ORIGINAL ARTICLE

Cathepsin B regulates non-canonical NLRP3 inflammasome pathway by modulating activation of caspase-11 in Kupffer cells

Nan Chen^{1,2} | Zhibing Ou¹ | Wenfeng Zhang¹ | Xiwen Zhu² | Peizhi Li¹ | Jianping Gong¹

¹Chongqing Key Laboratory of Hepatobiliary Surgery, Department of Hepatobiliary Surgery, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China

²Department of Anesthesia, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China

Correspondence

Peizhi Li and Jianping Gong, Department of Hepatobiliary Surgery, The Second Affiliated Hospital of Chongqing Medical University, 74 Linjiang Road, Chongqing 400010, China. Emails: lipeizhi@163.com; gongjianping11@126.com

Funding information

National Natural Science Foundation of China, Grant/Award Number: No. 81601715, No. 31370753 and No. 81401622; Basic Science and Frontier Technology Research Foundation of Chongqing science & technology commission, Grant/Award Number: No. cstc2015jcy jBX0070

Abstract

Objectives: The non-canonical inflammasome pathway was described which engages caspase-11 to mediate pyroptosis and the subsequent release of IL-1 α , IL-1 β and IL-18 in TLR4-independent way. Cathepsin B is capable of activating caspase-11 under cell-free conditions which may regulate non-canonical NLRP3 inflammasome pathway. In this study, we aimed to further investigate cathepsin B as potential activators of proinflammatory caspases which may be released upon proinflammatory stimuli and regulate non-canonical NLRP3 inflammasome pathway by modulating the activity of caspase-11.

Methods: Pharmacological and gene-silencing approaches were used to evaluate the impact of cathepsin B on regulating non-canonical NLRP3 inflammasome pathway in wild-type and TLR4^{-/-} Kupffer cells. A sepsis model was also created to investigate the effect of cathepsin B on survival. Meanwhile, cathepsin B activity and the expression level of caspase-4 were detected in human peripheral blood mononuclear cells (PBMC) which were separated from patients suffered from SIRS or sepsis and healthy volunteers.

Results: LPS stimulation caused cathepsin B activity and caspase-11 expression increase in TLR4^{-/-} mice. Cathepsin B activity inhibition reduced the activation of caspase-11 and inflammasome and benefited survival in TLR4^{-/-} mice. Upregulation of cathepsin B activity and caspase-4 activation was found in PBMC of patients with SIRS or sepsis.

Conclusion: Our results suggest a critical role for cathepsin B as activators of proinflammatory caspases-11 and the regulatory effect in LPS-induced caspases-11-dependent necrosis.

1 | INTRODUCTION

Lipopolysaccharide (LPS), a component present in many Gram-negative bacteria, is usually sensed exclusively through Toll-like receptor 4 (TLR4) a member of the Toll-like receptor (TLR) family dedicated to the detection of infectious microorganisms, inducing

production of the proinflammatory cytokines.¹⁻³ However, our previous work have found that blocking TLR4 signalling pathway did not completely eliminate the LPS signal recognition and conduction which show that LPS can also be sensed in a TLR4-independent manner in the cytoplasm of host cells.

The inflammasomes are a group of protein complexes that consist of an inflammasome sensor molecule, the adaptor protein ASC and caspase-1. Once the protein complexes have formed, the

Nan Chen and Zhibing Ou are the authors who have equal contributions to the study.

inflammasomes engage and activate caspase-1, which activates the proinflammatory cytokines interleukin-1 β (IL-1 β) and IL-18 as well as cell death termed "pyroptosis."^{4,5} The best-studied inflammasome contains the protein NLRP3 which engages the adapter molecule ASC then recruiting and activating the effector enzyme caspase-1.⁶ Although most activators of NLRP3 lead to caspase-1 engagement by canonical inflammasome activation,⁷ recent studies during infections with Gram-negative (but not Gram-positive) bacterial pathogens engagement of caspase-11 (human as caspase-4) was essential to facilitate NLRP3-ASC-dependent caspase-1 activation and IL-1 β maturation.⁸ Rathinam et al proposed that procaspase-11 undergoes autoprocessing when expressed at significant levels through TLR4/Trif-mediated type-I-IFN production.⁹ Kayagaki et al show that higher quantities of LPS, which reach the cytoplasm can also be sensed in a TLR4-independent manner through an as-yet-unidentified receptor which leads to the activation of caspase-11.¹⁰ In addition to activating caspase-1, caspase-11 can cause host-cell death in a caspase-1-independent way which results in non-canonical NLRP3 inflammasome activation.¹¹ What is the identity of the as-yet-unidentified receptor? Early research revealed that the lysosomal protease cathepsin B is capable of activating caspase-11 under cell-free conditions.^{12,13} However, whether cathepsin B has the same effect in vivo is still unknown.

About one-third of patients with sepsis shock result from Gram-negative bacterial infections with a high mortality rate.¹⁴ Highly specific TLR4-inhibiting drugs recently failed to reduce deaths from sepsis in human clinical trials.¹⁵ A better understanding of the non-canonical NLRP3 inflammasome activation in Gram-negative bacterial recognition may improve the outcome of sepsis.

Our previous experiments showed synchronous increase of cathepsin B activity and caspase-11 in LPS-primed Kupffer cells (KCs). We propose a new assumption: cytoplasmic LPS will facilitate cathepsin B activation and subsequently cleave proinflammatory caspase-11 into enzymatic active caspase-11 results in non-canonical NLRP3 inflammasome activation.

2 | MATERIALS AND METHODS

2.1 | Reagents

LPS (*Escherichia coli* O111:B4) and FITC-LPS were purchased from Sigma (Saint Louis, MO, USA). FuGENE transfection reagent was procured from Promega (Madison, WI, USA). The inhibitor N-[[[(2S,3S)-3-[(propylamino)carbonyl]-2-oxiranyl] carbonyl]-L-isoleucyl-L-proline, methyl ester (CA-074-Me) was obtained from Cayman chemical (Ann Arbor, MI, USA). The mouse IL-1 β ELISA kit and mouse IL-1 α ELISA kit were purchased from Boster Biological Technology (Pleasanton, CA, USA). The human IL-18 ELISA kit, human IL-1 α ELISA kit and human IL-1 β ELISA kit were purchased from Boster Biological Technology (Pleasanton, CA, USA). The mouse IL-18 ELISA kit was from NeoBioscience (Shenzhen, China) and used as described in the manufacturer's instructions. The antibodies against mouse caspase11

(ab10454), human caspase 4 (ab22687) and cathepsin B (ab58802) were from Abcam (Cambridge, MA, USA). The anti-procaspase-1 and caspase-1 p20 (NBP1-45433) were from Novus Biologicals (Littleton, CO, USA). The antibodies against NLRP3(#15101) was from CST (Danvers, MA, USA). The antibodies against β -actin (sc-47778) were from Santa Cruz Biotechnology (Dallas, TX, USA). Cathepsin B shRNA lentiviral particles (sc-29933-v) and puromycin dihydrochloride (sc-108071) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2 | KCs experiments

KCs were isolated separately from liver of wild-type (WT) C57BL/6 mice and TLR4^{-/-} mice by collagenase digestion and differential centrifugation as described in the literature of Pei-zhi Li et al.¹⁶ KCs were cultured in 6-well plates at a density of 1×10^6 cells/well in DMEM (Hyclone, USA) containing 10% FBS (Hyclone, USA) and 1% antibiotics (100 U/mL of penicillin G and 100 mg/mL of streptomycin sulphate) at 37°C in a humidified incubator. KCs were randomly divided into 5 groups: control group, low-dose LPS group, large dose LPS group, CA-074-Me group (CA-074 group) and cathepsin B shRNA transfection group (shRNA group). The KCs of shRNA group were transfected to cathepsin B shRNA plasmid (Santa Cruz, USA) according to the shRNA transfection protocol. The KCs of CA-074 groups were pretreated with 100 μ M/L CA-074-Me 1 hour prior to LPS stimulation. Then, cells in low-dose LPS group were added fresh medium containing LPS (10 ng/mL) and except the control group, the remaining 3 groups were added fresh medium containing LPS (10 μ g/mL) and harvested at 0, 2, 4 and 6 hours, respectively. To observe the intracellular uptake of LPS, FITC-LPS (10 μ g/mL) was used to stimulate KCs and FITC-LPS (1 μ g/mL) was encapsulated by transfection reagents and transfected KCs. KCs were transfected with 1 μ g/mL LPS plus 0.25% v/v FuGENE HD.¹⁷

2.3 | Animals experiments

Male WT C57BL/6 mice (8-10 weeks old) and TLR4^{-/-} mice were purchased from Experimental Animal Center of Chongqing Medical University. TLR4^{-/-} mice are all on the C57BL/6 background. Animals were maintained in accordance with the National Institutes of Health guidelines for animal research and the legal requirements in China. The WT and TLR4^{-/-} mice were divided randomly into the following groups (n = 10/group): NS (normal saline) group, LPS group, CA-074 group (the cathepsin B inhibitor) and CA-074 + LPS group. Mice were pretreated with intraperitoneal injection of 10 mg/kg CA-074-Me in CA-074 group and CA-074 + LPS group and an equal volume of sterile normal saline was injected intraperitoneally in NS group and LPS group. One hour after the first injection, mice in LPS group and CA-074 + LPS group were intraperitoneally injected of 20 mg/kg LPS. 0, 3, 6 and 12 h after LPS injection, mice was sacrificed and the samples of the liver and blood were harvested. KCs and serums were immediately isolated for Western blot analysis, ELISA, etc. Meanwhile, WT and TLR4^{-/-} mice were pretreated as described

above and challenged with a lethal dose of LPS (54 mg/kg). Survival was monitored once per 12-h period for 96 h and the samples of the liver and blood were harvested 6 h after LPS stimulation in NS group and LPS group.

2.4 | Human peripheral blood mononuclear cells separation

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Committee for Human Subjects of Chongqing Medical University. Peripheral blood samples were obtained from patients suffered from SIRS or sepsis. Peripheral blood samples of healthy volunteers were considered as control. PBMCs were isolated from 10 mL of EDTA anticoagulated blood on Ficoll density gradient centrifugation method using Histopaque-1077 (Sigma-Aldrich). Briefly, blood was carefully laid onto Histopaque-1077 in a 15-mL conical centrifuge tube. After centrifugation at $400 \times g$ for 30 min at room temperature, the layer containing PBMCs was carefully transferred and washed 3 times with phosphate-buffered saline (PBS). The plasma samples were obtained for detection of proinflammatory cytokines.

2.5 | Transfection of cathepsin B shRNA

Cultured KCs, isolated from liver of WT and TLR4^{-/-} mice, were transfected to cathepsin B shRNA plasmid (Santa Cruz) according to the shRNA transfection protocol. Briefly, KCs were cultured with complete optimal medium (with serum and antibiotics) in a 12-well plate (3×10^5 cells/well) 24 hours prior to viral infection. Remove media from plate wells and replace with 1 mL of Polybrene/media mixture (5 μ g/mL) per well. Then, shRNA lentiviral particles were added and incubated overnight. Cells were cultured for an additional 48 hours in complete medium without Polybrene. Stable clones expressing the shRNA were selected via puromycin dihydrochloride (6 μ g/mL).

2.6 | Cytotoxicity assays

KCs were treated as described above and cytotoxicity was quantitated by measurement of lactate dehydrogenase (LDH) using the LDH Cytotoxicity Assay Kit (Beyotime Biotechnology) according to the manufacturer's instructions.

2.7 | Cytosolic cathepsin B activity assays

For the extraction of cytosolic protein without disruption of lysosomes, treated KCs, KCs isolated from treated mice or PBMC were lysed by a described digitonin extraction method.¹⁸ After the removal of the medium, extraction buffer (25 μ g/mL digitonin, 250 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L pefablock, pH 7.5) was added and incubated on ice for 5 min. The lysis was centrifuged (1 min, $14\,000 \times g$, 4°C) and the resulting supernatant was

transferred and assayed for cathepsin B activity using Cathepsin B Activity Fluorometric Assay Kit (Biovision) according to the protocol.

2.8 | ELISA

ELISA was used to assay levels of IL-18, IL-1 α and IL-1 β in supernatants harvested from treated KCs or serums obtained from mice to patients according to the protocol.

2.9 | Western blot analysis

Treated KCs, KCs isolated from treated mice and PBMC were directly lysed in a buffer containing 0.42 mmol/L NaCl, 15 mmol/L $MgCl_2$, 20 mmol/L HEPES, 25% glycerol, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 0.5 mmol/L dithiothreitol. The cell lysis buffer above was centrifuged (20 min, $14\,000 \times g$, 4°C). Protein concentration of supernatants was determined by Bradford Assay Kit (Bio-Rad, USA). Protein samples were boiled, separated by SDS-PAGE and transferred polyvinylidene fluoride (PVDF) membrane. Primary antibodies against cathepsin B, caspase-1 (caspase-1 p20), NLRP3, mouse caspase11, human caspase 4 and β -actin were used. Finally, the membranes were developed using enhanced chemiluminescence detection kit (Pierce, USA) and exposed to autoradiographic film (Kodak, USA).

2.10 | Immunofluorescence and histological analysis

The protein expressions of cathepsin B and caspase-11 were detected by IF. In detail, KCs were seeded in a 24-well chamber slides at a density of 1×10^5 cells/well and incubated overnight. Cells were treated according to the above method mentioned in "KCs experiments." Then, the cells were fixed by 4% paraformaldehyde (PFA) for 10 min and incubated in goat serum for 1 h to block non-specific protein-protein interactions. The cells were then incubated overnight at 4°C with primary antibody diluted as indicated on datasheet (Abcam, Cambridge, MA, USA). Liver tissues harvested from treated mice were made into paraffin section and stained with haematoxylin and eosin (HE).

2.11 | Transmission electron microscope observation

Liver tissues harvested from treated mice were cut into a 1 millimetre cube and fixed in 2% glutaraldehyde. These specimens were delivered to the Electron Microscopy Center of Chongqing Medical University and observed under TEM.

2.12 | Statistics

All data are shown as means $\pm$ SD. Statistical significance was determined using the software spss 13.0 (SPSS, Inc., Chicago, IL, USA) by the one-way analysis of variance (ANOVA) for multiple comparisons and Student's *t* test for single comparisons. *P* < .05 was considered statistically significant.

FIGURE 1 The impact of a lethal dose of lipopolysaccharide (LPS) on cathepsin B activity, NLRP3 inflammasome activation and pro-inflammatory cytokines levels in both TLR4^{-/-} and wild-type (WT) mice. A, Kaplan-Meier survival curve for TLR4^{-/-} and WT mice challenged with 54 mg/kg LPS. Experiments used 6 mice of each genotype. B, Immunoblot analysis of cathepsin B and inflammasome proteins NLRP3 expression in Kupffer cells (KCs) of the NS group and LPS (54 mg/kg) group. C, Cytosolic cathepsin B activity assays in KCs. KCs were from 3 different mice. D, Enzyme-linked immunosorbent assay analyses of interleukin 1 α (IL-1 α), interleukin 18 (IL-18) and interleukin 1 β (IL-1 β) concentration changes in serums obtained from TLR4^{-/-} and WT mice 6 h after LPS (54 mg/kg) stimulation. NS refers to saline group. The results are depicted as means $\pm$ SD (n = 3). *P < .05

FIGURE 2 Triggering of the non-canonical inflammasome by large dose of LPS (10 μ g/mL) in Kupffer cells (KCs). Cells were isolated separately from wild-type (WT) and TLR4^{-/-} mice and divided into the following 2 groups: low dose of LPS group, in which cells were pretreated with 10 ng/mL LPS and large dose of LPS group, in which cells were pretreated with 10 μ g/mL LPS. A, Immunofluorescence detection for intracellular uptake of FITC-LPS in WT and TLR4^{-/-} KCs 4 h after large doses of LPS challenge and LPS transfection. B, Western blots to detect caspase-11 and inflammasome proteins NLRP3 expression in WT and TLR4^{-/-} KCs 0, 2, 4 and 6 h after low or large doses of LPS challenge. C, Enzyme-linked immunosorbent assay analyses of interleukin 1 α (IL-1 α), interleukin 18 (IL-18) and interleukin 1 β (IL-1 β) concentration changes in cells culture medium 0, 2, 4 and 6 h after low or large doses of LPS challenge. D, Cytosolic cathepsin B activity assays in KCs 0, 2, 4 and 6 h after low or large doses of LPS challenge. E, Cell death (% cytotoxicity) was measured by LDH release 0, 2, 4 and 6 h after low or large doses of LPS challenge. The results are depicted as means $\pm$ SD (n = 3). *P < .05

3 | RESULTS

3.1 | Cathepsin B activity and the activation of caspase-11 were upregulated in TLR4^{-/-} mice stimulated with large doses of LPS

TLR4 plays a central role in detecting LPS, but our in vivo experiment demonstrated that lethal dose of LPS challenge caused morbidity in all TLR4^{-/-} and WT mice within 84 hours (Figure 1A). TLR4-independent mechanisms for sensing LPS contribute to this

model of lethal sepsis. Western blotting analysis showed that no significant changes in levels of NLRP3, caspase-1 and processed caspase-1 (caspase-1 p10) after large doses of LPS stimulation in TLR4^{-/-} mice, whereas processed caspase-11 (caspase-11 p30) was significantly increased (Figure 1B). There was no significant difference of cathepsin B protein expression, while the cathepsin B activity in the cytosol was dramatically upregulated in both TLR4^{-/-} and WT mice (Figure 1C). proinflammatory cytokines including IL-1 α , IL-18 and IL-1 β were rapidly released in response to large

dose LPS stimulation in both TLR4^{-/-} and WT mice, but the IL-1 β and IL-18 levels in TLR4^{-/-} mice were significantly lower than that in WT mice (Figure 1D). These data suggest that large doses of

LPS stimulation in TLR4^{-/-} mice could still led to immune response which may be associated with the activation of caspase-11 and increased cathepsin B activity.

FIGURE 3 The effects of cathepsin B inhibition or knockdown on NLRP3 inflammasome activation and pro-inflammatory cytokines levels in both TLR4^{-/-} and WT Kupffer cells (KCs). Cells were isolated separately from WT and TLR4^{-/-} mice and divided into the following 4 groups: the Control group, the LPS group and the CA-074 + LPS group, in which cells were infected with control shRNA lentiviral particles; and the shRNA + LPS group, in which cells were transfected with cathepsin B-shRNA lentiviral particles. When stable clones expressing the shRNAs were established, the cells in the LPS group, the CA-074 + LPS group and the shRNA + LPS group were stimulated with large dose of lipopolysaccharide (LPS) (10 µg/mL) and the KCs of CA-074 + LPS group were pretreated with 100 µM/L CA-074-Me 1 h prior to LPS stimulation. A, Immunoblot analysis of cathepsin B and inflammasome proteins NLRP3 expression in KCs 4 h after LPS stimulation. B, Enzyme-linked immunosorbent assay analyses of interleukin 1α (IL-1α), interleukin 18 (IL-18) and interleukin 1β (IL-1β) concentration changes in cells culture medium 4 h after LPS challenge. C, Cell death (% cytotoxicity) was measured by LDH release 4 h after large doses of LPS challenge. D, Cytosolic cathepsin B activity assays in KCs 4 h after large doses of LPS challenge. E, Laser confocal fluorescence detection of cathepsin B and caspase-11 protein colocalization. The results are depicted as means ± SD (n = 3). *P < .05

3.2 | Cathepsin B contributes to inflammasome activation in response to large doses of LPS by activating caspase-11

We explored further the connection between cathepsin B and caspase-11 in inflammasome activation on both TLR4^{-/-} and wild-type (WT) KCs stimulated with LPS at varying doses in vitro. We stimulated KCs with FITC-LPS (10 µg/mL) and observed the intracellular uptake of FITC-LPS. We also encapsulated FITC-labelled LPS by transfection reagents and transfected KCs which mimicked LPS internalization by uptake of bacterial microvesicles. Green fluorescent particles appeared in cytoplasm (Figure 2A). Caspase-11 and caspase-1 were found to be processed, respectively, releasing a fragment of 30 kDa and 20 kDa in large dose LPS-primed WT KCs and increased time dependently and peaked at 4 h. Meanwhile, NLRP3 expression was significantly increased in WT KCs. However, only caspase-11 was processed in large dose LPS-primed TLR4^{-/-} KCs (Figure 2B). The production of inflammatory cytokines were significantly upregulated in cell culture supernatant of large dose LPS-primed TLR4^{-/-} KCs (Figure 2C). Cathepsin B activity in the cytosol increased time dependently and peaked at 4 h after large dose LPS stimulation (Figure 2D). Extensive cell death was observed and increased over time in large dose LPS-primed TLR4^{-/-} and WT KCs (Figure 2E). These data suggest that large doses of LPS (10 µg/mL) induce inflammatory cytokines generation and cell death in both TLR4^{-/-} and WT KCs which were related to caspase-11 activation and increased activity of cathepsin B.

3.3 | Inhibition of Cathepsin B activity or Cathepsin B knockdown reduce caspase-11 activation and inflammasome generation

To further verify the role of cathepsin B in caspase-11 activation and inflammasome generation, the activity and expression of cathepsin B were downregulated by the cathepsin B inhibitor and gene knockdown. TLR4^{-/-} and WT KCs were pretreated with the cathepsin B inhibitor CA-074-Me and were transfected to cathepsin B shRNA plasmid for the inhibition of cathepsin B expression. Transduction of cathepsin B shRNA led to a significant decrease in cathepsin B protein levels. However, no significant reduction in cathepsin B protein levels was observed after pretreated with CA-074-Me (Figure 3A). The cathepsin B activities were greatly inhibited after pretreated with CA-074-Me

and cathepsin B shRNA transduction (Figure 3D). Large doses of LPS induced processed caspase-11 (caspase-11 p30) and cell death in TLR4^{-/-} KCs, which were blocked by the cathepsin B inhibitor CA-074-Me and cathepsin B gene knockdown (Figure 3A,C). Meanwhile, the production of inflammatory cytokines was significantly downregulated in the CA-074 group and Cathepsin B-shRNA group (Figure 3B). The result of IF showed that the expression of caspase-11 protein decreased simultaneously with cathepsin B in a more intuitive manner (Figure 3E). These data suggest that inhibition of cathepsin B activity or cathepsin B knockdown fails to induce the activation of proinflammatory caspase-11 and inflammatory cytokines.

3.4 | Cathepsin B inhibition benefits survival in TLR4^{-/-} mice

TLR4^{-/-} mice pretreated with CA-074-Me were more resistant to lethal dose of LPS challenge. However, all the 6 TLR4^{-/-} mice pretreated with CA-074-Me were alive at 36 h after LPS injection, all the 6 WT mice had succumbed within 12 h and all the 6 WT mice pretreated with CA-074-Me were alive at 24 h after LPS injection (P < .05; Figure 4A). Western blotting analysis showed that CA-074-Me pre-treatment reduced the activation of caspase-11 (Figure 4B). Histopathologic changes of liver tissue of TLR4^{-/-} mice in CA-074 + LPS group showed less evidence of acute inflammatory injury compared with LPS group after 12 hours of large doses of LPS challenge. Mild ballooning degeneration of hepatocytes, spotty necrosis and few inflammatory infiltrating the portal area were observed in CA-074 + LPS group. However, severe cellular swelling and more inflammatory cells infiltrating the portal area were observed in LPS group (Figure 4C). Meanwhile, ultrastructures of liver tissues were obtained by TEM. Fewer mitochondrion oedema, fragmentation of cristae or vacuolation were observed in TLR4^{-/-} CA-074 + LPS group as compared to the TLR4^{-/-} LPS group (Figure 4D). Moreover, TLR4^{-/-} KCs in LPS group presented primary lysosome increasing and discontinuity of lysosomal membrane (Figure 4D). Serum IL-1α, IL-18 and IL-1β levels were markedly reduced in TLR4^{-/-} CA-074 + LPS group (Figure 4E). Cathepsin B activity in the cytosol was significantly decreased in TLR4^{-/-} CA-074 + LPS group (Figure 4F). Our data suggest that cathepsin B inhibition benefits survival of lethal dose of LPS strike by indirectly inhibiting the activity of caspase-11.

FIGURE 4 The impact of cathepsin B inhibition on cathepsin B activity, NLRP3 inflammasome activation, histopathological and ultrastructural changes in liver tissues and pro-inflammatory cytokines levels in TLR4^{-/-} mice. TLR4^{-/-} mice were divided into the following 4 groups: the NS group and the LPS group, in which mice were pretreated with intraperitoneal injection of 10 mg/kg sterile normal saline; the CA-074 group and CA-074 + LPS group, in which mice were pretreated with intraperitoneal injection of 10 mg/kg CA-074-Me. One hour after the first injection, mice in LPS group and CA-074 + LPS group were intraperitoneally injected of 20 mg/kg lipopolysaccharide (LPS). 0, 3, 6 and 12 h after LPS injection, mice were sacrificed, the samples of the liver and blood were harvested and Kupffer cells (KCs) were isolated. A, Kaplan-Meier survival curve for TLR4^{-/-} and wild-type (WT) mice challenged with 20 mg/kg LPS. Experiments used 6 mice of each genotype. B, Immunoblot analysis of cathepsin B and inflammasome proteins NLRP3 expression in TLR4^{-/-} mice. C, Histopathological changes in liver tissues 12 h after LPS challenge (haematoxylin and eosin stain; original magnification $\times 400$). Arrows indicate damaged liver cells and triangles indicate infiltrating inflammatory cells. D, Transmission electron microscope (TEM) observation for Ultrastructural changes of lysosomes in TLR4^{-/-} KCs (original magnification $\times 10\,000$). Red arrows indicate discontinuity of lysosomal membrane. E, Enzyme-linked immunosorbent assay analyses of interleukin 1 α (IL-1 α), interleukin 18 (IL-18) and interleukin 1 β (IL-1 β) concentration changes in serums obtained from TLR4^{-/-} mice 12 h after LPS stimulation. F, Cytosolic cathepsin B activity assays in TLR4^{-/-} KCs 12 h after LPS challenge. The results are depicted as means $\pm$ SD (n = 3). *P < .05

3.5 | Cathepsin B activity and protein expression is upregulated in PBMC of patients with endotoxaemia

Western blotting analysis showed that caspase-4, processed caspase-4 (caspase-4 p19) and caspase-1 expression in the Patient group was much higher than that in the control group (Figure 5A). Cathepsin B activity in the cytosol increased significantly and the level of cathepsin B protein expression increased slightly in the patient group (Figure 5A,C). Meanwhile, the production of inflammatory cytokines was significantly increased in the patient group (P < .05; Figure 5C). These data suggest an association between upregulation of cathepsin B activity and sepsis.

4 | DISCUSSION

Many bacterial toxins promote inflammasome activation¹⁹⁻²¹ and LPS—a prototypical endotoxin, elicit strong immune responses on hosts infected with Gram-negative bacteria which ultimately can lead to septic shock.^{22,23} Research has shown that Gram-negative bacteria elicit non-canonical inflammasome activation which depends on inflammatory pro-caspase 11 in mice.²⁴ Caspase-11 contributes both caspase-1-dependent and -independent outputs in inflammasome-triggered pyroptosis which highlights a unique proinflammatory role for caspase-11 in the innate immune response to bacterial infections.⁸ Consistent with previous research, our previous work demonstrated that LPS can be sensed in a TLR4-independent manner in the cytoplasm of host cells through an as-yet-unidentified receptor which leads to the activation of caspase-11.¹⁰ We used ultra-high concentration of FITC-labelled LPS (10 μ g/mL) to stimulate TLR4^{-/-} KCs and observed that LPS was taken up into the cytoplasm. We speculated that it might be related to the internalization of LPS. Hydrophilic polysaccharides and hydrophobic lipids located at both ends of LPS molecules make high concentration of LPS-forming polymer particles in aqueous solution and are easily phagocytosed and internalized by macrophages. We also encapsulated FITC-labelled LPS by transfection reagents and transfected KCs which mimicked LPS internalization by uptake of bacterial microvesicles.²⁵⁻²⁷

We observed that the intracellular uptake of FITC-LPS was greater than simple ultra-high concentration LPS stimulation. However, its specific mechanism is still not clear. Jon A. Hagar et al found that cytoplasmic LPS activates caspase-11 which can promote IL-1 β secretion by triggering the canonical NLRP3 pathway.²⁸ Some research

FIGURE 5 Cathepsin B activity, caspase-4 activation and pro-inflammatory cytokines levels in peripheral blood mononuclear cells (PBMC) of patients with endotoxaemia. A, Immunoblot analysis of cathepsin B and caspase-4 activation in PBMC of patients with endotoxaemia and healthy controls. B, Enzyme-linked immunosorbent assay analyses of interleukin 1 α (IL-1 α), interleukin 18 (IL-18) and interleukin 1 β (IL-1 β) concentration changes in serums obtained from patients with endotoxaemia and healthy controls. C, Cytosolic cathepsin B activity assays in PBMC of patients with endotoxaemia and healthy controls. The results are depicted as means $\pm$ SD (n = 3). *P < .05

suggest that caspase-11 forms a complex together with NLRP3/ASC and procaspase-1;^{8,9} others indicate that caspase-11 is upstream of NLRP3 activation and controlled the assembly of NLRP3–ASC complexes.^{21,29} Our results showed significantly increased IL-1 β and IL-18 production in TLR4^{-/-} KCs which proved that cytoplasmic LPS could mediate the activation of canonical NLRP3 inflammasome through activated caspase-11.

Meanwhile, Peter Schotte et al reported the lysosomal cysteine protease cathepsin B as a caspase-11-activating enzyme in vitro.¹³ Similarly, our pre-experiments showed that the levels of cathepsin B activity and caspase-11 protein were simultaneously elevated in LPS-primed TLR4^{-/-} KCs. This finding led us to speculate that cytoplasmic LPS cause the release of cathepsin B that activates caspase-11. Therefore, we examined whether the downregulation of cathepsin B in LPS-primed TLR4^{-/-} KCs affected caspase-11 expression and non-canonical inflammasome activation. We found that cathepsin B inhibition or knockdown block caspase-11 activation and inflammatory cytokines maturation in LPS-primed TLR4^{-/-} KCs. In vivo, caspase-11 expression was significantly suppressed in endotoxaemia model of TLR4^{-/-} mice pretreated with CA-074-Me. Our results demonstrated that cathepsin B contributed to non-canonical inflammasome activation in response to large doses of LPS by activating caspase-11.

Lasse Foghsgaard et al discovered TNF induces a translocation of cathepsin B from lysosomes to cytosol in ME-180 cervical carcinoma cells.³⁰ We speculated that disruption of the lysosomal membrane, caused by cytoplasmic LPS or phagocytosis of particulate matter resulted in cathepsin B release which mediated the activation of the proinflammatory caspase-11 and inflammatory cytokines in LPS-primed TLR4^{-/-} mice. Our experiments discovered that TLR4^{-/-} KCs in LPS group presented primary lysosome increasing and discontinuity of lysosomal membrane. Cellular redistribution of cathepsin B seemed to be found by IF. These data demonstrated that cytoplasmic LPS induced changes in the stability of the lysosomal membrane that caused cathepsin B outflow. Cytoplasmic cathepsin B became activated then primed pro-caspase-11 and ultimately led to the activation of the non-canonical inflammasome. However, additional studies are needed to confirm the specific molecular mechanism of cathepsin B redistribution and changes in the stability of lysosomal membrane caused by cytoplasmic LPS.

We also performed cathepsin B activity and analysed caspase-4 expression in the PBMC of patients with sepsis. We found that cathepsin B activity was significantly higher in sepsis samples as compared to healthy volunteers. Meanwhile, caspase-4 was found to be processed in sepsis samples. These human study data generally suggest an association between cathepsin B upregulation and caspase-4 activation in Gram-negative bacterial sepsis.

In conclusion, for the first time, our experiments demonstrated the role of cathepsin B regulating non-canonical inflammasome pathway by modulating the activity of caspase-11 in 3 ways—cells, animals and patients. Our results highlight the contribution of cathepsin B to non-canonical inflammasome activation that may benefit patients suffering from Gram-negative sepsis.

ACKNOWLEDGEMENTS

This study was supported by grants from the National Natural Science Foundation of China (No. 81401622, No. 31370753 and No. 81601715), Basic Science and Frontier Technology Research Foundation of Chongqing science & technology commission (No. cstc2015jcyjBX0070).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ORCID

Nan Chen <http://orcid.org/0000-0001-9825-2369>

REFERENCES

1. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 1998;282:2085-2088.
2. Meng J, Gong M, Björkbacka H, Golenbock DT. Genome-wide expression profiling and mutagenesis studies reveal that lipopolysaccharide responsiveness appears to be absolutely dependent on TLR4 and MD-2 expression and is dependent upon intermolecular ionic interactions. *J Immunol*. 2011;187:3683-3693.
3. Rathinam VA, Fitzgerald KA. Immunology: lipopolysaccharide sensing on the inside. *Nature*. 2013;501:173-175.
4. Schroder K, Tschopp J. The inflammasomes. *Cell*. 2010;140:821-832.
5. Jo EK, Kim JK, Shin DM, Sasakawa C. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol Immunol*. 2016;13:148-159.
6. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol*. 2010;29:707-735.
7. Gagliani N, Palm NW, de Zoete MR, Flavell RA. Inflammasomes and intestinal homeostasis: regulating and connecting infection, inflammation and the microbiota. *Int Immunol*. 2014;26:495-499.
8. Kayagaki N, Warming S, Lamkanfi M, et al. Non-canonical inflammasome activation targets caspase-11. *Nature*. 2011;479:117-121.
9. Rathinam VA, Vanaja SK, Waggoner L, et al. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell*. 2012;150:606-619.
10. Kayagaki N, Wong MT, Stowe IB. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science*. 2013;341:1246-1249.
11. Casson CN, Copenhaver AM, Zwack EE, et al. Caspase-11 activation in response to bacterial secretion systems that access the host cytosol. *PLoS Pathog*. 2013;9:e1003400.
12. Vancompernelle K, van Herreweghe F, Pynaert G, et al. Atractyloside-induced release of cathepsin B, a protease with caspaseprocessing activity. *FEBS Lett*. 1998;438:150-158.
13. Schotte P, Van Crielinge W, Van de Craen M, et al. Cathepsin B-mediated activation of the proinflammatory caspase-11. *Biochem Biophys Res Comm*. 1998;251:379-387.
14. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA*. 2016;315:801-810.
15. Opal SM, Laterre P-F, Francois B, et al. High-volume versus standard-volume haemofiltration for septic shock patients with acute kidney injury (IVOIRE study): a multicentre randomized controlled trial. *Intensive Care Med*. 2013;39:1535-1546.

16. Li PZ, Li JZ, Li M, Gong JP, He K. An efficient method to isolate and culture mouse Kupffer cells. *Immunol Lett*. 2013;158:52-56.
17. Cheng KT, Xiong S, Ye Z, et al. Caspase-11-mediated endothelial pyroptosis underlies endotoxemia-induced lung injury. *J Clin Invest*. 2017;127:4124.
18. Foghsgaard L, Wissing D, Mauch D, et al. Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J Cell Biol*. 2001;153:999-1010.
19. Koizumi Y, Toma C, Higa N, et al. Inflammasome activation via intracellular NLRs triggered by bacterial infection. *Cell Microbiology*. 2012 Feb;14:149-154.
20. Pellegrini C, Antonioli L, Lopez-Castejon G, et al. Canonical and non-canonical activation of NLRP3 inflammasome at the cross-road between immune tolerance and intestinal inflammation. *Front Immunol*. 2017;8:36.
21. Vanaja SK, Rathinam VA, Fitzgerald KA. Mechanisms of inflammasome activation: recent advances and novel insights. *Trends Cell Biol*. 2015;25:308-315.
22. Broz P, Ruby T, Belhocine K, et al. Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1. *Nature*. 2012;490:288-291.
23. Aachoui Y, Leaf IA, Hagar JA, et al. Caspase-11 protects against bacteria that escape the vacuole. *Science*. 2013;339:975-978.
24. Vasconcelos NMD, Opdenbosch NV, Lamkanfi M. Inflammasomes as polyvalent cell death platforms. *Cell Mol Life Sci*. 2016;73:2335-2347.
25. Cheng KT, Xiong S, Ye Z, et al. Caspase-11-mediated endothelial pyroptosis underlies endotoxemia-induced lung injury. *J Clin Invest*. 2017;127:4124.
26. Hagar JA, Powell DA, Aachoui Y, et al. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science*. 2013;341:1250-1253.
27. Shi J, Zhao Y, Wang Y, et al. Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature*. 2014;514:187-192.
28. Hagar JA, Powell DA, Aachoui Y, et al. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science*. 2013;341:1250-1253.
29. Sebastian R, Petr B. Caspase-11 activates a canonical NLRP3 inflammasome by promoting K(+) efflux. *Eur J Immunol*. 2015;45:2927-2936.
30. Foghsgaard L, Wissing D, Mauch D, et al. Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J Cell Biol*. 2001;153:999-1009.

How to cite this article: Chen N, Ou Z, Zhang W, Zhu X, Li P, Gong J. Cathepsin B regulate non-canonical NLRP3 inflammasome pathway by modulating activation of caspase-11 in Kupffer cells. *Cell Prolif*. 2018;51:e12487. <https://doi.org/10.1111/cpr.12487>