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Rutaecarpine ameliorated sepsis-induced peritoneal resident macrophages apoptosis and inflammation responses

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ABSTRACT

Background: Sepsis is a life-threatening organ dysfunction disease caused by a dysregulated host response to infection. Rutaecarpine is an important alkaloid component of *Evodia rutaecarpa*. There has been no study on the therapeutic effects of rutaecarpine in sepsis.

Methods: Mice were randomly assigned into four groups: sham, sepsis, sepsis plus vehicle and sepsis plus rutaecarpine groups. Mice in sepsis were administered CLP surgery. Rutaecarpine or vehicle was injected intraperitoneally 1 h after CLP. The liver damage, bacterial infection, survival rate and weight loss were observed, and changes in the ratio of peritoneal resident macrophages were analyzed by flow cytometry and immunofluorescence microscopy. Western blotting was used to identify the levels of NF-κB signaling pathway, ER stress and apoptosis related proteins. TUNEL and Annexin V/PI assay were used to detect the apoptosis of liver tissues and peritoneal resident macrophages, respectively. ELISA and qRT-PCR were used to detect the inflammatory factors.

Results: Rutaecarpine alleviated weight loss, bacterial infection and liver injury, and regulated inflammation homeostasis, enhancing survival rate induced by sepsis. Population of peritoneal resident macrophages ($CD11b^{+}F4/80^{hi}MHCII^{low}$) was significantly decreased in sepsis mice, which was resulted from ER stress-induced apoptosis through caspase-12 signaling pathway. Rutaecarpine restored the ratio of peritoneal resident macrophages and the level of GATA6 in $CD11b^{+}$ peritoneal macrophages. Rutaecarpine could also attenuate sepsis–induced inflammatory responses through inhibiting the activation of ER stress/NF-κB pathway.

Conclusion: Rutaecarpine ameliorated sepsis-induced peritoneal resident macrophages apoptosis and inflammation responses through inhibition of ER stress-mediated caspase-12 and NF-κB pathways. Our study provided new insights for drug development against sepsis.

1. Introduction

Sepsis is a life-threatening organ dysfunction disease caused by a dysregulated host response to infection [1,2]. Over 18 million cases of sepsis happened each year in the world, with the mortality rate as high as 40%, which was the leading cause of death in intensive care units [3]. In most cases, sepsis induces organ dysfunction such as liver and lung, leading to a chronic disease state. Due to the development of early diagnosis, effective antibiotics, and supportive care of patients, much research has been carried out on the pathogenesis of sepsis [4,5]. However, patients with sepsis still have a poor long-term prognosis [2,6], and several treatments have failed in clinical trials. Therefore, the detailed mechanisms remain more intensive study. Also, there is no effective therapeutic method for sepsis yet.

Macrophages can produce immune mediators, growth factors and opsonin, and maintain multi-functions including phagocytosis, antimicrobial function, vascular modulatory function and erythropoiesis [7,8]. Tissue-resident macrophages originate from prenatal progenitors, and keep responsibility for maintaining local environment and homeostasis [9]. Recent studies have classified peritoneal macrophages into two sub-populations: larger peritoneal macrophages (LPMs, expressing high level of F4/80 and low level of MHCII) and small peritoneal macrophages (SPMs, expressing low level of F4/80 and high level of MHCII). The function of SPMs is still unclear, but there are more and more studies focusing on the function of LPMs. LPMs, also named as peritoneal resident macrophages, specifically expressed transcription

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factor GATA6 [10,11]. Studies have demonstrated the significant role of peritoneal resident macrophages in maintaining the function of intestine. The decreased ratio of peritoneal resident macrophages was triggered in sepsis mouse model [12–14]. However, its specific mechanism remains unclear.

Endoplasmic reticulum (ER) stress has been linked to the pathogenesis of sepsis recently [15]. Studies have shown that ER stress can specifically activate caspase-12-mediated apoptosis. It has been demonstrated in mouse model of sepsis, ER stress contributes to abnormal apoptosis of lymphocytes, indicating that ER stress-mediated apoptosis pathway may be a novel target in clinical prevention and therapy of sepsis [16]. In addition, ER stress can also activate NF-κB signaling pathway and regulate inflammatory response [17].

Rutecarpine is a significant alkaloid component of *Evodia rutaecarpa*. It has extensive pharmacological effects including anti-thrombotic, anti-cancer, anti-inflammatory, anti-allergic, muscle relaxant, photo-aging protection, gastric mucosa protection, heart protection. It can also improve hyperlipidemia and affect immune and allergic reactions [18–21]. Previous researches have shown that rutaecarpine can inhibit inflammatory cell infiltration, exudation and tissue hyperplasia [21,22]. In addition, rutacarpine inhibited the protein level of proinflammatory cytokines TNF-α and IL-4 caused by the IgE-antigen complex [23]. However, there has been no study on the therapeutic effects of rutacarpine in sepsis.

Our study demonstrated that rutaecarpine ameliorated sepsis-induced peritoneal resident macrophages apoptosis and inflammation response through inhibiting ER stress-mediated caspase-12 and NF-κB pathways. This is the first report about rutacarpine in ameliorating sepsis. Our study provided new insights for drug development against sepsis.

2. Materials and methods

2.1. Cecal ligation and puncture (CLP) surgery

Male C57BL/6 mice of 8 weeks old were used in this study. All the animals were purchased from SJA Laboratory Animal company (Hunan, China) and maintained in accordance with Guidelines for the Care and Use of Laboratory Animals of the third Xiangya hospital (Hunan, China). All procedures were performed according to the internationally accepted ethical guidelines.

Cecal ligation and puncture (CLP) surgery was used to establish sepsis model according to the previous reference (24). In brief, mice were randomly divided into four groups (17 mice per group): sham surgery group, CLP surgery group, CLP surgery with vehicle (PBS) injection group and CLP surgery with rutaecarpine injection group. Mice were anesthetized with 40 mg/kg intraperitoneal injection of 10 g/L sodium pentobarbital. For CLP surgery groups, a 1.5 cm long longitudinal incision was made along the midline of the abdomen to separate the skin and subcutaneous tissue layer. The rectus abdominis and peritoneum were dissected at the abdominal white line, and the cecal and its surrounding adjacent intestines were exposed. The cecum was gently pulled out, and the cecum and mesenteric vessels were ligated from the end of the cecum to the entire cecum length of 1/3 to 1/2 using a 4th surgical line. Before the caecum perforation, the content of the cecum was gently pushed to the distal cecum. In the middle position between the ligation and the cecum tip, a single penetration puncture through the cecum was performed using a 21G needle. Then the cecal part of the ligation was perforated, and a small amount of intestinal contents were slightly squeezed out, so as to ensure that the puncture hole was unobstructed. All the intestines were then returned to the abdominal cavity, and the surgical incision was sutured layer by layer with the 4th surgical line. Animals were resuscitated by injecting prewarmed normal saline (37 °C, 5 mL per 100 g body weight) subcutaneously. Buprenorphine (0.05 mg per kg body weight) was injected every 6 h for at least 2 d for postoperative analgesia. Mice in sham surgery group had abdominal incision and cecum exposure without ligation and puncture. 20 mg/kg rutaecarpine (Sigma-Aldrich St Louis, MO, USA), which was dissolved in PBS, was administered intraperitoneally 1 h after the surgery. After 24 h, 5 mice from each of the above 4 groups were randomly selected to extract peritoneal resident macrophages for follow-up experiments, and others continued to observe survival rate and weight of mice. Mice were sacrificed by cervical dislocation after 12 days. Liver samples were obtained for analysis.

2.2. Bacterial load detection

Peritoneal lavage fluid or blood was used as a stock solution, and diluted 10 times with sterile physiological saline. 20 μL of different concentrations of bacterial dilutions were inoculated on aerobic culture dishes and anaerobic culture dishes (anaerobic culture dishes were placed in anaerobic culture bags). Aerobic and anaerobic culture dishes were cultured in a 37 °C incubator for 24 and 48 h, respectively. Count the colonies after the end of the culture and calculate the number of colony forming units (CFU) per mL of blood or peritoneal lavage fluid.

2.3. Inflammatory factors in peritoneal fluid

ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to detect the inflammatory factors TNF-α, IL-6 and IL-10 in peritoneal fluid from each group of mice, and strictly followed the ELISA kit instructions.

2.4. HE staining

The liver tissue sections were first de-paraffinized by two xylenes for 15 min and soaked in high-to-low-concentration ethanol (100%, 90%, 80% and 70%) for 3 min each. Then, hematoxylin was stained for 1–3 min, 1% ethanol for 10 s, dilute ammonia for 20 s, and eosin staining for 3 min. Each step above was rinsed with distilled water for 1 min. After dehydration with low to high concentration gradients (70%, 80%, 90% and 100%) ethanol for 5 min, xylene was allowed to clear twice for 15 min, and the solution was sealed with a neutral resin. The staining results were observed by an optical microscope (Olympus, Center Valley, PA, USA) and photographed. Histopathological changes were scored to four levels based on the intensity of necrosis and diffusion of the inflammatory cells according to previous research [25]. The four scored levels were interpreted as: Zero, no necrosis and inflammation; One, mild hepatocyte necrosis with mild inflammatory reaction; Two, diffuse hepatocyte necrosis and intralobular necrotic bridges along with inflammatory reaction; Three, complete destruction of lobules, diffuse hepatic necrosis along with diffuse interlobular inflammatory reaction.

2.5. TUNEL assay

The in-situ terminal labeling kit (Solarbio, Beijing, China) was used based on the operation manual. The procedures were briefly described as follows. The liver tissue samples were washed 3 times with PBS solution. Then, 100 μL of the 10% proteinase K solution was added and incubated at 37 °C for 30 min, followed by washing 3 times with PBS. Then, 50 μL of TdT enzyme solution was added and incubated at 37 °C for 60 min. After washing 3 times with PBS, the sample was mixed with 5 μL of streptavidin-fluorescein solution and 45 μL of labeling buffer and stored at 37 °C for 30 min in the dark. After washing with PBS and staining with DAPI, the samples were then observed and photographed under a fluorescence microscope (Carl Zeiss Microscopy, Jena, Germany).

2.6. Peritoneal macrophages isolation and culture

Five mice from each group were sacrificed 24 h after the surgery by

cervical dislocation and sterilized by 75% ethanol. Peritoneal exudate cells were immediately isolated by fractional peritoneal lavage with 5 mL (fractional lavage, a total of volume 5 mL) washing buffer (sterile PBS with 5% newborn calf serum and 0.5 mM EDTA). For cell phenotype identification, cells were directly washed and stained with indicated antibodies. For immunofluorescence microscopy and other experiments, the extracted solution was centrifuged at 300 ×*g* for 10 min and isolated cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD, USA) plus 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco).

2.7. Phenotyping analysis

Peritoneal exudate cells were washed twice with PBS-F (PBS containing 0.1% NaN_3 and 3% FBS), then stained with FITC labeled anti-CD11b (eBioscience), PE labeled anti-F4/80 and APC labeled anti-MHCII (eBioscience) for 20 min. After washing with PBS-F, cells were fixed with 4% paraformaldehyde in PBS and then analyzed by flow cytometer (BD Pharmingen, San Diego, CA, USA).

2.8. Immunofluorescence staining

Peritoneal macrophages harvested from indicated groups were cultured in glass-bottomed dishes. Then, cells were fixed in 4% paraformaldehyde, permeabilized with methanol on ice, and immunostained with AlexaFluor488-CD11b in combination with GATA6 antibody (Cell Signaling Technology, Danvers, MA, USA) followed by CF568-conjugated goat-anti-rabbit IgG, highly cross-absorbed (Biotium, Hayward, CA). Nuclei were stained by Hoechst 33342. Cells were observed by Zeiss microscope (Carl Zeiss Microscopy, Jena, Germany). Fluorescence images were captured and analyzed with Zeiss ZEN software.

2.9. Annexin V/PI assay

Peritoneal exudate cells were washed twice with PBS-F (PBS containing 0.1% NaN3 and 3% FBS). Then the cells were cultured and stained by using Annexin V-FITC and propidium iodide (PI) kit (KeyGen Biotech, Nanjing, China) according to the protocol of manufacture. Finally, cell apoptosis was quantified by flow cytometry (BD Pharmingen).

2.10. Western blot analysis

Western blotting was performed as previously described [26]. Briefly, 40 μg total proteins of peritoneal resident macrophages from indicated groups were isolated and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then proteins on the gel were transferred into polyvinylidene difluoride (PVDF) membranes (GE Healthcare Life Science, USA). After that, the membranes were incubated in blocking buffer and then replaced by primary antibodies caspase-12, caspase-9, caspase-8, caspase-3, PARP, GPR78, p-PERK, PERK, p-IRE-1, IRE-1, ATF-6, CHOP, IκBα, p65, GAPDH and Lamin B (Cell Signaling Technology, Danvers, MA, USA) in 4 °C overnight. On the next day, membranes were washed carefully and incubated with indicated secondary antibodies for 2 h in the room temperature. The bands were revealed with a chemiluminescence kit (Tiangen, Beijing, China) and imaged by Bio-rad (Hercules, CA, USA) gel image system. Analysis and quantification were performed by ImageJ software.

2.11. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from isolated cells using Trizol reagent (Invitrogen, USA). cDNA was synthesized by reverse transcription using the PrimerScript RT reagent Kit (TaKaRa, Japan). Real-time PCR was performed to quantify relative RNA levels using SYBR Green RT-qPCR SuperMix Kit (Thermo, USA) with customized primers (Table 1) on an AB7300 thermo-cycler (Applied Biosystems, USA). GAPDH was used as internal reference for mRNA. Relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ approach.

Table 1
Primer sequences for qRT-PCR.

Gene		Sequence(5′ → 3′)
GAPDH	Forward	TGAGGCCGGTGCTGAGTATGT
	Reverse	CAGTCTTCTGGGTGGCAGTGAT
IL-6	Forward	GAAATCGTGGAAATGAG
	Reverse	TAGGTTTGCCGAGTAGA
TNF-α	Forward	AGGGTCTGGGCCATAGAACT
	Reverse	CCACCACGCTCTTCTGTCTAC
IL-10	Forward	CCCATTCCTCGTCACGATCTC
	Reverse	TCAGACTGGTTTGGGATAGGTTT

2.12. Statistical analysis

All experiments were repeated at least in triplicate, with one representative experiment shown. Statistical analysis was performed with SPSS software. Data were expressed as mean ± standard deviation (SD). For survival analysis, Kaplan-Meier log-rank was performed. Statistical evaluation was performed using student's *t*-test between two groups or one-way analysis of variance (ANOVA) for multiple comparison. P values < 0.05 were considered as statistically significant.

3. Results

3.1. Rutaecarpine alleviated weight loss, bacterial infection and liver injury and enhanced survival rate induced by sepsis

In order to demonstrate the effects of rutaecarpine on animal model of sepsis, CLP surgery was performed to establish the model of sepsis, and mice were treated with rutaecarpine or not in this study. As shown in Fig. 1A and B, all mice survived within 12 days in sham group, while the survival rate was dramatically decreased to 25%–35% after 3 days in both CLP surgery alone group and CLP surgery plus vehicle group. In contrast, mice treated with rutaecarpine after CLP surgery displayed a much higher survival rate (about 70%), keeping alive after 8 days. Meanwhile, CLP surgery significantly caused continuous decrease of mice body weight until day 12, while rutaecarpine treatment restored the weight of mice from day 3. To investigate rutaecarpine effect on inflammatory factors in peritoneal fluid, the concentration of TNF-α, IL-6 and IL-10 in the peritoneal fluid of the four groups were tested. As indicated in Fig. 1C, the levels of TNF-α, IL-6 and IL-10 in peritoneal fluid were significantly enhanced in mice with sepsis, while levels of TNF-α and IL-6 were decreased in rutaecarpine treated group compared with sepsis group. However, the level of IL-10 was further increased in rutaecarpine group compared with in sepsis group. Statistics on bacterial infection showed bacterial infection in peritoneal fluid and blood was significantly induced in mice with sepsis, while it was decreased in rutaecarpine treated group compared with sepsis group (Fig. 1D). Subsequently, we studied the effect of rutaecarpine on liver injury. Mice in sham group showed normal cellular architecture of hepatic tissue. Vacuolization, conspicuous foci of inflammation and necrosis, and tissue disorder were observed in the sepsis group compared with the sham group, while rutaecarpine relieved liver pathological damage induced by sepsis (Fig. 1E and F). Furthermore, we also investigated whether rutaecarpine affected the sepsis-induced apoptosis in liver tissues by TUNEL assay. As shown in Fig. 1G, rutaecarpine inhibited the ratio of $TUNEL^+$ cells and relieved sepsis-induced liver tissues apoptosis. These results indicated positive effects of rutaecarpine on sepsis,

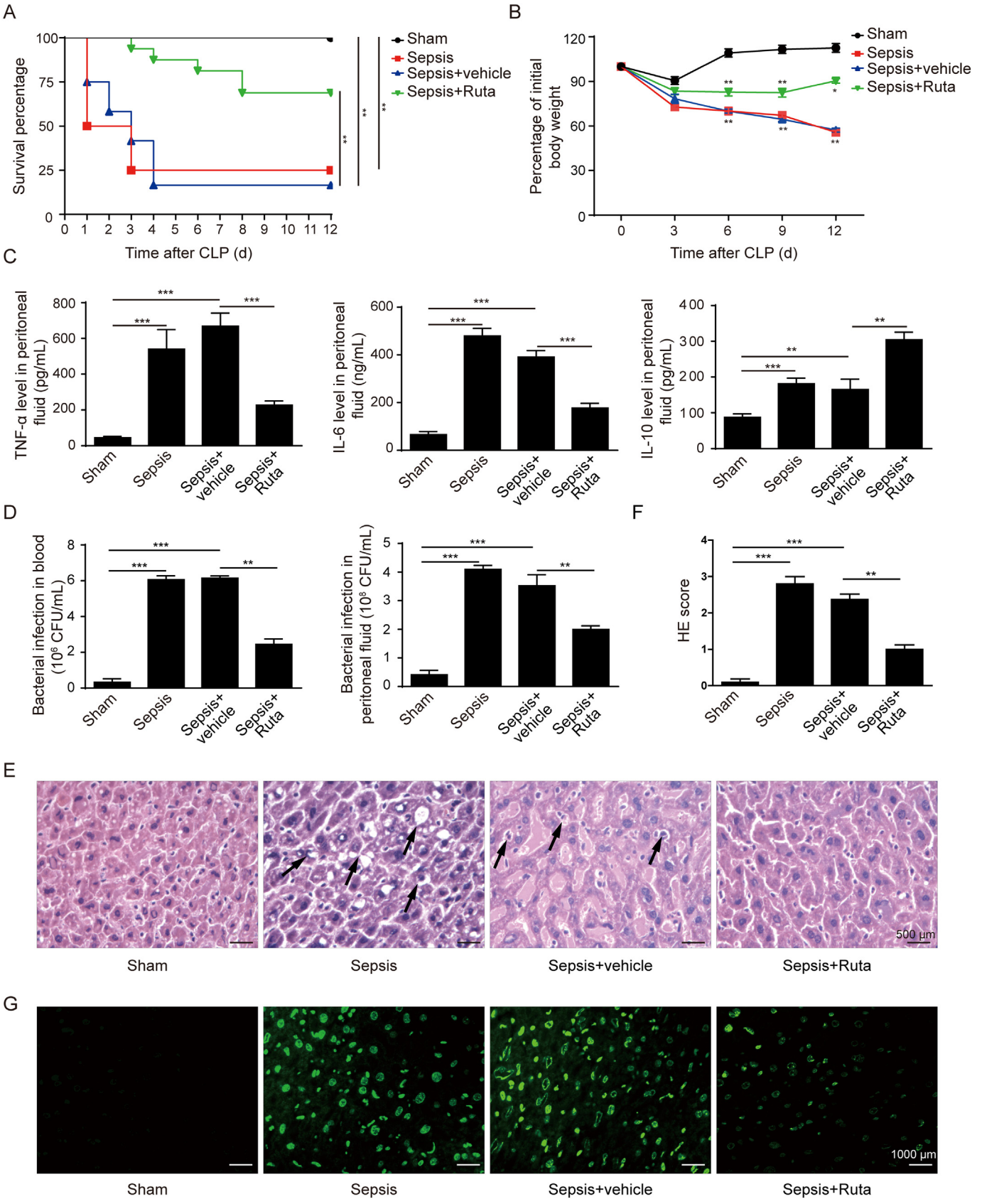

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Fig. 1. Rutaecarpine alleviated weight loss, bacterial infection and liver injury and enhanced survival rate induced by sepsis. (A) Kaplan-Meier survival curves were used to estimate survival rate in each group. (B) Effect of rutaecarpine administration on mice body weight. (C) Rutaecarpine effect on inflammatory factors in peritoneal fluid. (D) The bacterial load in blood and peritoneal fluid from septic mice after Rutaecarpine treatment. (E) Representative of HE staining in liver tissues. Arrows indicate conspicuous foci of inflammation and necrosis, and tissue disorder. (F) The histopatological score of HE in liver tissues. (G) Representative image of TUNEL assay in liver tissues. Mice were randomly divided into four groups (17 mice per group): sham surgery group, CLP surgery group, CLP surgery with vehicle (PBS) injection group and CLP surgery with rutaecarpine injection. After 24 h, 5 mice from each of the above 4 groups were randomly selected to extract peritoneal macrophages for follow-up experiments and other observations were continued. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

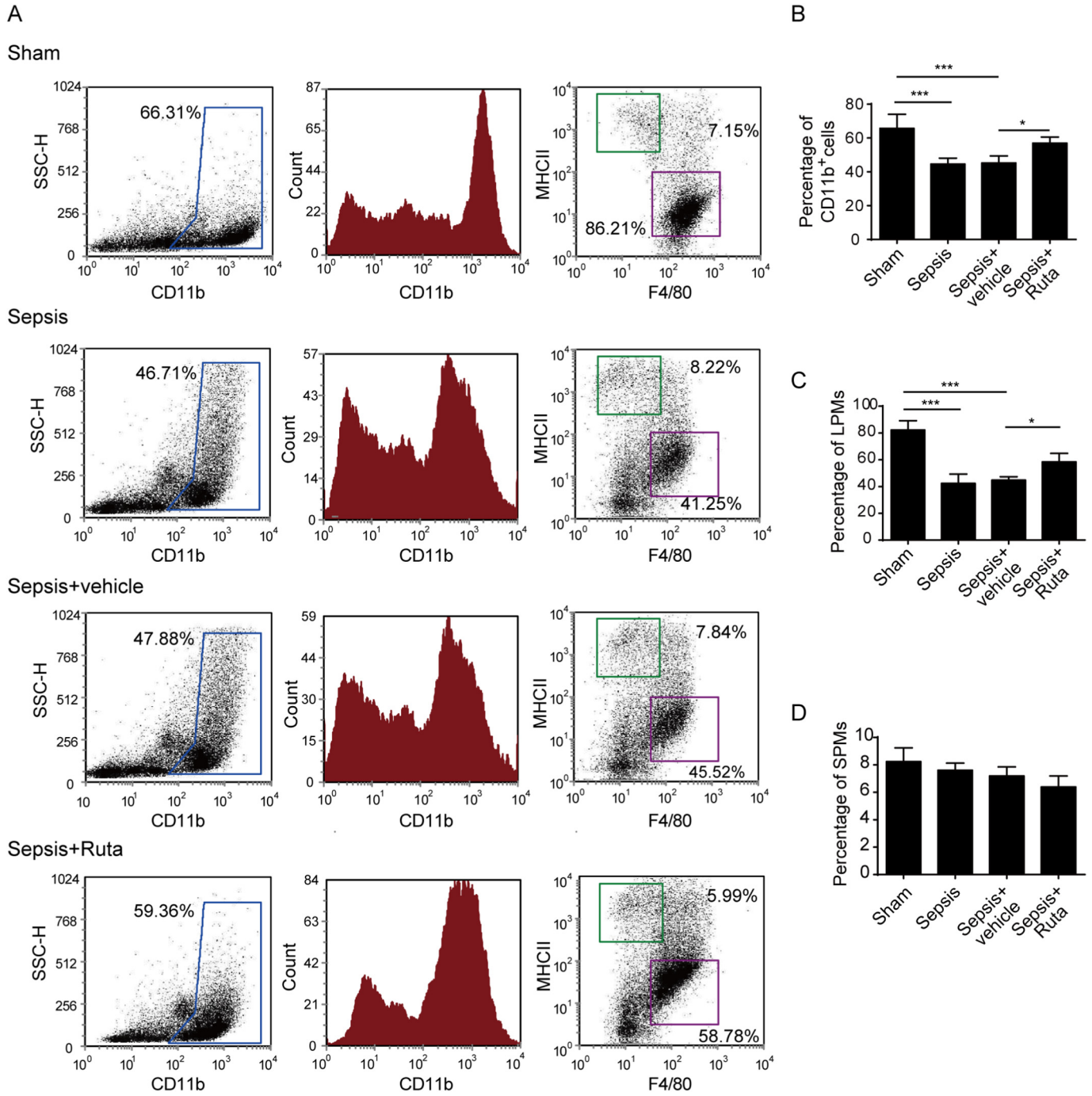

Fig. 2. Rutaecarpine elevated the ratio of peritoneal resident macrophages in sepsis mice. (A) Flow cytometry analysis of the phenotypes of CD11b positive peritoneal macrophages, large peritoneal macrophages (peritoneal resident macrophages) and small peritoneal macrophages (SMPs), which were identified by CD11b, F4/80 and MHCII staining. (B–D) Statistical results of flow cytometry analysis. LPMs and SPMs percentages are fraction of the $CD11b^{+}$ population. Mice were randomly divided into four groups (17 mice per group): sham surgery group, CLP surgery group, CLP surgery with vehicle (PBS) injection group and CLP surgery with rutaecarpine injection. After 24 h, 5 mice from each of the above 4 groups were randomly selected to extract peritoneal macrophages for follow-up experiments and other observations were continued. $^{*}P < 0.05$ and $^{***}P < 0.001$.

rutaecarpine alleviated weight loss and liver injury, regulated inflammation homeostasis and enhanced survival rate induced by sepsis.

3.2. Rutaecarpine elevated the ratio of peritoneal resident macrophages in sepsis mice

We investigated the influence of sepsis on the ratio of peritoneal resident macrophages and the effects brought by rutaecarpine treatment. Our results demonstrated that CLP surgery caused a decline of CD11b positive macrophages in the peritoneal cavity of mice, from about 70% to 45% (Fig. 2A and B). Meanwhile, the population of peritoneal resident macrophages ($F4/80^{hi}$ $MHCII^{low}$) was significantly decreased after surgery (about 40%) compared with sham group (about 85%, Fig. 2A and C). The mice from vehicle group remained the similar ratios of CD11b positive cells and peritoneal resident macrophages to sepsis group. There was a significant increase in the ratio of CD11b positive macrophages from mice in rutaecarpine treatment group (about 60%) compared with CLP surgery group (about 45%) (Fig. 2A and B). The same trend was observed in the population of peritoneal resident macrophages (Fig. 2A and C). Unlike the CD11b positive macrophages and peritoneal resident macrophages, no significant difference was identified in the ratio of SPMs among four groups (Fig. 2A and D). Taken together, these results revealed that sepsis could induce remarkable decrease in the ratios of CD11b positive macrophages and peritoneal resident macrophages. Rutaecarpine could partially restore the ratios of such two kinds of cells in peritoneal cavity.

3.3. Rutaecarpine restored the level of GATA6 in CD11b positive peritoneal macrophages after CLP surgery

To validate the flow cytometry results, we next used immunofluorescence staining to identify the ratio of peritoneal resident macrophages ($CD11b^+/GATA6^+$) in mice with different treatment. $CD11b^+/GATA6^+$ peritoneal macrophages were almost undetectable in the sepsis group and vehicle group compared with sham group (Fig. 3A and C). However, GATA6 level partly recovered in CD11b positive peritoneal macrophages after rutaecarpine treatment, which was consistent with the result of flow cytometry (Fig. 3A and C). Most interestingly, CD11b positive macrophages from sepsis and vehicle groups were significantly decreased compared with sham group, while those from rutaecarpine group were increased compared with sepsis group (Fig. 3A and B). Therefore, these results further demonstrated that rutaecarpine could inhibit reduction of sepsis-induced CD11b positive macrophages and peritoneal resident macrophages.

3.4. Rutaecarpine protected peritoneal resident macrophages from sepsis-induced apoptosis

To reveal the reason for the reduction of peritoneal resident macrophages in sepsis, we further investigated the apoptosis situation of peritoneal macrophages under different treatments. Our results demonstrated that the cell apoptosis was triggered in the peritoneal macrophages of mice after CLP surgery, which was proved by the increase in the cleaved protein level of caspase-3, 8, 9 and 12. (Fig. 4A–E). And there was a significant decrease of cleaved caspase-3 and 12 in rutaecarpine treated group, indicating that rutaecarpine inhibited apoptosis process by inactivating caspase-12/3 pathway (Fig. 4A, B and E). Furthermore, we also tested the level of uncleaved level of PARP, which was the substrate of caspase-3. In contrast to the level of cleaved-caspases, CLP surgery caused a remarkable decrease of uncleaved PARP, while rutaecarpine treatment significantly restored the uncleaved PARP level (Fig. 4A and F). Furthermore, there was a significant increase in the apoptosis rate of peritoneal macrophages compared with the sham group. Decreased cell apoptosis was observed from rutaecarpine treated group (Fig. 4G–H). Thus, sepsis could trigger the decrease of peritoneal resident macrophages population through

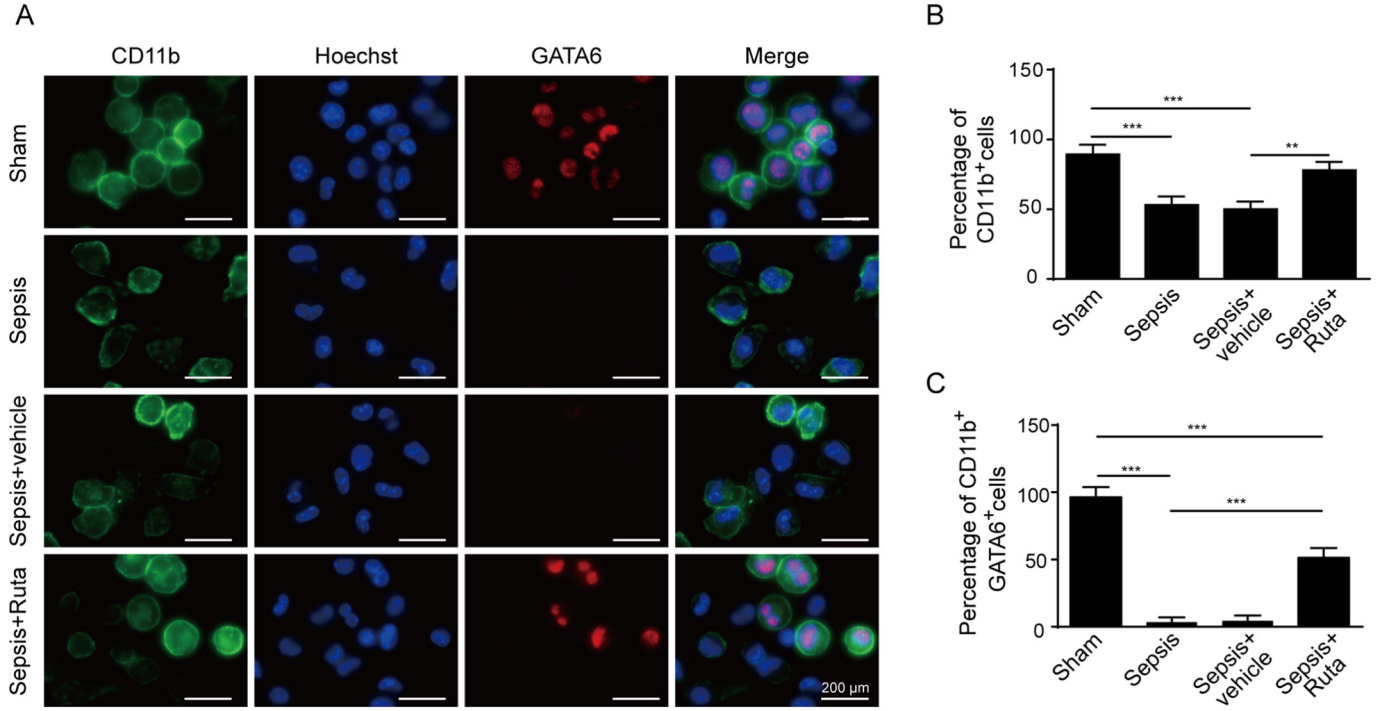

Fig. 3. Rutaecarpine restored the level of GATA6 in CD11b positive peritoneal macrophages after CLP surgery. (A) Immunofluorescent staining of CD11b (green) and GATA6 (red) in peritoneal resident macrophages after indicated treatments. Nuclei were stained by Hoechst 33342 (blue). (B and C) Statistical results of CD11b and GATA6 positive cells from each group. Mice were randomly divided into four groups (17 mice per group): sham surgery group, CLP surgery group, CLP surgery with vehicle (PBS) injection group and CLP surgery with rutaecarpine injection. After 24 h, 5 mice from each of the above 4 groups were randomly selected to extract peritoneal macrophages for follow-up experiments, and other observations were continued. $^{**}P < 0.01$ and $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

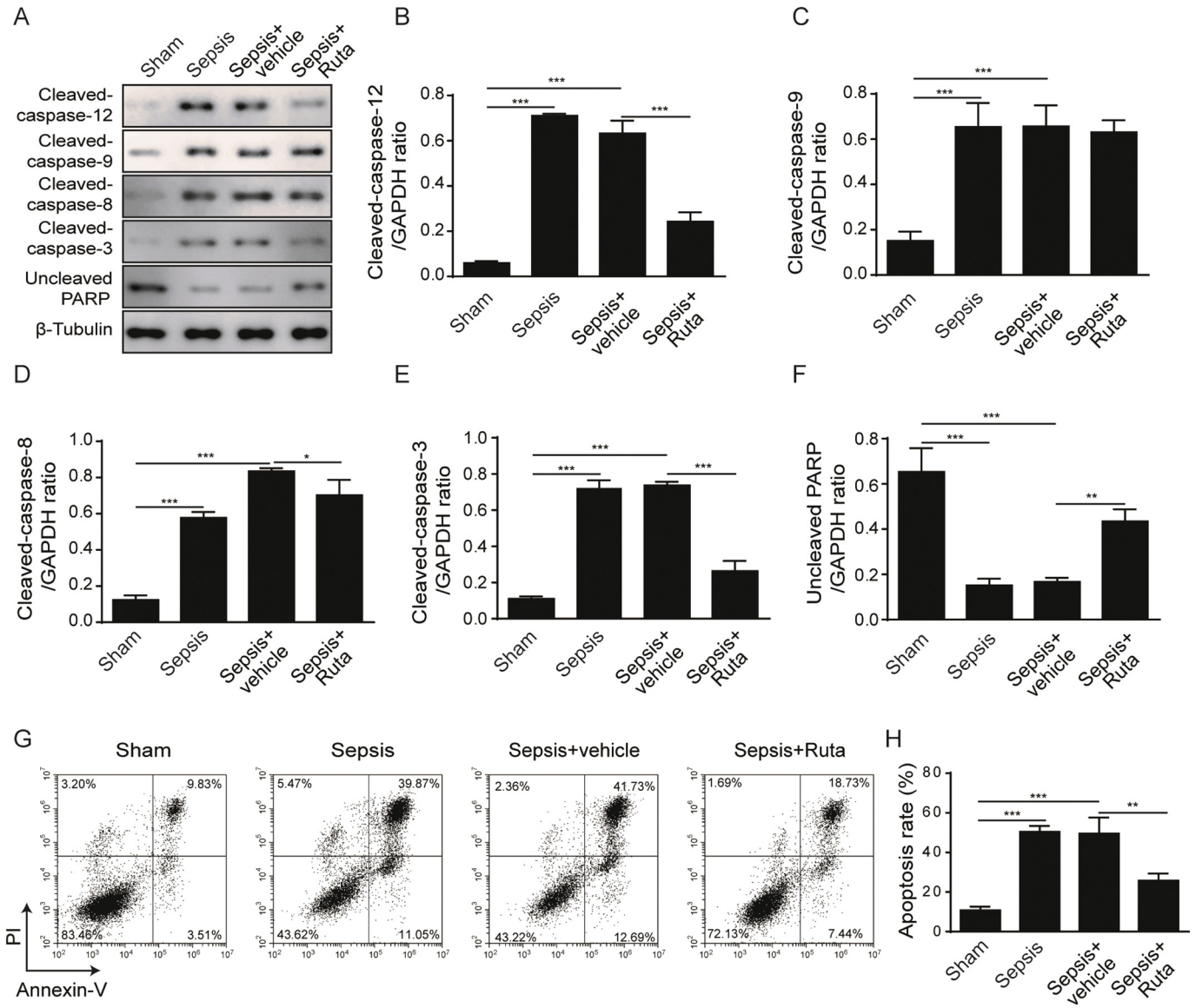

Fig. 4. Rutaecarpine protected peritoneal resident macrophages from sepsis-induced apoptosis. (A) Western blotting results of apoptosis associated proteins after CLP surgery from indicated treatments. (B–F) Statistical results of indicated protein levels in A. (G) Flow cytometry was used to evaluate the effect of rutaecarpine on apoptosis of peritoneal resident macrophages. (H) Statistical results of indicated peritoneal resident macrophages apoptosis levels in G. Mice were randomly divided into four groups (17 mice per group): sham surgery group, CLP surgery group, CLP surgery with vehicle (PBS) injection group and CLP surgery with rutaecarpine injection. After 24 h, 5 mice from each of the above 4 groups were randomly selected to extract peritoneal macrophages for follow-up experiments, and other observations were continued. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

inducing apoptosis. Rutaecarpine alleviated peritoneal resident macrophages apoptosis by inhibiting caspase-12/3-dependent apoptosis pathway.

3.5. Rutaecarpine inhibited sepsis-induced ER stress

Because of the association with caspase-12 and ER stress, we detected the protein levels associated with ER stress. As indicated in Fig. 5A, B and F, the protein levels of GRP78 and CHOP were significantly enhanced in mice with sepsis. But they were inhibited remarkably after rutaecarpine treatment. The same trend was observed on the activity of p-PERK, p-IRE-1, and cleaved-ATF-6, which were the three main pathways of ER stress (Fig. 5A and C–E). These results demonstrated that CLP surgery-induced sepsis could activate ER stress in peritoneal resident macrophages. Rutaecarpine was able to inhibit ER stress related pathways and protect peritoneal resident macrophages against this process. It was the main factor for rutaecarpine protecting peritoneal resident macrophages from sepsis-induced apoptosis.

3.6. Rutaecarpine attenuated inflammatory response via NF-κB signaling pathway of peritoneal resident macrophages in sepsis mice

As local and systemic inflammatory response was considered as the direct and initial factor of sepsis, and ER stress could induce inflammatory response via NF-κB signaling pathway. TNF-α, IL-10, IL-6 mRNA levels in the peritoneal macrophages were measured. As shown in Fig. 6A, compared with the sepsis group, the mRNA levels of TNF-α and IL-6 in the rutaecarpine treated group were significantly decreased, and the expression of IL-10 was increased. To investigate the mechanism by which rutaecarpine inhibited the inflammatory response in the peritoneal resident macrophages during sepsis, the expression and activation of NF-κB (p65) which was a key regulator of pro-inflammatory factors were detected. Our results demonstrated that cytoplasmic p65 of peritoneal macrophages was significantly decreased,

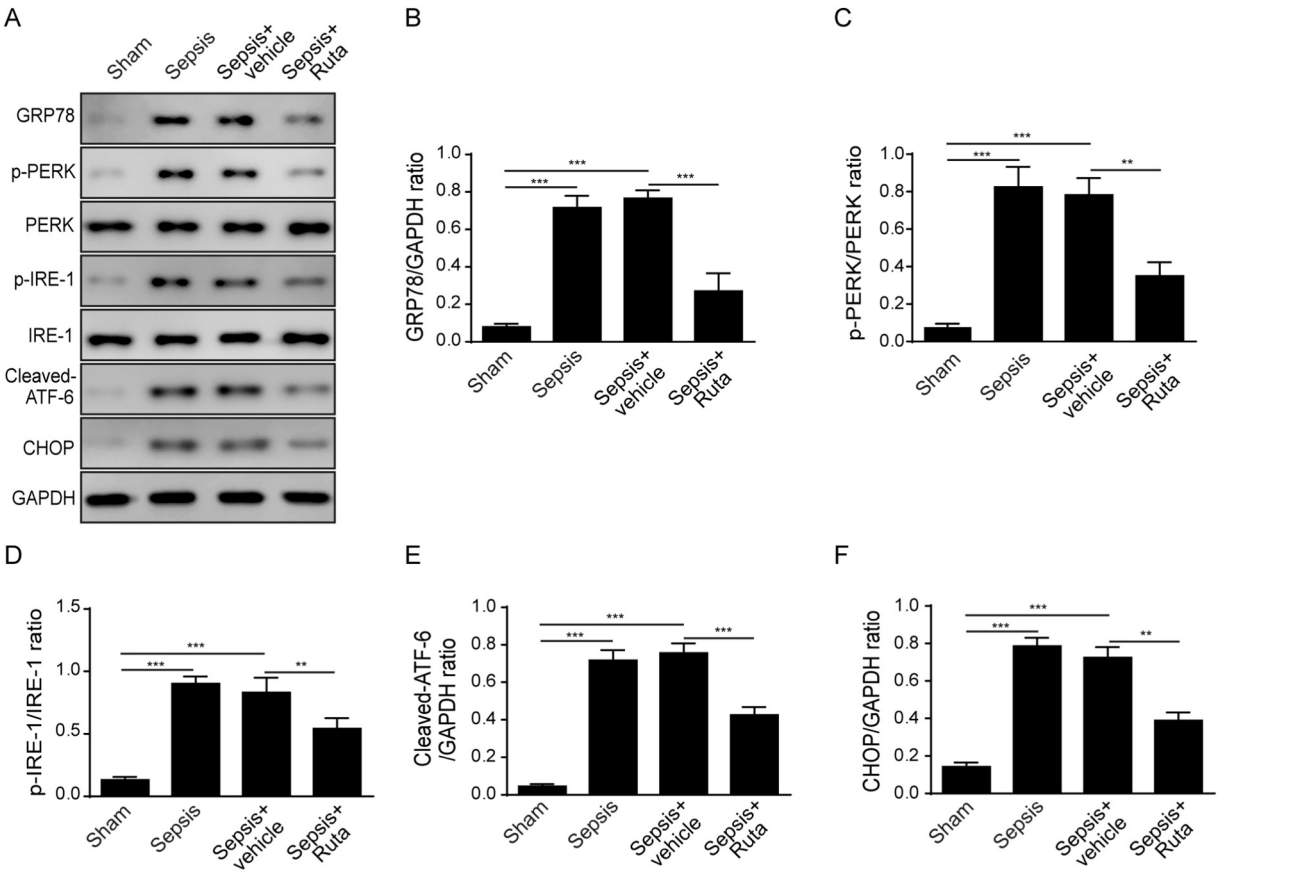

Fig. 5. Rutaecarpine inhibited sepsis-induced ER stress. (A) Western blotting analysis of ER stress associated proteins after CLP surgery from indicated treatments. (B–F) Statistical results of indicated protein levels in A. Mice were randomly divided into four groups (17 mice per group): sham surgery group, CLP surgery group, CLP surgery with vehicle (PBS) injection group and CLP surgery with rutaecarpine injection. After 24 h, 5 mice from each of the above 4 groups were randomly selected to extract peritoneal macrophages for follow-up experiments, and other observations were continued. $^{**}P < 0.01$, $^{***}P < 0.001$.

and the nuclear distribution of p65 was significantly increased in sepsis group compared with sham group. In addition, IκBα degradation of peritoneal macrophages was significantly increased in sepsis group compared with sham group. Furthermore, the nuclear distribution of p65 and IκBα degradation was also significantly inhibited in rutaecarpine treated group compared with sepsis group (Fig. 6B–F). These results indicated that rutaecarpine attenuated sepsis-induced inflammatory response in the peritoneal resident macrophages through inhibiting the activation of NF-κB pathway.

4. Discussion

Despite the fact that many efforts have been made for the therapy of sepsis, the mortality of severe sepsis remains high [27]. Consequently, studying the underlying mechanisms and discovering novel approaches for sepsis treatment are still of significance. Our study revealed that rutaecarpine ameliorated sepsis-induced peritoneal resident macrophages apoptosis and inflammation responses through inhibition of ER stress-mediated caspase-12 and NF-κB pathways. Our study provided new insights for drug development against sepsis.

Rutaecarpine is a kind of alkaloidal component in Chinese traditional medicine Evodia, which has such functions as anti-thrombotic function, anti-cancer function, anti-inflammatory function, anti-allergic function, muscle relaxant, photo-aging protection, gastric mucosa protection, heart protection, improving hyperlipidemia, as well as affecting immune and allergic reactions [18–21]. However, there has been no study on the therapeutic effects of rutaecarpine in sepsis. In this study, we first tested rutaecarpine's influence on liver damage, bacterial infection, survival rate and weight loss in sepsis mice. Consistent with our hypothesis, rutaecarpine administration could significantly relieve sepsis-induced liver injury, enhance the survival rate, alleviate bacterial infection and increase the weight of mice after CLP surgery. This is the first study of rutaecarpine on sepsis treatment.

Studies have demonstrated that in mice model of sepsis, the population of peritoneal resident macrophages was significantly decreased, indicating a potential role of peritoneal resident macrophages in protecting homeostasis and organs function [14]. He et al. showed that Isoalantolactone inhibited LPS-induced inflammation via NF-κB inactivation in peritoneal resident macrophages and improved survival in sepsis [28]. In addition, studies by Zheng et al. noted that bone marrow mesenchymal stem cells could reduce the pathological inflammatory responses in the lung of rats with sepsis and inhibit peritoneal resident macrophages from polarizing into M1 phenotype [29]. Our results indicated that rutaecarpine's beneficial effects on sepsis were associated with the increase of peritoneal resident macrophages population. Rutaecarpine restored the level of GATA6 and elevated the ratio of peritoneal resident macrophages in sepsis mice.

It is important to note that during sepsis, significant apoptosis levels of lymphocyte subsets and dendritic cells have been discovered [30,31]. Studies have indicated that rutaecarpine has anti-apoptotic effects. Lee et al. investigated the inhibitory effects of rutaecarpine on DNA strand break and apoptosis induced by hydrogen peroxide (H_2O_2) in murine Hepa-1c1c7 cells [32]. Meanwhile, reports have shown that rutaecarpine prevents hypoxia-reoxygenation-induced myocardial cell apoptosis via inhibition of NADPH oxidases [33]. Consequently, we speculated if rutaecarpine could have benefits for sepsis-induced apoptosis. In this study, we detected several markers for apoptosis to further illustrate the mechanism of rutaecarpine's function. Results

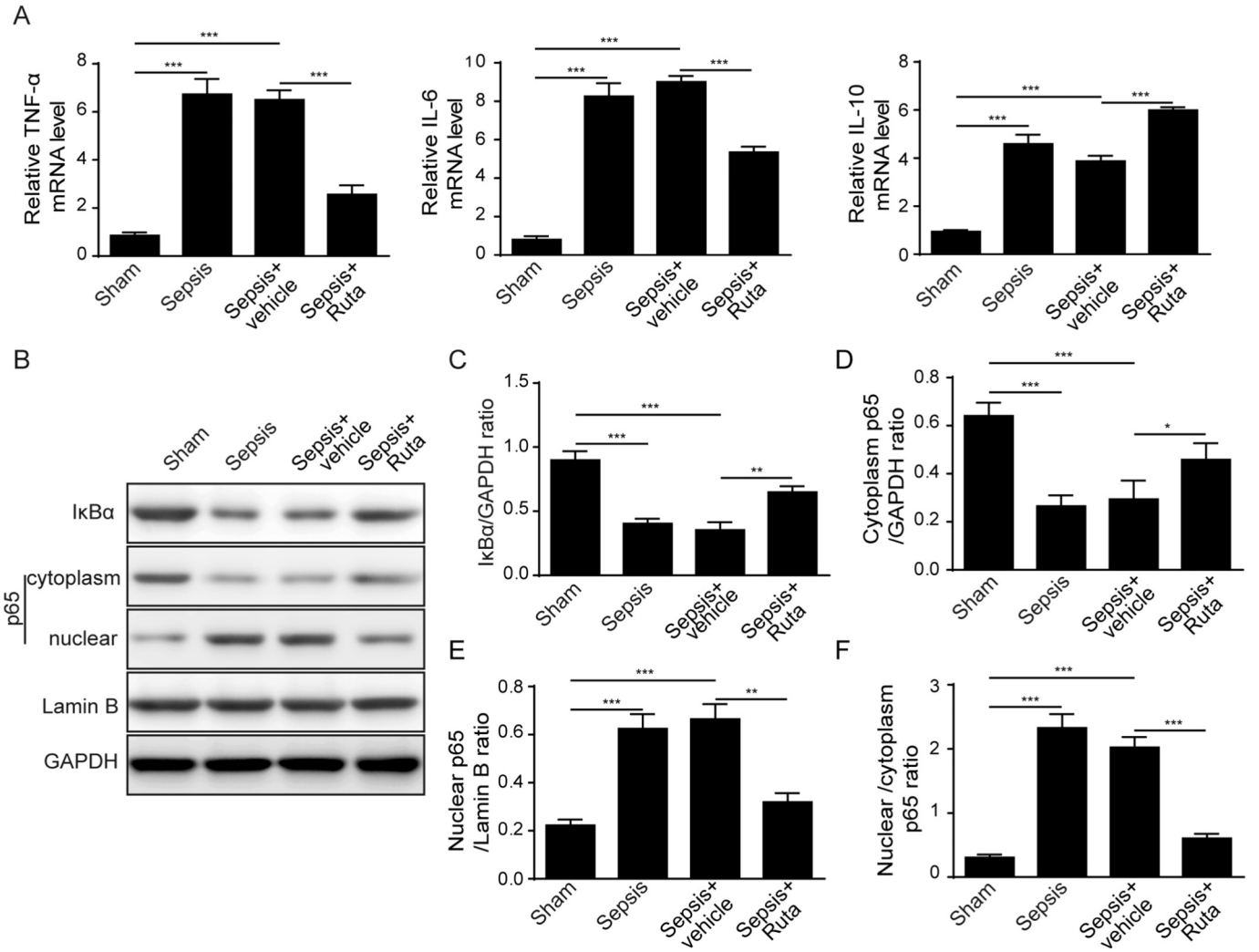

Fig. 6. Rutaecarpine attenuated inflammatory response via NF-κB signaling pathway of peritoneal resident macrophages in sepsis mice. (A) Quantitative RT-PCR analysis of relative TNF-α, IL-10, IL-6 mRNA levels of peritoneal resident macrophages. (B) Western blot analysis of the protein levels of NF-κB signaling pathway of peritoneal resident macrophages. (C–F) Statistical results of indicated protein levels in B. Mice were randomly divided into four groups (17 mice per group): sham surgery group, CLP surgery group, CLP surgery with vehicle (PBS) injection group and CLP surgery with rutaecarpine injection. After 24 h, 5 mice from each of the above 4 groups were randomly selected to extract peritoneal macrophages for follow-up experiments, and other observations were continued. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

demonstrated that the level of apoptosis in peritoneal resident macrophages was elevated by CLP surgery, represented by the increase of cleave-caspase-12/9/8/3 and the decrease of uncleaved PARP. And rutaecarpine alleviated peritoneal resident macrophages apoptosis by inhibiting caspase-12/3-dependent apoptosis pathway. We also found the pathway of ER stress was activated in sepsis group and was significantly inhibited by rutaecarpine. Rutaecarpine was sufficiently effective to inhibit sepsis-induced apoptosis via the inhibition of ER stress.

In severe sepsis, inflammatory cells, particularly neutrophils and macrophages, are recruited and activated in tissues as endotoxin stimulating the body. A series of inflammatory mediators such as cytokines, chemokines, oxygen free radicals, and proteases are then secreted and fed into each other to continuously expand the inflammatory response [33,34]. From the perspective of the entire structure of the inflammatory network, interventions at any or all of the inflammatory network pathways may affect the outcome of sepsis injury. In this study, rutaecarpine reduced the levels of inflammatory cytokines TNF-α and IL-6 as well as increased expression of immunosuppressive cytokine IL-10 in peritoneal lavage fluid of sepsis mice. And rutaecarpine also has the same effect in peritoneal macrophages. IL-10 was up-regulated in sepsis as an anti-inflammatory factor [35,36]. With an in-depth understanding of the pathogenesis of sepsis, it is thought that organism has an anti-inflammatory reaction spontaneously in the process of inflammatory response. Pathogenic microorganisms stimulate macrophages, and then recognize toll-like receptors (TLRs), allowing macrophages to secrete anti-inflammatory cytokines (such as IL-10) to initiate innate immune responses. In the meantime, pathogenic microorganisms induced by sepsis can also promote the secretion of IL-6, TNF-α and other pro-inflammatory cytokines, that is, the body is in a complex immune disorder [37,38]. Rutaecarpine may mediate the homeostasis of the body by regulating the balance between pro-inflammatory and anti-inflammatory factors. Studies have also shown that activation of TLR activates a cascade of signal transduction pathways in the cell to activate nuclear transcription factors such as NF-κB, which in turn produces large amounts of inflammatory cytokines TNF-α, IL-1β, and IL-6 [39]. Based on these features, in this study, we proved that rutaecarpine attenuated sepsis–induced inflammatory response in the peritoneal resident macrophages through inhibiting the activation of NF-κB pathway. The expression of IL-10 can be induced by TLR or non-TLR signaling in macrophages. Activation of TLRs and their adaptor molecules can also result in the activation of the extracellular signal-

regulated kinase 1 (ERK1) and ERK2, p38 pathways. Activation of these pathways also results in the induction of IL-10 expression, in addition to pro-inflammatory cytokines [40]. Our study showed higher level of IL-10 still happened after the rutaecarpine treatment since there was a reduction in NF-κB activation. This suggested that IL-10 secretion during rutaecarpine therapy of sepsis might be not only through the NF-κB signaling pathway, but also through other mechanisms such as ER1, ERK2 and p38 pathways [40]. However, the specific mechanism of IL-10 up-regulation still needs further research.

5. Conclusions

In summary, we demonstrated that ER stress-induced apoptosis and inflammatory response were the main causes for the decrease of peritoneal resident macrophages in sepsis model. Rutaecarpine could protect peritoneal resident macrophages against sepsis-induced apoptosis through inhibiting ER stress. Rutaecarpine could also attenuate sepsis-induced inflammatory response in the peritoneal resident macrophages through inhibiting the activation of ER stress/NF-κB pathways. These findings could provide us a better understanding of the pathogenesis, providing novel insights and approaches for the treatment of sepsis.

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Conflicts of interests

The authors declare that there are no conflicts of interest.

References

[1] M. Singer, et al., The third international consensus definitions for sepsis and septic shock (Sepsis-3), JAMA 315 (8) (2016) 801–810.
[2] L. Zeng, et al., ALK is a therapeutic target for lethal sepsis, Sci. Transl. Med. 9 (412) (2017).
[3] V.Y. Dombrovskiy, A.A. Martin, J. Sunderram, H.L. Paz, Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003, Crit. Care Med. 35 (5) (2007) 1244–1250.
[4] B. Yang, et al., Intraintestinal administration of ulinastatin protects against sepsis by relieving intestinal damage, J. Surg. Res. 211 (2017) 70–78.
[5] J. Zhou, et al., Epidemiology and outcome of severe sepsis and septic shock in intensive care units in mainland China, PLoS One 9 (9) (2014) e107181.
[6] Q. Feng, et al., Characterization of sepsis and sepsis-associated encephalopathy, J. Intensive Care Med. (2017) (885066617719750).
[7] R.J. Snyder, et al., Macrophages: a review of their role in wound healing and their therapeutic use, Wound Repair Regen. 24 (4) (2016) 613–629.
[8] S. Gordon, L. Martinez-Pomares, Physiological roles of macrophages, Pflugers Arch. 469 (3–4) (2017) 365–374.
[9] L.C. Davies, S.J. Jenkins, J.E. Allen, P.R. Taylor, Tissue-resident macrophages, Nat. Immunol. 14 (10) (2013) 986–995.
[10] M. Rosas, et al., The transcription factor Gata6 links tissue macrophage phenotype and proliferative renewal, Science 344 (6184) (2014) 645–648.
[11] E.L. Gautier, et al., Gata6 regulates aspartoacylase expression in resident peritoneal macrophages and controls their survival, J. Exp. Med. 211 (8) (2014) 1525–1531.
[12] T.E. Williams, A. Ayala, I.H. Chaudry, Inducible macrophage apoptosis following sepsis is mediated by cysteine protease activation and nitric oxide release, J. Surg. Res. 70 (2) (1997) 113–118.
[13] R. Liu, H. Jiang, Y. Tian, W. Zhao, X. Wu, Astragaloside IV protects against polymicrobial sepsis through inhibiting inflammatory response and apoptosis of lymphocytes, J. Surg. Res. 200 (1) (2016) 315–323.
[14] A. Dahdah, et al., Mast cells aggravate sepsis by inhibiting peritoneal macrophage phagocytosis, J. Clin. Invest. 124 (10) (2014) 4577–4589.
[15] M.M. Khan, W.L. Yang, P. Wang, Endoplasmic reticulum stress in sepsis, Shock 44 (4) (2015) 294–304.
[16] T. Ma, et al., The endoplasmic reticulum stress-mediated apoptosis signal pathway is involved in sepsis-induced abnormal lymphocyte apoptosis, Eur. Surg. Res. 41 (2) (2008) 219–225.
[17] M.L. Schmitz, M.S. Shaban, B.V. Albert, A. Gokcen, M. Kracht, The crosstalk of endoplasmic reticulum (ER) stress pathways with NF-kappaB: complex mechanisms relevant for cancer, inflammation and infection, Biomedicine 6 (2) (2018).
[18] S. Jia, C. Hu, Pharmacological effects of rutaecarpine as a cardiovascular protective agent, Molecules 15 (3) (2010) 1873–1881.
[19] Y. Kobayashi, K. Hoshikuma, Y. Nakano, Y. Yokoo, T. Kamiya, The positive inotropic and chronotropic effects of evodiamine and rutaecarpine, indoloquinazoline alkaloids isolated from the fruits of Evodia rutaecarpa, on the guinea-pig isolated right atria: possible involvement of vanilloid receptors, Planta Med. 67 (3) (2001) 244–248.
[20] T.C. Moon, et al., A new class of COX-2 inhibitor, rutaecarpine from Evodia rutaecarpa, Inflamm. Res. 48 (12) (1999) 621–625.
[21] X.Q. Nie, et al., Rutaecarpine ameliorates hyperlipidemia and hyperglycemia in fat-fed, streptozotocin-treated rats via regulating the IRS-1/PI3K/Akt and AMPK/ACC2 signaling pathways, Acta Pharmacol. Sin. 37 (4) (2016) 483–496.
[22] J.W. Yang, et al., Effects of rutaecarpine on inflammatory cytokines in insulin resistant primary skeletal muscle cells, Zhongguo Zhong Yao Za Zhi 39 (15) (2014) 2930–2935.
[23] Y.W. Shin, E.A. Bae, X.F. Cai, J.J. Lee, D.H. Kim, In vitro and in vivo antiallergic effect of the fructus of Evodia rutaecarpa and its constituents, Biol. Pharm. Bull. 30 (1) (2007) 197–199.
[24] D. Rittirsch, M.S. Huber-Lang, M.A. Flierl, P.A. Ward, Immunodesign of experimental sepsis by cecal ligation and puncture, Nat. Protoc. 4 (1) (2009) 31–36.
[25] F. Amiri, et al., Autophagy-modulated human bone marrow-derived mesenchymal stem cells accelerate liver restoration in mouse models of acute liver failure, Iran. Biomed. J. 20 (3) (2016) 135–144.
[26] D.Y. Ouyang, et al., Autophagy is differentially induced in prostate cancer LNCaP, DU145 and PC-3 cells via distinct splicing profiles of ATG5, Autophagy 9 (1) (2013) 20–32.
[27] D.C. Angus, The search for effective therapy for sepsis: back to the drawing board? JAMA 306 (23) (2011) 2614–2615.
[28] G. He, et al., Isoalantolactone inhibits LPS-induced inflammation via NF-kappaB inactivation in peritoneal macrophages and improves survival in sepsis, Biomed. Pharmacother. 90 (2017) 598–607.
[29] Y.H. Zheng, et al., Effects of allogeneic bone marrow mesenchymal stem cells on polarization of peritoneal macrophages in rats with sepsis, Zhonghua Shao Shang Za Zhi 33 (4) (2017) 217–223.
[30] A. Ayala, I.H. Chaudry, Immune dysfunction in murine polymicrobial sepsis: mediators, macrophages, lymphocytes and apoptosis, Shock 6 (Suppl. 1) (1996) S27–S38.
[31] M.C. Lu, T.A. Liu, M.R. Lee, L. Lin, W.C. Chang, Apoptosis contributes to the decrement in numbers of alveolar macrophages from rats with polymicrobial sepsis, J. Microbiol. Immunol. Infect. 35 (2) (2002) 71–77.
[32] S.J. Lee, H. Ahn, K.W. Nam, K.H. Kim, W. Mar, Effects of rutaecarpine on hydrogen peroxide-induced apoptosis in murine hepa-1c1c7 cells, Biomol. Ther. (Seoul) 20 (5) (2012) 487–491.
[33] M.H. Bao, W. Dai, Y.J. Li, C.P. Hu, Rutaecarpine prevents hypoxia-reoxygenation-induced myocardial cell apoptosis via inhibition of NADPH oxidases, Can. J. Physiol. Pharmacol. 89 (3) (2011) 177–186.
[34] M.J. Delano, P.A. Ward, Sepsis-induced immune dysfunction: can immune therapies reduce mortality? J. Clin. Invest. 126 (1) (2016) 23–31.
[35] K.A. Tanaka, S. Kurihara, T. Shibakusa, Y. Chiba, T. Mikami, Cystine improves survival rates in a LPS-induced sepsis mouse model, Clin. Nutr. 34 (6) (2015) 1159–1165.
[36] A.E. Ferreira, et al., PPAR-gamma/IL-10 axis inhibits MyD88 expression and ameliorates murine polymicrobial sepsis, J. Immunol. 192 (5) (2014) 2357–2365.
[37] N.J. Shubin, S.F. Monaghan, A. Ayala, Anti-inflammatory mechanisms of sepsis, Contrib. Microbiol. 17 (2011) 108–124.
[38] T.S. Wang, J.C. Deng, Molecular and cellular aspects of sepsis-induced immunosuppression, J. Mol. Med. (Berl) 86 (5) (2008) 495–506.
[39] M. Levi, The coagulant response in sepsis and inflammation, Hamostaseologie 30 (1) (2010) 10–12, 14-16.
[40] M. Saraiva, A. O'Garra, The regulation of IL-10 production by immune cells, Nat. Rev. Immunol. 10 (3) (2010) 170–181.