Front. Med. 2021, 15(4): 594–607
https://doi.org/10.1007/s11684-020-0809-2

RESEARCH ARTICLE

Bavachin enhances NLRP3 inflammasome activation induced by ATP or nigericin and causes idiosyncratic hepatotoxicity

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Abstract **Psoraleae Fructus (PF) is a well-known traditional herbal medicine in China, and it is widely used for osteoporosis, vitiligo, and other diseases in clinical settings. However, liver injury caused by PF and its preparations has been frequently reported in recent years. Our previous studies have demonstrated that PF could cause idiosyncratic drug-induced liver injury (IDILI), but the mechanism underlying its hepatotoxicity remains unclear. This paper reports that bavachin isolated from PF enhances the specific stimuli-induced activation of the NLRP3 inflammasome and leads to hepatotoxicity. Bavachin boosts the secretion of IL-1β and caspase-1 caused by ATP or nigericin but not those induced by poly(I:C), monosodium urate crystal, or intracellular lipopolysaccharide. Bavachin does not affect AIM2 or NLRC4 inflammasome activation. Mechanistically, bavachin specifically increases the production of nigericin-induced mitochondrial reactive oxygen species among the most important upstream events in the activation of the NLRP3 inflammasome. Bavachin increases the levels of aspartate transaminase and alanine aminotransferase in serum and hepatocyte injury accompanied by the secretion of IL-1β via a mouse model of lipopolysaccharide-mediated susceptibility to IDILI. These results suggest that bavachin specifically enhances the ATP- or nigericin-induced activation of the NLRP3 inflammasome. Bavachin also potentially contributes to PF-induced idiosyncratic hepatotoxicity. Moreover, bavachin and PF should be evaded among patients with diseases linked to the ATP- or nigericin-mediated activation of the NLRP3 inflammasome, which may be a dangerous factor for liver injury.**

Keywords Psoraleae Fructus; bavachin; idiosyncratic drug-induced liver injury; caspase-1; IL-1β; NLRP3 inflammasome

Introduction

Idiosyncratic drug-induced liver injury (IDILI) is an occasional, serious disease that occurs only in a small number of individual patients exposed to drugs. The occurrence of IDILI is dependent on individual susceptibility factors, such as host genetic and immunologic factors, rather than drug dose, route, or the duration of administration [1]. Therefore, IDILI is difficult to study effectively using current preclinical drug safety evaluation methods. Growing evidence has indicated that various cases of IDILI are induced by the immune response, and several immune-related hypotheses have emerged to explain the etiological mechanism of IDILI. Inflammatory mediators, such as lipopolysaccharide (LPS) and tumor necrosis factor (TNF), have been used to mimic inflammatory-related IDILI susceptibility factors in animal models [2]. Some features of human IDILI are reproduced

Received January 1, 2020; accepted June 3, 2020
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in animals with concurrent exposure to LPS, and a number of drugs can cause IDILI, including diclofenac [3], ranitidine [4], halothane [5], and trovafloxacin [6]. The combination of anti-CTLA-4 and Pd-1$^{-/-}$ mouse antibodies [7] also revealed that amodiaquine can induce IDILI [7,8].

The inflammasome, which consists of innate immune sensors, ASC, and pro-caspase-1, is a type of multiprotein complex in the cytoplasm that participates in the immune response [9]. The sensors of the innate immune system contain NOD-like receptor (NLR) family members, such as NLRP1, NLRP3, and NLRC4. Similar to non-NLR receptors, AIM2 and IFI16 can be assembled to form multiple subtypes of inflammasomes, of which the NLRP3, AIM2, and NLRC4 inflammasomes are the most widely studied and most closely related to many diseases [10]. The NLRP3 inflammasome is triggered by endogenous or exogenous factors, such as ATP, and by exposure to nigericin, MSU, and poly(I:C) [11]. AIM2 assembles with caspase-1 and ASC to form the AIM2 inflammasome in response to the recognition of the double-stranded DNA of pathogenic microorganisms or host cells [12], whereas the NLRC4 inflammasome is stimulated by flagella proteins in bacteria, such as those in *Salmonella*, *Legionella*, and *Pseudomonas aeruginosa* [13]. Three types of inflammasomes cause pyroptosis and induce the production of IL-1β and IL-18, resulting in many inflammatory diseases, including systemic lupus erythematosus [14], rheumatoid arthritis [15], gout [16], type 2 diabetes [17], and Alzheimer's disease [18].

The activation of inflammasomes requires two signaling pathways in coordination [10,11,19]. In the first signaling pathway, the TLR4 receptor binds to LPS, stimulating NF-κB to induce the production of pro-IL-1β and pro-IL-18, which is considered a priming event. Then, the expression levels of NLRP3 inflammasome constituent proteins, such as NLRP3, pro-IL-1β, and pro-IL-18, are upregulated. The second signaling pathway is based on the priming event. Under the stimulation of damage-related molecular patterns (DAMPs), such as ATP, and pathogen-related molecular patterns (PAMPs), such as nigericin, a multimeric complex assembled by ASC, NLRP3, and pro-caspase-1 induces the self-shear of pro-caspase-1. Then, pro-IL-1β and pro-IL-18 are cleaved by the activated caspase-1 to become their mature forms, extracellular IL-1β and IL-18, respectively, which participate in numerous inflammatory responses aside from inducing cell death.

Recent studies have indicated that abnormal activation of the NLRP3 inflammasome is associated with liver diseases, such as hepatic fibrosis, viral hepatitis, drug-induced liver injury, and nonalcoholic fatty liver disease [20]. In particular, recent studies have manifested that the NLRP3 inflammasome is extensively involved in idiosyncratic liver damage induced by drugs, such as nevirapine, amodiaquine [21], and carbamazepine [22].

With the rapidly increasing use of herbal medicine worldwide, liver injury induced by herbal medicines (DILI) has been reported frequently in recent years [23]. Psoraleae Fructus (PF), which is the dried ripe fruit of the leguminous plant *Psoralea corylifolia* L., is a traditional medicine for diseases, such as osteoporosis and vitiligo [24,25]. Various traditional Chinese medicine preparations containing PF are currently widely used in clinics in China [26]. Recent studies have shown that PF induces liver injury, raising serious concern about its safety [27]. Similarly, our previous study demonstrated that PF can cause liver injury and inflammasome activation in a mouse model of LPS-mediated susceptibility to IDILI [28]. However, the mechanism underlying the hepatotoxicity of PF remains unclear. The present study showed that bavachin, a compound of PF, can induce idiosyncratic liver injury by accelerating specific stimulus-mediated NLRP3 inflammasome activation, which may potentially contribute to PF-induced idiosyncratic hepatotoxicity.

Materials and methods

Animals

Female C57BL/6 mice (6–8 weeks) weighing 18–20 g were obtained from SPF Biotechnology Co., Ltd. (License No. SCXK2016-0002, Beijing, China). The female mice had access to water and food *ad libitum* in a room of 20–22 °C with 45%–50% relative humidity and a 12 h dark/light cycle for a week before the test. The animals in this study were used with the approval of the Ethics Committee of the Fifth Medical Center of the People's Liberation Army of Chinese. Bavachin was dissolved in 10% Tween 80 and 90% PBS (vehicle), and LPS was dissolved in saline solution.

Reagents and antibodies

Isobavachin, bavachin, backuchiol, psoralidin, bavachinin, and neobavaisoflavone were obtained from Med Chem Express (USA). MCC950 was purchased from TargetMol (USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin solution were obtained from HyClone (USA). ATP, Nigericin, SiO_2, MSU, dimethyl sulfoxide (DMSO), LPS (*Escherichia coli*, 055: B5), polyinosinic acid:polycytidylic acid (poly(I:C)), and poly(deoxyadenylic-thymidylic) acid sodium salt (poly(dA:dT)) were supplied by Sigma (Germany). Pam3CSK4 was attained from InvivoGen (France). Dextran sulfate sodium salt (DSS) was obtained from MP Biomedical (USA). Macrophage colony-stimulating factor (M-CSF) was gained from R&D Systems (USA). *Salmonella* was provided by Dr. Tao Li of the National Center of Biomedical Analysis. The following antibodies were used for Western blot: anti-mouse caspase-1 (1:1000, AG-20B-0042) from Adipogen

(USA) and anti-NLRP3 (1:2000, 15101S) and anti-mouse IL-1β (1:1000, 12507) from Cell Signaling Technology (USA). Anti-ASC (1:1000, sc-22514-R) was acquired from Santa Cruz Biotechnology (USA), and anti-GAPDH (1:2000, 60004-1-1g) was obtained from Proteintech (USA).

Cell culture

Bone marrow-derived macrophages (BMDMs) were collected from female C57BL/6 mice (10 weeks) and incubated in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (P/S), and 50 ng/mL M-CSF. The cells were cultured in humidity 5% CO_2 at 37 °C.

NLRP3 inflammasome activation *in vitro* model

BMDMs were cultured in a 24-well plate at a density 8.5×10^5 cells/mL. After preculturing for 12 h in an incubator, the cells were pretreated with 50 ng/mL LPS for 4 h, and then Opti-MEM serum-free medium containing bavachin was added. At 1 h after pretreatment, the BMDMs were induced with ATP (5 mmol/L), nigericin (7.5 μmol/L) for 1 h, MSU (200 mg/mL), *Salmonella* for 4 h or transfected with poly(I:C) (2 μg/mL) or poly(dA:dT) (2 μg/mL) for 6 h. For noncanonical inflammasome activation, the BMDMs were induced with Pam3CSK4 (1 μg/mL) for 4 h, incubated with Opti-MEM containing bavachin for 1 h, and then stimulated and transfected with LPS (1 μg/mL) for 6 h.

Caspase-1 activity assay

Caspase-1 activity was tested by Caspase-Glo® 1 inflammasome assay (Promega, Beijing, China). Culture supernatants were transferred to a 96-well white plate, and then Caspase-Glo reagent was added to each well and incubated for 1 h at room temperature protected from light. The amount of caspase-1 luminescence was measured by a microplate reader.

Western blot

Protein was extracted from the culture supernatants as described above. The expression levels of IL-1β p17, caspase-1 p20, pro-IL-1β, pro-caspase-1, NLRP3, and ASC were measured using Western blot. GAPDH was simultaneously used as a loading control. The samples were separated in a 10% or 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane, and then blocked with 5% skimmed milk for 1 h. The membrane was incubated overnight with primary antibodies at 4 °C, washed three times with tri-buffered saline Tween-20, and then probed with the appropriate HRP-coupled secondary antibody. The membrane was washed again and then conjugated with peroxidase for enhanced chemiluminescence detection (Amersham Pharmacia Biotech).

Cytokine analysis by ELISA

Supernatants were obtained from the cell culture, and the cytokine levels of serum were measured using their corresponding ELISA kits for mouse IL-1β or TNF-α (Dakawe, Beijing, China) following the manufacturer's description.

ASC oligomerization

ASC oligomerization assay was conducted as described previously [29].

Intracellular potassium determination

The assay for intracellular potassium was conducted as described previously [30].

Confocal microscopy

Confocal microscopy analysis was performed as previously described to determine mitochondrial damage [30].

Serum biochemistry

The levels of serum alanine aminotransferase (ALT) and aspartate transaminase (AST) were evaluated using ALT and AST assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively.

Mitochondrial reactive oxygen species assay

The assay for mitochondrial reactive oxygen species was performed as described previously [22].

LPS/bavachin cotreatment-induced liver injury *in vivo*

Female C57BL/6 (6–8 weeks) mice received LPS (2 mg/kg) in a saline vehicle by intravenous injection into the tail for 2 h. Then, the mice were injected intraperitoneally with bavachin (25 mg/kg). The serum was harvested from the mice 6 h after bavachin was administered.

In the second experiment, female C57BL/6 (6–8 weeks) mice were pretreated with MCC950 (50 mg/kg) or its vehicle for 1 h by intraperitoneal injection, and then the mice were administered with LPS (2 mg/kg) or its saline vehicle by intravenous injection into the tail. After 2 h, bavachin (25 mg/kg) or its vehicle was treated via intraperitoneal injection. Mouse serum was collected 6 h after bavachin was administered.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) in each experimental group. Statistical analysis was conducted with unpaired Student's *t*-test or one-way ANOVA (GraphPad San Diego, CA, US). Statistical significance was considered at $P < 0.05$.

Results

Bavachin specifically promotes the activation of the NLRP3 inflammasome triggered by ATP or nigericin

In this study, we selected compounds from PF (angelicin, isobavachin, bavachin, backuchiol, psoralidin, bavachinin, neobavaisoflavone, and psoralen) to determine whether or not these components affect the activation of the NLRP3 inflammasome in LPS-primed BMDMs. None of these compounds directly induced caspase-1 activation (Fig. 1A). However, when LPS-primed BMDMs were performed with these compounds before being stimulated with ATP, bavachin promoted the production of the caspase-1 p20 triggered by ATP, suggesting that bavachin promotes ATP-induced NLRP3 inflammasome activation (Fig. 1B).

To explore the impact of bavachin on the NLRP3 inflammasome, we next examined the dose–effect relationship of bavachin with ATP-induced NLRP3 inflammasome activation. Results indicated that bavachin enhanced the maturation of caspase-1 p20, IL-1β p17 induced by ATP in LPS-primed BMDMs in a dose-dependent manner (Fig. 2A – 2C). We also examined the effect of bavachin on nigericin-stimulated NLRP3 inflammasome activation, and results showed that bavachin facilitated the production of caspase-1 and IL-1β that was triggered by nigericin in LPS-primed BMDMs in a dose-dependent manner (Fig. 2D – 2F). However, the expression of inflammasome proteins, including NLRP3, pro-caspase-1, pro-IL-1β, and ASC, was not affected by bavachin in the cell lysates (Fig. 2A and 2D). TNF-α, an inflammasome-independent cytokine, was not impaired by bavachin (Fig. 2G and 2H).

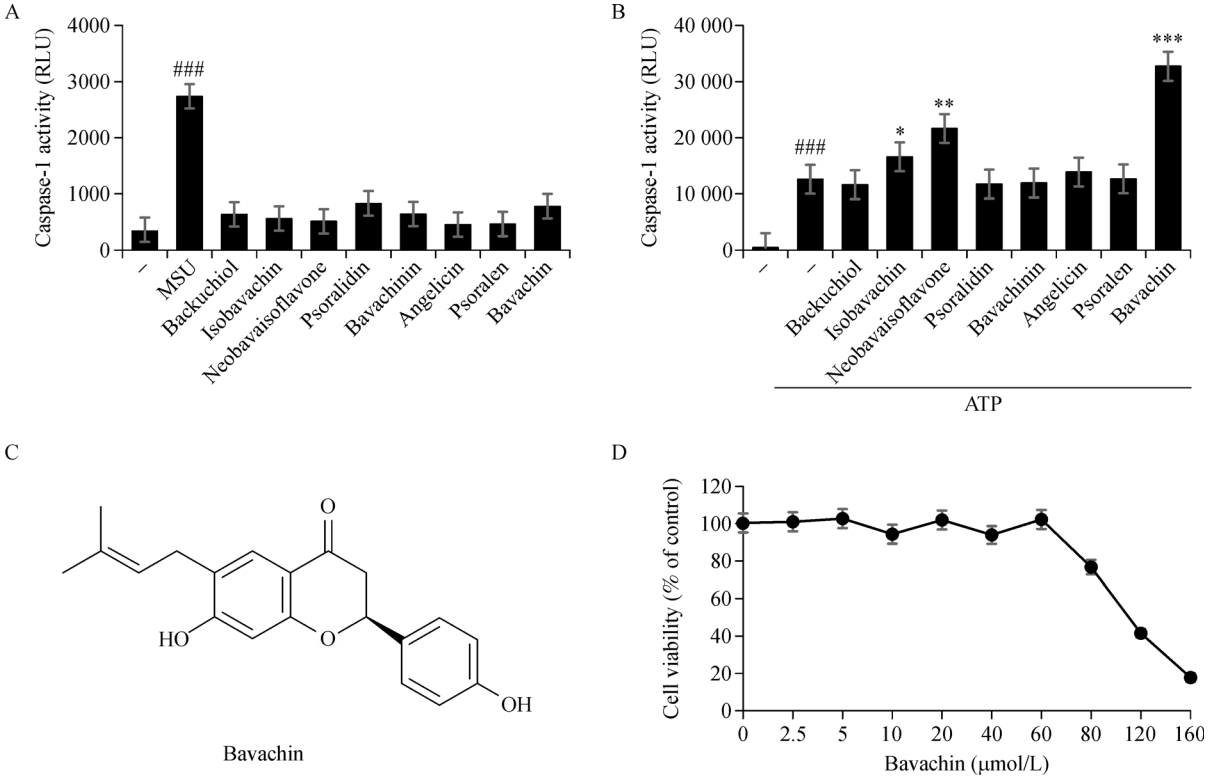

Fig. 1 NLRP3 inflammasome activation on main ingredients isolated from *Psoralea corylifolia* L. (A) Activity of caspase-1 in culture supernatants (Sup.) from LPS-primed BMDMs treated with MSU (200 μg/mL) or eight ingredients (10 μmol/L) from PL for 24 h. (B) Activity of caspase-1 in the Sup. from LPS-primed BMDMs treated with eight ingredients (10 μmol/L) from PL for 1 h and then stimulated by ATP for 1 h. (C) Bavachin structure. (D) Cytotoxicity in BMDMs treated with bavachin (2.5, 5, 10, 20, 40, 60, 80, 120, and 160 μmol/L). Data are expressed as the mean ± SEM of at least three independent experiments. $^{###}P < 0.001$ vs. the control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the LPS plus ATP group.

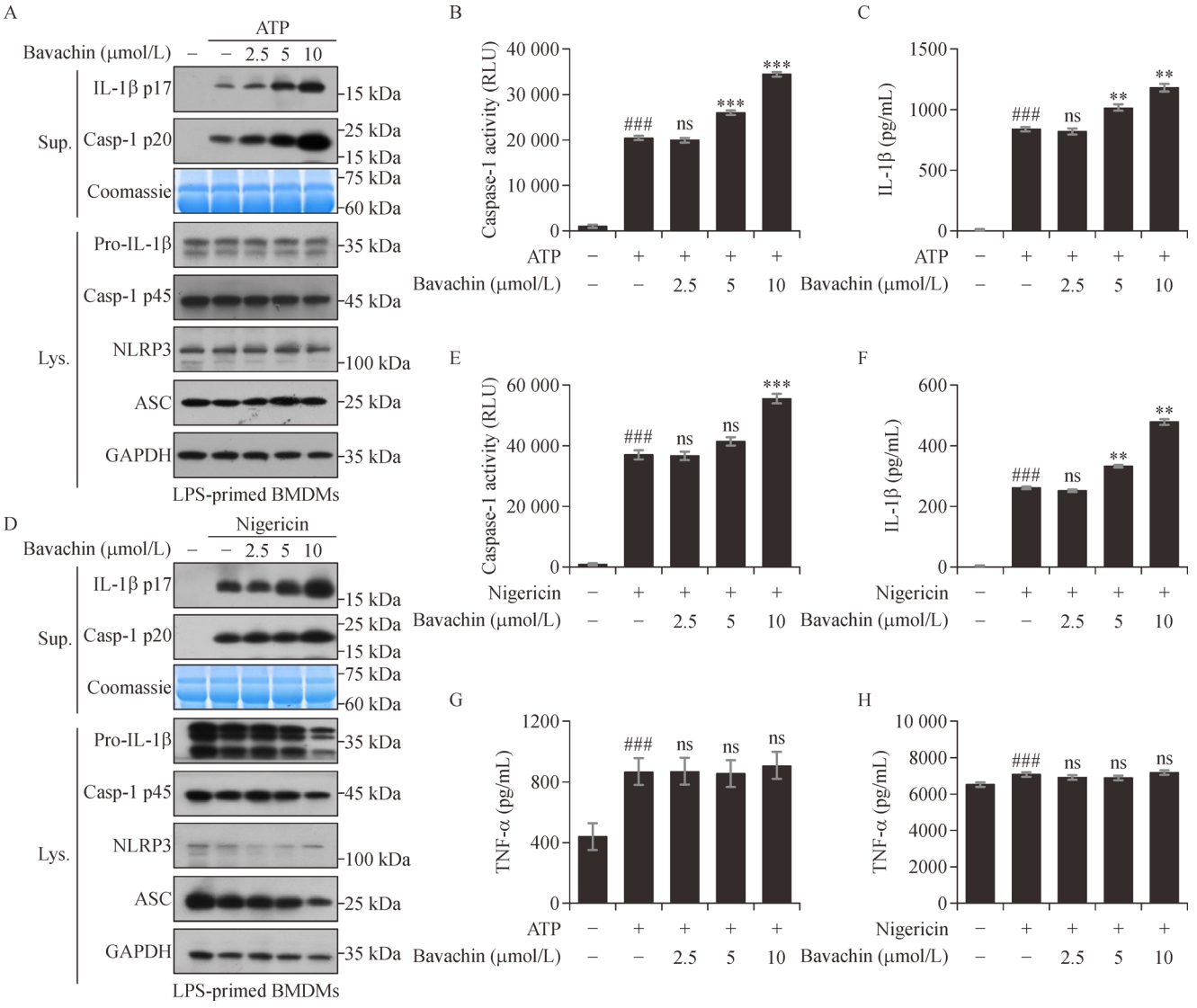

Fig. 2 Bavachin promotes the activation of the NLRP3 inflammasome triggered by ATP or nigericin. (A) LPS-primed BMDMs were treated with various doses of bavachin (2.5, 5, and 10 μmol/L) and then stimulated with ATP. Western blot analysis of IL-1β (p17) and caspase-1 (Casp-1 p20) in culture supernatants (Sup.) and pro-IL-1β, caspase-1 (Casp-1 p45), NLRP3, and ASC in cell lysates (Lys.). (B, C) Activity of caspase-1(B) and production of IL-1β (C) in the Sup. from BMDMs described in (A). (D) LPS-primed BMDMs were stimulated with nigericin after being treated with different concentrations (2.5, 5, and 10 μmol/L) of bavachin. Western blot analysis of IL-1β (p17) and caspase-1 (Casp-1 p20) in the Sup. and pro-IL-1β, caspase-1 (Casp-1 p45), NLRP3, and ASC in cell Lys. (E, F) Activity of caspase-1 (E) and production of IL-1β (F) in the Sup. from BMDMs described in (D). (G) TNF-α in the Sup. from BMDMs described in (A). (H) TNF-α in the Sup. from BMDMs described in (D). Coomassie blue staining was provided as the loading control for the Sup. (A and D). GAPDH was provided as the loading control for Lys. (A and D). Data are expressed as the mean ± SEM of at least three independent experiments. $^{###}P < 0.001$ vs. the control group. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the LPS plus ATP or nigericin group. ns, not significant.

Bavachin exerted no effect on NLRP3 inflammasome activation induced by other stimuli or on NLRC4 or AIM2 inflammasome activation

Apart from ATP and nigericin, MSU and poly(I:C) also induce canonical NLRP3 inflammasome activation [31,32]. To determine whether or not bavachin is a broad-spectrum enhancer of NLRP3 inflammasome activation, we examined the effect of bavachin on NLRP3 inflammasome activation induced by other stimuli. As shown in Fig. 3, bavachin was unrelated to the caspase-1 cleavage or IL-1β secretion induced by MSU or poly(I:C) (Fig. 3A, 3C, and 3D). These results suggest that bavachin can specifically promote canonical NLRP3 inflammasome

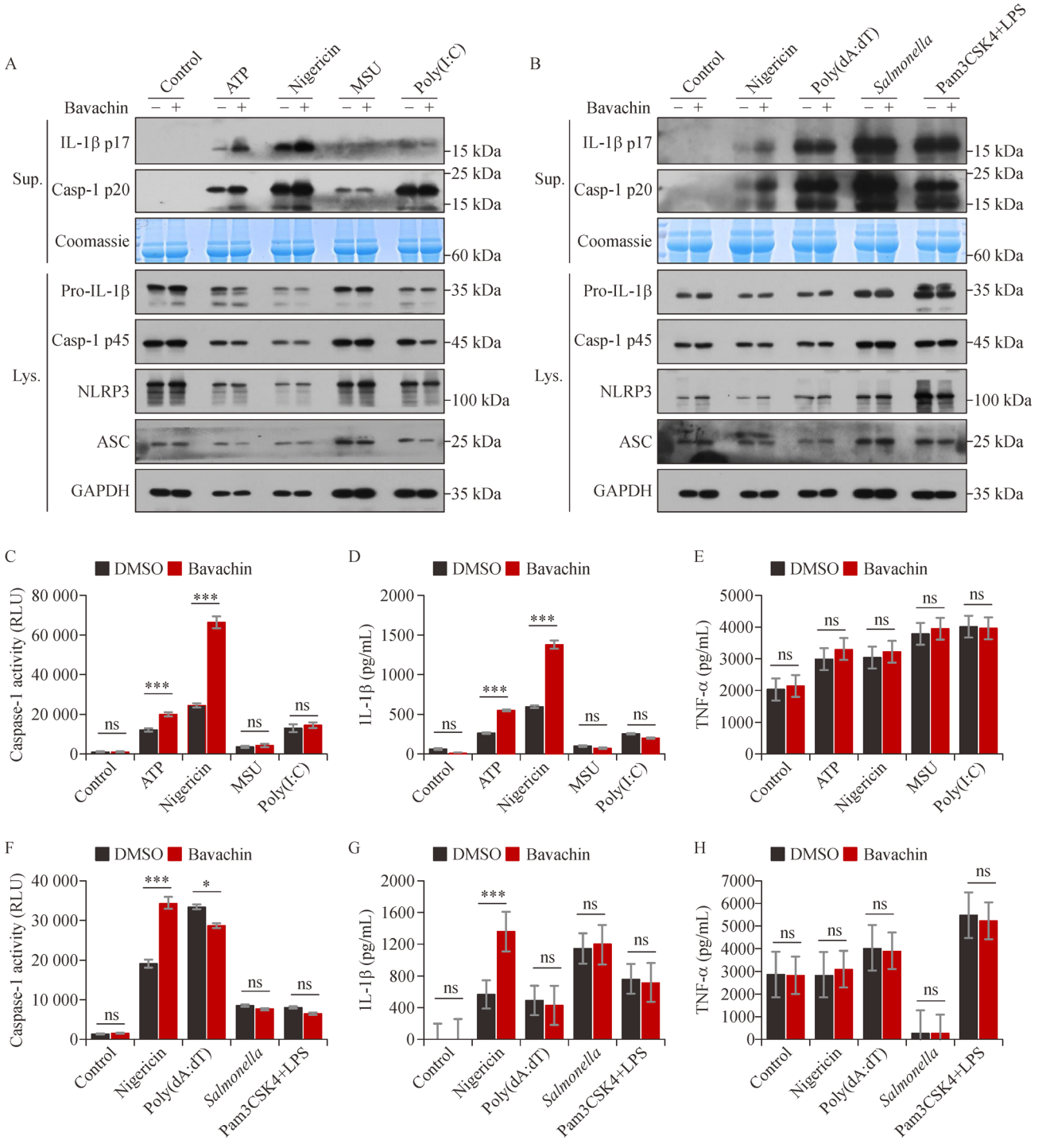

Fig. 3 Bavachin has no effect on other stimuli-induced NLRP3 inflammasome activation and NLRC4, AIM2 inflammasome activation. (A) Western blot analysis of IL-1β (p17) and caspase-1 (Casp-1 p20) in culture supernatants (Sup.) and pro-IL-1β, caspase-1 (Casp-1 p45), NLRP3, and ASC in cell lysates (Lys.) from LPS-primed BMDMs treated with bavachin (10 μmol/L) or not and then stimulated with ATP, nigericin, MSU, and poly(I:C). (B) Western blot analysis of IL-1β (p17), caspase-1 (Casp-1 p20) in the Sup. and pro-IL-1β, caspase-1 (Casp-1 p45), NLRP3, and ASC in cell Lys. from LPS-primed BMDMs treated in the presence or absence of bavachin (10 μmol/L) and then stimulated with nigericin, poly(dA:dT), *Salmonella*, or Pam3CSK4-primed BMDMs treated with bavachin (10 μmol/L) and then transfected with LPS (1 μg/mL). (C–E) Activity of caspase-1 (C), production of IL-1β (D) and TNF-α (E) in the Sup. from BMDMs described in (A). (F–H) Activity of caspase-1 (F), release of IL-1β (G), and TNF-α (H) in the Sup. from BMDMs described in (B). Coomassie blue staining was provided as the loading control for the Sup. (A and B). GAPDH was provided as the loading control for the Lys. (A and B). Data are expressed as the mean ± SEM of at least three independent experiments. $*P < 0.05$, $***P < 0.001$. ns, not significant.

activation induced by ATP or nigericin but not that induced by MSU or poly(I:C). Nonclassical NLRP3 inflammasome activation does not depend on the activation of the TLR4 signaling pathway. Caspase-11 directly recognizes transfected intracellular LPS to promote NLRP3 inflammasome activation and the subsequent release of IL-1β and mediating pyroptosis [33]. Caspase-1 and caspase-11 are aspartate-specific cysteine proteases that induce pyroptosis and increase the secretion of proinflammatory IL-1β and IL-18, resulting in apoptosis [9,34]. We also investigated whether or not bavachin affects noncanonical NLRP3 inflammasome activation. BMDMs were first primed with Pam3CSK4 after being pretreated with bavachin and then transfected with LPS. Results demonstrated that bavachin did not alter caspase-1 cleavage or IL-1β secretion. We treated LPS-primed BMDMs with bavachin and then stimulated them with poly(dA:dT) and *Salmonella*, which can activate the AIM2 and NLRC4 inflammasomes, respectively. Bavachin did not affect caspase-1 activation or IL-1β secretion in response to poly(dA:dT) and *Salmonella* treatment (Fig. 3B, 3F, and 3G). In addition, the expression levels of pro-IL-1β, pro-caspase-1, NLRP3, and ASC in whole cell lysates and TNF-α processing were not influenced by bavachin treatment (Fig. 3A, 3B, 3E, and 3H). These data demonstrate that bavachin can specifically promote ATP- or nigericin-induced NLRP3 inflammasome activation but does not affect the NLRC4, AIM2 inflammasome, or NLRP3 inflammasome activation induced by other stimuli.

Bavachin specifically facilitates ASC oligomerization triggered by ATP or nigericin

We next examined how bavachin facilitates the ATP- or nigericin-stimulated activation of the NLRP3 inflammasome. ASC oligomerization is a critical step in inflammasome activation and is a vital index that can reflect pyroptotic cell death or the secretion of inflammatory cytokines [35]. To determine whether or not ASC oligomerization participates in bavachin-mediated NLRP3 inflammasome activation, we cross-linked ASC with DSS and assessed ASC oligomerization in the lysates by immunoblotting. Results showed that ASC condensed into dimers, trimers, and oligomers after stimulation with ATP or nigericin in the LPS-primed BMDMs (Fig. 4A). Similarly, bavachin promoted the secretion of caspase-1 and IL-1β that is triggered via ATP or nigericin rather than other agonists (Fig. 3). Bavachin also promoted ATP- or nigericin-stimulated ASC oligomerization in LPS-primed BMDMs but had no influence on MSU or intracellular LPS-mediated ASC oligomerization (Fig. 4A and 4B). Moreover, ASC oligomerization triggered by poly(dA:dT) and *Salmonella* was not affected by bavachin treatment (Fig. 4B). Considering that ASC oligomerization is crucial for the NLRP3 inflammasome activation induced by all stimuli, we speculated that bavachin enhances the ATP- or nigericin-stimulated activation of the NLRP3 inflammasome by acting on upstream events of ASC oligomerization.

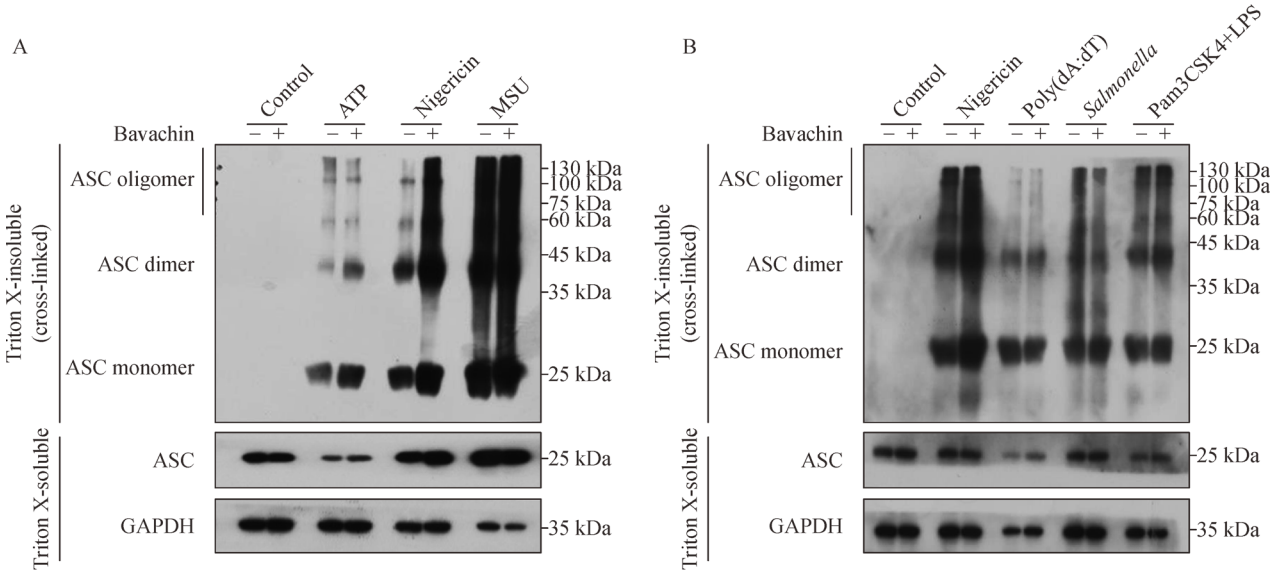

Fig. 4 Bavachin facilitates ASC oligomerization. (A) Western blots analysis of cell lysates and cross-linked cytosolic pellets of LPS-primed BMDMs stimulated with bavachin (10 μmol/L) and then stimulated with ATP, nigericin, and MSU. (B) Western blots analysis of cell lysates and cross-linked cytosolic pellets of LPS-primed BMDMs treated with bavachin (10 μmol/L) before stimulating nigericin, poly(dA:dT), *Salmonella*, or Pam3CSK4-primed BMDMs treated with bavachin (10 μmol/L) and then transfection with LPS. GAPDH served as a loading control for the Lys.

Bavachin does not affect the LPS-induced priming stage of NLRP3 inflammasome activation or K^+ efflux but specifically promotes nigericin-induced mitochondrial ROS production

The LPS-mediated NF-κB pathway is necessary in the priming events of the regulation of inflammasome protein expression [36]. Therefore, we examined whether or not bavachin influences NLRP3 inflammasome activation by regulating NF-κB-dependent NLRP3 and pro-IL-1β expression. BMDMs were pretreated with bavachin at different concentrations (2.5, 5, and 10 μmol/L) and then induced with LPS. Results showed that bavachin exerted no influence on NLRP3, pro-IL-1β, or TNF-α production (Fig. 5A and 5B). Previous studies indicated that K^+ efflux and mitochondrial damage are involved in the activation of the NLRP3 inflammasome [37]. Mitochondrial injury is also widely involved in the activation of inflammasomes. Therefore, we evaluated the effect of bavachin on mitochondrial injury by immunofluorescence assay, and the results showed that bavachin treatment did not cause mitochondrial dysfunction or an increase in the mitochondrial injury induced by nigericin (Fig. 5C). Subsequently, we explored whether or not bavachin can influence the K^+ efflux triggered by inflammasome agonists. Results showed that bavachin promoted the activity of caspase-1 but did not affect the K^+ efflux induced by ATP (Fig. 2B and 5D). Mitochondrial ROS (mtROS) is an upstream regulator of NLRP3 inflammasome activation. The level of mtROS produced by nigericin or SiO_2 treatment with bavachin was evaluated by MitoSOX red mitochondrial superoxide indicator assay. Results showed no changes in mtROS production induced by bavachin alone; interestingly, bavachin strengthened the mtROS production stimulated by nigericin but not SiO_2 in LPS-primed BMDMs (Fig. 5E and 5F). These results demonstrate that ROS production is an important part of the effect of bavachin on the NLRP3 inflammasome initiated.

Bavachin induces liver injury in LPS-mediated IDILI mouse model

LPS, as an inflammatory mediator, can induce the expression of immune inflammatory cytokines and mimic the immune susceptibility to IDILI in mice [38]. Several studies have shown that IDILI can be reproduced in an animal model with a nonhepatotoxic dose of LPS and that drugs such as trovafloxacin [6] and diclofenac [3] can induce IDILI. Our previous studies have shown that PF can cause IDILI without inducing the toxic effects induced by LPS [28]. In addition, LPS plays a major role in the priming event of NLRP3 inflammasome activation [39], which can directly lead to excessive NLRP3 inflammasome activation *in vivo*; therefore, it can mimic the immune susceptibility to the IDILI that is mediated by the NLRP3 inflammasome. We explored whether or not bavachin can cause liver damage in a mouse model of LPS-induced susceptibility to IDILI. Treatment with bavachin or LPS alone did not increase ALT or AST plasma levels compared with those in the control mice. Nevertheless, the administration of bavachin after LPS injection substantially increased the plasma levels of ALT and AST in the mice compared with the mice injected with LPS alone (Fig. 6A and 6B). Similarly, the administration of bavachin increased the serum concentrations of the inflammatory factors IL-1β and TNF-α in the LPS-treated mice (Fig. 6C and 6D). These results suggest that bavachin induces liver damage in a mouse model of LPS-induced susceptibility to IDILI by abetting the activation of the NLRP3 inflammasome. To explore the participation of the NLRP3 inflammasome in LPS/bavachin-induced liver injury, we pretreated female mice with MCC950 [40], the most well-characterized inhibitor of NLRP3 inflammasome, and then treated them with LPS and bavachin. As shown in Fig. 7, MCC950 pretreatment before LPS/bavachin decreased the serum levels of ALT, AST, IL-1β, and TNF-α. Therefore, blockade of the NLRP3 inflammasome pathway could reverse the LPS/bavachin-induced liver injury. The results suggest that NLRP3 inflammasome mediates bavachin-induced liver injury.

Discussion

The occurrence of IDILI is unpredictable and based on individual factors or medicinal properties, and this unpredictability has become an obstacle to drug development and the evaluation of drug safety in the clinic. The NLRP3 inflammasome can be stimulated by DAMPs and PAMPs, leading to the secretion and activation of inflammatory cytokines, such as IL-1β and IL-18, which are closely implicated in liver diseases. Drugs that can induce IDILI, including amodiaquine and nevirapine, can activate the NLRP3 inflammasome by triggering the release of risk factors from dead and injured hepatocytes *in vitro* [21]. The ability of Zhuangguguanjie pills [41] and Xianlinggubao capsules [42], both of which contain PF, to induce liver injury, has been repeatedly reported by the Chinese Food and Drug Administration, and the number of cases of liver damage is increasing in China and in other countries [43]. PF is a traditional nontoxic Chinese herbal medicine that is widely used in the treatment of orthopedic and skin diseases. Our previous studies confirmed that PF is the main substance in Zhuangguguanjie pills that induces idiosyncratic liver injury, and PF could lead to liver injury in a mouse model of LPS-mediated IDILI [28]. In the present study, we found that bavachin, a major bioactive component of PF, can enhance the NLRP3 inflammasome activation mediated by ATP or nigericin. In addition, bavachin can cause liver damage by facilitating

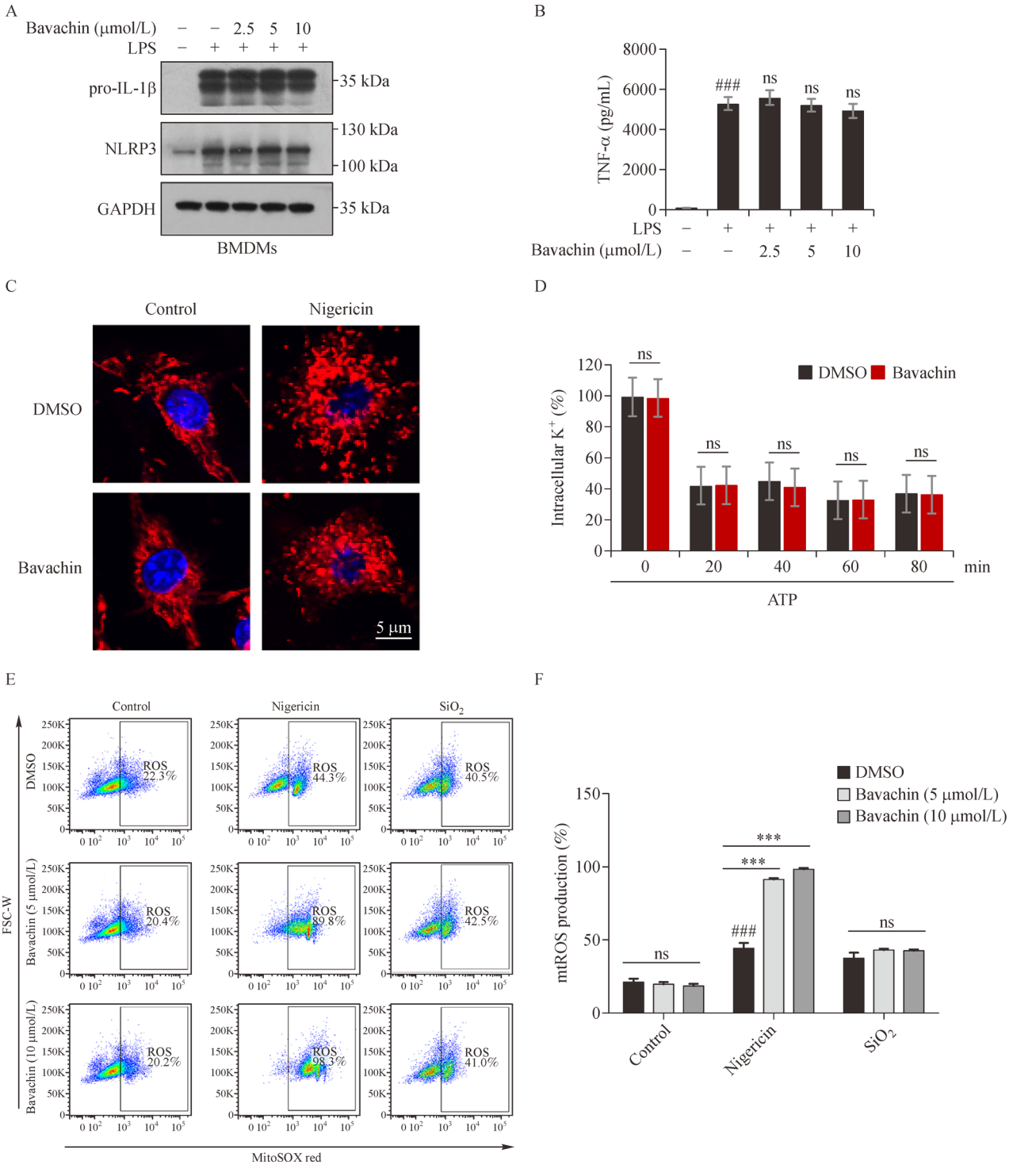

Fig. 5 Bavachin does not affect the LPS-induced NLRP3 priming, K^+ efflux but specifically promotes nigericin-induced mitochondrial ROS production. (A) Western blot analysis of pro-IL-1β and NLRP3 in cell lysates from BMDMs treated with different doses of bavachin (2.5, 5, 10 μmol/L) for 1 h and then stimulated with LPS for 4 h. (B) Production of TNF-α (as measured by ELISA) in culture supernatants from BMDMs described in (A). (C) Immunofluorescence analysis in LPS-primed BMDMs treated with bavachin (10 μmol/L) and then left stimulated with nigericin, followed by staining with Mitotracker red and DAPI. (D) Relative level of intracellular K^+ determined by inductively coupled plasma optical emission spectrometry in LPS-primed BMDMs and stimulated with ATP at different times. (E, F) LPS-primed BMDMs were treated with bavachin (5 and 10 μmol/L) and then stimulated with nigericin or SiO_2. BMDMs were loaded with MitoSOX red mitochondrial superoxide indicator (Ex/Em: 510/580 nm) (E). After staining and washing, flow cytometry was conducted to test mtROS production (F). Data are expressed as the mean ± SEM of at least three independent experiments. $^{###}P < 0.001$ vs. the control, $^{***}P < 0.001$ vs. LPS plus nigericin. ns, not significant.

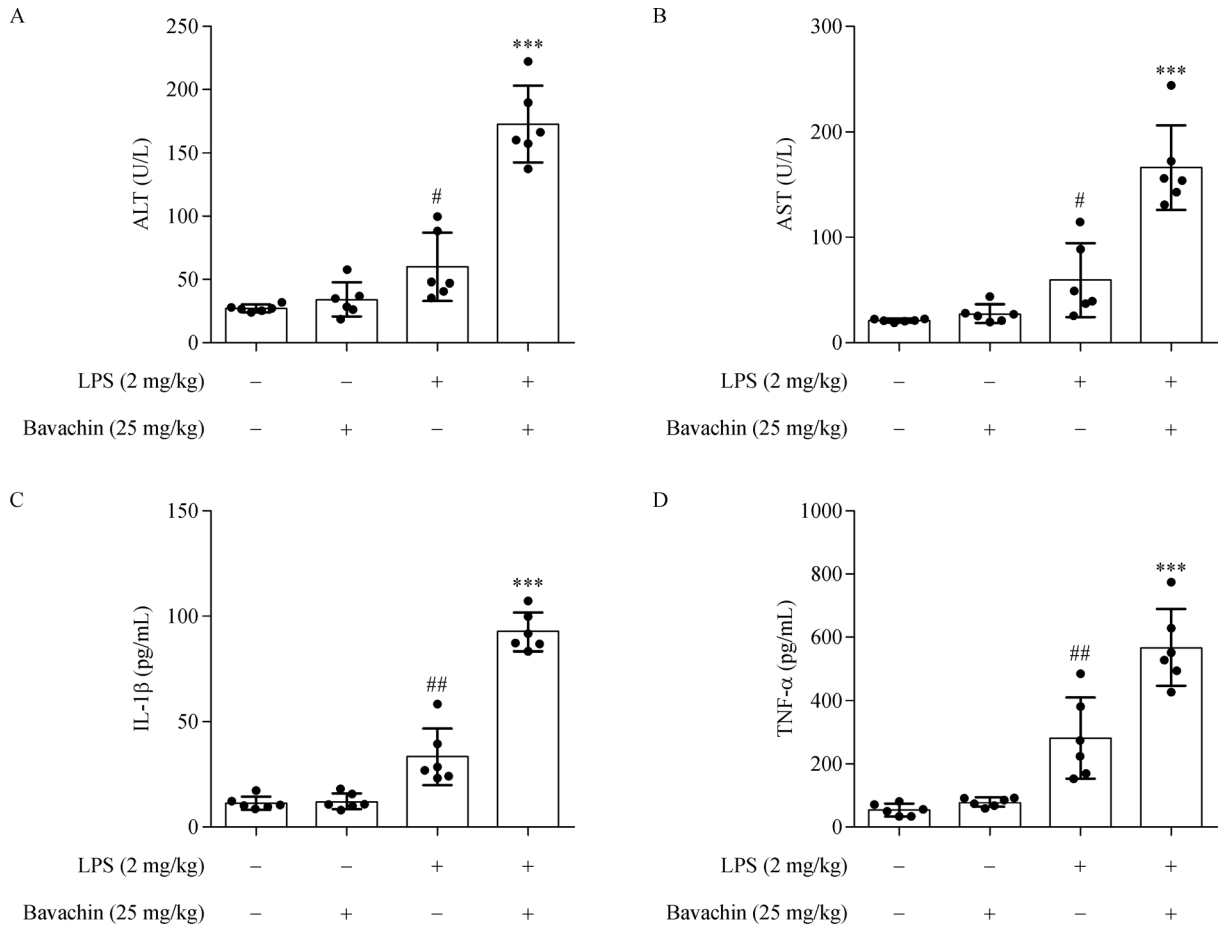

Fig. 6 Bavachin induces liver injury in LPS-mediated IDILI mouse model. Female C57BL/6 mice were treated with LPS (2 mg/kg) or its saline vehicle through the tail vein. After 2 h, bavachin (25 mg/kg) or its vehicle was administered through intraperitoneal injection. Mice were sacrificed 6 h later (n = 6 animals per group). (A) Serum ALT activity, (B) AST activity, (C) IL-1β, and (D) TNF-α were measured using their assay kits. Data are expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the control; $^{***}P < 0.001$ vs. LPS alone.

the activation of the NLRP3 inflammasome in a LPS-mediated mouse model. The results suggest that bavachin contributes to PF-induced liver damage by promoting the activation of the NLRP3 inflammasome.

In the liver, macrophages can be divided into Kupffer cells (KCs) and monocyte-derived macrophages (MDMs) according to different sources. KCs are tissue-resident microphage populations with self-sustainability, local proliferation, and tolerance; they reside in the liver and are usually different from MDMs. MDMs are divided by monocytes circulating in peripheral blood [44,45]. Previous studies have shown that KCs and recruited macrophages can mediate acute and chronic liver injury [46]. At present, similarities exist in the mechanisms of liver diseases between KCs and recruited macrophages [47]. BMDMs are usually selected for experiments because of their easy preparation. In addition, BMDMs are commonly used to evaluate the NLRP3 inflammasome activation in liver diseases [48–50]. Hence, BMDMs were selected in the present study to evaluate the activity of bavachin in regulating the NLRP3 inflammasome.

Excessive NLRP3 inflammasome activation has been linked to many autoinflammatory or autoimmune diseases, such as multiple sclerosis, Parkinson's disease, and atherosclerosis [51]. In addition, NLRP3 inflammasome activation serves an essential function in alcoholic and non-alcoholic fatty liver diseases, ischemia–reperfusion-induced liver injury, paracetamol-induced liver injury, HCV infection, and liver fibrosis [52]. In recent years, the NLRP3 inflammasome has been widely involved in idiosyncratic liver injury caused by various chemical drugs, such as isoniazid and nevirapine [53]. This study shows that bavachin does not induce NLRP3 inflammasome activation alone. However, bavachin specifically enhances the ATP- and nigericin-mediated activation of the NLRP3 inflammasome. Furthermore, bavachin can

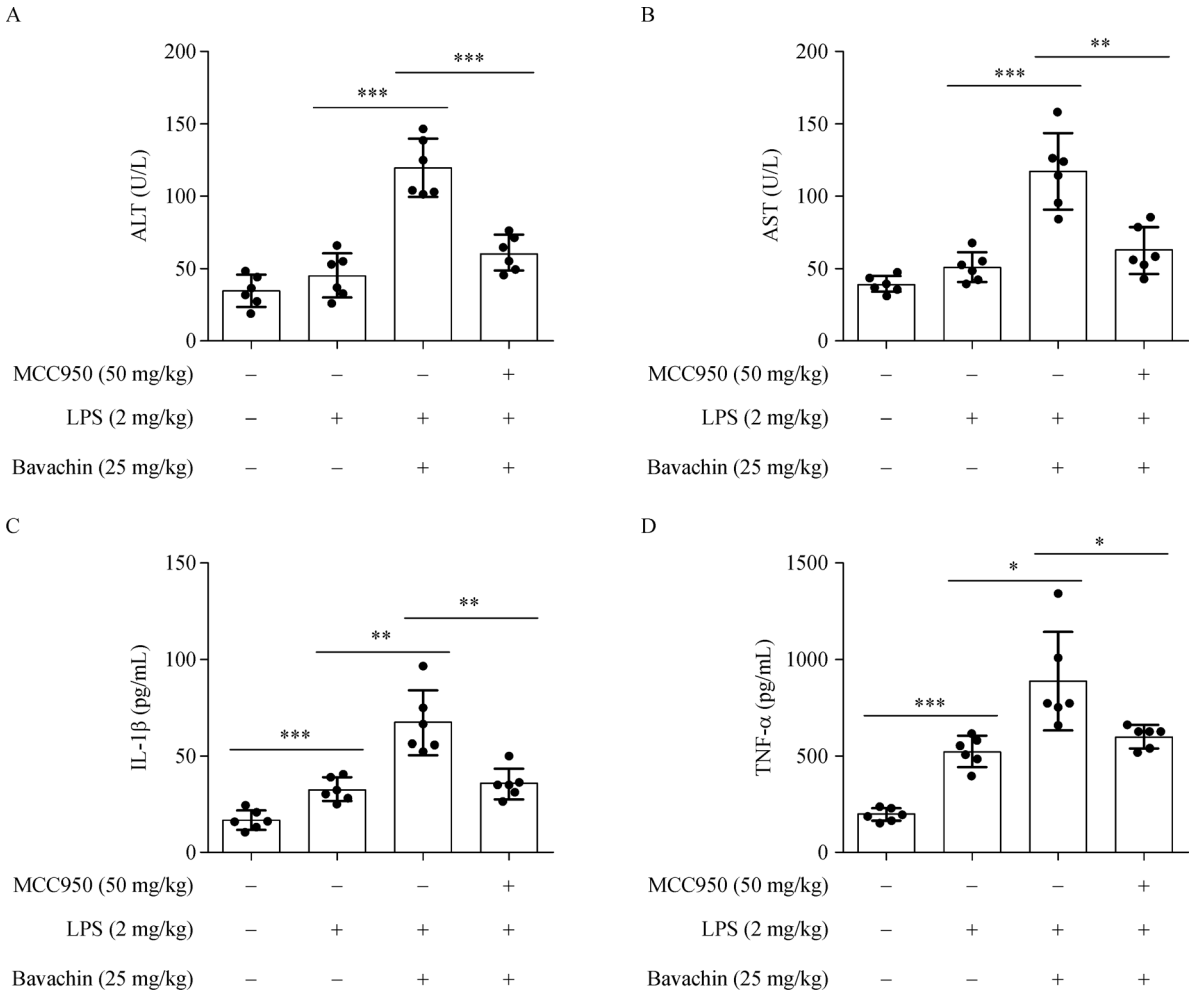

Fig. 7 MCC950 pretreatment suppresses liver injury induced by LPS/bavachin. Female C57BL/6 mice were given MCC950 (50 mg/kg) or its vehicle through intraperitoneal injection for 1 h. Then, female C57BL/6 mice received LPS (2 mg/kg) or its saline vehicle, intravenous injection via a tail vein. After 2 h, bavachin (25 mg/kg) or its vehicle was treated by intraperitoneal injection. After 6 h of bavachin administration ($n = 6$ animals per group), the serum levels of ALT (A), AST(B), IL-1β (C), and TNF-α (D) were measured using their assay kits. Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

increase LPS-mediated NLRP3 inflammasome and induce liver injury *in vivo*. Hence, liver in a state of NLRP3 inflammasome activation is crucial for bavachin-induced liver injury, which means that PF-induced liver injury only occurs in a specific population with NLRP3 inflammasome activation in the liver.

In the present study, bavachin can promote the NLRP3 inflammasome activation after ATP or nigericin stimulation but not after MSU or poly(I:C) stimulation (Figs. 2 and 3). Bavachin exerted no effect on the activation of the NLRC4 or AIM2 inflammasome or the nonclassical NLRP3 inflammasome. These data suggest that bavachin specifically increases the ATP- or nigericin-induced activation of the NLRP3 inflammasome. Next, we explored the mechanism by which bavachin affects NLRP3 inflammasome activation. ASC oligomerization plays a key role in NLRP3 inflammasome activation. Bavachin promoted the ASC oligomerization induced by ATP or nigericin but not that induced by MSU or intracellular LPS. However, ASC oligomerization is necessary for the NLRP3 inflammasome activation induced by all the stimuli tested, suggesting that bavachin acts on the upstream signaling events of ASC oligomerization to exacerbate ATP or nigericin-induced NLRP3 inflammasome activation. The priming stage of NLRP3 inflammasome activation is regulated by the NF-κB signaling pathway and results in the expression of NLRP3 and pro-IL-1β [54]. Our results also demonstrated that bavachin did not influence LPS-mediated pro-IL-lβ or NLRP3 expression (Fig. 5A and 5B). In addition, K^+ efflux [37] is crucial in NLRP3 inflammasome activation and mitochondrial damage [55], which may be sensed by NLRP3 via the oxidative stress caused by mtROS or the cytosolic release of oxidized mitochondrial DNA and

cardiolipin [56,57]. Our results indicated that bavachin did not affect mitochondrial damage or K^+ efflux (Fig. 5C and 5D). Although it did not directly induce mtROS production, bavachin specifically enhanced mtROS production induced by nigericin (Fig. 5E and 5F). These results suggest that the synergistic induction of mtROS contributes to the strengthened influence of bavachin on the nigericin-induced activation of the NLRP3 inflammasome. Previous reports indicated that ROS-generating mitochondria can be removed by autophagy [58], which protects cells from damage. Blocking autophagy can lead to the accumulation of disrupt ROS-generating mitochondria, which then stimulates the NLRP3 inflammasome [59]. The role of ROS has always been controversial. Most studies indicated that the mitochondria are the source of ROS, which participate in NLRP3 inflammasome activation [60]. However, a report showed that ROS only stimulate the priming event of the NLRP3 inflammasome and not the activation process [61]. Hence, support for the supposition that bavachin promotes nigericin-induced mitochondrial ROS production requires further research and exploration.

Bavachin can induce hepatocyte damage mainly through endoplasmic reticulum stress *in vitro* [62]. However, hepatocyte damage was detected only when the concentration of bavachin reached 40 μmol/L, which is much higher than the content of bavachin in PF or the dose used clinically [62]. In the present study, 5 μmol/L bavachin promoted NLRP3 inflammasome activation *in vitro*. Importantly, the administration of a single dose of 25 mg/kg bavachin (Fig. 6) induced liver damage in a mouse model of LPS-mediated susceptibility to IDILI and was accompanied by inflammasome activation. These data suggest that the ATP- or nigericin-induced activation of the NLRP3 inflammasome is a susceptibility factor for bavachin- or PF-induced liver injury patients. Thus, bavachin or PF should be avoided by patients with diseases related to NLRP3 inflammasome activation. In summary, our results demonstrate that bavachin can facilitate the ATP- and nigericin-stimulated activation of NLRP3 inflammasome and cause liver injury in a mouse model of LPS-medicated susceptibility to IDILI. Thus, bavachin might be a potential risk factor for the idiosyncratic liver injury induced by PF.

Increase in NLRP3 inflammasome activation and pyroptosis is not necessarily alarming because the inflamed cells could hence be removed through pyroptosis [11], which prevents chronic inflammation. This phenomenon may explain why PF is a popular herbal medicine for bone disease in China. Increasing evidence has linked inflammasome-driven inflammation to tissue damage and drug-induced liver injury [22,63,64]. In the present study, bavachin did not induce NLRP3 activation alone, but it can particularly enhance ATP- or nigericin-mediated NLRP3 inflammasome activation. It can also enhance LPS-mediated NLRP3 inflammasome activation and then induce liver injury *in vivo*. Liver in a state of NLRP3 inflammasome activation is crucial for bavachin-induced liver injury, which means that PF-induced liver injury only occurs in a specific population with NLRP3 inflammasome activation in the liver. Bavachin induces liver damage by continuously accelerating NLRP3 inflammasome activation. It may also promote inflamed cell death by NLRP3 inflammasome activation in bone diseases. These results demonstrate that bavachin plays a role in treating bone diseases. Therefore, the relationship between the toxicity and effects of bavachin in different tissues and diseases is still worthy of further study.

Acknowledgements

This work has been supported by the Beijing Nova Program (No. Z181100006218001), the National Natural Science Foundation of China (Nos. 81874368, 81630100, 81903891, and 81573676), the National Science and Technology Major Project "Key New Drug Creation and Manufacturing Program" (Nos. 2017ZX09301022 and 2018ZX09101002-001-002), and the Innovation Groups of the National Natural Science Foundation of China (No. 81721002).

Compliance with ethics guidelines

Nan Qin, Guang Xu, Yan Wang, Xiaoyan Zhan, Yuan Gao, Zhilei Wang, Shubin Fu, Wei Shi, Xiaorong Hou, Chunyu Wang, Ruisheng Li, Yan Liu, Jiabo Wang, Haiping Zhao, Xiaohe Xiao, and Zhaofang Bai declare that they have no conflict of interest. This study was ratified by the Experimental Animal Center of the Fifth Medical Centre, Chinese PLA General Hospital in Beijing, China. Approval for the animal experimental research was in accordance with the ethical standards of the Ethics Committee in the Fifth Medical Centre, Chinese PLA General Hospital.

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