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Original Article

LPS pretreatment ameliorates D-galactosamine/lipopolysaccharide-induced acute liver failure in rat

Jin-Zhong Dong[1*], Li-Ping Wang[2*], Sai-Nan Zhang[3], Ke-Qing Shi[3], Shao-Long Chen[4], Nai-Bin Yang[3], Shun-Lan Ni[3], Jian-Hua Zhu[1], Ming-Qin Lu[3]

Departments of [1]Intensive Care Unit, [2]Infection Disease, Ningbo First Hospital, Ningbo, China; [3]Department of Infection Disease, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China; [4]Department of Infectious Diseases, Huashan Hospital, Fudan University, Shanghai, China. [*]Equal contributors.

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Abstract: Acute liver failure (ALF) remains an extremely poor prognosis and high mortality; with no effective treatments. The endotoxin tolerance (ET) phenotype has been reported to exhibit protective activities in several sepsis models. We now investigated the effects and underlying intraperitoneal injection of the same volume of pyrogen-free 0.9% sodium chloride instead of LPS for five consecutive days before D-GalN/LPS injection in rats. The serum levels of TNF-α, IL-6, ALT, AST and TBiL from ET + ALF group and ALF group were measured at different time points. Our results showed that ET + ALF group markedly reduced the serum levels of TNF-α, IL-6, ALT, AST and TBiL and histological features in the ET + ALF group were improved significantly. Furthermore, LPS pre-treatment inhibited D-GalN/LPS-induced NF-κB activation, Bax activation, signal transducer and activator of transcription-1 (STAT1) and signal transducer and activator of transcription-3 (STAT3) activities. LPS pre-treatment also significantly enhance the expression of suppressors of cytokine signaling 1 (SOCS1) and suppressors of cytokine signaling 3 (SOCS3). Our experimental data indicated that ET might alleviate D-GalN/LPS-induced ALF by inhibiting the inflammatory response, inactivation of STAT1 and STAT3 and up-regulation of SOCS1 and SOCS3.

Keywords: D-galactosamine, lipoplysaccharide, inflammatory cytokines, acute hepatic failure, inflammatory cytokines, JAK/STAT, rat

Introduction

Acute liver failure (ALF) is the appearance of severe complications rapidly after the first signs of liver disease (such as jaundice), and indicates that the liver has sustained severe damage (loss of 80-90% function of liver cells) [1]. As we know, massive hepatocytes necrosis and apoptosis in the liver parenchymal cells will increase systemic inflammatory syndrome which will lead to ALF [2-4]. In clinic, about 60% of all ALF patients fulfill the criteria for systemic inflammatory syndrome irrespective of presence or absence of infection, resulting in multiple organ dysfunction syndrome (MODS) [5].

Endotoxin, as a gram-negative bacterial lipopolysaccharide (LPS), stimulates a wide variety of inflammatory mediators, which are considered to be related to the development of ALF [6-8]. Recent studies showed that reduction of endotoximia could prevent the death of hepatocytes, which could alleviate the development of ALF in experimental models [9-11]. Prior exposure of innate immune cells like monocytes/macrophages to minute amounts of endotoxin caused them to become refractory to subsequent endotoxin challenge, a phenomenon called endotoxin tolerance (ET) [12-14]. Therefore, we hypothesized that ET rat might have a lower mortality in D-GalN/LPS-induced ALF. However, the mechanism of ET in ALF still has not been fully clarified.

In this study, we investigated the effect of ET pretreatment on ALF in rat. Moreover, the mechanisms of ET in progression of ALF, including JAK/STAT pathway and cytokines were also explored.

Materials and methods

Animal grouping and treatment

One hundred-six Male SPF Sprague-Dawley rats (200-220 g) were obtained from the Shanghai

Laboratory Animal Center (Shanghai, China). The rats were housed under normal laboratory conditions (21 ± 2°C, 12 h light-dark cycle) with free access to standard pellet diet and water. All animal procedures were conducted in accordance with the standards set forth in the guidelines for the care and use of experimental animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals and the National Institutes of Health. The study protocol was approved by the Animal Ethics Committee of Wenzhou Medical University.

Experimental protocols

Animals were randomly divided into the control group (n = 6), the ALF group (n = 70), ET + ALF group (n = 30). In ET + ALF group, rats were treated with intraperitoneal injection of 0.1 mg/kg LPS for five consecutive days for induction of ET. As in the ALF group, rats were injected with the same volume of pyrogen-free 0.9% sodium chloride instead of LPS for five consecutive days. On the six day, all the model group animals were given an intraperitoneal injection of 500 μL saline containing 800 mg/kg D-GalN (Sigma, USA) and 8 μg LPS (Sigma, USA). In a separate experiment, survival rate was monitored for 72 hours after ALF in eighty rats.

Plasma biochemistry and Enzyme-linked immune sorbent assays

In the model groups, animals were anesthetized with urethane (1.0 g/kg, intraperitoneally (i.p.)) at 2, 6, 12, 24, 48 hours, respectively, the blood samples from abdominal aorta were drawn into heparinized injectors, and centrifuged at 1500 rpm at 4°C for 10 min. The serum levels of alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBiL) were measured according to the manufacturer's instructions. Plasma concentrations of cytokines, including tumor necrosis factor-α (TNF-a) and interleukin-6 (IL-6) were assayed using enzyme-linked immune sorbent assay (ELISA) kits according to the manufacturer's instructions.

Liver histological examination

After finishing with the blood collection, the experimental animals were sacrificed, liver samples were dissected and washed with ice-cold saline, then they were immediately stored at -80°C for further analysis. Partial liver specimens were fixed in 10% neutral formalin and embedded in paraffin. The tissue sections were then cut into 4-μm sections and histological changes were assessed by using H&E staining. Images from different groups were obtained using a video microscope.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from preserved liver tissue from each group using RNAiso Plus reagent, according to the manufacturer's protocol. The RNA samples were then reverse-transcribed into cDNA by reverse transcription reaction according to the following protocol. The above product was then used for amplification of cDNA, and the reaction system totaled 20 μl volume per sample, which consisted of The RNA samples were then reverse-transcribed into cDNA by reverse transcription reaction which contained 1 μl RNA sample, 1 μl Oligo dT-Adaptor primer (50 μM), 1 μl dNTP mixture (10 mM each), 4 μl 5× first-strand buffer, 1 μl Power M-MuL Reverse Transcriptase (200U/μl), 1 μl RNase inhibitor (40 U/μl), 11 μl RNase-free dH2O. The above product was then used for amplification of cDNA, and the reaction system was 25 μl volume per sample, which consisted of 12.5 μl 2X Master Mix, 0.5 μl forward primers, 0.5 μl reverse primers, 2.5 μl cDNA sample and 1 μl RNase-free dH2O. The primers of the different cell cytokines were as follows: SOCS1 (350 bp): forward primers: 5'-CCACTCTGATTACCGGCGCATC-3' and reverse primers: 5'-GCTCCTGCAGCGGCCGCACG-3'. SOCS3 (514 bp): forward primers: 5'-ATGGTCACCCACAGCAGTTTC-3' and reverse primers: 5'-CGCCCCCAGAATAGATGTAGTAAG-3' P65 (505 bp): forward primers: 5'- TTGAGCAGCCCAAGCAGCGG-3' and reverse primers: 5'-GCAGTGTTGGGGGCACGGTT-3' β-actin (149 bp): forward primers 5'-CAAGTTCAACGGCACAGTCAA-3' and reverse primers 5'-TGGTGAAGACGCCAGTAGACTC-3'.

The PCR protocol was as follows: the mixture was first denatured at 94°C for 5 min, 35 cycles at 94°C for 30 seconds. 58°C for 30 seconds (P65), 60°C for 30 seconds (SOCS1 and SOCS3), 61°C for 30 seconds (β-actin) followed by 72°C for 20 seconds and a final extension step at 72°C for 5 min. The PCR products were finally analyzed in a 2% agarose gel containing ethidium bromide (EB), and β-actin was used as a loading control.

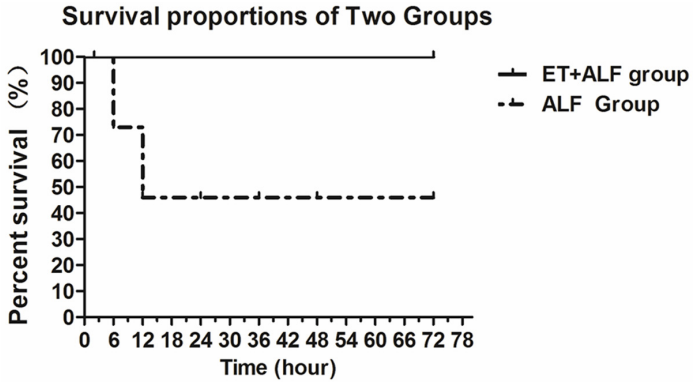

Figure 1. Effect of LPS pretreatment on survival rate after ALF. Rats were pretreated with either five consecutive daily injections of LPS at the dosage of 0.1 mg/kg (ET + ALF, n = 40) or normal saline (ALF, n = 40). ALF was performed on the sixth day and then survival was monitored for 72 hours. LPS pretreatment resulted in a significant improvement in survival compared with ALF group ($P < 0.05$).

Protein isolation and Western blotting

Total protein was isolated using lysis buffer supplemented with a protease inhibitor. Following heat denaturation at 95°C for 5 min, the samples (15 μg protein each) were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto a nitrocellulose membrane. The membrane was then blocked by skimmed milk for 90 min at room temperature. The primary antibodies against STAT1 (cst), STAT3 (cst), SOCS1 (cst), SOCS3 (cst), P65 (Santa Cruz), Bax (anbo) and β-actin (Santa Cruz) were used for incubation with the membrane overnight at 4°C, respectively. After being washed with three times of TBST, the membrane was incubated with the secondary antibody at room temperature for 1 hour. Then the film exposure was performed.

Statistical analysis

All data were expressed as the means ± standard deviation (SD). Statistical significances were determined using one-way analysis of variance (ANOVA) or the least significant difference (LSD) test. SPSS19.0 software (IBM, USA) was used for statistical analyses; $P < 0.05$ was considered to have statistical significance.

Results

Effect of LPS pretreatment on the survival rate of rats after ALF

In a separate experiment, eighty rats were randomly divided into two groups: ALF group (40) and ET + ALF (40), and survival rate was monitored for 72 hours after ALF. In the ALF group, twenty rats died in the first 12 hours. Among them, four rats died during 12-48 hours. The mortality rate reached 60% at 72 hours. However, in the ET + ALF group, all rat alive. The mortality rate was 0% at 72 hours (**Figure 1**).

Liver histopathology after D-GalN/LPS injection.

Histopathological study of the liver was performed to investigate events after D-GalN/LPS injection. In ALF group, hepatocytes become to swell, a large number of inflammatory cells infiltrated at 6 hours, and significantly degeneration and necrosis were observed at the later time (**Figure 2E**). However, in ET + ALF group,thehistopathologicalinjuriescausedbyD-GalN/LPS were significantly alleviated (**Figure 2E**).

Plasma indicators for liver function (ALT, AST, TBIL) after D-GalN/LPS injection.

Following the administration of D-GalN/LPS, serum ALT, AST levels of rats in both the ALF and ET + ALF group became to increase at 2 hours and were significantly elevated at 12 hours. Meanwhile, serum TBIL levels continuously increased after 2 hours. However, the expressions of all above factors in ALF group were higher compared to the ET + ALF group at each time point ($P < 0.01$, **Figure 2B**, **2D**).

Cytokines production after D-GalN/LPS injection.

After the administration of D-GalN/LPS, serum TNF-α and IL-6 levels became to increase at 2 hours and were significantly elevated at 24 hours both in ALF group and ET + ALF group compare to control group ($p < 0.05$, **Figure 1**). In addition, the expressions of all above data in ALF group remained higher than in ET + ALF group at each time point ($P < 0.05$, **Figure 3A**, **3B**)

Expression of P65, SOCS1 and SOCS3 genes after D-GalN/LPS injection.

To clarify the effect of D-GalN/LPS on the expression of P65, SOCS1 and SOCS3, liver tis-

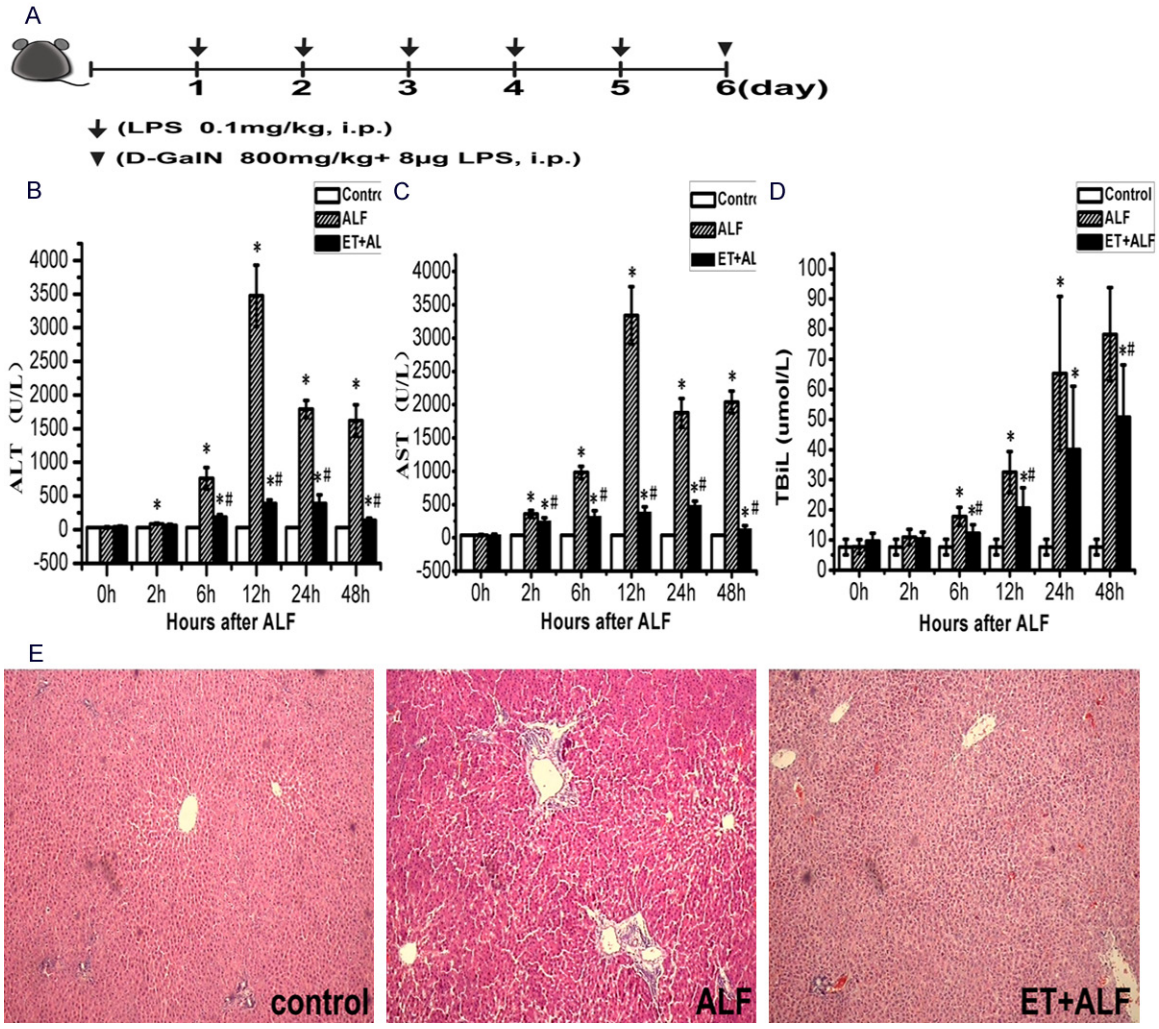

Figure 2. LPS pretreatment attenuates liver damage and inhibited the release of cytokines induced by D-GalN/LPS. A. Rats were treated with pyrogen-free 0.9% sodium chloride or LPS (0.1 mg/kg). Five days later, D-GalN/LPS (800 mg/kg D-GalN + 8 μg LPS) was injected. Serum samples were collected at indicated time points after D-GalN/LPS injection. ALT (B), AST(C) and TBIL (D) in serum were assayed by using an automated blood chemistry analyzer. (*significant compared to control group, $P < 0.05$; #significant compared to each subgroup, $P < 0.05$). Liver samples were collected 12 h after D-GalN/LPS injection for HE staining (original magnification: ×100) (E).

sues of ALF group and ET + ALF group were assayed by RT-PCR. In the ALF group, the mRNA levels of P65 began to rise at 2 hours after D-GalN/LPS injection and significantly elevated at 12 hours, and the mRNA levels of SOCS1 and SOCS3 increased gradually at 2 hours after D-GalN/LPS injection and significantly elevated at 12 hours, and 6 hours, respectively, then were down-regulated (**Figure 4B**, **4D**). Additionally, the above expression pattern of P65, SOCS1 and SOCS3 were also observed in the ET + ALF group. The mRNA levels of P65 was lower than in ALF group, while the mRNA levels of SOCS1 and SOCS3 were both higher than in ALF group (**Figure 4B**, **4D**).

Protein expression of STAT1, STAT3, SOCS1, SOCS3 P65 and Bax after D-GalN/LPS injection.

To further investigate the underlying mechanisms, the protein expressions of STAT1, STAT3 (**Figure 5B**, **5C**), SOCS1, SOCS3 (**Figure 6B**, **6C**), P65 and Bax (**Figure 7B**, **7C**) both in the ALF group and ET + ALF group are detected by Western Blotting, then all the above data was normalized against those of β-actin. In the ALF group, the protein levels of STAT1 and STAT3 increased at 2 hours after D-GalN/LPS injection. And the protein levels of P65 and Bax also increased at 2 hour after D-GalN/LPS injection.

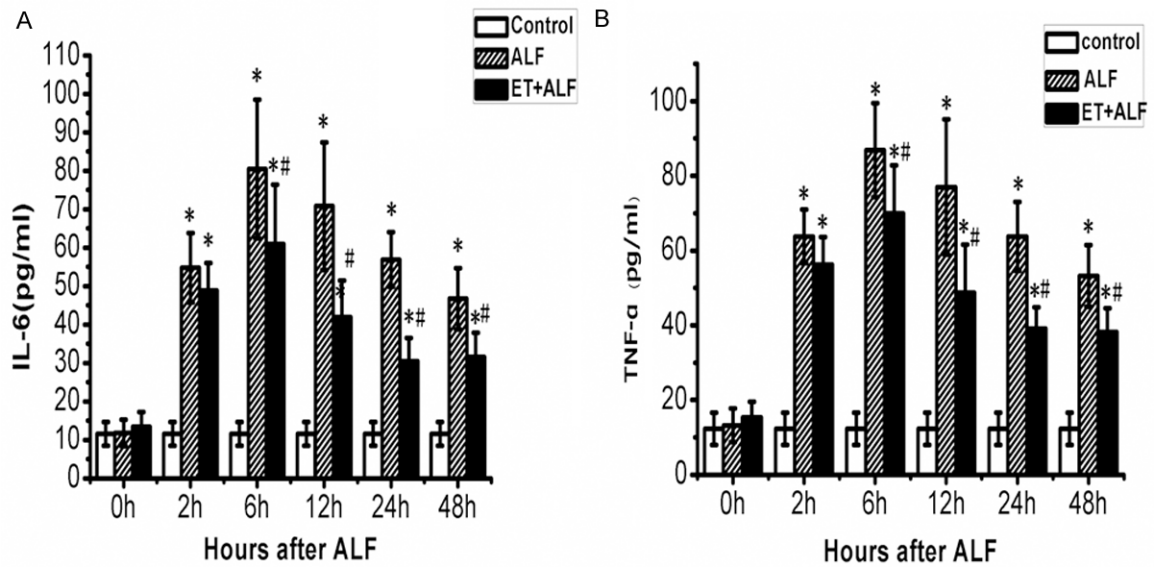

Figure 3. LPS pretreatment inhibited the release of cytokines induced by D-GalN/LPS. Serum samples were collected at indicated time points after D-GalN/LPS injection.IL-6 (A), and TNF-α (B) in serum were assayed by ELISA. *significant compared to control group, $P < 0.05$; #significant compared to each subgroup, $P < 0.05$).

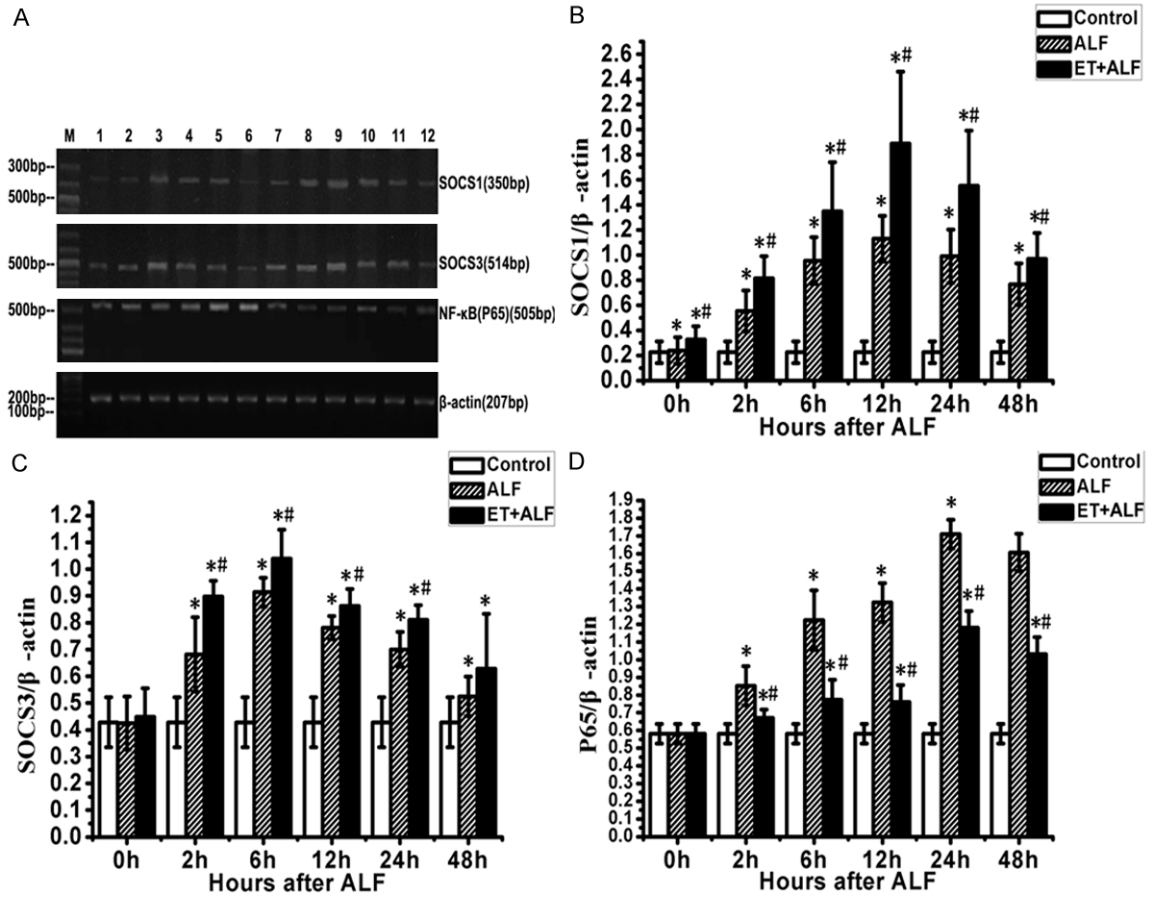

Figure 4. Effect of LPS pretreatment on the changes of SOCS1 (B), SOCS3 (C) and P65 (D) mRNA expression in liver of rat. A. M: marker; 1: The control group; 2: ALF 2 h group; 3: ALF 6 h group; 4: ALF 12 h group; 5: ALF 24 h group; 6: ALF 48 h group; 7: ETT 2 h group; 8: ETT 6 h group; 9: ETT 12 h group; 10: ETT 24 h group; 11: ETT 48 h group

Liver samples were collected at indicated time points after D-GalN/LPS (800 mg/kg D-GalN + 8 μg LPS) injection to determine the mRNA level of P65, SOCS1 and SOCS3 mRNA by RT-PCR. Data represent means ± SD. $^{*}P < 0.05$ (n = 6). *significant compared to control group, #significant compared to each subgroup, $P < 0.05$.

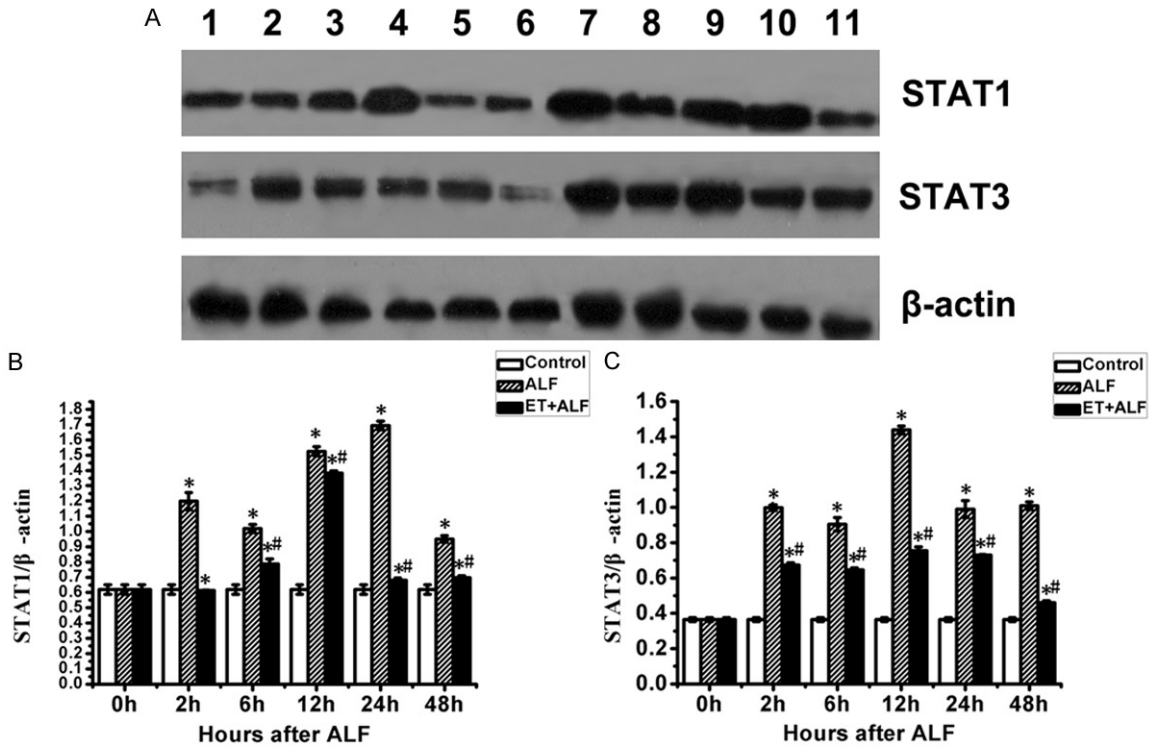

Figure 5. Effect of LPS pretreatment on the changes of STAT1 (B) and STAT3 (C) protein expression in liver of rat. A. 1: The control group; 2: ET 2 h group; 3: ET 6 h group; 4: ET 12 h group; 5: ET 24 h group; 6: ET 48 h group; 7: ALF 2 h group; 8: ALF 6 h group; 9: ALF 12 h group; 10: ALF 24 h group; 11: ALF 48 h group. Liver samples were collected at indicated time points after D-GalN/LPS (800 mg/kg D-GalN + 8 μg LPS) injection to determine the protein level of STAT1 and STAT3 by Western blot. Data represent means ± SD. $^{*}P < 0.05$ (n = 6). #significant compared to each subgroup, $P < 0.05$.

A similar result was found in the ET + ALF group. Our data showed that the protein levels of STAT1, STAT3, P65 and Bax in the ALF group were higher than the ET + ALF group. SOCS1 and SOCS3 in the ET + ALF group elevated gradually at 2 hours after D-GalN/LPS injection and significantly elevated at 12 hours, those levels were higher than ALF group.

Discussion

Endotoxin is a gram-negative bacterial lipopolysaccharide (LPS) and releases a wide variety of inflammatory mediators, which are considered to be related to the development of acute liver failure as well as to multiple organ failure. D-GalN is a specific hepatotoxic agent that induces liver injury by selective depletion of uridine nucleotides in hepatocytes. Taken together, can dramatically increase their susceptibility to the lethal effects of LPS and may leads to fulminant hepatic. Hence, combined administration of D-GalN/LPS together has been widely used as to mimic the sequences of events in human hepatitis [8, 15, 16]. In our study, we found that pretreated of rats with low dose LPS for five days could significantly ameliorate D-GalN/LPS-induced liver injury. ET significantly increased the survival rate in rats from 40% to 100% after D-GalN/LPS injection. Both in ALF group and ET + ALF group, the serum ALT, AST levels increased at 2 hour and were significantly elevated at 12 hour, and serum TBIL levels increased persistently from 2 hour. Additionally, we examined the expression of Bax proteins in liver. However, serum ALT, AST, and TBIL levels in the ET + ALF group were significant lower than HF group at each time point. Hence, our results demonstrated that pre-exposure of a sub-lethal dose of LPS in rats not

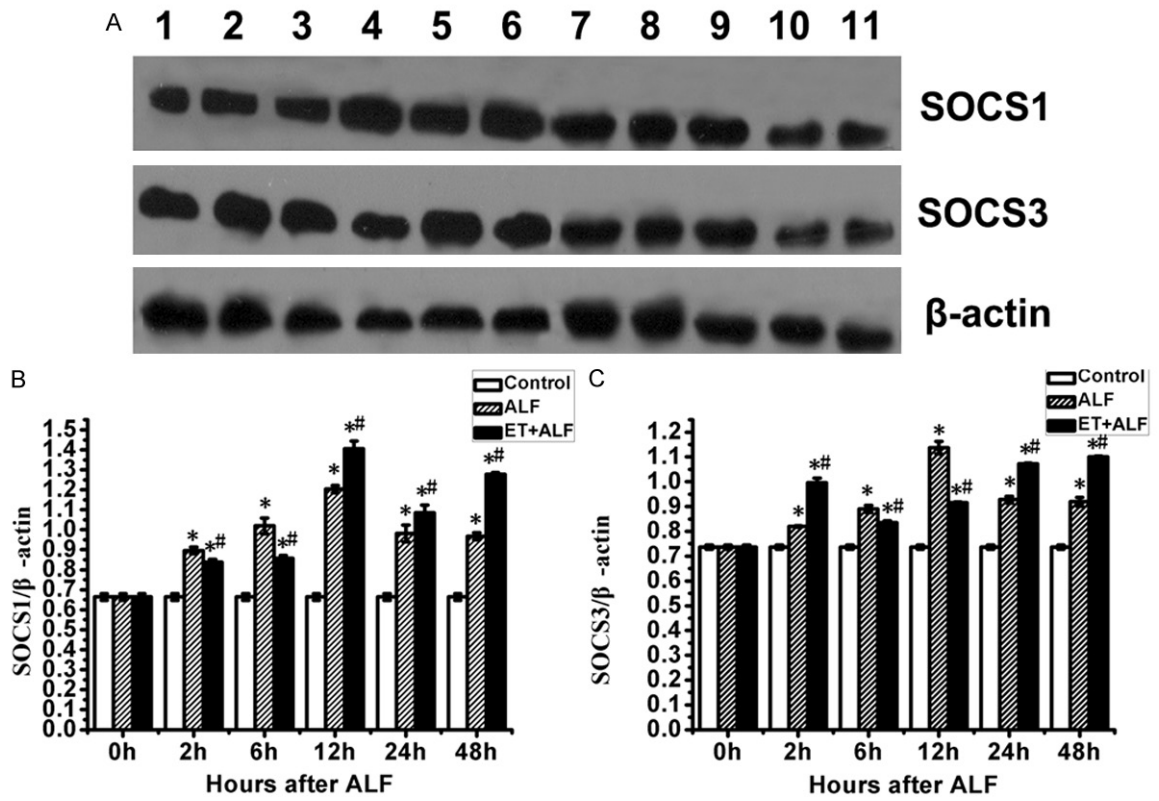

Figure 6. Effect of LPS pretreatment on the changes of SOCS1 (B) and SOCS3 (C), protein expression in liver of rat. A. 1: The control group; 2: ET 2 h group; 3: ET 6 h group; 4: ET 12 h group; 5: ET 24 h group; 6: ET 48 h group; 7: ALF 2 h group; 8: ALF 6 h group; 9: ALF 12 h group; 10: ALF 24 h group; 11: ALF 48 h group. Liver samples were collected at indicated time points after D-GalN/LPS (800 mg/kg D-GalN + 8 μg LPS) injection to determine the protein level of SOCS1 and SOCS3 by Western blot. Data represent means ± SD. $^{*}P < 0.05$ (n = 6). $^{\#}$significant compared to each subgroup, $P < 0.05$.

only attenuated histopathological injuries but also increased thier survive rate caused by D-GalN/LPS.

As we know, various cytokines and inflammatory mediators are involved in the pathogenesis of ALF. TNF-α, one of the most pivotal mediators in the pathogenesis of LPS and Gram-negative bacterial infection, has been widely investigated [8, 17, 18]. In addition, NF-kappa B plays a key role in the regulation of TNF-α transcription stimulated by LPS [19, 20]. After exposure to LPS, NF-kappa B is translocated to the nucleus and binds to NF-kappa B promoter sites on DNA, activating gene transcription of cytokines such as TNF-α, and accelerating liver injury [21, 22]. In our study, we exhibited that the plasma levels of TNF-α increased at 2 hours and were significantly elevated at 24 hours in ALF group. In ET + ALF group, TNF-α level was lower than in ALF group. Additionally, Bax protein encoded by Bax gene belongs to the BCL2 protein family. which leads to the loss in membrane potential and the release of cytochrome c, leading to cell death [23]. Our data showed that Bax protein levels in ALF group continuously increased after 2 hour. However, the expressions of Bax in ET + ALF group were lower compared to the ALF group at each time point, except at 12 hours. Those results indicated that mitochondrial in the ALF may be are serious losses and massive hepatic cell death. Therefore, pre-exposure of a sub-lethal dose of LPS significantly increased the survival rate via depressing the levels of pro-inflammatory mediators.

Following liver injury, liver cells, including Kupffer cells [24], hepatocytes [25, 26], natural killer cells [27], and dendritic cells [28], could produce pro-inflammatory cytokines and anti-inflammatory cytokines. IL-6 can activated the STAT3, which playing key roles in inducing the acute-phase response in the liver, protecting against hepatocellular damage, and promoting liver regeneration [29]. Furthermore, studies

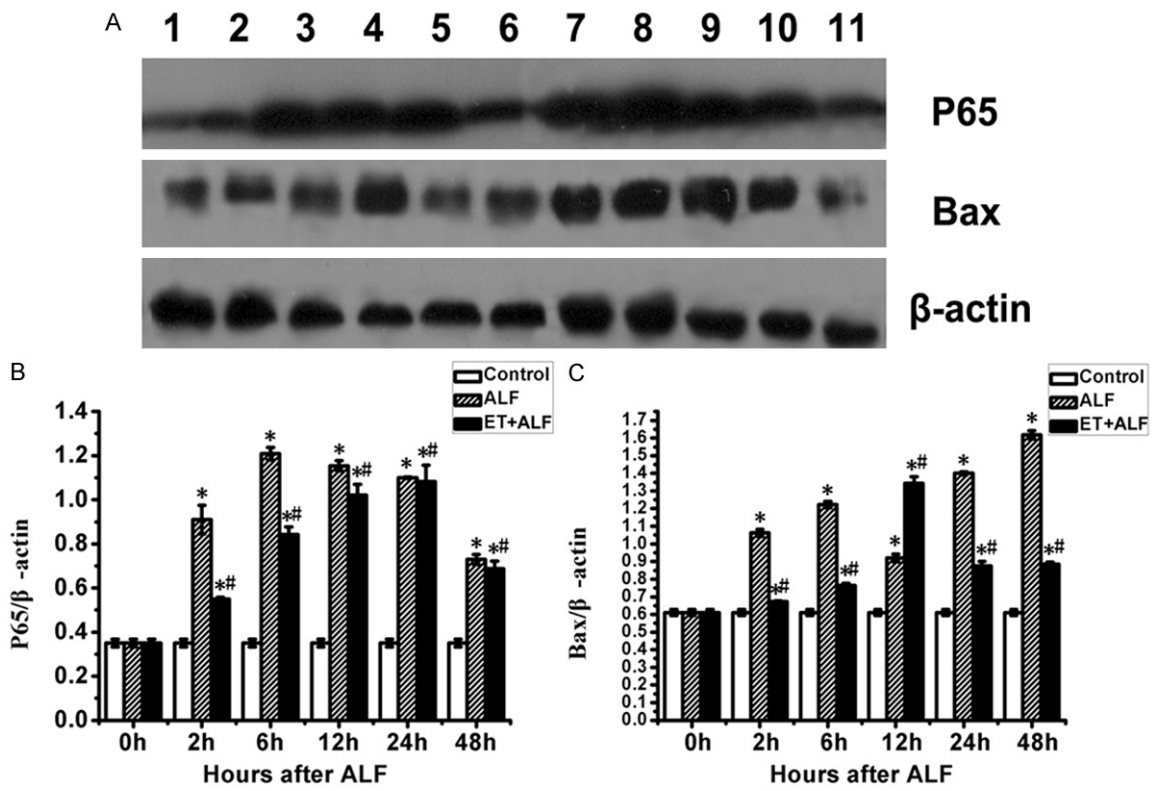

Figure 7. Effect of LPS pretreatment on the changes of P65 (B) and Bax (C) protein expression in liver of rat. A. 1: The control group; 2: ET 2 h group; 3: ET 6 h group; 4: ET 12 h group; 5: ET 24 h group; 6: ET 48 h group; 7: ALF 2 h group; 8: ALF 6 h group; 9: ALF 12 h group; 10: ALF 24 h group; 11: ALF 48 h group. Liver samples were collected at indicated time points after D-GalN/LPS (800 mg/kg D-GalN + 8 μg LPS) injection to determine the protein level of P65 and Bax by Western blot. Data represent means ± SD. $^{*}P < 0.05$ (n = 6). $^{\#}$significant compared to each subgroup, $P < 0.05$.

had shown that STAT1-deficient mice could abolish D-GalN/LPS-induced liver injury [30, 31]. Our study demonstrated that after injection of D-GalN/LPS, STAT1 was rapidly activated and highly induced liver damage, IL-6 and STAT3 in ALF group increased at 2 hour and peaked 12 hour, but those proteins in ET + ALF group were increased slightly at 2 hour. Those data indicated prior injection of rat with a low dose of LPS might reduce the levels of TNF-α and IL-6 then reduced the activation of STAT1 and STAT3 in the liver, which was involved in promoting liver regeneration. Suppressors of cytokine signaling (SOCS) proteins function as feedback inhibitors of the JAK/STAT signaling pathway, terminating innate and adaptive immune responses [32-34]. In our previous study, we observed in ET + ALF group hepatic SOCS1 and SOCS3 mRNA and proteins elevated higher than ALF group, which indicated SOCSs may involve in the mechanism of LPS pretreatment in ALF.

To conclude, our results suggested that LPS pretreatment has potent protection against D-GalN/LPS-induced ALF. It significantly decreased the high lethality, ameliorated the liver injuries, inhibited hepatocytes apoptosis and reduced hepatic inflammatory responses including down-regulating the level of STAT1, STAT3 and up-regulating the level of SOCS1, SOCS3. Those protective effects might be involved in suppression of NF-κB and Bax activation, which inhibited TNF-α and IL-6 production. Therefore, LPS pretreatment might be considered as a promising new strategy to regulate the inflammatory response in improving the prognosis of ALF and prolong life in patients with liver damage.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ming-Qin Lu, Department of Infection and Liver Diseases, Liver Research Center, The First Affiliated Hospital of Wenzhou Medical University, Nanbaixiang Street, Wenzhou, Zhejiang, P. R. China. E-mail: lmq0906@163.com

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