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Original Article

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USP9x promotes CD8+ T-cell dysfunction in association with autophagy inhibition in septic liver injury

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Abstract

Sepsis is a life-threatening condition manifested by concurrent inflammation and immunosuppression. Ubiquitin-specific peptidase 9, X-linked (USP9x), is a USP domain-containing deubiquitinase which is required in T-cell development. In the present study, we investigate whether USP9x plays a role in hepatic CD8+ T-cell dysfunction in septic mice. We find that CD8+ T cells are decreased in the blood of septic patients with liver injury compared with those without liver injury, the CD4/CD8 ratio is increased, and the levels of cytolytic factors, granzyme B and perforin are downregulated. The number of hepatic CD8+ T cells and USP9x expression are both increased 24 h after cecal ligation and puncture-induced sepsis in a mouse model, a pattern similar to liver injury. The mechanism involves promotion of CD8+ T-cell dysfunction by USP9x associated with suppression of cell cytolytic activity via autophagy inhibition, which is reversed by the USP9x inhibitor WP1130. In the *in vivo* studies, autophagy is significantly increased in hepatic CD8+ T cells of septic mice with conditional knockout of mammalian target of rapamycin. This study shows that USP9x has the potential to be used as a therapeutic target in septic liver injury.

Key words sepsis, liver injury, USP9x, CD8+ T cells, autophagy

Introduction

Sepsis is a life-threatening condition caused by an uncontrolled immune response to infection. It has high morbidity and mortality and is mainly characterized by multiple pathological or physiological abnormalities, such as cytokine storms, hyperinflammation, immunosuppression, and tissue damage [1]. As the second most commonly affected organ in sepsis, the liver plays a decisive role in maintaining homeostasis during host defense against pathogens, which affects the outcome of sepsis in patients [2]. The incidence of liver dysfunction is up to 46% and that of liver failure is up to 22% in septic patients [3]. Liver injury occurs as early as 1 h after cecal ligation and puncture (CLP) surgery in animal models [4]. Although much progress has been made in the study of sepsis, the precise cellular and molecular mechanisms involved in septic liver injury are still unclear.

Immune imbalance exists in sepsis and is manifested by concurrent inflammation and immunosuppression, which facilitates the development of persistent infection and a poor outcome [5]. Immunosuppression in sepsis is related to T-cell exhaustion and increased levels of apoptosis of various kinds of immune cells, including T cells, B cells, natural killer cells, dendritic cells, and macrophages [6,7]. In septic patients, the decrease in the numbers of splenic CD4+ and CD8+ T cells is associated with a reduced ability to elicit immune responses to viral infection [8]. Studies focusing on the number of liver-derived CD8+ T cells in septic animals revealed that the frequency of CD8+ T cells in the livers of septic mice was significantly increased at 20 h [9], and the CD4/CD8 ratio was decreased 3 h after CLP [10]. However, the dynamic change and function of hepatic CD8+ T cells in septic liver injury have not been described.

Ubiquitin-specific peptidase (USP) 9, X-linked (USP9x) is a USP domain-containing deubiquitinase (DUB) that is reported to regulate multiple cellular functions [11], and the role of USP9x in T cells has recently attracted increasing attention. Naik *et al.* [12] reported that USP9x is a positive regulator in CD4+ T-cell activation and homeostasis by controlling zeta-chain-associated protein kinase 70 (ZAP70) ubiquitination and protein kinase C beta kinase activity. Depletion of USP9x *in vivo* markedly attenuates CD4+ T-cell-mediated inflammatory responses by blocking T-cell receptor (TCR)-induced nuclear factor kappa B activation [13]. Both findings are consistent with another report showing that USP9x is required for normal T-cell development and that USP9x-deficient T cells are hypoproliferative [14]. As such, studies indicated that USP9x is required for CD4+ T-cell activity. Whether USP9x participates in the regulation of CD8+ T cells and in the process of sepsis is worthy of further investigation.

It is of interest that USP9x expression is found to be correlated with mammalian target of rapamycin (mTOR). mTOR is a ubiquitous protein kinase with two distinct complexes, mTORC1 and mTORC2. mTORC1 is involved in the regulation of cell cycle progression and autophagy, while mTORC2 is primarily required for the control of cell proliferation and survival. Decrease of USP9x has been shown to result in the inhibition of mTORC1 signaling in neural progenitors [15]. In addition, USP9x was shown to stimulate mTORC2 assembly and activity in multiple cell lines, including HeLa, HEK293FT, and HepG2 cells and mouse embryonic fibroblasts [16]. It is known that mTOR is closely associated with cell autophagy, with inhibition of mTOR leading to upregulated autophagy. As decreasing T-cell autophagy was shown to result in enhancement of apoptosis and immunosuppression in a mouse model of sepsis [17], we hypothesize that USP9x may regulate hepatic CD8+ T-cell dysfunction via autophagy in septic liver injury.

In the present study, we found that the number of CD8+ T cells was significantly decreased and the CD4/CD8 ratio was increased in the peripheral circulation of septic patients with liver injury. In a CLP-induced mouse sepsis model, the number of hepatic CD8+ T cells and the expression of USP9x in hepatic CD8+ T cells were both significantly increased 24 h after surgery. The pattern was similar to that associated with liver injury. More importantly, we demonstrated that USP9x promoted CD8+ T-cell dysfunction in association with autophagy inhibition in septic liver injury.

Materials and Methods

Clinical samples

Seventeen septic patients with liver injury and 24 septic controls hospitalized at Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China) were recruited between September 2018 and August 2021. Serum samples were collected following the protocols approved by the Ethics Committee at Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (No. 2021-KY-012-K). Written informed consent was obtained from each participant.

Patients who were 18–80 years of age with an intensive care unit stay of at least 24 h, at least three systemic inflammatory response-syndrome criteria, and the simultaneous identification of focal infection by clinical, radiological, or microbiological criteria of the Society of Critical Care Medicine/European Society of Intensive Care Medicine were eligible for inclusion [18]. The diagnosis of liver injury required at least two of the following three observations: (1) total bilirubin > 43 μM (2.5 mg/dL); (2) serum alanine aminotransferase (ALT) > 2 times the upper limit of normal; and (3) a prothrombin time 1.5 times longer than the reference value, or an international normalized ratio 1.5 times longer than the reference value. Pregnant women and patients with connective tissue diseases such as vasculitis, thrombotic diseases, or basic liver disease, including hepatitis, cirrhosis, or liver cancer, were excluded.

Cell lines

HEP1-6 mouse liver cancer cells were purchased from the Cell Bank/Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in complete medium made of RPMI 1640 (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Invitrogen, Carlsbad, USA) and 100 μg/mL streptomycin (Invitrogen) in a humidified atmosphere containing 5% CO_2 at 37°C.

Mice

Wild-type (WT) C57BL/6 mice were purchased from SLAC Laboratory Animal Co., Ltd (Shanghai, China). Estrogen receptor (ER)-Cre mice, which express Cre as a fusion protein with tamoxifen-responsive ER, and $mTOR^{flox/flox}$ mice were generously provided by Professor Yong Zhao of the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). mTOR conditional knockout mice ($mTOR^{\Delta ER}$ mice) were generated by mating $mTOR^{flox/flox}$ mice with ER-Cre mice. Deletion of mTOR was induced by intraperitoneal injection of 50 mg/kg tamoxifen (Sigma-Aldrich, St Louis, USA) for 5 consecutive days. Experiments were performed on mice at 8 weeks of age and undertaken 3 weeks after tamoxifen injection. PCR was used to genotype the mice. All animal experiments were performed following the guidelines of the Care and Use of Laboratory Animals of the Laboratory Animal Ethical Commission of Fudan University and were approved by the Animal Ethical Committee of Zhongshan Hospital, Fudan University (Shanghai, China).

Sepsis induction

A CLP-induced sepsis mouse model was established as previously described [9]. In brief, WT or $mTOR^{\Delta ER}$ mice were anesthetized with 1% pentobarbital, and a 0.5-cm incision was made to expose the cecum, which was punctured once with a 22 gauge needle. Mice subjected to the same procedure except for CLP were used as the sham group. All mice were immediately given 1 mL of 0.9% saline subcutaneously for fluid resuscitation after CLP. In some procedures, WP1130 (30 mg/kg; Selleck Chemicals, Houston, USA) was injected intravenously into mice 2 h after surgery. Serum and liver tissue were obtained from mice at 0, 6, 24, and 72 h after CLP.

Histology analysis

Liver specimens were fixed in 10% neutral buffered formalin and paraffin-embedded. Deparaffinized sections (5 μm) were stained with hematoxylin and eosin, observed with a digital slide scanner and camera system (C13220-0; Hamamatsu Photonics, Tokyo, Japan) and evaluated by a pathologist who was blinded to the group assignments. The pathological score was assessed as previously reported [19]. In brief, inflammation, necrosis, and thrombus formation were graded on a scale of 0 to 4 (from absent to severe),

respectively. The total score was the sum of each parameter, with a maximum score of 12.

Liver function assays

Serum was obtained from mice. Alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were measured using commercial kits (Sigma-Aldrich) according to the manufacturer's instructions and reported as U/L.

Flow cytometry

Monoclonal antibodies against CD8 (Alexa Fluor 647; Clone: 53-6.7) and isotype IgG2a (Alexa Fluor 647; Clone: RTK2758) were obtained from BioLegend (San Diego, USA). Multiple-color flow cytometry was performed with an Aria fluorescence-activated cell sorter (BD Biosciences, San Diego, USA).

Purification of CD8$^+$ T cells

Mouse livers were excised, minced, and digested with collagenase IV (Sigma-Aldrich) for 30 min at 37°C. Intrahepatic nonparenchymal cells were subject to Percoll (GE Healthcare, Piscataway, USA) density gradient centrifugation at 425 *g* for 30 min at 20°C. CD8$^+$ T cells were sorted by flow cytometry. In this study, the purity of the sorted cells was consistently more than 99%. Human CD8$^+$ T cells were magnetically purified from mononuclear cells of blood (#130-045-201; Miltenyi Biotec, Auburn, USA) following the manufacturer's recommendations after Ficoll density gradient centrifugation. The purity of the sorted cells was consistently more than 90%.

Quantitative PCR (qPCR)

Total RNA was extracted from CD8$^+$ T cells using Trizol (Invitrogen) and subsequently reverse-transcribed using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Lafayette, USA) according to the manufacturer's protocol. qPCR was performed using Hieff qPCR SYBR Green Master Mix (Yeasen, Shanghai, China) on an ABI Prism 7900HT (Applied Biosystems, Foster City, USA). Thermocycler conditions included a 2-min incubation at 50°C and 95°C for 10 min; a two-step PCR program of 95°C for 15 s; and 40 cycles at 60°C for 60 s each. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers are listed in Table 1.

Table 1. Sequences of primers used for qPCR

Name	Sequence (5′→3′)
Human	
GAPDH forward	GGATTTGGTCGTATTGGG
GAPDH reverse	GGAAGATGGTGATGGGATT
Perforin forward	GACTGCCTGACTGTCGAGG
Perforin reverse	TCCCGGTAGGTTTGGTGGAA
Granzyme B forward	CCCTGGGAAAACACTCACACA
Granzyme B reverse	GCACAACTCAATGGTACTGTCG
Mouse	
β-actin forward	TGTCCACCTTCCAGCAGATGT
β-actin reverse	AGCTCAGTAACAGT-CCGCCTAGA
Atg5 forward	GCTGCGGTTCACTCTGGTT
Atg5 reverse	TCGGTCGGGTTCTGTCTGC
Atg7 forward	TGACGAGGAGACTGTCTGAAGC
Atg7 reverse	CTGGTGAGATGGCACAGGAAA
Beclin-1 forward	AGCTGGAGTTGGATGACGAAC
Beclin-1 reverse	TGATTGTGCCAAACTGTCCG
LC3II forward	CCCACCAAGATCCCAGTGAT
LC3II reverse	CCAGGAACTTGGTCTTGTCCA
Granzyme B forward	TGTGAAGCCAGGAGATGTGTGCTA
Granzyme B reverse	TCAGCTCAACCTCTTGTAGCGTGT
Perforin forward	GCAGGTCAGGCCAGCATAAG
Perforin reverse	GCAGTCCTGGTTGGTGACCTT
USP5 forward	GCTGCTGTCAGTATTACCGAC
USP5 reverse	AAAGCCCAGAAACGTGTTCATA
USP9x forward	ACGTTCAGATAATTGCAGCCC
USP9x reverse	AACCCGCGAAGCTAAATTCTT
USP14 forward	ATGCCACTCTACTCTGTTACAGT
USP14 reverse	AACACCATTGGAGGTTCATCAG
USP37 forward	CTCATCAGTGTTGTCAGT
USP37 reverse	TCCAGGTCATTGTAAGTG
UCHL1 forward	GGCCAACAACCAAGACAAGC
UCHL1 reverse	GTCATCTACCCGACACTGGC

Western blot analysis

Cells were lysed, and proteins were quantified using BCA reagent (Beyotime Biotechnology, Shanghai, China). Proteins (20 μg) were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dried milk plus 0.1% Tween 20 for 2 h at room temperature and exposed to primary antibodies diluted 1000-fold that recognize USP9x (#14898; Cell Signaling, Danvers, USA), Atg5 (#12994; Cell Signaling), Atg7 (#8558; Cell Signaling), Beclin-1 (ab207612; Abcam, Cambridge, USA), LC3II (ab192890; Abcam), mTOR (#2983; Cell Signaling), p-mTOR (#5536; Cell Signaling), p62 (#88588; Cell Signaling), p70S6K1 (#34475; Cell Signaling), p-p70S6K1 (#9204; Cell Signaling), 4E-BP1 (#9452; Cell Signaling), p-4E-BP1 (#13443; Cell Signaling), or β-actin (#3700; Cell Signaling) overnight at 4°C and subsequently washed. Following primary incubation, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; Cell Signaling) for 1 h at room temperature. Protein bands were visualized with an enhanced chemiluminescence system (Thermo Fisher Scientific). Protein band intensities were analysed with Image-Pro Plus software (version 6.0; Media Cybernetics, Bethesda, USA) and normalized against β-actin.

Enzyme-linked immunosorbent assay (ELISA)

The whole liver was homogenized in PBS using a homogenizer. The homogenate was then centrifuged at 12,000 *g* for 5 min. The levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the supernatant obtained were assessed using the corresponding ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Cytolysis assay

For the cytolysis assay, CD8$^+$ T cells (5×10^4 or 2×10^5) were used as the effector cells (E), and HEP1-6 cells (1×10^4) were used as the target cells (T), with E/T coculture ratios of 5:1 or 20:1. The culture supernatants were collected from each well to assay lactate

dehydrogenase (LDH) release from lysed target cells using the LDH assay kit (Roche, Basel, Switzerland) following the manufacturer's instructions. The cytolysis rate (%) was calculated as: cytolysis rate (%) = {[(effector/target cell mix–effector cell control)–low control]/(high control–low control)} × 100%.

Statistical analysis

Prism 7.0 (GraphPad Software, San Diego, USA) was used for the statistical analysis. Data are shown as the mean ± SEM, and survival analysis was performed by the Kaplan-Meier method. Normally distributed variables were compared by Student's *t* tests and one-way analysis of variance. The nonparametric Mann-Whitney test was used when the distribution was not normal. $P < 0.05$ was considered statistically significant.

Results

Peripheral CD8+ T cells were decreased in septic patients with liver injury

To identify the role of CD8+ T cells in septic patients with or without liver injury, we determined the percentage of CD8+ T cells and the CD4/CD8 ratio in the peripheral blood. The percentage of CD8+ T cells was significantly decreased (Figure 1A), and the CD4/CD8 ratio was increased in septic patients with liver injury compared with those without liver injury (Figure 1B). CD8+ T cells were then magnetically purified from human blood to test cytolytic activity. The levels of granzyme B and perforin were downregulated in CD8+ T cells derived from septic patients with liver injury (Figure 1C). The data showed that the percentage of CD8+ T cells from septic patients with liver injury was decreased, and these cells might lose cytolytic function.

The number of hepatic CD8+ T cells was increased in septic mice with liver injury

To determine the role of hepatic CD8+ T cells, livers were harvested from septic mice at 0, 6, 24, and 72 h after CLP. Histology analysis showed that inflammation, necrosis, and thrombus formation appeared at 6 h, peaked at 24 h, and decreased at 72 h after CLP (Figure 2A). The pathological changes were consistent with significant increases in serum ALT and AST at 24 h after CLP (Figure 2B). In addition, the secretion of the liver-derived proinflammatory cytokines TNF-α and IL-6 was significantly increased 6 h after CLP and decreased at 72 h (Figure 2C). The number of CD8+ T cells in the livers of septic mice was significantly increased at 24 h and decreased at 72 h after CLP (Figure 2D). The change in the number of hepatic CD8+ T cells was positively correlated with the severity of septic liver injury.

USP9x promoted CD8+ T-cell dysfunction in septic liver injury

To determine whether USP9x is involved in the decrease of CD8+ T cells, its expression in hepatic CD8+ T cells after CLP was assayed. As shown in Figure 3A,B, both the mRNA and protein levels of USP9x were increased at 6 h, significantly upregulated at 24 h, and decreased at 72 h after CLP. This result was consistent with the severity of septic liver injury. To further confirm the role of USP9x in septic liver injury, intravenous injection of WP1130, a USP9x inhibitor, 2 h after CLP improved survival following CLP-induced sepsis (Figure 3C). Vehicle-treated mice served as controls. Moreover, liver inflammation, necrosis, and thrombus formation were reduced in mice treated with WP1130 (Figure 3D). WP1130 also significantly reversed the altered liver function (Figure 3E) and downregulated the proinflammatory cytokines TNF-α and IL-6 in the livers of septic mice (Figure 3F). These data demonstrated that USP9x is involved in promoting the development of septic liver injury.

To further investigate the activity of USP9x, the percentage and function of CD8+ T cells were determined in liver tissue with or without WP1130 administration. The percentage of CD8+ T cells in the livers of septic mice was considerably decreased by WP1130 (Figure 3G). The cytolytic activity of hepatic CD8+ T cells from septic mice against tumor cells was significantly promoted by WP1130 (Figure 3H). CD8+ T cells from septic mice without WP1130 treatment lost cytolytic activity. Hepatic CD8+ T cells from septic mice treated with WP1130 actively expressed granzyme B and perforin (Figure 3I). These results are consistent with the promotion of CD8+ T-cell dysfunction by USP9x in septic liver injury.

USP9x inhibited the autophagy of hepatic CD8+ T cells

As the USP9x level is correlated with cell autophagy, we

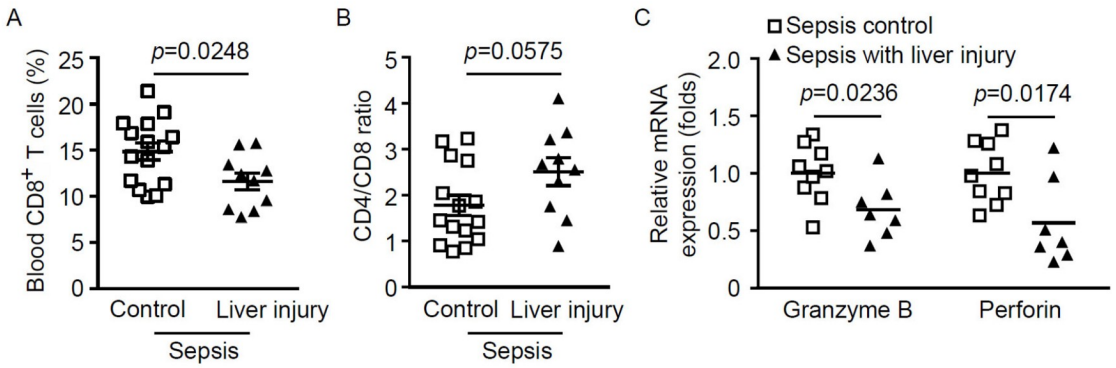

Figure 1. Peripheral CD8+ T cells were decreased in septic patients with liver injury Blood samples were collected from septic patients with or without liver injury. The percentage of CD8+ T cells (A) and CD4/CD8 ratio (B) are shown ($n = 15$ septic patients without liver injury; $n = 10$ septic patients with liver injury). (C) CD8+ T cells were then magnetically purified from blood, and the mRNA levels of granzyme B and perforin in cells were measured by qPCR. Data are normalized to the expression levels of the sepsis control group ($n = 9$ septic patients without liver injury; $n = 7$ septic patients with liver injury). Data are shown as the mean ± SEM. *P* values are shown in each panel.

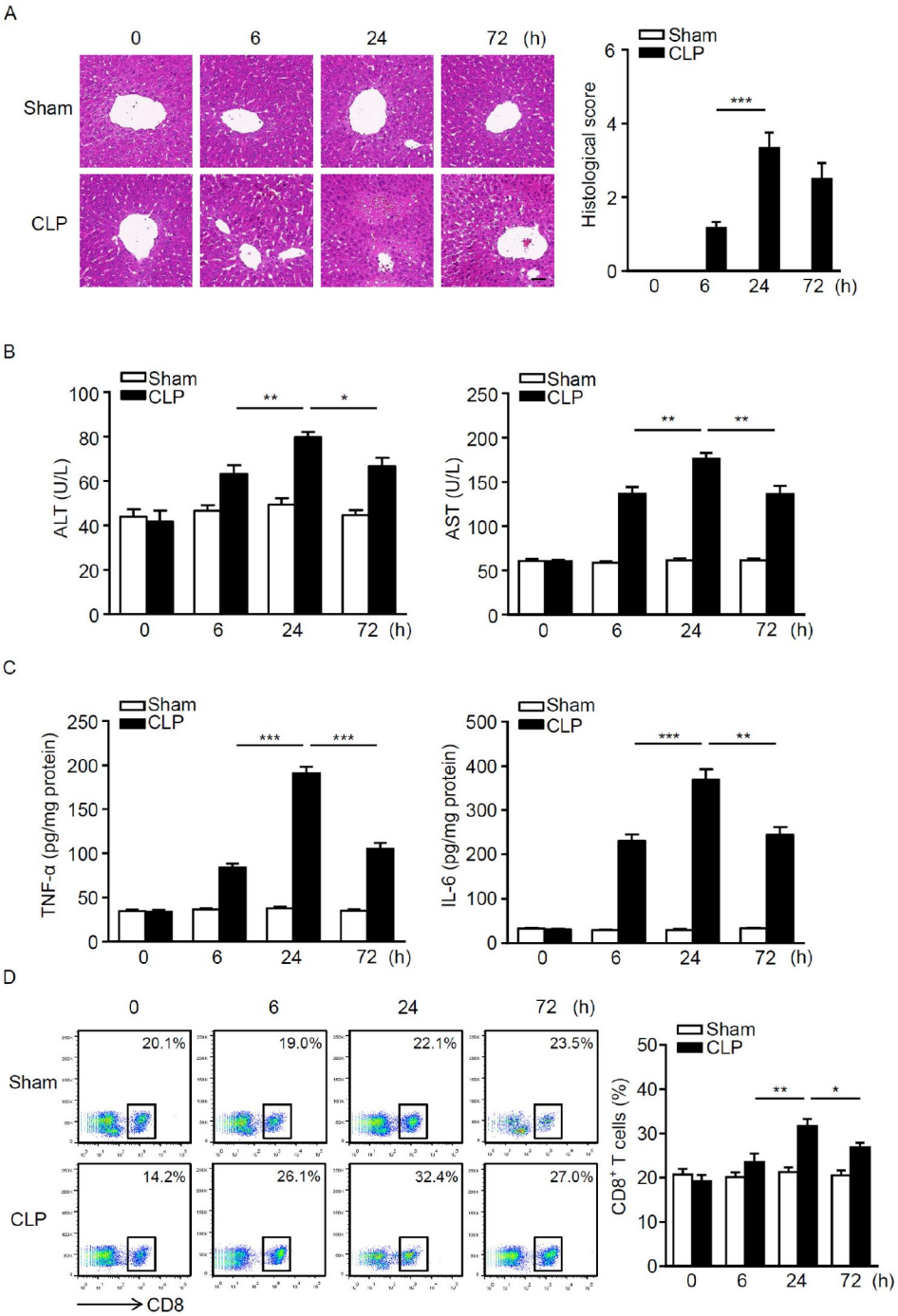

Figure 2. The number of hepatic CD8$^+$ T cells was increased in the livers of septic mice Serum and liver tissue from each group were sampled at 0, 6, 24, and 72 h after CLP surgery. (A) Representative liver tissue sections (scale bar: 100 μm) are shown on the left, and the pathology scores are shown on the right. (B) Serum levels of ALT and AST. (C) TNF-α and IL-6 levels in the liver tissue determined by ELISA. (D) The percentages of CD8$^+$ T cells in intrahepatic nonparenchymal cells were determined by flow cytometry. Representative images are shown on the left, and summarized results are shown on the right. Data are shown as the mean ± SEM of three independent assays ($n = 6$ mice per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

investigated whether blockade of USP9x influences autophagy in hepatic CD8$^+$ T cells from septic mice. As shown in Figure 4A,B, both the mRNA and protein expression levels of the autophagy-related proteins Atg5, Atg7, Beclin-1, LC3II and p62 were significantly reduced in hepatic CD8$^+$ T cells from septic mice, whereas WP1130 treatment reversed these changes. In addition, the phosphorylation of mTOR and the mTORC1 substrates p70S6K1 and 4E-BP1 was largely inhibited in hepatic CD8$^+$ T cells treated with WP1130 (Figure 4C). These data indicated that the dysfunction of hepatic CD8$^+$ T cells is resulted from USP9x-mediated autophagy

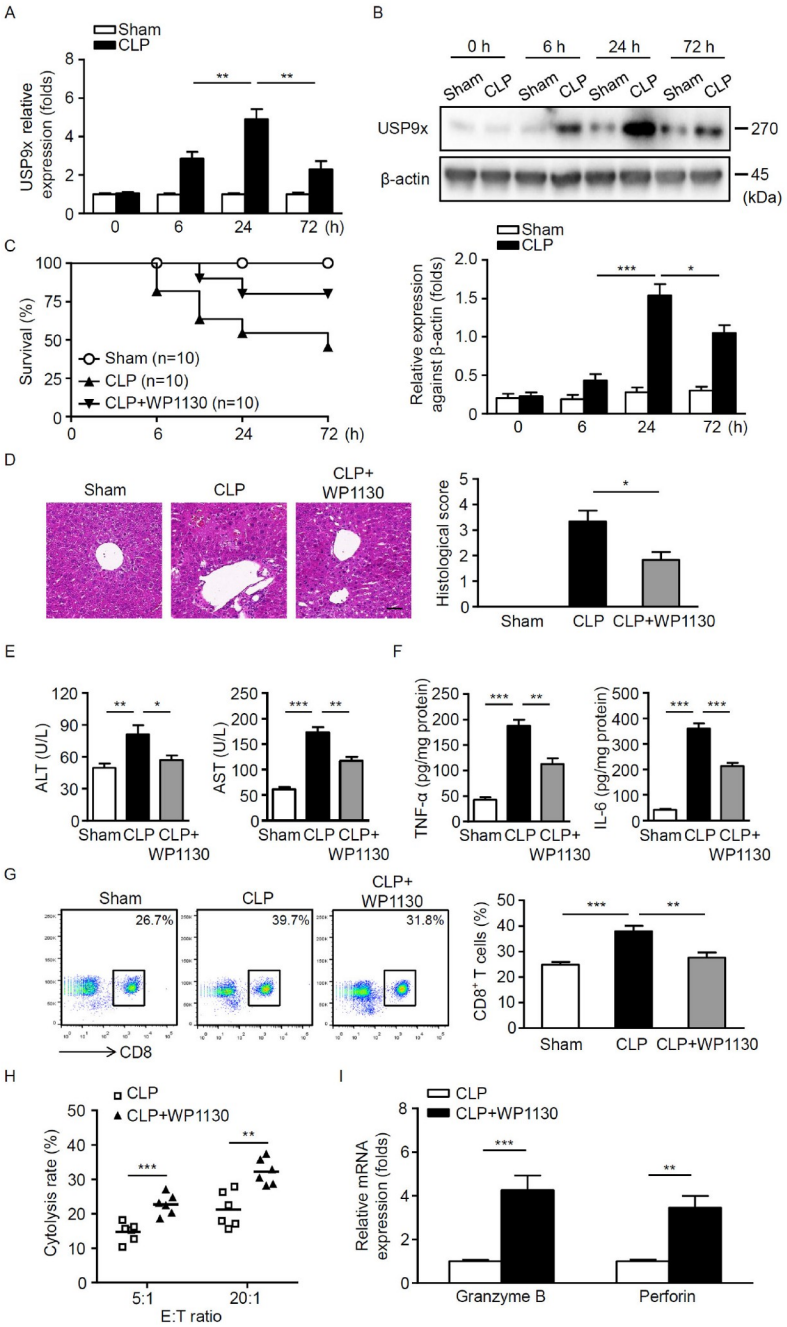

Figure 3. USP9x promoted CD8+ T-cell dysfunction in septic liver injury Serum and liver tissues from each group were sampled 0, 6, 24, and 72 h after CLP surgery in mice with or without WP1130 (30 mg/kg) treatment. CD8+ T cells in liver tissue were evaluated by fluorescence-activated cell sorting. (A) *USP9x* mRNA levels in hepatic CD8+ T cells were determined by qPCR. Data are normalized to its expression at 0 h. (B) Protein level of USP9x in hepatic CD8+ T cells was determined by western blot analysis. Representative images are shown on the top, and summarized results are shown at the bottom. (C) Cumulative survival of mice. Data from three independent experiments are combined ($n=10$ mice per group). (D) Representative images from liver tissue sections (scale bar: 100 μm) are shown on the left, and pathology scores are shown on the right. (E) Serum levels of ALT and AST. (F) TNF-α and IL-6 levels in the liver tissue determined by ELISA. (G) The percentage of CD8+ T cells in intrahepatic nonparenchymal cells was determined by flow cytometry. Representative images are shown on the left, and summarized results are shown on the right. (H) Cytolysis of HEP1-6 cells (target cells; T) by hepatic CD8+ T cells (effector cells; E) was determined by LDH activity assay. (I) *Granzyme B* and *perforin* mRNA levels in hepatic CD8+ T cells were measured by qPCR. Data were normalized to *CLP* expression in the control group. Data are shown as the mean ± SEM of three independent experiments ($n=6$ mice per group). *$P<0.05$, **$P<0.01$, ***$P<0.001$.

inhibition in septic liver injury.

As a partially selective DUB inhibitor, WP1130 not only inhibits USP9x activity but also suppresses the deubiquitinating activity of USP5, USP14, USP37, and UCHL1 [20]. We then investigated the effect of WP1130 treatment on the expressions of all these DUBs. As shown in Figure 4D, the mRNA levels of USP9x and UCHL1 were

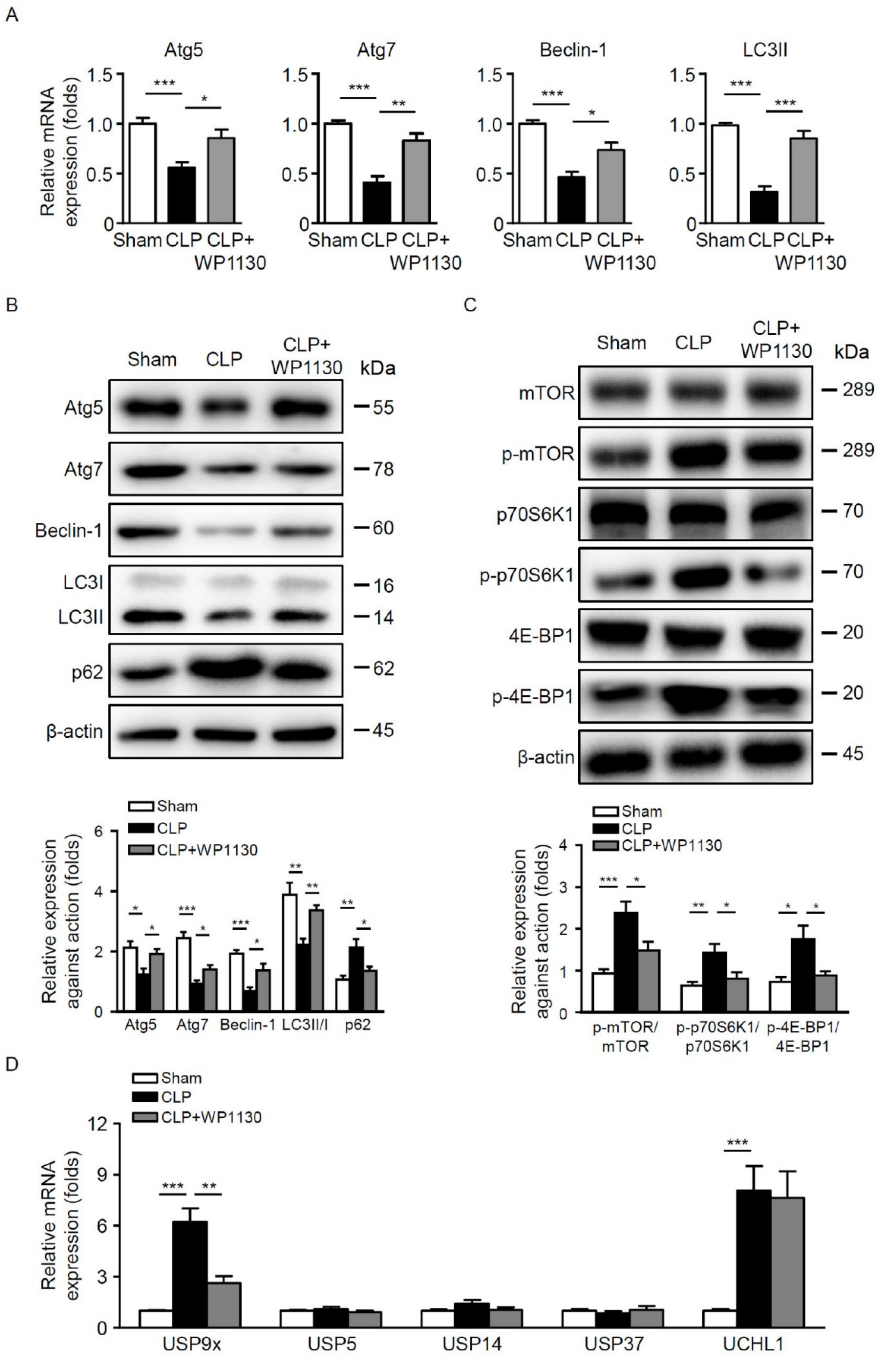

Figure 4. USP9x inhibited the autophagy of hepatic CD8+ T cells Liver tissues were harvested 24 h after CLP surgery from mice with or without WP1130 (30 mg/kg) treatment. Hepatic CD8+ T cells were evaluated by fluorescence-activated cell sorting. (A) The mRNA levels of *Atg5*, *Atg7*, *Beclin-1*, and *LC3II* in hepatic CD8+ T cells were determined by qPCR. Data were normalized to expression levels in the sham group. (B) The protein levels of Atg5, Atg7, Beclin-1, LC3II, p62, and β-actin in hepatic CD8+ T cells were determined by western blot analysis. Representative images are shown at the top, and summarized results are shown at the bottom. (C) The protein levels of mTOR, p-mTOR, p70S6K1, p-p70S6K1, 4E-BP1, p-4E-BP1 and β-actin in hepatic CD8+ T cells were determined by western blot analysis. Representative images are shown on the top, and summarized results are shown at the bottom. (D) The mRNA levels of *USP9x*, *USP5*, *USP14*, *USP37* and *UCHL1* in hepatic CD8+ T cells were determined by qPCR. Data were normalized to their expression levels in the sham group. Data are shown as the mean ± SEM from three independent assays ($n = 6$ mice per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

significantly upregulated in hepatic CD8+ T cells from septic mice, whereas WP1130 treatment inhibited USP9x expression but not UCHL1 expression. USP14 expression was slightly increased in CLP mice, and WP1130 treatment inhibited its expression to some extent. In addition, there were no significant changes in USP5 or USP37 in any treatment group, showing that the protective role of WP1130 in septic liver injury may partially be attributed to USP9x inhibition.

mTOR deficiency reversed the effect of USP9x on CD8+ T-cell dysfunction

Autophagy in hepatic CD8+ T cells was compared between WT and mTOR$^{\Delta ER}$ mice with CLP surgery. The survival of septic mice with mTOR deficiency was improved compared with that of WT mice (Figure 5A). mTOR deficiency also resulted in significant down-

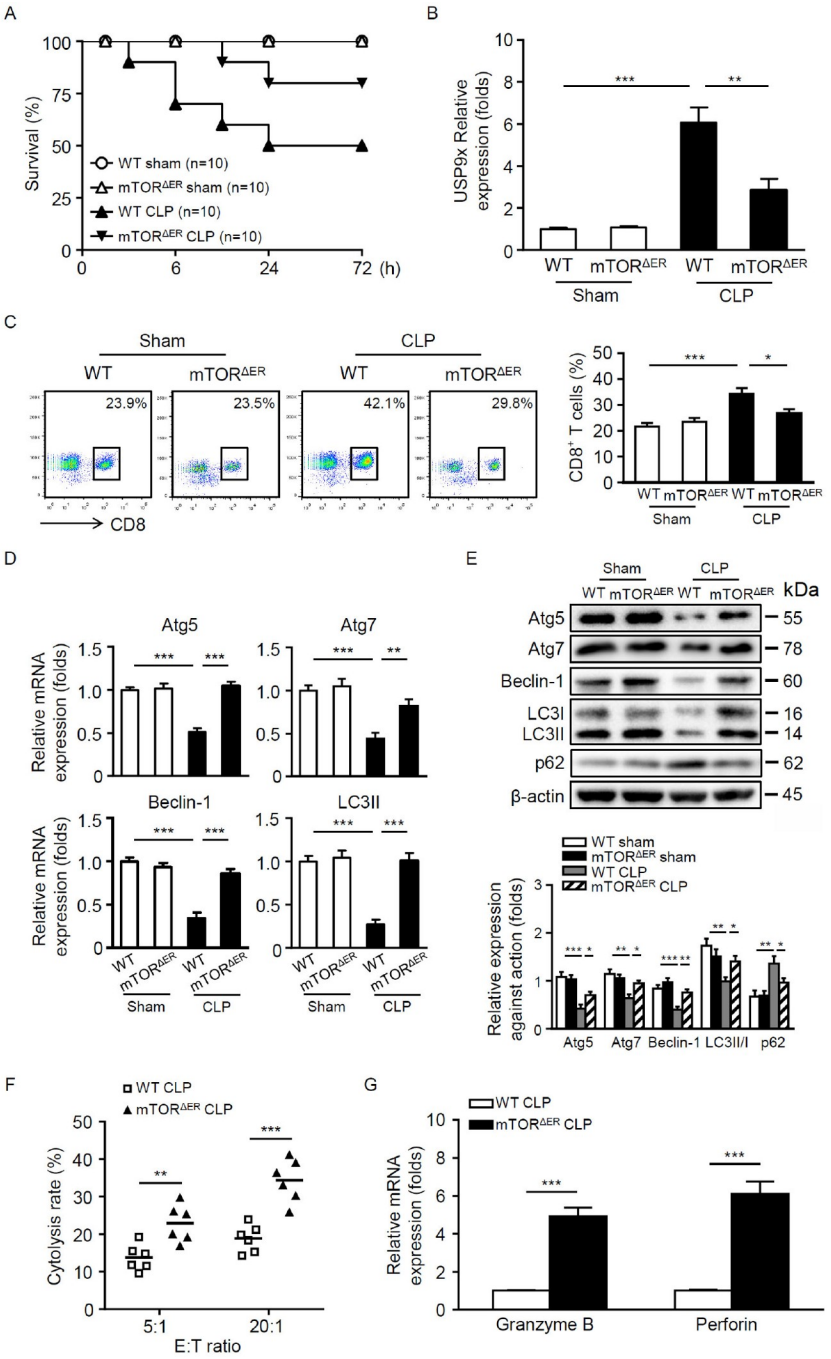

Figure 5. mTOR deficiency reversed the effect of USP9x on CD8+ T-cell dysfunction Serum and liver tissue were harvested from each group of WT or mTOR$^{\Delta ER}$ mice 24 h after CLP surgery, and CD8+ T cells from liver tissue were evaluated by fluorescence-activated cell sorting. (A) Cumulative survival was estimated using combined data from three independent experiments ($n = 10$ mice per group). (B) *USP9x* mRNA level in hepatic CD8+ T cells was measured by qPCR. Data were normalized to its expression in the WT sham group. (C) The percentage of CD8+ T cells in intrahepatic nonparenchymal cells was determined by flow cytometry. Representative images are shown on the left, and summarized results are shown on the right. (D) The mRNA levels of *Atg5*, *Atg7*, *Beclin-1*, and *LC3II* in hepatic CD8+ T cells were determined by qPCR. Data were normalized to their expressions in the WT-sham group. (E) The protein levels of Atg5, Atg7, Beclin-1, LC3II, p62, and β-actin in hepatic CD8+ T cells were measured by western blot analysis. Representative images are shown on the top, and summarized results are shown at the bottom. (F) Cytolysis of HEP1-6 cells (target cells; T) by hepatic CD8+ T cells (effector cells; E) was determined by LDH activity assay. (G) *Granzyme B* and *perforin* mRNA levels in hepatic CD8+ T cells were measured by qPCR. Data are normalized to mRNA expression in the WT CLP group. Data are shown as the mean ± SEM from three independent experiments ($n = 6$ mice per group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

regulation of USP9x expression (Figure 5B) and the percentage of CD8$^+$ T cells (Figure 5C) in the livers of septic mice 24 h after CLP. We evaluated autophagy in CD8$^+$ T cells and found that both the mRNA and protein expression levels of Atg5, Atg7, Beclin-1, LC3II, and p62 were significantly reversed in hepatic CD8$^+$ T cells from mTOR$^{\Delta ER}$ mice with CLP (Figure 5D,E). Cytolysis assays of hepatic CD8$^+$ T cells in septic mice (Figure 5F,G) revealed that mTOR deficiency is not associated with the loss of cytolytic activity of hepatic CD8$^+$ T cells in septic mice, which express granzyme B and perforin. These results indicated that conditional knockout of *mTOR* effectively reversed the effect of USP9x on CD8$^+$ T-cell dysfunction in septic mice.

Discussion

We found that CD8$^+$ T cells were decreased in the peripheral blood of septic patients with liver injury and that their expressions of cytolytic factors were decreased. The percentage of hepatic CD8$^+$ T cells and USP9x level in hepatic CD8$^+$ T cells started to increase at 6 h, peaked at 24 h, and then decreased at 72 h after CLP. USP9x promoted CD8$^+$ T-cell dysfunction by suppressing cell cytolytic ability and the expression of effective factors in association with autophagy inhibition in septic liver injury.

Sepsis is characterized by concurrent unbalanced hyperinflammation and immunosuppressive aberrations. The extent of proinflammatory and immunosuppressive responses varies in individual patients, with distinct contributions at different pathological stages [21]. The functions of almost all kinds of immune cells are impaired with the development of sepsis, including defective neutrophil chemotaxis at infection sites [22], loss of cytokine secretion and T-cell activation by antigen-presenting cells [23], and increased T-cell apoptosis and dysfunction [24]. In the present study, the number of CD8$^+$ T cells in the livers of septic patients with liver injury was found to be significantly decreased compared with that in septic patients without liver injury. CD8$^+$ T cells were increased in the livers of mice with CLP-induced sepsis, which is consistent with our previous report that CD4$^+$ T cells migrated from the periphery into target organs in a heat-killed pathogen-induced inflammatory liver injury mouse model [25]. Although it is difficult to confirm that the decrease of CD8$^+$ T cells in peripheral blood is attributed to their recruitment into the liver in septic patients, the increased number of hepatic CD8$^+$ T cells in our mouse model may, to some extent, explain the phenomena. Nevertheless, the results showed that both peripheral and hepatic CD8$^+$ T cells were dysfunctional, which was manifested by decreased expressions of granzyme B and perforin, leading to loss of cytolytic potency.

USP9X was reported to be an oncogene that promotes tumorigenesis and chemoresistance by regulating tumor cell apoptosis [26] and participating in the activation of TCR signaling. USP9x was found to be indispensable for lymphocyte activation, either by controlling ZAP70 ubiquitination or modulating the carma1-bcl10-malt1 complex, but the molecular mechanisms are not clear [12–14]. Intriguingly, T cells with excessive or constant activation by chronic viral infection or repeated antigen stimulation displayed dysfunction, manifested by exhaustion [14]. In this CLP-induced sepsis mouse model, dysfunction of hepatic CD8$^+$ T cells was observed, even though their number was increased. Blockade of USP9x by WP1130 effectively downregulated the percentage of hepatic CD8$^+$ T cells during sepsis, suggesting that upregulation of USP9x might be correlated with CD8$^+$ T-cell exhaustion during sepsis. This conclusion is consistent with a recent clinical study demonstrating that exhaustion markers 2B4, PD-1, and CD160 on CD8$^+$ T cells on day 1 identified septic patients and were correlated with poor outcome [27]. Although we cannot exclude the possibility that WP1130 may act on other DUBs, *e.g.*, USP14, this study shows that WP1130 significantly decreases the expression of USP9x in hepatic CD8$^+$ T cells from septic mice 24 h after CLP, indicating that USP9x is an important target of WP1130 in sepsis-induced liver injury.

Autophagy is necessary for maintaining cellular homeostasis, in which cellular components are degraded and recycled. T-cell autophagy deficiency has been shown to suppress immune responses and increase sepsis mortality [17]. Inhibition of autophagy by small-molecule drugs or gene depletion was shown to promote apoptosis of CD4$^+$ T cells in sepsis [28,29]. Depletion of mTOR in T cells enhanced splenic CD8$^+$ T-cell autophagy by increasing splenic autophagosomes in lethal fungal sepsis [30]. The function of CD8$^+$ T cells in the liver was not described in that study, and increased autophagy of splenic CD8$^+$ T cells was to some extent consistent with our findings that autophagy was not significantly impaired in hepatic CD8$^+$ T cells from mTOR$^{\Delta ER}$ mice with septic liver injury. As mTOR deficiency-mediated autophagy still exists in the whole body *in vivo*, we cannot exclude the possibility that cells other than CD8$^+$ T cells may be involved in the amelioration of sepsis. mTOR kinase is the catalytic component of mTORC1 and mTORC2, both of which were reported to be regulated by USP9x, and USP9x co-immunoprecipitated mTOR with both the regulatory-associated protein of mTOR (RAPTOR) and rapamycin-insensitive companion of mTOR (RICTOR) components of mTORC1 and mTORC2 [31]. Bridges *et al.* [15] found that the level of the

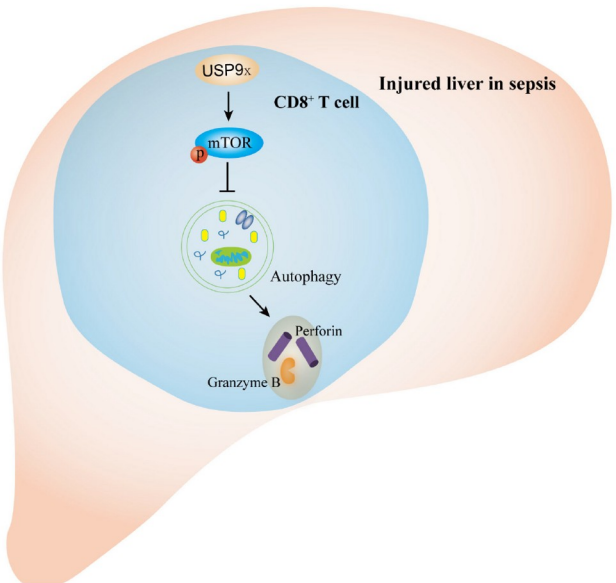

Figure 6. Schematic representation of septic liver injury in which USP9x promotes hepatic CD8$^+$ T-cell dysfunction by inhibiting autophagy via mTOR phosphorylation With the progression of sepsis, the number of hepatic CD8$^+$ T cells and cellular USP9x expression are both increased. Upregulation of USP9x inhibits CD8$^+$ T cell-autophagy by promoting mTOR phosphorylation, and subsequently downregulate the levels of cytolytic factors, granzyme B and perforin, leading to T-cell dysfunction.

canonical mTORC1 protein RAPTOR was directly proportional to USP9x and that USP9x deubiquitylating activity inhibited the proteasomal degradation of RAPTOR. In addition, USP9x can remove Lys63-linked ubiquitin from RICTOR to facilitate mTORC2 signaling through its interaction with mTOR [16]. However, the cellular and molecular mechanisms involved in USP9x regulation of mTOR in septic CD8+ T cells need further investigation.

In summary, this study shows that inhibition of autophagy, in association with USP9x via mTOR phosphorylation, leads to CD8+ T-cell dysfunction and the subsequent development of septic liver injury (Figure 6). The results increase our understanding of the regulation of USP9x and provide evidence of potential new therapeutic targets for the management of septic liver injury.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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