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Review

The Impact of the NLRP3 Pathway in the Pathogenesis of Non-Alcoholic Fatty Liver Disease and Alcohol-Related Liver Disease

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Abstract: The presence of hepatic steatosis and inflammation is increasingly associated with both metabolic and alcohol-related liver conditions. Both are on the increase globally and, apart from liver transplantation, there are no licensed therapies that target the full complement of disease features. The presence of some shared pathogenic mechanisms and histological features in NAFLD and ALD suggests that it may be possible to develop markers for prognostication or staging, or indeed new therapeutic tools to treat both conditions. One such example of an approach exists in the form of the NACHT-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome pathway. Activation of the NLRP3 inflammasome results in hepatocyte pyroptosis, persistence, and amplification of liver inflammation and activation of profibrogenic signaling cascades. Thus, targeting elements of the pathway in NAFLD and ALD may provide a tractable route to pharmacological therapy. In this review, we summarize the contribution of this inflammasome to disease and review the current options for therapy.

Keywords: NAFLD; ALD; steatosis; NLRP3; inflammasome

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1. Introduction

The presence of hepatic steatosis and inflammation is increasingly associated with both metabolic and alcohol-related liver conditions. Both non-alcoholic fatty liver disease (NAFLD) and alcohol-related liver disease (ALD) are chronic conditions driven by multifactorial mechanisms. Patients with either disease are at increased mortality risk and have an increased likelihood of developing primary liver cancer. Non-alcoholic fatty liver disease exists as a spectrum from simple fatty liver injury to non-alcoholic steatohepatitis (NASH), fibrosis, and end-stage liver cirrhosis. NAFLD is the most common liver disease, with a worldwide prevalence of 25% [1]. Within those individuals with NAFLD, the prevalence of NASH ranges from 7% to 30%, with an estimated 16.5 million individuals affected in the United States alone in 2015 [2]. Like NAFLD, alcoholic liver disease also embraces a spectrum of disease, but here, consumption of alcoholic beverages drives the hepatocellular damage and inflammation. Mortality from ALD has risen by 450% in the last 30 years [3], and alcohol-related liver disease is particularly prevalent in European countries [4]. In both situations, although the causative triggers differ, one of the earliest pathophysiological changes observed in the liver is steatosis. This plus signal, arising from generation of damage-associated molecular patterns (DAMPs) or gut derived pathogen associated molecular patterns (PAMPs), contributes to the development of hepatocellular damage and liver inflammation. Thus, the likelihood of some shared pathogenic mechanisms in NAFLD and ALD means that it may be possible to develop markers for prognostication or staging, or indeed new therapeutic tools to treat both conditions. One such example of an approach exists in the form of the NACHT-, LRR-, and

pyrin domain-containing protein 3 (NLRP3) inflammasome pathway. Inflammasomes are intracellular signaling pathways linked to receptors that respond to PAMP and DAMP signals. This leads to eventual immune response activation and death of the affected cell. Although these pathways are common to immune cell types, elements of inflammasomes are also found in some epithelial cell populations, where they maintain barrier homeostasis or respond to local pathogen exposure [5]. Thus, in this review article, we focus on the NLRP3 inflammasome and consider its relevance to both NAFLD and ALD to demonstrate the potential therapeutic implications of targeting the response. We begin by providing an outline of the pathogenesis of alcohol and non-alcohol-related metabolic liver injury.

2. The Pathogenesis of NAFLD

NAFLD is the liver component of metabolic syndrome, which is characterized by obesity, insulin resistance, and cardiovascular risk factors, including elevated blood pressure and dyslipidemia (low HDL/elevated serum TG). The hallmark hepatic feature is steatosis, encompassing >5% of hepatocytes in the absence of significant alcohol consumption [6]. This steatosis is a consequence of an imbalance of hepatic lipid turnover. There is increased hepatic fatty acid synthesis and *de novo* lipogenesis, which is, in part, due to increased fatty acid influx to the liver from lipolysis in adipose tissue. Insulin resistance accompanies the hepatic steatosis, and this, combined with exposure to a high carbohydrate diet [7,8], particularly one rich in fructose [9], further amplifies hepatic *de novo* lipogenesis. Steatosis by itself may be relatively benign [6] but sensitizes hepatic cells to subsequent damaging insult [10] in the form of reactive oxygen species or other proinflammatory signal. This may arise because of hepatocyte lipotoxicity and lipid peroxidation, which promote mitochondrial dysfunction and reactive oxygen species (ROS) generation [11]. Consequent hepatocyte damage initiates the release of DAMPs, which trigger a reparative inflammatory response. NAFLD is also associated with changes in gut microbiota, including elevations in *Proteobacterial* species [12], which may contribute to systemic and hepatic inflammation. This may be either a direct response to bacterial LPS or as a consequence of changes in metabolite production and bile acid composition, which can alter signaling through FXR [13].

Inflammation is a normal physiological defense mechanism to any type of injury or infection, which induces secretion of numerous inflammatory cytokines, chemokines, and other factors. However, dysregulation of inflammation leads to bystander parenchymal cell damage and sustained activation of the profibrogenic response. All of this explains the characteristic histological features of NASH, in terms of hepatic steatosis and lobular inflammation, and ballooning of hepatocytes. These correlate with elevated serum aminotransferases as a measure of hepatic necroinflammatory activity [14]. The main cell types that have pro-inflammatory roles in NASH are the liver resident Kupffer cells (KCs), natural killer (NK) cells, NK T cells, lymphoid cells, sinusoidal endothelial cells, and hepatic stellate cells (HSCs). Under normal conditions in the liver, there is a crosstalk between immune and parenchymal cells to control the inflammatory tone and metabolic homeostasis. However, in NAFLD, proinflammatory signals as described above, support the maintenance and progression of inflammation. The liver-resident macrophages, including Kupffer cells (KCs), are highly phagocytic [15] and reside in the hepatic sinusoid, where they scavenge microbial products from the intestine along with other waste molecules and components from cell death and renewal. Upon phagocytosis or activation of PAMPs, these macrophages can produce KCs and release chemokines and proinflammatory cytokines, such as TNFα, IL-1, and IL-6 [16]. These not only amplify hepatocyte damage but also activate endothelial cells and promote further proinflammatory cell infiltration into the parenchyma. Recruited neutrophils are commonly observed in NAFLD liver biopsies and numbers correlated with extent of fibrosis and ductular reaction [17]. These cells can aggravate NASH by direct hepatocyte damage, proinflammatory cytokine, chemokine production, and NET release [18]. They also produce IL-17, which can activate stellate cells and prime fibrosis [19]. Local cytokine production and oxidative stress also promote

T cell recruitment and activation [20] and deletion of more regulatory types of lymphocytes [21]. Hence, in NASH, there is excessive Th1- and Th17-derived IFNγ and IL-17 production, respectively, but a deficiency in Th2-derived IL-4, IL-5, and IL-13. In turn, cytotoxic CD8+ T cells are supported by type I IFN responses and lead to the production of further IFNγ and TNFα [22]. The consequential sustained proinflammatory environment leads to activation of hepatic stellate cells (HSC) and initiation of the fibrotic response.

It is clear that signals derived from the intestinal microbiome are key to pathogenesis in NAFLD [23], and indeed evidence from rodent models suggests that transfer of microbes collected from obese mice into lean recipients is associated with metabolic alterations [24]. Similar transient effects linked to improvement in insulin sensitivity and repopulation of the gut, with more beneficial species, has been observed in obese humans treated with microbiota from lean individuals [25]. Clearly, the individual species of bacteria present are key, since administration of specific bacterial species (including *Ruminococcaceae)* can have beneficial effects on liver damage, steatosis, and fibrogenesis in rodent models. Meanwhile, elevated populations of *Fermicutes* and reduced intestinal bacterial diversity is often associated with obesity [23].

3. The Pathogenesis of Alcohol Related Liver Disease

Alcoholic and non-alcoholic steatosis can be hard to distinguish histologically and clinically [26] as many common features are observed. Here, steatosis is characterized by the accumulation of triglycerides, phospholipids, and cholesterol esters in the hepatocytes. Depending on the extent and duration of alcohol consumption, initial steatosis may be accompanied by hepatocyte damage. In acute and significant exposure, disease can present as the more severe 'alcoholic hepatitis', where hepatocyte loss and degeneration is accompanied by neutrophilic inflammation and patients are at significant mortality risk. The toxic and severe proinflammatory effects of binge alcohol exposure are also seen in nearly half of patients with a more chronic disease. Here, the steatohepatitis is accompanied by chronic inflammation and fibrogenesis, and, as with NAFLD, patients are at risk of progression to cirrhosis and liver cancer. The initiating stimuli that trigger these responses are multiple. Within hepatocytes, alcohol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH) in the cytosol. In turn, the acetaldehyde is metabolized by mitochondrial aldehyde dehydrogenase (ALDH). Both alcohol and aldehyde are highly reactive and can form various protein and DNA adducts, which are toxic to hepatocytes [27]. Acetaldehyde is carcinogenic and promotes generation of proinflammatory neoantigens [28]. The damaged hepatocytes in turn release DAMPS, which activate cellular pattern recognition receptors, recruit innate and adaptive immune cells, and produce proinflammatory cytokines, thereby driving hepatic inflammation. Here, tissue-resident macrophages (Kupffer cells) are particularly important for orchestrating the damaging immune response [29,30]. Ethanol metabolism also primes lipogenesis within hepatocytes due to changes in cellular NADH and redox potential [31], altered expression, and activation of genes and transcription factors involved in lipid metabolism and inhibition of fatty acid oxidation [32,33]. Changes in hepatocellular expression of fatty acid transporters upon exposure to ethanol also add to the lipid accumulation [31]. In situations of repeated or significant levels of ethanol exposure, cytochrome P450 2E1 (CYP2E1) [34], which has higher capacity for ethanol binding than ADH, generates a significant amount of ROS, which amplifies cellular damage, oxidant stress, and lipid peroxidation [35].

Exposure to ethanol increases hepatic production of proinflammatory cytokines and chemokines, which increase hepatic inflammation and the progression from alcoholic steatosis to alcoholic steatohepatitis (ASH). This is accompanied by hepatocyte apoptosis, which can be driven by ER stress, local interferon-induced signaling, and caspase activation [36]. In common with NAFLD, continued drinking also activates the profibrogenic response, since continued exposure of Kupffer cells to acetaldehyde, endotoxin, ROS, and local DAMPs leads to production of profibrogenic cytokines that activate stellate cells [28]. In alcohol-related liver disease, the liver is also exposed to PAMPs arising from the gut

microbiome and DAMPs produced by cells damaged by ethanol or lipotoxicity [37]. Alcohol exposure also causes a dysbiosis (decreased diversity of species) within the intestine and compromises the epithelial barrier function. In particular, an increased preponderance of *Proteobacteria* and *Fermicutes* and reduced abundance of *Bacteroides* species drive hyperpermeability [38]. Reduced *Bacteroides* representation is also seen in the colon of patients with ALD [39]. Translocation of bacterial PAMPs, such as LPS, to the liver cause LPS binding to TLR4. This triggers NF-κb activation, which promotes not only further inflammation but also the release of cytokines, such as IL-1β and TNFα. Local CCL2 and IL-8 production, in particular, recruits macrophages and neutrophils into the damaged liver. This proinflammatory response is caspase-dependent and may also relate to signaling arising as a consequence of increased Uric acid and ATP levels in the injured liver [40].

4. The NLRP3 Inflammasome

The mechanisms described above show a commonality in that, in both NAFLD and ALD, the liver cells are exposed to DAMPs and PAMPs because of cell damage, lipotoxicity, and alterations in the gut microbiome. This leads to a persistent and damaging chronic inflammatory response and fibrogenic activation. The innate immune system is the first line of host defense that initiates the processes of clearance or repair after exposure to harmful stimuli, dead/dying cells, or infections [41]. Inflammasomes are important multi-protein complexes that play role in these processes, and one of the most significant is the NLRP3 (NACHT-, LRR-, and pyrin domain-containing protein 3) inflammasome [42], which has been extensively studied in macrophages. The purpose of NLRP3 activation is to generate signals that trigger controlled cell death and clearance. The key components of the pathway are summarized in Figure 1, and it is composed of elements involved in sensing damage (NLRP3), adaptor molecules (ASC), and a signaling caspase complex. The combination of these elements constitutes the NLRP3 inflammasome. Assembly of the complex results from a two-step process referred to as priming and activation. Under normal conditions, the NLRP3 is repressed, owing to an internal interaction between one domain of NLRP3 (NACHT) and another (LRR), which are ubiquitinated to prevent activation. These are deubiquitinated in the presence of pathogen-associated molecular patterns (PAMPs) from microorganisms or damage-associated molecular patterns (DAMPs), which permit NLRP3 to oligomerize and recruit apoptosis-associated speck-like protein containing a CARD (ASC; also known as PYCARD) and pro-caspase 1. This triggers the activation of caspase 1 and the maturation and secretion of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and IL-18. Upon activation in a target cell, the NLRP3 inflammasome also promotes inflammatory cell death in the form of pyroptosis, which is regulated by the N-terminal domain of gasdermin D (GSDMD) by forming pores in the plasma membrane [43,44]. This leads to cell swelling and eventual lysis, with the release of cytoplasmic contents.

Activation of the NLRP3 inflammasome can be triggered by multiple ligands, which may be different in terms of what 'primes' and what 'activates' the response [45]. Activation can also be considered as 'canonical' when a downstream caspase-1 activation is triggered (Figure 1) or 'non-canonical' if other caspases (typically 4/5 in humans or caspase 11 in rodents) are targeted [46,47]. In the canonical context, TLR or cytokine receptors are triggered to prime the response to drive NF-κB activation and NLRP3/pro IL-1β expression. This is followed by a second stage, where the components of the NLRP3 inflammasome assemble, caspase 1, is activated and IL-1β and IL-18 are processed and released. As well as TLR ligands and cytokines, changes in intracellular ion concentrations arising from stimulation by mediators, including ROS, degraded ECM components, cholesterol, and misfolded proteins, are also potent activators. However, as noted above, activation can also be induced relatively rapidly by post-translational modification of components of the NLRP3 complex. In 'non-canonical' activation, TLR signals are again triggered but often by gram-negative bacteria [48]. These activate alternate pathways, such as TRIF or MyD88. This again culminates in NF-κb activation and indirect transcription of IL-1β and Il-18, but there is also transcription of IFNα/β and JAK/STAT activation. However,

membrane pores are again initiated through GSDMD cleavage. Hence, activation of the NLRP3 inflammasome results in hepatocyte pyroptosis, maintenance of liver inflammation, and activation of profibrogenic signaling cascades [49], and thus, targeting elements of the pathway in NAFLD and ALD may provide a tractable route to pharmacological therapy.

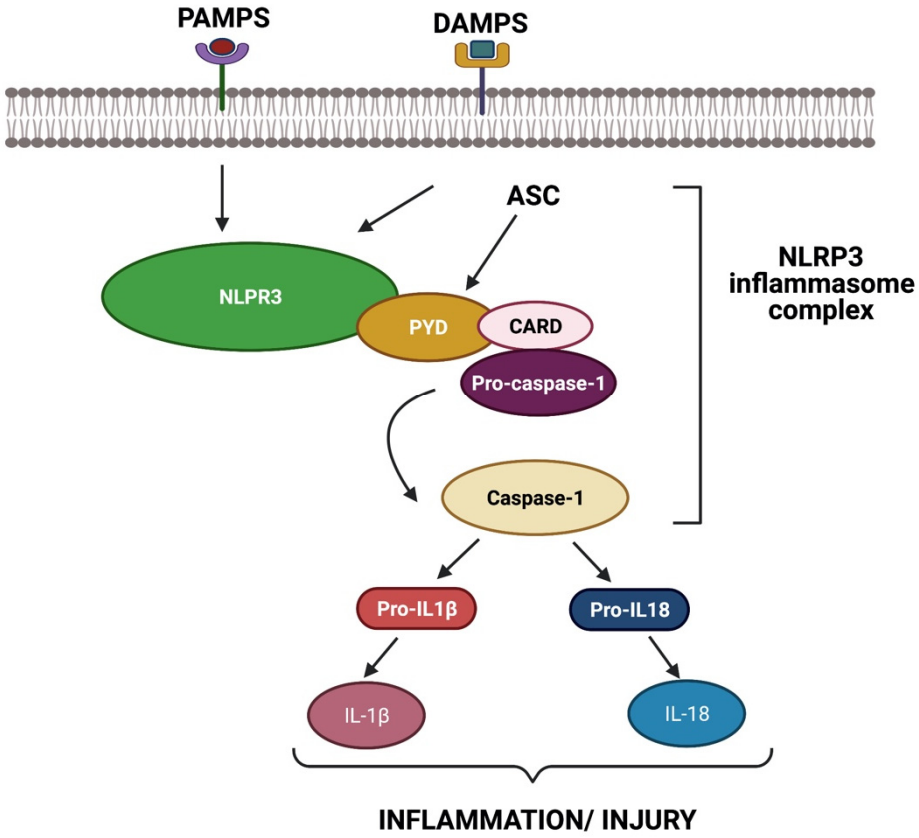

Figure 1. Schematic representation of the canonical NLRP3 inflammasome. The presence of pathogen-associated molecular patterns (PAMPs) from microorganisms or damage-associated molecular patterns (DAMPs) activates cell surface receptors, such as TLR4, which cause NLRP3 to oligomerize and recruit apoptosis-associated speck-like protein containing a CARD (ASC; also known as PYCARD) and pro-caspase 1. This triggers the activation of caspase 1 and the maturation and secretion of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and IL-18. Image created in Biorender.

5. The Contribution of NLRP3 Inflammasome to the Pathogenesis of NAFLD and ALD

Inflammasomes are multiprotein complexes that can sense and recognize various exogenous and endogenous danger signals, eventually activating IL-1β and IL-18. The role of inflammasomes and IL-1 signaling has been demonstrated in acute and chronic liver injury, such as acetaminophen-induced liver damage [50], NASH [51], liver fibrosis [52], and immune-mediated liver injury [53], but has not been studied thoroughly in alcoholic liver disease. The impact of NLRP3 activation in the context of ethanol or metabolic liver disease is summarized in Figure 2. Elements of the NLRP3 inflammasome are abundantly expressed in cells of the myeloid lineages, and so, within the hepatic parenchyma, KCs express the highest levels of inflammasome components among liver cell types. Expression of NLRP3, Caspase-1, IL-1β, and IL-18 in CD14+ peripheral blood monocytes also increases upon initial stimulus by LPS. However, cells become refractory upon repeated stimulation [54], which may contribute to immune depression in sepsis associated with liver disease. In more chronic conditions, such as hepatitis B infection, myeloid cells in the periphery show a reduced expression of NLRP3 components, which becomes more pronounced as the disease develops [54] and is associated with a concurrent increase in expression in hepatic myeloid populations. Similarly, activation or damage to hepatocytes

via fatty acid signaling in steatosis leads to the production of IL-1β, which then activates the NLRP3 inflammasome in neighboring cells. Such activation also sensitizes the cells to endotoxin [55]. Hence, mice fed a high fat diet or MCD diet demonstrate elevated hepatic inflammasome mRNA and serum IL-1β levels [56], which arise from myeloid cell infiltration into the damaged livers. These cells then contribute to fibrogenesis in a caspase-1 dependent manner [57], and so caspase-1 inhibitor treatment reduces development of fibrosis and NASH in rodent models [58]. This NLRP3 activation in KCs promotes IL-1β secretion, driving the progression of NASH [59], and hence, both pro-IL-1β and pro-IL-18 are increased in the liver of patients with NASH [49]. Elevated liver expression of inflammasome components, such as IL-1β, IL-18, and caspase-1, in patients is also correlated with liver injury in patients with ALD [60].

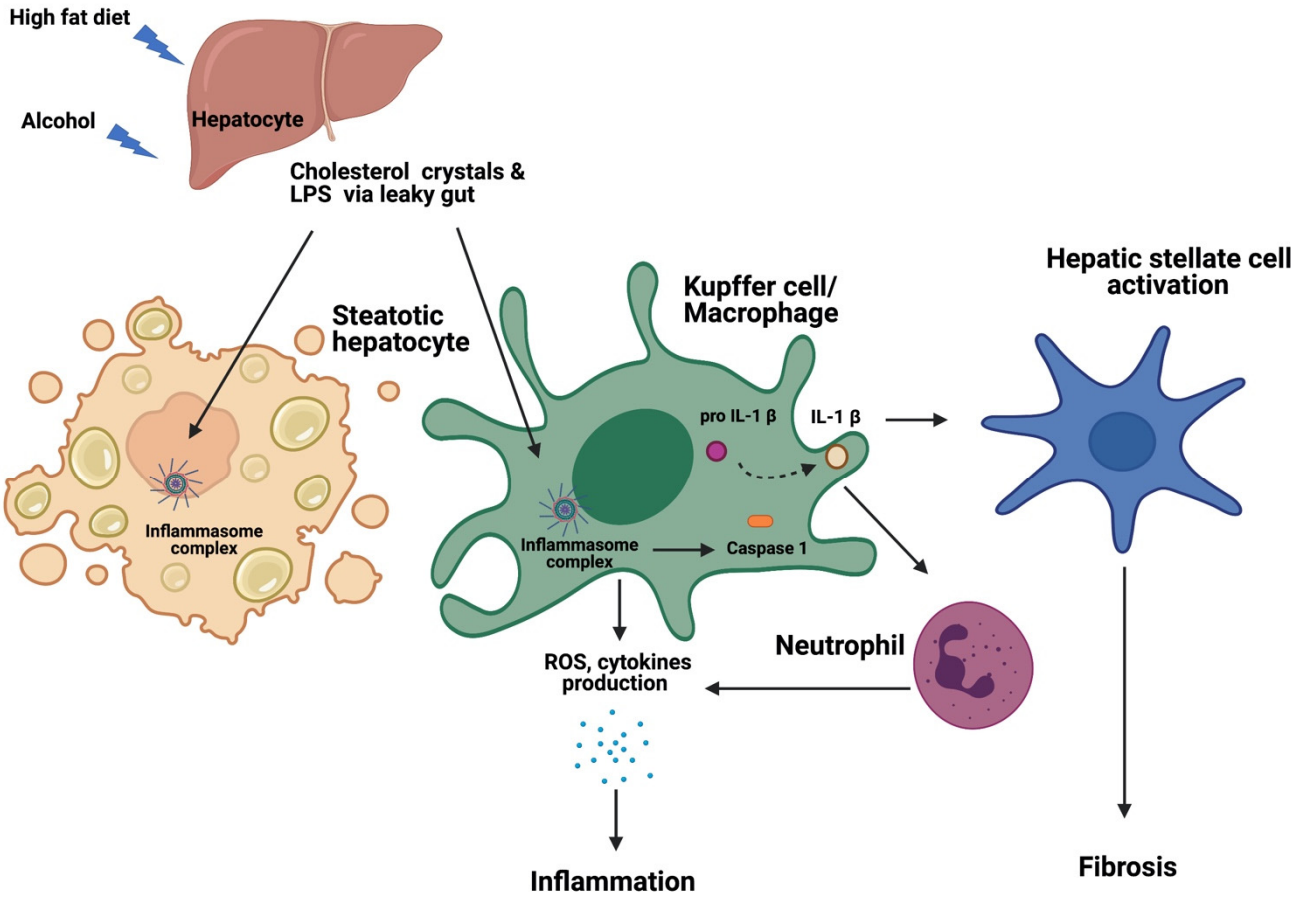

Figure 2. The impact of NLRP3 activation in the context of ethanol or metabolic liver disease. Liver injury causes production of multiple agonists for the NLRP3 inflammasome. For example, both LPS and cholesterol crystals increase expression of NLRP3 inflammasome in hepatocytes, leading to ER stress and hepatocyte death. Activation of the NLRP3 inflammasome in hepatic macrophages by DAMP or PAMP signals drives production of mature Il-1B, in addition to other proinflammatory molecules and cytokines. These promote further immune cell recruitment into the hepatic parenchyma and also cause activation of hepatic stellate cells. Thus, persistent activation of the pathway amplifies liver injury and promotes fibrosis. Image created in Biorender.

Disease severity is an important consideration however, as it appears, steatosis alone may be insufficient to drive full inflammasome activation in some contexts. NLRP3 IL-1β and IL-18 expression is elevated in the liver of human patients with NASH, compared to matched controls with early NAFLD [49]. Gasdermin D levels are also elevated [61] and correlate with the NAS score and extent of fibrosis. Intriguingly, intrahepatic cholesterol crystals are formed during administration of MCD and in human NAFLD [62,63], and these are potent activators of NLRP3 in macrophages [62]. Neutrophillic inflammation

also contributes to the extent and outcome of inflammasome activation. MicroRNA-223 is selectively expressed in neutrophils, acts as a negative regulator of inflammasome activity, and is important for promoting regeneration after liver injury [64]. Here, miR-223 is secreted within extracellular vesicles from activated neutrophils. Once taken up by macrophages, this post-transcriptionally regulates NLRP3 expression in macrophages *via* interactions with the 3′-UTR of NLRPs. This then primes alternate activation and generation of a restorative macrophage phenotype [64]. MiR-223 is dramatically downregulated in the livers of patients who have NASH and mice exposed to CCL_4. Similarly, mice that lack MiR-223 exhibit worse liver injury. Thus, it appears that neutrophils silence NLRP3 activation in hepatic macrophages via MiR-223 and this promotes alternative macrophage activation and generation of 'restorative' phenotype [64] in some situations.

In support of the key role of NLRP3 activation in pathogenesis, NLRP3 knockout mice are protected from the effects of CDAA-induced injury [49]. Here, neutrophil and macrophage recruitment, hepatic chemokine expression, and expression of Caspase-1 and IL-1β were all reduced in deficient animals compared to wild type animals. There was also a reduction in fibrogenesis (αSMA, Col1a1, MMP2, and TIMP1 expression, plus Sirius red staining) in the knockout animals, which also occurs in caspase-1-deficient animals [57,65]. Gasdermin D-deficient mice are protected from the effects of MCD [61] and CDAA diets [49]. A similar pattern emerges in models of alcohol injury. Expression of NLRP3, caspase-1, and IL-1β dramatically increase in mice exposed to alcohol, and deficient mice are protected from its effects [66,67]. Using three different mouse models, the caspase-1, ASC, and IL-1R1 knockout mice, Perasek et al., showed amelioration of steatosis and fibrosis and a decrease in pro-inflammatory cytokines in ethanol fed mice. It has been suggested that IL-1β leads to the recruitment of invariant natural killer T (iNKT) cells in alcoholic hepatitis, which promotes the influx of neutrophils that exacerbates the hepatitis [68]. Exposure to alcohol has been shown to increase hepatic expression of components of the NLRP3 inflammasome and KC NLRP3 activation [67]. Here again, mice deficient in caspase-1 or ASC are protected against steatosis, inflammation, and fibrogenesis on an ethanol feeding protocol [67]. Importantly, mice recover faster if they are exposed to an ethanol diet to induce injury and then treated with an agent to block activation of the IL-1 receptor [67].

However, the picture is complex as some groups suggest that mice that are genetically modified to lack components of the NLRP3 inflammasome suffer a dysbiosis phenotype and demonstrate more severe liver injury on MCD [65] and a high fat high cholesterol diet [24]. In addition, the absence of NLRP3 signaling in deficient animals has been associated with a change in intestinal bacterial species compared to wild type littermates [69]. This reiterates the importance of PAMP activation of NLRP3, highlights the importance of the gut liver axis [70], and also provides a means to link the microbiome to the outcome. The bacterial species within the intestine play an important role in both gut and liver bile acid metabolism and diversity because of their metabolism of primary bile acids [13]. FXR is a transcription factor that is activated upon exposure of cells to a variety of signals, including endogenous and synthetic bile acids. Nuclear receptors, such as FXR and TGR5 (Takeda G protein coupled receptor 5, also known as G-protein coupled bile acid receptor-1), have been implicated in the pathogenesis of hepatocyte damage during ethanol-induced injury. Treatment of mice exposed to ethanol with FXR/TGR5 agonists reduces steatosis and inflammation *via* effects on fatty acid synthase expression and caspase-1 activation [71]. The diversity of microbial species and stool bile acid metabolites is related to the fibrosis stage in NAFLD [72]. NLRP3-deficient animals show altered gut microbiome, reduced *Bacteroides* species, and enhanced *Ruminococcus* species [73]. NAFLD severity in humans is also associated with altered abundance of *Veillonellaceae* and *Ruminococcaceae*. Here, a reduction in *Ruminococcaceaea* species is associated with more severe fibrosis in non-obese populations with NAFLD [72] and administration of *R faecalis* to mice fed MCD resulted in a reduction in disease severity.

The microbiome composition can impact on the metabolic drivers of disease progression. Infusion of microbiota from lean individuals to recipients with metabolic syndrome to increase microbial diversity improved insulin sensitivity and glucose metabolism [25]. Activation of FXR has also been linked to improved insulin sensitivity, glucose homeostasis, and cellular integrity. Expression of FXR inversely correlates with components of the NLRP3 inflammasome [74] in mice and humans, and transcript expression decreases with increasing disease severity. This suggests that FXR activity may suppress activation of NLRP3 and is supported by the observation that FXR-KO mice have increased NLRP3 activation and that activation of FXR using pharmacological agonists or bile acids protects hepatocytes from ER stress and NLRP3 activation [74].

In ALD exposure of liver cells to gut derived-LPS activates TLR4 and primes IL-1β production. This not only sensitizes hepatocytes to apoptosis but also promotes both inflammation and fibrosis [28]. Alcohol exposure is also associated with increased expression of NLRP3 components in the gut (NLRP3/Casp-1, Il-1, ASC) [75], which can be reduced by administration of antibiotics. Understandably, much of the focus in NLRP3 biology has been placed on the role of macrophages in disease pathogenesis. However, it is important to note that, in particular, 'non-canonical' activation of the NLRP3 inflammasome is common to both myeloid and non-myeloid cells [5] and that Gasdermin D components of other inflammasome complexes are present in epithelial cells. [76]. Here, the function of inflammasome activation is intended to help maintain epithelial integrity and barrier function in the context of exposure to pathogenic organisms. Again, this will be of significance in both the intestine and liver [77]. Thus, dietary steatosis is accompanied by inflammasome activation in both myeloid cells and hepatocytes [56] (Figure 2) and IL-1β production by non-myeloid cells contributes to the hepatocellular damage and immune activation [56]. Here, activation of the NACHT, LRR, and NLRP3 inflammasomes in hepatocytes has been highlighted as playing a central role in liver disease [74]. Studies using mice expressing hyperactive mutant forms of NLRP3 confirm that hepatocyte pyroptosis as a consequence of liver damage is key to subsequent inflammation and fibrogenesis [49].

Patients who regularly binge on ethanol are also at risk of developing acute pancreatitis. Release of ATP and genomic and mitochondrial DNA by pancreatic acinar cell damage in pancreatitis activates TLR9 on pancreatic macrophages, initiating NLRP3 activation *via* NFκB [78] and P2X7 [79] signaling and IL-1β release. This again supports the interplay between NLRP3 activation on both parenchymal and immune cells in disease development.

6. Can Targeting of the NLRP3 Inflammasome Be of Benefit in Human Disease?

The evidence reviewed above links NLRP3 inflammasome activation to the pathogenesis of both alcohol and metabolic-related liver disease. This supports the concept that targeted intervention may be therapeutically viable and reduce the need for transplantation. This is of clinical relevance, since there are currently no licensed therapies that manage all the features of NASH [80] and treatment of alcohol-related disease (and particularly alcoholic hepatitis) [81] remains challenging. To that end, there is much research activity in preclinical models and some human studies targeting elements of the NLRP3 inflammasome or modifying its activation. There are multiple means by which the NLRP3 inflammasome could be targeted therapeutically. These include direct targeting of the NLRP3 complex with pharmacological agents or approaches, which inhibit downstream products of NLRP3 activation, including IL-1β and IL-18. As noted above, it is also possible that modification of the patient microbiome, either by transplanting bacteria or administration of probiotics, could restore gut homeostasis and reduce hepatic NLRP3 activation. For example, LPS blockers and probiotics to modify the intestinal microbiome in ALD are in early stages of investigation as potential therapies [82]. Similarly, interference with bile acid signal transduction through the use of obeticholic acid as an FXR activator is being investigated in both alcoholic hepatitis [83] and NAFLD [84].

To manage the metabolic drivers of NAFLD and ALD, one could focus on systemic nutrient homeostasis or specifically hepatocyte lipid handling. Accumulation of lipid

within hepatocytes after exposure to ethanol is linked to AMPK-dependent inhibition of acetyl-CoA carboxylase (ACC), activation/increased expression of sterol regulatory-element-binding protein 1 (SREBP1), and altered fatty acid synthesis and oxidation. Here, plant derived dihydroflavones, such as dihydroquercetin, have been suggested to ameliorate ethanol-induced lipogenesis and P2X7-dependent NLRP3 activation [85]. Drugs currently used to treat diabetic complications also have potential in this context. For example, administration of the GLP-1 analogue Liraglutide improves insulin resistance and steatosis in a mouse high fat diet-induced model of NAFLD. Here, liver damage was reduced by treatment and there was some degree of normalization of systemic lipid parameters. This was accompanied by a reduction in serum TNFα and IL-1β and reduced activation of components of the NLRP3 signaling cascade (NLRP3, ASC, and Caspase activation), as determined by western blotting [86]. Importantly, whilst these responses were detected in the liver and periphery, additional *in vitro* experiments using isolated Kupffer cells exposed to lipid confirmed that liraglutide reduced mitochondrial dysfunction and NLRP3 activation in these cells. Thus, it is likely that a combination of indirect effects on causative mechanisms (e.g., improved insulin responsiveness, lipid handling, and weight loss) and direct effects on target cells lead to improvement in disease parameters after liraglutide treatment. This is supported by the publication of a recent phase 2 trial of the GLP-1 receptor agonist semaglutide, which caused a significant resolution of NASH compared to placebo [87]. It may be possible to interfere with the association between NLRP3 and other members of the inflammasome complex. For example, the anti-allergy drug 3′4′dimethoxycinnamoly-anthranilic acid (Tranilast) has been suggested to bind to the NACHT domain of NLRP3, preventing subsequent oligomerization with ASC [42,88]. Treatment of mice fed HFD with Tranilsast reduced weight gain, systemic glucose, IL-1β levels, and hepatic TNFα levels [88]. Similar effects were observed in diabetic mice, suggesting that Tranilast could be effective at managing both T2DM and NAFLD. OLT177 is another orally bioavailable NLRP3 inhibitor, which is safe and has been shown to correct metabolic parameters, including fatty acid metabolism and mitochondrial function, and reduce IL-1β production in a murine inflammatory disease model [89]. This molecule has been through Phase 1 and 2 trials for degenerative arthritis [90] and thus has significant potential for use in human metabolic disease.

Alternatively, there are several other NLRP3 inhibitors under investigation that have yet to make it to human trials. One molecule, MCC950, was found to be a potent and selective inhibitor in several disease contexts, including inflammatory bowel disease and NASH [91]. It was suggested to block both canonical and non-canonical NLRP3 activation and IL-1B production by reducing ASC oligomerization [90]. MCC950 functions by binding to the Walker B motif in the NACHT domain of NLRP3 and inhibits NLRP3-mediated ATP hydrolysis [92,93]. Since its discovery, it has been used as a tool compound in the field [32,94] and has been shown to reduce Kupffer cell activation, IL-1β release, and features of NAFLD in mice fed a MCD diet [62]. Use of MCC950 to block NLRP3 inflammasome reduces inflammatory recruitment and fibrosis [62] in NASH. Another similarly selective molecule, CY-09, was tested in experimental NAFLD, in which inhibiting NLRP3 reduced hepatic steatosis, alleviated insulin resistance, and improved glucose metabolism [95]. It has been suggested to act by inhibiting caspase-1-dependent production of IL-1β *via* its interaction with the same Walker B motif in the NACHT domain of NLRP3 as MCC950 [90].

A few molecules, often adapted from the structure of tool compounds, such as MCC950, are starting to be investigated in human patients [96]. Novartis has an NLRP3 antagonist in the phase 1 trial for NASH and other chronic inflammatory conditions and NodThera has another, which is currently undergoing preclinical safety evaluation [96].

However, in terms of early human studies, the most advanced candidate molecules often focus on the downstream proinflammatory consequences of NLRP3 activation. It was observed that antibody-based inhibition of IL-1β in mice fed a Lieber de Cali ethanol diet reduces inflammation and liver injury and increases recovery [37]. Similarly, the IL-1R

antagonist anakinra has been tested in in alcoholic steatohepatitis and reduced effects of ethanol exposure in rodent models [67] and such molecules are now being examined in early trials of human inflammatory disease [97] and alcoholic hepatitis [83].

In conclusion, there is much evidence to support the contribution of the NLRP3 inflammasome to the pathogenesis of NASH and ALD. This has informed much preclinical research activity and several drugs are under investigation that target elements of the inflammasome, its activation, or its downstream products. However to date, none of the lead compounds have regulatory approval, and the safety and efficacy of the more developmental compounds remains to be established. It is important to consider potential detrimental effects of NLRP3 targeting molecules. Inconsistencies in the research data thus far may be linked to maintenance of effective gut barrier function and healthy microbiomes [66]. There are also other inflammasome pathways that may impact on efficacy. Nevertheless, the NLRP3 pathway remains of significant interest, and derivatives of current experimental tool compounds with improved tolerability and pharmacokinetics may show great promise in the future.

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