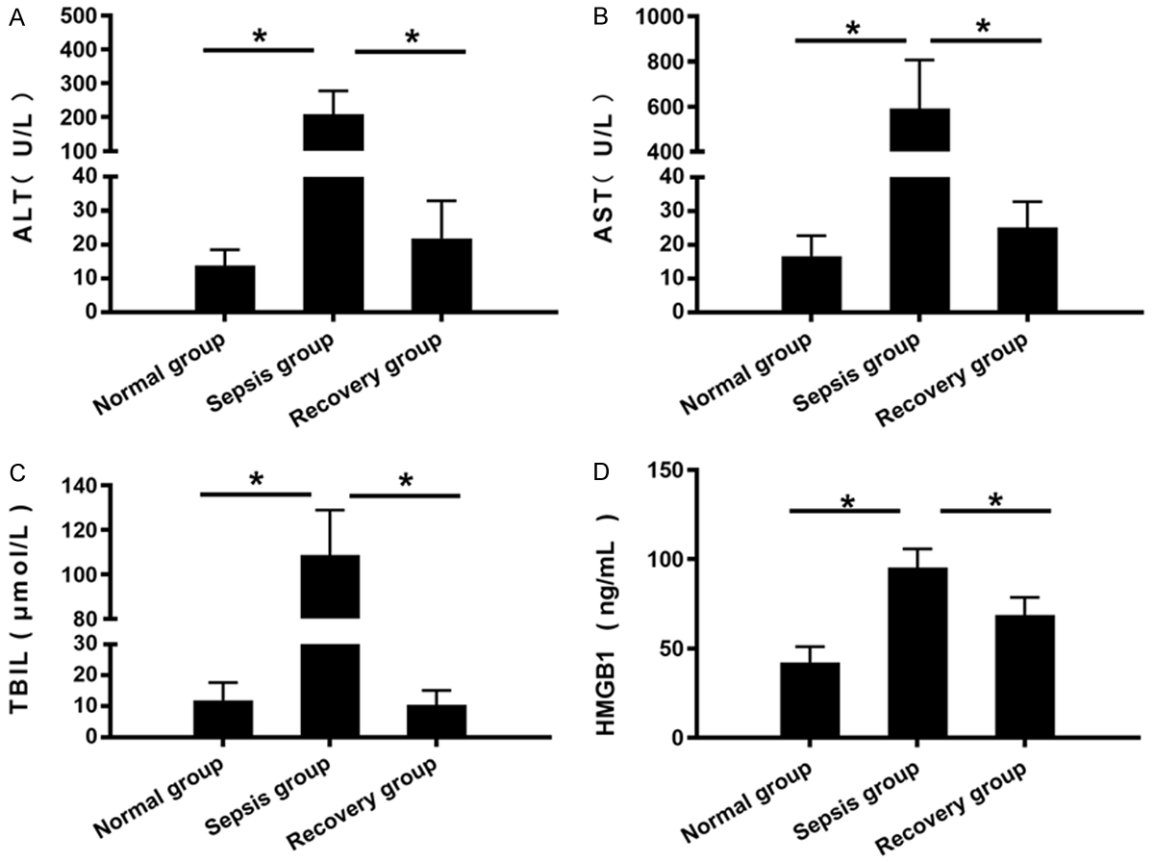

Figure 1. Liver injury in patients with sepsis. The serum samples were prepared and tested for levels for ALT (A), AST (B), TBIL (C), HMGB1 (D) in normal group, sepsis group and recovery group (n = 10; *, P<0.05). Values are presented as the mean ± S.D.

before loading. FACSCalibur flow cytometer was use to analyze the stained cells, and the FlowJo software was to analyze the data.

Statistical analysis

Data were expressed as means ± deviation (SD). Differences were analyzed by one-way ANOVA, and between-groups comparisons were examined by t-test. The survival curve was estimated according to the method of Kaplan-Meier. *P*-values less than 0.05 were considered as statistically significant. All calculations were performed using GraphPad Prism 8.

Results

Liver injury in patients with sepsis

In the intensive care unit (ICU) of our hospital, the liver function indexes in early septic patients were shown as **Figure 1**. We found the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum of sepsis group were markedly higher than that of normal group (n = 10, P<0.05; **Figure 1A**, **1B**). When patients recovered, the levels of ALT and AST returned to normal. All above were typically used to detect cell injury in liver. Otherwise, as signs of hepatic metabolic disorder, total bilirubin (TBIL) were detected. Compared to the normal group, the levels of TBIL increased significantly and returned after recovery from sepsis (n = 10, P<0.05; **Figure 1C**). We guessed that liver may be a point in the treatment of sepsis refrained from multiple organ dysfunction syndrome (MODS). While there was a similar trend for HMGB1 (n = 10, P<0.05; **Figure 1D**), it suggested that HMGB1 may regulate the liver injury of sepsis.

Sepsis-induced acute liver injury modeling in mice

To simulate acute liver injury of sepsis, we established the CLP model of sepsis in mice. We monitored the survival in mice in which sepsis was induced via CLP and the mortality rate

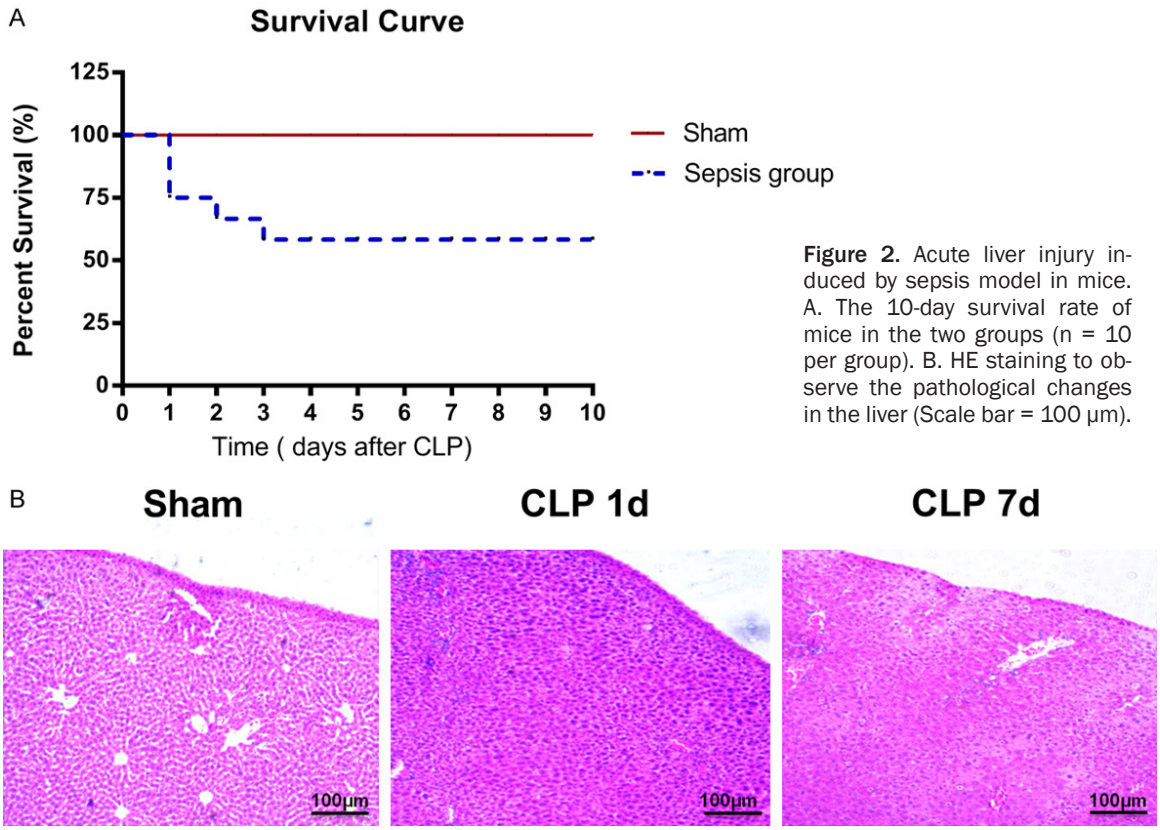

Figure 2. Acute liver injury induced by sepsis model in mice. A. The 10-day survival rate of mice in the two groups (n = 10 per group). B. HE staining to observe the pathological changes in the liver (Scale bar = 100 μm).

was approximately 50% (**Figure 2A**). The pathological changes were evaluated by HE staining. It showed hyperaemia in central vein and hepatic sinusoid. Many inflammatory cells were found to infiltrate in the periportal and pericentral regions of the liver on the first day after CLP and reduced at the 7th day (**Figure 2B**). The content of ALT, AST in mice serum was determined by Elisa assay. Compared to the sham group, the levels of ALT and AST at the first day after CLP model were higher significantly while the damage recovered at 7 d after CLP (**Figure 3A**, **3B**). We considered the first day after CLP as the point of acute liver injury because of the inflammatory reaction in the liver itself. The levels of HMGB1, IL-1β in the serum and the relative expression of mRNA in the liver showed that the first day of CLP was an acute phase of inflammation (**Figure 3C-F**). Therefore, the liver injury at this point was considered as the sepsis induced acute liver injury.

Pyroptosis of hepatic macrophage in sepsis

To the best of our knowledge, sepsis can cause multiple organ dysfunctions and the related liver damage mainly results from the hepatocytes. It is very important to find out the relevant cell, because it is not certain which kind of cell plays a major role in the acute liver injury. We distinguished the several cell types from the hepatocytes by forward scatter (FSC) and side scatter (SSC) profiles such as lymphocytes and macrophages (**Figure 4A**). We found the rate of pyroptotic cells in liver was about 18.19%, while 16.29% in macrophages gated by FSC and SSC (**Figure 4B-D**). All these findings indicated that pyroptosis of hepatic macrophage occupied a dominant position in sepsis-induced acute liver injury.

Discussion

Sepsis is defined as a systemic inflammatory response to infection with multiple organ dysfunction syndrome and death [12]. According to relevant statistical reports, the incidence and mortality of sepsis have been increasing year by year in China in recent years, and effective interventions against acute liver injury in sepsis have not been summarized in the clinical field [13, 14].

CLP model is the most commonly used as sepsis model in animal model. It can lead to severe

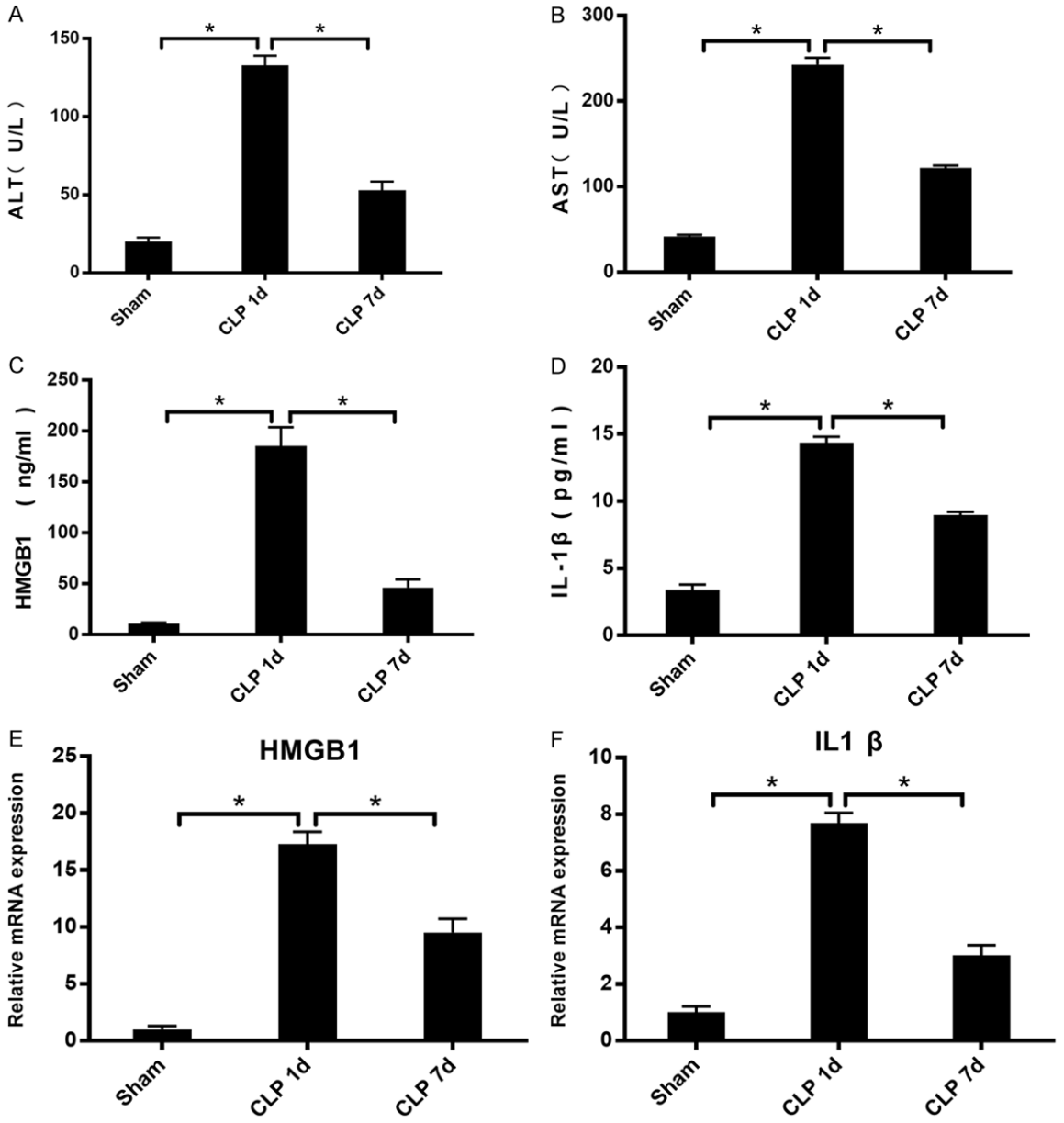

Figure 3. Acute liver injury after CLP model. The activity of ALT (A) and AST (B) in the serum of the sham group and the CLP group at the 1st day and 7th day after CLP. Levels of HMGB1 (C) and IL-1β (D) in the serum of mice in different groups. Relative mRNA expression of HMGB1 (E) and IL-1β (F) in the liver of three groups. (n = 6; *, $P<0.05$. All bars represent mean ± S.D).

peritonitis and multiple organ dysfunction, including the liver, lung and kidney, the occurrence and development of this process are consistent with the pathological process of sepsis in clinics [15]. In this study, we applied the CLP model to simulate the sepsis successfully, and the mortality was stably at about 50%. It showed that acute liver injury we can observe on the first day after CLP, appearing a large number of liver cell edema, necrosis and infiltration of the inflammatory cells, ALT and AST increased. However, on the 7th day after CLP, the edema alleviated, inflammation levels decreased, ALT and AST fell. It illustrates that is in the stage of recovery. Therefore, first day after CLP is as the time point for our study of acute liver injury. Rudiger A et al. have found decreased hepatic blood, liver dysfunction, damaged mitochondrial structure and function, scavenging activity and detoxification ability declined at the early stage of sepsis induced by cecal ligation and puncture (CLP) in rats [16].

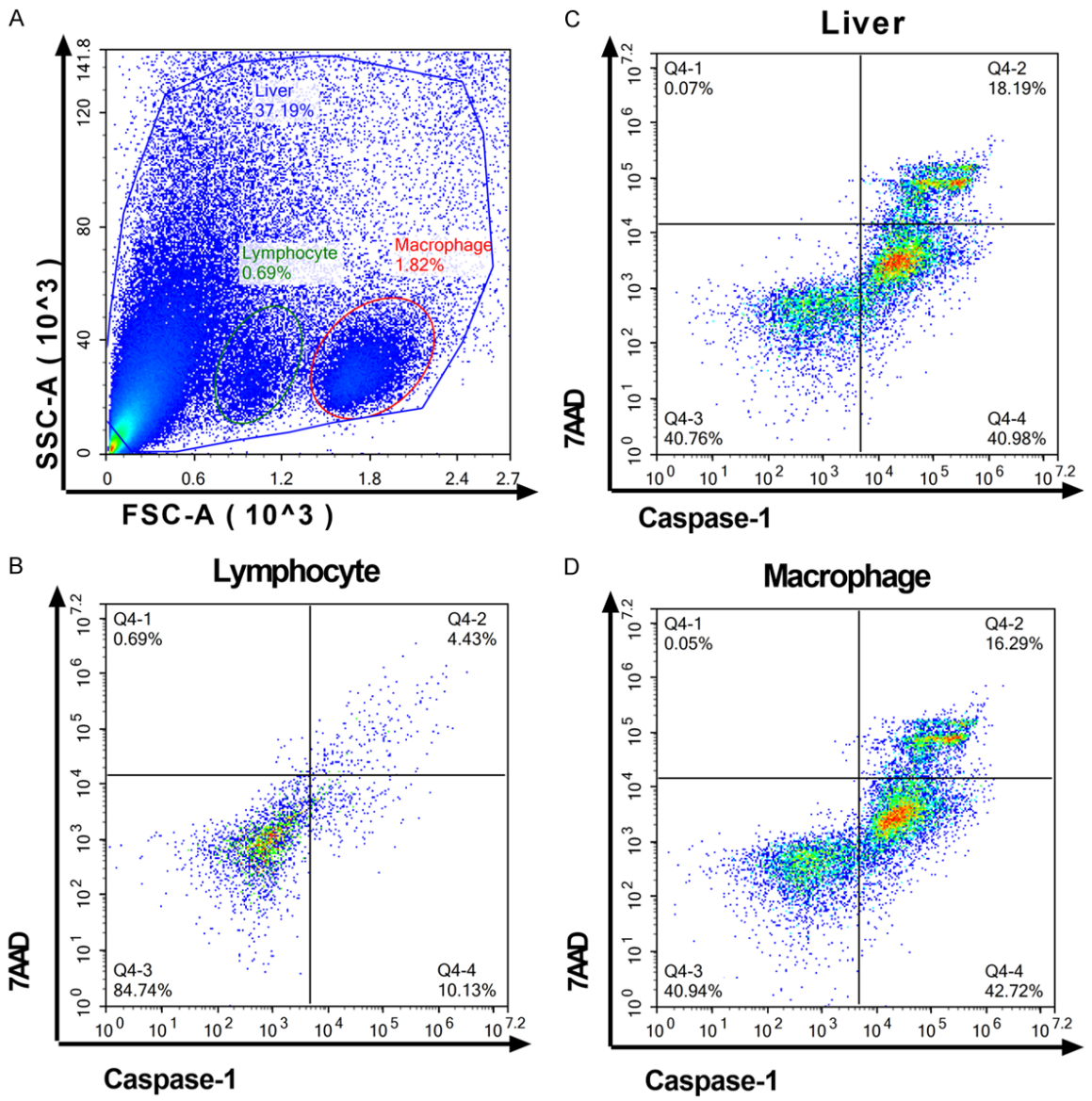

Figure 4. Pyroptosis of hepatic macrophage in sepsis. A. The hepatocytes were gated by forward scatter (FSC) and side scatter (SSC). B. The whole liver cells were stained with 7AAD and caspase-1, while the cells in 7AAD (+) FLICA (+) called as pyroptosis. C. Lymphocytes were stained with 7AAD and caspase-1. D. Macrophages were stained with 7AAD and caspase-1, and pyroptotic cells were detected by flow cytometry. Results are representative of three separate independent experiments.

It is generally known that there are a variety of different cells in liver, including macrophages known as kupffer cells, parenchymal cells, lymphocytes, sinusoidal endothelial cells and so on. They play a significant role in immune response, anti-infection and metabolism [17]. Especially kupffer cells, which are abundant in the liver, occupy 70% of all the macrophages in organism. The activated kupffer cells are significant in liver injury induced by sepsis. Moreover, the activated kupffer cells release various inflammatory cytokines such as TNFα, IL-6, IL-1β, and a variety of membrane receptor on liver cells. Wang et al. reported that HMGB1 is a key inflammatory factor for acute liver injury induced by D-amino semi-lactose and LPS, liver tissue damage and cell necrosis leading to a large release of HMGB1, and that HMGB1 and TNF-α aggravated acute liver injury [18]. It has been found that HMGB1 can interact with inflammatory factors as IL-1β, IL-8 etc. to form a complete circuit, stimulate each other, and

cause the inflammatory response [19, 20]. As we showed the levels of HMGB1 and IL-1β are consistent with the degree of liver damage in mice. So we supposed that as the release of cytosolic contents, pyoptosis has a severe inflammatory response observed in the liver during sepsis in contrast to apoptosis. Moreover our study found that macrophages in the liver are the most important target cells in acute liver injury induced by sepsis.

In summary, our study has demonstrated that HMGB1 may mediate acute liver injury in sepsis through pyroptosis of liver macrophages and indicate an effective target for the clinical treatment. But future studies are recommended to define the pathways and factors that induce pyroptosis during acute liver injury in sepsis.

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Disclosure of conflict of interest

None.

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