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Original Article

Ginsenoside Rb1 Reduces D-GalN/LPS-induced Acute Liver Injury by Regulating TLR4/NF-κB Signaling and NLRP3 Inflammasome

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Abstract

Background and Aims: The effect of ginsenoside Rb1 on D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced acute liver injury (ALI) is unknown. The aim of this study was to evaluate the effect of ginsenoside Rb1 on ALI and its underlying mechanisms. ***Methods:*** Mice were pretreated with ginsenoside Rb1 by intraperitoneal injection for 3 days before D-GalN/LPS treatment, to induce ALI. The survival rate was monitored every hour for 24 h, and serum biochemical parameters, hepatic index and histopathological analysis were evaluated to measure the degree of liver injury. ELISA was used to detect oxidative stress and inflammatory cytokines in hepatic tissue and serum. Immunohistochemistry staining, RT-PCR and western blotting were performed to evaluate the expression of toll-like receptor 4 (TLR4), nuclear factor-kappa B (NF-κB), and NLR family, pyrin domain-containing 3 protein (NLRP3) in liver tissue and Kupffer cells (KCs). ***Results:*** Ginsenoside Rb1 improved survival with D-GalN/LPS-induced ALI by up to 80%, significantly ameliorated the increased alanine and aspartate transaminase, restored the hepatic pathological changes and reduced the levels of oxidative stress and inflammatory cytokines altered by D-GalN/LPS. Compared to the control group, the KCs were increased in the D-GalN/LPS groups but did not increase significantly with Rb1 pretreatment. D-GalN/LPS could upregulate while Rb1 pretreatment could downregulate the expression of interleukin (IL)-1β, IL-18, NLRP3, apoptosis associated speck-like protein containing CARD (ASC) and caspase-1 in isolated KCs. Furthermore, ginsenoside Rb1 inhibited activation of the TLR4/NF-κB signaling pathway and NLRP3 inflammasome induced by D-GalN/LPS administration. ***Conclusions:*** Ginsenoside Rb1 protects mice against D-GalN/LPS-induced ALI by attenuating oxidative stress and the inflammatory response through the TLR4/NF-κB signaling pathway and NLRP3 inflammasome activation.

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Introduction

The liver plays a variety of roles in the metabolic, hematopoietic and immune systems in the body. However, it is vulnerable to injury from various internal and external pathogenic factors, such as trauma, infection, drugs and autoimmune abnormalities. Acute liver injury (ALI) is one of the most common diseases that threatens the lives of patients. Damage and necrosis of liver cells may lead to abnormal liver function, which may subsequently develop into acute liver failure. The liver is also a frequent target of dysregulated inflammation in infectious diseases, promoting the development and occurrence of inflammation.[1] Previous studies reported that the mortality of sepsis combined with ALI is higher than that of other diseases.[2,3] Lipopolysaccharide (LPS) and Kupffer cells (KCs) may play an important part in the establishment of sepsis-related ALI. D-galactosamine (D-GalN)/ LPS-induced hepatocellular toxicity is a well-established animal model of ALI.[4] D-GalN/LPS activates KCs and stimulates the secretion of a large number of inflammatory cytokines, leading to an extensive inflammatory response and ALI.

The NLR family, pyrin domain-containing 3 protein (NLRP3) inflammasome is a recently identified pattern-recognition receptor that is mainly expressed in hepatocytes and KCs.[5,6] The NLRP3 inflammasome is activated by a variety of pathogens, such as bacteria, viruses and fungi, and promotes the maturation and secretion of inflammatory cytokines, including IL-1β and IL-18.[7] Wang *et al*.[8] showed that NLRP3 was activated in KCs in the ALI model. Fur-

Keywords: Ginsenosides; Chemical and drug induced liver injury; Toll-like receptor 4; NLR family, pyrin domain-containing 3 protein.

Abbreviations: ALI, acute liver injury; ALT, alanine transaminase; ASC, apoptosis associated speck-like protein containing CARD; AST, aspartate transaminase; D-GalN, d-galactosamine; GSH-px, glutathione-peroxidase; IHC, immunohistochemistry; IL, interleukin; KCs, Kupffer cells; LPS, lipopolysaccharide; MDA, malondialdehyde; MPO, myeloperoxidase; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-kappa B; NLRP3, NLR family, pyrin domain-containing 3 protein; NPC, nonparenchymal cell; ROS, reactive oxygen species; SOD, superoxide dismutase; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-alpha.

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thermore, Liu *et al*.[9] found that a reduction in the NLRP3 inflammasome activation in KCs suppressed the systemic inflammatory response caused by infection. Therefore, inhibition of NLRP3 inflammasome activation may be an effective strategy to alleviate the inflammatory response in ALI.

However, few effective drugs are available for sepsis-related ALI. *Panax ginseng* is the most extensively used traditional herbal medicine, with a usage history of over 2,000 years; it exhibits protective effects on oxidative stress, inflammation, hypoxia injury, central nervous disease, and aging.[10,11] Ginsenoside Rb1 is one of the major bioactive components of ginseng, and previous studies have shown that ginsenoside Rb1 can reverse the pathological and physiological changes caused by oxidative damage.[12,13] Liu *et al*.[14] reported that ginsenoside Rb1 may protect retinal ganglion cells against oxidative stress-induced apoptosis through a caspase-9-dependent mitochondrial pathway. Guo *et al*.[15] found that ginsenoside Rb1 prevents ischemia-reperfusion injury in the liver through the reactive oxygen species (ROS)-nitric oxide-HIF pathway. Wu *et al*.[16] reported that ginsenoside Rb1 protects the liver and lung during septic shock by downregulating the expression of toll-like receptor 4 (TLR4) mRNA. However, the effect and the underlying mechanism of ginsenoside Rb1 on D-GalN/LPS-induced ALI have not been examined. In this study, we investigated the ability of ginsenoside Rb1 to protect the liver against D-GalN/LPS-induced ALI and explored its relationship with the TLR4/ nuclear factor-kappa B (NF-κB) signaling pathway and NLRP3 inflammasome.

Methods

Animals and ethical approval

Mice (male sex, C57BL/6 strain, 8–10 weeks of age, 25±5 g body weight) were purchased from the laboratory animal resources of the Chinese Academy of Sciences and raised in the animal laboratory of Fudan University. Mice had an adaptation period of 1 week (temperature of 18–26°C, humidity of 45–55%) under standard lighting conditions (12/12 h light/dark). All animals received food and water without limitation. The study protocol was approved by the Ethics Committee of Zhongshan Hospital of Fudan University. All of our experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Experimental design

All animals were randomly assigned into the following four groups (n=10 each): Control group; D-GalN/LPS group; D-GalN/LPS+30 mg/kg Rb1 group; and D-GalN/LPS+60 mg/kg Rb1 group. For the induction of ALI, mice received D-GalN (400 mg/kg) and LPS (50 μg/kg) by intraperitoneal injection. The dose of the mixture was in accordance with previous studies.[17,18] First, mice received 30 or 60 mg/kg Rb1 by intraperitoneal injection for 3 days, while the control group and D-GalN/LPS group received the same volume of 0.9% saline for 3 days. On the third day, 1 h after the final Rb1 or 0.9% saline injection, the D-GalN/LPS group, the D-GalN/LPS+30 mg/kg Rb1 group and the D-GalN/LPS+60 mg/kg Rb1 group received D-GalN/LPS injection for the induction of ALI, while the control group was given the same volume of 0.9% saline. The survival rate was obtained by recording the number of dead mice every hour for 24 h. After D-GalN/LPS administration at 6 h, mice were anesthetized with ketamine IP (150 mg/kg) and euthanized, and liver and blood samples were collected for experimental analysis.

Reagents

Ginsenoside Rb1 (purity approximately 98%) was obtained from Meilun Pharmaceuticals Inc. (Dalian, China). D-GalN and LPS were obtained from Sigma (St. Louis, MO, USA). Enzyme-linked immunosorbent assay kits were purchased from BioLegend (San Diego, CA, USA). Antibodies against interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), TLR4, myeloid differentiation factor 88 (MyD88), IkBα, p-IkBα, NF-kB p65, NLRP3, apoptosis associated speck-like protein containing CARD (ASC), caspase-1, and GAPDH were purchased from Abcam (Cambridge, MA, USA).

Serum biochemical parameters

After sampling, the blood specimens were placed in a refrigerator at 4°C for 1 h and then centrifuged at 3,500 rpm for 15 m. The levels of alanine transaminase (ALT) and aspartate transaminase (AST) in serum were measured using a specific kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Detection of hepatic index

The hepatic indices of different groups were calculated using the following equation: hepatic index=liver wet weight/mouse body weight×100%.

Histopathological examination

Excised liver tissue was fixed with 10% formaldehyde solution. The ethanol-dehydrated and paraffin-embedded sections were cut into 5-μm thick slices and subjected to hematoxylin-eosin staining. The pathological changes in liver tissue were observed with a light microscope (Olympus, Tokyo, Japan), and micrographs were randomly selected and analyzed according to Suzuki's criteria as reported previously.[19] The severity of liver injury was scored on a scale from 0 to 4.

Detection of oxidative stress and inflammatory cytokines

Liver tissue from each group was collected and transferred into liquid nitrogen. The liver tissue was homogenized, the lysate was centrifuged at 3,000 rpm at 4°C for 15 m, and the supernatant was collected. The supernatant of liver tissue was used for measurements of ROS, myeloperoxidase (MPO), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione-peroxidase (GSH-px) levels according to the requirements of the instructions provided in reagent kits. The levels of IL-6, TNF-α, IL-1β and IL-18 in serum and liver tissue were analyzed using enzyme-linked immunosorbent assay kits according to the manufacturer's instructions.

Isolation of KCs

KCs were isolated using the method described by Yue *et al*.[20] Briefly, the portal vein was exposed, a surgical thread was inserted in the portal vein and the liver was perfused with HBSS solution prewarmed at 37°C. The perfusion solution was then switched to type IV collagenase for 20 m, until the liver had slightly swelled. The liver was removed and washed

with phosphate-buffered saline for three times, and then the liver was finely chopped and digested with collagenase solution for 20 m by vigorous shaking at 37°C. The resultant cell suspension was teased through a cell strainer (100 μm) and the hepatocytes in the supernatant was removed by three 2 m centrifugations at 50×*g*. Then the nonparenchymal cells (NPCs) were layered onto a 50%/25% two-step Percoll gradient (Sigma) and centrifuged at 2,000×*g* for 10 m at 4°C. KCs in the middle layer were collected.

Immunohistochemistry (IHC) staining

Liver tissue sections 5-μm thick were rehydrated and incubated with 3% hydrogen peroxide to block endogenous peroxidase activity. Then, the sections were incubated overnight at 4°C with primary antibodies against F4/80 (1:1,000; Proteintech, Rosemont, IL, USA), IL-6 (1:200; Abcam, Cambridge, UK), TNF-α (1:200; Abcam), TLR4 (1:200; Abcam), and NLRP3 (1:200; Abcam). The sections were washed with phosphate-buffered solution three times and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 30 m. Finally, the sections were incubated with diaminobenzidine tetrahydrochloride and then counterstained with Mayer's hematoxylin. The slides were subjected to microscopic (Olympus) analysis.

Real-time RT-PCR

Liver tissue (20 mg) and KCs were collected, and total RNA was isolated using TRIzol and reverse transcribed into cDNA according to the manufacturer's instructions for the TaKaRa kit (TaKaRa Biotechnology, Shiga, Japan). RT-PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Thermo Scientific, Waltham, MA, USA). The PCR protocol conditions were as follows: 95°C for 10 m, followed by 40 cycles at various temperatures/times (95°C for 20 s, 60° for 20 s, and 72°C for 20 s). A melting curve analysis was conducted at the end of the amplification period. The fold-changes of the expression of the candidate genes relative to β-actin were analyzed using the normalized expression (ΔCt) method. The primer sequences used to amplify a specific mouse gene fragment were as follows: mouse TLR4 sense primer: 5′- TTGAAGACAAGGCATGGCATGG -3′, mouse TLR4 antisense primer: 5′- TCTCCCAAGATCAACCGATG -3′; mouse MyD88 sense primer: 5′- GTTGTGTGTGTCCGACCGT -3′, mouse MyD88 antisense primer: 5′- GTCAGAAACAACCACCACCATGC -3′; mouse NLRP3 sense primer: 5′- GTGGTGACCCTCTGTGAGGT -3′, mouse NLRP3 antisense primer: 5′- TCTTCCTGGAGCGCTTCTAA -3′; mouse ASC sense primer: 5′- AGACATGGGCTTACAGGA -3′, mouse ASC antisense primer: 5′- CTCCCTCATCTTGTCTTGG -3′; mouse caspase-1 sense primer: 5′- TATCCAGGAGGGAATATGTG -3′, mouse caspase-1 antisense primer: 5′- ACAACACCACTCCTTGTTTC -3′; mouse IL-1β sense primer: 5′-GCAACTGTTCCTGAACTCAACT -3′, mouse IL-1β antisense primer: 5′-ATCTTTTGGGGTCCGTCAACT -3′; mouse IL-18 sense primer: 5′- GACCTGGAATCAGACAACTTTGG -3′, mouse IL-18 antisense primer: 5′- GCCTCGGGTATTCTGTTATGGA -3′; mouse β-actin sense primer: 5′- CCGTGAAAAGATGACCCAGA -3′, and mouse β-actin antisense primer: 5′- TACGACCAGAGGCATACAG -3′.

Western blot analysis

Protein was extracted from liver tissue and KCs using RIPA lysis buffer (Beyotime, Shanghai, China). Cytoplasmic and nuclear proteins were extracted using a nuclear and cytoplasmic protein extraction kit (Sangon Biotech, Shanghai, China), according to the manufacturer's instructions. The protein concentrations were subsequently determined using a BCA kit (Beyotime). Equal amounts of proteins were separated via sodium dodecyl sulfonate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Membranes were blocked in 5% nonfat dry milk for 2 h at room temperature, and then incubated with primary antibodies overnight at 4°C. The primary antibodies used in our study included anti-TLR4 (1:1,000; Abcam), anti-MyD88 (1:1,000; Abcam), anti-IkBα (1:1,000; Abcam), anti-p-IkBα (1:1,000; Abcam), anti-NF-κB p65 (1:1,000; Abcam), anti-NLRP3 (1:1,000; Abcam), anti-ASC (1:1,000; Abcam), anti-caspase-1 (1:1,000; Abcam), and anti-GAPDH (1:1,000; Abcam). Membranes were then incubated with secondary antibodies that were conjugated with horseradish peroxidase for 1 h at room temperature. The immunoreactive bands were visualized using an enhanced chemiluminescent detection kit and exposure to film. The densitometric values of the bands were analyzed using the ImageJ program version 1.47e (National Institutes of Health, Bethesda, MD, USA).

Statistical analyses

The results are presented as the mean±standard deviation. The Kaplan-Meier method was utilized to analyze the survival rate of mice. Differences between experimental groups were analyzed by one-way analysis of variance followed by post hoc Tukey's test or Student's *t*-test (SPSS version 17.0; SPSS Inc., Chicago, IL, USA). A p-value of <0.05 was considered to be statistically significant.

Results

Ginsenoside Rb1 protected mice against ALI-induced by D-GalN/LPS

At 4 h after intraperitoneal injection of D-GalN/LPS, we observed death of mice in the D-GalN/LPS group. After 24 h of observation, the survival rate in the D-GalN/LPS group was 20%, while the survival rate of the control group was 100%. Notably, pretreatment of ginsenoside Rb1 improved the survival rate of mice to 80% (Fig. 1A), and the 24-h survival rate in D-GalN/LPS+ginsenoside Rb1 group was significantly higher than that in the D-GalN/LPS group ($p<0.05$). The levels of ALT and AST in serum are important indexes of liver functional abnormalities. Both the liver damage markers ALT and AST were markedly increased in the D-GalN/LPS group compared with the control group. In contrast, the levels were significantly decreased in the D-GalN/LPS+ginsenoside Rb1 group compared with the D-GalN/LPS group (Fig. 1B, C). Pretreatment with ginsenoside Rb1 significantly reduced the D-GalN/LPS-induced increase of hepatic index compared with the control group (Fig. 1D). The pathological changes of ALI include inflammatory cell infiltration, hepatocyte cytoplasm vacuolization and parenchymal necrosis.[21] While hepatocyte necrosis and vacuolation were observed in the D-GalN/LPS group, pretreatment of ginsenoside Rb1 improved the liver architecture, as determined by Suzuki scoring (Fig. 1E, F). Taken together, these results indicate that pretreatment with ginsenoside Rb1 exerts a protective effect against D-GalN/LPS-induced ALI.

Ginsenoside Rb1 reduced oxidative stress damage in ALI

To evaluate the oxidative stress burden in the liver tissue

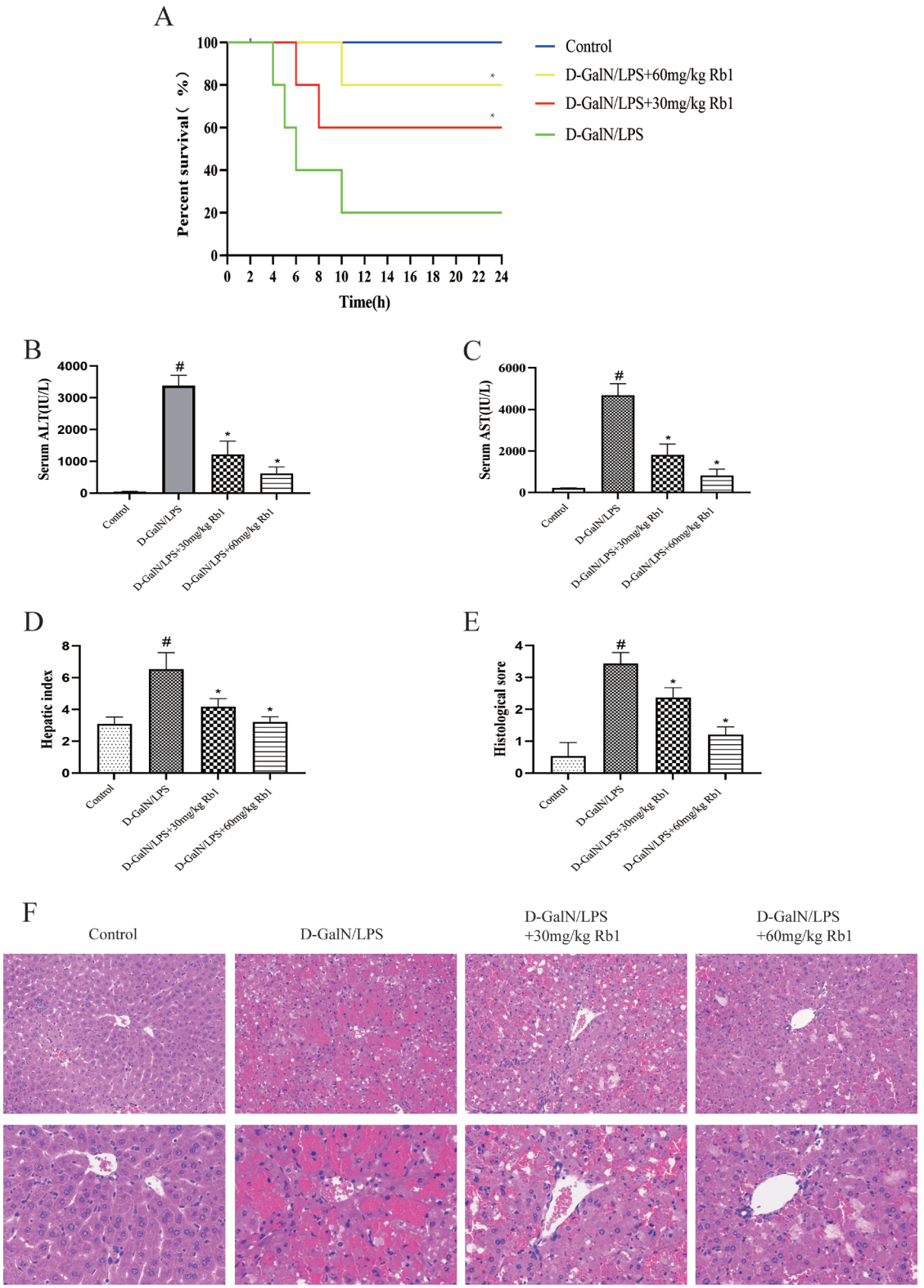

Fig. 1. Rb1 protected mice against ALI induced by D-GalN/LPS. (A) The survival rate of mice was continuously monitored every hour for 24 h. (B-C) The levels of liver damage markers ALT and AST in serum. (D) Hepatic index calculated in different groups. (E) Histological score used to evaluate the severity of liver injury according to the Suzuki's criteria. (F) Hematoxylin-eosin staining (left, 200× original magnification; right, 400× original magnification). Data are shown as mean±standard error of the mean (n=10). $^{\#}p$<0.05 vs. control group, $^{*}p$<0.05 vs. D-GalN/LPS group. ALI, acute liver injury; ALT, alanine transaminase; AST, aspartate transaminase; D-GalN, d-galactosamine; LPS, lipopolysaccharide.

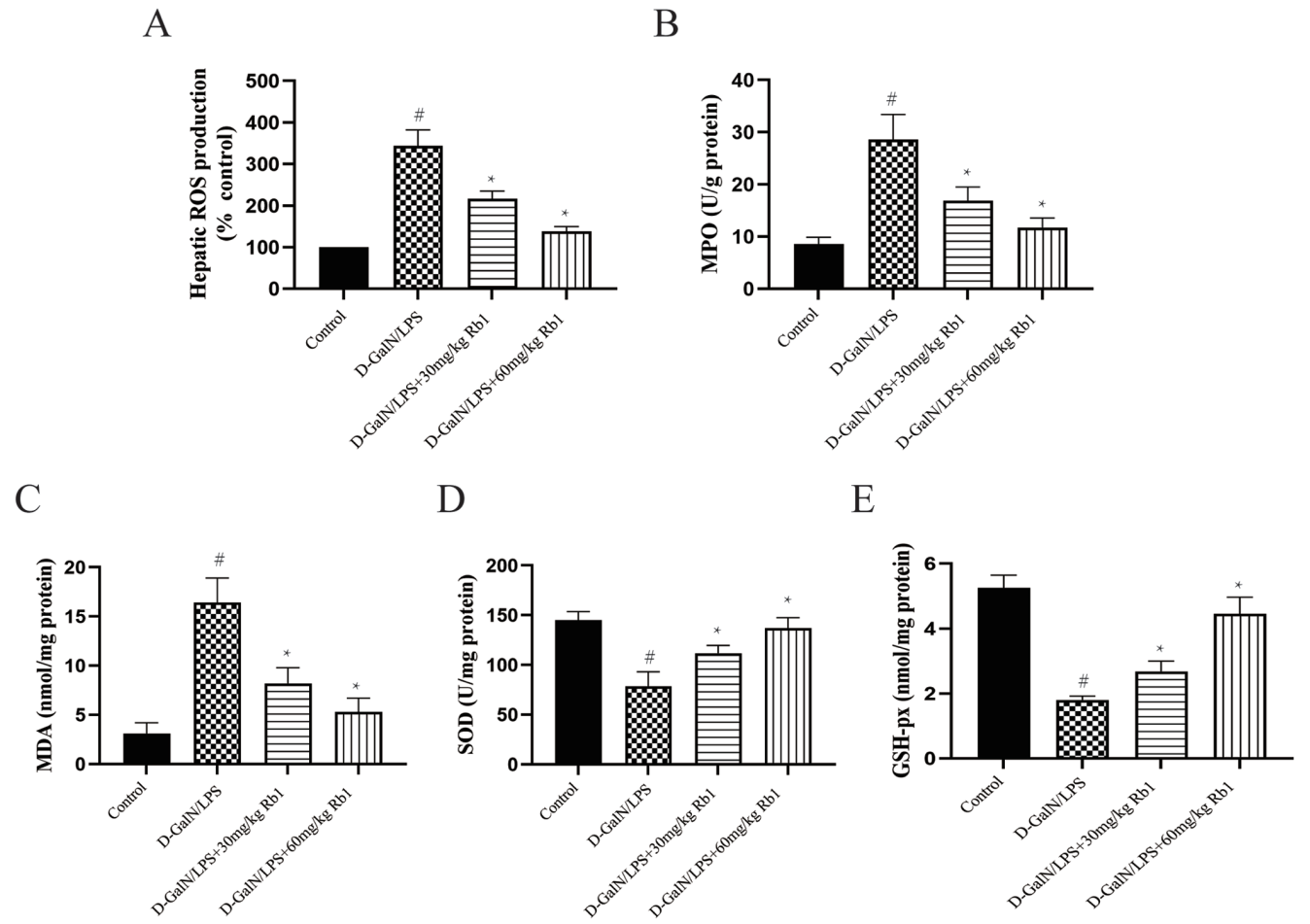

Fig. 2. Effect of Rb1 on oxidative stress in liver tissue of ALI mice. The pro-oxidation markers such as ROS, MPO and MDA, and antioxidant markers such as SOD and GSH-px were determined by commercial reagent kits in fresh liver tissues. Tests were performed at least three times. Data are shown as mean±standard error of the mean and are expressed as % of normal, U/g of protein, nmol/mg of protein, U/mg of protein and nmol/mg of protein, respectively. $^{\#}p<0.05$ vs. control group, $^{*}p<0.05$ vs. D-GalN/LPS group. ALI, acute liver injury; D-GalN, d-galactosamine; GSH-px, glutathione-peroxidase; LPS, lipopolysaccharide; MDA, malondialdehyde; MPO, myeloperoxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

of ALI, we examined pro-oxidation markers, such as ROS, MPO and MDA, antioxidant markers, such as SOD and GSH-px, in fresh liver tissues from the mice in each treatment group. As shown in Figure 2, the levels of hepatic ROS, MPO and MDA were significantly increased in the D-GalN/LPS group, while the activities of SOD and GSH-Px were significantly decreased compared with the control group. In liver tissues from mice pretreated with ginsenoside Rb1, we observed a significantly reduced production of oxidative stress indicators and increased levels of endogenous antioxidant indicators. These results indicate that ginsenoside Rb1 significantly relieves the oxidative stress burden in the liver tissue of ALI mice.

Ginsenoside Rb1 ameliorated the inflammatory responses of ALI

Overproduction of inflammatory cytokines may be involved in regulation of the acute-phase response to liver injury and infection.[22] Therefore, we evaluated the effect of ginsenoside Rb1 on inflammatory responses; the levels of inflammatory cytokines, such as IL-6, TNF-α, IL-1β and IL-18, in serum and liver tissue were analyzed. As shown in Figure 3A–H, the levels of inflammatory cytokines were significantly increased in the D-GalN/LPS group compared with the control group. In contrast, the group given pretreatment with ginsenoside Rb1 showed suppressed production of inflammatory cytokines. Corresponding with the serum levels of inflammatory cytokines, IHC staining showed lower expression of IL-6 and TNF-α in the ginsenoside Rb1 pretreatment group compared with the D-GalN/LPS group (Fig. 3I). These results show that ginsenoside Rb1 protects mice against inflammatory responses induced by D-GalN/LPS.

Ginsenoside Rb1 alleviated D-GalN/LPS-induced ALI by suppressing inflammatory cytokine expression and NLRP3 inflammasome activation in KCs

The F4/80+ KCs were evaluated by IHC. In the D-GalN/LPS group, along with hepatocyte necrosis and vacuolation, F4/80+ KC infiltration were increased. With the Rb1 pretreatment, F4/80+ KCs did not increase significantly after the D-GalN/LPS intervention (Fig. 4A).

Because the administration of D-GalN/LPS may activate KCs to secrete inflammatory cytokines, the inflammatory

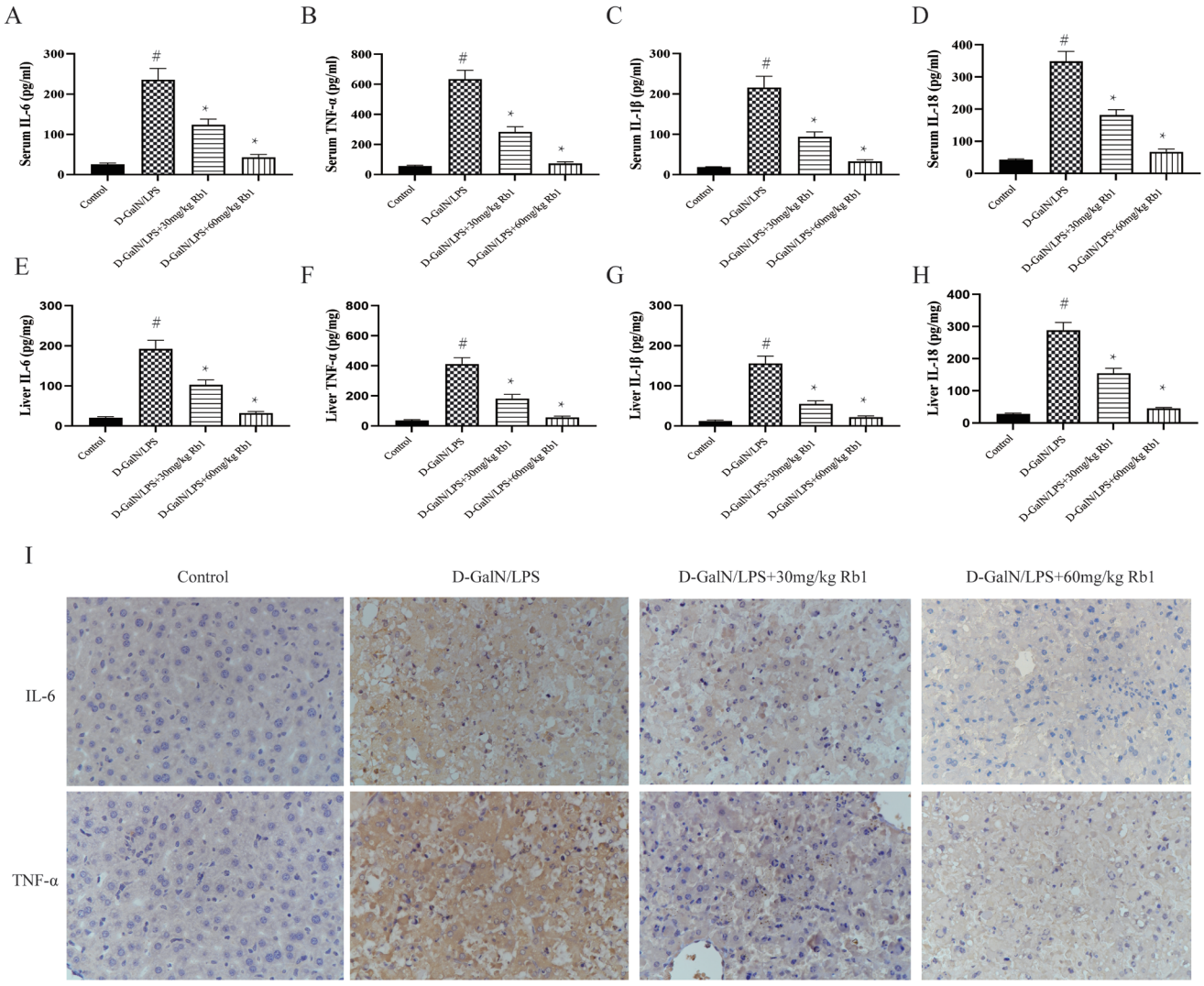

Fig. 3. Effect of Rb1 on inflammatory cytokine expression in serum and liver tissue of ALI mice. (A-H) Serum and hepatic levels of IL-6, TNF-α, IL-1β and IL-18 were evaluated by commercial reagent kits. (I) IHC was used to evaluate the expression of IL-6 and TNF-α in liver tissue (400× original magnification). Tests were performed at least three times. Data are shown as mean±standard error of the mean and are expressed as pg/mL and pg/mg, respectively. $^{\#}p<0.05$ vs. control group, $^{*}p<0.05$ vs. D-GalN/LPS group. ALI, acute liver injury; ALT, alanine transaminase; D-GalN, d-galactosamine; IHC, immunohistochemistry; IL, interleukin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-alpha.

cytokine expression and NLRP3 inflammasome activation in KCs were evaluated. As shown in Figure 4B and C, the mRNA expressions of IL-1β and IL-18 in KCs were upregulated in the D-GalN/LPS group compared with the control group, and levels were downregulated by pretreatment with ginsenoside Rb1 ($p<0.05$). Western blot analysis showed that ginsenoside Rb1 at 30 mg/kg and 60 mg/kg significantly inhibited the D-GalN/LPS-induced upregulation of NLRP3, ASC and caspase-1 expression (Fig. 4D, E). These data demonstrated that KCs were activated in D-GalN/LPS-induced ALI and that ginsenoside Rb1 significantly inhibited the expression of inflammatory cytokines and activation of NLRP3 inflammasome in KCs during ALI induced by D-GalN/LPS.

Ginsenoside Rb1 inhibited the activation of the TLR4/NF-κB signaling pathway in ALI mice

As a component of the outer membrane of Gram-negative cells, LPS activates the TLR4/MD-2 complex, which triggers MyD88-dependent production of inflammatory cytokines,[23] such as IL-6 and TNF-α.[24] Therefore, we next evaluated activation of the TLR4/NF-κB signaling pathway in liver tissue. IHC staining revealed that the TLR4-positive area dramatically decreased in the ginsenoside Rb1 pretreatment group compared with the D-GalN/LPS group (Fig. 5A). Both the mRNA and protein levels of TLR4 and MyD88 were upregulated in the D-GalN/LPS group compared with the control group, and the administration of ginsenoside Rb1 reversed the effects of D-GalN/LPS induction (Fig. 5B). Phosphorylation and degradation of IκBα plays an important role in the translocation of NF-κB p65 from the cytoplasm to the nucleus;[25,26] both are crucial steps in the TLR4/NF-κB signaling pathway. Western blot analysis of liver tissue in D-GalN/LPS mice showed upregulated p-IκBα levels and downregulated total IκBα expression, while the liver tissues from mice pretreated with administration of ginsenoside Rb1 showed reduced phosphorylation of IκBα. Moreover, translocation of

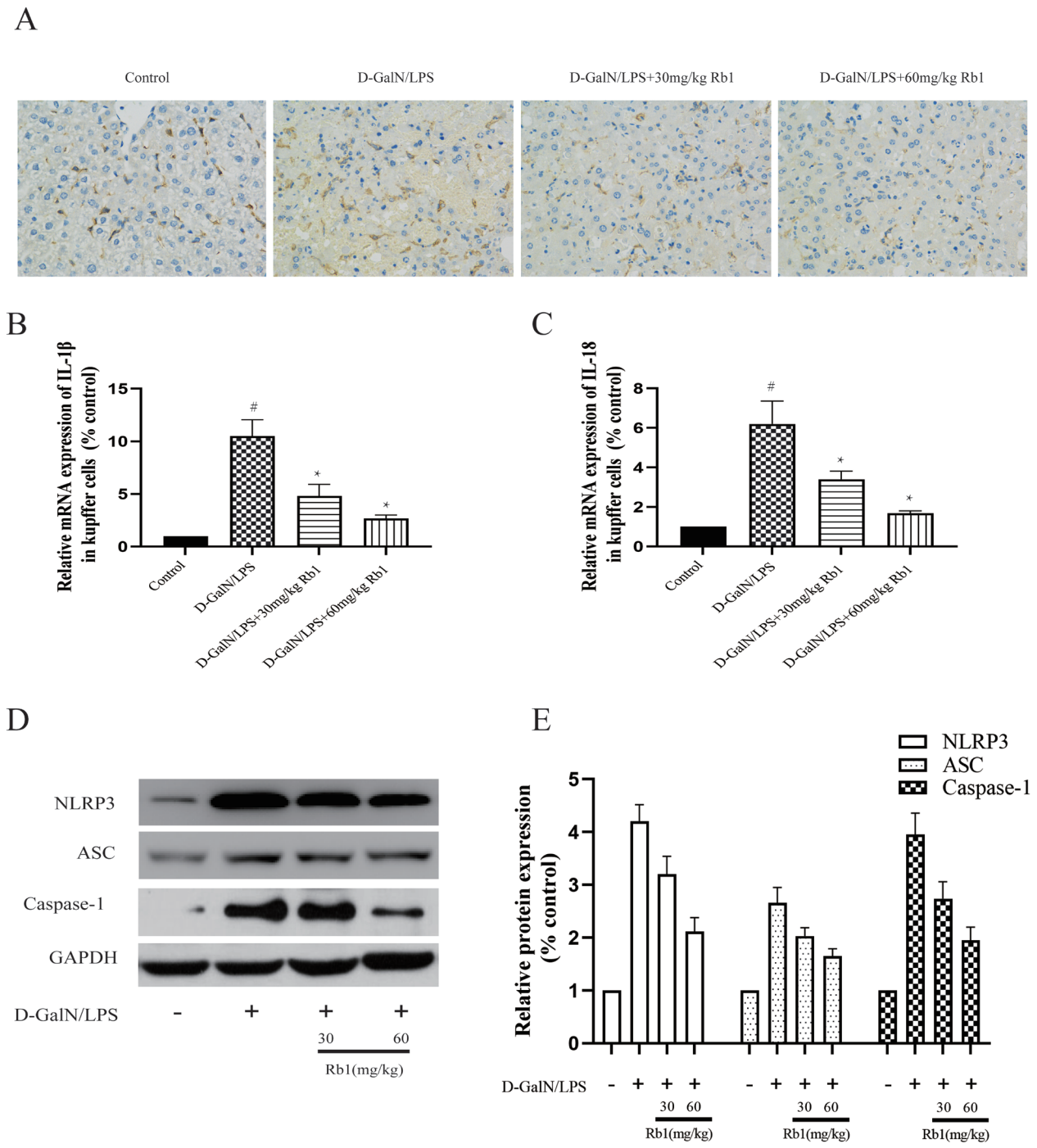

Fig. 4. KCs accumulation, and inflammatory cytokine expression and NLRP3 inflammasome activation in KCs among different groups. (A) F4/80+ KCs were evaluated by IHC staining of hepatic tissues. (B) mRNA expressions of IL-1β. (C) mRNA expressions of IL-18. (D) Western blot analysis of NLRP3, ASC and caspase-1 in KCs. (E) Relative protein content was quantified by densitometry. Tests were performed at least three times. Data are shown as mean±standard error of the mean. $^{\#}p<0.05$ vs. control group, $^{*}p<0.05$ vs. D-GalN/LPS group. ASC, apoptosis associated speck-like protein containing CARD; D-GalN, d-galactosamine; IHC, immunohistochemistry; IL, interleukin; KCs, Kupffer cells; LPS, lipopolysaccharide; NLRP3, NLR family, pyrin domain-containing 3 protein.

NF-κB p65 from the cytoplasm to the nucleus was observed in the D-GalN/LPS group, while ginsenoside Rb1 blocked the translocation of p65 into the nucleus (Fig. 5C, D). Taken together, these results indicate that pretreatment with ginsenoside Rb1 inhibits activation of the TLR4/NF-κB signaling pathway in ALI mice.

Ginsenoside Rb1 inhibited activation of the NLRP3 inflammasome in the livers of ALI mice

Previous studies showed that ROS may serve as a triggering factor to activate NLRP3 inflammasomes, resulting in

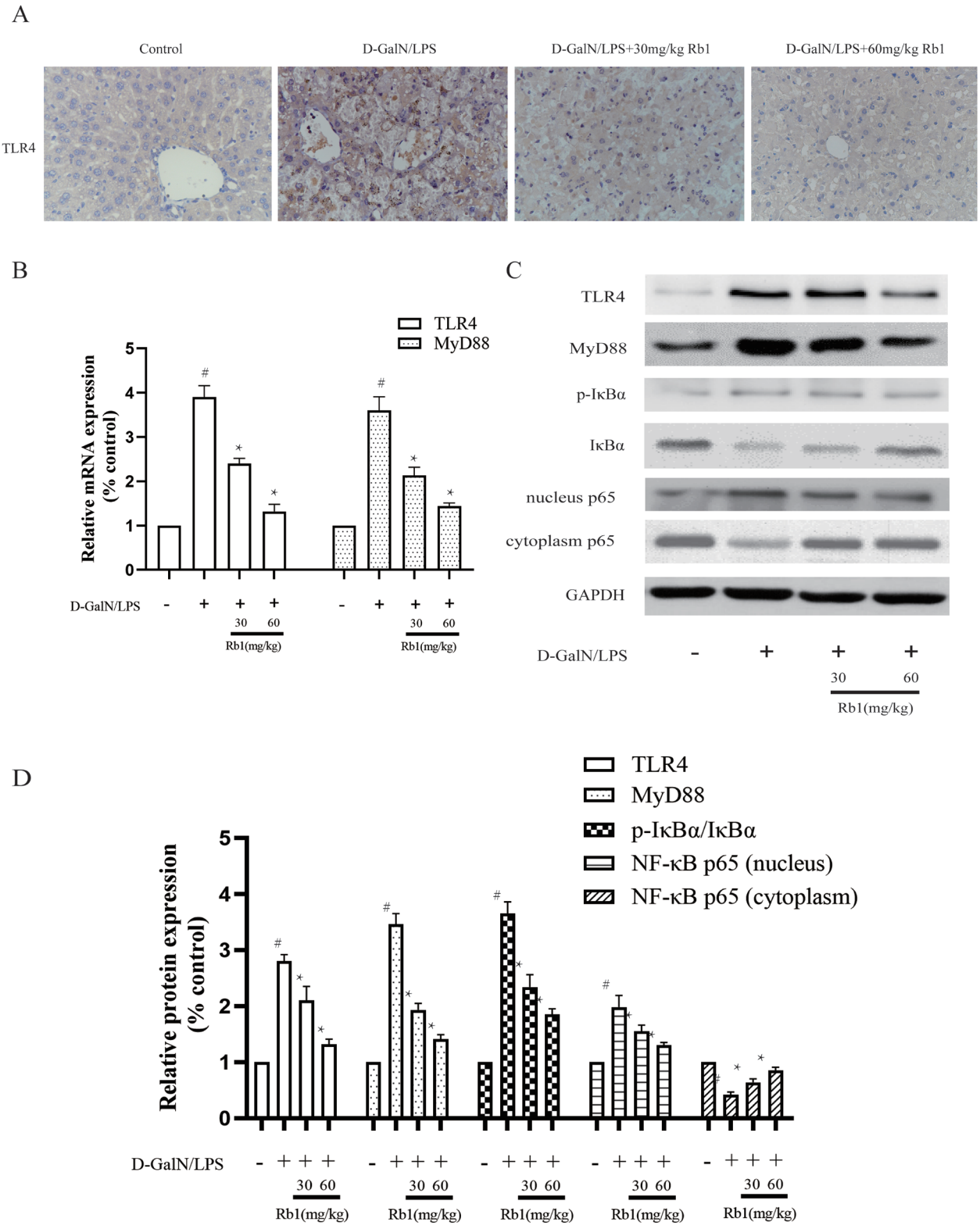

Fig. 5. Effect of Rb1 on TLR4/NF-κB signal pathway in D-GalN/LPS-induced ALI mice. (A) IHC analysis of TLR4 (400× original magnification). (B) mRNA expressions of TLR4 and MyD88. (C) Western blot analysis of TLR4, MyD88, p-IκBα, IκBα, nucleus p65 and cytoplasm p65 expression in liver tissue. (D) Relative protein content was quantified by densitometry. Tests were performed at least three times. Data are shown as mean±standard error of the mean. $^{\#}p<0.05$ vs. control group, $^{*}p<0.05$ vs. D-GalN/LPS group. ALI, acute liver injury; D-GalN, d-galactosamine; IHC, immunohistochemistry; LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; TLR4, toll-like receptor 4.

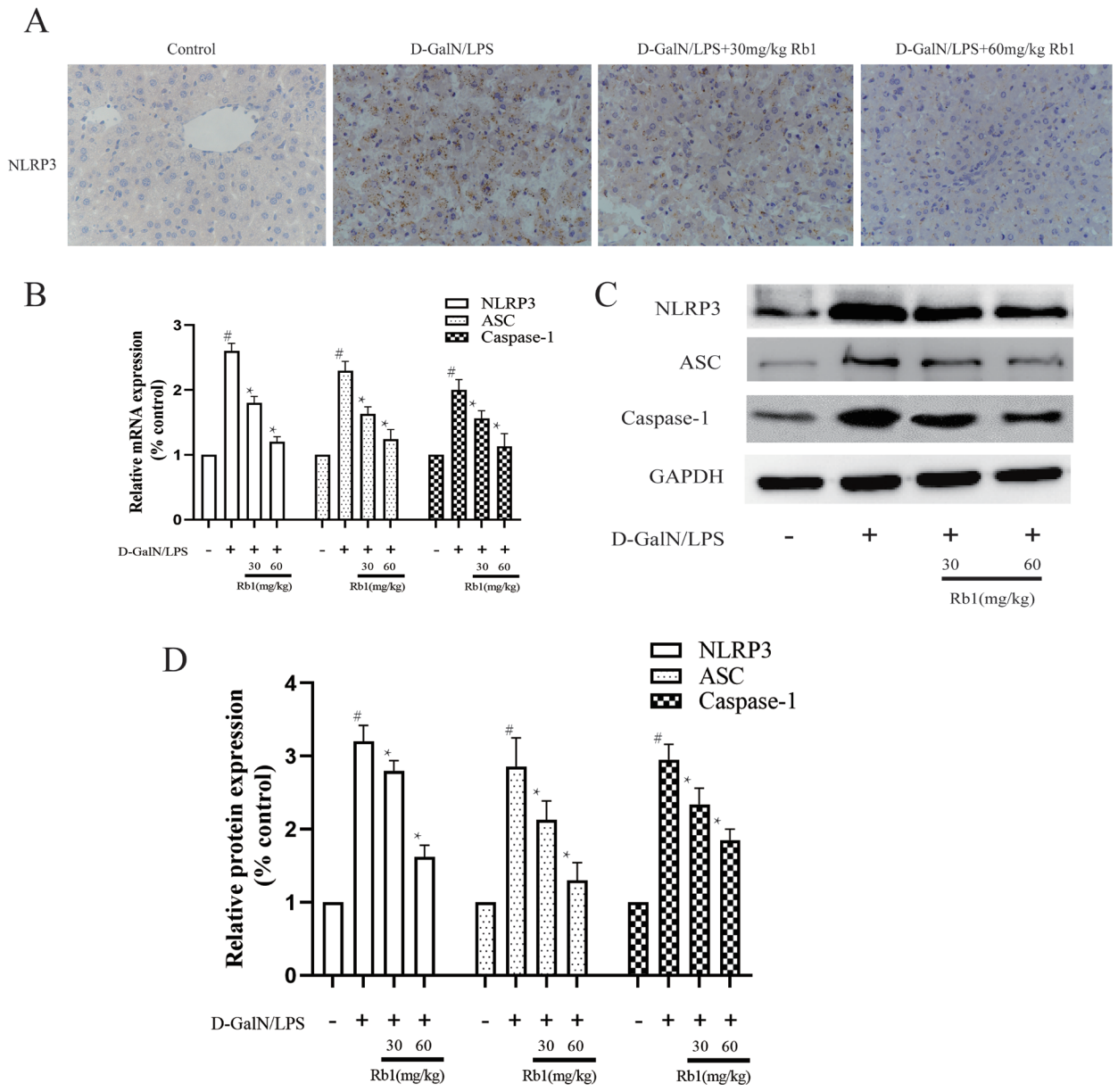

Fig. 6. Effect of Rb1 on the activation of NLRP3 inflammasome in D-GalN/LPS-induced ALI mice. (A) IHC analysis of NLRP3 (400× original magnificatios). (B) The mRNA expressions of NLRP3, ASC and caspase-1. (C) Western blot analysis of NLRP3, ASC and caspase-1 in liver tissue. (D) Relative protein content was quantified by densitometry. Tests were performed at least three times. Data are shown as mean±standard error of the mean. $^{\#}p<0.05$ vs. control group, $^{*}p<0.05$ vs. D-GalN/LPS group. ALI, acute liver injury; ASC, apoptosis associated speck-like protein containing CARD; D-GalN, d-galactosamine; IHC, immunohistochemistry; LPS, lipopolysaccharide; NLRP3, NLR family, pyrin domain-containing 3 protein.

hepatocellular toxicity.[27] Therefore, we measured NLRP3 expression in liver tissues from ALI mice via IHC staining. The NLRP3-positive area was increased in the D-GalN/LPS group compared with the control group; however, pretreatment with ginsenoside Rb1 attenuated the increase in the NLRP3-positive area (Fig. 6A). The mRNA levels of NLRP3, ASC and caspase-1 were significantly upregulated in the D-GalN/LPS group compared with the control group, while pretreatment with ginsenoside Rb1 resulted in significantly downregulated mRNA levels (Fig. 6B). Consistent with the mRNA expression results, western blot analysis showed that ginsenoside Rb1 significantly suppressed the upregulation of NLRP3, ASC and caspase-1 induced by D-GalN/LPS (Fig, 6C, D). These results indicate that ginsenoside Rb1 inhibits activation of the NLRP3 inflammasome to alleviate liver injury in mice.

Discussion

The pathogenesis of ALI includes hepatic blood flow variation, microcirculation disorders, impaired endothelial function, mitochondrial dysfunction, oxidative stress and excessive inflammatory response. Several studies have revealed that oxidative stress, excessive inflammatory responses and hepatocellular toxicity play a key role in the pathogenesis of D-GalN/LPS induced ALI.[28,29] However, a limited number

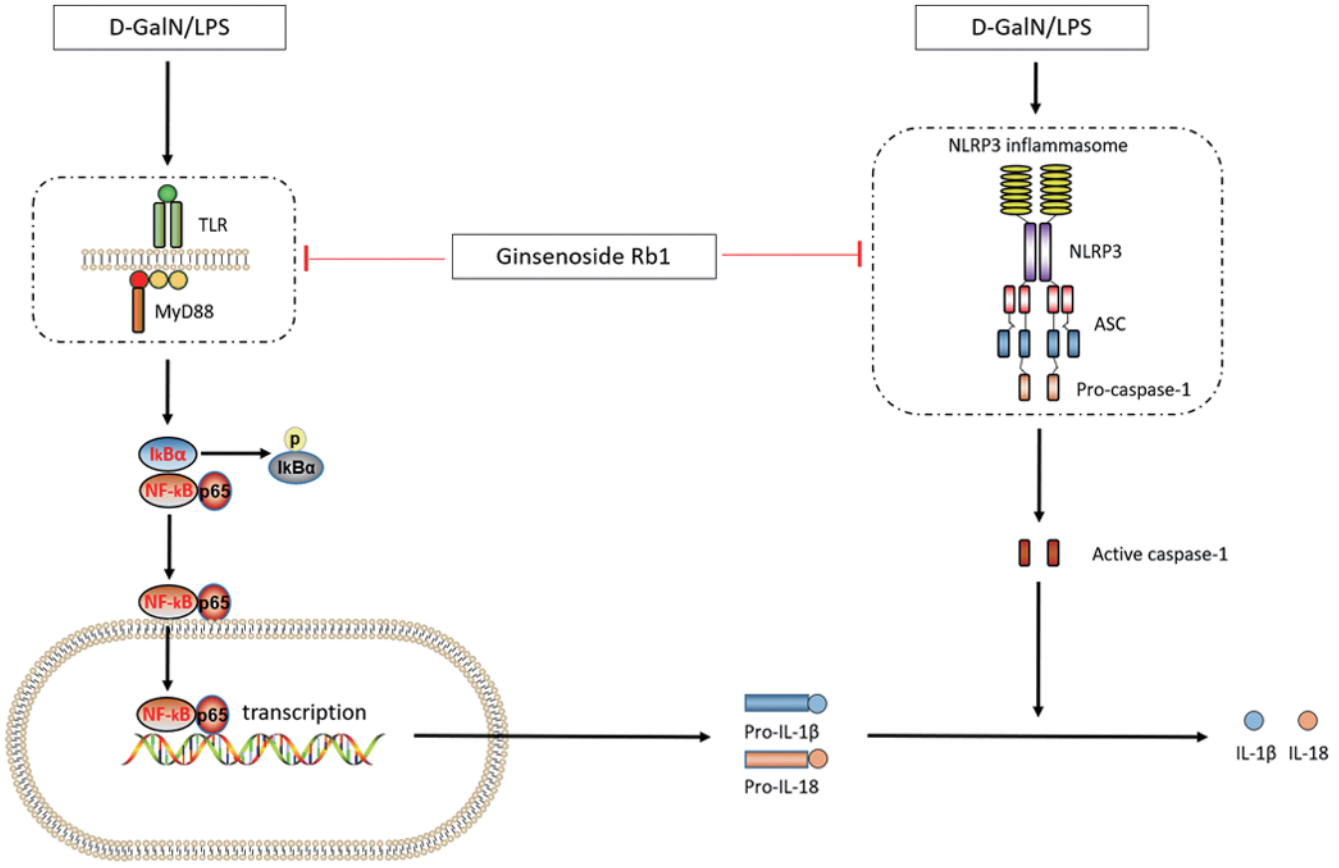

Fig. 7. Working model of the protective effects of Rb1 against D-GalN/LPS induced ALI: Rb1 inhibition of the TLR4/MyD88/NF-κB signaling pathway and NLRP3 inflammasome activation. ALI, acute liver injury; D-GalN, d-galactosamine; LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-kappa B; NLRP3, NLR family, pyrin domain-containing 3 protein; TLR4, toll-like receptor 4.

of effective agents are available for liver diseases.[30] Currently, a large number of inflammatory factors are secreted by KCs in response to microbial or viral pathogens to initiate and promote the inflammatory response and ALI. Hence, we evaluated the therapeutic effects of ginsenoside Rb1 on mice ALI induced by D-GalN/LPS and explored the relationship between ginsenoside Rb1 and KCs.

In this study, the animal model of ALI was successfully established by intraperitoneal injection of D-GalN/LPS. We found that the survival rate was only 20% at 24 h after the intraperitoneal injection of D-GalN/LPS, and the liver damage markers ALT and AST were markedly increased in the D-GalN/LPS group. We observed severe hepatocyte necrosis and vacuolation in the D-GalN/LPS group. In addition, pro-oxidation markers, such as ROS, MPO and MDA, and the levels of inflammatory cytokines, such as IL-6, TNF-α, IL-1β and IL-18, were significantly increased, while antioxidant markers, such as SOD and GSH-Px, were significantly decreased in the D-GalN/LPS group. These were in line with findings from a previous study which indicated that the systemic balance of prooxidants and antioxidants in ALI mice was disrupted with the accumulation of prooxidants and the depletion of endogenous antioxidants.[31] In addition, previous studies[32,33] have also showed an excessive inflammatory response in ALI mice, and increased levels of inflammatory cytokines, such as IL-6, TNF-α, IL-1β and IL-18, were detected in serum and liver tissue. Furthermore, our results revealed that ginsenoside Rb1 reduced serum ALT and AST levels, pro-oxidation markers and inflammatory cytokines, while improving hepatic necrosis and increasing the level of antioxidants. Consequently, we indicated that ginsenoside Rb1 had a protective effect on liver, which could reduce oxidative stress damage and ameliorated the overproduction of inflammatory cytokines in ALI induced by D-GalN/LPS. To the best of our knowledge, this was the first study on the effect of ginsenoside Rb1 on D-GalN/LPS-induced ALI and its underlying mechanisms.

The underlying mechanism of the protective effect of ginsenoside Rb1 on D-GalN/LPS-induced ALI has remained unclear. In this study, we demonstrated that D-GalN/LPS upregulated TLR4 and MyD88, induced phosphorylation of IκBα, and promoted the translocation of NF-κB p65 from the cytoplasm to the nucleus, and these effects were abolished by pretreatment with ginsenoside Rb1. TLR4 is a pattern-recognition receptor expressed on the surface of various types of cells that regulates the activation of NF-κB. When microbial or viral pathogens translocate to the liver, they bind to TLR4 and recruit MyD88, triggering a downstream signaling cascade. Activation of the IκB kinase leads to the phosphorylation of the inhibitory protein IκB-α, which typically binds to NF-κB p65 in the cytoplasm of non-stimulated cells as an inactive complex.[34] Phosphorylation of the inhibitory protein IκB-α leads to its degradation, allowing for the translocation of NF-κB p65 from the cytoplasm to the nucleus and resulting in the secretion of inflammatory cytokines, such as IL-6 and TNF-α.[35] Wang *et al*.[36] found that ginsenoside Rb1 reduced liver injury induced by intestinal ischemia-reperfusion by regulating NF-κB activation. Therefore, we indicated that ginsenoside Rb1 protects mice against D-GalN/LPS-induced ALI through the inhibition of the TLR4/NF-κB signaling pathway (Fig. 7).

Currently, we also found that the protein and mRNA expressions of NLRP3, ASC and caspase-1 were increased after the treatment with D-GalN/LPS, and reversed by pretreatment with ginsenoside Rb1. NLRP3 is another partner involved in the TLR4/NF-κB signaling pathway. It is polymerized and bound to the ASC adapter to induce translocation and activation of caspase-1. Activation of caspase-1 is related to the maturation and secretion of IL-1β and IL-18. This mechanism was also validated in the models of ALI induced by other causes. Li *et al*.[37] demonstrated that the TLR4/NF-κB/NLRP3 signaling pathway was involved in cholestatic liver injury, and that methane-rich saline suppressed the TLR4/NF-κB/NLRP3 pathway, leading to a reduction in inflammatory factor excretion. Liu *et al*.[38] reported NLRP3 inflammasome activation and subsequent inflammatory factor excretion in paraquat-induced ALI, and showed that hydrogen sulfide ameliorated the toxic effects of paraquat on the liver by suppressing ROS-induced NLRP3 inflammasome activation. Therefore, we proposed that NLRP3 inflammasome activation was also involved in the D-GalN/LPS-induced ALI, while ginsenoside Rb1 could inhibit of the NLRP3 inflammasome activation, and finally block the TLR4/NF-κB signaling pathway (Fig. 7).

In addition, compared to the control group, KCs were increased in the D-GalN/LPS groups, but did not appear to be increased significantly after Rb1 pretreatment. Previous studies also found that extensive activation of KCs was involved in D-GalN/LPS-induced inflammation in liver injury.[33] We also found the expression of NLRP3, ASC, caspase-1 and inflammatory cytokine in isolated KCs to be upregulated by D-GalN/LPS and downregulated by Rb1 pretreatment. As a member of the NLR family, the NLRP3 inflammasome had been demonstrated in a previous study to be essential to caspase-1 activation and inflammatory cytokine secretion in KCs following stimulation with LPS.[39] Accordingly, we indicated that increased activation of KCs participated in D-GalN/LPS-induced ALI, and Rb1 mainly effected KCs to protect the ALI.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (MJ, NS), acquisition of data (YmL, NL, YjL), analysis and interpretation of data (YmL, NL, HH, ZL, WL), drafting of the manuscript (YmL, NL, YjL), critical revision of the manuscript for important intellectual content (MJ, NS), administrative, technical, or material support, study supervision (MJ, NS).

Data sharing statement

No additional data are available.

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