Bidirectional Role of NLRP3 During Acute and Chronic Cholestatic Liver Injury

Mick Frissen, Lijun Liao, Kai Markus Schneider, Sonja Djudjaj, Johannes Haybaeck, Alexander Wree, Ulrike Rolle-Kampczyk, Martin von Bergen, Eicke Latz, Peter Boor, and Christian Trautwein

BACKGROUND AND AIMs: Cholestatic liver injury leads to cell death and subsequent inflammation and fibrosis. As shown for primary biliary cholangitis (PBC), the mechanisms and circuits between different cell death pathways leading to disease progression are incompletely defined. Common bile duct ligation (BDL) is a well-established murine model to mimic cholestatic liver injury. Here, we hypothesized that pyroptotic cell death by the Nucleotide-Binding Domain, Leucine-Rich-Containing Family, Pyrin Domain-Containing-3 (Nlrp3) inflammasome plays an essential role during human and murine cholestasis.

APPROACH AND RESULTS: NLRP3 activation was analyzed in humans with cholestatic liver injury. Wild-type (WT) and Nlrp3−/− mice were subjected to BDL for 2 or 28 days. Chronic cholestasis in humans and mice is associated with NLRP3 activation and correlates with disease activity. Acute BDL in Nlrp3-deficient mice triggered increased inflammation as well as liver injury, associated with stronger apoptotic and necroptotic cell death. In contrast, NLRP3 deletion led to decreased liver injury and inflammation in chronic cholestasis. Moreover, bridging fibrosis was observed in WT, but not in NLRP3 knockout, mice 28 days after BDL. In contrast, lack of NLRP3 expression attenuated kidney injury and fibrosis after acute and chronic BDL. Importantly, administration of MCC950, an NLRP3 small molecule inhibitor, reduced BDL-induced disease progression in WT mice.

CONCLUSIONS: NLRP3 activation correlates with disease activity in patients with PBC. NLRP3 has a differential role during acute and chronic cholestatic liver injury in contrast to kidney injury. Disease progression during chronic cholestasis can be targeted through small molecules and thus suggests a potential clinical benefit for humans, attenuating liver and kidney injury. (Hepatology 2021;73:1836-1854).

Functionally, the bile, including the enterohepatic circulation of bile acids, has been increasingly recognized to have a role beyond its essential function during digestion, which includes the vital mechanism of absorbing fat and fat-soluble vitamins. Impaired bile formation or flow to the bile ducts, referred to as “cholestasis,” is known to trigger liver disease. Cholestatic liver injury leads to cell death, followed by inflammation and fibrosis.

Cholestatic liver injury is relevant for a variety of diseases, such as obstructive gallstones, primary biliary cholangitis (PBC), primary sclerosing cholangitis, and drug toxicity. However, the mechanisms and different cell death pathways leading to disease progression are still not fully understood. In the liver, cells undergo two main forms of cell death: apoptosis and necroptosis.
However, other forms, such as ferroptosis and pyroptosis, may also have an impact on liver injury.

A well-established murine model of cholestatic liver injury is induced by common bile duct ligation (BDL). Acute, up to 3 days, BDL injury is characterized by initiation of inflammation and immune cell infiltration, while the chronic phase, more than 10 days, leads to disease progression characterized by changes in immune cell infiltration, inflammation, and advanced fibrogenesis. In both phases, inflammatory mechanisms are essential in order to perpetuate cholestatic liver injury. In this process, not only hepatocytes but also nonparenchymal liver cells (e.g., endothelial cells, Kupffer cells, and cholangiocytes) and infiltrating immune cells are involved.

BDL is known to cause apoptosis and necrosis, but the actual importance of specific cell death mechanisms is still controversial and largely unknown. Bile acids are considerably elevated in liver and serum after BDL. Bile acids are one of the major initiators of an inflammatory response involved in cholestatic liver injury.

Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) lead to activation of the innate immune response by complex formation of nonobese diabetic–like receptors (NLRs), apoptosis–associated speck-like protein containing a carboxy-terminal caspase recruitment domain, and the effector procaspase-1. Procaspase-1 is cleaved, which in turn cleaves pro-interleukin 1-beta (IL-1β) and pro-IL-18. Activation of the inflammasome leads to inflammatory cell death, referred to as “pyroptosis,” which also triggers fibrosis.

NLR family pyrin domain containing 3 (NLRP3) is one of the NLRs and a well-characterized member of the inflammasome family that is activated by a variety of stimuli. NLRP3 activation needs two distinct signals: the first signal acts as a priming step through toll-like receptors (TLRs), tumor necrosis factor (TNF) receptor, or IL-1R activating nuclear factor kappa B (NF-κB), which up-regulates the expression of NLRP3, pro-IL-1β, and pro-IL-18. The second signal can be any PAMP or DAMP triggering inflammasome assembly and subsequent downstream effector activation. Earlier studies reported that in rats the NLRP3 inflammasome is already activated 2 days after BDL.

In the present study, we hypothesized that pyroptotic cell death through the NLRP3 inflammasome plays an essential role during cholestasis in humans and mice, especially due to the fact that toxic bile acids serve as a DAMP, likely mediating this effect. Interestingly, we found that the NLRP3 inflammasome is involved in mediating time-dependent disease progression after
BDL, as well as bile cast nephropathy, and that it can be targeted therapeutically.

**Methods**

**HUMAN STUDIES**

Human samples of cholestatic liver injury (PBC, n = 12) and control liver, noninflammatory, or fibrotic resection tissue (n = 8) were collected retrospectively at the Institute of Pathology, RWTH Aachen University (Aachen, Germany); the Department of Pathology, Otto-von-Guericke University (Magdeburg, Germany); and the Department of Pathology, Neuropathology and Molecular Pathology of the University of Innsbruck (Innsbruck, Austria), under approval of the local ethics committees with informed consent (no. 61/18, Magdeburg, and no. 1115/2019, Innsbruck). Board-certified liver pathologists blinded to the clinical details confirmed the diagnosis histologically.

**ANIMALS**

All mouse experiments were performed as approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (84-02.04.2013.A184). All mouse genotypes—wild-type (WT), Nlrp3−/−, and Il-18−/−—were bred on a C57BL/6 background and housed in filter-top cages in the yellow barrier of the animal facility of the University Clinic RWTH Aachen. BDL was performed on 8-week-old mice as described. Animals were sacrificed after 2 days (acute cholestasis model) and 28 days (chronic cholestasis model). Blood serum was used for the measurement of transaminases, glutamate dehydrogenase (GLDH), alkaline phosphatase (AP), bilirubin, and cholesterol. Livers were collected for histology, immunohistochemistry (IHC), protein, and RNA analysis. NLRP3 inhibitor MCC950 was injected intraperitoneally 3 days after BDL, 20 mg/kg in 0.9% NaCl every second day, sacrificing the mice at day 28.

**HISTOLOGY**

Directly after taking the liver, macroscopic pictures were taken with a Discus Z16 APO (Leica, Wetzlar, Germany) camera. Furthermore, tissue sections were frozen in Tissue-Tec or fixed in 4% paraformaldehyde (PFA). PFA sections were embedded in paraffin and cut (2 μm), after which they were stained with hematoxylin–eosin (H&E) or Sirius red. Quantifications were performed with ImageJ (National Institutes of Health); bile infarct areas were measured on H&E staining and confirmed by a board-certified pathologist.

In addition, IHC or immunofluorescent staining on paraffin and frozen sections was performed for cluster of differentiation 11b (cd11b; BD), receptor interacting protein 3 (RIP3; Prosci), cytoketer 19 (Santa Cruz), cleaved caspase-3 (Cell Signaling), alpha-smooth muscle actin (αSMA; Sigma), terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL; Roche), fluorochrome inhibitor of caspases using FAM-YVAD-FMK (FAM-FLICA caspase-1; ImmunoChemistry Technologies), F4/80 (SerotecAbD), and cleaved caspase-8 (Cell Signaling).

For histopathological analyses, renal tissue was fixed in methyl Carnoy’s solution and embedded in paraffin. Sections (1 μm thick) were stained with periodic acid–Schiff (PAS) reagent and counterstained with hematoxylin (PAS] to assess overall kidney damage; H&E was used for quantification of bile casts. Tubulointerstitial injury, defined as tubular damage and atrophy, interstitial inflammation, and fibrosis, was scored semiquantitatively depending on the extent of the whole renal cortex affected as follows: 0 = no damage, 1 = 1%-25%, 2 = 26%-50%, 3 = 51%-75%, 4 = 76%-100%.

In addition, computer-assisted quantification of IHC staining was performed for collagen type III (Southern Biotech), F4/80 (SerotecAbD), lymphocyte 6 antigen G (Ly6G; BD Bioscience), and neutrophil gelatinase-associated lipocalin (NGAL)/lipocalin-2 (R&D Systems). All analyses were performed in a blinded fashion.

**QUANTITATIVE PCR ANALYSIS**

RNA was isolated from liver with TRIzol (Invitrogen), and complementary DNA was generated with Omniscript (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Samples were run with Fast SYBR green on QuantStudio 6 (Applied Biosystems) and normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
WESTERN BLOT ANALYSIS

Liver tissue was homogenized in Nonidet P40 lysis buffer by using a BeadBug microtube homogenizer to extract proteins. All concentrations were adjusted to 2 μg/μL, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a 0.2-μm nitrocellulose membrane by Trans-Blot Turbo or Criterion Blotter (BioRad), followed by analysis using immunoblotting. Blocking was performed by 2.5% bovine serum albumin, followed by incubation of primary antibodies for RIP1 (BD), RIP3 (Prosci), phosphorylated mixed lineage kinase domain-like protein (pMLKL; Abcam), αSMA (Sigma), and GAPDH (Invitrogen).

FLUORESCENCE-ACTIVATED CELL SORTING ANALYSIS

Leukocytes were isolated from fresh liver tissue, as described by Karlmark et al. (21); livers were perfused with phosphate-buffered saline, minced with scissors, digested with collagenase type IV (Worthington) at 37°C, and pressed through 70-μm cell strainers. Leukocytes were then collected by centrifugation, washed, and stained for CD45, CD11b, CD11c, F4/80, Gr11, Ly6G, and Hoechst. Subsequently, samples were processed in the FACScanto (BD) and analyzed using Flowjo software (Treestar).

BILE ACID PROFILING

The analysis was performed using the validated Bile Acid Kit (Biocrates Life Sciences, Innsbruck, Austria) as described in Pham et al. (22). The kit measures 16 human-specific and 19 rodent-specific bile acids. Sample preparation was done according to the manufacturer’s instructions. For quantitation, seven external calibration standards (each containing all 20 bile acids) and 10 isotope-labeled internal standards were used. Liquid chromatography–tandem mass spectrometry analysis was carried out by multiple reaction monitoring acquisition using a Waters Acquity UPLC System coupled with QTRAP 5500 (AB Scieix, Concord, Canada). Mobile phase (MP) A consisted of 10 mM ammonium acetate and 0.015% formic acid, while MP B was a mixture of acetonitrile/methanol/water (65/30/5;v:v:v), 10 mM ammonium acetate, and 0.015% formic acid. Data processing was carried out with the provided quantitation method kit (Biocrates Life Sciences AG, Innsbruck, Austria).

STATISTICAL ANALYSIS

All bars show mean with standard error of mean, and groups are described in each graph. Statistical significance was evaluated with GraphPad Prism by performing one-way analysis of variance with Bonferroni’s posttest, with between-group significance shown as *P < 0.05, **P < 0.01, and ***P < 0.001 or as explained in the figure legends.

Results

CHRONIC CHOLESTASIS IN HUMANS AND MICE IS ASSOCIATED WITH NLRP3 ACTIVATION

Chronic cholestasis-induced liver injury triggers inflammation, which is an essential driver of disease progression. We first tested whether the NLRP3 inflammasome might be involved in mediating inflammation in human cholestatic liver injury. Here, we investigated IL-1β protein expression in patients with PBC (Supporting Table S1). IL-1β staining in PBC livers revealed stronger positivity compared with healthy controls. Statistical analysis of patients (n = 12) with chronic cholestatic liver injury compared with healthy controls (n = 8) showed that the difference in IL-1β expression was significantly higher in the PBC group (Fig. 1A). Increased AP levels in patients with PBC is a biomarker of stronger disease activity. Interestingly, we found a significant correlation between higher AP levels and increased IL-1β positivity in livers of patients with PBC (Fig. 1B). These results suggest that increased disease activity in patients with PBC is associated with stronger NLRP3 activation.

We next tested if NLRP3 inflammasome activation may also apply to chronic cholestasis in mice. We thus performed BDL in mice and investigated IL-1β expression 28 days after surgery. As found in patients with PBC, chronic cholestasis in mice induced IL-1β expression 28 days after BDL compared with sham-operated animals (Fig. 1C). Hence, our analysis suggests that the NLPR3 inflammasome is involved in
Because the NLRP3 inflammasome is primarily expressed in myeloid cells and is highly inducible, we tested more specifically which cell type shows stronger IL-1β expression after BDL and thus NLRP3 activation. By performing F4/80 and IL-1β costaining experiments, we found that IL-1β expression is especially found in macrophages (Supporting Fig. S1a).\(^{(23,24)}\)

**FIG. 1.** Cholestatic liver injury activates the inflammasome in humans and mice, and the severity of cholestasis correlates with increased inflammasome activation. (A) Human PBC (n = 12) compared to healthy control liver (n = 8), representative H&E and IL-1β staining images at ×40 magnification. IL-1β quantified by measuring positive area. (B) Linear regression analysis of IL-1β area versus AP; increased IL-1β staining correlates with increased AP values. (C) Mouse BDL (n = 6) representative images for H&E staining at ×10 magnification and IL-1β staining at ×20 magnification. IL-1β quantified by measuring positive area. *P < 0.05, **P < 0.01.

**INCREASED LIVER INJURY AFTER ACUTE BDL IN Nlrp3-DEFICIENT MICE**

In further experiments, we tested the functional relevance of these findings. BDL was performed in Nlrp3-deficient (Nlrp3\(^{-/-}\)) mice and respective WT controls. We first investigated the implication of NLRP3 for acute cholestatic liver injury 48 hours after BDL. At this time, Nlrp3\(^{-/-}\) animals showed increased...
liver injury compared to WT controls, as evidenced by elevated liver enzymes (Fig. 2A). Additionally, cholestatic serum parameters were significantly elevated in Nlrp3−/− mice (Fig. 2B; Supporting Fig. S1b), while there was no significant difference between WT and Nlrp3−/− livers on the macroscopic level (Supporting Fig. S1c).

H&E staining evidenced a significant increase in amount, but not in size, of bile infarcts in Nlrp3−/− compared to WT animals 48 hours after BDL (Fig. 2C; Supporting Fig. S1d). Furthermore, both fluorescence-activated cell sorting (FACS) analysis and CD11b immunostaining revealed that there was a significantly higher influx of CD11b+Ly6G+ neutrophils in Nlrp3−/− (39.2%) compared to WT livers (29.25%) (Fig. 2D,E; Supporting Fig. S1e). Proinflammatory cytokines Tnf, Il-6, Il-1β, and monocyte chemoattractant protein 1 (Mcp-1) were all significantly increased in Nlrp3−/− mice 48 hours after BDL, while Il-18 was decreased in Nlrp3-deficient mice, which is in line with the stronger influx of inflammatory cells (Fig. 2F).

**FIG. 2.** Acute BDL induces significantly more liver injury and inflammation in Nlrp3−/− compared to WT mice. (A) Serum ALT and GLDH levels after acute BDL. (B) Cholestasis markers AP and bilirubin after acute BDL. (C) Bile infarct amount and size after acute BDL, quantified by measurement on H&E staining. (D) Quantification of FACS analysis for Ly6G+CD11b+ cell compartment shown as percentage of total CD45+ living cells. (E) Representative images for stained CD11b+ infiltrating neutrophils at ×10 magnification. (F) Relative expression of inflammasome and inflammation-related genes compared to WT control normalized for GAPDH. *P < 0.05.
LACK OF Nlrp3 EXPRESSION TRIGGERS INCREASED APOPTOTIC AND NECROPTOTIC CELL DEATH EARLY AFTER BDL

NLRP3 mediates pyroptotic cell death; hence, we investigated the impact on cell death pathways in WT compared to Nlrp3−/− livers 48 hours after BDL. TUNEL staining revealed an increase in positive cells 48 hours after BDL. However, no difference in TUNEL-positive cells was found between the two mouse strains (Fig. 3A; Supporting Fig. S2a).

Pyroptotic cell death requires inflammasome activation, and different NLRP family members may mediate this effect. We studied whether BDL triggers pyroptosis in WT animals and to what extent this effect is Nlrp3-dependent in the respective knockout (KO) animals. FLICA assay demonstrated increased caspase-1 positivity in WT mice early after BDL. In contrast, no increase in caspase-1-positive cells was observed in Nlrp3−/− livers (Fig. 3B; Supporting Fig. S2b). This result suggests that acute BDL induces pyroptotic cell death and that Nlrp3 is predominantly involved in mediating this effect.

As liver injury was more prominent in Nlrp3−/− livers after BDL despite lack of pyroptotic cell death, we then addressed the question whether other forms of cell death, e.g., apoptosis or necroptosis, might contribute to the compensation for this effect. We first investigated the effect on apoptotic cell death by caspase-3 western blot. At 48 hours after BDL, there was a significantly higher proportion of apoptotic cell death in Nlrp3−/− compared to WT livers, indicating a stronger apoptotic response in KO animals (Fig. 3C).

Next, we investigated the abundance of necrototic cell death during acute choledochal liver injury by analyzing RIP3 and its substrate MLKL. Here, we specifically analyzed pMLKL expression as its phosphorylation by RIP3 is essential to trigger necroptosis. IHC staining revealed that Nlrp3−/− livers show increased pMLKL expression in bile ducts compared to WT controls 48 hours after BDL (Fig. 3D). This finding was further confirmed by RIP3 immunostaining and western blot analysis, showing higher expression of both proteins after acute choledochal liver injury (Fig. 3D).

The increase in apoptotic and necrototic cell death after BDL in Nlrp3−/− livers was also associated with a significantly increased oval cell response as evidenced by K19 and A6 staining (Supporting Fig. S2c,d). Therefore, lack of Nlrp3-dependent pyroptotic cell death early after BDL triggers increased liver injury by pronounced activation of apoptosis and necroptosis.

Nlrp3 DELETION LEADS TO DECREASED LIVER INJURY AND INFLAMMATION IN CHRONIC CHOLESTATIS

After showing that the Nlrp3 inflammasome was an important protective mediator of liver injury early after BDL, we investigated the relevance of Nlrp3 during chronic cholestatic liver injury. Animals were sacrificed 28 days after BDL. At this point, we found significant differences compared to acute BDL-induced liver injury. Interestingly, liver enzymes were significantly decreased in Nlrp3−/− animals after chronic BDL (Fig. 4A). Furthermore, this held true for cholestatic serum parameters such as AP, bilirubin, and cholesterol (Fig. 4B; Supporting Fig. S3a). Macroscopically WT livers showed larger bile infarcts, which were also observed on the liver surface, compared to Nlrp3−/− livers (Supporting Fig. S3b).

Histologically H&E staining evidenced an overall decrease in size and amount of bile infarcts in Nlrp3−/− livers. However, there was no difference between WT and Nlrp3−/− mice (Fig. 4C,D; Supporting Fig. S3c).

Next, FACS analysis demonstrated a significant difference in infiltrating CD11b+Ly6G+ immune cells. Nlrp3−/− livers showed significantly reduced CD11b+Ly6G+ cells compared to WT mice (Fig. 4E; 33.8% versus 17.8%; Supporting Fig. S3d). We confirmed this finding by staining liver sections for CD11b (Fig. 4F).

Moreover, stronger infiltration of CD11b+Ly6G+ cells in WT livers was associated with increased mRNA expression of the inflammatory mediators Tnf, Mcp-1, Il-6, and Il-1β, but not Il-18, displaying a drastic reduction in the overall inflammatory profile of Nlrp3−/− mice after chronic BDL. Additionally, F4/80 was significantly reduced in Nlrp3−/− compared to WT animals during chronic cholestasis (Fig. 4G).
FIG. 3. The mechanism of cell death is altered by the lack of Nlrp3 expression, triggering increased apoptotic and necroptotic cell death after acute BDL. (A) TUNEL staining quantified as positive cells per ×20 viewfield. (B) FAM-FLICA caspase-1 assay, percentage of positive FLICA area normalized by 4′,6-diamidino-2-phenylindole area. (C) Western blot for cleaved Caspase-3, 17 kDa, quantified by normalizing for GAPDH, 37 kDa. (D) Necroptotic cell death analysis by RIP3 staining at ×20 magnification and RIP3 western blot, 57 kDa, quantified by normalizing for GAPDH, 37 kDa. pMLKL staining at ×40 magnification, quantified by counting positive cells/bile duct. *P < 0.05, **P < 0.01.
The effect of Nlrp3 deficiency on different cell death mechanisms was investigated 28 days after BDL. Overall cell death as assessed by TUNEL staining was significantly reduced in Nlrp3−/− compared to WT controls (Fig. 5A). At 48 hours after BDL, pyroptosis was present in WT mice but not in Nlrp3−/− mice. Therefore, we investigated the effect during chronic cholestasis. The FLICA assay showed a low number of caspase-1-positive cells in both phenotypes, with no difference in caspase-1 positivity between Nlrp3−/− and WT controls (Fig. 5B; Supporting Fig. S4a). This suggests that pyroptosis was an acute-phase response in WT, while it was not very prominent.

**FIG. 4.** Nlrp3−/− mice have significantly decreased liver injury and inflammation after chronic cholestasis. (A) Serum ALT and GLDH levels after chronic BDL. (B) Cholestasis markers AP and bilirubin after chronic BDL. (C) Bile infarct amount and size after chronic BDL, quantified by measurement on H&E staining. (D) Representative images for H&E staining. (E) Quantification of FACS analysis for Ly6G+CD11b+ cell compartment shown as percentage of total CD45+ living cells. (F) Representative images for stained-CD11b+ infiltrating neutrophils. (G) Relative expression of inflammasome and inflammation-related genes compared to WT control normalized for GAPDH. *P < 0.05, **P < 0.01.

**CELL DEATH AFTER CHRONIC BDL IS AMELIORATED IN Nlrp3−/− MICE**

The effect of Nlrp3 deficiency on different cell death mechanisms was investigated 28 days after BDL. Overall cell death as assessed by TUNEL staining was significantly reduced in Nlrp3−/− compared to WT controls (Fig. 5A).
FIG. 5. Overall cell death is ameliorated in Nlrp3−/− mice after chronic BDL; WT livers still exhibit pyroptotic cell death. (A) TUNEL staining quantified as positive cells per ×20 viewfield. (B) FAM–FLICA caspase-1 assay; percentage of positive FLICA area normalized by 4′,6-diamidino-2-phenylindole area. (C) Relative expression of cell death and inflammasome pathway-related genes compared to WT control normalized for GAPDH. (D) Caspase-3 activity assay and cleaved caspase-3 staining, quantified by positive cells per ×20 viewfield. (E) pMLKL staining quantification by positive cells/bile duct. *P < 0.05, **P < 0.01, ***P < 0.001. Abbreviations: AFC, 7-amino-4-trifluoromethyl coumarin; Aim2, absent in melanoma 2; AFC, Asc, activating signal cointegrator.
in either phenotype after chronic BDL. Most likely, the slight pyroptotic activity in Nlrp3−/− mice was caused by other NLRs or noncanonical pathways. Investigation of inflammasome-related genes showed a significant down-regulation of all genes analyzed such as Caspase-1, Caspase-11, Asc, Nlrp1, absent in melanoma 2, as well as the TLRs 2, 4, and 9 in Nlrp3−/− livers (Fig. 5C).

As we found differences in apoptotic and necroptotic cell death in acute cholestatic injury, we first evaluated caspase-3 activity. Here, caspase-3 staining revealed clusters of caspase-3-positive cells in WT but not in Nlrp3−/− livers 28 days after BDL. This result was confirmed by a caspase-3 assay, which demonstrated significantly less caspase-3 activity 28 days after BDL (Fig. 5D).

As shown during acute BDL, necroptotic cell death was shown by IHC. pMLKL expression was significantly increased in both WT and Nlrp3−/− livers 28 days after BDL but more pronounced in Nlrp3−/− (Fig. 5E; Supporting Fig. S4b). Taken together these results suggest that reduced apoptotic and pyroptotic cell death contributes to reduced liver injury and TUNEL-positive cells in Nlrp3−/− compared to WT livers during chronic cholestasis.

**CHOLESTATIC LIVER INJURY LEADS TO BRIDGING FIBROSIS IN WT, BUT NOT IN Nlrp3 KO, MICE**

Persistent inflammation during chronic liver injury is an essential mediator triggering fibrogenesis. Thus, we analyzed fibrosis markers in WT and Nlrp3−/− livers during chronic cholestasis. Sirius red staining evidenced bridging fibrosis in WT in contrast to Nlrp3−/− livers (Fig. 6A). In addition, hepatic gene expression of several profibrotic factors, such as collagen type I alpha 1 chain, transforming growth factor-beta, and aSMA was significantly higher in WT compared to Nlrp3−/− mice (Fig. 6B). These findings were further supported by the significant reduction of aSMA-positive staining in Nlrp3−/− livers compared to WT controls (Fig. 6C). Similar results were found using western blot analysis, demonstrating high aSMA expression only in WT livers 28 days after BDL (Fig. 6D).

To better understand the contribution of Il-1β and Il-18 to the observed phenotype of Nlrp3−/− mice 28 days after BDL, we included Il−1R and Il−18R KO mice in our analysis. Unexpectedly, both KO strains showed a more severe phenotype after BDL. Therefore, we were forced to terminate these experiments 21 days after BDL according to our animal laws. At this stage the mice had higher serum transaminases compared to Nlrp3−/− mice 28 days after BDL (Supporting Fig. S5a) but especially a higher abundance of bile infarcts as well as fibrosis (Supporting Fig. S5b,c).

**Nlrp3 DELETION CHANGES BILE ACID METABOLISM BEFORE AND AFTER BDL**

Bile acid profiling was performed on controls as well as BDL-operated WT and NLRP3−/− animals 2 and 28 days after surgery. This analysis revealed a distinct bile acid profile between WT and NLRP3−/− mice in serum as well as liver. Remarkably, NLRP3−/− control mice have a reduced amount of bile acids in liver and serum (Supporting Fig. S6a).

However, 2 days after BDL, NLRP3−/− mice have a marked increase in conjugated bile acids in serum and unconjugated bile acids in liver and serum compared to WT mice (Supporting Fig. S6a). At 28 days after BDL, in WT and NLRP3−/− livers bile acid levels revert to the level found in control. Though conjugated bile acids in the serum of WT mice initially increase more slowly than in NLRP3−/− mice, they continuously increase at 28 days after BDL (Supporting Fig. S6a).

The primary bile acid cholic acid (CA) is higher in serum and livers of WT mice compared to NLRP3−/− mice (control). Unexpectedly, 2 days after BDL CA increases significantly in NLRP3−/− livers and is at this time point higher than in WT livers, while 28 days after BDL CA returns to the situation found in control (Supporting Fig. S6b). Interestingly, mice fed a 1% CA diet are known to develop cholestasis. Therefore, the strong increase in CA expression in NLRP3−/− mice 2 days after BDL might contribute to increased liver injury at this time point.

Next, we specifically evaluated the expression of genes involved in bile acid synthesis and its transport. Interestingly, cytochrome P450 7A1 (Cyp7a1) was up-regulated in Nlrp3−/− livers before BDL, while it was down-regulated in both genotypes 48 hours after BDL. Additionally, the bile transporter...
LACK OF Nlrp3 EXPRESSION ATTENUATES KIDNEY INJURY AND FIBROSIS AFTER BDL

After BDL, toxic bile acids are involved in triggering tubular epithelial injury and consecutive kidney damage, leading to kidney fibrosis. Histomorphologically, BDL-associated kidney injury closely reflects bile cast nephropathy, a relatively...
common complication of cholestasis in patients. We first verified whether lack of Nlrp3 expression might have an impact on acute kidney injury, having in mind that liver damage is more severe in Nlrp3−/− animals after acute BDL. Although the extent of injury was mild, it was detectable already at this very early stage. Notably, compared to WT controls, we found significantly reduced kidney injury in Nlrp3−/− mice, as evidenced by decreased granulocyte infiltration and reduced expression of tubular damage markers kidney injury molecule 1 (KIM-1) and NGAL (Fig. 7A,B). These results indicated that, despite increased liver injury, lack of Nlrp3 expression ameliorated acute cholestasis-induced kidney injury.

Next, we studied the impact of long-term BDL on kidney injury. At this stage, i.e., 28 days after BDL, a full-blown bile cast nephropathy was observed in WT mice (Fig. 7A). Remarkably, we found significantly fewer bile casts in Nlrp3−/− kidneys compared to WT animals (Fig. 7A). This was associated with less tubular damage and significantly reduced expression of tubular damage markers KIM-1 and NGAL (Fig. 7A,B).

The Nlrp3-dependent effect on cholestasis-induced tubular damage was further investigated by analyzing F4/80-positive cells 28 days after BDL. Here Nlrp3−/− kidneys showed a significantly lower number of F4/80-positive cells (Fig. 7C). Finally, we also found reduced kidney fibrosis assessed by collagen accumulation in Nlrp3−/− kidneys compared to WT controls after BDL (Fig. 7D).

In summary, these results show that NLRP3 is involved in triggering cholestasis-induced kidney damage not only in the acute but also in the chronic phase independent of ongoing liver injury.

AN Nlrp3 INHIBITOR PROTECTS FROM CHRONIC BDL-INDUCED LIVER INJURY

As NLRP3 triggers injury and fibrogenesis during chronic cholestasis, we addressed the question of whether this effect can be therapeutically targeted using the NLRP3 inhibitor MCC950. Therefore, we performed BDL and started to treat the animals with MCC950 72 hours after BDL (Fig. 8A). At 28 days after BDL, MCC950-treated mice showed significantly reduced liver injury compared to solvent-treated controls as evidenced by significantly reduced aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (Fig. 8B). To test the functionality of our finding, we performed FAM-FLICA caspase-1 assays in both groups. Here, we saw a decrease in caspase-1 activity between MCC950-treated livers compared to solvent controls (Fig. 8C).

Reduction in liver injury was also associated with a significant decrease in inflammasome markers. Il-1β and Caspase-1 were significantly reduced in MCC950-treated mice compared to solvent controls. Il-18 expression shows the same trend as found in Nlrp3−/− mice during chronic cholestasis.

As shown before in Nlrp3−/− animals, fibrosis progression was attenuated in MCC950-treated liver 28 days after BDL compared to solvent-treated controls as shown by Sirius red staining as well as αSMA expression (Fig. 8D,E).

Hence, our results demonstrate that NLRP3 is essentially involved in mediating chronic liver injury after BDL and that this effect can be specifically targeted by therapeutic NLRP3 inhibition.

Discussion

Our study demonstrates that NLRP3 is activated in humans suffering from cholestatic liver injury, e.g., PBC, and correlates with disease activity. In murine studies using the BDL model, we tested the functional relevance of NLRP3 and postulated that these findings could be therapeutically relevant.(22)

Unexpectedly, we found that in acute cholestatic liver injury, Nlrp3−/− mice showed stronger liver injury but less severe kidney injury. At present, the reasons for the discrepancy in these findings are unclear. Therefore, we can only speculate why a lack of NLRP3 expression aggravates hepatic injury, while being protective for the kidney already in the acute situation.

Possibly, lack of NLRP3 activation during acute cholestasis might be harmful because the absence of pyroptotic cell death in Nlrp3−/− animals activates compensatory mechanisms of cell death. Based on previous studies, there is increasing evidence that there is a bidirectional crosstalk between the different forms of cell death. For example, the pyroptosis-mediating substrate gasdermin D (GSDMD) is the only caspase-1 activating substrate. During apoptosis, caspases 3 and 7 specifically cleave GSDMD at a distinct site from caspase-1, and consequently
FIG. 7. Lack of Nlrp3 expression significantly attenuates kidney injury and fibrosis after acute as well as chronic BDL. (A) Representative pictures for H&E, NGAL, Ly6G, and PAS staining at ×40 magnification. H&E quantified by percentage plaque area, NGAL by percentage stained area, Ly6G by positive cells per ×40 viewfield, and PAS by tissue infiltration scoring. (B) Relative expression of kidney injury genes compared to WT control normalized for GAPDH. (C) Macrophage infiltration by F4/80 staining at ×40 magnification, quantified by percentage stained area. (D) Collagen type III staining at ×40 magnification, quantified by percentage stained area. *P < 0.05.

Abbreviations: Col III, collagen type III; TIS, tissue infiltration score.
pyroptosis is blocked. Therefore, during acute cholestatic liver injury, NLRP3 activation has a protective function as it seems to act by inhibiting other cell death pathways, e.g., apoptosis and necroptosis. (27,28) When blocking caspase activation with a pan-caspase inhibitor, mice are protected against multidrug resistance protein 2–deficient associated cholestatic liver injury; in addition, hepatocyte-specific caspase-8 KO

FIG. 8. MCC950 treatment protects WT mice from chronic BDL-induced liver injury. (A) Experimental setup for MCC950 treatment; start of MCC950 intraperitoneal injection 72 hours after BDL. (B) Serum ALT and AST levels after chronic BDL. (C) FAM-FLICA caspase-1 assay; percentage of positive FLICA area normalized by 4′,6-diamidino-2-phenylindole area. (D) Relative expression of inflammasome and fibrosis markers compared to WT control normalized for GAPDH. (E) Sirius red overview staining, x5 magnification. *P < 0.05. (F) Graphical presentation of proposed mechanism of cholestatic liver injury and protection provided by NLRP3 deletion. Cholestatic liver injury leads to bile infarcts and inflammation, causing cell death through three distinct mechanisms. In WT this is mainly pyroptosis and necroptosis, while in Nlrp3−/− mice mainly necroptosis and apoptosis are involved. This leads to a stronger perpetuation of inflammation in WT mice compared to KO mice. In addition, the stronger initial inflammatory reaction in KO mice leads to activation of oval cells to start regeneration. MCC950 acts on the pyroptosis pathway and thus leads to a less severe inflammatory response and subsequent fibrosis. Abbreviation: RIPK3, RIP3 kinase.
protects mice against BDL-associated cholestatic liver injury.(7,29)

Moreover, the direct impact of elevated bile acids in the liver may play an important role because \textit{Nlrp3}−/− animals show increased expression of hepatic \textit{Cyp7A1}, the rate-limiting step in bile acid synthesis, before BDL. In combination with decreased bile acid exporter \textit{Abcb11}, this finding likely contributes to the increased number of bile infarct areas, especially as we found that the primary bile acid CA is strongly elevated in the liver 2 days after BDL. A 1% CA diet has been shown to trigger cholestatic liver injury.(25)

The increased number of bile infarct areas triggers higher reactive oxygen species production, representing a site where neutrophil serine proteases can effectively kill the damaged cells.(30) Furthermore, MCP-1 is produced at sites with oxidative stress, which attracts more neutrophils.(31) Because \textit{Nlrp3}-deficient mice show an increase in bile infarcts in the acute phase, neutrophil influx as well as protease activity are exacerbated, contributing to the increased inflammatory profile and apoptotic and necroptotic response.(32,33) The stronger injury in the \textit{Nlrp3}−/− liver after acute BDL also explains the stronger activation of oval cells because inflammatory cytokines, such as IL-6 and TNF, as well as impaired hepatocyte regeneration, are known to activate precursor cells.(34,35)

In contrast, during chronic cholestatic liver injury, the inability of cells to undergo pyroptotic cell death has a protective effect. Recent studies suggest that the chronic inflammatory response activated by NLRP3 has a major impact on pyroptosis, on the subsequent inflammatory profile, and, as a consequence, on fibrogenesis.(16,36,37) Notably, the NLRP3 inhibitor MCC950 also reduced the fibrotic phenotype after chronic BDL, defining NLRP3 as a therapeutic target during chronic cholestasis. MCC950 has been shown to attenuate inflammation in distinct injury models, e.g., brain, colon, and intestine. However, for chronic cholestatic liver injury, the beneficial effect has not yet been clarified.(38-41) Furthermore, Henao-Mejia et al.(42) found inflammasome components negatively regulating steatohepatitis progression after a methionine/choline-deficient (MCD) diet. Nonetheless, the effect seen there was most likely due to the time-dependent difference in \textit{Nlrp3}−/− animals because a 24-day MCD diet led to an acute, and not to a chronic, nonalcoholic steatohepatitis phenotype.

For the findings during chronic cholestasis, bile acids most likely play a prominent role. Bile acids serve as a DAMP and priming signal for the inflammasome, leading to up-regulation of NF-κB-dependent genes.(10) In WT mice, bile acids also act as a second signal activating the inflammasome complex and thus eventually leading to pyroptosis. Because the bile acid metabolism is dysregulated in \textit{Nlrp3}−/− mice, with a rapid increase in bile acids in the liver and serum after BDL, this can lead to an initial increased activation of damage-inducing pathways. However, in \textit{Nlrp3}−/− mice, the inflammasome complex cannot be formed, and only an Nlrp3-independent response can take place through caspase-11. The caspase-11-mediated, noncanonical inflammasome activation was shown to be responsible for lipopolysaccharide-induced lethal septic shock, while the NLRP3-activating signal cointegrator–caspase-1 axis amplified this response.(43) A similar effect likely might occur during chronic cholestatic liver injury because the inflammasome complex is required for further perpetuating the response.(37,41)

Moreover, we found reduced \textit{Tlr4} expression in \textit{Nlrp3}−/− mice 28 days after BDL, which is in line with the finding that \textit{Tlr4}−/− animals are protected against BDL-induced fibrosis. Because TLRs act as a first signal, increasing overall inflammasome-related gene expression, the reduction in \textit{Tlr4} expression supports the lower expression of the other genes and the decrease of fibrosis in \textit{Nlrp3}−/− mice.(44)

Furthermore, similar to our findings, DeSantis et al.(45) found that \textit{Il-18} and \textit{Il-1β} show a differential expression in \textit{Nlrp3}−/− mice. Yet, in contrast to their observations, we found \textit{Nlrp3} deficiency to be beneficial. As Finotto et al. reported, \textit{Il-18} transgenic mice show increased apoptosis through the Fas pathway. Paradoxically, we found decreased \textit{Il-18} during the early phase in \textit{Nlrp3}−/− mice but an increase in apoptosis. WT mice, however, showed an increase in \textit{Il-18} in the early phase but a decrease in the chronic phase, which might cause the increase of inflammatory response genes in WT mice after chronic cholestatic liver injury through NF-κB activation. To better understand the relevance of \textit{Il-18}, as well as \textit{Il-1β}, regulation, we included \textit{Il-1R} and \textit{Il-18R} KO mice in our experiments. Interestingly, both strains were severely injured after BDL and showed more liver damage and fibrosis, suggesting
a different role compared to NLRP3 during cholestatic liver injury.

Our recent experiments revealed an essential role of caspase-8 during cholestatic liver injury. Hepatocyte-specific caspase-8 KO animals were protected after chronic BDL. In agreement with Cubero et al., caspase-3 activity was elevated in WT mice but considerably reduced in Nlrp3−/− mice after chronic BDL. Furthermore, increases in caspase-8 and MLKL activity led to inflammasome activation and subsequent pyroptosis, which was blocked in Nlrp3−/− mice. In addition, mitochondrial stress and apoptosis can be sensed by the inflammasome in several ways; NLRP1 can be bound by the proapoptotic member B-cell lymphoma-2, preventing NLRP1 from binding adenosine triphosphate and oligomerization, which in turn, reduces pyroptosis, another way to directly activate the inflammasome by the release of danger signals from dying cells. This crosstalk can thus exacerbate the inflammatory response in WT mice.

Petrasek et al. found attenuated liver inflammation, fibrosis, and injury in alcohol-induced liver injury in Caspase-1 and Il-1β KO mice. Our results show a similar effect as both Caspase-1 and Il-1β cannot be activated in Nlrp3−/− mice and are down-regulated after chronic BDL. In line with their finding, pharmacological intervention, in our case with MCC950, protects against injury. Nlrp3−/− is protective in several models of acute and chronic kidney diseases, substantiating a crucial role of NLRP3 inflammasome in kidney injury. The BDL-associated kidney injury closely reflects bile cast nephropathy in humans, a relatively common and partly severe complication of cholestasis. Our study shows the crucial role of NLRP3 inflammasome in cholestatic kidney injury. Strikingly and in contrast with liver injury, kidney injury was ameliorated in the acute as well as the chronic phase. These data suggest that targeting NLRP3 inflammasome might be a potential effective treatment option for bile cast nephropathy.

Finally, we found that administration of MCC950 protected against cholestasis induced fibrosis. Because MCC950 was proven to have a protective function in various other injury models and diseases, such as colitis, lung ischemia–reperfusion, and traumatic brain injury, it is likely that there is a potential therapeutic function in human cholestatic liver and kidney injury. Hence, it might be interesting to introduce Nlrp3 inhibitors in humans with chronic cholestatic liver injury, e.g., patients with PBC.

In summary, our study highlights the relevance of NLRP3 activation in human PBC. NLRP3 has a differential role in disease progression in acute and chronic cholestatic liver injury after BDL. Especially during chronic cholestasis, inhibition of NLRP3 activation is an attractive target as it attenuates the inflammatory response and liver fibrogenesis. Additionally, this effect can be targeted by small molecules and has a beneficial effect on bile cast nephropathy. We summarize the mechanism through which NLRP3 deletion protects against cholestasis-induced liver injury in Fig. 8F.

Acknowledgment: Open access funding enabled and organized by Projekt DEAL.

Author Contributions: M.F. was responsible for writing the manuscript and performing all liver experiments. L.L. was responsible for performing BDL and sacrificing animals. K.M.S. was responsible for performing FACS analysis and sacrificing animals. J.H. was responsible for providing the human PBC samples and stainings. S.D. was responsible for performing the kidney experiments. A.W. was responsible for providing the inflammasome-related critical revision. U.R.K. and M.B. were responsible for performing the bile acid analysis. P.B. was responsible for reviewing stainings and providing critical revision. C.T. was responsible for providing funding, concept and design of the work, as well as critical revision.

REFERENCES

1) Schneider KM, Albers S, Trautwein C. Role of bile acids in the gut–liver axis. J Hepatol 2018;68:1083–1085.
2) Malhi H, Guicciardi ME, Gores GJ. Hepatocyte death: a clear and present danger. Physiol Rev 2010;90:1165–1194.
3) Poupon R, Chazouillères O, Poupon RE. Chronic cholestatic diseases. J Hepatol 2000;32:129–140.
4) Woolbright BL, Antoine DJ, Jenkins RE, Bajt ML, Park BK, Jaeschke H. Plasma biomarkers of liver injury and inflammation demonstrate a lack of apoptosis during obstructive cholestasis in mice. Toxicol Appl Pharmacol 2013;273:524–531.
5) Tag CG, Sauer-Lehnen S, Weiskirchen S, Borkham-Kamporst E, Tolba RH, Tacke F, et al. Bile duct ligation in mice: induction of inflammatory liver injury and fibrosis by obstructive cholestasis. J Vis Exp 2015;96:52438.
6) Kosters A, Karpen SJ. The role of inflammation in cholestasis: clinical and basic aspects. Semin Liver Dis 2010;30:186–194.
7) Cubero FJ, Peng J, Liao L, Su H, Zhao G, Zoubek ME, et al. Inactivation of caspase 8 in liver parenchymal cells confers protection against murine obstructive cholestasis. J Hepatol 2018;69:1326-1334.

8) Gucciardi ME, Malhi H, Mott JL, Gores GJ. Apoptosis and necrosis in the liver. In: Terjung R, ed. Comprehensive Physiology. Hoboken, NJ: John Wiley & Sons Inc; 2013.

9) Luedde T, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. Gastroenterology 2014;147:765-783.e4.

10) Cai S-Y, Ouyang X, Chen Y, Soroka CJ, Wang J, Mennone A, et al. Bile acids initiate cholestatic liver injury by triggering a hepatocyte-specific inflammatory response. JCI insight 2017;2:e90780.

11) Zhang Y, Hong J-Y, Rockwell CE, Coppel BL, Jaeschke H, Klaassen CD. Effect of bile duct ligation on bile acid composition in mouse serum and liver. Liver Int 2012;32:58-69.

12) Fickert P, Krüner E, Thüeringer A, Moustafa T, Silbert D, et al. Bile acids trigger cholemic nephropathy in common bile-duct-ligated mice. Hepatology 2013;58:2056-2069.

13) de Zee MR, Palm NW, Zha S, Flavell RA. Inflammasomes. Cold Spring Harb Perspect Biol 2014;6:a016287.

14) He Y, Hara H, Núñez G. Mechanism and regulation of NLRP3 inflammasome activation. Trends Biochem Sci 2016;41:1012-1021.

15) Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-1β. Mol Cell 2002;10:417-426.

16) Wree A, Eguchi A, McGough MD, Pena CA, Johnson CD, Canbay A, et al. NLRP3 inflammasome activation results in hepatocyte pyroptosis, liver inflammation, and fibrosis in mice. Hepatology 2014;59:899-910.

17) Groslandm P, My BF. Spotlight on the NLRP3 inflammasome pathway. J Inflamm Res 2018;11:359-374.

18) Bieghs V, Trautwein C. The innate immune response during liver inflammation and metabolic disease. Trends Immunol 2013;34:446-452.

19) Jo E-K, Kim JK, Shin D-M, Sasakawa C. Molecular mechanisms regulating NLRP3 inflammasome activation. Cell Mol Immunol 2016;13:148-159.

20) Boanis SG, Borkham-Kamphorst E, Tihiba L, Haas U, Weiskirchen R. Expression analysis of inflammasomes in experimental models of inflammatory and fibrotic liver disease. J Inflamm (Lond) 2012;9:49.

21) Karin K, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. Hepatology 2009;50:261-274.

22) Pham HT, Arnhand K, Asad YJ, Deng L, Felder TK, St John-Williams L, et al. Inter-laboratory robustness of next-generation bile acid study in mice and humans: International Ring Trial involving 12 laboratories. J Appl Lab Med 2019;1:129-142.

23) Guarda G, Zenger M, Yazdi AS, Schroder K, Fickert P, Menu E, et al. Differential expression of NLRP3 among hematopoietic cells. Cell Mol Immunol 2013;10:319-330.

24) Jha S, Bricky WJ, Ting JP-Y. Inflammasomes in myeloid cells: warriors within. Microbiol Spectr 2017;5. https://doi.org/10.1128/microbiolspec.MCHD-0049-2016.

25) Kulkarni SR, Soroka CJ, Hagey LR, Boyer JL. Sirtuin 1 activation alleviates cholestatic liver injury in a cholic acid-fed mouse model of cholestasis. Hepatology 2016;64:2151-2164.

26) Krones E, Polleitmeier MJ, Rosenbergkrantz AR, Fickert P. Cholemic nephropathy—historical notes and novel perspectives. Biochem Biophys Acta 2018;1864:1356-1366.

27) Jorgensen I, Miao EA. Pyroptotic cell death defends against intracellular pathogens. Immunol Rev 2015;265:130-142.

28) He S, Wang X. RIP kinases as modulators of inflammation and immunity. Nat Immunol 2018;19:912-922.

29) Liao I, Schneider KM, Galvez EJC, Frissen M, Marschall H-U, Su H, et al. Intestinal dysbiosis augments liver disease progression via NLRP3 in a murine model of primary sclerosing cholangitis. Gut 2019;68:1477-1492.

30) Jaeschke H. Mechanisms of liver injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. Am J Physiol Gastrointest Liver Physiol 2006;290:G1083-G1088.

31) Deshmule SM, Kremelev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res 2009;29:313-326.

32) Wiedow O, Meyer-Hoffert U. Neutrophil serine proteases: potential key regulators of cell signalling during inflammation. J Intern Med 2005;257:319-328.

33) Gujrati JS, Farooq M, Bajt ML, Jaeschke H. Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. Hepatology 2003;38:355-363.

34) Erker L, Grompe M. Signaling networks in hepatic oval cell activation. Stem Cell Res 2008;1:90-102.

35) Best J, Dollé L, Manka P, Coombes J, van Grunsven LA, Syn W-K. Role of liver progenitors in acute liver injury. Front Physiol 2013;4:258.

36) Wree A, McGough MD, Peña CA, Schlattman M, Li H, Inzaugarat ME, et al. NLRP3 inflammasome activation is required for fibrosis development in NALFD. J Mol Med 2014;92:1069-1082.

37) Inzaugarat ME, Johnson CD, Holtmann TM, McGough MD, Trautwein C, Papouchado BG, et al. NLR family pyrin domain-containing 3 inflammasome activation in hepatic stellate cells induces liver fibrosis in mice. Hepatology 2019;69:845-859.

38) Perera AP, Fernando R, Shivde T, Gundamaraju R, Southam B, Sohal SS, et al. MCC950, a specific small molecule inhibitor of NLRP3 inflammasome attenuates colonic inflammation in spontaneous colitis mice. Sci Rep 2018;8:8618.

39) Ismael S, Zhao L, Nasoohi S, Ishrat T. Inhibition of the NLRP3-inflammasome as a potential approach for neuroprotection after stroke. Sci Rep 2018;8:5971.

40) Mridha AR, Wree A, Robertson AAB, Yeh MM, Johnson CD, van Rooyen DM, et al. NLRP3 inflammasome blockade reduces liver inflammation and fibrosis in experimental NASH in mice. J Hepatol 2017;66:1037-1046.

41) Broderick L, Hoffman HM. cASCading specks. Nat Immunol 2014;15:698-700.

42) Henao-Mejía J, Elnav E, Jin C, Hao L, Mehal WZ, Strowig T, et al. Inflammasome-mediated dysbiosis regulates progression of NALFD and obesity. Nature 2012;482:179-185.

43) Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, et al. Non-canonical inflammasome activation targets caspase-11. Nature 2011;479:117-121.

44) Seki E, de Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-β-signalling and hepatic fibrosis. Nat Med 2007;13:1324-1332.

45) DeSantis DA, Ko C-W, Liu Y, Liu X, Hise AG, Nunez G, et al. Alcohol-induced liver injury is modulated by Nlrp3 and Nlrc4 inflammasomes in mice. Mediators Inflamm 2013;2013:751374.

46) Finotto S, Siebler J, Hausding M, Schipp M, Wirz S, Klein S, et al. Severe hepatic injury in interleukin 18 (IL-18) transgenic mice: a key role for IL-18 in regulating hepatocyte apoptosis in vivo. Gut 2004;53:392-400.

47) Vince JE, Silke J. The intersection of cell death and inflammasome activation. Cell Mol Life Sci 2016;73:2349-2367.

48) Horvath GL, Schram JC, de Nardo CM, Latz E. Intracellular sensing of microbes and danger signals by the inflammasomes. Immunol Rev 2011;243:119-135.

49) Latz E, Xiao TS, Sturza A. Activation and regulation of the inflammasomes. Nat Rev Immunol 2013;13:397-411.
50) Petrasek J, Bala S, Csak T, Lippai D, Kodys K, Menashy V, et al. IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice. J Clin Invest 2012;122:3476-3489.

51) Wu X, Dong L, Lin X, Li J. Relevance of the NLRP3 inflammasome in the pathogenesis of chronic liver disease. Front Immunol 2017;8:1728.

52) Hutton HL, Ooi JD, Holdsworth SR, Kitching AR. The NLRP3 inflammasome in kidney disease and autoimmunity. Nephrology (Carlton) 2016;21:736-744.

53) Xu K-Y, Wu C-Y, Tong S, Xiong P, Wang S-H. The selective Nlrp3 inflammasome inhibitor Mcc950 attenuates lung ischemia–reperfusion injury. Biochem Biophys Res Commun 2018;503:3031-3037.

54) Hong P, Li F-X, Gu R-N, Fang Y-Y, Lai L-Y, Wang Y-W, et al. Inhibition of NLRP3 inflammasome ameliorates cerebral ischemia–reperfusion injury in diabetic mice. Neural Plast 2018;2018:9163521.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31494/suppinfo.