The NLRP3 inflammasome mediates liver failure by activating procaspase-1 and pro-IL-1β and regulating downstream CD40-CD40L signaling

Zenghui Li and Jianning Jiang

Abstract

**Objectives:** In this prospective case–control study, we explored the regulatory roles of the NLRP3 inflammasome in hepatitis B virus-associated acute-on-chronic liver failure (HBV-ACLF).

**Methods:** Thirty patients with HBV-ACLF, 30 patients with chronic hepatitis B, and 30 healthy individuals were enrolled. Real-time reverse transcription polymerase chain reaction was used to assess mRNA levels in peripheral blood mononuclear cells and serum protein levels were assessed by enzyme-linked immunosorbent assay.

**Results:** Serum levels of alanine aminotransferase, asparagine aminotransferase, total bilirubin, and direct bilirubin in patients with HBV-ACLF were increased. Transcript levels of NLRP3 and ASC and protein levels of interleukin (IL)-1β, IL-18, and sCD40L were elevated in patients with HBV-ACLF. Expression of the NLRP3 inflammasome signaling pathway components procaspase-1 and pro-IL-1β was elevated in patients with HBV-ACLF.

**Conclusions:** This prospective case-control study demonstrated that significant activation of the NLRP3 inflammasome occurs in patients with HBV-ACLF. The activated NLRP3 inflammasome mediated liver failure by stimulating procaspase-1 and pro-IL-1β and regulating downstream CD40-CD40L signaling.

**Keywords**

NLRP3, hepatitis B virus, liver failure, inflammasome, CD40-CD40L, case–control study

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Introduction
Liver failure occurs when the liver is damaged and is no longer able to function because of hepatocyte necrosis. Liver failure develops rapidly and leads to severe illness and high mortality. The pathogenesis of liver failure is complex and is related to many factors including viral infections, alcohol, drugs, autoimmune diseases, hepatotoxic substance damage, and inflammatory immune damage. The major cause of liver failure in China is hepatotropic viral infection, especially by hepatitis B virus (HBV). The main clinical manifestation of hepatotropic viral infection is acute-on-chronic liver failure (ACLF), which has showed increasing incidence in recent years. The mechanisms underlying HBV-associated ACLF (HBV-ACLF) are complex. Therefore, studies investigating the mechanisms of HBV-ACLF are needed.

Inflammatory responses are characteristic manifestations of acute and chronic liver diseases. Inflammasomes are multi-protein complexes in cells that are involved in inflammation and immune responses during infection, diabetes, atherosclerosis, and cancer. Inflammasomes can prevent infection and maintain homeostasis by promoting activation of cellular factors such as interleukin (IL)-18 and IL-1β, inducing programmed cell death, initiating inflammatory responses, and activating immune responses. There are two main receptor proteins for inflammasomes: the NOD-like receptor (NLR) protein family and the AIM2-like receptor (ALR) protein family. NLRs are mainly expressed in immune cells and modulate inflammatory responses and natural immune responses through recognition of intracellular pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns. There are many activation signals for NLR family proteins. The activation signals for NLRP3 are the most fully understood and include components of pathogens (e.g., bacteria, viruses, and fungi) as well as non-pathogen molecules (e.g., adenosine triphosphate, uric acid, silicon crystal, and amyloid β). Recognition of PAMPs by NLRP3 inflammasomes plays important roles in antibacterial immune responses.

NLRP3 is an important component of inflammasomes, and can stimulate the maturation of precursors of the caspase-1-dependent cytokines IL-1β and IL-18 (pro-IL-1β and pro-IL-18). The NLRP3 inflammasome has been connected to various hepatopathies, and its activation is implicated in hepatic injury. Levels of NLRP3 in normal hepatic tissue are low. However, following injury, the numbers of neutrophils and monocytes in liver tissue are significantly increased, and NLRP3 expression in nonparenchymal cells such as Kupffer cells was increased in a CCL4-induced model of liver injury. Following ischemia/reperfusion injury, reactive oxygen species mediate the activation of NLRP3 and ALR inflammasomes to induce inflammatory responses. Kupffer cells also play important roles in this process. NLRP3 inflammasomes can potentiate inflammatory responses induced by galactosamine (GalN)/lipopolysaccharide (LPS) following the interaction between thioredoxin interacting protein and NLRP3. By contrast, heme oxygenase-1 can protect the liver by inhibiting NLRP3-associated signaling and antagonizing inflammation induced by GalN/LPS. Therefore, NLRP3 may be an important regulator of oxidative stress and inflammatory diseases. After stimulation of monocytes with LPS, expression of IL-1β in the hepatic tissues of patients with ACLF was elevated compared with healthy individuals, while IL-1β levels in patients with advanced ACLF are decreased. Therefore, understanding the role of NLRP3 in hepatic failure is crucial.
This prospective case–control study investigated the role of NLRP3 inflammasome activation in liver failure. The results may be helpful in developing novel therapies for hepatic failure.

**Materials and methods**

**Patients**

HBV-ACLF patients were selectively recruited. In addition, patients with chronic hepatitis B (CHB) with a similar age range were also enrolled. Healthy individuals were enrolled as the healthy control (HC) group. There was no special screening for sex in the three groups. We used the Model for End Stage Liver Disease (MELD) scoring system to grade liver disease. The system is mainly based on the international normalized ratio (INR), serum total bilirubin (TBIL), and serum creatinine. Patients with cirrhosis had clinical manifestations of decompensated liver function prior to inclusion. Patients with a previous history of HBV or hepatitis B surface antigen (HBsAg) positive test results for more than 6 months as well as current positive results for HBsAg and/or HBV DNA were diagnosed with CHB. The study was approved by The First Affiliated Hospital of Guangxi Medical University (ID: IACUC-20170301-03). Written informed consent was obtained from all patients and/or their families.

The inclusion criteria were: (i) age 18 to 65 years; (ii) history of HBV or positive HBsAg test for more than 6 months; and (iii) clinical manifestations of decompensated liver function (TBIL ≥ 171.1 μmol/L and INR ≥ 1.5) meeting the standards of the Guideline of Prevention and Treatment for CHB (2015 update) and Guidelines for Diagnosis and Treatment of Liver Failure (2018 edition). The exclusion criteria were: (i) other hepatitis virus infections; (ii) alcoholic, drug or autoimmune liver diseases; (iii) tumors; and (iv) serious systemic diseases. Peripheral blood was collected and centrifuged at 3500 × g for 5 minutes to separate serum or 1000 × g for 10 minutes to isolate plasma. To isolate peripheral blood mononuclear cells (PBMCs), 5 mL of plasma was thoroughly mixed with 5 mL of phosphate-buffered saline, then gently dispensed on top of 4 mL of Ficoll. After centrifugation at 2500 × g for 20 minutes, the middle PBMC layer was aspirated and diluted to 10 mL with phosphate-buffered saline. After centrifuging the sample at 1500 × g for 10 minutes, the upper layer of liquid was removed. PBMCs were mixed with cryopreservation solution and stored at −80°C.

We followed the EQUATOR guidelines for reporting observational studies ([https://www.equator-network.org/reporting-guidelines/reporting-participation-in-case-control-studies/](https://www.equator-network.org/reporting-guidelines/reporting-participation-in-case-control-studies/)).

**Quantitative reverse transcription polymerase chain reaction (RT-qPCR)**

TRIzol reagent (1 mL) was used to lyse 3 × 10⁶ cells. Total RNA was obtained by phenol chloroform extraction. The concentration and quality of RNA was assessed using a Nanodrop ND2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA (1 μg) was reverse transcribed into cDNA using the TIANScript II cDNA First Strand Synthesis Kit (Tiangen, Beijing, China) and stored at −20°C. To assess mRNA abundance of NLRP3, ASC, procaspase-1, and pro-IL-1β, RT-qPCR was carried out using the SuperReal PreMix (SYBR Green) qRT-PCR kit (Tiangen). Glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. The primer sequences for NLRP3 were 5’-CTATCTGTCTATATCCACTGTCG-3’ (forward) and 5’-GAGGAAGAGGATTCTGGAGG GT-3’ (reverse). The primer sequences for
ASC were 5’-CGCCGAGGAGCTCAAG
AAGT-3’ (forward) and 5’-GCTCGGC
GCCGTAGGTCTC-3’ (reverse). The
primer sequences for GAPDH were 5’-
TGACGTGGACATCCGCAAAG-3’ (for-
ward) and 5’-CTGGAAGGTGGACAG
CGAGG-3’ (reverse). Each RT-qPCR reac-
tion consisted of 10 µL of SYBR Premix
EXTaq, 0.5 µL of forward primer, 0.5 µL
of reverse primer, 2 µL of cDNA, and
7 µL of ddH2O. The reactions were incubat-
ed at 94°C for 52 minutes (initial denatur-
ation) followed by 46 cycles of 94°C for 30 s
(denaturation), 55°C for 30 s (annealing);
and 72°C for 30 s (elongation). To deter-
mine relative abundance of NLRP3
mRNA compared with GAPDH mRNA,
the 2−ΔΔCq method was employed (n = 3).

**Statistical analyses**

All data were analyzed using SPSS version
22.0 (IBM Corp., Armonk, NY, USA).
Data were expressed as means ± standard
deviations. To assess differences between
two groups, independent sample t-tests
were carried out. Comparisons among
more than two groups was performed
using one-way analysis of variance followed
by Student–Newman–Keuls post-hoc tests.
Values of P < 0.05 were considered statisti-
cally significant. Sample size was calculated
using a sample calculator (https://www.
powerandsamplesize.com/) and yielded an
estimate of at least 24 samples.

**Results**

**Patient characteristics**

Thirty patients with HBV-ACLF were
selectively recruited (18 men and 12
women). The HBV-ACLF patients were
aged from 18 to 65 years old (mean
45.6 ± 8.6 years). Thirty patients with
CHB with a similar age range as well as
30 healthy individuals were also enrolled.
Among the patients with HBV-ACLF,
eight showed cirrhosis with decompensated
liver function. The clinical characteristics of
participants are shown in Table 1.

**Biochemical indicators of liver function
are elevated in patients with HBV-ACLF**

Patients with HBV-ACLF had higher levels
of alanine aminotransferase, asparagine
aminotransferase, total bilirubin, and
direct bilirubin compared with patients
with CHB and HCs (P < 0.05) (Figure 1a–d).
These data suggested that biochemical
indices of liver function were elevated in
patients with HBV-ACLF.
**Table 1.** Clinical characteristics of study participants.

| Indicators                        | HC             | CHB            | HBV-ACLF        |
|----------------------------------|----------------|----------------|-----------------|
| MELD                             | Not tested     | 6.32 ± 1.45    | 23.43 ± 3.76    |
| INR                              | Not tested     | 0.87 ± 0.56    | 2.34 ± 0.39     |
| WBC (10⁹/L)                      | 6.45 ± 2.67    | 5.98 ± 1.45    | 8.27 ± 6.19     |
| Ascites                          | No             | No             | Yes             |
| Previous decompensations         | No             | No             | Yes             |

Data represent means ± standard deviations.

HC, healthy control; CHB, chronic hepatitis B; HBV-ACLF, hepatitis B virus-associated acute-on-chronic liver failure; MELD, model for end stage liver disease; INR, international normalized ratio; WBC, white blood cells.

**Figure 1.** Biochemical indicators of liver function in patients with HBV-ACLF, patients with CHB, and HCs. Serum levels of (a) ALT, (b) TB, (c) AST and (d) DB were measured. *, P < 0.05; ***, P < 0.01; n.s., not significant. HC, healthy control; CHB, chronic hepatitis B; HBV-ACLF, hepatitis B-associated acute-on-chronic liver failure; ALT, alanine aminotransferase; AST, asparagine aminotransferase; TB, total bilirubin; DB, direct bilirubin.

**NLRP3 and ASC mRNA and protein levels are increased in patients with HBV-ACLF**

We used RT-qPCR and ELISA to assess levels of NLRP3 and ASC mRNA and protein. PBMCs from patients with HBV-ACLF had higher levels of NLRP3 and ASC mRNA compared with those of patients with CHB or HCs (P < 0.05) (Figure 2a and b). ELISA showed that
NLRP3 and ASC levels in the sera of patients with HBV-ACLF were significantly increased compared with those of patients with CHB or HCs \((P < 0.05)\) (Figure 2c and d). These data showed that levels of NLRP3 and ASC were increased in patients with HBV-ACLF.

Patients with HBV-ACLF have elevated levels of IL-1β, IL-18, and sCD40L

Levels of immune-related molecules were assessed by ELISA. The sera of patients with HBV-ACLF had increased levels of IL-1β, IL-18 and sCD40L compared with those of patients with CHB and HCs \((P < 0.05)\) (Figure 3a, b and d). Interestingly, serum levels of sCD40 in patients with HBV-ACLF were increased compared with HCs but decreased compared with patients with CHB \((P < 0.05)\) (Figure 3c). These data show that levels of the immune-related molecules IL-1β, IL-18, and sCD40L were increased in patients with HBV-ACLF.

Expression of NLRP3 inflammasome signaling pathway molecules is increased in patients with HBV-ACLF

To further study the mechanisms of NLRP3 in HBV-ACLF, we examined mRNA and protein levels of the NLRP3 inflammasome signaling pathway molecules, procaspase-1 and pro-IL-1β, in PBMCs and serum. Procaspase-1 and pro-IL-1β mRNA and protein levels were significantly
increased in PBMCs from patients with HBV-ACLF compared with those of patients with CHB or HCs (P < 0.05) (Figure 4a–d). These data showed that levels of NLRP3 inflammasome signaling pathway molecules were increased in patients with HBV-ACLF compared with patients with CHB or HCs.

**Discussion**

Liver failure is a heterogeneous and serious disease that is characterized by major harms, treatment difficulties, poor prognosis, and high mortality. Liver failure can be categorized into acute liver failure, subacute liver failure, ACLF, and chronic liver failure; ACLF is the most common. HBV-ACLF can lead to metabolic disorders and further impairment of multiple organ functions. The mechanisms underlying HBV-ACLF are complex and include immune damage and inflammatory responses.

The NLRP3 inflammasome is an important intracellular protein complex and plays important roles in liver diseases. Following stimulation, NLRP3 combines with ASC and procaspase-1 to form inflammasomes. Inactive procaspase-1 is activated to form caspase-1, which then converts inactive IL-1β and IL-18 into their active state and mediating inflammatory apoptosis. NLRP3 inflammasomes can be activated by many different stimuli including LPS, nucleic acids, hyaluronic
Acid, and heparin sulfate. However, the specific molecular mechanisms of activation remain unclear. Activation of the NLRP3 inflammasome is associated with intracellular potassium outflow, lysosomal damage, intracellular cAMP reduction, and increased calcium concentration. The occurrence of ACLF is associated with many confounding factors. For example, high dynamic and low dynamic circulation in patients with liver cirrhosis, as well as systemic infection, are high risk factors for fatal ACLF. There appears to be no correlation between severity of liver disease and cardiac insufficiency. Acute decompensation can lead to deposition of type III collagen and poor prognosis in patients with liver cirrhosis. When CD40L expressed on activated T cells interacts with CD40 on macrophages, this can inhibit the activation of NLRP3 inflammasomes in macrophages. CD40L alone can also inhibit the activation of macrophage NLRP3 inflammasomes by activators such as alum. Our data demonstrated that the mRNA levels of the NLRP3 inflammasome-associated molecules ASC, NLRP3, and caspase-1 were significantly increased in PBMCs from patients with HBV-ACLF compared with patients with CHB and HCs.

The regulatory network of the CD40-CD40L costimulatory pathway is also complex. Serum sCD40L and sCD40 have key

Figure 4. Levels of procaspase-1 and pro-IL-1β in patients with HBV-ACLF, patients with CHB, and HCs. (a and c) Relative expression of (a) procaspase-1 and (c) pro-IL-1β mRNA in PBMCs measured by RT-qPCR. (b and d) Levels of (b) procaspase-1 and (d) pro-IL-1β proteins in serum measured by ELISA. *, P < 0.05; **, P < 0.01.

HC, healthy control; CHB, chronic hepatitis B; HBV-ACLF, hepatitis B-associated acute-on-chronic liver failure; IL, interleukin; PBMC, peripheral blood mononuclear cell; RT-qPCR, quantitative reverse transcription polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.
functions in ACLF.\textsuperscript{37} sCD40L can bind to CD40 on monocytes, stimulating CD40-CD40L signaling and activating the cell.\textsuperscript{37} The interaction between sCD40 and CD40L inhibits immunoglobulin production and T cell activation, playing an immunosuppressive role.\textsuperscript{38} When inflammation and necrosis are aggravated in liver tissues of patients with CHB, levels of sCD40 are increased significantly in serum. However, levels of sCD40 in the sera of patients with liver failure are similar to those of CHB patients with level 1 inflammation and necrosis, but lower than those of CHB patients with level 2 or 3 inflammation and necrosis.\textsuperscript{39} Another study showed that CD40 and CD40L expression was significantly higher in liver tissue from patients with fulminant liver failure compared with patients with chronic liver disease or healthy individuals. LPS stimulates human liver macrophages to up-regulate CD40L expression.\textsuperscript{39} It has recently been shown that inflammasome activation in both PBMCs and in the liver results in IL-1 production.\textsuperscript{40,41} We did not investigate liver-derived mediators in this study. Additionally, we did not examine levels of IL-1α or gasdermin, which will be the focus of a future study. We also plan to carry out animal experiments to explore the mechanisms of NLRP3 in liver disease. The role of NLRP3 in HBV-ACLF needs to be further investigated. In future studies, we plan to investigate how NLRP3 regulates the CD40-CD40L pathway and how caspase-1, IL-1β, and NLRP3 interact with the CD40-CD40L pathway to regulate the occurrence and development of HBV-ACLF.

In conclusion, this prospective case-control study showed significant activation of the NLRP3 inflammasome in patients with HBV-ACLF. In addition, the activated NLRP3 inflammasome may participate in liver failure by activating procaspase-1 and pro-IL-1β as well as by regulating downstream CD40-CD40L signaling. Future studies will address how NLRP3 interacts with procaspase-1 and pro-IL-1β to regulate the CD40-CD40L pathway.

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Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions
ZL and JJ contributed to the design of the study. ZL performed the experiments. JJ analyzed the data. ZL and JJ interpreted results and prepared the manuscript. The final version of the manuscript was read and approved by both authors.

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