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Review

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Review

Gut–liver axis and sterile signals in the development of alcoholic liver disease

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Abstract

Background: Innate immunity plays a critical role in the development of alcohol-induced liver inflammation. Understanding the inter-relationship of signals from within and outside of the liver that trigger liver inflammation is pivotal for development of novel therapeutic targets of alcoholic liver disease (ALD).
Aim: The aim of this paper is to review recent advances in the field of alcohol-induced liver inflammation.
Methods: A detailed literature review was performed using the PubMed database published between January 1980 and December 2016.
Results: We provide an update on the role of intestinal microbiome, metabolome and the gut–liver axis in ALD, discuss the growing body of evidence on the diversity of liver macrophages and their differential contribution to alcohol-induced liver inflammation, and highlight the crucial role of inflammasomes in integration of inflammatory signals in ALD. Studies to date have identified a multitude of new therapeutic targets, some of which are currently being tested in patients with severe alcoholic hepatitis. These treatments aim to strengthen the intestinal barrier, ameliorate liver inflammation and augment hepatocyte regeneration.
Conclusion: Given the complexity of inflammation in ALD, multiple pathobiological mechanisms may need to be targeted at the same time as it seems unlikely that there is a single dominant pathogenic pathway in ALD that would be easily targeted using a single target drug approach.
Short summary: Here, we focus on recent advances in immunopathogenesis of alcoholic liver disease (ALD), including gut–liver axis, hepatic macrophage activation, sterile inflammation and synergy between bacterial and sterile signals. We propose a multiple parallel hit model of inflammation in ALD and discuss its implications for clinical trials in alcoholic hepatitis.

INTRODUCTION

Approximately two-thirds of the US adult population drink alcohol on a regular basis, and in up to 20% of individuals, alcohol intake is considered excessive (Mandayam *et al.*, 2004). Excessive (heavy) drinking is defined as >14 drinks per week for men and 7 drinks per week for women (Mandayam *et al.*, 2004). Up to 90% of individuals drinking alcohol in excess will develop liver steatosis, 50% will develop inflammation and fibrosis (steatohepatitis), and 25% will develop liver cirrhosis (Younossi and Henry, 2016), the final stage of alcoholic liver disease (ALD). Overall, ALD affects 5–7 million Americans, and is responsible for healthcare-associated costs of around $185 billion per year (Saberi *et al.*, 2016). Because of the steady disease burden and lack of effective therapies, there is an urgent need for new drug development for the management of ALD and more importantly alcoholic

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Table 1. Review criteria

A detailed literature review was performed using the PubMed database in December 2016 for papers published between January 1980 and December 2016, with the following search terms: 'inflammation', 'innate immunity', 'microbiome', 'inflammasome', 'alcoholic liver disease', 'steatosis' or 'hepatitis'. Relevant English-language papers were evaluated by both authors of this manuscript.

hepatitis (AH). AH is a rare but often deadly complication of ALD, with short-term mortality reaching up to 50% (Crabb *et al.*, 2016).

In this review, we will focus on liver inflammation in ALD, which is a critical step in the progression from steatosis to steatohepatitis, fibrosis and cirrhosis, and a defining feature of AH. The review criteria are detailed in Table 1. First, we will discuss mechanisms of liver inflammation triggered by bacterial components translocated from the gut to the liver. Second, we will review the emerging knowledge about distinct roles of resident liver macrophages (Kupffer cells, KC) versus infiltrating bone marrow (BM)-derived monocytes/macrophages in liver inflammation. Finally, we will discuss sterile inflammation in the liver, a concept based on the evidence that hepatocytes are damaged by alcohol release sterile pro-inflammatory signals. It is likely that inflammation in ALD results from the synergy of responses to microbial and sterile signals along with the shift from immune-tolerant KC to pro-inflammatory BM-derived monocytes/macrophages. This multiple parallel hit hypothesis will be discussed in the concluding section of this paper, along with its therapeutic implications.

GUT–LIVER AXIS IN ALCOHOL-INDUCED LIVER INFLAMMATION

Due to its unique anatomy and blood supply the liver receives blood from the intestine, exposing cells in the liver not only to nutrients but also to gut-derived microbial products. These products include bacterial components and bacterial metabolites (Wang *et al.*, 2012; Kirpich *et al.*, 2016). The gut mucosal epithelium serves as an interface between the vast microbiota, internal host tissues and the immune system. In normal homeostasis, a balance between gut barrier function, gut permeability and equilibrium of commensal and pathogenic microorganisms in the gut lumen is maintained that prevents harmful microbial translocation from the gut (Rao, 2009). The small amounts of gut-derived bacterial products that physiologically translocate from the gut to the liver are eliminated by KC, without triggering liver inflammation (Benacerraf *et al.*, 1959; Nolan, 2010; David *et al.*, 2016). This delicate balance is disturbed by alcohol at multiple levels and interconnected steps at the level of gut microbiome, gut permeability, exposure of the liver to microbial products and alteration of differentiation and activity of liver macrophages, thus resulting in a pro-inflammatory environment in the liver.

Alcohol alters the gut microbiome and gut metabolome

Increased intestinal permeability and load of bacterial products in the portal blood are common features in alcoholics and individuals with alcoholic liver cirrhosis; a phenomenon that is reproducible in animal models of ALD (Parlesak *et al.*, 2000; Bajaj *et al.*, 2014). There is evidence that alcohol intake leads to intestinal bacterial overgrowth in humans and animals and that alcohol causes changes in the taxonomic composition of the intestinal microbiome (intestinal dysbiosis) (Bode *et al.*, 1984; Yan *et al.*, 2011) and (Szabo, manuscript in preparation). Intestinal dysbiosis was correlated with the amount of alcohol consumed (Leclercq *et al.*, 2014), and the term 'cirrhosis dysbiosis ratio' indicating an imbalance of specific bacterial families has been associated with a extent of endotoxemia in patients with cirrhosis (Bajaj *et al.*, 2014). Recently, the increase in gut permeability in ALD has been related to dysbiosis-induced intestinal inflammation (Chen *et al.*, 2015), emphasizing the role for intestinal microbiota in ALD. There is an increase in pro-inflammatory mediators and innate immune cell activation particularly in the small bowel after chronic alcohol feeding in mice (Lippai *et al.*, 2014).

Alterations in gut microbiome

Intestinal dysbiosis triggers inflammation in ALD by compromising intestinal barrier and by increasing translocation of bacterial products to the liver. Metagenomic analysis showed that alcohol feeding in mice decreases diversity of intestinal microbiome and shifts the representation of bacterial phyla over time (reviewed in Engen *et al.*, 2015; Szabo, 2015). In mice on a controled diet, the majority of intestinal bacteria were in the Bacteroides and Firmicutes phylum, whereas alcohol feeding significantly increased the presence of Actinobacteria and Proteobacteria, and increased the proportion of Firmicutes over Bacteroides (Bull-Otterson *et al.*, 2013; Engen *et al.*, 2015). These changes were associated with intestinal hyperpermeability and endotoxemia, and were further aggravated by feeding the alcohol-exposed mice with unsaturated fat diet (corn oil) (Kirpich *et al.*, 2016). One of the early events in an alcohol-related shift within the gut microbiome is the reduction of Akkeremansia both in a mouse model and in humans with ALD (Szabo, manuscript in preparation and Herbert Tilg personal communication).

In humans that have been exposed to excessive amounts of alcohol, the intestinal microbial community was significantly altered, with higher abundance of Proteobacteria and the potentially pathogenic bacteria from the families Prevotellaceae, Enterobactericeae, Veillonellaceae and Strepotococcaceae, and with lower abundance of Bacteroides. The presence of dysbiotic microbiota is correlated with a high level of endotoxin in the blood (Mutlu *et al.*, 2012; Engen *et al.*, 2015).

The causality of altered intestinal dysmicrobia in the pathogenesis of ALD was demonstrated in a study by Perlemuter's group showing that alcohol-induced liver inflammation is a trait transmissible by intestinal microbiota (Llopis *et al.*, 2016). In this study, germ-free mice fed with alcohol received transplant of fecal microbiota isolated from healthy human controls or from humans with varying severity degrees of ALD (Llopis *et al.*, 2016). The research team found that alcohol-fed mice harboring the intestinal microbiota from patients with severe AH developed more severe liver inflammation and necrosis, associated with increased translocation of bacteria from the gut to the liver, compared to alcohol-fed mice that were transplanted with intestinal microbiota either from control individuals or alcoholics without liver disease. Furthermore, in alcohol-fed mice humanized with the intestinal microbiota from severe AH patients, a subsequent transfer of intestinal microbiota from patients with no AH improved alcohol-induced liver lesions. Key deleterious bacterial species involved in transmission of the severe AH phenotype included altered Bacteroides phylum as well as Bilophila, Alistipes, Butyricimonas, Clostridium, Proteus and *Escherichia coli* (Llopis *et al.*, 2016). In contrast, intestinal microbiota from patients with severe AH showed relative lack of Faecalibacterium Prausnitzii, a bacterial species known for its anti-inflammatory and mucosal-protective properties (Sokol *et al.*, 2008).

The decrease in intestinal bacterial diversity observed in alcoholics and in patients with ALD (Bull-Otterson *et al.*, 2013) seems

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to be a general phenomenon frequently observed in patients with advanced liver disease, irrespective of etiology (Engen *et al.*, 2015). Metagenomic analysis indicated that gene richness in intestinal microbiota was much lower in patients with liver cirrhosis than in healthy individuals, and it is thought that decreased diversity of intestinal microbiome is associated with relative lack of protective species, abundance of harmful species and decreased integrity of intestinal mucosa (Qin *et al.*, 2014). Surprisingly, the decreased diversity of intestinal microbiota in patients with cirrhosis was associated with a relative abundance of taxa such as Veillonella, Streptococcus and Clostridia which are known to include species of oral origin, indicating that oral commensals invade the gut of patients with liver cirrhosis. Possibly, altered bile production in cirrhosis renders the gut more permissible to 'foreign' bacteria, as bile resistance may be required for survival in the human gut (Saarela *et al.*, 2000; Merritt and Donaldson, 2009; Qin *et al.*, 2014).

Alterations in gut metabolome

Alcohol alters the metabolic composition in the gastrointestinal content, which changes the source of nutrition for microbes (Xie *et al.*, 2013). For example, alcohol feeding to mice resulted in a decrease in all amino acids in the gut, including the branched chain amino acids that are known to be decreased in patients with hepatic encephalopathy (Xie *et al.*, 2013; Gluud *et al.*, 2015; Kirpich *et al.*, 2016). There also were changes in fecal lipid metabolites, including the decrease in short-chain fatty acids such as butyrate, valerate and propionate (Kirpich *et al.*, 2016), all of which represent a source of energy for intestinal epithelia and are required for integrity of intestinal barrier and mucosal immune tolerance (Scheppach, 1994; Maslowski *et al.*, 2009; Kim *et al.*, 2013). These findings were confirmed in humans drinking alcohol (Couch *et al.*, 2015) and were consistent with studies showing that patients with liver cirrhosis have low abundance of Coprococcus and Faecalibacterium prausnitzii, both of which contribute to gut integrity through butyrate production (Cotillard *et al.*, 2013; Le Chatelier *et al.*, 2013; Qin *et al.*, 2014). In addition, a peptidomic analysis showed that Faecalibacterium prausnitzii secretes a 15-kDa protein, which has anti-inflammatory properties and is found in a 'healthy' gene-rich microbiome (Quevrain *et al.*, 2016).

Available evidence supports a possible involvement of volatile organic compounds in pathogenesis of steatosis or liver inflammation. For example, changing the microbiota using prebiotic treatment has decreased hepatic lipogenesis and plasma triglycerides, showing a metabolic link between microbiota and liver biology in humans (Letexier *et al.*, 2003). The fermentation of prebiotics by gut microbes increases the abundance of short-chain fatty acids in the cecum and also in the portal blood, where the concentration of both acetate and propionate is doubled (Roberfroid *et al.*, 2010; Bindels *et al.*, 2012; Everard *et al.*, 2014). Published evidence suggests that propionate may contribute to reduced hepatic lipogenesis, whereas acetate is a lipogenic substrate (Demigne *et al.*, 1995; Lin *et al.*, 1995).

It is not entirely known whether intestinal metabolome changes are specific to ALD or whether they represent general phenomenon in advanced liver disease. In a recent study published in *Nature*, metabolomic changes in intestinal microbiome in advanced liver disease were put into the context with liver cirrhosis-related complications (Qin *et al.*, 2014). In that study, the microbial metabolic pathways most enriched in patients with cirrhosis included assimilation of nitrates to form ammonia, manganese transport systems and pathways of gamma-aminobutyric acid (GABA) synthesis and transport (Qin *et al.*, 2014). The enrichment of microbial metabolic modules for ammonia production suggests a potential role of gut dysmicrobia in hepatic encephalopathy, a complication of liver cirrhosis that is accompanied by increased levels of ammonia in blood. Manganese transport system modules enriched in patients with cirrhosis may contribute to the changes in the accumulation of manganese within the basal ganglia, which is another pathogenic mechanisms contributing to hepatic encephalopathy (Krieger *et al.*, 1995). Finally, the modules for GABA synthesis enriched in microbiota of cirrhotic patients represent another potential mechanism contributing to hepatic encephalopathy as GABA levels are increased in the blood of patients with cirrhosis (Ferenci *et al.*, 1983; Minuk *et al.*, 1985).

Although further studies are needed to clarify whether altered intestinal microbiome is the cause or consequence of advanced liver disease including ALD, it is possible that microbiome modulation may provide new therapeutic options for treatment of ALD and liver cirrhosis. Multiple randomized controlled trials addressing the therapeutic modulation of intestinal microbiota in hepatic encephalopathy, fibrosis progression, metabolic consequences of liver disease and in the outcome of severe AH are currently underway (trials number NCT02485106, NCT02862249, NCT01069133, NCT02400216, NCT02496390, NCT02424175 and NCT01968382 at www.clinicaltrials.gov).

Mechanisms of increased gut permeability in ALD

The integrity of the intestinal mucosa is determined by the protective layer of defensins on the intraluminal surface of the intestinal epithelium, tight junction proteins between enterocytes, the gut immune cells located in the submucosa and protective factors released by intestinal microflora such as short-chain fatty acids (butyrate) and anti-inflammatory peptides (Marteau, 2013; Szabo, 2015; Quevrain *et al.*, 2016).

A single administration of ethanol will cause intestinal epithelial damage only if used in a very high dose (Lippai *et al.*, 2014). In chronic and repetitive exposure to ethanol, disruption of the intestinal barrier is explained by reduced expression of proteins involved in tight junction between enterocytes, such as occludin and zonula occludens protein ZO-1 (Dunagan *et al.*, 2012; Wang *et al.*, 2014). These changes are attributable to acetaldehyde, a product of ethanol oxidation, in circulating blood (Rao, 2009). Gut permeability may also be increased by tumour necrosis factor (TNF)-α derived from inflamed liver (Yajima *et al.*, 2009) or by miR212, that was upregulated in colon biopsy samples of patients with ALD which downregulates proteins of the zonula occludens in intestinal cell culture (Tang *et al.*, 2008). More recently, studies utilizing a mouse model of ALD showed that bacterial translocation was found even before changes in the intestinal microbiome and the bacterial translocation was associated with reduced expression of the bactericidal c-type lectins, Reg3b and Reg3g, in the small intestine (Yan *et al.*, 2011). Mucin-2, a mucus layer protein, secreted by goblet cells of the intestine, was found to be a critical regulator of intestinal Reg3b and Reg3g in a mouse model of ALD (Hartmann *et al.*, 2013). The decreased expression of Reg3b in the intestine of alcohol-fed mice was associated with increased expression of miR-155, and miR-155-deficient mice that were protected from alcohol-induced inflammation in the small intestine (Lippai *et al.*, 2014).

Most recent studies have demonstrated the role of bile acids in gut bacterial translocation and inflammation. Depriving the intestines of bile through bile duct ligation, or silencing the bile acid receptor farnesoid X receptor (FXR) increased bacterial overgrowth,

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bowel permeability and bacterial translocation (Kalman and Goldberg, 2016). It has been hypothesized that decreased amounts of bile produced in patients with cirrhosis dysregulates intestinal microbiome and disrupts intestinal integrity. This hypothesis was confirmed in a study utilizing a rat model of liver cirrhosis induced by repetitive injections of carbon tetrachloride (Ubeda *et al.*, 2016). In this study, treatment of cirrhotic rats with obeticholic acid (OCA), a potent agonist of the bile acid receptor, FXR, significantly reduced bacterial translocation from the intestine to the blood. Treatment with OCA stimulated FXR pathways in cirrhosis, and upregulated antimicrobial proteins angiogenin-1 and α-5 defensin, as well as tight junction proteins ZO-1 and occludin. In addition, OCA shifted the composition of intestinal microbiome toward Firmicutes (including Lactobacillus), whereas the proportion of Proteobacteria (such as *E. coli* and Shigella) was reduced. These changes were associated with improvement in local and systemic inflammation and subsequent improvement in hepatic fibrosis. The mechanism behind the beneficial effect of OCA is thought to be due to the reparative effect of OCA on the small intestinal barrier, OCA-mediated increases in the secretion of bile acids by the liver, and by inhibitory effect of FXR signaling on hepatic fibroblasts (Kalman and Goldberg, 2016; Laleman *et al.*, 2016; Ubeda *et al.*, 2016).

Gut-derived bacterial products in the pathogenesis of ALD

Gut-derived bacteria contribute to the pathogenesis of ALD by their structural components (pathogen-associated molecular patterns, PAMPs) that activate innate immune cells in the liver, or by their metabolites that alter gut mucosal integrity. A host of bacterial PAMPs activates the cells of innate immune systems via binding to specific receptors, including the Toll-like receptors (TLRs) (reviewed in Pandey *et al.*, 2014). Four bacterial PAMPs have been studied in alcohol-induced liver inflammation so far: lipopolysaccharide (LPS), an activator of TLR4, bacterial hypomethylated (CpG) DNA, an activator of TLR9, flagellin, an activator of TLR5, and lipoteichoic acid, an activator of TLR2 (reviewed in Petrasek *et al.*, 2010; Wang *et al.*, 2013; Roh *et al.*, 2015). Two of these, LPS and bacterial DNA, are increased in the plasma in humans exposed to alcohol (Bala *et al.*, 2014; Michelena *et al.*, 2015). No studies published to date report on plasma levels of lipoteichoic acid or flagellin in the plasma of patients with ALD.

For historical reasons, mechanistic studies on the role of bacterial PAMPs in alcohol-induced liver inflammation have focused predominantly on LPS/TLR4 (discussed in detail below), whereas less attention has been paid to CpG-DNA/TLR9, flagellin/TLR5 or lipoteichoic acid/TLR2 in the development of alcohol-induced liver inflammation. For example, bacterial DNA was found in serum and ascites patients with advanced liver cirrhosis leading to increased cytokine production in peritoneal macrophages (Frances *et al.*, 2004a, 2004b). In mouse models of ALD, alcohol feedings sensitized to TLR9 ligand CpG to enhance TNF-α production (Gustot *et al.*, 2006). Mice deficient in TLR9 or TLR2 showed protection from alcohol-induced liver inflammation, but detailed mechanistic studies on the role of TLR9 and TLR2 in ALD are lacking (Roh *et al.*, 2015).

Gut-derived LPS (endotoxin) in the pathogenesis of ALD

Early in the 20th century, endotoxin (later characterized as LPS) was the first bacterial component linked with pathophysiological consequences of bacterial infections, including sepsis (Old, 1987). As reviewed by (Nolan, 2010), a causal relationship between LPS and liver inflammation and injury has been known for at least the past 50 years, and clinical studies have demonstrated correlations of LPS levels with extrahepatic manifestations of alcoholic cirrhosis, such as the hepatorenal syndrome and clotting abnormalities (Clemente *et al.*, 1977; Michelena *et al.*, 2015). In addition, the increased levels of LPS in peripheral circulation after exposure to alcohol were consistently demonstrated in humans (Rao, 2009; Bala *et al.*, 2014; Michelena *et al.*, 2015).

Gut-derived LPS activates hepatic macrophages via TLR4

Upon entering the portal blood, LPS is recognized by the TLR4 receptor complex expressed on hepatic macrophages and other liver immune and parenchymal cells (Szabo, 2015; David *et al.*, 2016). In the normal liver, hepatic macrophages show tolerance in small amounts of gut-derived endotoxin. However, in the pathogenesis of alcohol-induced liver inflammation, hepatic macrophages lose their quiescent phenotype and become activated. Multiple lines of evidence demonstrate that activation of hepatic macrophages in ALD involves TLR4-dependent mechanism activated by gut-derived LPS (Adachi *et al.*, 1995; Enomoto *et al.*, 1998; Thurman, 1998; Nagy, 2003). While TLR4 cannot directly bind LPS its co-receptors, CD14 and MD-2, bind LPS and upon LPS-binding activate TLR4 (Park *et al.*, 2009). The association between LPS and CD14 is facilitated by LPS-binding protein (LBP), which is a soluble shuttle protein (Wright *et al.*, 1989).

TLR4, CD14 and LBP are critical in alcohol-induced liver injury. Alcoholic liver inflammation was prevented in C3H/HeJ mice (Uesugi *et al.*, 2001), which have functional mutation in the *TLR4* gene and have defective response to bacterial endotoxin (Sultzer, 1968). Prevention of alcohol-induced liver inflammation and injury in C3H/HeJ mice was associated with decreased TNF-α expression, compared to wild-type mice. Similar protection from alcohol-induced liver inflammation and injury was observed in mice deficient for LBP (Uesugi *et al.*, 2002) and CD14 (Yin *et al.*, 2001), whereas mice transgenic for human CD14 were hypersensitive to LPS (Ferrero *et al.*, 1993).

MECHANISMS LEADING TO ACTIVATION OF HEPATIC MACROPHAGES IN ALD

Gut-derived LPS is captured by hepatic macrophages within minutes from entering the portal circulation, and under baseline conditions, this process does not result in hepatic macrophage activation (David *et al.*, 2016). Three major mechanisms, each supported by ample evidence, have been suggested to explain activation of liver macrophages in ALD. First, studies utilizing mouse models of ALD and *ex vivo* stimulation of human or murine mononuclear cells indicate that the switch from tolerant cells to pro-inflammatory cells (also known as loss of LPS tolerance) is a process intrinsic to KC secondary to repetitive exposure to LPS and ethanol. Second, studies utilizing cell fate-mapping strategies (Irish, 2014) suggest that rather than to KC, the pro-inflammatory activation in the liver may be attributable to BM-derived monocytes/macrophages that infiltrate the liver and further polarize following liver injury (Polarization of hepatic macrophages in ALD). Finally, studies using cell-specific knockouts, gnotobiotic approaches and *ex vivo* co-cultures of macrophages and hepatocytes indicate that in ALD, activation of liver macrophages is dependent on alterations of liver microenvironment secondary to the direct effect of ethanol, attributable to the release of hepatocyte-specific sterile signals that sensitize liver macrophages to gut-derived

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LPS. This will be discussed below in the section on sterile inflammation. These mechanisms are not mutually exclusive and may play a role in concert in ALD.

Loss of LPS tolerance in hepatic macrophages in ALD

Under normal circumstances, KCs prevent gut-derived LPS from reaching the systemic circulation, without themselves being activated (David *et al.*, 2016), a phenomenon called LPS tolerance. It is important to note that hepatocytes were also shown to play a 'detoxification' role in taking up LPS delivered by the portal blood thereby contributing to liver homeostasis (Shao *et al.*, 2012). However, long-term administration of alcohol to rats sensitized KCs to secrete high levels of inflammatory cytokines after isolation and *ex vivo* exposure to LPS (Hansen *et al.*, 1994).

Induction of TLR4 signaling is dependent on the mode of LPS exposure. When LPS challenge is provided following an initial insult with LPS, induction of TNF-α (a prototypical cytokine induced by LPS/TLR4 pathway) is severely attenuated, a phenomenon called 'LPS tolerance'. Studies have demonstrated that upregulation of negative regulators of TLR signaling plays a central role in TLR tolerance (Huang *et al.*, 1995; De Nardo *et al.*, 2009; Piao *et al.*, 2009). Experimental evidence suggests, however, that TLR tolerance can be broken by multiple sequential LPS administration *in vivo* and *in vitro* (Medvedev *et al.*, 2006). When mice were injected with a single dose of LPS, a second LPS challenge failed to induce significant serum TNF-α induction compared to the initial dose demonstrating TLR4 tolerance (Dolganiuc *et al.*, 2007). However, when LPS was given in 3-day intervals for five repeated times, the TLR tolerance was lost and serum TNF-α levels induced by the last dose of LPS were comparable to TNF-α induced by a single LPS administration (Roth *et al.*, 1994). The bimodal effects of LPS on TNFα production are reminiscent to the opposite modulation of inflammation by acute and prolonged alcohol use.

At the molecular level, acute alcohol administration inhibited while chronic alcohol use increased production of pro-inflammatory cytokines (Messingham *et al.*, 2002). The opposing effects of acute and chronic alcohol can be partly linked to loss of a key regulator of LPS tolerance in macropahges, IRAK-M (Mandrekar *et al.*, 2009). In a study utilizing murine macrophages *in vitro* and a mouse model of acute alcohol administration, acute alcohol treatment induced TLR4/LPS tolerance through the induction of Bcl-3, a negative regulator of TNF-α transcription via its association with NF-κB p50/p50 dimers (Bala *et al.*, 2012).

In vitro studies showed that prolonged alcohol exposure of monocytes for 4 days or longer augmented LPS-induced TNF-α production compared to alcohol-naïve cells (Mandrekar *et al.*, 2009). The involvement of the TLR4 signaling pathway was suggested by increased IKK kinase activity, increased NF-kB nuclear translocation and DNA transactivation in human monocytes (Mandrekar *et al.*, 2009). This upregulation of TLR4 signaling occurred in the presence of diminished expression of IRAK-M, a negative regulator of TLR4 signaling, in monocytes after prolonged alcohol treatment. Overexpression of IRAK-M prevented the increased LPS-induced TNF-α production in chronic alcohol-treated cells suggesting that loss of IRAK-M is likely to contribute to the loss of TLR tolerance in monocytes after prolonged alcohol exposure (Mandrekar *et al.*, 2009).

The diversity of innate immune cell populations in the liver in ALD

The hepatic environment physiologically harbors a vast population of innate immune cells such as KC (resident liver macrophages), BM-derived infiltrating monocytes/macropahges and dendritic cells. In order to accomplish critical innate immune functions, KCs are located in the sinusoidal lumen, where they constantly survey blood content, ingesting aging erythrocytes and catching pathogens, including bacterial components such as LPS, out of the bloodstream (David *et al.*, 2016). Dendritic cells are located in hepatic parenchyma but are not known to contribute to the baseline surveillance and phagocytic activity; their gene expression profile is different from that of the KC, favouring antigen processing and presentation but not phagocytosis (David *et al.*, 2016).

As discussed above, data from the past 15 years support the hypothesis that in pathogenesis of ALD, repetitive exposure to gut-derived LPS converts KC from a state of immunotolerance to a state of immune activation, resulting in the expression of inflammatory cytokines and liver inflammation (Mandrekar *et al.*, 2009; Nolan, 2010; Bala *et al.*, 2012). More recent data do not contradict this notion but suggest that the scenario is more complex and that there is functional distinction between KC and infiltrating monocytes/macrophages: whereas KC maintain tolerance, BM-derived macrophages are either pro-inflammatory (M1 macrophages) or profibrogenic (M2 macrophages) (Ju and Mandrekar, 2015; Xu *et al.*, 2015b; Saha *et al.*, 2016).

Studies have shown that KC represent only one of the multiple subpopulations of monocytes and macrophages in the liver (Yona *et al.*, 2013; Ju and Mandrekar, 2015; David *et al.*, 2016). In a study of severe liver injury induced by acetaminophen, KC removal actually augmented the extent of liver inflammation and injury, likely secondary to impaired mechanisms of tissue repair (David *et al.*, 2016). In addition, the activation of monocytes/macrophages in the liver is not entirely dependent on instructions from microbiota but requires endogenous mediators from a hepatic microenvironment as well (Szabo and Petrasek, 2015; David *et al.*, 2016; Llopis *et al.*, 2016). The complexity of the differential role of KCs versus BM-derived macrophages in liver inflammation needs further investigation.

Differential function of KCs versus BM-derived liver monocytes/macrophages in alcohol-induced liver inflammation

Recent studies on the origin of KCs have shown that macrophages seed the liver during embryogenesis from yolk sac progenitors, and in the absence of liver injury, this resident pool of KCs is maintained in adulthood predominantly via self-renewal of intrahepatic precursors and, to a lesser extent, recruitment of BM-derived cells (Schulz *et al.*, 2012; Yona *et al.*, 2013; Scott *et al.*, 2016). The uptake of gut-derived LPS by KC in undamaged livers happens in a manner that prevents cell damage or inflammation (Rao, 2009). In a mouse study using intravital microscopy (David *et al.*, 2016), time-lapse imaging revealed that *E. coli* (carrying large quanta of LPS) flowing in liver hepatic sinusoids were immediately immobilized to the KC membrane and cleared from the circulation at first contact, and no major changes in KC morphology or activation were necessary for bacterial arrest (David *et al.*, 2016).

Although the pool of KC undergoes repletion from intrahepatic precursors under physiological circumstances, the situation dramatically changes if liver damage occurs. Recently published data have shown that significant liver injury is associated with depletion of KC, which generates intrahepatic niche available for BM-derived circulating monocytes to engraft in the liver. Over the period of 2–8 weeks, these newly engrafted BM-derived monocytes/macrophages gradually adopt the transcriptional profile of KC and become

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long-lived self-renewing cells (David *et al.*, 2016; Scott *et al.*, 2016). However, during the process of liver engraftment, immigrating BM-derived monocytes/macrophages lack the tolerogenic properties of KC (David *et al.*, 2016). That translates into a temporary altered response to injury, including a transitory pro-inflammatory signature after acute liver injury, decreased LPS clearance after exposure to exogenous *E. coli*, and augmented response to LPS (David *et al.*, 2016).

Based on the cell fate-mapping studies in the liver, it cannot be fully ruled out that in the pathogenesis of liver disease, the pro-inflammatory phenotype historically attributed to KC is actually more pertinent to BM-derived monocytes/macrophages, although further studies will be needed to differentiate this in the context of ALD. The distinction between KC and BM-derived monocytes/macrophages in the liver could not be made in previous studies as routine markers of KC, such as F4/80, cannot distinguish between these two sets of cells, and it has been impossible to selectively deplete only one population of tissue macrophages, without disturbing the entire mononuclear phagocyte system (David *et al.*, 2016). Cell fate-mapping strategies (Irish, 2014) or the use of Clec4F (C-type lectin domain family 4, member F), a recently discovered marker specific to murine KC (Scott *et al.*, 2016), could provide a tool to specifically evaluate the contribution of individual liver macrophage subpopulations to the pathogenesis of ALD.

Although the source and differential contribution of KC versus BM-derived monocytes/macrophages to the pathogenesis of ALD await further elucidation, there is ample evidence supporting the role of activated macrophages and inflammatory cytokines in alcohol-induced liver inflammation. Treatment of alcohol-fed rats or mice with gadolinium chloride or clodronate to deplete liver macrophages almost completely protected from alcohol-induced liver inflammation (Adachi *et al.*, 1994; Roh *et al.*, 2015). Similarly, mice deficient in macrophage-derived inflammatory cytokines or chemokines TNF-a, interleukin (IL)-1, MCP-1, MMF (macrophage migration inhibitory factor) showed significant protection from alcohol-induced liver inflammation (summarized in Nagy, 2015). With the exception of MMF, all the above-mentioned cytokines were significantly increased in patients with severe AH and their level in the serum was associated with disease severity and survival in these patients (McClain *et al.*, 1986; Degre *et al.*, 2012; Michelena *et al.*, 2015; Yeluru *et al.*, 2016).

Polarization of hepatic macrophages in ALD

Published evidence suggests that BM-derived circulating monocytes can be recruited to the site of injury early during inflammation in the liver, where they differentiate into macrophages (Irish, 2014; Ju and Mandrekar, 2015; David *et al.*, 2016; Scott *et al.*, 2016). Infiltrating monocytes/macrophages alter their phenotypes and functions depending on tissue microenvironmental cues, such as growth factors, PAMPs and damage-associated molecular patterns (DAMPs). This process, also referred to as macrophage polarization, results in different phenotypic macrophage populations with the final extremes of M1 and M2 macrophages. However, *in vivo* and in dynamic environments as the alcoholic liver, macrophage phenotypes change rapidly within this spectrum and rarely show the prototypic M1 or M2 phenotype. M1 macrophages are induced by Th1 cytokines and LPS, have pro-inflammatory effects and mediate the initial defense against bacteria and viruses. In addition, they are important for the response to tissue injury. The M1 macrophages produce pro-inflammatory mediators such as IL-1, TNF-α, IFN-γ, IL12, IL-18 and reactive oxygen species (Murray and Wynn, 2011). Once the infection or tissue injury is controlled, M2 macrophages, under the influence of Th2 cytokines, render anti-inflammatory effects and promote fibrogenesis and wound healing by way of anti-inflammatory and profibrogenic mediators including IL-10, TGF-β, matrix metalloproteinases, tissue inhibitors of matrix metalloproteinases, arginase and VEGF (Ju and Mandrekar, 2015).

Both subsets of macrophages (M1 and M2) are present in the livers of patients with ALD (Lee *et al.*, 2014) and are involved in response to tissue injury triggered by alcohol (Ju and Mandrekar, 2015). In addition, they have distinct metabolic profiles: M1 use glucose for energy through glycolysis while M2 use fatty acid oxidation (Galvan-Pena and O'Neill, 2014). An explanation for metabolic reprogramming of M1 macrophages towards glycolysis has recently been provided by Tsukamoto's group utilizing a mouse model of ALD combined with a high-fat diet (Xu *et al.*, 2015b). The study showed that differentiation of macrophages into the M1 phenotype is dependent on NOTCH1, a transmembrane receptor that mediates cell–cell communication and that is activated by inflammatory signals related to alcohol consumption, including gut-derived LPS. The M1 macrophage polarization was induced by coupling the LPS/NOTCH-mediated upregulation of the M1 gene transcription with reprogramming of the mitochondrial metabolism toward enhanced glucose oxidation and oxidative phoshophorylation (OXPHOS). The enhanced expression of OXPHOS genes encoded by mitochondrial DNA lead to enhanced production of mitochondrial reactive oxygen species, which in turn augmented expression of M1 genes by way of hypoxia-inducible factor-1α and NF-κB (Xu *et al.*, 2015a). This mechanism had functional impact on alcohol-induced liver inflammation. Pharmacological inhibition or genetic deficiency of NOTCH1 significantly attenuated alcohol-induced liver inflammation and decreased oxidative stress in the liver. Cell fate-mapping experiments showed that the amelioration of hepatic inflammation seen in Notch1 knockout mice was a result of reduced migration of BM-derived monocytes to the liver and their inability of M1 differentiation. Importantly, preexisting resident KC were not involved in this process. In addition, the expression of M1 or M2 genes in resident KC was not affected by alcohol or high-fat diet feeding, supporting the role of BM-derived monocytes, but not of KC, in NOTCH1-dependent M1 polarization and inflammatory activation in ALD (Xu *et al.*, 2015a).

In addition to inducing M1 polarization, consumption of ethanol leads to M2 polarization as well. Recent studies demonstrated that differentiation of alcohol-exposed monocytes to M2 macrophages is dependent on Kruppel-Like Factor-4, which upregulated M2 genes, and requires miR-27a, which activates the ERK signaling pathway and IL-10 secretion via targeting the ERK inhibitor, Sprouty-2 (Saha *et al.*, 2015a, 2015b). In addition, miR27 is present in extracellular vesicles derived from M2-polarized monocytes and these miR27-containing extracellular vesicles signal naive monocytes to differentiate into M2 macrophages (Saha *et al.*, 2016).

Although the M1 versus M2 macrophage polarization is a novel concept in ALD, it clearly indicates that infiltrating macrophages respond to various signals through different pathways and subsequently undergo a phenotypic switch that determines their role in inflammation. From a clinical perspective, this work provides new potential therapies that require further direct evaluation of selective modulators of M1 or M2 activation. For example, DAPT, a gamma-secretase inhibitor, inhibited NOTCH1 pathway, prevented M1 differentiation and ameliorated alcohol injury in a mouse model of ALD (Xu *et al.*, 2015a) as well as liver fibrosis in an alternative

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models of cirrhosis (Chen *et al.*, 2012). No M2 macrophage-specific inhibitors have been evaluated in *in vivo* models of ALD to date.

Mechanisms of alcohol-induced liver inflammation driven by sterile signals

In addition to microbial signals, liver immune cells are commonly exposed to sterile (i.e. non-microbial) molecules derived from the host, which are released from damaged hepatocytes and other cells in the liver (known as DAMPs) (Kubes and Mehal, 2012). Under normal circumstances, DAMPs remain hidden from the extracellular environment and are released when tissues are injured (Kubes and Mehal, 2012). Several DAMPs (ATP, uric acid, cholesterol crystals, beta amyloid, calcium pyrophosphate dehydrate crystals and cytosolic DNA) are known to trigger the assembly of a cytosolic protein complex termed 'the inflammasome', which activates the serine protease Caspase-1 (CASP-1) and leads to the secretion of cytokines such as IL-1β and IL-18 (Martinon *et al.*, 2002; Ogura *et al.*, 2006; Szabo and Csak, 2012).

The role of inflammasomes in ALD

Inflammasomes are multiprotein complexes that sense danger signals via nucleotide-binding oligomerization domain receptors (commonly known as NOD-like receptors [NLRs]) (Schroder and Tschopp, 2010). NLRs contain a ligand recognition domain, a central domain that is responsible for oligomerization, and an N-terminal activation domain (Kumar *et al.*, 2011). Following its activation by inflammatory signals, NLR forms a complex with the effector molecule, pro-CASP-1. The inflammasome can then oligomerize and activate CASP-1 that, in turn, results in the maturation of pro-inflammatory cytokines pro-IL-1β and pro-IL-18 to IL-1β and IL-18 (Schroder and Tschopp, 2010). Among the best-characterized inflammasome-activating signals in liver diseases are ATP, uric acid, palmitic acid, cholesterol crystals and reactive oxygen species (Mariathasan *et al.*, 2006; Csak *et al.*, 2011; Vandanmagsar *et al.*, 2011; Wen *et al.*, 2011; Matsuzaka *et al.*, 2012; Rock *et al.*, 2013). Uric acid, ATP and reactive oxygen species have been shown to be involved in alcohol-induced liver inflammation to date, and the role of other inflammasome-activating signals in ALD remains to be elucidated.

Patients with AH have increased serum levels of IL-1, TNF-α and IL-8, elevated expression of CASP-1 and NLRP3 in the liver, neutrophilia, and activation of monocytes and macrophages (McClain *et al.*, 1986; O'Shea *et al.*, 2010; Peng *et al.*, 2014). Notably, patients with the most severe forms of AH have a substantial increase in serum levels of IL-1β, an inflammasome-driven cytokine, compared with healthy individuals (McClain *et al.*, 1986), and increased levels of the inflammasome components NLRP3, ASC, CASP-1, correlating with the presence of Mallory–Denk bodies in liver pathology (Peng *et al.*, 2014). The presence of increased IL-1β and neutrophilia, pathognomonic for sterile inflammation, indicates inflammasome activation (Gao and Bataller, 2011).

The key role of inflammasome activation in ALD has been confirmed in mouse models. Chronic administration of ethanol to wild-type mice has induced steatosis, liver injury and increased hepatic expression of Il-1β as well as expression of the inflammasome components pro-Casp-1, Asc and Nlrp3 (Petrasek *et al.*, 2012). Similarly, exposure of mice to ethanol has increased Casp-1 activity in the liver, indicating inflammasome activation (Petrasek *et al.*, 2012). Mice deficient in IL-1 receptor, inflammasome activator Nlrp3, inflammasome adaptor Asc or the inflammasome executioner component Casp-1 were protected from ethanol-induced inflammasome and Il-1β activation, and displayed attenuation of ethanol-induced liver injury and steatosis (Petrasek *et al.*, 2012, 2015). The absence of inflammasome activation also prevented accumulation of inflammatory cells in the liver. Daily injections of an Il-1 receptor antagonist (Il-1ra, Anakinra) ameliorated alcohol-induced liver inflammation with a dose-dependent decrease in steatosis and liver injury (Petrasek *et al.*, 2012). In addition, when mice were treated with Il-1ra after 2 weeks of ethanol administration, steatosis and liver injury were also attenuated, and the same protective effect was observed when Il-1ra was administered during recovery from acute-on-chronic ethanol exposure (Petrasek *et al.*, 2012).

Analysis of primary murine cells demonstrated that expression of the inflammasome components Casp-1, Asc and NLRP3 is ~20-fold higher in liver immune cells than in primary hepatocytes. In further experiments, the administration of ethanol to wild-type mice induced cleavage of Casp-1 in liver immune cells but not in hepatocytes (Petrasek *et al.*, 2012). Using mice with a cell-specific deletion of Casp-1, liver macrophages were found to be the main cell types that mediate inflammasome-dependent ALD progression (Petrasek *et al.*, 2012). Collectively, the current available data indicate that the pathogenic role of the inflammasome and IL-1 in ALD is mediated by its activation in hepatic macrophages. Inhibition of IL-1 signaling in humans with severe AH is currently being tested in clinical trials (NCT01809132 and NCT01903798 at www.clinicaltrials.gov).

Activators of hepatic inflammasomes in ALD

Activators of the inflammasome in ALD have not yet been fully defined. Gut-derived LPS, which signals through TLR4 (Gao *et al.*, 2011; Szabo, 2015), is likely to be the first signal that induces expression of pro-IL-1β (Inokuchi *et al.*, 2011; Petrasek *et al.*, 2012). Experiments using mice with cell-specific deficiency of Casp-1 demonstrated that inflammasome activation and IL-1β secretion in ALD is specific to liver macrophages (Petrasek *et al.*, 2012). The list of second activating signals that release active IL-1β has not been fully characterized but alcohol-induced mitochondrial dysfunction is associated with changes in the metabolism of uric acid and ATP, two known activators of the NLRP3 inflammasome, suggesting the possibility that they could be the source of an inflammasome-activating signal (Lieber *et al.*, 1962; Hoek *et al.*, 2002; Stiburkova *et al.*, 2014). Indeed, individuals exposed to ethanol have increased serum levels of uric acid (Lieber *et al.*, 1962) and treatment of alcohol-exposed rats with allopurinol, an inhibitor of uric acid synthesis, ameliorates liver inflammation, steatosis and injury (Kono *et al.*, 2000). In our own work, we have observed a lack of alcohol-induced inflammasome activation in the livers of mice with a genetic deficiency in the ATP receptor P2X7, and in mice depleted of uric acid as a result of uricase overexpression (Iracheta-Vellve *et al.*, 2015). Consequently, uric acid and ATP likely represent second signals for inflammasome activation in ALD, although we cannot exclude the possibility that other host-derived molecules are also involved in this process. For example, the non-histone chromosomal protein high mobility group box-1 (HMGB-1), an alarmin that is released predominantly from damaged hepatocytes and recognized by liver immune cells, is a strong pro-inflammatory signal in ALD and might activate the inflammasome (Ge *et al.*, 2014). HMGB-1 activates TLR4 on the surface of liver immune cells which leads to the induction of inflammatory cytokines (Yu *et al.*, 2006). This

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process requires the endocytosis of HMGB-1 and is dependent on CASP-1, which indicates an interaction between HMGB-1 and inflammasome signaling (Xu *et al.*, 2014).

SYNERGY BETWEEN BACTERIAL AND STERILE SIGNALS IN ALCOHOL-INDUCED LIVER INFLAMMATION

Liver inflammation in ALD was initially viewed as a linear process triggered by translocation of LPS from the gut to the liver resulting in dose-dependent activation of KC (Enomoto *et al.*, 1998; Thurman, 1998; Hoek and Pastorino, 2002). With the emergence of new data on intestinal microbiome/metabolome, diverse roles of liver macrophage populations and the role of inflammasome in liver disease, it is becoming clear that alcohol-induced inflammation in the liver results from an intricate network of multiple signals from the gut and from the liver that can vary in their timing, cellular location, intensity and duration.

For example, a recent study published by Menezes' group (David *et al.*, 2016) demonstrated that liver-derived factors shape KC function in response to liver injury. This finding was consistent with the report of Perlemutter's group (Llopis *et al.*, 2016) in which intestinal microbiome from humans with severe AH (sAH) was transplanted to conventional mice fed control or alcohol diet. Transplantation of sAH microbiome to mice significantly increased gut permeability and translocation of intestinal microbiota to the liver, but was not sufficient to cause liver inflammation or damage, unless mice were fed with alcohol diet. This finding was consistent with our observations that primary liver insult is required for liver inflammation to develop (Iracheta-Vellve *et al.*, 2015; Petrasek *et al.*, 2015), and confirmed our hypothesis that in alcohol-induced liver, there is a synergistic interaction between gut-derived bacterial signals and hepatocyte-derived sterile signals (Szabo and Petrasek, 2015).

We have previously postulated that only certain constellations of multiple signals that involve the inflammasome will enable the liver immune system to be activated in the pathogenesis of ALD (Szabo and Petrasek, 2015). A definitive answer on whether the interaction between the gut and the liver takes place parallel or consecutively will likely be provided by future studies. Previous reports on the pathogenesis of non-alcoholic steatohepatitis proposed that translocation of bacterial pathogens combined with the underlying steatosis is required to trigger liver inflammation in steatohepatitis ('two hit theory' proposed by Day and James, 1998). The data available to date support the concept that at least two signals are *required* for liver inflammation, but, at the same time, indicate that they may not be *sufficient* to trigger inflammation. The complete set of mechanisms that enable liver immune cells distinguish pro-inflammatory noxious signals from physiological background is likely broader and is awaiting its full elucidation. Based on available evidence, we and others believe that inflammation in ALD is determined by the presence of multiple parallel hits (Szabo and Petrasek, 2015; Mandrekar *et al.*, 2016). This concept has been reflected in current ongoing clinical trials in AH, as will be discussed below.

THERAPEUTIC IMPLICATIONS

sAH represents one of the deadliest complications of ALD, with short-term mortality between 20 and 50% (Thursz and Morgan, 2016). sAH is an example of acute-on-chronic liver injury, in which the majority of patients have already established alcoholic cirrhosis, and in which consumption of alcohol in binges leads to deterioration of liver function, manifesting as jaundice, encephalopathy and coagulopathy (Crabb *et al.*, 2016). Available evidence demonstrates that sAH is driven by a surge of inflammatory activity in the liver and by impairment of hepatocyte regeneration (Dubuquoy *et al.*, 2015; Louvet and Mathurin, 2015). Historically, the anti-inflammatory medication prednisone was the mainstay of treatment of sAH, but its efficacy is limited and its use is complicated by opportune infections (Thursz *et al.*, 2015).

Until recently, sAH received very little attention from policy makers, pharmaceutical companies and funding agencies. Due to improved pre-clinical models on ALD (Mandrekar *et al.*, 2016), novel data on pathobiology of liver inflammation (Yeluru *et al.*, 2016), updated consensus on definitions for sAH (Crabb *et al.*, 2016) and changing views on sAH this is now perceived as a major public health issue (Mandrekar *et al.*, 2016), multicentric clinical trials have been recently initiated in the US and in Europe in which novel therapeutic targets are being tested in patients with sAH (Mandrekar *et al.*, 2016). In line with the multiple parallel hit hypothesis discussed above, the prevailing concept embedded in these clinical trials is to target multiple pathobiological mechanisms at the same time. For example, the therapeutic approaches focus on modulation of gut microbiome by probiotics or antibiotics, prevention of gut leakage by administering zinc sulfate, inhibition of inflammatory signals including the inflammasome using IL-1 or caspase inhibitors, and promotion of hepatocyte regeneration using IL-22 (Mandrekar *et al.*, 2016). For detailed information about specific therapies currently tested in clinical trials, we refer to recently published reviews on this topic (Arsene *et al.*, 2016; Mandrekar *et al.*, 2016; Petrasek and Szabo, 2016; Mitchell *et al.*, 2017).

CONCLUSIONS

In the pathogenesis of ALD, liver inflammation represents a transition point from simple steatosis to steatohepatitis, fibrosis and cirrhosis. In the past few years, we have witnessed significant advances in the elucidation of mechanisms behind alcohol-induced inflammation in the liver, some of which are currently tested in clinical trials. With integrative approaches and new data on alcohol-induced liver inflammation, we may be approaching a point at which our understanding of inflammation in ALD will change from mere enumeration of mechanisms required for inflammation to an integrated view of signaling circuits along with their critical regulatory checkpoints that would be amenable to novel therapeutic approaches. Carefully designed clinical trials to test these targets in patients with ALD are urgently needed.

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CONFLICT OF INTEREST STATEMENT

None declared.

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