

Original Article

Picroside II ameliorates acute liver injury induced by LPS/D-GalN in mice

Shangping Fang^{1,2*}, Yang Jiao^{1*}, Yiyang Chen^{2*}, Wei Fang³, Yu Cao⁴, Shaoxia Duan⁵, Zhenzhou He⁵, Xueyin Shi^{1,6}

¹Department of Anesthesiology, Changzheng Hospital, Second Military Medical University, Shanghai, China; ²Shanghai Pulmonary Hospital, School of Medicine, Tongji University, Shanghai, China; ³Department of Medicine, Hebei North University, Zhangjiakou, Hebei, China; ⁴Ningxiang Hospital Affiliated to Hunan University of Chinese Medicine, Changsha, Hunan, China; ⁵Department of Anesthesiology and ICU, Renji Hospital South Campus, School of Medicine, Shanghai Jiaotong University, Shanghai, China; ⁶Department of Anesthesiology, Shanghai Xinhua Hospital, School of Medicine, Jiaotong University, Shanghai, China. *Equal contributors.

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Abstract: Background: Acute liver injury (ALI) due to massive hepatocyte death can lead to hepatic metabolic dysfunction and high mortality. Picroside II (Picro II), an iridoid compound extracted from the roots of *Picrorhiza*, has been demonstrated to possess anti-inflammatory and anti-apoptotic activities. Objective: To determine whether Picro II could protect against ALI by inhibiting hepatocytic apoptosis and pyroptosis in a lipopolysaccharide (LPS) and D-galactosamine (GalN)-induced ALI mouse model. Methods: Male C57BL/6 mice aged 6-8 weeks were randomized into three groups: a control group, a LPS/D-GalN group, and a LPS/D-GalN+20 mg/kg Picro II group. The ALI model was established by intraperitoneal (i.p.) injection of 700 mg/kg D-GalN/10 ug LPS. Picro II (20 mg/kg) was injected i.p. at 1, 6, 12 and 24 h prior to LPS/D-GalN exposure. HE staining was used to determine the liver injury level. AST, ALT levels in plasma and IL-6, TNF- α , IL-1 β , IL-18 in serum or supernatant were examined by ELISA. Besides, RNA levels in liver tissue were detected by RT-qPCR. To assay cell death rate, flow cytometry was used by double staining with Annexin V-PE and 7-AAD. To detect whether caspase-1 and caspase-3 were activated, western blot was used. Results: The results showed that pretreatment with Picro II decreased the mortality and alleviated liver injury of the ALI mice, as demonstrated by a significant decrease in alanine aminotransferase (ALT) and aspartate transaminase (AST) levels and amelioration of the histological changes as represented by less hemorrhage and inflammatory cell infiltration. In addition, Picro II protected hepatocytes against apoptosis by inhibiting the expression of Bax and increasing the expression of Bcl-2, protected hepatocytes against pyroptosis by inhibiting the expression of caspase-1, and prevented inflammatory response by inhibiting the activation of NF- κ B. Conclusion: Our results suggest that Picro II could protect the liver in LPS/D-GalN-induced ALI. This finding may provide a new therapeutic option for the clinical treatment of human septic liver injury.

Keywords: Picroside II, acute liver injury, inflammation, apoptosis, pyroptosis

Introduction

Acute liver injury (ALI) is a common clinical syndrome due to massive hepatocyte death, accounting for an incidence of 50-80% [1, 2]. The clinical prognosis of ALI is extremely obscure. Other than liver transplantation, there is no effective therapy for ALI at present [3]. As the liver is the most important immune organ and the metabolism center, any structural, functional and metabolic abnormality of or injury to the liver may cause multiple organ dysfunction syndrome (MODS) [4]. The model of hepatic injury

induced by simultaneous injection of D-GalN and LPS has been widely used to imitate ALI, because it can produce hepatic apoptosis and other types of cell death [5, 6].

Picroside II (Picro II) is an iridoid compound extracted from *Picrorhiza* and has long been used as a traditional medicine to treat inflammatory diseases [7, 8]. Recent studies have demonstrated that Picro II has antioxidant and anti-inflammatory activities [9-11]. Picro II was reported to inhibit the overexpression of inflammatory factors and improve microcirculation

in various diseases. Wang et al [8, 10, 12] reported that Picro II could protect the brain and kidney by inhibiting oxidative stress and inflammatory response. In addition, Picro II has a therapeutic effect on cell apoptosis and pyroptosis in septic mice. The aim of the present study was to determine whether Picro II also had anti-inflammatory, anti-apoptotic and anti-pyroptotic activities in LPS/D-GalN-induced ALI.

Materials and methods

Animals and treatment

Male C57BL/6 mice aged 6-8 weeks (Shanghai SLAC laboratory Animal co., Ltd) were housed in the animal facility of the Second Military Medical University (Shanghai, China) under specific pathogen-free conditions and received humane care according to the Animal Care Committee guidelines of the said university.

The mice in the model groups were injected intraperitoneally (i.p.) with 700 mg/kg D-GalN and 10 µg LPS (Sigma) dissolved in normal saline (NS). Picro II (Nanjing Senbeijia Co., Ltd, CAS No: 39012-20-9, HPLC>98%) or NS was suspended in PBS and injected i.p. bid at a dose of 200 ng in 2 ml PBS per injection prior to LPS/D-GalN treatment. Mice in the control group received the same amount of NS only. Five hours after LPS/D-GalN injection, the mice were anesthetized and blood was collected. There were 3 mice in each group.

The liver was harvested for the following uses: a) a representative section was fixed in 10% formalin for 24 h and paraffin embedded; b) a 50 µg sample was placed in 1 ml of RNeasy Lysis Solution (Qiagen, Carlsbad, CA, USA); c) a small section was used for flow cytometry; and d) the remainder was quickly frozen in liquid nitrogen and stored at -80°C.

Cell culture

Hepatocytes were isolated from the wild-type (WT) mouse liver tissue as previously described [13], and cultured in a 6-well plate in RPMI1640 medium at a density of 1×10^6 cells per well. The cells were pretreated with caspase-1 inhibitor or Picro II, added with 100 ng/mL LPS for 3 h, and then stimulated with 5 mmol/L ATP for 30 min. The supernatant was harvested and

frozen at -80°C for enzyme linked immunosorbent assay (ELISA), and cells were harvested for flow cytometry or Western blot.

Hematoxylin-eosin (HE) staining

The liver tissue was immersion-fixed in 10% neutral buffered formalin for 24 h, trimmed, processed, embedded, sectioned, HE stained routinely, and finally analyzed by light microscopy.

RNA extraction and quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

For liver tissue RNA extraction, 50 µg liver was harvested into 1 mL TRIzol reagent. RNA was extracted using the TRIzol Kit according to manufacturer's protocol. For RNA extraction from cultured hepatocytes, RNA was harvested using RNeasy Lysis Kit according to manufacturer's instruction. cDNA synthesis was performed using AKARA PrimeScript™ RT Master Mix Kit with oligo(dT) primers. qRT-PCR reactions were performed on a CFX96 Real-Time System using SYBR Premix Ex Taq II (Takara).

ELISA

Blood was harvested from mice by retro-orbital bleeding and plasma was isolated using heparin coated plasma separator tubes. Serum or supernatant AST, ALT, IL-6, TNF-α, IL-1β and IL-18 levels were determined by ELISA kits (R&D Systems) following the manufacturer's instructions.

Flow cytometry

A small part of the liver in each group was extracted, ground to 200 mesh and prepared to a single-cell suspension. After red blood cell (RBC) lysis, the remaining cells were washed with PBS twice and stained with FAM-FLICA™ in vitro Caspase Detection Kit protected from light at 37°C for 30 min and then stained with Annexin V-PE and 7AAD for apoptosis assay away from light at room temperature for 15 min by following the manufacturer's protocol (BD559763). For tissue analysis, at least 1×10^6 cells were acquired within the singlet live gate, as defined by size, granularity, and pulse-width. The stained cells were acquired on a FACSCalibur flow cytometer and the data with FlowJo software.

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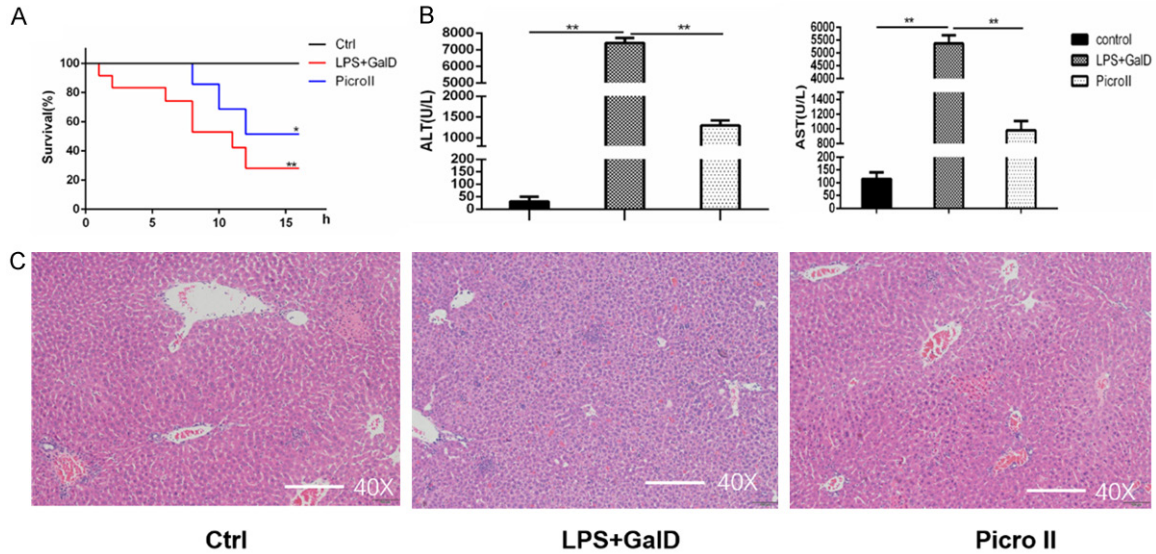


Figure 1. Picro II alleviated acute liver injury induced by LPS/D-GalN. A. The survival rate within 16 h in each group, showing that the survival rate in Picro II group was significant higher than that in LPS/D-GalN group. B. Serum concentrations of ALT and AST. C. HE staining of the liver tissue from each group (40 ×). * $P < 0.05$; Picro II vs. Ctrl. ** $P < 0.01$ LPS/D-GalN vs. Ctrl.

Western blot analysis

Proteins were extracted from the liver tissue in RIPA buffer together with phosphatase and protease inhibitors. Equal amounts of protein (40 ug sample) in SDS-loading buffer were loaded per well of a SDS-12% polyacrylamide gel electrophoresis (PAGE) and run in $1 \times$ MOPS buffer. Proteins were transferred to the PVDF membrane (Bio-Rad, Hercules, CA), and blocked in 5% milk in TBST for 1 h. The membrane was incubated with monoclonal rabbit antibodies against cleaved-Caspase-3, cleaved-Caspase-1, β -actin mAb at 4°C overnight, washed for 15 min \times 3 with TBST, incubated with the secondary antibodies for 1.5 h at room temperature, washed again with TBST three times, and finally analyzed with an ECL chemiluminescence system. After short exposure to X-rays, proteins were quantified by densitometry using ImageJ software.

Statistical analysis

All data were expressed as mean \pm SD. Statistical analyses were performed using Student's t test and one-way analysis of variance (ANOVA) test with multiple comparisons where appropriate using Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Kaplan Meier survival curves were compared using log-rank Mantel-

Cox test. A p value smaller than 0.05 was considered statistically significant.

Results

Picro II improves the survival rate and attenuates hepatotoxicity induced by LPS/D-GalN

To dissect the role of Picro II in ALI, the effects of Picro II on the mortality and hepatic histopathology of ALI mice were illustrated. The survival rate was monitored within 16 h after LPS/D-GalN injection. It was found that the survival rate in the control group was 100% vs. 25% in LPS/D-GalN group. Pretreatment with Picro II significantly improved the survival rate of LPS/D-GalN group (* $P < 0.05$, ** $P < 0.01$; **Figure 1A**).

To determine the effect of Picro II against hepatic injury, serum ALT and AST levels were determined. As expected, ALT and AST levels were increased markedly in LPS/D-GalN-challenged group. However, the elevation of ALT and AST was significantly suppressed by Picro II pretreatment (** $P < 0.01$; **Figure 1B**).

Histological examination of the liver sections showed severe hemorrhage and massive inflammatory cell infiltration in LPS/D-GalN group as compared with those in the control group. Consistently, these histological lesions were

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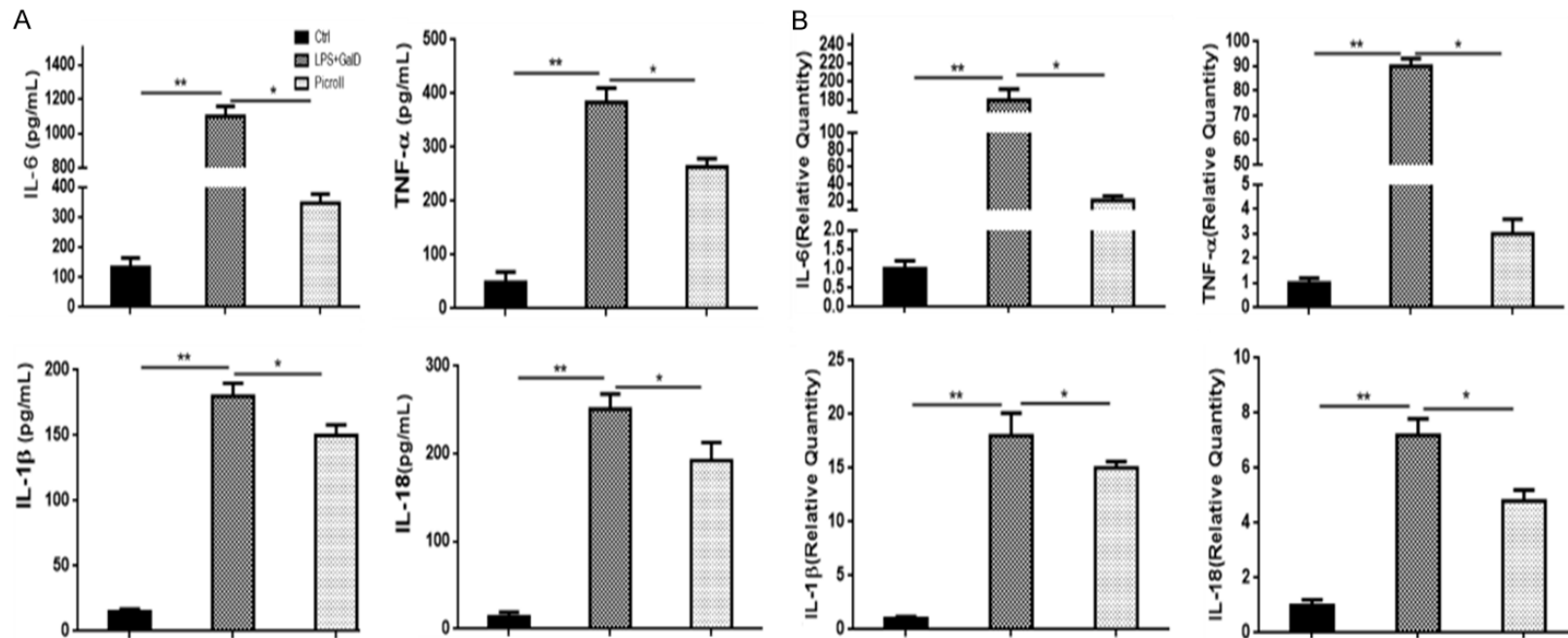
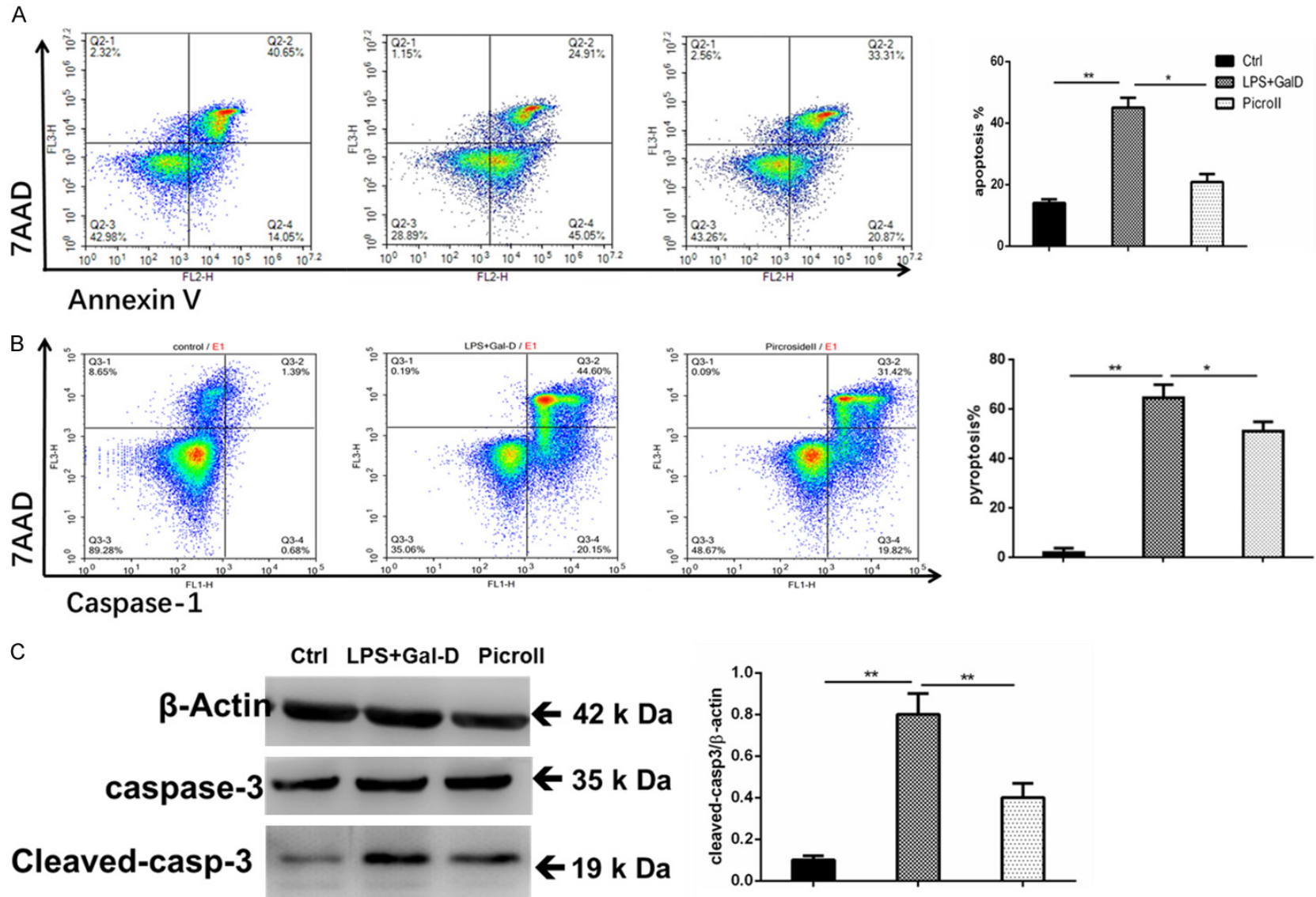


Figure 2. Picoside II decreased the inflammatory response. A. The serum concentrations of IL-6, TNF- α , IL-1 β and IL-18 were examined by ELISA. B. The relative quantity of IL-6 mRNA, TNF- α mRNA, IL-1 β mRNA and IL-18 mRNA in the liver were examined by RT-qPCR. All results are representative of three independent experiments. The graphs show the mean and SEM, n=3. Significances between groups were determined by using independent-samples two-tailed Student's t-test. **P<0.01 LPS/D-GalN vs. Ctrl. *P<0.05 PicroII vs. LPS/D-GalN.

Picroside II ameliorates ALI



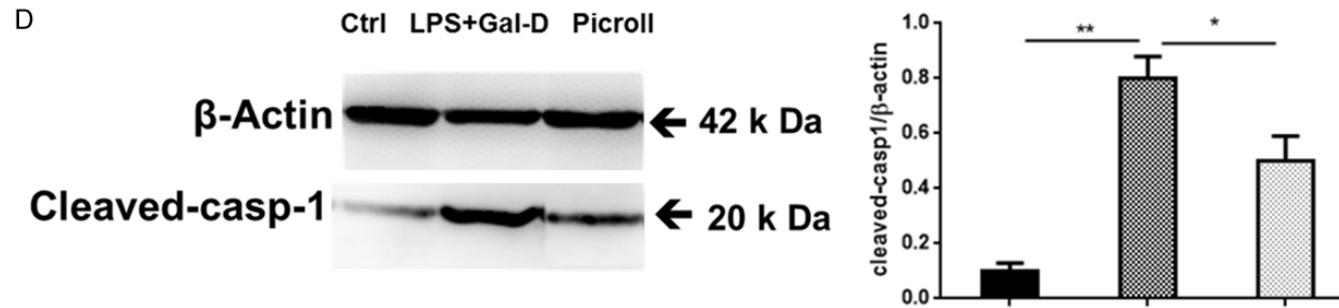


Figure 3. Picro II reduced the apoptosis and pyroptosis in acute liver injury induced by LPS+D-GalN. A. Hepatic cell apoptosis by flow cytometry. B. Hepatic cell pyroptosis by flow cytometry, left: two-dimensional graph, right: histogram; C. The expression of cleaved-caspase 3 protein by Western blot. D. The expression of cleaved-caspase-1 (p20) proteins by Western blot, left: Western blot; right: quantitative analysis representation, and expressed as the optical density ratio to β -actin. All results are representative of three independent experiments. The graphs show the mean and SEM, n=3. Significances between groups were determined by using independent-samples two-tailed Student's t-test. **P<0.01 LPS/D-GalN vs. Ctrl. *P<0.05 Picroll vs. LPS/D-GalN.

alleviated in mice treated with Picro II, as represented by decreased liver injury (**Figure 1C**).

Picro II alleviates the inflammatory response

To explore whether Picro II could further improve systemic and hepatic inflammatory responses, serum IL-6, TNF- α , IL-1 β and IL-18 levels were detected by ELISA, and the related mRNA expression of the above inflammatory cytokines in the liver tissue were detected by RT-qPCR. The results illustrated that IL-6, TNF- α , IL-1 β and IL-18 were significantly elevated in LPS+D-GalN group. All the serum cytokine levels and mRNA levels in the liver tissue were reduced after Picro II treatment (*P<0.05, **P<0.01; **Figure 2A, 2B**).

Picro II suppresses hepatocyte apoptosis and pyroptosis in vivo

We next investigated the protective effect of Picro II on the liver tissue by inhibiting apoptosis. Flow cytometry showed that the apoptosis rate and the percentage of pyroptosis of hepatocytes in LPS/D-GalN group were higher than those in the control group. However, the apoptosis rate was ameliorated after Picro II administration. Likewise, the percentage of pyroptosis was decreased (*P<0.05, **P<0.01; **Figure 3A, 3B**), which further confirmed the activation of caspase-3 in the liver of the mice. Activation of Caspase-3 is known as an indicator of apoptotic event occurrence, and caspase-1 plays an important role in hepatocyte pyroptosis. Western blot analysis indicated that LPS/D-GalN-challenge markedly increased the expression of cleaved-caspase 3 in the liver, while Picro II suppressed the induction of apoptotic protein markedly. In line with cleaved caspase-3, Picro II also inhibited the activation of caspase 1 (*P<0.05, **P<0.01; **Figure 3C, 3D**).

The mechanism of Picro II in reducing the pyroptosis of hepatocytes in vitro

Mouse hepatocytes were isolated and cultured in vitro. A cell pyroptosis mouse model of ALI induced by LPS+ATP was established. Hepatocytes were pretreated with caspase-1 (AC-YVAD-CMK) inhibitors or Picro II in 6-well plates. The supernatant was collected; cells were harvested for flow cytometry; and the total protein was extracted for Western blot. The results showed that the expression of cleaved-cas-

pase-1 was significantly increased after LPS+ATP treatment. Cleaved-caspase 1 was decreased after pretreatment with AC-YVAD-CMK. In addition, pretreatment with Picro II had the similar effect on caspase 1 splitting (*P<0.05, **P<0.01; **Figure 4A**). Consistently, flow cytometry using caspase-1-FITC, 7AAD as the pyroptotic marker showed that the pyroptosis rate in AC-YVAD-CMK group was significantly lower than that in the positive group. Likewise, Picro II treatment also reduced the degree of pyroptosis (*P<0.05, **P<0.01; **Figure 4B**). Then, we detected the concentration of IL-1 β and IL-18 in the supernatant by ELISA. Compared with LPS+ATP group, the concentration of IL-1 β and IL-18 after inhibition of caspase-1 was significantly decreased prior to addition with AC-YVAD-CMK and Picro II (*P<0.05, **P<0.01; **Figure 4C**), suggesting that Picro II had the same mechanism as AC-YVAD-CMK on hepatocyte pyroptosis.

The mechanism of Picro II in inhibiting hepatocyte apoptosis

Total protein was extracted from the liver tissue of each group, and the expression level of p-NF- κ B (p65) was determined, knowing that it is an indicator of the inflammatory pathway. The result showed that NF- κ B was obviously phosphorylated in LPS/D-GalN group, and the expression of pNF- κ B (p65) was reduced after pretreatment with Picro II (*P<0.05, **P<0.01; **Figure 5A**). Next, we investigated the effects of Picro II on the expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins, knowing that they are two representative apoptosis modulators in the liver tissue. Western blot analysis indicated that LPS/D-GalN-challenge markedly increased the expression of Bax in the liver, while pretreatment with Picro II significantly suppressed the induction of Bax. In contrast, Bcl-2 level was decreased in LPS/D-GalN group, and increased after pretreatment with Picro II (*P<0.05, **P<0.01; **Figure 5B**).

Discussion

Acute liver injury is a dramatic clinical disease due to release of abundant inflammatory factors, causing massive hepatocyte death including apoptosis, necrosis, autophagy and other forms of cell death [2, 14]. Apoptosis is the most common form of programmed cell death that plays a vital role in liver injury induced by

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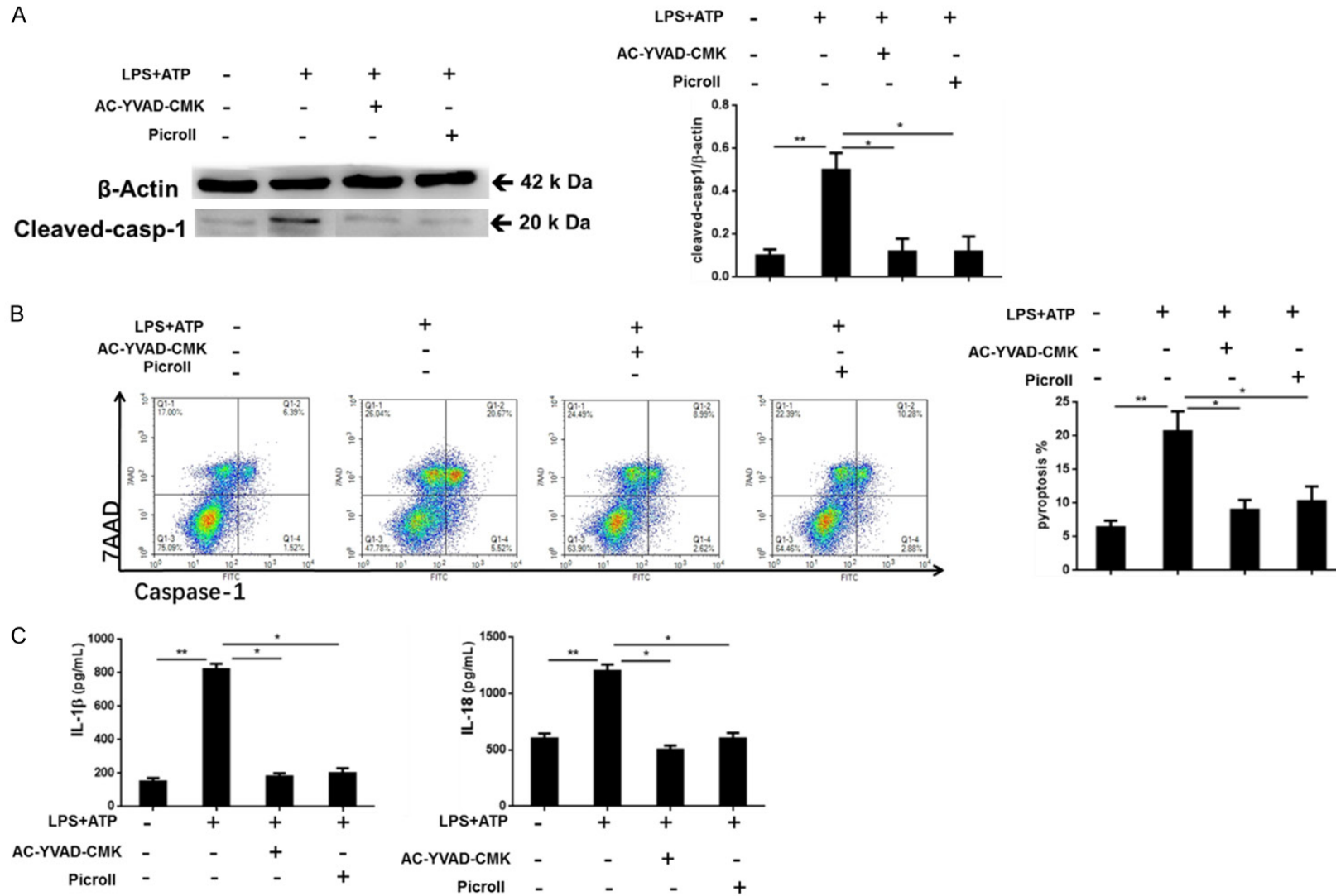


Figure 4. Picro II reduced the pyroptosis in vivo. Isolation and culture of normal mouse hepatic cells to establish the cell model of acute liver injury. A. The expression of cleaved-caspase 1 protein in each group by Western blot, left: Western blot; right: quantitative analysis representation; B. Hepatic cell pyroptosis by flow cytometry, left: two-dimensional graph, right: histogram; C. The concentration of IL-1 β and IL-18 in the supernatant of each group by ELISA. All results are representative of three independent experiments. The graphs show the mean and SEM, n=3. Significances between groups were determined by using independent-samples two-tailed Student's t-test. **P<0.01 LPS/D-GalN vs. Ctrl. *P<0.05 Picroll vs. LPS/D-GalN.

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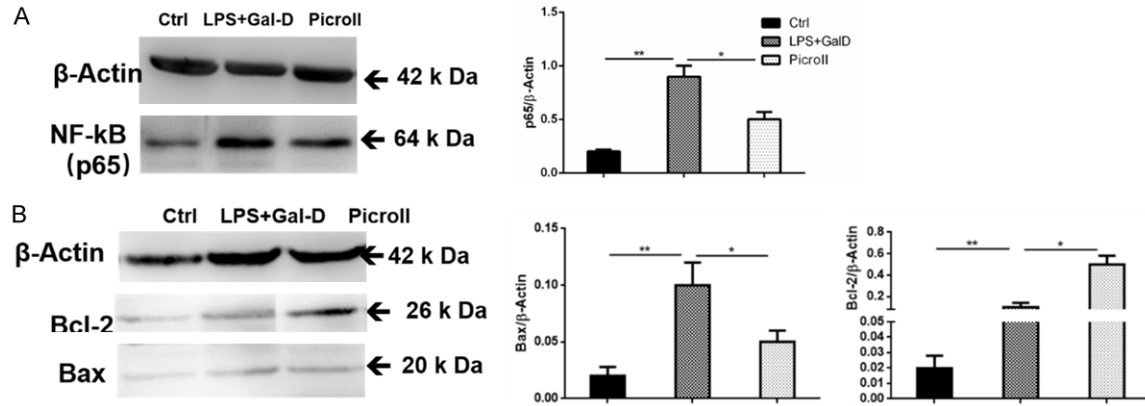


Figure 5. Picro II inhibited inflammation and hepatocyte apoptosis by inhibiting the expression of Bax but increasing the expression of Bcl-2. **A.** The expression of NF- κ B protein in each group by Western blot, left: Western blot; right: quantitative analysis representation; **B.** The expression of Bax and Bcl-2 gene in each group by Western blot, left: Western blot; right: quantitative analysis representation. All results are representative of three independent experiments. The graphs show the mean and SEM, $n=3$. Significances between groups were determined by using independent-samples two-tailed Student's t -test. $**P<0.01$ LPS/D-GalN vs. Ctrl. $*P<0.05$ PicroII vs. LPS/D-GalN.

sepsis [15, 16]. However, little is known about pyroptosis, another significant form of programmed cell death that participates in ALI induced by LPS/D-GalN [16]. LPS-induced upregulation of pro-inflammatory cytokines is known to play a critical role in liver injury. In the present study, we demonstrated that Picro II played a critical role in ALI through anti-inflammatory and anti-apoptosis but not anti-pyroptosis activities. Concurrently, the concentrations of serum ALT and AST were reduced, and the survival rate was decreased.

Several studies have illustrated the anti-inflammatory effect of Picro II, and this effect seems to be associated with the suppression of NF- κ B signaling pathway. Activation of the NF- κ B pathway may trigger the expression of cytokines, adhesion molecules, inflammatory reaction enzymes. Joshi et al [17] showed that NF- κ B activity in peripheral monocytes and neutrophils of sepsis group was obviously higher than that in the control group, and that NF- κ B was also a prognostic indicator of inflammation, indicating that NF- κ B activation mediates the pathophysiology of inflammatory liver injury. It was found in our study that treatment with Picro II markedly reduced the levels of TNF- α , IL-6, and p65 in the liver of LPS/D-Gal-exposed mice.

Hepatocyte apoptosis induced by deleterious cytokines such as TNF- α and IL-6 plays another crucial role in the development of LPS/D-Gal-induced liver injury [18]. There are two apop-

totic pathways: intrinsic and extrinsic [14, 19]. The intrinsic pathway is induced through the release of mitochondrial cytochrome C and activation of caspase-9, while the extrinsic pathway is activated by the receptors of FasL, Fas and caspase-8. Both pathways finally activate caspase-3. Several studies demonstrated that Picro II exerted obvious effects on the apoptosis of cells in sepsis. In the present study, picroside II markedly abrogated hepatocyte apoptosis in LPS/D-Gal-challenged mice as evidenced by suppressed activities of caspase-3 and decreased level of cleaved caspase-3. In addition, treatment with Picro II significantly suppressed the induction of pro-apoptotic Bax, but the level of anti-apoptotic Bcl-2 was elevated. As a result, the Bax/Bcl-2 ratio was decreased and the LPS/D-Gal-induced apoptosis was suppressed.

Pyroptosis is another significant form of programmed cell death that is involved in liver injury [20]. Wu et al showed that pyroptosis occurred in large numbers of alveolar macrophages in ALI [21], and that Caspase-1 inhibitor AC-YVAD-CMK could ease the process of pyroptosis [22]. Cao et al [23] found that AC-YVAD-CMK could reduce the acute kidney induced by cecal ligation and puncture (CLP). In vitro experiments showed that inhibitors of Caspase-1 (AC-YVAD-CMK) decreased the hepatocyte pyroptosis rate as represented by reduced levels of pyroptosis-related inflammatory cytokines IL-1 β and IL-18. So, Caspase-1 inhibitor

AC-YVAD-CMK is a positive control. Our study demonstrated that Picr II could cleave caspase-1 and promote the release of IL-1 β and IL-18.

Conclusion

In summary, Picro II protected against ALI induced by LPS/D-GalN in mice by decreasing the expression of pro-inflammatory cytokines, suppressing hepatocyte apoptosis, and easing the process of pyroptosis. However, the molecular mechanisms underlying the anti-inflammatory, anti-apoptosis and anti-pyroptosis activities of Picro II need to be further investigated.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xueyin Shi, Department of Anesthesiology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China; Department of Anesthesiology, Shanghai Xinhua Hospital, School of Medicine, Jiaotong University, 1665 Kongjiang Road, Shanghai 200092, China. Tel: +86-021-25078712; Fax: +86-021-25078712; E-mail: shixueyin1128@163.com; Dr. Zhenzhou He, Department of Anesthesiology and ICU, Renji Hospital South Campus, School of Medicine, Shanghai Jiaotong University, 2000 Jiangyue Road, Shanghai 201114, China. Tel: +86-021-34506542; Fax: +86-021-34506542; E-mail: dsxiii@126.com

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