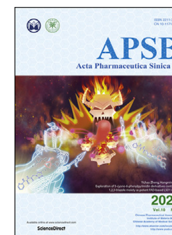
Acta Pharmaceutica Sinica B 2020;10(9):1619–1633

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

Icariside II, a main compound in Epimedii Folium, induces idiosyncratic hepatotoxicity by enhancing NLRP3 inflammasome activation

Zhilei Wang[a,b†], Guang Xu[b†], Hongbo Wang[c†], Xiaoyan Zhan[b], Yuan Gao[d], Nian Chen[a], Ruisheng Li[e], Xueai Song[f], Yuming Guo[f], Ruichuang Yang[e], Ming Niu[b], Jiabo Wang[b], Youping Liu[a,*], Xiaohe Xiao[b,f,*], Zhaofang Bai[b,*]

[a]*School of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China*
[b]*China Military Institute of Chinese Materia, the Fifth Medical Centre, Chinese PLA General Hospital, Beijing 100039, China*
[c]*Department of Hepatobiliary Surgery Center, the Fifth Medical Centre, Chinese PLA General Hospital, Beijing 100039, China*
[d]*School of Chinese Materia Medica, Capital Medical University, Beijing 100029, China*
[e]*Research Center for Clinical and Translational Medicine, the Fifth Medical Centre, Chinese PLA General Hospital, Beijing 100039, China*
[f]*Integrative Medical Center, the Fifth Medical Centre, Chinese PLA General Hospital, Beijing 100039, China*

Received 17 January 2020; received in revised form 20 February 2020; accepted 9 March 2020

KEY WORDS

Epimedii Folium;
Icariside II;
Idiosyncratic drug-induced liver injury;
NLRP3 inflammasome;
Reactive oxygen species;
Mitochondria

Abstract Idiosyncratic drug-induced liver injury (IDILI) is an infrequent but potentially serious disease that develops the main reason for post-marketing safety warnings and withdrawals of drugs. Epimedii Folium (EF), the widely used herbal medicine, has shown to cause idiosyncratic liver injury, but the underlying mechanisms are poorly understood. Increasing evidence has indicated that most cases of IDILI are immune mediated. Here, we report that icariside II (ICS II), the major active and metabolic constituent of EF, causes idiosyncratic liver injury by promoting NLRP3 inflammasome activation. ICS II exacerbates NLRP3 inflammasome activation triggered by adenosine triphosphate (ATP) and nigericin, but not silicon dioxide (SiO_2), monosodium urate (MSU) crystal or cytosolic lipopolysaccharide (LPS).

*Corresponding authors.
E-mail addresses: liuyouping@163.com (Youping Liu), pharmacy_302@126.com (Xiaohe Xiao), baizf2008@hotmail.com (Zhaofang Bai).
†These authors made equal contributions to this work.
Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2020.03.006
2211-3835 © 2020 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Additionally, the activation of NLRC4 and AIM2 inflammasomes is not affected by ICS II. Mechanistically, synergistic induction of mitochondrial reactive oxygen species (mtROS) is a crucial contributor to the enhancing effect of ICS II on ATP- or nigericin-induced NLRP3 inflammasome activation. Importantly, *in vivo* data show that a combination of non-hepatotoxic doses of LPS and ICS II causes the increase of aminotransferase activity, hepatic inflammation and pyroptosis, which is attenuated by *Nlrp3* deficiency or pretreatment with MCC950 (a specific NLRP3 inflammasome inhibitor). In conclusion, these findings demonstrate that ICS II causes idiosyncratic liver injury through enhancing NLRP3 inflammasome activation and suggest that ICS II may be a risk factor and responsible for EF-induced liver injury.

© 2020 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Idiosyncratic drug-induced liver injury (IDILI) is a severe adverse reaction that significantly increases the expenditure of drug development on account of a variable latency to onset and a lack of clear dose dependency[1,2]. Recently, several studies have pointed out that traditional Chinese medicines (TCMs) and anti-tuberculosis drugs are the primary causes of IDILI in China[3,4]. Specifically, liver injury caused by TCMs, such as *Polygonum multiflorum*, *Gynura segetum*, and *Ageratina adenophora*, has occurred frequently in recent years[5−7]. Although emerging evidence has demonstrated that some TCMs can lead to IDILI, the precise pathogenesis underlying this role is still undefined.

NLRP3 inflammasome comprises a sensor molecule NLRP3, an adaptor protein ASC, and an effector molecule pro-caspase-1[8]. NLRP3 inflammasome senses pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which leads to pro-caspase-1 cleavage, and subsequently induces pyroptosis and triggers inflammatory responses[9−11]. Recent researches have demonstrated that excessive activation of NLRP3 inflammasome could lead to various liver diseases, such as IDILI, nonalcoholic/alcoholic steatohepatitis and liver fibrosis[12−14], and plays a critical role in determining the progression, severity and pathology *via* amplifying the inflammatory response.

Epimedii Folium (EF) is a well-known herbal medicine that has been employed extensively as a tonic, aphrodisiac, and anti-rheumatic[15]. In recent years, the possibility that EF and its preparations cause IDILI has been reported frequently[16,17], and clinical safety issues associated with EF have drawn increasing attention. In our previous research[18], we demonstrated that EF could induce idiosyncratic liver injury in a non-hepatotoxic dose of lipopolysaccharide (LPS)-mediated susceptibility mouse model of IDILI. In addition, interleukins-1β (IL-1β) is a special inflammatory cytokine in EF-induced liver injury. Therefore, we speculated that EF likely induces liver injury by promoting the activation of NLRP3 inflammasome. In the current study, we showed that icariside II (ICS II), the major active and metabolic constituent of EF, could specifically enhance NLRP3 inflammasome activation induced by adenosine triphosphate (ATP) or nigericin, thereby leading to an uncontrolled immune response and the induction of idiosyncratic liver injury.

2. Materials and methods

2.1. Mice

Female wild-type (WT) C57BL/6 mice (6−8-week-old) were obtained from SPF Biotechnology Co., Ltd (Beijing, China). *Nlrp3* knock-out (*Nlrp3*$^{-/-}$) mice were from National Center of Biomedical Analysis (NCBA, Beijing, China) supplied by Dr. Tao Li. All mice were permitted water and food freely and held under a 12-h dark/light cycle at 22 ± 2 °C. All of the experimental procedures in our experiments were carried out on the basis of the Guidelines Of Laboratory Animals Care And Use and authorized by the Animal Ethics Committee Of the Fifth Medical Centre, Chinese People's Liberation Army (PLA) General Hospital (Beijing, China).

2.2. Cell culture

Using WT or *Nlrp3*$^{-/-}$ female C57BL/6 mice (10-week-old), bone marrow cells were extracted into Dulbecco's modified Eagle's medium (DMEM, Macgene, Beijing, China) containing 1% penicillin/streptomycin (Macgene) and 10% fetal bovine serum (Gibco, Rockford, IL, USA). Moreover, 50 ng/mL murine macrophage colony-stimulating factor (MedChemExpress, Monmouth, NJ, USA) was added to DMEM to differentiate cells into bone marrow-derived macrophages (BMDMs). Human THP-1 cells, which were supplied by Dr. Tao Li from NCBA, were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Macgene) containing 1% penicillin/streptomycin (Macgene) and 10% fetal bovine serum (FBS), and they were stimulated using 100 nmol/L phorbol-12-myristate-13-acetate (PMA, MedChemExpress) overnight to differentiate into macrophages. All of the cultured cells were placed in a humidified 5% CO_2 atmosphere at 37 °C.

2.3. Inflammasomes activation

For inducing NLRP3, NLRC4 and AIM2 inflammasomes activation, BMDMs at 1 × 10^6 cells/mL were seeded 0.5 mL in 24-well plates overnight. Then, 50 ng/mL LPS-PG Ultrapure (InvivoGen, San Diego, CA, USA) or 1 μg/mL Pam3CSK4 (InvivoGen) was used to stimulate BMDMs for 4 h, after which the medium was changed to Opti-MEM (Gibco) containing the

main components from EF (epmedin A, epmedin A1, epmedin B, epmedin C, icariin, icaritin, icariside I, ICS II, anhydroicaritin; TargetMol, Boston, MA, USA), hydrogen peroxide (H_2O_2, Sigma, Darmstadt, Germany) or *N*-acetyl-L-cysteine (NAC, MedChemExpress). One hour later, the cells were stimulated for 1 h with ATP (5 mmol/L; Sigma) and nigericin (7.5 μmol/L; InvivoGen) or for 6 h with monosodium urate (MSU, 200 μg/mL; InvivoGen), silicon dioxide (SiO_2, 250 μg/mL; InvivoGen) or Lfn-Flic (200 ng/mL, supplied by Dr. Tao Li). Cells were transfected with poly (I:C) (2 μg/mL; InvivoGen), poly(dA:dT) (2 μg/mL; InvivoGen) or LPS (1 μg/mL; InvivoGen) for 6 h through the use of Lipofectamine 2000 (Thermo Fisher Scientific, Rockford, MI, USA) according to the manufacturer's protocols. PMA-primed THP-1 cells were seeded at 1×10^6 cells/mL in 24-well plates overnight. Then, the cells were pretreated with ICS II. One hour later, the cells were stimulated with nigericin (7.5 μmol/L) for 1 h. Cell extracts and precipitated supernatants were analyzed by immunoblot.

2.4. *Western blot analysis*

Cell extracts and precipitated supernatants were lysed with 1 × loading buffer (Dingguo Changsheng Biotechnology, Beijing, China) containing radio immunoprecipitation assay buffer (RIPA, Thermo Fisher Scientific). The samples were denatured in 105 °C for 15 min, and equal amounts of the protein samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.2 μm polyvinylidene fluoride membranes. Then, the membrane was blocked by 5% non-fat milk for 1 h at room temperature. The indicated primary antibodies were incubated at 4 °C overnight and then treated with anti-rat IgG (1:3000; Cell Signaling Technology, Danvers, MA, USA), anti-mouse IgG (1:5000; Jackson ImmunoResearch, West Grove, PA, USA) or anti-rabbit IgG (1:5000; Jackson ImmunoResearch) for 1 h at room temperature. The signals were analyzed using the enhanced chemiluminescent reagent (Promega, Beijing, China). The primary antibodies anti-NLRP3 (1:1000) and anti-mouse caspase-1 (1:1000) were bought from Adipogen (San Diego, CA, USA). Anti-mouse cleaved IL-1β (1:2000) was from R&D systems (Minneapolis, MN, USA). Anti-human caspase-1 (1:2000), anti-mouse caspase-11 (1:1000), anti-mouse cleaved GSDMD (1:500), and anti-human cleaved IL-1β (1:1000) were obtained from Cell Signaling Technology (Boston, MA, USA). Anti-ASC (1:1000) was purchased from ABclonal (Wuhan, China). Anti-GAPDH (1:2000; Proteintech, Chicago, IL, USA) was used as an internal control.

2.5. *Caspase-1 activity assay*

The activity of caspase-1 in cell culture supernatants was evaluated by Caspase-Glo® one Inflammasome Assay on the basis of the manufacturer's direction (Promega).

2.6. *Lactate dehydrogenase (LDH) assay*

BMDMs or THP-1 cells were primed with LPS or PMA, and the cells were treated with ICS II before stimulated with the inflammasomes stimuli. LDH was measured by an LDH cytotoxicity assay kit (Promega).

2.7. *ASC oligomerization*

BMDMs at 1×10^6 cells/mL were seeded with 1.0 mL medium in 12-well plates overnight. The following day, the medium was replaced, and cells were primed with 50 ng/mL LPS or 1 μg/mL Pam3CSK4 for 4 h. Next, the medium was changed to Opti-MEM containing ICS II. The method to induce inflammasomes activation has been described in Section 2.3. Next, the cells were lysed by Triton Buffer [50 mmol/L Tris−HCl (pH 7.5), 150 mmol/L NaCl, 0.5% Triton X-100, and 1% EDTA-free protease inhibitor cocktail (TargetMol)]). A small amount (2.5%) of the lysates were collected as whole cell lysates. The rest of the lysates were centrifuged at 6000×g for 10 min at 4 °C. The pellets were washed with one mL of ice-cold phosphate buffered saline (PBS) and resuspended in 200 μL PBS. Two millimolar disuccinimidyl suberate (DSS, Abcam, Cambridge, UK) was added to the resuspended pellets, which were incubated at 37 °C for 30 min with rotation. Samples were then centrifuged at 6000×g for 10 min at 4 °C. The cross-linked pellets were resuspended in 60 μL sample buffer and then were boiled and analyzed by immunoblot.

2.8. *Intracellular potassium detection*

To measure the intracellular potassium, BMDMs at 1×10^6 cells/mL were seeded with 1.0 mL medium overnight in 12-well plates. After being primed with 50 ng/mL LPS for 4 h, cells were treated with ICS II for 1 h and then stimulated with different NLRP3 stimuli. Culture medium was thoroughly aspirated and lysed with 3% ultrapure HNO_3. Intracellular K^+ measurements were performed by inductively coupled plasma optical emission spectrometry.

2.9. *Immunoprecipitation assay*

For the exogenous NLRP3−NLRP3 interaction experiment, HEK-293T cells (3×10^5 cells/mL) were transfected with Flag-NLRP3 and Myc-NLRP3 in 6-well plates *via* Lipofectamine 2000. Twelve hours later, ICS II was given to assess its impact on NLRP3−NLRP3 interaction. Then, cells were gathered with lysis buffer containing protease inhibitor after 24 h. Next, the lysate was immunoprecipitated with anti-Flag antibody and beads, and then Western blot was used to evaluate the NLRP3−NLRP3 interaction.

2.10. *Confocal microscopy*

Confocal microscopy analysis, which was carried out to test mitochondrial damage, has been described previously[19]. BMDMs were plated at 5×10^5 cells/mL on coverslips in 24-well plates overnight. Then, the cells were stimulated with 50 ng/mL LPS for 4 h. After that, ICS II was added as described for another 1 h. BMDMs were stimulated by ATP for 1 h and stained with Mito-Tracker Red (50 nmol/L, Thermo Fisher Scientific), then cells were washed three times by PBS and fixed with 4% paraformaldehyde in PBS for 15 min. After that, cells were washed with PBST three times. Confocal microscopy analysis was carried out by using a Zeiss LSM 700.

2.11. *Cell viability assay*

The cell counting kit-8 (CCK-8; Dojindo, Shanghai, China) assay was applied to detect the viability of cells. BMDMs were seeded in 96-well growth-medium plate overnight at 1×10^5 cells/well. Then, LPS-primed BMDMs were incubated at 37 °C followed by treatment with H_2O_2, then these cells were cultured with CCK-8 for 30 min. The optical density (O.D.) values at the wavelength of 450 nm were determined.

2.12. *Mitochondrial reactive oxygen species assay*

BMDMs were put onto 100 mm diameter culture dish tubes and primed with LPS (50 ng/mL) for 4 h. Then, the LPS-primed BMDMs were transferred into 1.5 mL tubes and treated with ICS II for 1 h. Next, the cells were stimulated with ATP, nigericin or SiO_2, after which the cells were washed with PBS. For mitochondrial ROS (mtROS) measurement, BMDMs were loaded with 4 μmol/L MitoSOX red mitochondrial superoxide indicator (Invitrogen, Ex/Em: 510/580 nm) for 20 min and washed with PBS. After staining and washing, cells were resuspended in PBS and flow cytometry was conducted to test mtROS production.

2.13. *Alanine aminotransferase (ALT) and aspartate transaminase (AST)*

Serum ALT and AST levels were measured according to the GPT (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and GOT (Nanjing Jiancheng Bioengineering Institute) assay kit directions.

2.14. *Enzyme-linked immunosorbent assay (ELISA)*

ELISA measurements of mouse IL-1β (Dakewe, Beijing, China), human IL-1β (Dakewe), mouse tumor necrosis-α (TNF-α, Dakewe), and human TNF-α (Dakewe) in cell culture supernatant and mouse serum IL-1β (R&D systems) were executed on the basis of the manufacturer's directions.

2.15. *Levels of oxidative stress parameter*

Mouse liver tissue oxidative stress parameter malondialdehyde (MDA) assay (Beyotime, Shanghai, China) was detected according to the manufacturer's directions. Liver samples were homogenized and treated in accordance with the manufacturer's recommendations.

2.16. *Assessment of the effects of LPS/ICS II cotreatment-induced DILI in vivo*

Six to eight-week-old female C57BL/6 mice fasted for 24 h were given 2 mg/kg LPS (from *Escherichia coli* O55:B5, Sigma) or its saline vehicle *via* a tail vein (i.v.). Two hours later, ICS II (25 and 50 mg/kg, TargetMol) or its vehicle (10% tween 80 + 90% PBS) was administered through intraperitoneal injection. Mice serum was collected 6 h after ICS II treatment. The serum IL-1β, TNF-α, ALT and AST levels were tested.

In the second experiment, 6−8-week-old female C57BL/6 WT and *Nlrp3*$^{-/-}$ mice fasted for 24 h were given 2 mg/kg LPS (Sigma) or its saline vehicle *via* a tail vein (i.v.). Two hours later, ICS II (50 mg/kg) or its vehicle (10% tween 80 + 90% PBS) was administered through intraperitoneal injection. Mice serum and a fraction of liver samples fixed in 10% formalin neutral buffer solution were collected 6 h after ICS II treatment. Hematoxylin and eosin (H&E), TUNEL staining and the serum IL-1β, TNF-α, ALT and AST levels were tested. Moreover, liver homogenate was used to detect the activity of caspase-1 after normalization processing of bicinchoninic acid (BCA) protein quantification kit (Solarbio, Beijing, China).

In the third experiment, 6−8-week-old female C57BL/6 mice were fasted for 24 h. Then, MCC950 (50 mg/kg, TargetMol) or its vehicle (10% tween 80 + 90% PBS) was administered through intraperitoneal injection. One hour later, the mice were given 2 mg/kg LPS (Sigma) or its saline vehicle *via* a tail vein (i.v.). Two hour later, ICS II (50 mg/kg, TargetMol) or its vehicle (10% tween 80 + 90% PBS) was administered through intraperitoneal injection. Mice serum and a fraction of liver samples were collected after 6 h. The serum IL-1β, TNF-α, ALT and AST levels, and level of oxidative stress parameter MDA was tested. Moreover, liver homogenate was used to detect the expression of caspase-1, IL-1β and cleaved GSDMD by Western blot after normalization processing using a BCA protein quantification kit (Solarbio).

2.17. *Statistical analyses*

The software Prism six and SPSS statistics 21.0 were used for statistics and analysis. The testing data are presented as mean ± standard error of mean (SEM). A two-tailed unpaired Student's *t*-test for two groups or one-way ANOVA for multi groups was conducted to evaluate the significant differences. A *P* value less than 0.05 was considered to be significant.

3. Results

3.1. *Numerous ingredients derived from EF enhance ATP-induced caspase-1 activation and IL-1β production*

Nine compounds (epmedin A, epmedin A1, epmedin B, epmedin C, icariin, icaritin, icariside I, ICS II and anhydroicaritin) derived from EF were selected to test whether they initiated an immune response by activating NLRP3 inflammasomes. First, LPS-primed BMDMs were tested with these compounds (10 μmol/L) for 24 h, but none of them induced caspase-1 maturation and IL-1β secretion (Supporting Information Fig. S1A and S1B), which suggested that they did not serve as direct agonists to induce inflammasome activation. Next, LPS-primed BMDMs were pretreated with these components (10 μmol/L) before ATP stimulation, which is a NLRP3 inflammasome stimulator. The results show that epmedin B, icariside I and ICS II significantly promoted caspase-1 maturation and IL-1β production that was triggered by ATP in LPS-primed BMDMs (Fig. 1A−C). Besides, TNF-α, an inflammasome-independent cytokine, was not influenced by these compounds under this condition (Fig. 1D). Among these EF constituents, ICS II (Supporting Information Fig. S2A), which exhibits the most potent effect on NLRP3 inflammasome activation, may be the main contributor to EF-induced liver injury.

3.2. *ICS II specifically accelerates NLRP3 inflammasome activation triggered by ATP and nigericin*

Next, we evaluated the impact of ICS II on NLRP3 inflammasome activation. In the following experiment, we first tested the synergistic effects of ICS II, ATP, and LPS on NLRP3 inflammasome

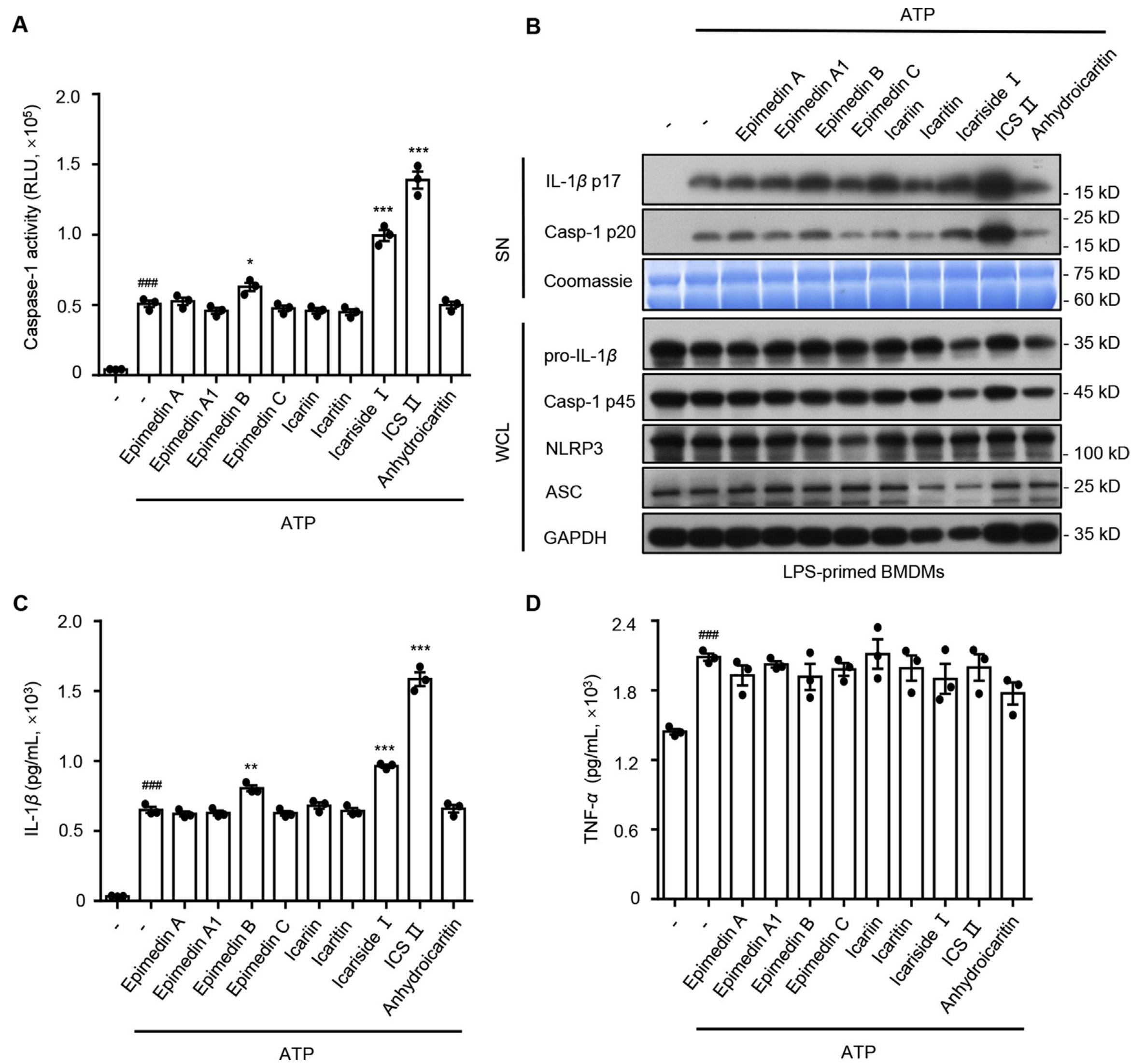


Figure 1 Effect of the main compositions from Epimedii Folium on NLRP3 inflammasome activation. (A) Caspase-1 activity in supernatants (SN) from LPS-primed BMDMs treated with the main components from EF (10 μmol/L) and then stimulated with ATP (1 h). (B) Western blot analysis of IL-1*β* (p17), caspase-1 (p20) in culture supernatants (SN) and pro-IL-1*β*, caspase-1 (p45), NLRP3, ASC in whole cell lysates (WCL) of LPS-primed BMDMs treated with the main components from EF (10 μmol/L) and then stimulated with ATP (1 h). ELISA of IL-1*β* (C) and TNF-*α* (D) in SN described in (B). RLU, relative light units. Data are means ± SEM ($n = 3$); $^{###}P < 0.001$ *vs.* the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* the LPS plus ATP group.

activation. The expression of NLRP3 and pro-IL-1*β* induced by LPS treatment, but not ATP or ICS II alone (Fig. S2B and S2C). We subsequently treated LPS-primed BMDMs with ICS II alone. The result show that NLRP3 inflammasome was not activated in the absence of ATP (Fig. S2B and S2C). Caspase-1 maturation and IL-1*β* secretion were induced when BMDMs were cotreat with LPS and ATP. Interestingly, ICS II significantly promoted ATP-induced caspase-1 activation and IL-1*β* production in LPS-primed BMDMs (Fig. S2B, S2C, Supporting Information Fig. S5A and S5B), which was blocked by *Nlrp3* deficiency (Fig. S2D−S2H). Then, we assessed the dose−effect relationship of ICS II on NLRP3 inflammasome activation triggered by ATP. The results indicated that ICS II dose-dependently promoted caspase-1 cleavage and IL-1*β* secretion that was induced by LPS and ATP in BMDMs, but it had no influence on TNF-*α* production (Fig. 2A−C and Supporting Information Fig. S3A).

Next, we assessed the impact of ICS II on NLRP3 inflammasome activation in BMDMs and THP-1 cells evoked by nigericin. The results show that ICS II also dose-dependently enhanced caspase-1 maturation, IL-1*β* production, GSDMD cleavage and LDH release but had no impact on TNF-*α* production in response to nigericin in LPS-primed BMDMs (Figs. 2D−F, S3B, S3C, S5A, and S5B) or PMA-primed THP-1 cells (Figs. 2G−I, S3D, and S3E). In addition, NLRP3 inflammasome can be activated by MSU, SiO_2, and cytosolic LPS. To determine whether ICS II was a common accelerant for NLRP3 inflammasome, we detected the effect of ICS II on NLRP3 inflammasome activation induced by those stimuli. Unexpectedly, pretreatment with ICS II had no influence on caspase-1 maturation,

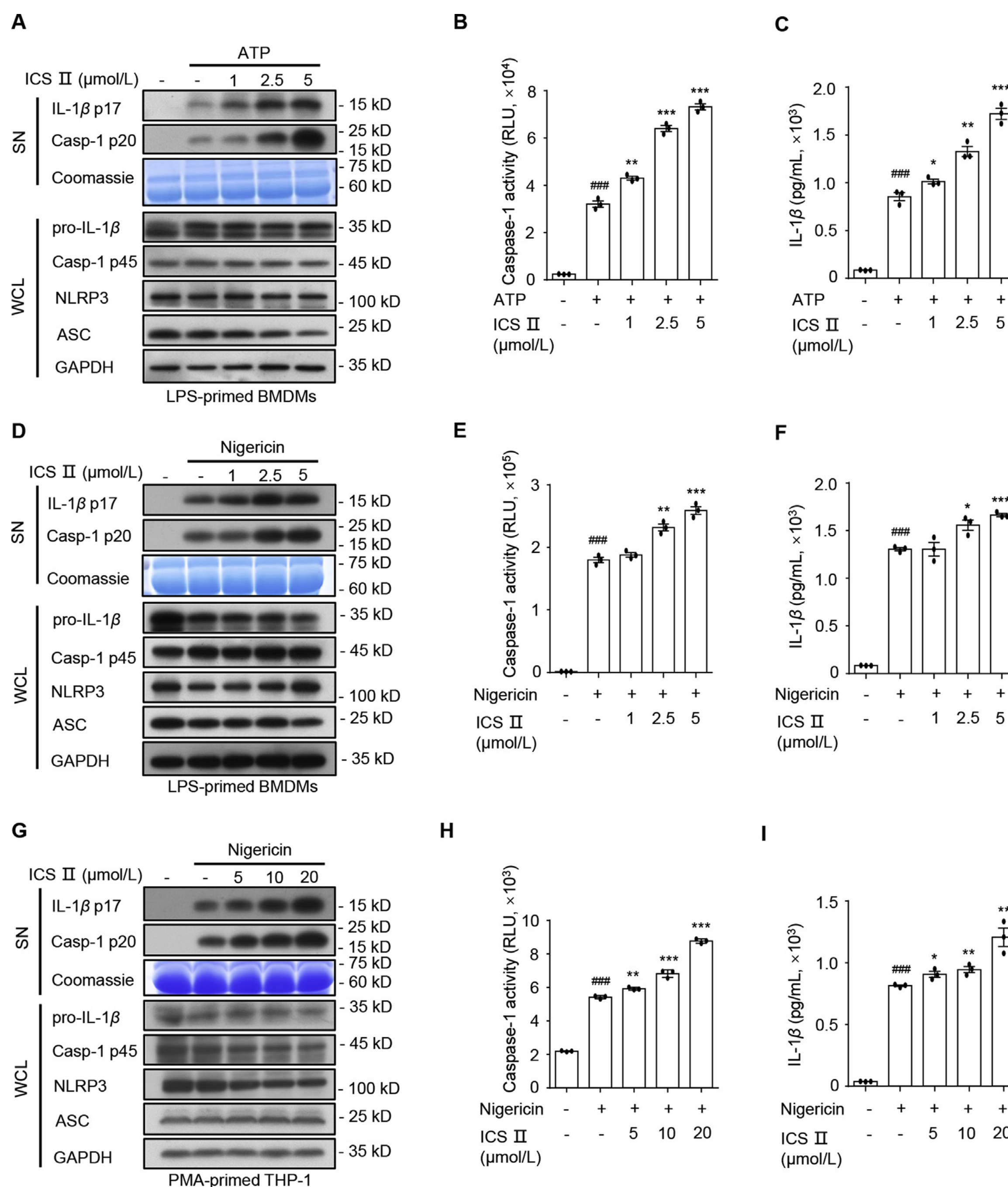


Figure 2 ICS II promotes NLRP3 inflammasome activation stimulated by ATP and nigericin. (A)−(C) Western blot analysis of IL-1β (p17), caspase-1 (p20) in SN and pro-IL-1β, caspase-1 (p45), NLRP3, ASC in WCL (A), caspase-1 activity (B) in SN and ELISA of IL-1β (C) in SN from LPS-primed BMDMs treated with various doses of ICS II and then stimulated with ATP (1 h). (D)−(F) Western blot analysis of IL-1β (p17), caspase-1 (p20) in SN and pro-IL-1β, caspase-1 (p45), NLRP3, ASC in WCL (D), caspase-1 activity (E) in SN and ELISA of IL-1β (F) in SN from LPS-primed BMDMs treated with various doses of ICS II before nigericin (1 h) stimulation. (G)−(I) Western blot analysis of IL-1β (p17), caspase-1 (p20) in SN and pro-IL-1β, caspase-1 (p45), NLRP3, ASC in WCL (G), caspase-1 activity (H) in SN and ELISA of IL-1β (I) in SN from PMA-primed THP-1 cells treated with various doses of ICS II before nigericin (1 h) stimulation. Data are means ± SEM (n = 3); $^{###}P < 0.001$ *vs*. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. the LPS/PMA plus ATP/nigericin group.

IL-1β secretion and TNF-α production triggered by MSU (Fig. 3A−C, and Supporting Information Fig. S4A), SiO_2 (Fig, S4B−D), or cytosolic LPS (Figs. 3D−F, and S4E). Moreover, ICS II had no impact on AIM2 and NLRC4 inflammasomes activation induced by poly(dA:dT) and Lfn-Flic, respectively (Figs. 4G−I, S4F, and S4G). These results indicate that ICS II acts as a specific promoter to increase ATP- and nigericin-induced NLRP3 inflammasome activation.

3.3. *ICS II promotes ATP- or nigericin-induced ASC oligomerization, but has no effect on the NLRP3−NLRP3 interaction and potassium efflux*

Next, we further investigated how ICS II affected NLRP3 inflammasome activation induced by ATP and nigericin. ASC oligomerization is the pivotal step for NLRP3, NLRC4 and AIM2 inflammasome activation. LPS- or Pam3CSK4-primed BMDMs were handled with ICS II before various activators stimulation. Then, ASC was crosslinked by disuccinimidyl suberate (DSS), and ASC oligomerization was detected through Western blot. The results show that ICS II dose-dependently promoted ASC oligomerization that was induced by ATP (Fig. 4A) and nigericin (Supporting Information Fig. S6). However, ICS II had no impact on ASC oligomerization induced by MSU, poly(dA:dT), Lfn-Flic or cytosolic LPS (Fig. 4B), which indicated that ICS II acted upstream event of ASC oligomerization to exacerbate ATP- or nigericin-induced NLRP3 inflammasome activation.

We then investigated whether ICS II affected the direct NLRP3−-NLRP3 interaction. HEK-293T cells were handled with ICS II before being transfected with Flag-NLRP3 and Myc-NLRP3, and a co-immunoprecipitation assay was carried out to assess NLRP3−NLRP3 interaction. The results show that ICS II did not alter the NLRP3−NLRP3 interaction in HEK-293T cells (Fig. 4C). Potassium efflux is an important upstream event for the activation of NLRP3 inflammasome. We therefore examined whether ICS II affect potassium efflux triggered by these stimuli, and the results show that ICS II promoted potassium efflux induced by nigericin (Supporting Information Fig. S7A), but not ATP (Fig. 4D) or SiO_2 (Fig. S7B). Therefore, potassium efflux may be not responsible for the enhancement effect of ICS II on ATP- and nigericin-induced NLRP3 inflammasome activation.

3.4. *ICS II facilitates ATP- or nigericin-induced NLRP3 inflammasome activation by increasing mitochondrial ROS (mtROS) production*

Mitochondrial damage and mtROS are thought to be important upstream events for NLRP3 inflammasome activation[20]. Next, the impact of ICS II on mitochondrial damage was evaluated in the following experiment. The results show that mitochondrial damage was not observed in BMDMs that were treated with ICS II (5 μmol/L) (Supporting Information Fig. S8A). The MitoSOX Red Mitochondrial Superoxide Indicator assay was used to record the amount of mtROS production during the course of ATP, nigericin or SiO_2 treatment in the presence or absence of ICS II. The results show that ICS II treatment alone did not induce the mtROS production (Fig. 5A). However, ICS II obviously potentiated mtROS production that was triggered by ATP and nigericin rather than SiO_2 in LPS-primed BMDMs (Fig. 5A and B). This suggested that synergistic induction of ROS production is a crucial contributor to the enhancement effect of ICS II on NLRP3 inflammasomes triggered by ATP and nigericin. To evaluate the role of ROS production on the activation of NLRP3 inflammasomes triggered by ATP or nigericin, LPS-primed BMDMs were treated with oxidizing agent H_2O_2 or NAC (a scavenger of ROS) before ATP, nigericin or SiO_2 stimulation. Western blot analysis show that H_2O_2 enhanced caspase-1 maturation and IL-1β production in a certain dose range triggered by ATP or nigericin, but not SiO_2 (Supporting Information Fig. S9A−S9E). Similarly, NAC treatment suppressed mtROS production significantly (Supporting Information Fig. S10C) and inhibited nigericin-induced caspase-1 activation and IL-1β secretion in a dose-dependent manner (Fig. S10A and S10B). Most importantly, NAC pretreatment eliminated the increase of caspase-1 maturation and IL-1β production when LPS-primed BMDMs were treated with ICS II and then stimulated with ATP or nigericin (Fig. 5C and D). These results confirmed that ICS II facilitates ATP- or nigericin-induced NLRP3 inflammasome activation by increasing mtROS production.

3.5. *Combination of LPS and ICS II induces liver injury in WT mice but not in Nlrp3$^{-/-}$ mice*

Coexisting inflammatory mediators such as LPS should be considered to be determinants of susceptibility to IDILI[21]. Non-hepatotoxic doses of LPS decrease the threshold for toxicity and/or increase the magnitude of response[22]. Numerous studies have demonstrated that IDILI can be mimicked in animals through co-exposure to non-hepatotoxic doses of LPS and drugs with the ability to induce IDILI[23−26]. A previous study demonstrated that EF alone does not cause liver injury *in vivo*, but EF combined with non-hepatotoxic doses of LPS could induce hepatotoxicity[18]. We therefore evaluated whether ICS II, an active constituent that promotes NLRP3 inflammasome activation, could result in hepatoxicity in an LPS-mediated susceptibility mouse model of IDILI. The results show that mice treated with ICS II alone did not have altered plasma ALT and AST levels compared with those of control mice. However, co-treatment with LPS and ICS II resulted in significant increases in the plasma levels of ALT and AST, and this was accompanied by increases in the level of inflammatory cytokines IL-1β and TNF-α compared to those of LPS group (Supporting Information Fig. S11A−S11D). These results suggested that ICS II can induce liver injury in an LPS-mediated susceptibility mouse model of IDILI.

To further confirm the role of the NLRP3 inflammasomes on ICS II-induced liver injury, WT and *Nlrp3*$^{-/-}$ mice were used to explore the relationship between NLRP3 inflammasome and the liver injury caused by ICS II. The results show that co-treatment with LPS and ICS II led to elevation of the serum ALT, AST, IL-1β and TNF-α production in WT mice but not in *Nlrp3*$^{-/-}$ mice (Fig. 6A−D). Histological analysis of the mouse liver tissues was performed using H&E staining assay. As illustrated in Fig. 6G, co-exposure to LPS and ICS II resulted in pathological changes including inflammatory infiltration and hepatocyte focal necrosis, which was not occurred in control group, ICS II group, LPS group or any of the *Nlrp3*$^{-/-}$ groups. Moreover, caspase-1 activity was increased in the livers of WT mice but not in the livers of *Nlrp3*$^{-/-}$ mice after LPS/ICS II treatment (E). Similarly, TUNEL fluorescence staining showed that the TUNEL positive signal was significantly increased in WT mice that were co-exposed to LPS and ICS II compared with the other groups (Fig. 6F and G). Taken together, these results suggest that ICS II could induce liver injury by promoting NLRP3 inflammasome activation.

3.6. *MCC950 pretreatment reverses LPS/ICS II-induced liver injury*

To further prove the involvement of NLRP3 inflammasome in ICS II/LPS-induced liver injury, a specific NLRP3 inflammasome

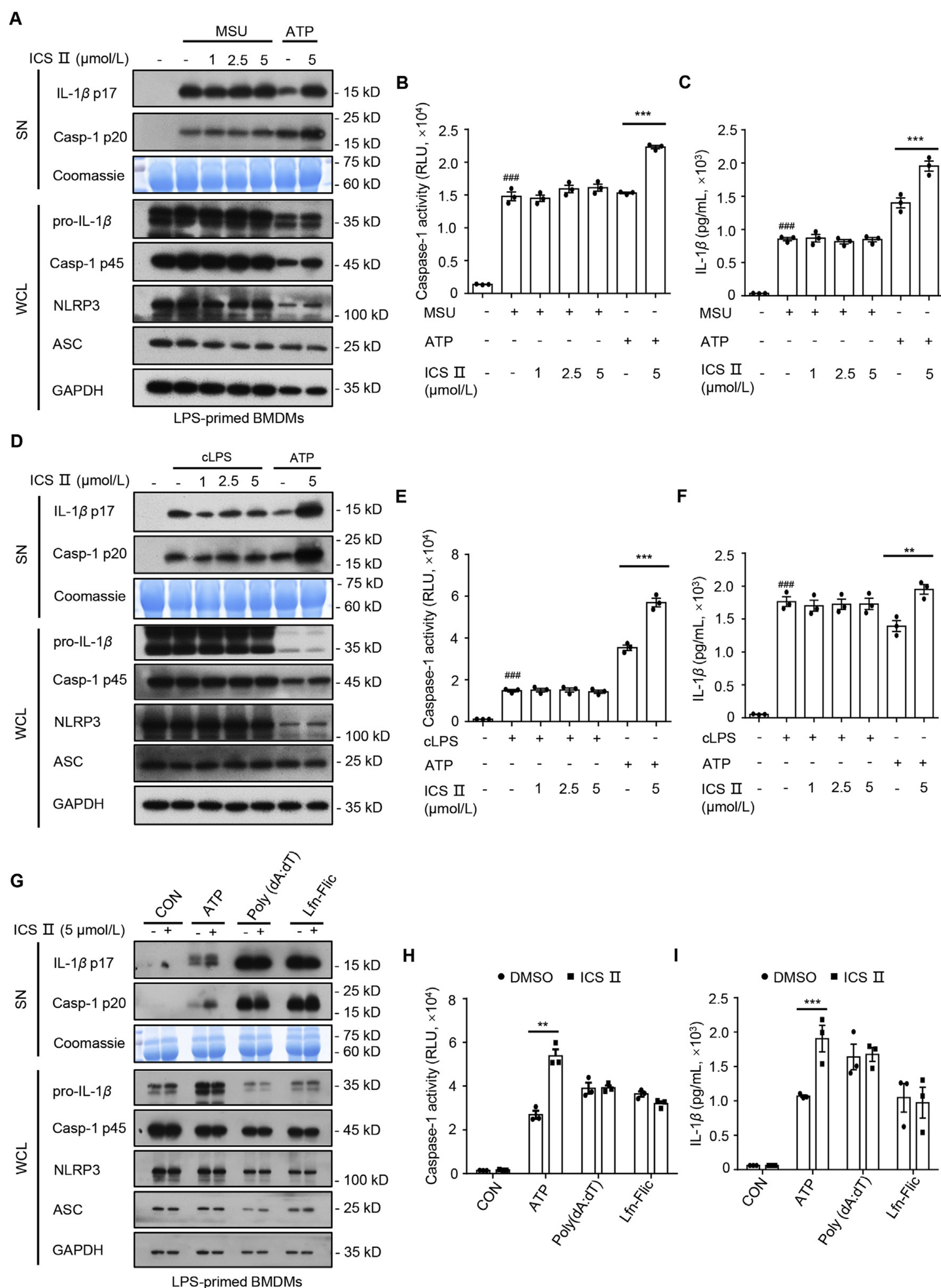


Figure 3 ICS II has no effect on the activation of NLRP3 inflammasome induced by MSU and cytosolic LPS (cLPS), as well as AIM2 and NLRC4 inflammasomes. (A)−(C) Western blot analysis of IL-1β (p17), caspase-1 (p20) in SN and pro-IL-1β, caspase-1 (p45), NLRP3, ASC in WCL (A), caspase-1 activity (B) in SN and ELISA of IL-1β (C) in SN from LPS-primed BMDMs treated with various doses of ICS II and then stimulated with MSU (6 h) or LPS-primed BMDMs stimulated with ATP (1 h) in the presence or absence of ICS II (5 μmol/L). (D)−(F) Western blot analysis of IL-1β (p17), caspase-1 (p20) in SN and pro-IL-1β, caspase-1 (p45), NLRP3, ASC in WCL (D), caspase-1 activity (E) in SN and ELISA of IL-1β (F) in SN from Pam3CSK4-primed BMDMs treated with various doses of ICS II and then transfected with LPS (6 h) or LPS-primed BMDMs stimulated with ATP (1 h) in the presence or absence of ICS II (5 μmol/L). (G)−(I) Western blot analysis of IL-1β (p17), caspase-1 (p20) in SN and pro-IL-1β, caspase-1 (p45), NLRP3, ASC in WCL (G), caspase-1 activity (H) in SN and ELISA of IL-1β (I) in SN

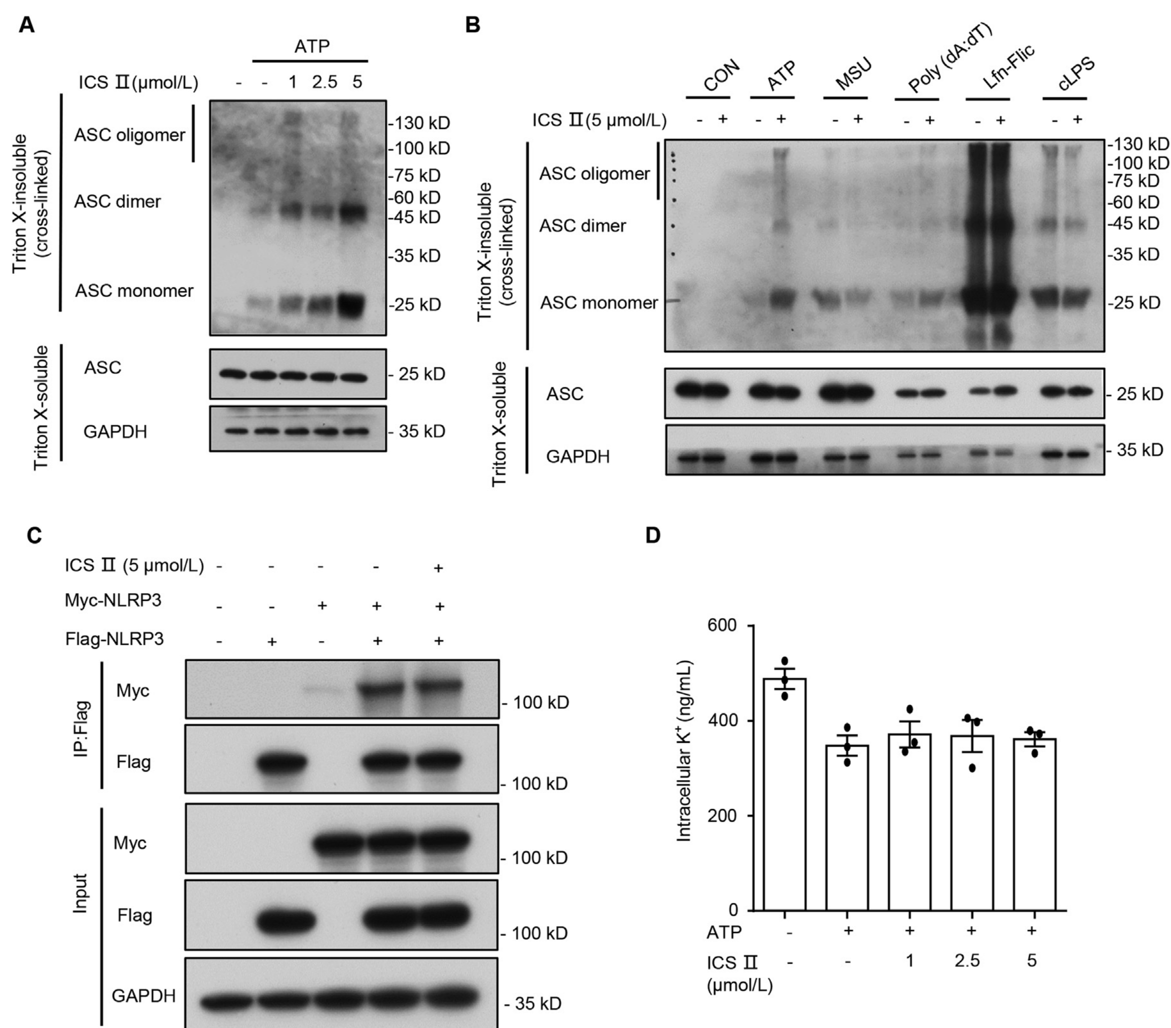


Figure 4 ICS II facilitates NLRP3-dependent ASC oligomerization, but has no impact on NLRP3−NLRP3 interaction and intracellular potassium. (A) Western blot analysis of ASC oligomerization in lysates from BMDMs stimulated with LPS and ATP (1 h) and treated with various doses of ICS II. (B) Western blot analysis of ASC oligomerization in lysates from LPS-primed BMDMs treated with ICS II (5 μmol/L) before ATP (1 h), MSU (6 h), poly(dA:dT) (6 h), Lfn-Flic (6 h) stimulation or Pam3CSK4-primed BMDMs treated with ICS II (5 μmol/L) and then transfected with LPS (6 h). (C) Western blots of cell lysates and flag-immunoprecipitation samples from HEK-293T cells transfected with Flag-NLRP3 and Myc-NLRP3. (D) Qualification of potassium efflux in LPS-primed BMDMs treated with various doses of ICS II and then stimulated with ATP (1 h). Data are means ± SEM ($n = 3$).

inhibitor (MCC950) was administered before ICS II and LPS treatment to suppress the activation of NLRP3 inflammasomes *in vivo*. *In vivo* data demonstrate that MCC950 pretreatment before LPS/ICS II suppressed serum ALT, AST, IL-1β and TNF-α production (Fig. 7A−D). Because mtROS plays an important role in ICS II/LPS-induced IDILI, we next evaluated the level of oxidative stress in the liver. MDA, the end-product of lipid peroxidation, is used widely as a sensitive marker of oxidative stress and contributes to the hepatocyte injury[27,28]. Similar to ALT and AST activity, the levels of MDA in the ICS II/LPS group were higher than that of the other groups (Fig. 7E). In addition, LPS/ICS II enhanced the production of IL-1β p17 and caspase-1 p20, and cleaved GSDMD (a downstream effector of pyroptosis) in the liver tissue (Fig. 7F). On the contrary, the above indicators in the liver decreased substantially when MCC950 was administered before LPS/ICS II treatment (Fig. 7F). These results suggest that NLRP3 inflammasomes play a critical role in ICS II/LPS-induced liver injury.

4. Discussion

IDILI caused by the wide use of conventional drugs is recognized broadly; however, there is deficient understanding of the possible liver injury of herbal preparations all over the world[29−31]. In recent years, the problem of IDILI caused by TCMs, especially the traditional non-toxic Chinese medicines, has become increasingly serious because of increasing awareness of risk recognition and prevention. Several studies have reported that hepatotoxicity can be induced by two Chinese materia medica

from LPS-primed BMDMs treated with ICS II (5 μmol/L) before ATP (1 h), poly(dA:dT) (6 h) or Lfn-Flic (6 h) stimulation. Data are means ± SEM ($n = 3$); $^{###}P < 0.001$ *vs*. the control group; $^{**}P < 0.01$, $^{***}P < 0.001$.

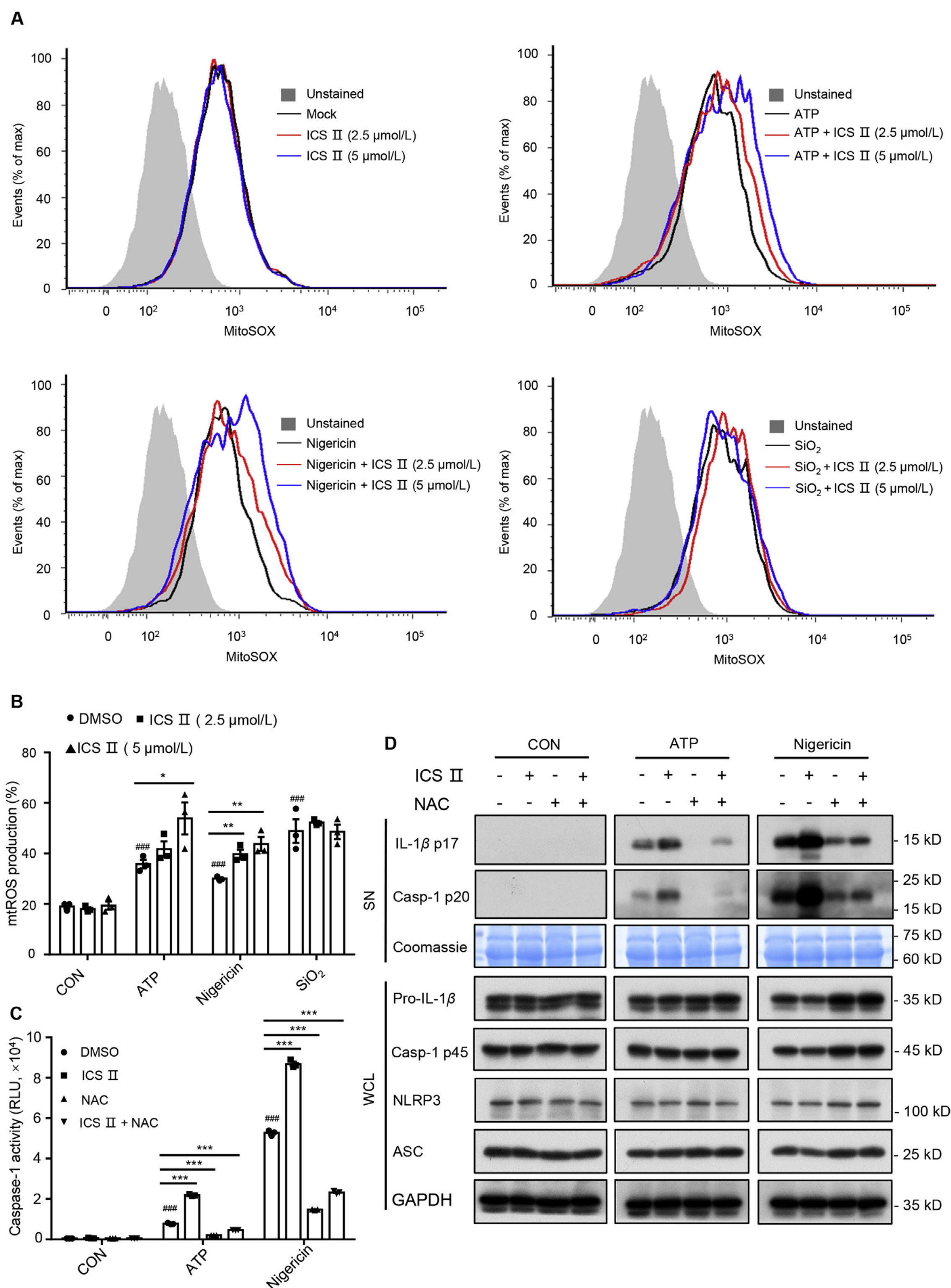


Figure 5 ICS II facilitates ATP/nigericin-induced NLRP3 inflammasome activation dependent on mitochondrial ROS production. LPS-primed BMDMs were untreated or treated with ICS II (2.5 and 5 μmol/L) before no stimulation (A) or stimulation with ATP (B), nigericin or SiO_2. BMDMs were loaded with MitoSOX red mitochondrial superoxide indicator (Ex/Em: 510/580 nm). After staining and washing, flow cytometry was conducted to test mtROS production. Caspase-1 activity (C) and Western blot analysis of IL-1β (p17), caspase-1 (p20) in SN and pro-IL-1β, caspase-1 (p45), NLRP3, ASC in WCL (D) from LPS-primed BMDMs treated with ICS II (5 μmol/L), NAC (2.5 mmol/L) or ICS II (5 μmol/L) plus NAC (2.5 mmol/L), and then stimulated with ATP, nigericin or SiO_2. Data are means ± SEM ($n = 3$); $^{###}P < 0.001$ *vs*. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

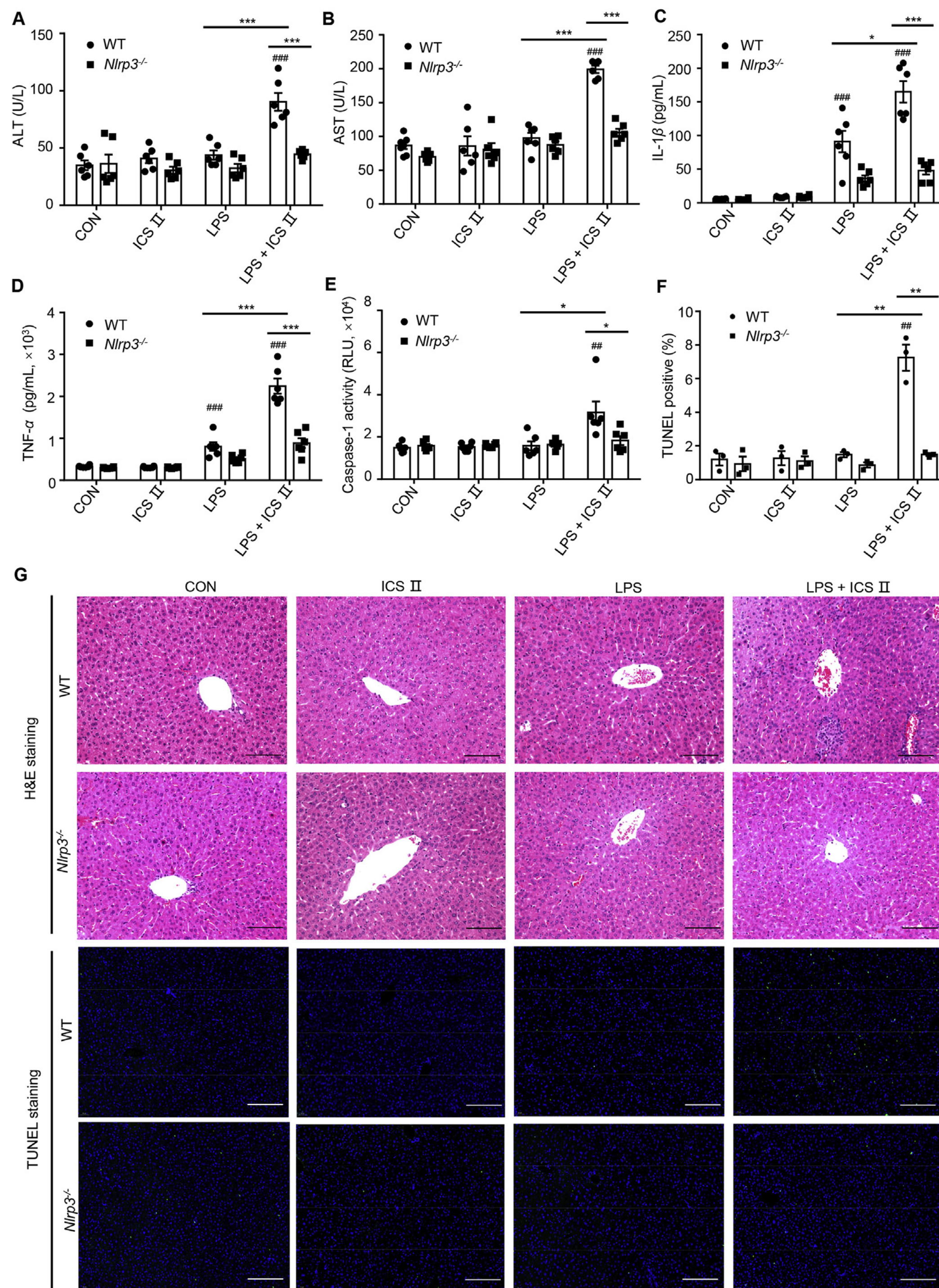


Figure 6 Early liver injury and inflammatory mediator production after LPS/ICS II cotreatment. (A)–(D) WT and *Nlrp3*$^{-/-}$ female C57BL/6 mice were pretreated with LPS (2 mg/kg) through the tail vein. Two hours later, intraperitoneal ICS II (50 mg/kg, $n = 6$) injection was conducted. Six hours after ICS II injection, serum levels of ALT (A), AST (B), IL-1β (C), TNF-α (D) were measured by assay kit. (E) Caspase-1 activity in the livers was detected after BCA protein quantification and normalization processing. H&E staining (G) and TUNEL staining (F) were conducted to observe liver injury and TUNEL positive. Data are means ± EM; $^{##}P < 0.01$, $^{###}P < 0.001$ *vs*. the control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bar: 100 μm.

preparations, namely Zhuang-gu-guan-jie pills and Xian-ling-gu-bao capsules, both of which contain EF[32–34]. Our previous studies demonstrated that EF may cause hepatotoxicity in an LPS-mediated susceptibility mouse model of IDILI. ICS II is the main component of EF, and it is also the major pharmacological metabolite of EF[35,36]. Previous studies have shown that some drugs with the ability to induce DILI, such as amodiaquine and nevirapine, may induce the release of DAMPs from hepatocytes,

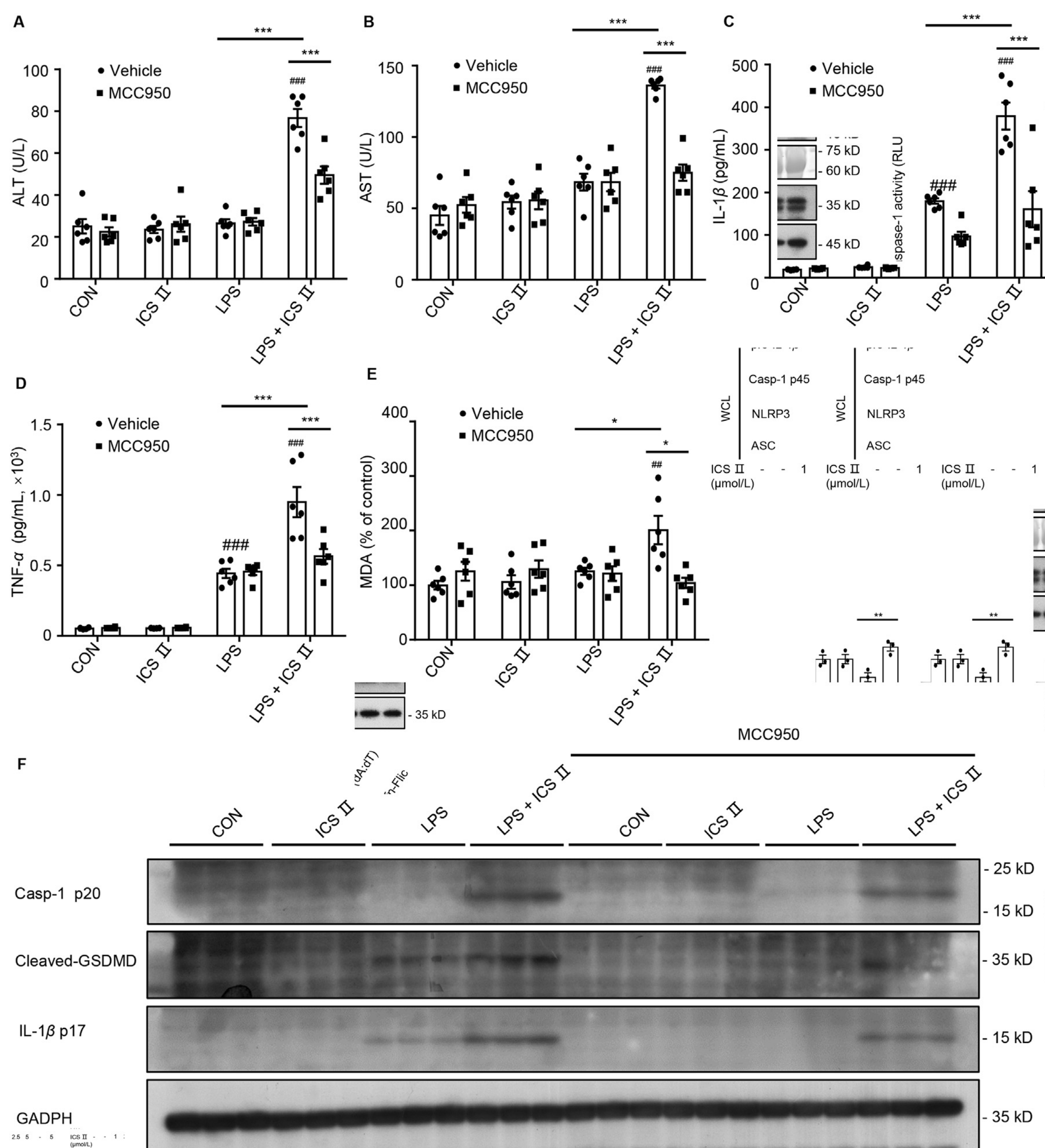


Figure 7 MCC950 pretreatment reverses LPS/ICS II-induced liver injury. (A)−(D) Female C57BL/6 mice were pretreated with MCC950 (50 mg/kg), then treated with LPS (2 mg/kg) through the tail vein. Two hours later, intraperitoneal ICS II (50 mg/kg, $n = 6$) injection was conducted. Six hours after ICS II injection, serum levels of ALT (A), AST (B), IL-1β (C), TNF-α (D) were measured by assay kit. (E) Mouse liver tissue oxidative stress parameter malondialdehyde (MDA) was detected from female C57BL/6 mice pretreated with MCC950 (50 mg/kg) before LPS (2 mg/kg) and ICS II (50 mg/kg, $n = 6$) injection. (F) Western blot analysis of IL-1β (p17), caspase-1 (p20) and cleaved GSDMD in the liver tissue. Data are means ± SEM; $^{##}P < 0.01$, $^{###}P < 0.001$ *vs*. the control; $^{*}P < 0.05$, $^{***}P < 0.001$.

which leads to the activation of NLRP3 inflammasomes in macrophages[37]. Although ICS II inhibits LPS-induced NF-κB signaling pathway *in vitro*[38,39], our research demonstrated that ICS II had no impact on pro-IL-1β expression and TNF-α production in our experiment conditions (stimulated with LPS for 4 h before ICS II treatment). We further found that none of the components of EF could directly activate NLRP3 inflammasome, but when LPS-primed BMDMs were pretreated with ICS II before stimulation with various NLRP3 inflammasome agonists, ICS II specifically enhanced ATP- or nigericin-induced NLRP3 inflammasome activation and pyroptosis.

NLRP3 inflammasome can sense PAMPs and DAMPs, such as extracellular ATP, nigericin, SiO_2, and MSU. Our research indicates that ICS II can specifically reinforce NLRP3 inflammasome activation and pyroptosis induced by ATP or nigericin, but not MSU, SiO_2, or cytosolic LPS. Furthermore, ICS II had no impact on AIM2 and NLRC4 inflammasomes activation. Evidence from well-designed studies has indicated that drugs

cause IDILI synergistically with other risk factors that include drug properties, host factors, and drug—host interaction, which contribute to influence the susceptibility to hepatotoxicity[40,41]. For example, methotrexate-related fatty liver disease and its severity levels have been connected with alcohol abuse, type two diabetes and obesity[42−44]. The inheritable variant of the HLA-B gene, HLA-B*1502, increases sensibility to Stevens—Johnson syndrome and toxic epidermal necrolysis induced by carbamazepine[45]. ICS II specifically amplified ATP- or nigericin-induced NLRP3 inflammasome activation, which suggested that ICS II should be avoided in treating diseases related to ATP- or nigericin-induced NLRP3 inflammasome activation. However, underlying the other agonist-induced NLRP3 inflammasome activation-related diseases, such as MSU-induced gout, SiO_2-induced silicosis, may not be the risk signal of ICS II-induced idiosyncratic liver injury.

To evaluate the mechanism by which ICS II enhanced NLRP3 inflammasome activation triggered by ATP or nigericin, we assessed the effects of ICS II on the upstream and downstream signaling that is associated with NLRP3 inflammasome activation. ICS II could promote ASC oligomerization triggered by ATP rather than SiO_2. However, ASC oligomerization is essential for all stimuli induction of NLRP3 inflammasome, which suggests that ICS II acts on upstream signaling of ASC oligomerization to exacerbate ATP- or nigericin-induced NLRP3 inflammasome activation. MtROS is thought to be important upstream events for NLRP3 inflammasome activation. Interestingly, ATP, nigericin or SiO_2 could induce the production of mtROS, but ICS II treatment intensified mtROS generation triggered by ATP or nigericin but not SiO_2, suggesting that ICS II may enhance ATP- or nigericin-induced NLRP3 inflammasome activation by promoting mtROS production. Further, oxidizing agent H_2O_2 enhanced caspase-1 maturation and IL-1β production triggered by ATP or nigericin, but not SiO_2. Similarly, ROS scavenger NAC and ICS II co-treatment suppressed caspase-1 maturation and IL-1β production when stimulated with ATP or nigericin. Therefore, induction of mtROS production contributes to the enhancement effect of ICS II on NLRP3 inflammasome activation triggered by ATP and nigericin that results in a continuous inflammatory response and ultimately induces liver injury.

NLRP3 inflammasome activation leads to caspase-1-dependent release of IL-1β and pyroptosis. IL-1β serves mainly as a proinflammatory mediator to activate and recruit neutrophils into the tissues[46] and subsequently trigger neutrophil tissue infiltration and liver damage[47,48]. In addition, IL-1β can induce the production of TNF-α[49,50], another key cytokine involved in amplifying and perpetuating the liver damage by triggering liver inflammation, neutrophils, and proinflammatory macrophage recruitment[51−54]. Moreover, NLRP3 inflammasome activation also leads to caspase-1-dependent pyroptosis[8], this distinct form of programmed cell death could mediate liver injury[47,55,56]. In addition, one of important upstream signaling of NLRP3 inflammasome activation is ROS production[57,58], which can lead to mitochondrial dysfunction through an intracellular oxidant stress in hepatocytes leading mainly to oncotic necrosis and less apoptosis[52,59]. Our *in vivo* data demonstrated that LPS/ICS II enhanced the production of IL-1β p17, TNF-α, cleaved GSDMD (the downstream effector of pyroptosis), and the level of oxidative stress in the liver tissue, accompanied by the rise of ALT and AST, which is reversed by MCC950 pretreatment or *Nlrp3* deficiency. These results demonstrated that NLRP3 inflammasome plays a critical role in ICS II/LPS-induced liver injury.

5. Conclusions

The study demonstrated that ICS II exacerbates NLRP3 inflammasome activation and pyroptosis triggered by ATP and nigericin, but not SiO_2, MSU or cytosolic LPS. Synergistic induction of mtROS is a crucial contributor to the enhancing effect of ICS II on ATP- or nigericin-induced NLRP3 inflammasome activation. Our *in vivo* data show that a combination of non-hepatotoxic doses of LPS and ICS II causes the increase of ALT and AST, hepatocyte necrosis and hepatic inflammation in WT mice, which is blocked by MCC950 or *Nlrp3* deficiency. The data suggest that ICS II causes idiosyncratic liver injury through enhancing NLRP3 inflammasome activation and may be a risk factor and responsible for EF-induced liver injury.

Acknowledgments

This work has been supported by National Natural Science Foundation of China (81874368, 81630100, and 81903891), Beijing Nova Program (Z181100006218001, China), National Science & Technology Major Project "Key New Drug Creation and Manufacturing Program" (2017ZX09301022 and 2018ZX09101002-001-002, China), the Innovation Groups of the National Natural Science Foundation of China (81721002). Thanks to pathologists Yanhong Tai and Lina Jiang from the Fifth Medical Centre, Chinese PLA General Hospital (Beijing, China) for the analysis of H&E staining.

Author contributions

Zhilei Wang, Guang Xu and Hongbo Wang: investigation, validation, visualization, software, and original draft. Xiaoyan Zhan and Yuan Gao: investigation, formal analysis, and data curation. Nian Chen: visualization and software. Ruisheng Li: methodology and formal analysis. Xueai Song and Yuming Guo: investigation and software. Ruichuang Yang, Ming Niu and Jiabo Wang: visualization and formal analysis. Youping Liu, Xiaohe Xiao and Zhaofang Bai: conceptualization, methodology, validation, resources, data curation, supervision, project administration, review & editing, and funding acquisition.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.03.006.

References

1. Fontana RJ. Pathogenesis of idiosyncratic drug-induced liver injury and clinical perspectives. *Gastroenterology* 2014;**146**:914−28.
2. Kullak-Ublick GA, Andrade RJ, Merz M, End P, Benesic A, Gerbes AL, et al. Drug-induced liver injury: recent advances in diagnosis and risk assessment. *Gut* 2017;**66**:1154−64.
3. Shen T, Liu Y, Shang J, Xie Q, Li J, Yan M, et al. Incidence and etiology of drug-induced liver injury in mainland China. *Gastroenterology* 2019;**156**:2230−41.
4. Bjornsson ES. Drug-induced liver injury: an overview over the most critical compounds. *Arch Toxicol* 2015;**89**:327−34.

5. Lin L, Ni B, Lin H, Zhang M, Li X, Yin X, et al. Traditional usages, botany, phytochemistry, pharmacology and toxicology of *Polygonum multiflorum* Thunb.: a review. *J Ethnopharmacol* 2015;**159**:158−83.
6. Lin G, Wang JY, Li N, Li M, Gao H, Ji Y, et al. Hepatic sinusoidal obstruction syndrome associated with consumption of *Gynura segetum*. *J Hepatol* 2011;**54**:666−73.
7. Sun W, Zeng C, Liu S, Fu J, Hu L, Shi Z, et al. *Ageratina adenophora* induces mice hepatotoxicity *via* ROS-NLRP3-mediated pyroptosis. *Sci Rep* 2018;**8**:16032.
8. He Y, Hara H, Nunez G. Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci* 2016;**41**:1012−21.
9. Elliott EI, Sutterwala FS. Initiation and perpetuation of NLRP3 inflammasome activation and assembly. *Immunol Rev* 2015;**265**:35−52.
10. Zhou X, Wu Y, Ye L, Wang Y, Zhang K, Wang L, et al. Aspirin alleviates endothelial gap junction dysfunction through inhibition of NLRP3 inflammasome activation in LPS-induced vascular injury. *Acta Pharm Sin B* 2019;**9**:711−23.
11. Wang Z, Xu G, Gao Y, Zhan X, Qin N, Fu S, et al. Cardamonin from a medicinal herb protects against LPS-induced septic shock by suppressing NLRP3 inflammasome. *Acta Pharm Sin B* 2019;**9**:734−44.
12. Wu X, Dong L, Lin X, Li J. Relevance of the NLRP3 Inflammasome in the pathogenesis of chronic liver disease. *Front Immunol* 2017;**8**: 1728.
13. Szabo G, Csak T. Inflammasomes in liver diseases. *J Hepatol* 2012;**57**: 642−54.
14. Wree A, Eguchi A, McGeough MD, Pena CA, Johnson CD, Canbay A, et al. NLRP3 inflammasome activation results in hepatocyte pyroptosis, liver inflammation, and fibrosis in mice. *Hepatology* 2014;**59**:898−910.
15. Ma H, He X, Yang Y, Li M, Hao D, Jia Z. The genus *Epimedium*: an ethnopharmacological and phytochemical review. *J Ethnopharmacol* 2011;**134**:519−41.
16. Zhang L, Wang T, Zhao BS, Zhang JX, Yang S, Fan CL, et al. Effect of 2″-*O*-rhamnosyl icariside II, baohuoside I and baohuoside II in herba Epimedii on cytotoxicity indices in HL-7702 and HepG2 cells. *Molecules* 2019;**24**:1263.
17. Zhong R, Chen Y, Ling J, Xia Z, Zhan Y, Sun E, et al. The toxicity and metabolism properties of Herba Epimedii flavonoids on laval and adult zebrafish. *Evid Based Complement Alternat Med* 2019;**2019**: 3745051.
18. Gao Y, Wang Z, Tang J, Liu X, Shi W, Qin N, et al. New incompatible pair of TCM: Epimedii Folium combined with Psoraleae Fructus induces idiosyncratic hepatotoxicity under immunological stress conditions. *Front Med* 2020;**14**:68−80.
19. He H, Jiang H, Chen Y, Ye J, Wang A, Wang C. Oridonin is a covalent NLRP3 inhibitor with strong anti-inflammasome activity. *Nat Commun* 2018;**9**:2550.
20. Dan Dunn J, Alvarez LA, Zhang X, Soldati T. Reactive oxygen species and mitochondria: a nexus of cellular homeostasis. *Redox Biol* 2015;**6**: 472−85.
21. Ganey PE, Luyendyk JP, Maddox JF, Roth RA. Adverse hepatic drug reactions: inflammatory episodes as consequence and contributor. *Chem Biol Interact* 2004;**150**:35−51.
22. Roth RA, Ganey PE. Animal models of idiosyncratic drug-induced liver injury—current status. *Crit Rev Toxicol* 2011;**41**:723−39.
23. Yee SB, Kinser S, Hill DA, Barton CC, Hotchkiss JA, Harkema JR, et al. Synergistic hepatotoxicity from coexposure to bacterial endotoxin and the pyrrolizidine alkaloid monocrotaline. *Toxicol Appl Pharmacol* 2000;**166**:173−85.
24. Shaw PJ, Ditewig AC, Waring JF, Liguori MJ, Blomme EA, Ganey PE, et al. Coexposure of mice to trovafloxacin and lipopolysaccharide, a model of idiosyncratic hepatotoxicity, results in a unique gene expression profile and interferon gamma-dependent liver injury. *Toxicol Sci* 2009;**107**:270−80.
25. Hassan HM, Guo H, Yousef BA, Ping-Ping D, Zhang L, Jiang Z. Dexamethasone pretreatment alleviates isoniazid/lipopolysaccharide hepatotoxicity: inhibition of inflammatory and oxidative stress. *Front Pharmacol* 2017;**8**:133.
26. Luyendyk JP, Maddox JF, Green CD, Ganey PE, Roth RA. Role of hepatic fibrin in idiosyncrasy-like liver injury from lipopolysaccharide-ranitidine coexposure in rats. *Hepatology* 2004;**40**:1342−51.
27. Bao W, Li K, Rong S, Yao P, Hao L, Ying C, et al. Curcumin alleviates ethanol-induced hepatocytes oxidative damage involving heme oxygenase-1 induction. *J Ethnopharmacol* 2010;**128**:549−53.
28. Xu L, Yu Y, Sang R, Li J, Ge B, Zhang X. Protective effects of taraxasterol against ethanol-induced liver injury by regulating CYP2E1/Nrf2/HO-1 and NF-κB signaling pathways in mice. *Oxid Med Cell Longev* 2018;**2018**:8284107.
29. Navarro VJ, Khan I, Bjornsson E, Seeff LB, Serrano J, Hoofnagle JH. Liver injury from herbal and dietary supplements. *Hepatology* 2017; **65**:363−73.
30. Teschke R, Frenzel C, Glass X, Schulze J, Eickhoff A. Herbal hepatotoxicity: a critical review. *Br J Clin Pharmacol* 2013;**75**:630−6.
31. Cho JH, Oh DS, Hong SH, Ko H, Lee NH, Park SE, et al. A nationwide study of the incidence rate of herb-induced liver injury in Korea. *Arch Toxicol* 2017;**91**:4009−15.
32. Cheng Y, Liu Y, Wang H, Li J, Ren J, Zhu L, et al. A 26-week repeated dose toxicity study of Xian-ling-gu-bao in Sprague−Dawley rats. *J Ethnopharmacol* 2013;**145**:85−93.
33. Wu H, Zhong Q, Wang J, Wang M, Fang F, Xia Z, et al. Beneficial effects and toxicity studies of Xian-ling-gu-bao on bone metabolism in ovariectomized rats. *Front Pharmacol* 2017;**8**:273.
34. Wu W, Wang T, Sun B, Liu D, Lin Z, Miao Y, et al. Xian-Ling-Gu-Bao induced inflammatory stress rat liver injury: inflammatory and oxidative stress playing important roles. *J Ethnopharmacol* 2019;**239**: 111910.
35. Xu W, Zhang Y, Yang M, Shen Z, Zhang X, Zhang W, et al. LC−MS/MS method for the simultaneous determination of icariin and its major metabolites in rat plasma. *J Pharmaceut Biomed Anal* 2007; **45**:667−72.
36. Teo YL, Cheong WF, Cazenave-Gassiot A, Ji S, Logan S, Lee ZXK, et al. Pharmacokinetics of prenylflavonoids following oral ingestion of standardized epimedium extract in humans. *Planta Med* 2019;**85**: 347−55.
37. Kato R, Uetrecht J. Supernatant from hepatocyte cultures with drugs that cause idiosyncratic liver injury activates macrophage inflammasomes. *Chem Res Toxicol* 2017;**30**:1327−32.
38. Song J, Feng L, Zhong R, Xia Z, Zhang L, Cui L, et al. Icariside II inhibits the EMT of NSCLC cells in inflammatory microenvironment *via* down-regulation of Akt/NF-κB signaling pathway. *Mol Carcinog* 2017;**56**:36−48.
39. Yan N, Wen DS, Zhao YR, Xu SJ. *Epimedium sagittatum* inhibits TLR4/MD-2 mediated NF-κB signaling pathway with anti-inflammatory activity. *BMC Compl Alternative Med* 2018;**18**:303.
40. Bjornsson ES. Epidemiology and risk factors for idiosyncratic drug-induced liver injury. *Semin Liver Dis* 2014;**34**:115−22.
41. Chalasani N, Reddy KRK, Fontana RJ, Barnhart H, Gu J, Hayashi PH, et al. Idiosyncratic drug induced liver injury in African−Americans is associated with greater morbidity and mortality compared to caucasians. *Am J Gastroenterol* 2017;**112**:1382−8.
42. Rosenberg P, Urwitz H, Johannesson A, Ros AM, Lindholm J, Kinnman N, et al. Psoriasis patients with diabetes type 2 are at high risk of developing liver fibrosis during methotrexate treatment. *J Hepatol* 2007;**46**:1111−8.
43. Laharie D, Seneschal J, Schaeverbeke T, Doutre MS, Longy-Boursier M, Pellegrin JL, et al. Assessment of liver fibrosis with transient elastography and FibroTest in patients treated with methotrexate for chronic inflammatory diseases: a case-control study. *J Hepatol* 2010;**53**:1035−40.
44. Aithal GP. Dangerous liaisons: drug, host and the environment. *J Hepatol* 2007;**46**:995−8.
45. Usui T, Naisbitt DJ. Human leukocyte antigen and idiosyncratic adverse drug reactions. *Drug Metabol Pharmacokinet* 2017;**32**:21−30.
46. Bajt ML, Farhood A, Jaeschke H. Effects of CXC chemokines on neutrophil activation and sequestration in hepatic vasculature. *Am J Physiol Gastrointest Liver Physiol* 2001;**281**:G1188−95.

47. Geng Y, Ma Q, Liu YN, Peng N, Yuan FF, Li XG, et al. Heatstroke induces liver injury *via* IL-1β and HMGB1-induced pyroptosis. *J Hepatol* 2015;**63**:622−33.
48. Huebener P, Pradere JP, Hernandez C, Gwak GY, Caviglia JM, Mu X, et al. The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis. *J Clin Invest* 2015;**125**:539−50.
49. Granowitz EV, Vannier E, Poutsiaka DD, Dinarello CA. Effect of interleukin-1 (IL-1) blockade on cytokine synthesis: II. IL-1 receptor antagonist inhibits lipopolysaccharide-induced cytokine synthesis by human monocytes. *Blood* 1992;**79**:2364−9.
50. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 2009;**27**:519−50.
51. Bieghs V, Trautwein C. The innate immune response during liver inflammation and metabolic disease. *Trends Immunol* 2013;**34**:446−52.
52. Schwabe RF, Brenner DA. Mechanisms of Liver Injury. I. TNF-α-induced liver injury: role of IKK, JNK, and ROS pathways. *Am J Physiol Gastrointest Liver Physiol* 2006;**290**:G583−9.
53. Wree A, McGeough MD, Inzaugarat ME, Eguchi A, Schuster S, Johnson CD, et al. NLRP3 inflammasome driven liver injury and fibrosis: roles of IL-17 and TNF in mice. *Hepatology* 2018;**67**:736−49.
54. Wang Z, Xu G, Zhan X, Liu Y, Gao Y, Chen N, et al. Carbamazepine promotes specific stimuli-induced NLRP3 inflammasome activation and causes idiosyncratic liver injury in mice. *Arch Toxicol* 2019;**93**:3585−99.
55. Zhang LY, Zhan DL, Chen YY, Wang WH, He CY, Lin Y, et al. Aflatoxin B1 enhances pyroptosis of hepatocytes and activation of Kupffer cells to promote liver inflammatory injury *via* dephosphorylation of cyclooxygenase-2: an *in vitro, ex vivo* and *in vivo* study. *Arch Toxicol* 2019;**93**:3305−20.
56. Chen YL, Xu G, Liang X, Wei J, Luo J, Chen GN, et al. Inhibition of hepatic cells pyroptosis attenuates CLP-induced acute liver injury. *Am J Transl Res* 2016;**8**:5685−95.
57. Minutoli L, Puzzolo D, Rinaldi M, Irrera N, Marini H, Arcoraci V, et al. ROS-mediated NLRP3 inflammasome activation in brain, heart, kidney, and testis ischemia/reperfusion injury. *Oxid Med Cell Longev* 2016;**2016**:2183026.
58. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* 2011;**469**:221−5.
59. Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury: present concepts. *J Gastroenterol Hepatol* 2011;**26**(Suppl 1):173−9.