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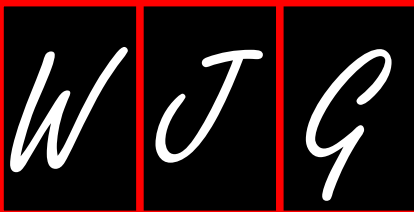
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Lipopolysaccharide induces and activates the Nalp3 inflammasome in the liver

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Abstract

AIM: To examine the activation of the Nalp3 inflammasome and its downstream targets following lipopolysaccharide (LPS)-induced stimulation in the liver.

METHODS: Six-to-eight-week-old C57BL/6 chow fed mice were injected intraperitoneally with 0.5 μ g/g bodyweight LPS and sacrificed 2, 4, 6, 18 or 24 h later. LPS-induced liver damage was confirmed by a biochemical assay to detect alanine aminotransferase (ALT) levels. To determine if LPS stimulation in the liver led to activation of the inflammasome, real-time quantitative polymerase chain reaction was used to evaluate the mRNA expression of components of the Nalp3 inflammasome. Enzyme-linked immunosorbent assays were used to determine the protein expression levels of several downstream targets of the Nalp3 inflammasome, including caspase-1 and two cytokine targets of caspase-1, interleukin (IL)-1 β and IL-18.

RESULTS: We found that LPS injection resulted in liver damage as indicated by elevated ALT levels. This was associated with a significant increase in both mRNA and protein levels of the proinflammatory cy-

tokine tumor necrosis factor (TNF)- α in the liver, as well as increased levels of TNFs in serum. We showed that LPS stimulation led to upregulation of mRNA levels in the liver for all the receptor components of the inflammasome, including Nalp3, Nalp1, pannexin-1 and the adaptor molecule apoptosis-associated speck-like, caspase recruitment domain-domain containing protein. We also found increased levels of mRNA and protein for caspase-1, a downstream target of the inflammasome. In addition, LPS challenge led to increased levels of both mRNA and protein in the liver for two cytokine targets of caspase-1, IL-1 β and IL-18. Interestingly, substantial baseline expression of pre-IL-1 β and pre-IL-18 was found in the liver. Inflammasome and caspase-1 activation was indicated by the significant increase in the active forms of IL-1 β and IL-18 after LPS stimulation.

CONCLUSION: Our results show that the Nalp3 inflammasome is upregulated and activated in the liver in response to LPS stimulation.

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Key words: Endotoxin; Nod-like receptor; Interleukin-1 β ; Interleukin-18; Caspase-1

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INTRODUCTION

The endotoxin lipopolysaccharide (LPS), a component of Gram-negative bacteria, plays an important role in

acute liver injury as well as chronic liver diseases including fatty liver associated with either alcohol consumption or metabolic syndrome and obesity^[1,2]. LPS has also been implicated in insulin resistance as well as in steatohepatitis in non-alcoholic fatty liver disease^[3-5]. Increasing evidence suggests that gut-derived LPS through the gut-liver axis affects the extent of liver damage in many different types of inflammatory liver diseases^[6]. LPS is a prototypical ligand for the pattern recognition receptor (PRR), Toll-like receptor 4 (TLR4). TLR4 induces downstream signaling *via* the MyD88 adapter molecule and induces production of proinflammatory cytokines through activation of the regulatory factor nuclear factor (NF)- κ B^[6]. In the liver, TLR4 is expressed in both parenchymal and immune cells, thereby providing potential for LPS-induced activation^[7].

Studies have shown that the Nalp3 inflammasome is activated by both pattern associated molecular patterns (PAMPs), including LPS and bacterial RNA, and danger-associated molecular patterns (DAMPs)^[8,9]. The Nalp3 inflammasome is a caspase-1 activating multiprotein complex, which has been implicated in numerous inflammatory processes and human diseases including gout, pseudogout, contact hypersensitivity, and most recently Alzheimer's disease^[10,11]. Furthermore, mutations in the *Nalp3* gene that lead to gain-of-function mutations, result in several hereditary syndromes including: Muckle-Wells syndrome, familial cold autoinflammatory syndrome, and chronic infantile neurological cutaneous and articular syndrome^[12].

In response to stimulation by either DAMPs or PAMPs, Nalp3 interacts with pro-caspase-1 through the adaptor molecule [apoptosis-associated speck-like, caspase recruitment domain (CARD)-domain containing protein (ASC)] to form the inflammasome, which leads to activation of caspase-1. Active caspase-1, previously known as interleukin (IL)-1 β converting enzyme, a heterodimer of p20 and p10 subunits, is the central effector protein of the inflammasome complex and promotes the cleavage of pro-IL-1 β , pro-IL-18 and pro-IL-33 to their biologically active, mature forms^[13]. In addition, active caspase-1 has been shown to cleave other substances, such as caspase-7 and sterol regulatory element-binding proteins, therefore playing a pivotal role in cell death and survival^[14].

The mRNA levels of the components the Nalp3 inflammasome are expressed in the eye, heart and lung in response to LPS stimulation^[15]. Although mRNA levels of IL-1 β have also been shown to increase in the liver in response to LPS stimulation, the role of the Nalp3 inflammasome in the liver in response to LPS stimulation has not been determined^[16]. In the liver, the Nalp3 inflammasome is upregulated upon acetaminophen-induced toxicity^[17] and the contribution of Nalp3 has been described in *Propionibacterium acnes* and LPS-induced acute liver injury^[18]; however, a role for Nalp3 in other liver conditions has not been identified.

Although the role of the proinflammatory cytokine, tumor necrosis factor (TNF) α , has been extensively studied in both alcoholic and non-alcoholic fatty liver disease, the role of IL-1 β and the inflammasome has yet to be explored. LPS plays an important role in liver disease; elevated LPS levels are detected in the portal and systemic blood of patients with alcoholic and non-alcoholic fatty liver disease^[19,20]. Furthermore, there is increased sensitivity to LPS in both alcoholic liver disease and fatty liver disease^[21]. Here, we examined if LPS induce the Nalp3 inflammasome in the liver. In this study, we showed that LPS stimulation led to induction of the components of the Nalp3 inflammasome at both the mRNA and protein level in the liver and this is associated with increased IL-1 β production.

MATERIALS AND METHODS

Mice

Six-to-eight-week-old wild-type C57BL/6 chow fed mice received intraperitoneal injections of 0.5 μ g/g LPS in PBS (Sigma, St Louis, MO, United States) (three mice per group) for 2, 4, 6, 18 and 24 h. Serum was separated from whole blood and stored at -80 °C. Liver samples were snap frozen in liquid nitrogen or stored in RNAlater (Qiagen Sciences, Germantown, MD, United States) for RNA extraction. All animals received proper care in agreement with animal protocols at the University of Massachusetts Medical School Institutional Animal Use and Care Committee.

Biochemical alanine aminotransferase assay

Serum alanine aminotransferase (ALT) was determined using a kinetic method (DTEK LLC, Bensalem, PA, United States).

RNA analysis

RNA was purified from livers using the RNeasy kit (Qiagen Sciences) and on-column DNA digestion. cDNA was transcribed with the Reverse Transcription System (Promega, Madison, WI, United States). Real-time quantitative polymerase chain reaction (qPCR) was performed using iCycler (Bio-Rad Laboratories, Hercules, CA, United States) as described previously^[22]. Primer sequences are shown in Table 1.

Cytokine enzyme-linked immunosorbent assay measurements

Whole cell lysates were extracted from liver tissue as previously described^[22]. These lysates were assayed for mature IL-1 β (R and D Systems, Minneapolis, MN, United States), TNF- α , total IL-18, and total IL-1 β (BD Biosciences, United States). Serum was also assayed for mature IL-1 β (R and D Systems).

Statistical analysis

Statistical significance was determined using Student's *t*

Target gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
18S	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CCG TAG TAG CG
TNF- α	GAA GTT CCC AAA TGG CCT CC	GTG AGG GTC TGG GCC ATA GA
IL-1 β	TCT TTG AAG TTG ACG GAC CC	TGA GTG ATA CTG CCT GCC TG
IL-1RA	TCA GAT CTG CAC TCA ATG CC	CTG GTG TTT GAC CTG GGA GT
Caspase-1	AGA TGG CAC ATT TCC AGG AC	GAT CCT CCA GCA ACT TC
ASC	GA GCT GCT GAC AGT GCA AC	GCC ACA GCT CCA GAC TCT TC
Nalp3	AGC CTT CCA GGA TCC TCT TC	CTT GGG CAG TTT CTT TC
Nlrc4	TGG TGA CAA TAG GGC TCC TC	CTG TTC CCT TTG CTC ACC TC
Nalp1	TGG CAC ATC CTA GGG AAA TC	TCC TCA CGT GAC AGC AGA AC
Pannexin-1	TGT GGC TGC ACA AGT TCT TC	ACA GAC TCT GCC CCA CAT TC

TNF: Tumor necrosis factor; IL: Interleukin; ASC: Apoptosis-associated speck-like, caspase recruitment domain-domain containing protein.

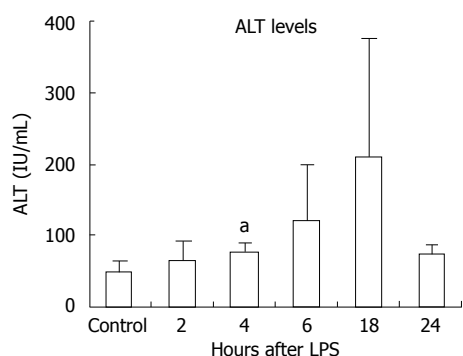


Figure 1 Lipopolysaccharide stimulation results in liver damage. C57BL/6 wild-type chow-fed mice (three per group) were injected intraperitoneally with lipopolysaccharide (LPS) for 2, 4, 6, 18 or 24 h. Serum was separated from whole blood and analyzed for alanine aminotransferase (ALT), which was significantly increased after 4 h of LPS stimulation. Mean \pm SD are shown. ^a $P < 0.01$.

test (two-tailed distribution). Data are presented as mean \pm SE and were considered significant at $P < 0.05$. ALT statistical significance was determined using the non-parametric Kruskal-Wallis test followed by the Mann-Whitney test.

RESULTS

LPS induces liver injury and inflammatory cytokines

Gut-derived LPS plays a role in several inflammatory liver diseases^[6]. To evaluate the effects of LPS on the liver, we injected wild-type mice with LPS for 2, 4, 6, 18 or 24 h and monitored ALT levels to evaluate liver injury. We found increased ALT levels in sera from mice injected with LPS at the 4-18-h time points, reaching statistically significant levels at 4 h, indicating LPS-induced liver damage (Figure 1).

It has been shown that LPS-induced signaling through TLR4 results in production of proinflammatory cytokines, one of which, TNF- α , has been linked to liver damage^[23,24]. We identified significant elevation in liver TNF- α mRNA expression at all time points after LPS stimulation compared to the unstimulated controls (Figure 2A). TNF- α protein levels were undetectable in liver tissue from unstimulated controls but were signifi-

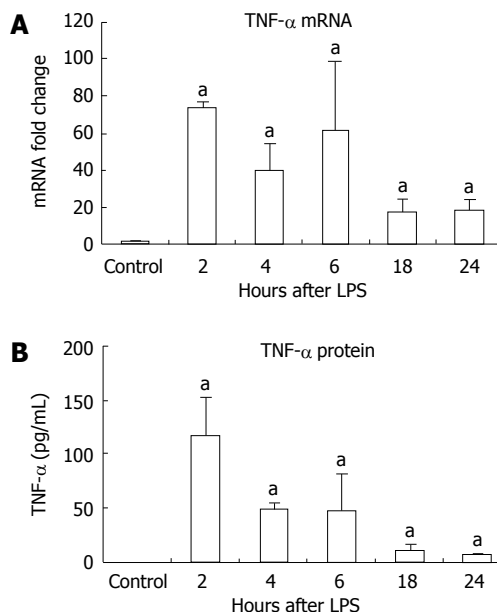


Figure 2 Inflammation in the liver is seen after lipopolysaccharide stimulation. A: Tumor necrosis factor (TNF)- α mRNA in liver tissue was significantly increased at all time points after lipopolysaccharide (LPS) stimulation. TNF- α mRNA was analyzed by real-time quantitative polymerase chain reaction and normalized to 18S. The values are shown as a fold change to the non-stimulated LPS control; B: Similarly, TNF- α protein was significantly elevated in liver tissue following LPS stimulation as detected by enzyme-linked immunosorbent assay. Protein levels were normalized to total protein concentration in each tissue sample. Mean \pm SD are shown. $n = 3$ for each group (except at 4 h LPS stimulation, where $n = 2$ due to an outlier), ^a $P < 0.01$.

cantly increased at all time points after LPS stimulation with a maximal increase at 2 h (Figure 2B).

LPS upregulates mRNA expression of Nalp3 inflammasome components

For complete function, the inflammasome requires expression of its various components including Nalp3, ASC (the adapter molecule), and caspase-1^[8,10]. We investigated the mRNA levels of the different types of inflammasomes, including the Nalp1 and Nlrc4 inflammasomes. Our examination revealed a significant increase in liver mRNA of *Nalp3* after ≥ 4 h LPS stimulation, with a peak at 6 h (Figure 3A). Upregulation of the inflammasome was not limited to *Nalp3*, and there was a significant increase in *Nalp1* (sevenfold) and *Nlrc4* (3.5-fold) mRNA at 24 h compared to the unstimulated controls ($P < 0.01$, $P = 0.04$, respectively, data not shown). We also analyzed the mRNA of other components of the inflammasome including the pyrin and CARD-domain-containing adaptor ASC; caspase-1, an inflammatory caspase; and pannexin-1, a hemi-channel that is recruited upon activation of the P2X7 receptor and is a necessary component for inflammasome activation^[25]. The mRNA levels of *ASC* gradually increased at each time point after LPS stimulation (Figure 3B), whereas caspase-1 levels peaked at 6 h and pannexin-1 mRNA levels peaked at 6 h (Figure 3C and D).

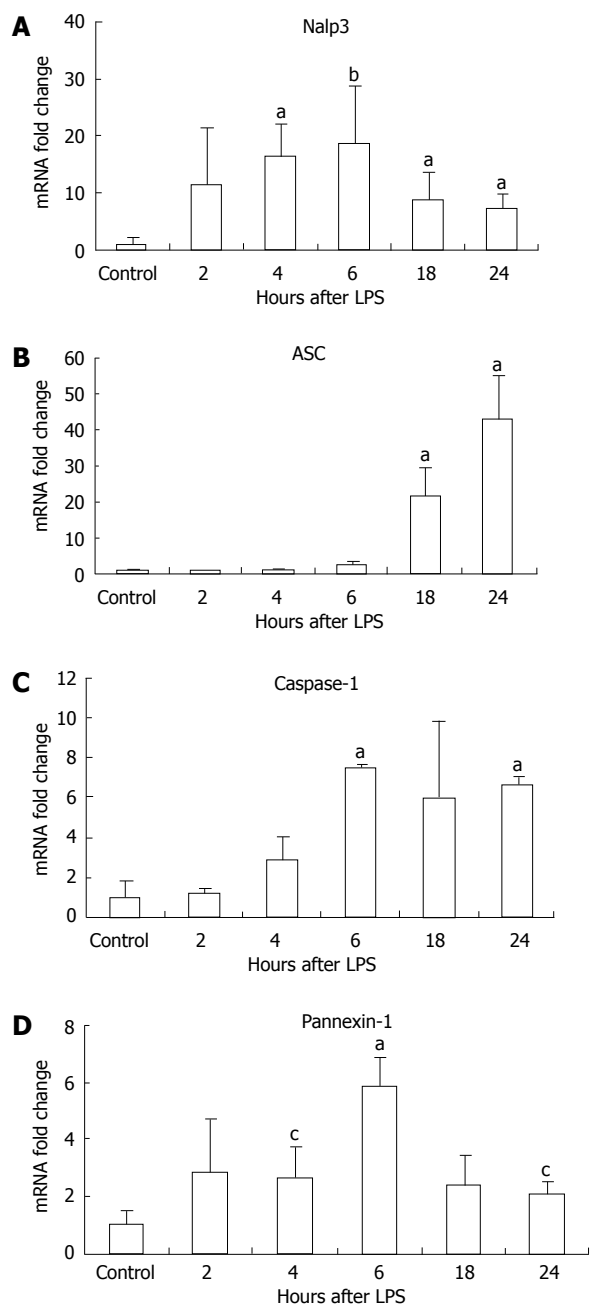


Figure 3 Lipopolysaccharide stimulation increased Nalp3 inflammasome mRNA expression in liver tissue. Liver RNA analysis of Nalp3 (A), apoptosis-associated speck-like, caspase recruitment domain-domain containing protein (ASC) (B), caspase-1 (C) and pannexin-1 (D) were analyzed by real-time quantitative polymerase chain reaction. The values were normalized to 18S and are shown as a fold increase to the non-lipopolysaccharide (LPS) stimulated control. Mean \pm SD are shown. $n = 3$ per group, ^a $P < 0.01$, ^b $P < 0.03$, ^c $P < 0.05$.

Activation of the inflammasome results in increased levels of total IL-18, total IL-1 β , and mature IL-1 β

LPS stimulation has been shown to increase levels of pro-IL-1 β transcripts^[26]. However, production of the biologically active IL-1 β and IL-18 requires the mature caspase-1 to cleave the precursor forms into their mature forms^[27]. First, we evaluated mRNA levels of IL-1 β and IL-18 and found that IL-1 β mRNA levels were significantly increased at all time points post-LPS stimulation

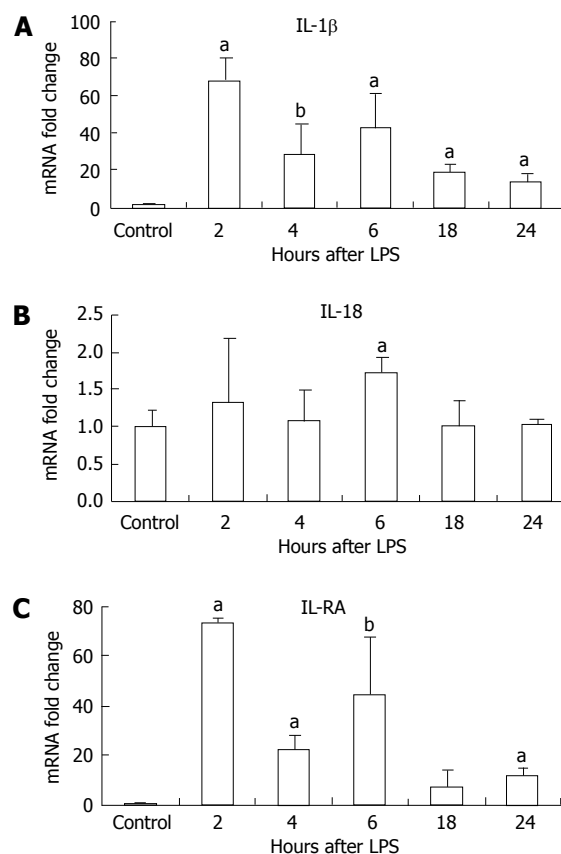


Figure 4 Lipopolysaccharide stimulation increased interleukin (IL)-1 β and IL-18 mRNA, and IL-1RA transcription in the liver. Liver mRNA levels of IL-1 β (A), IL-18 (B) and IL-1RA (C) were analyzed by real-time quantitative polymerase chain reaction and normalized to 18S. Mean \pm SD are shown. $n = 3$ per group [except at 2 h lipopolysaccharide (LPS) stimulation, where $n = 2$ due to outlier], ^a $P < 0.01$, ^b $P < 0.03$.

peaking at 2 h (Figure 4A). Induction of IL-18 mRNA by LPS was modest but reached statistical significance at the 6-h time point (Figure 4B). There was also induction of the mRNA for the IL-1 receptor antagonist after LPS stimulation in the liver (Figure 4C).

We sought to determine whether the increase in mRNA correlated with increased protein production for IL-1 β and IL-18. We found basal expression of pro-IL-1 β protein in the livers of unstimulated control mice and this was significantly upregulated at 2 h after LPS stimulation (Figure 5A). More importantly, we observed a significant increase in mature IL-1 β protein in liver tissue at all time points post-LPS stimulation compared to the controls, in which no mature IL-1 β was detected (Figure 5B). Consistent with inflammasome activation, we found increased serum IL-1 β levels in mice after stimulation with LPS for 2 and 6 h (Figure 5C). Investigation of IL-18, which is also cleaved by caspase-1, revealed a significant increase in total IL-18 in the liver at all time points following LPS injection (Figure 5D).

DISCUSSION

Although many molecules and compounds activate the

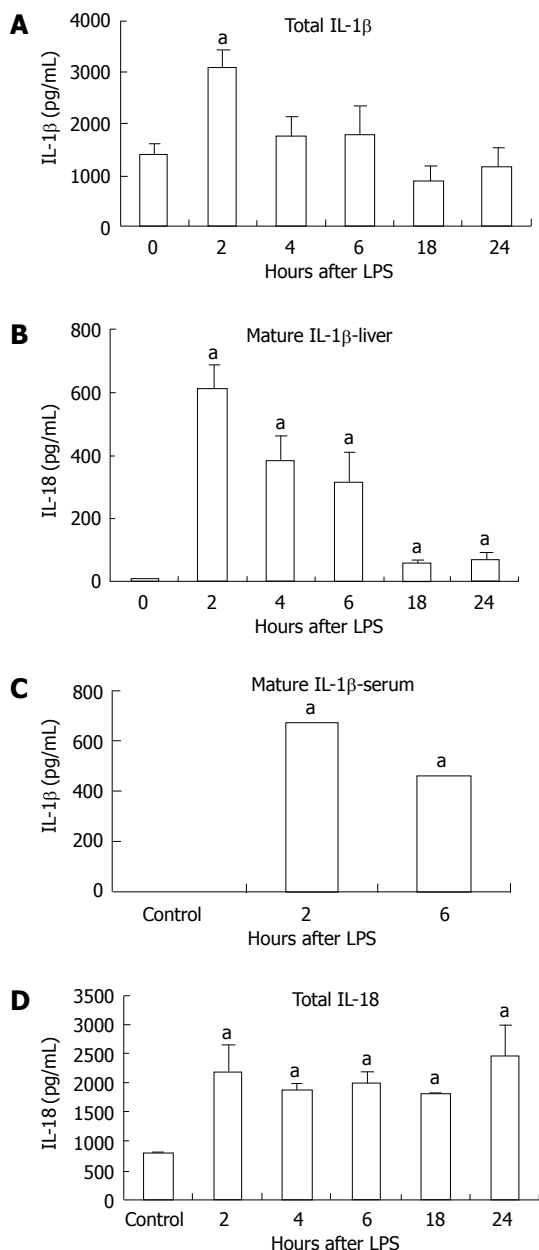


Figure 5 Lipopolysaccharide increased interleukin-1β and interleukin-18 protein. Protein level was detected in liver tissue by enzyme-linked immunosorbent assay for total-interleukin (IL)-1β (pro-IL-1β and cleaved IL-1β) (A), cleaved IL-1β in the liver tissue (B),cleaved IL-1β in the serum (C) and total IL-18 (pro-IL-18 and cleaved IL-18) (D) in liver tissue. The values shown are the fold change compared to non-Lipopolysaccharide (LPS) stimulated control. Protein levels were normalized to total protein concentrations in each tissue sample. Mean ± SD are shown. *n* = 3 per group (except at 2 h LPS stimulation, where *n* = 2 due to an outlier), ^a*P* < 0.01.

Nalp3 inflammasome both *in vitro* and *in vivo*^[8], the role of Nalp3 inflammasome in a healthy liver following LPS stimulation has not been determined. Our novel findings demonstrate upregulation of the Nalp3 inflammasome in the liver in response to LPS stimulation, both at the mRNA and protein levels. We also show for the first time that the components of the inflammasome pathway, including ASC and caspase-1, are upregulated in the liver after LPS challenge and this results in functional

activation of the inflammasome and caspase-1, indicated by IL-1β and IL-18 secretion.

LPS is recognized by TLR4, which induces an intracellular signaling cascade leading to activation of NF-κB, as well as the production of pro-IL-1β and pro-IL-18^[28]. Previous studies have suggested that signaling through the TLRs alone is insufficient to generate the production of mature IL-1β and IL-18, and that a second signal is needed that leads to ionic perturbation^[29]. These perturbations may include ATP signaling through the P2X7 channel, leading to changes in intracellular potassium concentrations and membrane perturbations. The physiological importance of ATP in IL-1β secretion is unclear and the second signal that leads to cleavage of pro-IL-1β in the liver is still under investigation^[30]. *In vitro* studies have shown that a second signal is needed in addition to LPS to obtain cleavage of pro-IL-1β^[31]. It is possible that a DAMP released by injured cells could act as the second signal. *In vivo* studies, however, have shown that LPS alone is sufficient to increase cleavage of IL-1β and IL-18^[31]. Our novel data demonstrate that, after *in vivo* LPS challenge, the inflammasome is activated in the liver and this results in increased inflammasome function and IL-1β secretion. We found that LPS stimulation not only increased *IL-1β* mRNA levels and the expression of pro-IL-1β protein, but it significantly increased the levels of the 18-kDa form of mature IL-1β in the liver. Secretion of mature IL-1β also resulted in elevated IL-1β in the serum. Interestingly, we found significant levels of pro-IL-1β protein expressed in the liver without exogenous stimulation. This suggests that the exogenous LPS probably provided the second signal for IL-1β production. The mechanisms for the presence of pro-IL-1β protein in the liver remain to be determined, however, one possibility is stimulation *via* PAMPs, such as LPS, which are being constantly supplied by the gut through the portal vein to the liver^[6].

Cleavage of pro-IL-1β is mediated by activated caspase-1; consistent with this, we found increased levels of IL-1β protein in the serum after LPS challenge. The other target of the caspase-1 complex and inflammasome activation is IL-18. Consistent with LPS-induced inflammasome and caspase-1 activation, we found an increase in IL-18 mRNA and total IL-18 levels in the liver. Due to limitations of the detection assay, we could not distinguish between the mature form of IL-18 and pro-IL-18, but the increase in total IL-18 after LPS stimulation was most likely due to an increase in both.

We found elevated basal levels of pro-IL-1β in the liver, and pro-IL-1β was further increased by LPS with the highest levels at 2 h following stimulation. It is likely that LPS-induced inflammasome activation can cleave the pre-existing pro-IL-1β to the mature form by caspase-1. In addition to activation of the Nalp3 inflammasome, our data suggests that other types of caspase-1-activating inflammasomes, Nalp1 and Nlr4, may also play a role in caspase-1 activation and subsequent IL-1β cleavage in the liver. Our data showed that both *Nalp1* and *Nlr4* mRNA

levels were upregulated in the liver following LPS stimulation. This suggests that other inflammasome-activating signals lead to full activation in the liver.

The contribution of the Nalp3 inflammasome to inflammation in the liver in response to LPS stimulation may have implications for several different liver diseases. Increased gut-derived LPS has been shown to contribute to liver inflammation in alcoholic and non-alcoholic fatty liver diseases, as well as other forms of liver damage such as hepatitis C virus infection^[32]. In healthy subjects, the liver plays a central role in elimination of gut-derived endotoxins and other pathogens to maintain immune homeostasis^[33]. Thus, it is tempting to speculate that gut-derived ligands of TLRs constantly entering the liver could contribute to the high basal expression of pro-IL-1 β found in the liver in our experiments. The high basal expression of pro-IL-1 β represents the first step of inflammasome activation, and this appears to provide a pre-activated state where even single LPS stimulation can result in inflammasome activation, as seen in the liver after *in vivo* LPS challenge in our experiments. The specific role of Nalp3 and the inflammasome remains to be evaluated in different types of liver diseases where inflammation is related to LPS and other pathogen-derived or endogenous danger signals.

ACKNOWLEDGMENTS

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COMMENTS

Background

The endotoxin lipopolysaccharide (LPS) is a component of Gram-negative bacteria, and plays an important role in both acute liver injury as well as chronic liver diseases, including fatty liver associated with either alcohol consumption or metabolic syndrome and obesity. Several pathogen-associated molecular patterns, including LPS, induce inflammasome activation, an intracellular multiprotein complex leading to release of inflammatory cytokines and cell death.

Research frontiers

Inflammasomes are required for effective clearance of several viral and bacterial pathogens. In addition, inflammasomes have been reported to play a major role in several diseases with sterile inflammation such as gout, Alzheimer's disease, atherosclerosis and type 2 diabetes. Here, the authors show the contribution of the Nalp3 inflammasome to LPS-induced inflammation in the liver.

Innovations and breakthroughs

Recently, the contribution of the Nalp3 inflammasome to *Propionibacterium acnes* plus LPS-induced acute liver injury has been described. Here, authors show the increased expression and role of the inflammasome in healthy liver following LPS stimulation.

Applications

The contribution of the Nalp3 inflammasome to inflammation in the liver in response to LPS stimulation may have implications for several liver diseases, including alcoholic and non-alcoholic fatty liver disease, as well as hepatitis C virus infection where the role of gut-derived endotoxin has been shown previously. A better understanding of the pathological mechanism may help future attempts to develop effective therapy.

Terminology

Nalp3, also called cryporin is a nucleotide oligomerization domain-like receptor

that forms one of the most characterized inflammasome complexes.

Peer review

This is a well done study that examines the role of the inflammasome in the liver following LPS exposure.

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