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NLRP3 inflammasome activation in hepatic stellate cells induces murine liver fibrosis

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Abstract

The NLRP3 inflammasome plays an important role in liver fibrosis development. However, the mechanisms involved in NLRP3-induced fibrosis are unclear. Our aim was to test the hypothesis that the NLRP3 inflammasome in hepatic stellate cells (HSC) can directly regulate their activation and contribute to liver fibrosis. Primary HSC isolated from WT, *Nlrp3*^{-/-}, or *Nlrp3*^{L351PneoR} knock-in crossed to inducible (estrogen receptor Cre - CreT) mice were incubated with LPS and ATP, or 4OH-tamoxifen, respectively. HSC-specific *Nlrp3*^{L351P} knock-in mice were generated by crossing transgenic mice expressing lecithin retinol acyltransferase (Lrat)-driven Cre and maintained on standard rodent chow for 6 months. Mice were then sacrificed; liver tissue and serum were harvested. *Nlrp3* inflammasome activation along with HSC phenotype and fibrosis were assessed by RT-PCR, Western blot, FACS, ELISA, immunofluorescence and immunohistochemistry. Stimulated WT HSC displayed increased levels of NLRP3 inflammasome-induced ROS production and Cathepsin B activity, accompanied by an upregulation of mRNA and protein levels of fibrotic makers, an effect abrogated in *Nlrp3*^{-/-} HSC. *Nlrp3*^{L351P} CreT HSC also showed elevated mRNA and protein expression of fibrotic markers 24h after inflammasome activation induced with 4OH-tamoxifen. Protein and mRNA expression levels of fibrotic markers were also found to be increased in isolated HSC and whole liver tissue from *Nlrp3*^{L351P} *Lrat* *Cre* mice compared to WT. Liver sections from 24 week-old *Nlrp3*^{L351P} *Lrat* *Cre* mice showed fibrotic changes with increased α SMA and desmin positive cells and collagen deposition, independent of inflammatory infiltrates; these changes were also observed after LPS challenge in 8 week-old *Nlrp3*^{L351P} *Lrat* *Cre* mice.

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Conclusion: Our results highlight a direct role for the NLRP3 inflammasome in the activation of HSC directly triggering liver fibrosis.

Keywords

liver fibrosis; inflammasome; NLRP3; hepatic stellate cells

Introduction

Inflammasomes are multiprotein complexes expressed in innate immune cells, and non-immune cells within the liver that sense danger signals via nucleotide-binding oligomerization domain (NOD) like receptors (NLR) and control the activation of Caspase (Casp-1)(1, 2). The most characterized and investigated member of the NLR inflammasome family is NLRP3, activation of which can occur in response to a wide range of stimuli, including pathogens and sterile insults (3) via the generation of mitochondrial-derived ROS or Cathepsin B activation, and deubiquitination, among other mechanisms (4, 5). Upon stimulation, components of the NLRP3 inflammasome are recruited and assembled leading to cleavage of pro-Casp1 into its active form, which in turn cleaves pro-interleukin (IL) -1β and pro-IL-18 into their mature forms (1). Non-canonical Casp11-dependent NLRP3 activation has been shown to involve gasdermin cleavage, and the release of high mobility group box (HMGB)-1 and IL-1 α (6).

Inflammasome activation and IL-1 β production have been associated with several chronic liver diseases, including fibrosis development (7). One of the central features of fibrotic changes within the liver is the augmented production of extracellular matrix proteins, including collagen fibers. Hepatic stellate cells (HSC) represent the major liver mesenchymal cell type playing a key role in fibrosis, due to their activation and subsequent transdifferentiation into myofibroblasts leading to extracellular matrix deposition and scar tissue formation upon sustained injury (8–10).

Recent studies from our lab using constitutively activated *Nlrp3* mutant mice demonstrated that NLRP3 activation is required for hepatic inflammation and fibrosis, potentially through TNF signaling, as well as for inducing the programmed inflammatory cell death known as pyroptosis (11–13). However, it remains unclear whether HSC activation is the result of intracellular NLRP3 activation or a consequence of inflammasome activation in the surrounding milieu. Thus, we aimed to test our working hypothesis that the NLRP3 inflammasome activated in HSC directly contributes to liver fibrosis development.

Material and Methods

Animal models

The following mouse strains were used in this study: C57BL/6 wildtypes (WT) and *Nlrp3*^{-/-}. *Nlrp3*^{L351PneoR} mice were generated as previously described, with a leucine 351 to proline (L351P) substitution and the presence of an intronic floxed neomycin resistance cassette, in which expression of the mutation does not occur unless the *Nlrp3*^{L351PneoR} mutants are first bred with mice expressing Cre recombinase (14). *Nlrp3*^{L351PneoR} mice were bred to B6.Cg-

Tg (Cre/Esr1)5Amc/J (CreT) mice generating conditional knock-in mice in which the mutation is inducible with tamoxifen or to transgenic mice expressing Lecithin retinol acyltransferase-driven Cre (Lrat Cre) coexpressing tdTomato+ in which hepatic expression of mutant *Nlrp3*^{L351P} is restricted to HSC. Eight male *Nlrp3*^{L351P/+} Lrat Cre and eight male littermate mice were maintained on a standard rodent chow for 24 weeks. At this time point mice were sacrificed and liver tissue and serum were harvested.

LPS challenge: Ten male Lrat-Cre *Nlrp3*^{L351P} and ten littermate control mice at 8 weeks of age were included. Half of the mice in each group were given a single intraperitoneal injection of lipopolysaccharides from Escherichia coli 0127:B8 (Sigma-Aldrich, St. Louis, MO, USA) while the other half were injected with saline solution. LPS was injected at a concentration of 2.5 mg/kg and mice were sacrificed and livers were collected six hours post-injection.

All procedures were approved by German local governmental authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, LANUV NRW) and the Institutional Animal Care and Use Committee at the University of California, San Diego. All efforts were made to minimize pain and distress during animal husbandry and experimental assessments.

Hepatic stellate cell isolation and stimulation

For isolation of hepatic stellate cells, 24 week old female or male mice were used. Briefly; mice were anesthetized by ketamine/xylazine injection and perfused *in situ* through the inferior vena cava with sequential Pronase E (0.4 mg/ml) and Collagenase D (0.8 mg/ml) solutions. Liver was removed and digested *in vitro* with Collagenase D (0.5 mg/ml), Pronase E (0.5 mg/ml) and DNase I (0.02 mg/ml). After 20 minutes, tissue was filtered through a 70 µm mesh. Cells were separated using a Nycodenz gradient centrifugation. The HSC were sorted by gating for tomato positive signaling or seeded into plastic tissue culture flasks in DMEM containing fetal serum, and incubated at 37 °C with CO₂ overnight. In order to deplete any macrophage contamination, HSC layer was treated with clodronate liposomes (5mg/ml) for 4 h at 37°C as previously described (15). The next morning, the culture medium was changed and inflammasome activation in HSC was induced with LPS (1 µg/ml) for 3 h and either adenosine triphosphate (ATP) (5 mM) for 1 h or with 4-hydroxytamoxifen (4-OH Tamoxifen), the active metabolite of tamoxifen, for 24 h. In addition, HSC were co-incubated with CA-074 -Me (20 µM) (Sigma Aldrich) to inhibit Cathepsin B activity. In an independent experiment, HSC were stimulated with TGF-β (100 ng/ml) for 4 h.

Histology, immunostaining and Sirius Red staining

Liver tissues were fixed, embedded in paraffin, and processed on slides for hematoxylin-eosin (H&E) or Sirius Red staining. Histopathological staging of liver fibrosis was performed by an experienced pathologist. Primary monoclonal antibodies used to perform immunostaining were: anti-F4/80 (a murine pan-macrophage marker) (AbDSerotec, Hercules, CA, USA), anti-myeloperoxidase (MPO) (Thermo Fisher Scientific, Waltham, MA, USA), alpha smooth muscle actin (αSMA) (Abcam, Cambridge, UK), anti-Collagen alpha 1a (BioTrend, Germany), anti-IL-1β (Abcam), anti-NLRP3 (AdipoGen, San Diego,

USA, anti-desmin (Thermo Scientific, Waltham, MA, USA), the negative controls in all procedures omitted primary antibody. Cells were fixed with pre-chilled methanol and blocked with 5% BSA in PBS-T while the slides were deparaffinized and hydrated in ethanol and the antigens were retrieved in citrate buffer pH 6.0 for 20 minutes at 95°C or treated with 2% BSA 1x Triton in TBS-T for 30 min at room temperature. Following overnight incubation with primary antibodies, horseradish peroxidase (HRP) or Alexa-Fluor 488 or Alexa-Fluor 546 conjugated second antibody (ThermoFisher, Waltham, MA, USA) was applied. For color reaction of HRP, Streptavidin-peroxidase complex 3, 3'-diaminobenzidine tetrahydrochloride was used as chromogen and the slides were counterstained with hematoxylin. Mounting solution containing DAPI (Vector Laboratories, Burlingame, CA, USA) was used to counterstain the nuclei.

For detecting cathepsin B activity, stimulated hepatic stellate cells were stained using cell-permeable fluorescently labeled Magic Red cathepsin B substrate (ImmunoChemistry Technologies, Bloomington, MN) and Hoechst stain according to the manufacturer's instructions. The slides containing the stained live cells were then mounted in a drop of PBS and examined within 30 min.

Photos were taken with Axioimager Z1 using Axio Vision 4.2 software (Carl Zeiss, Jena, Germany) and NanoZoomer 2.0HT Slide Scanning System (Hamamatsu, Japan) and analyzed using ImageJ software (National Institute of Health, USA). Confocal imaging was performed with LSM 710 confocal laser scanning microscope (Carl Zeiss) (Immunohistochemistry & Confocal Microscopy Facility, RWTH Uniklinik Aachen).

Real-time PCR

Total RNA was isolated using PeqGold TriFast following manufacture instruction (PeqLab, Germany). The reverse transcript (cDNA) was synthesized from total RNA using the iScript cDNA Synthesis kit (BioRad, Hercules, California, US). Real-time PCR quantification was performed using Fast Sybr-Green and QuantStudio 6 (Applied Biosystems, Massachusetts, US). The sequences of the primers used for quantitative PCR are given in supplemental table 1.

Flow cytometry analysis

After stimulation, hepatic stellate cells were harvested, permeabilized and incubated with Vimentin PE-conjugated antibody (R&D, Minneapolis, MN, USA). To assess caspase 1 activity, FLICA FAM-YVAD-FMK (FAM-FLICA® Caspase-1 Assay Kit, ImmunoChemistry Technologie) was used following manufacturer's instructions. In order to measure reactive oxygen species production, cells were incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) for 30 min at 37°C and analyzed by flow cytometry at 500 nm (BD Canto II). Macrophage contamination was evaluated by staining HSC with F4/80 PE (eBioscience, Hatfield, UK) for 30 min at 4°C.

Immunoblot analysis and ELISA

Immunoblot analysis was performed as previously described. Anti-LRAT (Donated by Dr. Palczewski, University of Washington, Seattle, Washington), anti-NLRP3(Adipogen), anti-

IL-1 β (Abcam), anti-Caspase1 (Santa Cruz Biotechnology, Dallas, TX, USA), anti- α SMA (Abcam), anti-CTGF (Abcam) antibodies were used in combination with appropriate peroxidase-conjugated secondary antibodies. Protein load was verified with GAPDH antibody (Abcam). Bands were visualized with the enhanced chemiluminescence substrate (BioRad, Hercules, California, US) and digitized using a CCD camera (ImageQuant LAS4000, GE Healthcare, Chicago, Illinois, US). Quantification of IL-1 β levels in supernatant was performed according to the manufacturer's instruction (Peprotech, Hamburg, Germany). Hydroxyproline levels were measured using a standard chemical assay.

Statistical Analysis

Analyses were performed with Graph Pad (version 7.0; Graph Pad, Graph Pad Software Inc., CA, USA). The significance level was set at $\alpha = 5\%$ for all comparisons. Non-parametric Mann-Whitney test was used for two-group comparison. For experiments involving three or more groups, data were evaluated using Kruskal-Wallis test and Dunn's Multiple Comparison Test. Unless otherwise stated; data are expressed as mean $\pm$ SEM.

Results

LPS triggers canonical NLRP3 inflammasome activation in isolated hepatic stellate cells

We first assessed the purity of isolated HSC and macrophage contamination. FACS analysis showed that the mean percentage of F4/80+ cells was 1.45%, which, along with the evaluation of blue autofluorescence of retinoid droplets containing Vitamin A, indicates that isolated HSC exceeded 98% purity (Supplemental Fig. 1A-D).

Next, to confirm that hepatic stellate cells possess a completely functional inflammasome, we first stimulated cells with LPS as a first signal for NLRP3 activation, followed by ATP as a second signal. Stimulation of WT cells with LPS induced upregulation of *Nlrp3* and pro-*Il1b* mRNA levels that were further increased when cells were subsequently incubated with ATP (Fig. 1A), an effect that was not present in *Nlrp3*^{-/-} HSC. Moreover, increased production of mature IL-1 β was observed in the supernatant of WT, but not *Nlrp3*^{-/-} HSC after incubation with LPS and ATP (Fig. 1B). Immunostaining confirmed that WT cells are able to increase IL-1 β expression when stimulated with LPS followed by ATP, while *Nlrp3*^{-/-} HSC failed to respond (Fig. 1C). In order to confirm that the NLRP3 inflammasome was active, Caspase 1 activity was measured by flow cytometry and quantified by normalizing the mean fluorescence intensity of stimulated cells to basal conditions. Increased caspase 1 activity was observed in WT, but not *Nlrp3*^{-/-} cells, after LPS and ATP stimulation (Fig. 1D). *Casp11*, *Il1a* and *Hmgbl* mRNA levels were unchanged after LPS or LPS/ATP stimulation ruling out the presence of non-canonical NLRP3 inflammasome activation (Fig. 1E). To rule out that the changes observed were due to an indirect effect via macrophage contamination and their interaction with HSC, HSC were treated with clodronate liposomes, which did not prevent the upregulation of *Nlrp3* and *Il1b* mRNA or protein levels or the increase of Caspase1 activity after LPS or LPS and ATP stimulation (Supplemental Fig. 2A-D).

Next, we assessed which mechanism may be involved in activating the inflammasome in HSC. Using immunofluorescence, we found that Cathepsin B was increased only after LPS/ATP treatment in WT HSC (Fig. 1F). Likewise, ROS production was augmented after LPS, and further elevated after subsequent ATP incubation, as evidenced by increased DCF intensity measured by FACS (Fig. 1G). To further dissect its involvement in NLRP3 inflammasome activation, Cathepsin B activity was inhibited by incubating HSC with CA-074 –Me – a selective Cathepsin B inhibitor – together with LPS and ATP. Immunostaining showed that WT HSC failed to upregulate Cathepsin B as well as *Nlrp3* and IL-1 β after LPS and ATP when CA-074-Me was present (Sup Fig 3A). Moreover, supernatant levels of mature IL-1 β were not changed in WT HSC after incubation with LPS followed by ATP in the presence of CA-074-Me when compared to basal conditions (Sup Fig 3B). No increase in caspase 1 activity was observed in WT after LPS and ATP stimulation in combination with Cathepsin B inhibitor (Supplemental Fig. 3C). Taken together, these results suggest that NLRP3 activation in HSC is mediated at least in part via increased ROS production and Cathepsin B activation secondary to the release of this protease from the lysosome into the cytosol.

Lastly, we examined the specificity of NLRP3 modulation in HSC and whether its activation is a common pathway during HSC transdifferentiation into a myofibroblast-like cell. To address this question we treated HSC with TGF- β , a known pro-fibrotic factor, and assessed NLRP3 inflammasome activation. Stimulation with TGF- β did not induce upregulation of *Nlrp3* or *Il1b* mRNA levels or increase the concentration of mature IL-1 β in the supernatant, even though WT HSC showed augmented *aSma* and *Ctgf* mRNA levels. (Supplemental Fig. 4)

Isolated hepatic stellate cells upregulate fibrotic markers after LPS-driven NLRP3 inflammasome activation

Immunostaining showed that when stimulated with LPS followed by ATP, WT HSC demonstrated changes in shape, with an increase in collagen production and α SMA expression, however these changes were not observed in *Nlrp3*^{-/-} HSC's (Fig. 2A). In addition, vimentin expression measured by flow cytometry was increased in WT stellate cells after LPS/ATP stimulation while *Nlrp3*^{-/-} HSC failed to upregulate this marker (Fig. 2B), suggesting that *Nlrp3* inflammasome activation leads to the upregulation of fibrosis markers. Along with NLRP3 activation, WT HSC exhibit augmented mRNA levels of *Ctgf*, *aSma*, *Tgfb*, *Vim*, and *Col1a1* (Fig. 2C) as well as *Timp1* and *Mmp9*, but not *Mmp2* (Supplemental Fig 5A), after LPS or LPS and ATP stimulation; this effect was abrogated in *Nlrp3*^{-/-} HSC's. Treatment of HSC with clodronate liposomes did not prevent the upregulation of these fibrotic markers in WT HSC (Supplemental Fig. 2E).

NLRP3 inflammasome overexpression induces a fibrotic profile in isolated hepatic stellate cells

To validate that HSC activation can be modulated by NLRP3 inflammasome activation we isolated cells from *Nlrp3*^{L351P} *CreT* mice – a mouse strain that allows for temporal NLRP3 inflammasome activation. After 24h 4OH-tamoxifen induction, NLRP3 inflammasome hyper-activation was confirmed by upregulated *Nlrp3* and pro-*Il1b* mRNA levels, as well as

increased caspase 1 activity, in *Nlrp3* mutant HSC (Supplemental Fig. 6). We also confirmed previous observations that tamoxifen does not have an effect on inflammasome expression or activation in HSC that do not express the *Nlrp3* mutation secondary to a lack of cre recombinase (Supplementary Fig. 7).

Interestingly, NLRP3 hyper-activation results in the upregulation of fibrosis markers in stimulated mutant HSC that further increases after LPS stimulation, as evidenced by elevated mRNA levels of *aSma*, *Col1a1*, *Vim*, *Ctgf* and *Tgfb* (Fig. 2F) as well as *Mmp9* and *Mmp2* (Supplemental Fig 5B) compared to the basal condition. Flow cytometry analysis revealed that vimentin expression was increased after inducing NLRP3 overexpression in mutant HSC, an effect that was enhanced when LPS was used to prime the cells (Fig. 2E). Furthermore, overactivation of *Nlrp3* in mutant HSC resulted in marked morphologic changes along with an observed upregulation in *Col1a1* and α SMA protein expression, which, again, were enhanced in LPS primed cells (Fig. 2D).

NLRP3 activation in HSC *in vivo* results in a phenotypic switch to myofibroblasts

As described before by Kluwe et al (16), we observed that LRAT is strongly expressed in HSC but absent in other hepatic cell types, such as hepatocytes and Kupffer cells (Supplemental Fig 8A). Furthermore, we confirmed that breeding *Nlrp3^{L351P/+}* mice onto *Lrat Cre* mice resulted in hyper-activation of NLRP3 within HSC, as evidenced by co-localization of tdTomato positive nucleus and augmented expression of NLRP3 in isolated HSC (Supplemental Fig 8B). Western blot of whole liver lysates showed that inflammasome components, specifically NLRP3, mature IL-1 β and cleaved p-10 Caspase 1 were increased in *NLRP3^{L351P/+} Lrat Cre* mice (Fig. 3A, B), which was further confirmed by elevated mRNA levels of *pro-Il1b* and *pro-Casp1* (Fig. 3C).

Isolated HSC from *Nlrp3^{L351P/+} Lrat Cre* mice showed increased protein expression levels of α SMA and desmin, as evidenced by immunofluorescence staining (Fig. 3E), which indicates a switch from a quiescent state to a myofibroblast phenotype that was not present in WT HSC. Moreover, a 3-fold increase in protein expression (Fig. 3F, G) as well as a 2-fold increase in mRNA levels (Fig. 3G) of α SMA was observed in whole tissue lysates from *Nlrp3^{L351P/+} Lrat Cre* mice compared to WT littermates.

Persistent NLRP3 activation in HSC results in liver fibrosis

In order to assess the effect of persistent NLRP3 activation on the phenotype of HSC, we used paraffin-embedded liver tissue and stained them with two markers of HSC. We could detect a significant increase in α SMA (Fig. 4A, B) as well as desmin (Fig. 4A, C) positive cells in *Nlrp3^{L351P/+} Lrat Cre* mice compared to WT littermates. Next, we determined the downstream effect of myofibroblast transdifferentiation, specifically collagen deposition. Sirius red staining showed an increase in collagen deposition and, moreover, further evaluation by a pathologist confirmed the presence of mild predominantly perisinusoidal fibrosis (Fig. 4A, D) in *Nlrp3^{L351P/+} Lrat Cre* mice. In addition, these changes were accompanied by the upregulation of mRNA levels of *Col1a1*, *Col3a1* and *Timp1* (Fig. 4E) when compared to WT. This increase in collagen production was further supported by an

increase in hydroxyproline protein concentration (Fig. 4F) in whole liver lysate from *Nlrp3^{L351P/+} Lrat* mice compared to littermates.

Fibrotic changes in the liver were independent of inflammation or injury

The assessment of basic liver histology did not reveal any signs of hepatocellular injury due to inflammation in *Nlrp3^{L351P/+} Lrat Cre* mice (Fig. 5A). In line with this finding, serum ALT levels were unchanged in *Nlrp3^{L351P/+} Lrat Cre* when compared to WT mice (Fig. 5B). TUNEL staining showed no evidence of increased hepatocyte apoptosis in the *Nlrp3^{L351P/+} Lrat Cre* mice compared to littermates (Fig. 5A). Interestingly, the fibrotic changes observed within the liver were also independent of macrophage infiltration as evidenced by the lack of alterations in F4/80 expression in liver sections of *Nlrp3^{L351P/+} Lrat Cre* when compared to WT mice (Fig. 5C,D). Analyses of markers for macrophage activation *Ly6c* and *Tnf* also did not differ in *Nlrp3^{L351P/+} Lrat* and WT mice (Fig. 5E). The expression of myeloperoxidase (MPO), used to assess infiltration of neutrophils, was similar in *Nlrp3^{L351P/+} Lrat Cre* and WT mice (Fig. 5C, D).

Nlrp3 hyper-activation leads to HSC activation and fibrotic changes in the liver after LPS challenge

In order to further investigate the effect of Nlrp3 activation in HSC on fibrosis development, we injected LPS intraperitoneally and observed the changes in liver fibrosis markers after 6 hours.

Even though Sirius red staining showed no significant difference in collagen deposition (Fig. 6A, B), HSC activation markers were increased in *Nlrp3^{L351P} Lrat Cre* mice compared to WT after LPS challenge as evidenced by the upregulation of mRNA levels of *Ctgf*, *Coll1a1*, *Tgfb aSma*, and *Vimentin* (Figure 6C). Moreover, a 4-fold increase in α SMA along with a 1.5-fold increase in CTGF (Fig. 4D) protein expression levels was observed in whole tissue lysates from LPS-injected *Nlrp3^{L351P/+} Lrat Cre* mice compared to WT.

Liver histology revealed a slightly increased inflammation in both *Nlrp3^{L351P/+} Lrat Cre* and WT after 6h LPS injection compared to WT but no difference between the two groups (Suppl. Fig. 9A). Immunohistochemistry of liver sections, as well as mRNA levels, showed that mutant mice do not display an increase in MPO, F4/80 or CD68 positive cells compared to littermate animals challenged with LPS (Suppl. Fig. 9A, C), even though overall levels were increased compared to WT mice. Serum AST and LDH levels were found to be increased compared to littermate controls but not different between mutant mice and littermate controls postLPS challenge (Suppl. Fig. 9B).

Discussion

The findings of the present study highlight NLRP3 inflammasome induction in HSC as sufficient to trigger their activation and subsequent fibrogenesis independent of inflammation. Our results demonstrate that when NLRP3 activation in HSC is induced by either inflammasome inducers, or through a point mutation causing a ligand-independent overactivation, HSC display upregulation of several fibrotic markers (*Tgfb*, *aSma*, *Vim*, *Coll1a1*, *Mmp9* and *Timp1*) and shift their phenotype to myofibroblasts. Moreover, HSC-

specific NLRP3 inflammasome overactivation initiated pathways *in vivo* related to fibrogenesis that resulted in signs of liver fibrosis and collagen deposition (*Col1a1*, *Col3a1*, *Timp1*) without evident changes in hepatic inflammatory infiltrates (F4/80, MPO positive cells), which can be further exacerbated by an LPS-challenge.(Fig. 7).

Fibrosis is the result of a sustained scarring response to chronic liver injury which gradually disrupts liver architecture and causes vascular distortion, eventually leading to cirrhosis (17). Several sources functioning as important players in liver fibrosis development have been identified. However, activated HSC represent the fundamental fibrogenic cell type involved in this process independent of the etiology of liver damage (9, 18). Upon sustained liver damage, HSC undergo an activation process that involves increased expression of TIMP-1, as well as extracellular matrix proteins such as collagens (19). Moreover, aberrant activation and proliferation of HSC together with exacerbated metabolism of extracellular matrix proteins are crucial factors in liver fibrosis initiation and development (20). Exploring cellular and molecular mechanisms that are responsible for liver fibrosis development may lead to the identification of novel anti-fibrotic approaches that could help improve the treatment and prognosis of chronic liver diseases (21). Growing evidence supports a central role of NLRP3 activation and downstream effectors, such as Caspase-1 activation and IL-1 β signaling in the development of liver fibrosis (7, 11–13, 22). It has been shown that the level of *Nlrp3* expression, among other inflammasomes, is augmented during experimental liver fibrosis (23). To date, there are only a few studies elucidating different stimuli that can trigger HSC activation and subsequent upregulation of fibrotic markers along with the activation of the NLRP3 inflammasome in both LX-2 cells, an immortalized human stellate cell line, and murine primary HSC (24–26). In particular, Watanabe et al. (27) not only showed that HSC expressed all of the NLRP3 inflammasome components, but also that activation of the NLRP3 inflammasome using monosodium urate crystals leads to a phenotypic switch from a quiescent state to collagen-producing myofibroblasts. These changes were absent in HSC derived from ASC-deficient mice. In line with these results, our data confirm that HSC indeed possess a fully intact NLRP3 inflammasome whose activation leads to a switch toward a myofibroblast profile. This was shown by the upregulation in mRNA and protein levels for NLRP3 and IL-1 β , as well as increased Caspase1 activity, along with an upregulation of α SMA and *Col1a1* after WT HSC were exposed to LPS/ATP. This point is further demonstrated by the fact that *Nlrp3*^{-/-} HSC failed to upregulate fibrotic markers while *Nlrp3*^{L351P/+} mutant HSC displayed a gain-of-function activated phenotype.

ROS have been demonstrated to contribute to chronic liver disease, including fibrosis, and to be one of many important NLRP3 inflammasome activators (28). Specifically in HSC, an upregulation of NOX4 expression, a generator of ROS, was found to be associated with NLRP3 inflammasome activation and increased collagen production (29). In connection to this, our results showed that NLRP3 inflammasome activation occurs along with increased ROS production after LPS administration and is further enhanced after subsequent treatment with ATP, representing a potential mechanism that could explain the link between NLRP3 inflammasome activation and fibrotic changes.

In the past few years, the association of the NLRP3 inflammasome to liver damage and fibrosis has been widely explored. IL-1 β levels were shown to be elevated in an

experimental murine fibrosis model while genetic depletion of IL-1ra results in an amelioration of the fibrotic phenotype (27). Moreover, mice deficient in Caspase1, ASC and NLRP3 have been shown to exhibit a protective effect on fibrosis development in several experimental murine models as evidenced by decreased TGF- β and Col1a1 expression, and reduced serum levels of TIMP1 and hyaluronic acid (13, 30–33). In contrast, persistent global activation of the NLRP3 inflammasome in mice leads to severe liver inflammation, hepatocyte pyroptotic cell death and liver fibrosis (11). However, the fact that these studies made use of global knock-out mice prevents us from dissecting the cell-specific role of inflammasome pathway activation, as well as whether the pro-fibrogenic mechanisms involved are due to direct effects on HSC or indirect consequences of increased inflammation and cell death in macrophages and hepatocytes, which then influence the activation of HSC (34). To address this issue, we used a gain-of-function model with selective expression of mutant hyperactive NLRP3 in HSC. Our results show for the first time that persistent activation of NLRP3 in HSC resulted in a marked upregulation of α SMA and desmin positive cells with a perisinusoidal / pericellular distribution in the liver, together with the spontaneous increase in collagen secretion and the development of liver fibrosis. Even more interesting was the finding that these fibrotic changes occur without the presence of inflammatory infiltrates, supporting a direct role for the NLRP3 inflammasome in HSC activation and liver fibrotic responses. These results have important translational implications for the various liver diseases such as nonalcoholic steatohepatitis (NASH) and alcoholic steatohepatitis (ASH) where the development and progression of liver fibrosis represents the major cause of liver related morbidity and mortality and identifies a novel anti-fibrotic approach by targeting NLRP3 Inflammasomes in activated myofibroblasts.

In summary, our study provides new insights into the cellular mechanisms by which the NLRP3 inflammasome contributes to HSC biology and fibrogenesis and carries implications related to the development of novel therapeutic strategies for the treatment of liver fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

α-SMA	alpha smooth muscle actin
ASH	alcoholic steatohepatitis
ATP	adenosine triphosphate
Col	Collagen
CTGF	connective tissue growth factor

F4/80	murine macrophage marker
HMGB1	high-mobility group box 1
HSC	hepatic stellate cells
IL	interleukin
LPS	lipopolysaccharides
Lrat	Lecithin retinol acyltransferase
MPO	myeloperoxidase
NASH	non-alcoholic steatohepatitis
NLRs	nucleotide-binding oligomerization domain (NOD) leucine-rich repeat containing receptors
TIMP1	tissue inhibitor of matrix metalloproteinase 1
TNF	Tumor necrosis factor
TGF-β	transforming growth factor beta
Vim	Vimentin

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Figure 1: Induction of canonical NLRP3 inflammasome activation accompanied by ROS production and cathepsin B in hepatic stellate cells by LPS/ATP.

Cultured HSC from WT mice, but not from *Nlrp3*^{-/-}, display augmented protein and mRNA expression levels for *Nlrp3* and *pro-Il1b* as well as mature IL-1 β (supernatant) after LPS and/or LPS/ATP stimulation (A-B). Flow cytometry analysis showed that WT HSC increase caspase1 activity after LPS/ATP stimulation, which was not seen in *Nlrp3*^{-/-} HSC (C). mRNA levels of *Casp11*, *Il1a* and *Hmgb1*, effectors of non-canonical activation of NLRP3, are not affected by LPS or LPS/ATP stimulation (D). LPS triggers ROS production in WT HSC, evidenced by increased DCF fluorescence, which was further enhanced after subsequent ATP stimulation, an effect not present in *Nlrp3*^{-/-} HSC (E). Increased cathepsin B was detected in WT HSC after LPS/ATP stimulation. Interestingly, *Nlrp3*^{-/-} HSC showed reduced cathepsin B levels compared to WT HSC. (6 replicates were included for all measured values, * = $p < 0.05$, ** = $p < 0.01$)

Figure 2: Effect of NLRP3 inflammasome activation in hepatic stellate cells fibrotic phenotype
 After LPS/ATP stimulation, HSC from WT but not *Nlrp3*^{-/-} mice showed an increase in collagen 1 and α SMA expression along with a change in the shape (A). Flow cytometry analysis showed that WT HSC displayed increased vimentin levels after LPS/ATP stimulation, an effect not present in *Nlrp3*^{-/-} HSC (B). mRNA levels of *Ctgf*, *aSma*, *Tgfb*, *Vim*, and *Coll1a1* were found to be increased after LPS, and further enhanced after subsequent ATP stimulation (C). HSC isolated from *Nlrp3*^{L351P/+} CreT showed a pronounced change in shape along with increased collagen 1 and α SMA expression (D), augmented vimentin expression measured by FACS (E) and elevated mRNA levels of *aSma*, *Coll1a1*, *Vim*, *Ctgf* and *Tgfb* (F) after NLRP3 inflammasome overactivation was induced by 4-OH-tamoxifen, effects that were intensified after the addition of LPS. (6 replicates were included for all measured values, * = $p < 0.05$, ** = $p < 0.01$)

Figure 3: Result of Lrat Cre-driven expression of mutant gain-of-function NLRP3 on hepatic stellate cell phenotype

Western blot of whole liver lysates showed augmented Nlrp3 inflammasome-related proteins in *Nlrp3^{L351P/+} Lrat Cre* mice (A), supported by quantification of protein levels of NLRP3, Caspase1 and IL-1β (B) as well as elevated mRNA levels of *pro-Il1b* and *pro-Casp1* (C). Immunofluorescence showed increased protein expression of αSMA and desmin in isolated HSC from *Nlrp3^{L351P/+} Lrat Cre* mice (D). Further analysis in whole liver lysates confirmed increased protein expression (E, F) as well as increased mRNA levels (F) of *αSma* in *Nlrp3^{L351P/+} Lrat Cre* mice compared to control littermates. (n= 8 mice per group for all measured values, * = p<0.05, ** = p<0.01)

Figure 4: Effect of persistent NLRP3 activation in hepatic stellate cells on liver fibrosis
 Representative pictures of α SMA, desmin and Sirius Red stained liver sections from *Nlrp3^{L351P} Lrat Cre* and control animal littermates (A). Quantification of stained sections showed an increased number of α SMA (B) and desmin (C) positive cells in *Nlrp3^{L351P} Lrat Cre* compared to littermates. Staging of fibrosis showed the presence of mild predominantly perisinusoidal fibrosis (D) in *Nlrp3^{L351P/+} Lrat Cre* mice accompanied by an upregulation of mRNA levels of *Col1a1*, *Col3a1* and *Timp1* (E) compared to littermate control animals. Increased hydroxyproline concentration in whole liver lysates of *Nlrp3^{L351P/+} Lrat Cre* mice compared to littermates (F). (n=3–5 mice per group for all measured values, * = p<0.05, ** = p<0.01)

Figure 5: Inflammation and liver injury after persistent NLRP3 activation in hepatic stellate cells

Representative pictures of H&E, TUNEL staining (A), F4/80 and MPO (C) stained liver sections from *Nlrp3^{L351P} Lrat Cre* and control littermates. Serum ALT levels were unchanged in *Nlrp3^{L351P/+} Lrat Cre* compared to littermate mice (B). Quantification of immunohistochemistry showed no detectable changes in F4/80 (D) or MPO (E) positive cells between *Nlrp3^{L351P/+} Lrat Cre* and the littermate control mice. (n=5 mice per group for all measured values, * = p<0.05, ** = p<0.01)

Figure 6.

Representative pictures of Sirius Red stained liver sections from LPS or Saline injected *Nlrp3^{L351P/+} Lrat Cre* and littermates (A). Sirius red staining quantification showed no changes in collagen deposition (B). Nevertheless, LPS-injected *Nlrp3^{L351P/+} Lrat Cre* displayed an upregulation of mRNA levels of *Ctgf*, *Col1a1*, *Tgfb*, *aSma* and *Vim* (E) compared to saline and LPS injected littermates. Increased α SMA and CTGF protein concentration in whole liver lysates of *Nlrp3^{L351P/+} Lrat Cre* mice compared to littermates (D). (n=4–6 mice per group for all measured values, * = p<0.05 vs. WT saline, # = p<0.01 vs. WT LPS)

Figure 7:
Summary of the role of Nlrp3 inflammasome in HSC activation and liver fibrosis development