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

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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 (SiO2), monosodium urate (MSU) crystal or cytosolic lipopolysaccharide (LPS).

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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\(^1\). Recently, several studies have pointed out that traditional Chinese medicines (TCMs) and anti-tuberculosis drugs are the primary causes of IDILI in China\(^2\). Specifically, liver injury caused by TCMs, such as Polygonum multiflorum, Gynura segetum, and Ageratina adenophora, has occurred frequently in recent years\(^3\). 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\(^9\). 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\(^12\), 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 through enhancing NLRP3 inflammasome activation and suggest that EF II may be a risk factor and responsible for EF-induced 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 mmol/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 \(1 \times 10^6\) 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, icarin, icaritin, icariside I, ICS II, anhydroicaritin; TargetMol, Boston, MA, USA), hydrogen peroxide (H$_2$O$_2$, 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(ethylene) (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 x 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 x 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 with 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 x 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 x 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 x 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 x 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 x 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. BMDMs were plated at 5 x 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 MitoTracker Red (50 nmol/L, Thermo Fisher Scientific), then cells were washed three times by PBS and fixed with 4% formaldehyde in PBS for 15 min. After that, cells were washed with PBS 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. BMDCs were seeded in 96-well growth-medium plate overnight at 1 × 10^5 cells/well. Then, LPS-primed BMDCs 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

BMDCs were put onto 100 mm diameter culture dish tubes and primed with LPS (50 ng/mL) for 4 h. Then, the LPS-primed BMDCs 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, BMDCs 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 were 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, LPS 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 hours 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 BMDCs 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 BMDCs 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 BMDCs (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
The expression of NLRP3 and pro-IL-1β 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-1β secretion were induced when BMDMs were cotreat with LPS and ATP. Interestingly, ICS II significantly promoted ATP-induced caspase-1 activation and IL-1β production in LPS-primed BMDMs (Figs. 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-1β 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-1β 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, SiO2, 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,
IL-1β secretion and TNF-α production triggered by MSU (Fig. 3A–C, and Supporting Information Fig. S4A), SiO₂ (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.

**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.
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. S7A). However, ICS II had no impact on ASC oligomerization induced by MSU, poly(dA:dT), Lfn-Flc 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 SiO₂ (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. 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 SiO₂ 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 SiO₂ 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 H₂O₂ or NAC (a scavenger of ROS) before ATP, nigericin or SiO₂ stimulation. Western blot analysis show that H₂O₂ enhanced caspase-1 maturation and IL-1β production in a certain dose range triggered by ATP or nigericin, but not SiO₂ (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. Non-hepatotoxic doses of LPS decrease the threshold for toxicity and/or increase the magnitude of response. 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. 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. We therefore evaluated whether ICS II, an active constituent that promotes NLRP3 inflammasome activation, could result in hepatotoxicity 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.
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. 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. 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

**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).
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 SiO2. 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), 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 SiO2. Data are means ± SEM (n = 3); ### P < 0.001 vs. the control group; * P < 0.05, ** P < 0.01, *** P < 0.001.
preparations, namely Zhuang-gu-guan-jie pills and Xian-ling-gu-bao capsules, both of which contain EF. 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. 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 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.
which leads to the activation of NLRP3 inflammasomes in macrophages. Although ICS II inhibits LPS-induced NF-κB signaling pathway in vitro, 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, SiO2, and MSU. Our research indicates that ICS II can specifically reinforce NLRP3 inflammasome activation and pyroptosis induced by ATP or nigericin, but not MSU, SiO2, or cytosolic LPS. Furthermore, ICS II had no impact on AIM2 and NLRC4 inflammasomes activation. Evidence from well-designed studies has indicated that drugs

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.
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\cite{40,41}. For example, methotrexate-related fatty liver disease and its severity levels have been connected with alcohol abuse, type two diabetes and obesity\cite{42–44}. The inheritable variant of the HLA-B gene, HLA-B*1502, increases sensitivity to Stevens–Johnson syndrome and toxic epidermal necrolysis induced by carbamazepine\cite{35}. 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, SiO2-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 SiO2. 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 SiO2 could induce the production of mtROS, but ICS II treatment intensified mtROS generation triggered by ATP or nigericin but not SiO2, suggesting that ICS II may enhance ATP- or nigericin-induced NLRP3 inflammasome activation by promoting mtROS production. Further, oxidizing agent H2O2 enhanced caspase-1 maturation and IL-1β production triggered by ATP or nigericin, but not SiO2. 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\cite{46} and subsequently trigger neutrophil tissue infiltration and liver damage\cite{47,48}. In addition, IL-1β can induce the production of TNF-α\cite{49,50}, another key cytokine involved in amplifying and perpetuating the liver damage by triggering liver inflammation, neutrophils, and proinflammatory macrophage recruitment\cite{51–54}. Moreover, NLRP3 inflammasome activation also leads to caspase-1-dependent pyroptosis\cite{5}, this distinct form of programmed cell death could mediate liver injury\cite{55,56}. In addition, one of important upstream signaling of NLRP3 inflammasome activation is ROS production\cite{57,58}, which can lead to mitochondrial dysfunction through an intracellular oxidant stress in hepatocytes leading mainly to oncocytic necrosis and less apoptosis\cite{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 SiO2, 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.

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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, Xiaohao 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.

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