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Original Article

Protective Effect of Silibinin on Lipopolysaccharide-Induced Endotoxemia by Inhibiting Caspase-11-Dependent Cell Pyroptosis*

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ABSTRACT **Objective:** To explore the protective effect and the underlying mechanism of silibinin (SIB), one of the active compounds from *Silybum marianum* (L.) Gaertn in endotoxemia. **Methods:** Mouse peritoneal macrophage were isolated via intraperitoneally injection of BALB/c mice with thioglycolate medium. Cell viability was assessed using the cell counting kit-8, while cytotoxicity was determined through lactate dehydrogenase cytotoxicity assay. The protein expressions of interleukin (IL)-1α, IL-1β, and IL-18 were determined by enzyme-linked immunosorbent assay. Intracellular lipopolysaccharide (LPS) levels were measured by employing both the limulus amoebocyte lysate assay and flow cytometry. Additionally, proximity ligation assay was employed for the LPS and caspase-11 interaction. Mice were divided into 4 groups: the control, LPS, high-dose-SIB (100 mg/kg), and low-dose-SIB (100 mg/kg) groups (n=8). Zebrafish were divided into 4 groups: the control, LPS, high-dose-SIB (200 μmol/L), and low-dose-SIB (100 μmol/L) groups (n=30 for survival experiment and n=10 for gene expression analysis). The expression of caspase-11, gasdermin D (GSDMD), and N-GSDMD was determined by Western blot and the expressions of caspy2, gsdmeb, and IL-1β were detected using quantitative real-time PCR. Histopathological observation was performed through hematoxylin-eosin staining, and protein levels in bronchoalveolar lavage fluid were quantified using the bicinchoninicacid protein assay. **Results:** SIB noticeably decreased caspase-11 and GSDMD-mediated pyroptosis and suppressed the secretion of IL-1α, IL-1β, and IL-18 induced by LPS (P<0.05). Moreover, SIB inhibited the translocation of LPS into the cytoplasm and the binding of caspase-11 and intracellular LPS (P<0.05). SIB also attenuated the expression of caspase-11 and N-terminal fragments of GSDMD, inhibited the relative cytokines, prolonged the survival time, and up-regulated the survival rate in the endotoxemia models (P<0.05). **Conclusions:** SIB can inhibit pyroptosis in the LPS-mediated endotoxemia model, at least in part, by inhibiting the caspase-11-mediated cleavage of GSDMD. Additionally, SIB inhibits the interaction of LPS and caspase-11 and inhibits the LPS-mediated up-regulation of caspase-11 expression, which relieves caspase-11-dependent cell pyroptosis and consequently attenuates LPS-mediated lethality.

KEYWORDS silibinin, caspase-11, endotoxemia, pyroptosis, inflammation

Endotoxemia is characterized by a global activation of uncontrollable inflammation, immune suppression, and tissue injury. This triad further leads to complications like aggravation or induction of shock and multi-organ failure.[1] The Gram-negative bacterial cell wall component, lipopolysaccharides (LPS), is known for initiating global inflammatory actions and is a very potent stimulus for many diseases; including septic shock, atherosclerosis, obesity, and metabolic syndrome.[2] Therefore, the endotoxemia model classically uses LPS to activate the immune system, leading to unregulated inflammation, microcirculatory disturbances, tissue injury, and death.[3]

Toll-like receptor 4 (TLR4) signal pathway plays a crucial role in response to the LPS, which is responsible for inflammatory disorders.[4] However, recent studies

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have revealed that intracellular LPS sensing leads to pyroptosis and NOD-like receptor thermal protein domain associated protein 3 (NLRP3) activation, which could cause uncontrollable inflammation in the absence of TLR4. These results revealed that the intracellular LPS sensing signal might be more critical in the development of excess inflammation than TLR4.[5,6] Extracellular LPS is delivered to the cytosol by guanylate-binding proteins and outer membrane vesicles (OMVs). Then LPS directly binds to its intracellular caspase-4/5/11 receptor to activate the gasdermin D (GSDMD). Then the N-terminal GSDMD fragment (N-GSDMD) translocates to the cell membrane to bind phospholipids, thereby accumulating on the cell membrane to form pore-like structures, which leads to pyroptosis. This is followed by the activation of NLRP3 inflammasome that leads to the maturation and secretion of interleukin (IL)-1β, IL-1α, and IL-18.[7-9] Therefore, inhibitors of these cytosolic LPS-triggered intracellular caspase-4/5/11 activation might be considered prospective drugs for treating uncontrolled inflammation.

Silibinin (SIB), a crude form of silymarin, is a biologically active flavonolignan derived from the milk thistle plant. It exerts a broad-spectrum efficacy in controlling cancers, like non-melanoma skin cancers, cervical cancer, breast cancer, and human fibrosarcoma by modulating the apoptosis, cell cycle progression, and autophagy pathways.[10-12] SIB also exerts different effects including antifibrotic and antioxidant properties.[13] Mounting evidence revealed that SIB has significant anti-inflammatory effects. SIB treatment effectively inhibited the upregulation of the mRNA expression of tumor necrosis factor (TNF)-α, IL-1β, and IL-6 in NCI-H292 airway epithelial cells induced by SiONPs.[14] Moreover, SIB could inhibit the expression of the IL-6/STAT3 signaling pathway, reduce the production of TNF-α, and IL-1β, and attenuate the injury of the colonic mucosal barrier in inflammatory bowel disease.[15] Furthermore, SIB has been approved by US Food and Drug Administration for the treatment of hepatic diseases in forms of tablet, capsule, liposome, etc.[16] However, the anti-inflammatory effects of SIB in endotoxemia and its underlying molecular mechanism need further exploration. This study establishes that SIB is protective against endotoxemia by inhibiting caspase-11-mediated pyroptosis.

METHODS

Reagents

SIB (purity>97%) was purchased from Meilunbio (No. MB1962, China). Antibodies against GSDMD (No. EPR20859), caspase-11 (No. EPR18628), and lactate dehydrogenase (LDH) cytotoxicity assay kit (No. ab65393) were obtained from Abcam (UK). Antibodies against N-GSDMD (No. 10137), GAPDH (No. 5174), caspase-11 (No. 14340), and goat anti-rabbit-IgG (No. 7074) were purchased from Cell Signaling Technology (USA). TRIzol reagents were purchased from Invitrogen (No. 15596026, USA). 4',6-diamidino-2-phenylindole were purchased from Beyotime (No. C1002, China). DOTAP liposomal transfection reagent was obtained from Roche (No.11202375001, Switzerland). Poly(I:C) (LMW, No. tlrl-picw) and Pam3CSK4 were obtained from Invivogen (No. tlrl-bpms, USA). Limulus amoebocyte lysate (LAL) assay kit was obtained from Xiamen Bio Endo Technology (No. G010030, China). *Chlamydia trachomatis* LPS antibody (fluorescein isothiocyanate, FITC) was purchased from GeneTex (No. GTX36872, Southern California, USA). PrimeScript RT reagent kit with gDNA Eraser (No. RR047Q) and TB Green® Premix Ex Taq (No. RR420Q) were purchased from Takara (Japan). IL-1α (No. P01582), IL-1β (No. P10749), and IL-18 (No. P70380) enzyme-linked immunosorbent assay (ELISA) kits were purchased from CUSABIO (China). Bicinchoninic acid protein assay kit (No. A55860) and enhanced chemiluminescence kit (No. 32106) were obtained from Thermo Fisher (USA). Fetal bovine serum (FBS, No. FSP500) was obtained from ExCell Biology (China). Alanine aminotransferase (ALT) kit (No. C009-2-1) was obtained from Nanjing Jiancheng Bioengineering Institute (China). All primers were designed and obtained from Sangon Biotech (China). LPS (055:B5, No. L2880), proximity-ligation assay (PLA) kit (No. DUO96030), and other reagents were purchased from Sigma-Aldrich (USA).

Mouse Peritoneal Macrophages Isolation

Mouse peritoneal macrophage isolation was effectuated by intraperitoneally injecting BALB/c mice with 2 mL of 5% thioglycolate medium. The cells were harvested after 72 h by performing peritoneal cavity lavage with 8 mL sterile Hanks' balanced salt solution and laid over 6-well culture plates (2×10^6 cells/well) in RPMI 1640 media containing 10% FBS and 1% penicillin/streptomycin.

Cell Viability Assay

Cell viability was determined by using the cell counting kit-8 (CCK-8). Mouse peritoneal macrophages were laid over in a 96-well plate (8×10^4 cells/mL)

and cultured for 24 h and were then incubated with SIB (12.5, 25, 50, 100, and 200 μmol/L) for another 24 h. Subsequently, cells were incubated for 1 h in the incubator after the addition of 10 μL CCK-8 per well. The absorbance at 405 nm was measured using the Multiskan™ FC Microplate Photometer (Thermo Fisher).

Cytotoxicity Assay

Mouse peritoneal macrophages were primed with Pam3CSK4 (1 μg/mL) or LPS (100 ng/mL) for 6 h and stimulated with or without SIB (25, 50, and 100 mmol/L). Subsequently, LPS (1 μg/mL) was transferred into cells in a 6-well culture plate by Dotap. After 16 h treatment, culture media were harvested and centrifuged for 5 min at 400 × *g*. The resulting supernatant was used to evaluate the cytotoxicity with an LDH cytotoxicity assay kit.

ELISA

Mouse peritoneal macrophages were primed with Pam3CSK4 (1 μg/mL) or LPS (100 ng/mL) for 6 h and stimulated in the absence or presence of SIB (25, 50, and 100 mmol/L). Subsequently, LPS (1 μg/mL) were transferred into cells by 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). After 16 h treatment, the supernatant was harvested and evaluated for the protein expressions of IL-1α, IL-1β, and IL-18 by using the ELISA kits as per the manufacturer's instructions.

LAL Assay

The isolation of cytosol fraction was done by washing mouse peritoneal macrophages with Dulbecco's phosphate-buffered saline (DPBS) and then digesting them with 0.25% trypsin.[17] Digestion was then halted with RPMI 1640 containing 10% FBS. Cells were washed 3 times by DPBS and incubated with 0.005% digitonin buffer for 20 min at 4 ℃. The supernatant containing cytosol was centrifuged at 15,000 × *g* and harvested after transferring to an empty tube. The levels of LPS in the cytosol fraction were analyzed using the LAL assay.

Flow Cytometry

For the detection of intracellular LPS, cells were primed with Pam3CSK4 (1 μg/mL) with or without SIB (100 μmol/L) for 6 h, and then treated with LPS (1 μg/mL), or LPS plus DOTAP. Macrophages were washed and stained with LPS antibody (FITC) 16 h after treatment, and intracellular fluorescence intensity was determined by Beckman Coulter flow cytometry (CytoFLEX, USA).

PLA

To determine the LPS and caspase-11 interaction, macrophages plated in confocal dishes were primed with Pam3CSK4 (1 μg/mL) with or without SIB (100 μmol/L) for 6 h, then cells were treated with LPS (1 μg/mL), or LPS plus DOTAP. Cells were washed 16 h after treatment and fixed with 4% methanol for 10 min. They were then treated with 0.1% Triton X-100 for 5 min and subsequently blocked for 1 h. Macrophages were incubated overnight with antibody pairs of two species directed to caspase-11 (rat monoclonal 17D9) and LPS (mouse monoclonal 2D7/1). The PLA was measured as per the manufacturer's instructions. Images were obtained using the LSM800 confocal microscope (Carl Zeiss, Germany).

Western Blot

Mouse peritoneal macrophages were seeded in 60 mm dishes (5×10^5 cells). The cells were then primed with Pam3CSK4 (1 μg/mL) or LPS (100 ng/mL) for 6 h with or without SIB (25, 50, and 100 μmol/L), and subsequently treated with LPS (1 μg/mL) plus DOTAP for 16 h. Methanol/chloroform (4:1) was used to precipitate the proteins in culture supernatants of mouse peritoneal macrophage. Western blot was employed as described previously.[18]

Real-Time Quantitative Polymerase Chain Reaction

The total RNA of zebrafish was extracted using TRIzol and was reversely transcribed into a complementary strand of DNA by using the RT reagent kit. Afterward, the samples were implemented as per the instructions of TB Green® Premix Ex Taq. The primer information was as follows: caspy2 (forward): CTGAGCGTGTAGTCACAAGAA; caspy2 (reverse): CGCTCCTCGGTGTGTAAATATC; gsdmeb (forward): GACTCTGAAGGTTTCCTGATCC; gsdmeb (reverse): TTCAATCACGCTGTAAGCAA; IL-1β (forward): TGGACTTCGCAGCACAAAATG; IL-1β (reverse): CACTTCACGCTCTTGGATGA; β-actin (forward): ATGGATGAGGAAATCGCTG; β-actin (reverse): ATGCCAACCATCACTCCCTG.

Animals

Sixty-four SPF BALB/c mice (male, 18–22 g) were provided from the Center of Experimental Animals of Southern Medical University (China). Zebrafish were

maintained at 28.5 ℃ in recirculating systems in a cycle of 14:10 h day/night and the embryos of zebrafish were collected and incubated as per the instructions described previously.[19] The Tg (coro1a: GFP; lyz: Dsred) zebrafish was provided by the South China University of Technology (China). All the zebrafish experiments were carried out in the Key Laboratory of Zebrafish Modeling and Drug Screening for Human Diseases Institute at Southern Medical University (China). All mice were maintained at Southern Medical University Laboratory Animal Center SPF facility, under controlled environment (12-h light/dark cycle; temperature, 22–24 ℃) and provided with standard food and water *ad libitum*. All mice and zebrafish experiments were conducted as per the National Institutes of Health guidelines issued for the care and use of laboratory animals approved by the Ethical Committee of the Southern Medical University (No. SMUL2020160).

Grouping and Modeling

Sixty-four mice were divided into 4 groups by simple random sampling: the control group, the LPS group, the high-dose-SIB group (H-SIB), and the low-dose-SIB group (L-SIB). The control group and LPS group were treated with normal saline (200 μL/d, intraperitoneal injections) for 7 constitutive days. H-SIB and L-SIB groups were treated with SIB [100 and 50 mg/(kg·d), intraperitoneal injections] for 7 constitutive days, respectively.

After 7 days, all groups, except the control group, were primed with poly (I:C, 10 mg/kg, intraperitoneal injections). After 3 h, H-SIB and L-SIB groups received 100 and 50 mg/kg SIB, intraperitoneal Injections, respectively. All the groups, except the control group, were treated with LPS (5 mg/kg, intraperitoneal injections) after 3 h. Subsequently, mortality within 96 h post-injection was observed for each group, with 8 mice in each group.

Eight mice in each group were anesthetized after 8 h with 1.5% (w/v) pentobarbital sodium and kill by exsanguination. The bronchoalveolar lavage fluid (BALF) was collected from the lungs. Lung, liver, kidney, and intestines were collected for histological observation and protein expression detection. Serum samples were harvested for the determination of IL-1β, IL-1α, IL-18, and ALT. The core temperatures of the mice were measured using a Minipa Digital Thermometer MT450.[20] Mortality was evaluated for up to 4 days in the survival experiments.

Zebrafish were divided into 4 groups by simple random sampling: the control group, the LPS group, the H-SIB group, and the L-SIB group (n=30 for survival experiment and n=10 for gene expression analysis). The zebrafish were anesthetized 3 days post-fertilization (dpf) using 0.02% tricaine. Then they were injected with 2 nL LPS (2 mg/mL) into the yolk to establish the lethal LPS-induced inflammatory model. PBS was used in the control group. After recovery from anesthesia, zebrafish in each group were divided into a 12-well culture plate. SIB groups were administrated with SIB (200 or 100 μmol/L). All zebrafish were maintained at a volume of 3 mL of egg water at 28.5 ℃. Zebrafish mortalities within 96 h post-injection (hpi) were observed.

Histopathological Observation

Histopathological observation of the pulmonary, hepatic, renal, and intestinal tissues was done as mentioned previously.[21]

Cell Count and Protein Levels Detection in BALF

After the sacrifice of mice, BALF samples were centrifuged (400×g, 4 ℃) for 10 min. The protein concentration in BALF was determined by the bicinchoninic acid protein assay kit. The cells in the BALF were re-suspended in 200 μL sterile PBS. A double-blinded method was used to total cells in BALF using a hemacytometer.

Statistical Analysis

Data were analyzed by SPSS Statistics (version 23.0; IBM, USA) and expressed as mean ± standard deviation ($\bar{x} \pm s$). All data were obtained in at least 3 independent experiments. Survival data were evaluated by the Kaplan-Meier method. The comparisons between groups were using the Log-rank test. Comparison among multiple groups was analyzed by one-way analysis of variance (ANOVA). Tukey's test was employed for multiple comparisons. P<0.05 was considered statistically significant.

RESULTS

SIB Inhibits Caspase-11 Expression and Pyroptosis Mediated by Intracellular LPS *in vitro*

SIB (12.5–100 μmol/L) revealed no obvious cytotoxicity in macrophages (Figure 1A). Hence, SIB was used in the subsequent experiments at this concentration.

As shown in Figures 1B and 1C, after LPS

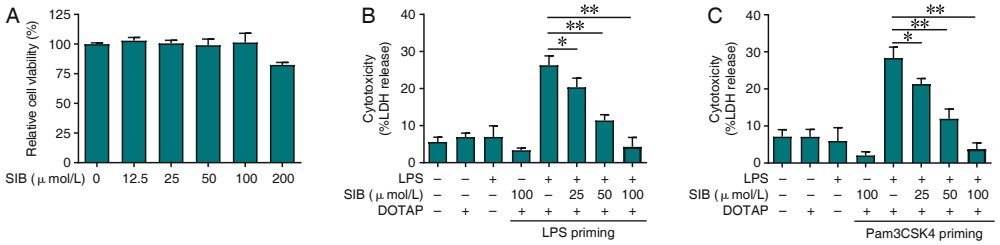

Figure 1. SIB Inhibits Intracellular LPS-Induced Pyroptosis in Macrophages ($\bar{x} \pm s$, n=3)

Notes: (A) SIB (12.5–100 μmol/L) showed no obvious cytotoxicity in macrophages; the cell cytotoxicity of SIB was determined by CCK8 assay; (B–C) SIB inhibits the intracellular LPS-induced macrophages pyroptosis; cytotoxicity was detected by LDH assay; $^{*}P$<0.05, $^{**}P$<0.01; SIB: silibinin, DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane, LDH: lactate dehydrogenase

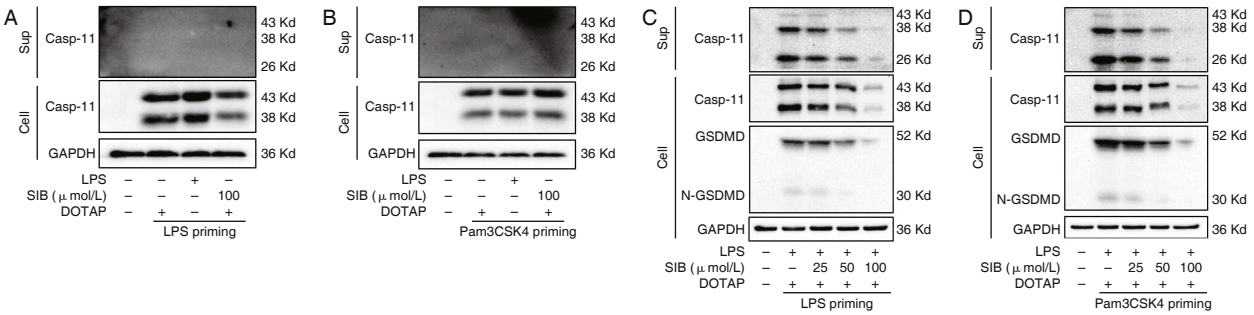

Figure 2. SIB Blocks Caspase-11 Expression and GSDMD Cleavage Mediated by Intracellular LPS

Notes: (A–B) DOTAP, LPS, or SIB (100 μmol/L) did not induce the GSDMD cleavage and the secretion of caspase-11 from primed macrophages. (C–D) SIB abolished intracellular LPS-mediated caspase-11 expression and the GSDMD cleavage. Protein expression was determined by Western blot; SIB: silibinin, DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane, LPS: lipopolysaccharides, GSDMD: gasdermin D, sup: cell supernatant

or PAM3CSK4 priming, transfection of LPS by using DOTAP significantly induced cell death in macrophages. SIB could inhibit the cell death of macrophages in a dose-independent manner.

Western blot revealed that LPS or Pam3CSK4 priming induced the expression of caspase-11 in macrophages. Furthermore, the treatment with DOTAP, LPS, or SIB (100 μmol/L) did not induce the secretion of caspase-11 from macrophages after priming (Figures 2A and 2B). However, transfection of LPS by using Dotap induced the up-regulation of N-GSDMD in cells and caspase-11 in cell supernatant. SIB inhibited LPS-induced up-regulation of caspase-11 and N-GSDMD (Figures 2C and 2D). SIB also noticeably inhibited the secretion of IL-1α, IL-1β, and IL-18 in a dose-dependent manner (Figure 3).

SIB Blocks LPS Translocation into Cytosol and Inhibits Caspase-11 Interaction with Intracellular LPS

It was significant that transfection of LPS by DOTAP increased the LPS level whereas SIB inhibited the intracellular translocation of LPS in the macrophages (Figures 4A and 4B).

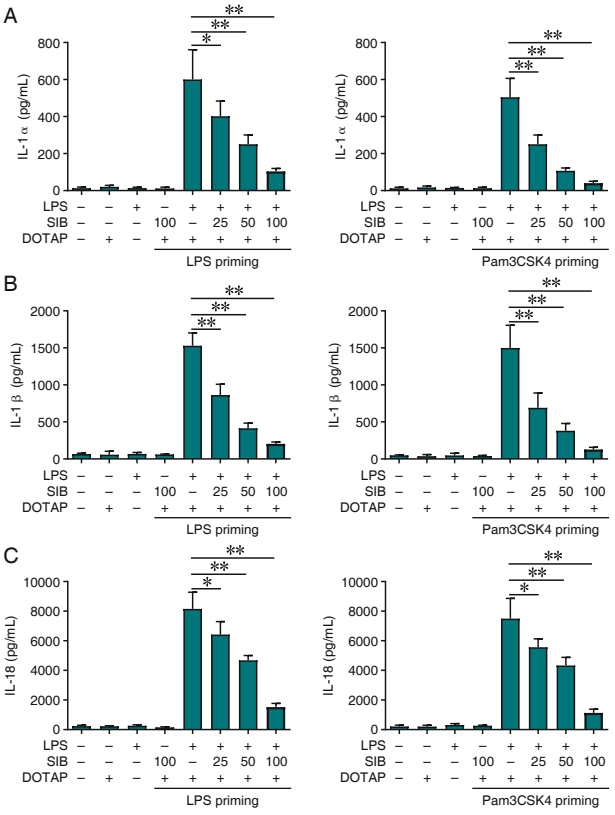

Figure 3. SIB Abolishes Release of IL-1α (A), IL-1β (B), and IL-18 (C) Induced by Intracellular LPS ($\bar{x} \pm s$, n=3)

Notes: $^{*}P$<0.05, $^{**}P$<0.01; IL: interleukin, SIB: silibinin, DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane, LPS: lipopolysaccharides

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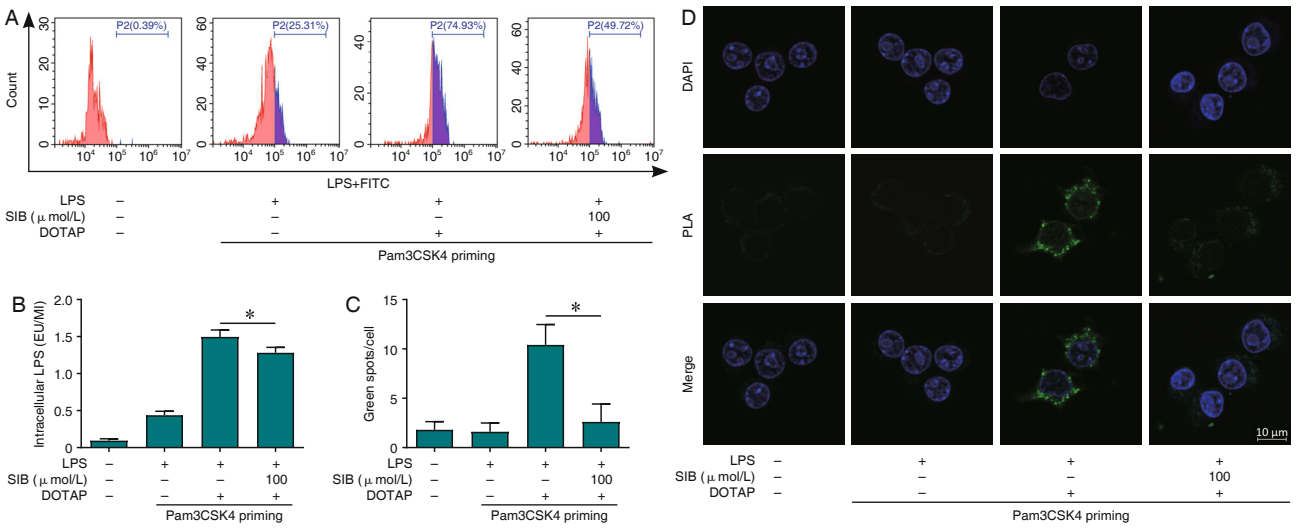

Figure 4. SIB Prevents LPS Translocating into Cytoplasm and Interaction of LPS and Caspase-11 in Cells

Notes: (A–B) The intracellular LPS was measured by LAL assay and flow cytometry; (C–D) The binding of caspase-11 and intracellular LPS was evaluated by PLA; Green spots in 5 random fields were quantified for each group under 100× magnification; Data are represented as $\bar{x} \pm s$ of 3 independent experiments. $^{*}P<0.05$; SIB: silibinin, LPS:lipopolysaccharides, LAL: limulus amoebocyte lysate, PLA: proximity-ligation assay

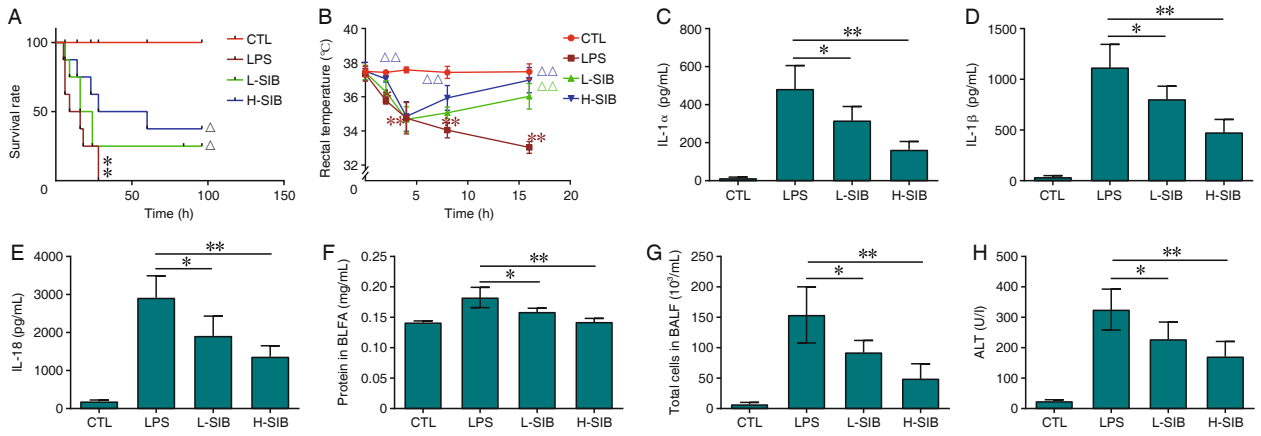

Figure 5. SIB Prevented Lethality and Inflammatory Response in Endotoxemic Mice

Notes: (A) The survival rate of endotoxemic mice with or without treatment of SIB, n=8 for each group; (B) SIB up-regulates the rectal temperature in endotoxemic mice, the rectal temperatures of the mice were measured using a Minipa Digital Thermometer MT450, n=3 for each group; $^{*}P<0.05$, $^{**}P<0.01$ *vs*. control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. LPS group; (C–E) Levels of IL-1α, IL-1β, and IL-18 in endotoxemia mice serum were determined by ELISA, n=8; (F–H) Protein content, total cells in BALF, and serum levels of ALT in endotoxemia mice were determined, n=8; Data are represented as the $\bar{x} \pm s$, $^{*}P<0.05$, $^{**}P<0.01$; IL: interleukin, SIB: silibinin, LPS: lipopolysaccharides, BALF: bronchoalveolar lavage fluid, ALT: alanine aminotransferase

The observation revealed that the interaction between caspase-11 and LPS decreased when cells were treated with SIB (Figures 4C and 4D).

SIB Protects Mice from Endotoxemia

SIB significantly improved the survival rate in the LPS-mediated mouse endotoxemia model ($P<0.05$, Figure 5A). In comparison to the control group, endotoxemia also lowered the mouse body temperature. However, the treatment of SIB decreased this effect (Figure 5B). Moreover, consistent with the findings of our *in vitro* study, in the mouse LPS-induced endotoxemia model, IL-1α, IL-1β, and IL-18 levels in the serum were largely reduced by SIB ($P<0.05$, Figures 5C–5E). SIB significantly attenuated hepatic and lung injury caused by LPS due to the reduction in protein level, total cells in BLAF, and serum levels of ALT ($P<0.05$, Figures 5F–5H) in endotoxemic mice. Additionally, in comparison to the endotoxemic mice who did not receive SIB treatment, sections of kidney, liver, lung, and intestinal tissues of endotoxemic mice who received SIB showed less tissue injury and hyperemia (Figure 6A). Consistent with the findings of this present *in vitro* experiments, the LPS-induced expression of caspase-11 and cleavage of GSDMD

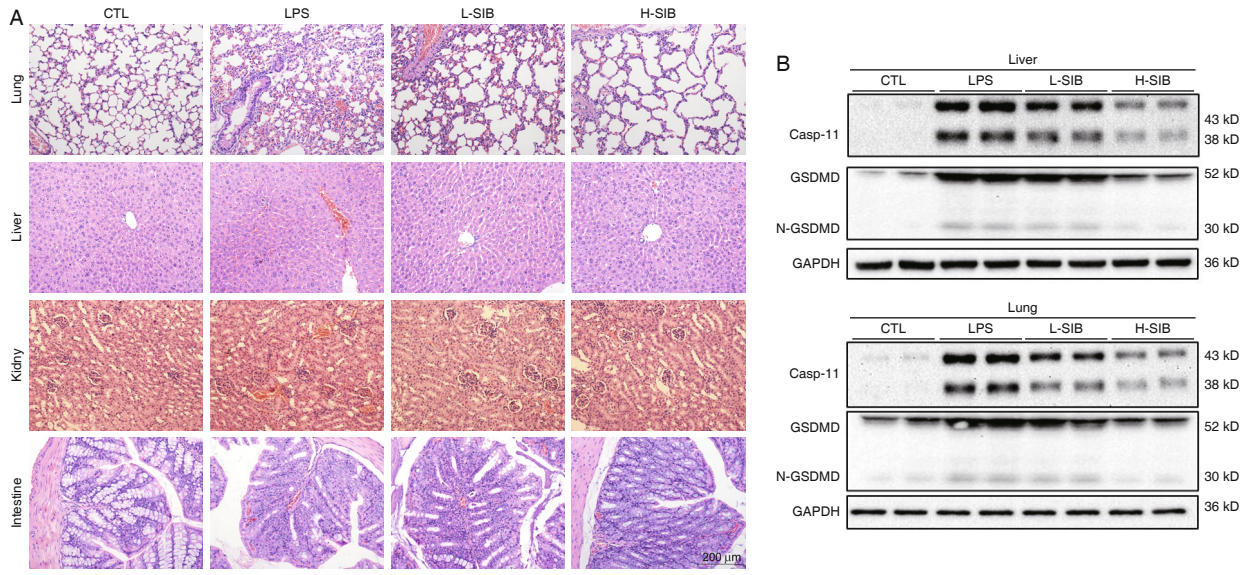

Figure 6. SIB Protects Against Endotoxemia by Inhibiting Non-canonical Inflammasome Activation

Notes: (A) SIB alleviates the pathological changes in LPS-induced endotoxemic mice. All tissue sections were observed after HE staining (200 ×); (B) SIB treatment significantly down-regulates the expression of caspase-11 and the cleavage of GSDMD in the liver and lung of endotoxemia mice; SIB: silibinin, LPS:lipopolysaccharides

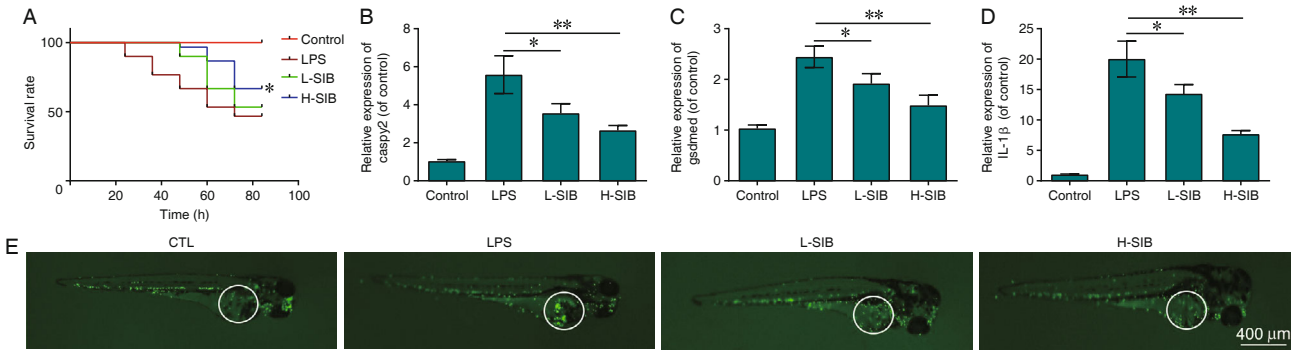

Figure 7. SIB Noticeably Improves Survival Rates of Zebrafish Induced by Lethal LPS through Inhibiting Caspy2 Signaling

Notes: (A) SIB protects zebrafish larvae infected by LPS, $^{*}P<0.05$ *vs*. LPS group; comparisons between groups were using the Log-rank test; (B–D) SIB exerts an inhibitory effect on caspy2 signaling; All zebrafish were harvested for measured mRNA expression by qPCR; Data are represented as the $\bar{x} \pm s$ of 3 independent experiments; (E) SIB reduced macrophage and neutrophil accumulation in LPS-infected larvae, marked by green fluorescence within the white-framed yolk sac; $^{*}P<0.05$, $^{**}P<0.01$

were significantly reduced in lung and liver after SIB treatment (Figure 6B).

SIB Protects Zebrafish Against Lethal LPS Infection

At 72 hpi, more than 50% of zebrafish infected with LPS (2 mg/mL) died, while 100% of zebrafish in the control group survived ($P<0.05$, Figure 7A). This indicated that lethal LPS-infected zebrafish inflammatory model could be successfully established. Meanwhile, different concentrations of SIB (100 and 200 μg/mL) increased the survival of LPS-infected zebrafish. Our data revealed that SIB treatment attenuated the expression of caspy2 and GSDMEb (homology to GSDMD) induced by LPS ($P<0.05$, Figures 7B and 7C). Moreover, the expression of IL-1β in LPS-infected zebrafish was inhibited by SIB treatment ($P<0.05$, Figure 7D). Evaluation was done to study the accumulation of macrophages and neutrophils in LPS-infected double-transgenic zebrafish line Tg (coro1a:GFP; lyz:Dsred). In this zebrafish line, macrophages and neutrophils are marked with green fluorescence. As shown in Figure 7E, in LPS-infected larvae, the green fluorescence noticeably increased at 12 hpi in the yolk. SIB decreased the accumulation of macrophages and neutrophils in LPS-infected larvae.

DISCUSSION

Endotoxemia, often triggered by the presence of LPS, leads to a cascade of inflammatory responses.

Elevated levels of pro-inflammatory cytokines such as TNF-α and IL-1β, along with increased expression of adhesion molecules, contribute to endothelial dysfunction and tissue damage. Concurrently, anti-inflammatory mediators like IL-10 may exhibit compensatory responses. The intricate interplay between these factors ultimately determines the severity and progression of endotoxemia, with imbalances contributing to the development of systemic inflammation and organ dysfunction. LPS is the most important mediator in endotoxemia. LPS triggers inflammatory responses through both extracellular and intracellular pathways. Classically, extracellular LPS first binds to the LPS binding protein (LBP). The LPS-LBP complex could initiate a pro-inflammatory downstream and can be located by CD14, MD2, and TLR4.[22] However, it has been documented that LPS could be internalized by macrophages and bonded to caspase-11. Active caspase-11 cleaves GSDMD to induce cell lysis and pyroptosis and then secretes IL-1β, IL-1α, and IL-18 because of pyroptosis during endotoxemia.[9,23,24] Therefore, inhibition of caspases-11 to block intracellular LPS signaling may be a potential therapeutic target for the clinical setting of endotoxemia. This study reveals that SIB can attenuate caspase-11-dependent cell pyroptosis by inhibiting the internalization of the LPS and the binding between caspase-11 and LPS. Furthermore, this study also reveals that SIB protects mice and zebrafish from lethal LPS infection.

SIB has shown notable inhibitory effects in inflammation. The previous studies had reported that SIB reduced the expression of IL-1β, IL-6, and TNF-α, which was dependent on inhibiting mitogen-activated protein kinase, nuclear factor kappa B, and signal transducer of activation signaling.[25-27] Moreover, SIB attenuated the LPS-induced expression of pro-inflammatory cytokines, like TNF-α, IL-1β, IL-6, and IL-8 *in vivo* and *in vitro*.[28,29] However, the detailed mechanism of SIB in endotoxemia and its protective role in endotoxemic damage remains unclear. In this present study, a novel mechanism that SIB could inhibit caspase-11-dependent cell pyroptosis by inhibiting the internalization of LPS and the binding of LPS and caspase-11 has been elucidated by us. This mechanism indicated that SIB protected endotoxemic mice from death by inhibiting the caspase-11 signal partially. Furthermore, SIB has been approved by US Food and Drug Administration for treating hepatic diseases.[16] SIB has the potential for the treatment of endotoxemia in a clinical setting in the future.

It has been documented that caspase-11 expression needs translocation and binding with LPS in the cytosol. In this study, it was foremost determined if SIB is functional in the internalization of LPS or the interaction of caspase-11 and LPS. The results of flow cytometry and LAL assay suggested that SIB prevented the transmembrane delivery of LPS, blocked the binding of caspase-11 and LPS, and consequently inhibited caspase-11 mediated cell pyroptosis. Consistent with the findings of our *in vitro* results, the expression of pro-caspase-11 in lung and liver was decreased by SIB treatment and the expression of N-GSDMD was almost attenuated in the LPS-mediated endotoxemic mice. Moreover, previous study has shown that caspy2 has the highest homology to human caspase-4/5, and it binds cytosolic LPS and then specifically cleaves GSDMEb to release its N terminus to mediate pyroptosis in zebrafish.[30,31] Therefore, the expressions of caspy2 and GSDMEb were determined by qPCR in zebrafish after SIB treatment. We found that SIB treatment attenuated the expression of caspy2 and GSDMEb, which were also consistent with the experiments conducted on mice.

Previous studies have reported the mechanism of the translocation of LPS into the cytoplasm. After being infected by Gram-negative bacteria, OMVs are internalized mediated by endocytosis, and then LPS is translocated into the cytoplasm from early endosomes. However, OMVs were not employed in the present study. Deng, et al[33] also reported that HMGB1 could bind to LPS and target its translocation into the lysosomes of, like macrophages and endothelial cells. Subsequently, after permeabilizing the phospholipid bilayer of lysosomes, HMGB1 led to the leakage of LPS into the cytoplasm and the activation of caspase-11.[32] Tsaroucha has reported that SIB inhibited HMGB1 expression in ischemia-reperfusion rat model. In this study, it was speculated that the inhibitory effect of translocation of LPS into the cytosol by SIB might partially depend on the depression of HMGB1. The mechanism of SIB responsible for the translocation of LPS into the cytosol would be explored in our next study.

To summarize, the results of this study validated that SIB could inhibit pyroptosis in the LPS-mediated endotoxemia model, at least in part, by inhibiting

the caspase-11-mediated cleavage of GSDMD. Additionally, this study has revealed a new mechanism by which SIB protects against endotoxemia. However, to promote the clinical application, future investigations need to be done to further explore the pharmacological mechanisms of SIB that are responsible for the inhibition of translocation of LPS into the cytoplasm, the binding of LPS, and caspase-11, and the location of the active site of caspase-11. Additionally, the underlying mechanism of SIB that was responsible for the reduction of caspase-11 to suppress the cleavage of GSDMD and subsequently result in a decrease of IL-1 α , IL-1 β , and IL-18 was also elucidated by us (Figure 8). The present study suggests a rationale for the clinical use of SIB in the treatment of endotoxemia and also reveals the critical role of caspase-11 in inflammation.

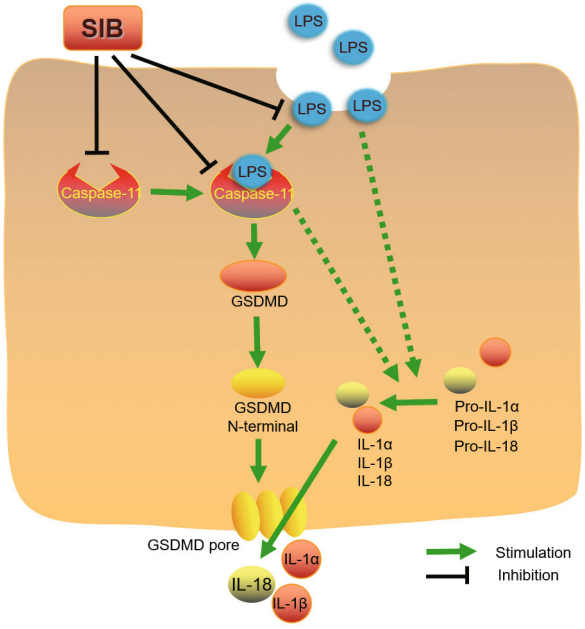

Figure 8. Anti-inflammatory Mechanism of SIB on LPS-Induced Endotoxemia

Notes: SIB exerts anti-inflammatory property by inhibiting caspase-11 expression and the cleavage of GSDMD, subsequently inducing reduction of the IL-1 α , IL-1 β , and IL-18; IL: interleukin, SIB: silibinin, LPS: lipopolysaccharides, GSDMD: gasdermin D

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Ou JY and Liu SH designed and conducted the experiment. Paper drafting and a portion of the *in vivo* experiment were carried out by Ou JY, Liu SH, Tang DK, Shi LZ, and Chen YY. Yan LJ, Huang JY, and You YT were responsible for data curation and visualization. Zou LF, You YT, Quan JY, and Yu LZ handled data curation, formal analysis, and methodology. Lu ZB provided supervision, research guidance, and reviewed the draft. All authors read and approved the final manuscript for publication.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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