Linking Pathogenic Mechanisms of Alcoholic Liver Disease With Clinical Phenotypes

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Abstract

Alcoholic liver disease (ALD) develops in approximately 20% of alcoholics, with a higher prevalence in females. ALD progression is marked by fatty liver and hepatocyte necrosis, as well as apoptosis, inflammation, regenerating nodules, fibrosis, and cirrhosis [1]. ALD develops via a complex process involving parenchymal and non-parenchymal cells, as well as recruitment of other cell types to the liver in response to damage and inflammation. Hepatocytes are damaged by ethanol, via generation of reactive oxygen species and induction of endoplasmic reticulum stress and mitochondrial dysfunction. Hepatocyte cell death via apoptosis and necrosis are markers of ethanol-induced liver injury. We review the mechanisms by which alcohol injures hepatocytes and the response of hepatic sinusoidal cells to alcohol-induced injury. We also discuss how recent insights into the pathogenesis of ALD will affect treatment and management of patients.

Keywords

alcoholic hepatitis; alcoholic liver disease; hepatic stellate cell

Ethanol leads to hepatocyte stress and injury through effects on hepatocytes and on intestinal epithelia. Toxicity is mediated through ethanol as well as ethanol metabolism and its

metabolites. What are the molecular mechanisms of these processes, and how do they affect disease development and phenotype?

Oxidative Stress

Ethanol is metabolized through 2 major oxidative and 2 minor non-oxidative pathways. Ethanol is oxidized by alcohol dehydrogenase (ADH) and cytosolic aldehyde dehydrogenase 1 (ALDH1) and mitochondrial ALDH2 [1], which converts NAD^+ into NADH. Ethanol is also oxidized by cytochrome P450 family 2, subfamily E, polypeptide 1 (CYP2E1) and catalase, which generates reactive oxygen species (ROS) and leads to cellular damage [2]. Although the liver metabolizes the most ethanol, other tissues can also metabolize alcohol [3]. For example, chronic ethanol intake induces CYP2E1 expression in Kupffer cells [4,5]. In addition to oxidative metabolism, 2 non-oxidative pathways can metabolize small amounts of ethanol [3]. However, the effects of these pathways on cell functions are not clear [6].

Ethanol induces oxidative stress via multiple pathways (reviewed in [7]). In hepatocytes, increased oxidative stress directly damages mitochondria to induce cell death or sensitize hepatocytes to cell death in response to inflammatory cytokines. Increased oxidative stress in Kupffer cells also increases their sensitivity to lipopolysaccharide (LPS) [5]. Livers from NADPH oxidase- and inducible nitric oxide synthase-knockout mice have decreased, whereas Cu,Zn-superoxide dismutase knockout-mice have increased, oxidative and nitrate-induced stress and injury after chronic ethanol feeding [8–10].

Strategies to downregulate oxidative stress pathways might therefore prevent or reduce development of ALD. Unfortunately, clinical trials in patients with ALD focusing on antioxidant therapies have generally not been successful. Rather than excluding a role for this approach in therapy, the results highlight the need to increase our understanding of oxidative stress and antioxidants, to guide appropriate selection of targets, as well as proper doses for humans. Other antioxidants that have received attention either in preclinical and small clinical trials and warrant further study include s-adenosyl methionine, betaine, folate, and methionine adenosyltransferase I, alpha (MAT1A).

Hypoxia

Alcohol consumption induces pericentral hypoxia due to increased oxygen consumption during ethanol metabolism [11,12]. Prolonged hypoxia promotes ROS production, impairs mitochondrial fatty acid oxidation, and stimulates expression of lipid synthesis genes, contributing to mitochondrial damage and cell death. Liver can adapt to hypoxia by activating transcription of genes that include hypoxia inducible factors (HIFs). Expression of HIF1A and HIF2A increases in liver with binge and chronic ethanol feeding. Unfortunately, although HIF1A protects the liver from hypoxia, a number of events also ensue that exacerbate alcohol-induced liver injury [13–15]. Mice with hepatocyte-specific disruption of *Hif1b* (*Arnt*) are resistant to chronic plus binge alcohol-induced steatosis and liver injury [16]. Activation of HIFs during alcohol-induced hypoxia therefore have complex effects on liver injury in mice. The roles of HIFs in human ALD have not been investigated.

Dysregulation of Lipid Synthesis

Alcohol consumption dysregulates lipid synthesis and metabolism, resulting in steatosis[17]. Alcohol exposure inhibits hepatic activity of sirtuin 1 (SIRT1), leading to increased acetylation and stability, as well as increased transcriptional activity of sterol regulatory element-binding protein-1 (SREBP1), which regulates lipogenesis [18, 19]. SREBP2, lipin 1 [20, 21], and ceramide also regulate lipogenesis. SIRT1 regulates fatty acid synthesis and oxidation; ethanol-induced decreases in SIRT1 affect multiple processes to lead to steatosis [22, 23, 24]. Because regulates lipid metabolism, it could be targeted to prevent and/or treat steatosis associated with ALD.

Mitochondria, Endoplasmic Reticulum (ER) stress, and Autophagy

Acute and chronic alcohol exposure alter liver mitochondria structure and function in animal models and humans [25]. Alcohol exposure also damages mitochondrial DNA and ribosomes and decreases rates of mitochondrial respiration (state III) and oxygen consumption, leading to mitochondrial-mediated apoptosis [11, 26]. However, liver can adapt to chronic alcohol-induced mitochondrial and metabolic stress by activating mitophagy [27], mitochondrial fusion, or mitochondrial respiration, as well as mitochondrial biogenesis (via peroxisome proliferator-activated receptor gamma, coactivator 1 alpha or PPARGC1A) in mice [28]. So, mitochondrial plasticity allows for a balance between alcohol-induced mitochondrial damage and repair or biogenesis.

Alcohol metabolism can result in the formation of a variety of protein adducts, as well as impair proper protein folding in the ER, resulting in accumulation of misfolded protein and ER stress[29]. Cells can adapt to ER stress by activating the unfolded protein response (UPR). The UPR attenuates ER stress and restores ER homeostasis by decreasing general protein translation and increasing protein folding capacity (by promoting expression of chaperone proteins). The UPR also promotes degradation of misfolded proteins by ER-associated protein degradation (ERAD) via the proteasome or by ER stress-mediated compensatory autophagy [30, 31]. However, chronic alcohol consumption inhibits hepatic proteasome activity [32]. As a result, damaged or misfolded proteins accumulate in cells and form insoluble protein aggregates that are resistant to degradation by the proteasome and require removal by autophagy. In support of this model, autophagy was observed to be activated in mouse liver after acute or chronic alcohol exposure [33, 34]. This process adds another layer of adaptive compensatory mechanisms for regulation of proteostasis in response to ER stress and impaired proteasome function after alcohol exposure.

Autophagy is a catabolic process involved in maintaining normal liver physiology and development of liver diseases [35]. Autophagy involves the formation of double-membrane autophagosomes that traffic and fuse with lysosomes to form autolysosomes, where autophagic cargo are degraded. Chronic alcohol impairs vesicular function and trafficking [36–39]. Cells can adapt to impaired vesicular function by promoting lysosome biogenesis and synthesis of early autophagosomes [40]. Therefore, autophagy could either decrease or increase depending on the balance of impaired cellular trafficking and lysosomes, and the compensatory activation of de novo autophagosome synthesis and

lysosome biogenesis after alcohol exposure. Although our knowledge in this area is far from complete, especially with regard to autophagy in human ALD, a number of newly developed and repurposed drugs have been shown to regulate the autophagy process and may eventually warrant evaluation for human ALD.

Interactions of Apoptosis, Necrosis, and Autophagy

Alcohol consumption leads to hepatocyte death via apoptosis, necrosis, or necroptosis (programmed necrosis). Ethanol induces apoptosis via the extrinsic (death-receptor regulated) or intrinsic (mitochondrial) pathways [41–43]. Necroptosis is similar in nature to necrosis but is initiated by death receptor activation and mediated by receptor-interacting protein kinase 1 (RIP1) and 3 (RIP3), and the downstream mixed lineage kinase domain-like protein (MLKL) [44–46].

Although multiple pathways can contribute to hepatocyte cell death in the context of ethanol exposure, tumor necrosis factor-α (TNF)-induced cell death has been the most well studied. After TNF binds to its receptor (TNFR1), it recruits downstream factors (see Figure 1)[47]. Formation of specific down-stream effector complexes determines whether TNF induces hepatocyte survival, apoptosis or necroptosis[47]. Recent reviews have summarized mechanisms that regulate formation of these effector complexes [48, 49]. RIP1 and RIP3 could have multiple roles in regulating apoptosis, necroptosis, cell survival, and inflammation (Figure 1) [50–52].

Because TNF mediates ethanol-induced hepatocyte death, as well as inflammation, its pathway has been a longstanding therapeutic target for human ALD. Unfortunately, results from studies of mice were not translated into success in clinical trials. In fact, inhibition of TNF in patients with ALD had detrimental effects. Findings from mouse studies are difficult to translate to humans. We require a more detailed understanding of TNF signaling pathways —particularly in the context of alcohol-induced steatosis and inflammation.

Strategies to disrupt apoptosis have also been tested in clinical trials—trials with caspase inhibitors are in planning stages. However, in contrast to TNF, results of studies of early stages of steatosis or inflammation do not uniformly indicate that apoptosis contributes to liver injury. Although hepatocyte apoptosis has been observed in mice fed alcohol and in humans with ALD, inhibition of apoptosis either by disruption of the *Bid* gene or a pharmacologic pan-caspase inhibitor (VX166) did not attenuate alcohol-induced liver injury and steatosis in mice [53]. Therefore, other forms of cell death are also involved in the pathogenesis of ALD. Increased levels of RIP3 protein were found in mouse livers after chronic ethanol feeding, and in livers of alcoholic patients [54]. Moreover, RIP3-knockout mice have less liver injury, steatosis, and inflammation than control mice after chronic ethanol feeding [54]. Studies of mice with liver-specific disruption of *Rip1* or *Rip3* mice, fed with alcohol, could help clarify the role of RIP1 and RIP3 in alcohol-induced necrosis and liver pathogenesis.

How would apoptosis and necrosis occur during the pathogenesis of ALD if apoptosis normally suppresses necrosis, via caspase-mediated cleavage of RIP3? Liver has unique

zones with different levels of oxygen, nutrients, and metabolic enzymes. Induction of autophagy varies among the different zones of mouse liver after acetaminophen administration [55]. It is therefore possible that apoptosis and necrosis also occur in different zones of the liver. Future studies are needed to elucidate these processes.

In addition to the mutual regulation of apoptosis and necrosis, autophagy and cell death can regulate each other. Many cell death stimuli induce cell death (apoptosis and necrosis) and autophagy at the same time. Autophagy might protect against ALD by selectively removing damaged organelles (such as mitochondria) and lipid droplets, as well as by releasing ER stress [27, 34]. Although autophagy can protect against cell death, apoptosis can suppress autophagy, inducing caspase-mediated cleavage of essential autophagy proteins such as Beclin 1 [56]. Moreover, RIP1 also represses basal autophagy by activating ERK and subsequently inhibits TFEB-mediated expression of autophagy-related and lysosome genes [57].

So, there is a complicated regulatory network among autophagy, apoptosis, and necroptosis. After alcohol exposure, a cell's fate is decided by the balance of autophagy vs apoptosis, necroptosis, or mitochondrial stress, as well as UPR vs ER stress. Disruption of the balance from cell survival (UPR and autophagy) towards cell injury (apoptosis or necroptosis) eventually leads to liver injury after alcohol exposure (Figure 2). Therefore, despite the negative results observed with TNF inhibitors in clinical trials of patients with ALD, studies of the pathways of hepatocyte apoptosis and necrosis will continue to provide new therapeutic targets. These might include strategies to inhibit caspases or factors in the TNF pathway that mediate liver injury.

Interactions Between Liver and Intestine

High-dose acute and chronic consumption of ethanol can damage the intestinal mucosa, causing loss of epithelial cells from villi tips and hemorrhagic erosions in the lamina propria [58]. Ethanol also affects the diversity of the intestinal microbiome (see Bajaj and Gillevet, ref XX). Junction proteins, including those in tight and adherens junctions, are disassembled by alcohol and acetaldehyde [59,59]. Alterations include redistribution of occludin and zonula occludens from intercellular junctions and dissociation from the actin cytoskeleton. Other alterations include disruption of the adherens junctions via redistribution of E-cadherin and β-catenin from intercellular junctions [59]. Ethanol can also modulate the peri-junctional actin and myosin filaments through activation of myosin light-chain kinase [58]. Ethanol activates nuclear factor (NF)-kB, leading to instability of the F-actin cytoskeleton and disruption of the intestinal barrier [58]. Additionally, ethanol initiates barrier dysfunction by affecting the transcription of intestinal circadian clock genes and activity of microRNAs [58]. Ethanol metabolism also induces oxidative stress, partly by increasing expression of CYP2E1, which increases gut permeability by damaging cells and modifying proteins [58, 60].

Disruption of the tight junction protein complex has pathologic potential, because it allows exogenous and endogenous agents to cross the epithelial barrier and initiate or perpetuate systemic inflammation. Experimental animal models of acute and chronic ethanol exposure

have associated increased permeability to gastrointestinal macromolecules and pathogen-associated molecular patterns with inflammatory conditions and organ injury [60–62]. Alcoholic patients without advanced-stage liver disease have increased intestinal permeability to macromolecules, so increased gut permeability appears to be caused by ethanol, rather than as a consequence of advanced alcoholic liver disease [63]. Drugs are being developed to reduce gut permeability for treatment of a number of luminal diseases, such as celiac disease and inflammatory bowel disease. These might also be used in patients with early-stage ALD, and reduce or reverse disease progression before severe liver injury and inflammation.

Hepatocyte Signaling in Response to Injury

When hepatocytes are injured or stressed, they communicate with other cells in their local environment, as well as to more distant organs, such as the bone marrow. These signals facilitate a protective or wound-healing response. We review some of the mechanisms by which hepatocytes communicate injury and coordinate either maintenance of a healthy liver or progression of chronic disease (see Figure 3).

Release of danger-associated molecular patterns (DAMPs)

Injured and dying hepatocytes release DAMPs (metabolites, proteins, carbohydrates and organelles), which signal to surrounding cells (Figure 3). DAMPs are primarily released from necrotic, necroptotic, or pyroptotic cells, but also undergo regulated release [64]. DAMPs can have paracrine or endocrine effects within the hepatic sinusoid, where they interact with immune cells, liver endothelial cells, and hepatic stellate cells (HSCs). For example, ethanol exposure results in the release of high mobility group box 1 (HMGB1) from hepatocytes; [65] disruption of *Hmgb1* in hepatocytes of mice prevents ethanol-induced hepatic steatosis [66]. HMGB1 also recruits HSCs and liver endothelial cells to sites of ethanol-induced injury [65].

Although apoptotic hepatocytes release fewer DAMPs than necrotic cells, they can activate quiescent HSCs [67]. ATP and uric acid, released from hepatocytes in response to ethanol, activated inflammasomes in immune cells in the hepatic sinusoid [68]. In humans, a single dose of alcohol increased serum levels of ATP and uric acid, indicating that DAMPs also signal to distant organs [68]. Agents that target the DAMP-mediated signaling may be effective at limiting the transition from hepatocyte injury to exacerbated inflammation and fibrosis in patients with ALD. For example, antagonists of the interleukin 1 beta (IL1B) receptor, such as anakinra and ramonaband, which broadly target inflammasome activity, are under evaluation in patients with alcohol-associated hepatitis.

Cytokine and chemokine signaling

Hepatocytes also produce cytokines and chemokines. Although immune cells produce the greatest levels of cytokines and chemokines, under some conditions, hepatocytes release IL1B and IL18, by upregulating components of the inflammasome [69, 70]. Hepatocytes also produce other inflammatory cytokines, including TNF and IL6. Chemokine (C-C motif) ligand 2 (CCL2 or MCP1) is a hypoxia-sensitive chemokine produced by immune cells and hepatocytes in response to chronic ethanol exposure [71, 14]. MCP1 also acts as a steatokine,

increasing the accumulation of triglycerides in hepatocytes via the liver X receptor (nuclear receptor subfamily 1, group H, member 3) α [72]. Many compounds have been identified that target specific chemokines, but these have not been tested in trials of patients with ALD.

Extracellular vesicles

Recent studies have focused on extracellular vesicles as a mechanism to mediate alcohol-related liver injury [73, 74]. Extracellular vesicles include exosomes, microparticles, and apoptotic bodies; these are distinguished based on size and marker proteins [75]. Ethanol stimulates release of extracellular vesicles from hepatocytes in vivo and in vitro, [73] and extracellular vesicles are increased in blood samples of patients with alcoholic hepatitis [73, 74]. Putative extracellular vesicle-associated molecules include microRNAs and proteins, such as CD40 ligand; these might contribute to inflammatory responses that develop after alcohol-induced hepatocyte injury. Strategies to prevent release of extracellular vesicles, such as those that target exocytic actin, the molecular motor machinery, or specific molecules within extracellular vesicles, might be developed as therapeutics.

Innate Immune Responses to Alcohol and Injured Hepatocytes

The innate immune response is activated during acute and chronic exposure to ethanol and contributes to alcoholic liver disease. It is a relatively low-grade form of inflammation that does not resolve quickly. Ethanol intake affects many cellular and soluble components of the innate immune system.

Kupffer cells and macrophage

Kupffer cells, which are macrophages of the liver, are important mediators of ALD. Following short-term and chronic exposure to alcohol, receptor-dependent signal transduction is dysregulated in these cells. Kupffer cells become sensitized to LPS and signal via TLR4, increasing production of inflammatory cytokines, chemokines, and other mediators of inflammation (reviewed in [76], see Figure 4). Production of these inflammatory mediators is regulated at transcriptional and post-transcriptional levels, including mRNA stability and cytokine secretion.

MicroRNAs determine the sensitivity of Kupffer cells to LPS [77]. For example, expression of microRNA155 is up-regulated in Kupffer cells and contributes to ethanol-induced stabilization of *TNF* mRNA [78]. Multiple microRNAs are affected by ethanol; [17, 79] studies should address the role of additional microRNAs in regulation of inflammatory responses to ethanol.

Although responses of Kupffer cells to pattern-associated recognition receptors have been well studied, less is known about the sensitivity of Kupffer cells to DAMPs. Given our growing knowledge of the types of DAMPs released by injured hepatocytes during ethanol exposure, studies of DAMP-mediated responses in Kupffer cells will be important.

The relative expression of inflammatory vs anti-inflammatory mediators depends, at least in part, on the state of Kupffer cells and polarization of macrophages in the liver. Alcohol shifts the balance of the M1 vs the M2 phenotype towards M1, characterized by increased

production of inflammatory cytokines and ROS [80, 81]. The mechanisms of this shift are not well understood, but metabolic reprogramming via NOTCH1 signaling has been implicated in ethanol-induced on M1 polarization [82]. Although gamma-secretase inhibitors inhibit the NOTCH pathway, researchers are developing more-selective agents that target NOTCH. Agents such as antibody-tagged corticosteroids, which selectively target macrophages, are also under evaluation and might reduce hepatic inflammation in patients with ALD.

Anti-inflammatory pathways

Multiple anti-inflammatory mediators control the resolution of inflammation [83, 84]. Chronic ethanol exposure may impair the ability of Kupffer cells to activate and/or respond to anti-inflammatory mediators. For example, the sensitivity of Kupffer cells to anti-inflammatory mediators that act via cAMP, such as adenosine and PGE_2 is decreased in chronic ethanol-fed rats [85] and associated with an increase in PDE4. Adiponectin is another anti-inflammatory molecule that may be decreased after chronic ethanol feeding [86]. Incubation of Kupffer cells from ethanol-fed rodents with adiponectin normalizes TLR4-medated signaling via MYD88-dependent and -independent pathways, via induction of heme-oxygenase 1 [87, 88]. Compounds such as PDE4 inhibitors, which promote resolution of inflammation, are being tested in trials of ALD.

Recruitment of immune cells to the liver

Although resident macrophages are important regulators of innate immune responses during the progression of ALD, peripheral monocytes and neutrophils are also recruited to the liver during disease progression. They are recruited by a variety of chemokines, which have salutary as well as detrimental effects. For example, macrophage inhibitor factor (MIF), a pluripotent cytokine and chemokine, recruits peripheral Ly6C+ monocytes to livers of mice during development of chronic ethanol-induced injury and carbon tetrachloride-induced fibrosis [89, 90]. MIF-mediated recruitment of monocytes contributes to inflammation and damage, but protects against fibrosis. This is likely because these cells activate scar-associated macrophages, which promote resolution of fibrosis [89, 91]. During alcohol-induced liver injury, chemokines of CXC and CC families recruit neutrophils, monocytes, and T and B cells [71, 92]. Adhesion molecules, such as E-selectin, also mediate infiltration of the liver by neutrophils during ethanol exposure [93].

Adaptive Immune Responses

Alcohol can impair the adaptive immune system, depending on the severity of ALD [94]. Patients with advanced ALD have circulating T cells and antibodies against epitopes generated by hydroxyethyl free radicals, produced during ethanol oxidation, as well as end products of lipid peroxidation [95]. IL17-producing T helper cells contribute to development of ALD by promoting infiltration of liver by neutrophils [96]. Regulatory T cells might also mediate progression of ALD—patients with alcohol-associated hepatitis have reduced numbers of circulating regulatory T cells [97].

Dendritic cells and natural killer (NK) cells

Hepatic dendritic cells are important antigen presenting cells in liver. Chronic ethanol consumption disrupts dendritic cell function, likely contributing to impaired cellular immune responses. For example, myeloid dendritic cells isolated from ethanol-fed mice have enhanced interleukin (IL) 1β and IL-10 production, but decreased secretion of TNF, IL12, interferon gamma, and IL6 [98, 99]. Similarly, myeloid dendritic cells generated in presence of ethanol produce increased amounts of IL10 production and express lower levels of the co-stimulatory molecules CD80 and CD86, compared to myeloid dendritic cells not exposed to ethanol[100].

NK regulate development liver fibrosis as well as hepatic immune tolerance. NK cells prevent fibrosis by killing activated stellate cells. Depletion of NK cells increases experimental liver fibrosis in mice [101]. NK cells indirectly maintain hepatic tolerance through interactions with dendritic cells [102]. However, NK cells also contribute to neutrophil infiltration of liver in mouse models of ALD. [103] Early-phase clinical trials are being designed to alter activities of NK cells in patients with ALD.

Fibrogenesis

Fibrogenesis culminates from alcohol-induced hepatocyte injury and its associated immune response. Mechanisms of fibrogenesis in ALD are probably similar to those of other fibrogenic liver diseases in that they are likely to be mediated by activated HSCs. During fibrogenesis, HSCs migrate, proliferate, and increase matrix deposition. HSC activation occurs via multiple pathways (see [104]), although some of these might be more specifically associated with development of ALD compared to in other diseases. For example, alcohol-induced dysbiosis of the intestinal microbiota and increased gut permeability appear to selectively activate the TLR4 signaling pathway, which leads to HSC activation [105]. Acetaldehyde has also been implicated in HSC activation and collagen expression.

Our mechanistic understanding of fibrogenesis in response to alcohol-related liver injury has been hindered by the lack of animal models that recapitulate human disease. Nonetheless, studies have identified several potential pathways for therapeutic intervention. For example, IL22, a hepato-protective cytokine produced by inflammatory cells that acts on hepatocytes and HSC [106], attenuates HSC activation and matrix production. Macrophages regulate fibrosis and may be therapeutic targets. Macrophages acquire a phenotype that promotes inflammation (polarization) in response to alcohol [107]. However, alternative phenotypes of macrophages, such as scar-associated macrophages, are important for fibrosis resolution [108]. The precise roles of macrophages in fibrogenesis and fibrosis resolution in the context of ALD require further study. Studies of natural killer cells indicated these cells may be an interesting target for therapy of alcohol-related fibrogenesis [109]. Finally, cell signaling within the hepatic sinusoid is implicated in fibrogenesis, including communication via molecules contained within extracellular vesicles . Interestingly, some contents of extracellular vesicles can activate HSC, whereas other cargo promote their quiescence [110].

Clinical Manifestations and Pathogenesis

Clinical trials.gov lists over 100 clinical trials related to ALD. However, we know little about the genetic and epigenetic factors that determine why some individuals who drink in excess do not develop histologic lesions beyond steatosis. Nonetheless, the increase in clinical trial activity is encouraging, given the dearth of trials for this disease in prior years.

From our understanding of the molecular and cellular pathogenesis of the disease, it is clear that the progression of ALD can be distinguished from other forms of chronic liver injury, except for non-alcoholic steatohepatitis (NASH), which produces liver lesions similar to those found in patients with ALD, based on histopathologic analysis. ALD is believed to progress from steatosis to inflammation and eventually to fibrosis. Histologic features of steatosis can be detected even after short alcohol binges by healthy individuals. Histologic markers of inflammation, which indicate alcoholic hepatitis, include Mallory hyaline, mega-mitochondria, balloon degeneration of hepatocytes, and infiltration by polymorphonuclear leukocytes. Advanced-stage disease is characterized by fibrosis. In reality, many of these stages of ALD occur concurrently. However, in many patients with advanced fibrosis, early-stage lesions can no longer be detected by histologic analysis, so the alcohol-associated etiology might not be obvious. What strategies are being developed to target these specific lesions? For more comprehensive reviews focused see [111],[112].

Steatosis is the earliest histopathologic marker of ALD. It cannot distinguish patients from ALD with those from non-alcoholic liver disease, so excess alcohol consumption must be confirmed. For patients with this lesion, interventions aimed at decreasing harmful drinking behaviors are an important first step. Ethanol metabolism mediates steatosis and subsequent injury. Increased oxidative stress, due, at least in part, to ethanol metabolism, as well as alcohol-induced changes in gut integrity and microbiome might also be targeted at this early stage. In fact, the link between gut permeability and pathogenesis of liver disease is more established for ALD than for other liver diseases. Therapeutic strategies to target these processes include neutralization of gut bacterial products (endotoxin), restitution of intestinal permeability (with zinc), and realignment of changes in microbiome (with probiotics or antibiotics) [111]. Increasing knowledge of how lipid droplet biology contributes to steatosis is likely to lead to therapies that act on steatosis itself, although steatosis itself might not have any detrimental effects on liver function.

As disease progresses, the situation becomes more complex. Inflammation spirals out of control, and hepatocyte injury and death continue. Agents that block a single inflammatory cytokines, such as TNF, have not been shown to be effective in patients with ALD. This is probably because most cytokines have inflammatory and hepato-protective activities. TNF is required for liver regeneration and protection from infection, which are each required for recovery from any severe liver insult. In fact, stimulating regeneration could be an important and understudied approach for treatment of ALD. Similarly, steroids, which reduce inflammation systemically, increase the risk of infection and sepsis.

Strategies aimed at decreasing hepatocyte cell death, by either apoptosis or necroptosis, are also of interest, but will need to be developed for specific stages and cell types. For example,

agents that prevent programmed cell death of hepatocytes might also prevent apoptosis of immune cells, which would exacerbate inflammation, or prevent death of activated HSCs, which would promote fibrosis.

Recent preclinical studies have focused on the cytokine IL22 [113, 114]. IL22 is produced by inflammatory cells and induces regeneration of hepatocytes via STAT proteins. IL22 might therefore be developed to promote hepatocyte regeneration in patients with ALD. IL22 has also been proposed to prevent HSC activation. Its lack of inhibitory effect on inflammatory cells indicates that it may not induce immunosuppression, although there have no clinical studies of IL22's effects.

Another compound of interest is anakinra, which is already approved for treatment of rheumatologic disorders. Anakinra disrupts IL1B signaling, which is required for induction of sterile necrosis, in which hepatocyte injury leads to recruitment of inflammatory cells. Studies of this compound are underway in patients with alcoholic hepatitis.

Future Directions

Unregulated inflammation stimulates a dysregulated wound healing response that leads to the development of fibrosis and eventual cirrhosis in response to chronic heavy ethanol consumption. In humans, long-term abstinence can attenuate this process. Potential therapies for early-stage ALD could overlap with those for liver cirrhosis of other etiologies. With the increasing prevalence of NASH, a number of trials are underway to target NASH-induced fibrosis. These provide an opportunity to evaluate compounds that might have effects in patients with ALD as well, given their similarities in histopathology. One example is the FXR agonist obeticholic acid [115]. This agent has been shown to have effect in patients with NASH. In mouse models, it prevents and even induces regression of fibrosis and reduces portal hypertension.

Our understanding of the mechanisms by which alcohol induced steatosis, inflammation, and fibrosis continues to grow at a rapid rate. New advances in animal models of ALD could more effectively guide identification of targets and development of new therapeutic agents for ALD. Combination therapies against multiple aspects of this complex disease might also be effective. These could include drugs that improve gut and kidney function, normalize (rather than eliminate) the immune response, and promote hepatocyte survival or regeneration. The National Institute on Alcohol Abuse and Alcoholism has increased support for clinical trials of ALD, which could lead to new treatments for ALD.

Acknowledgments

ABBREVIATIONS

ADH	alcohol dehydrogenase

ALDH1	aldehyde dehydrogenase 1
NAD	nicotinamide adenine dinucleotide
CYP2E1	cytochrome P450 family 2, subfamily E, polypeptide 1
ROS	reactive oxygen species
LPS	lipopolysaccharide
TNF	tumor necrosis factor-α
ALD	alcoholic liver disease
HIFs	hypoxia inducible factors
SREBP1	sterol regulatory element-binding protein-1
PPARGC1A	proliferator-activated receptor gamma, coactivator 1 alpha
ER	endoplasmic reticulum
UPR	unfolded protein response
ERAD	ER-associated protein degradation
IKK	I-κB kinase
IRF3	interferon regulator factor 3
RIP	receptor-interacting protein kinase
MIF	macrophage inhibitor factor
MLKL	mixed-lineage kinase domain-like protein
SIRT1	sirtuin 1
TNFR1	TNF receptor 1
TRADD	TNFR-associated death domain
TRAF2	TNFR-associated factor 2
cIAP1	cellular inhibitor of apoptosis proteins 1
TAK1	transforming growth factor β-activated kinase 1
FADD	fas-associated protein with a death domain
CFLAR	CASP8 and FADD-like apoptosis regulator
TAB2	TGF-beta activated kinase 1/MAP3K7 binding protein 2

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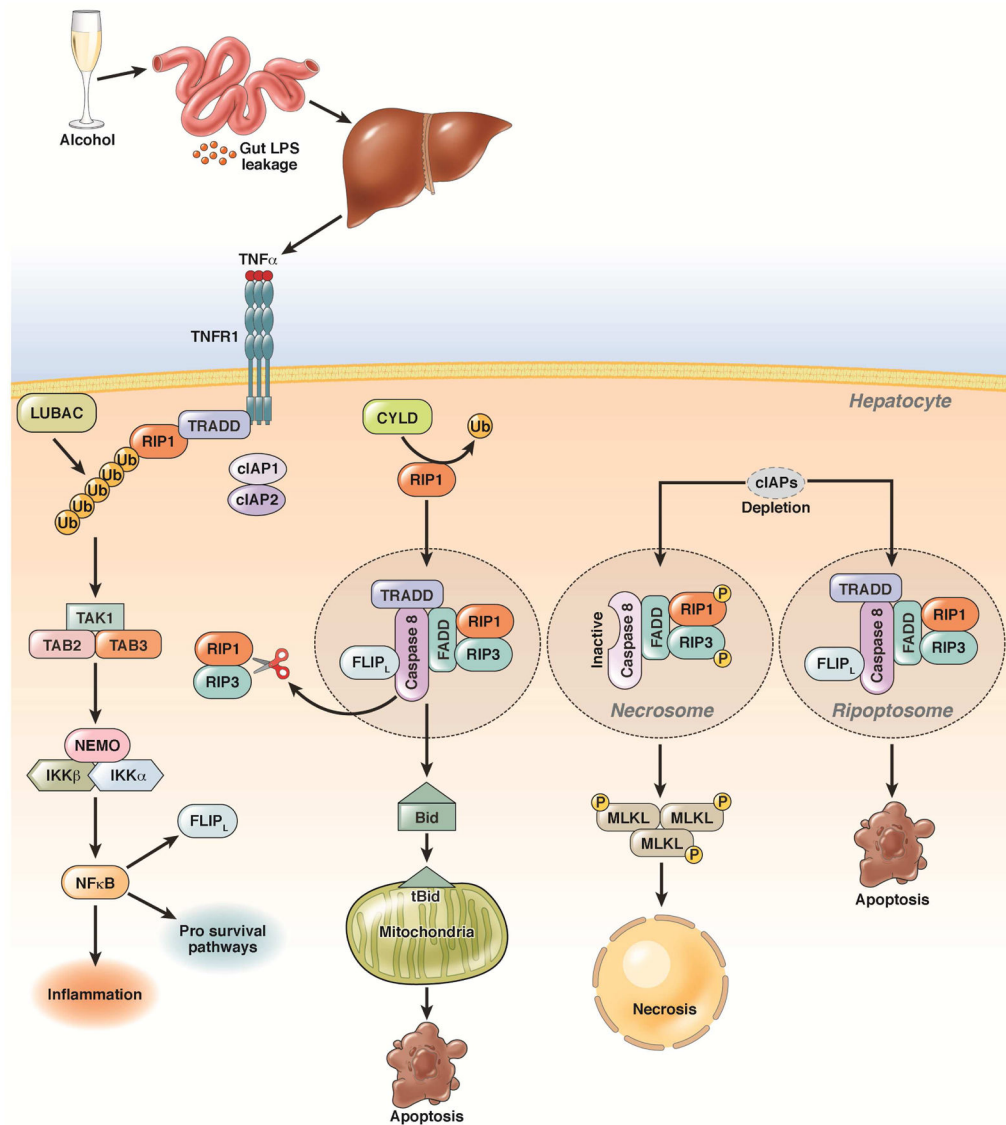
Figure 1. Pathways of alcohol-induced apoptosis and necrosis

Alcohol consumption increases intestinal permeability resulting in increased influx of LPS into the liver. LPS activates Kupffer cells to produce TNF. TNF binds to its receptor (TNFR1), which recruits TNFR-associated death domain (TRADD), RIP1, cellular inhibitor of apoptosis proteins 1 (cIAP1/2), and linear ubiquitin chain assembly complex (LUBAC) and promotes the linear ubiquitination of RIP1. This leads to activation of NF-κB by the ubiquitin chain-dependent recruitment of transforming growth factor beta-activated kinase 1/ MAP3K7 binding protein 2 (TAB2) and 3 (TAB3), transforming growth factor beta-activated kinase 1 (TAK1), and the I-κB kinase (IKK) complex. CYLD removes the ubiquitin chain from RIP1, and de-ubiquitinated RIP1 interacts with TRADD, fas-associated protein with a death domain (FADD), caspase-8, and the CASP8 and FADD-like apoptosis regulator (CFLAR), resulting in caspase-8 activation. Activated caspase-8 cleaves Bid to activate the mitochondrial apoptotic pathway and induce apoptosis; it also cleaves RIP1 and RIP3 to inactivate RIP1–RIP3-mediated necroptosis. When cIAPs are depleted and

caspase-8 is inhibited, RIP1 and RIP3 interact via RHIM domains to from the amyloid-like necrosome. Auto- and trans-phosphorylated RIP1 and RIP3 then recruit downstream MLKL to initiate necroptosis. In the absence of cIAPs, RIP1, RIP3, TRADD, caspase-8, and CFLAR form a complex called the ripoptosome, which activates caspase-8 and apoptosis and requires RIP1 kinase activity.
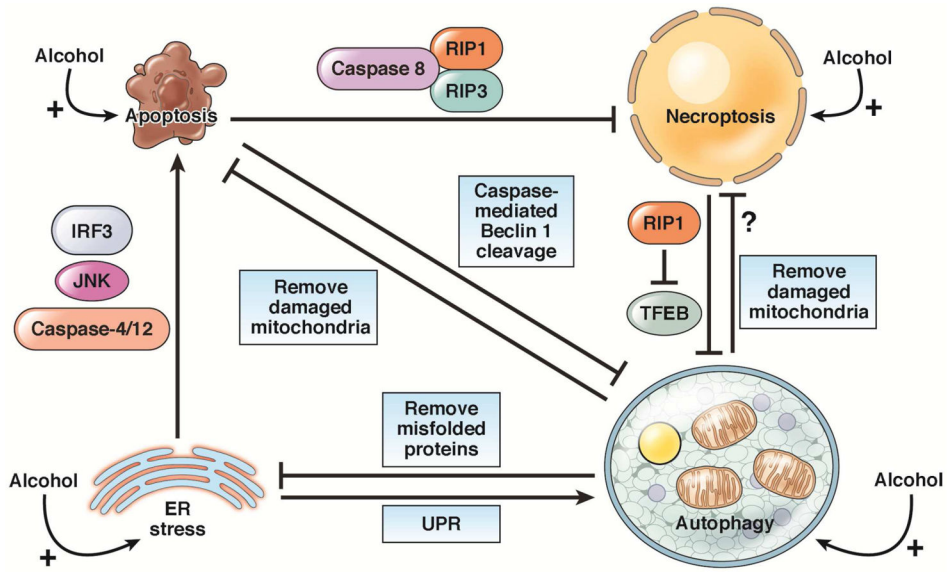
Figure 2. Interactions among apoptosis, necrosis, ER stress, and autophagy
Apoptosis suppresses necroptosis via caspase-8 cleavage and inactivation of RIP1 and RIP3.
Apoptosis also inhibits autophagy by caspase-mediated cleavage of Beclin 1. The
necroptosis protein RIP1 negatively regulates TFEB to repress autophagy. ER stress induces
apoptosis by activating caspase-12 and -4, JNK, and the interferon regulator factor 3 (IRF3)
signaling pathway. ER stress can also induce autophagy via UPR signaling. Autophagy
inhibits apoptosis and necrosis by removing damaged mitochondria. Autophagy also relieves
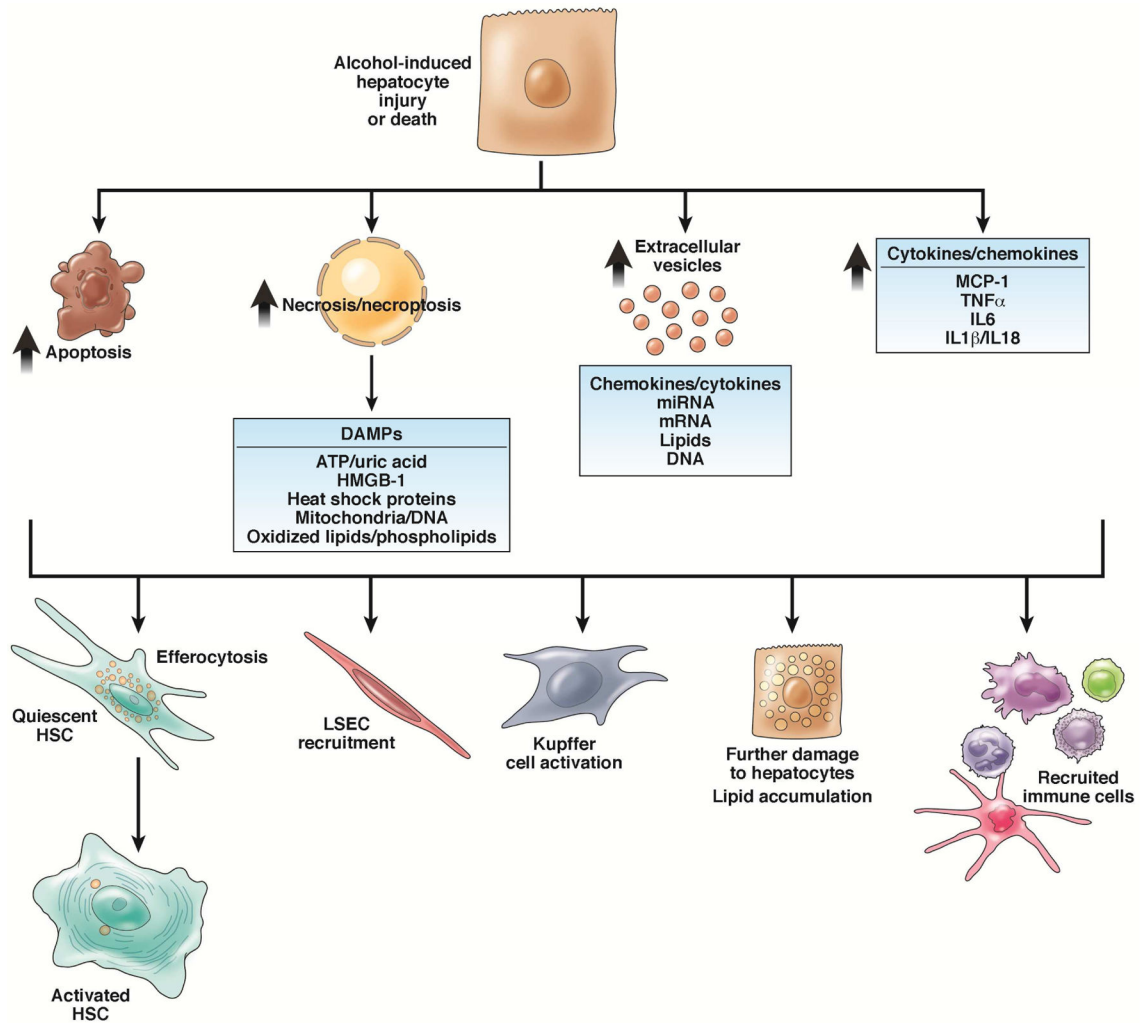ER stress by degrading misfolded proteins and protein aggregates.

Figure 3. Injured hepatocytes signal to cells within the hepatic sinusoid

Cells within the hepatic sinusoid respond to hepatocytes dying via apoptosis and necrosis/necroptosis, either via efferocytosis or phagocytosis of apoptotic bodies or DAMP-mediated signaling. Injured hepatocytes can also signal to their surrounding environment via the release of extracellular vesicles, containing a number of regulatory factors, or the direct expression of cytokines and chemokines.
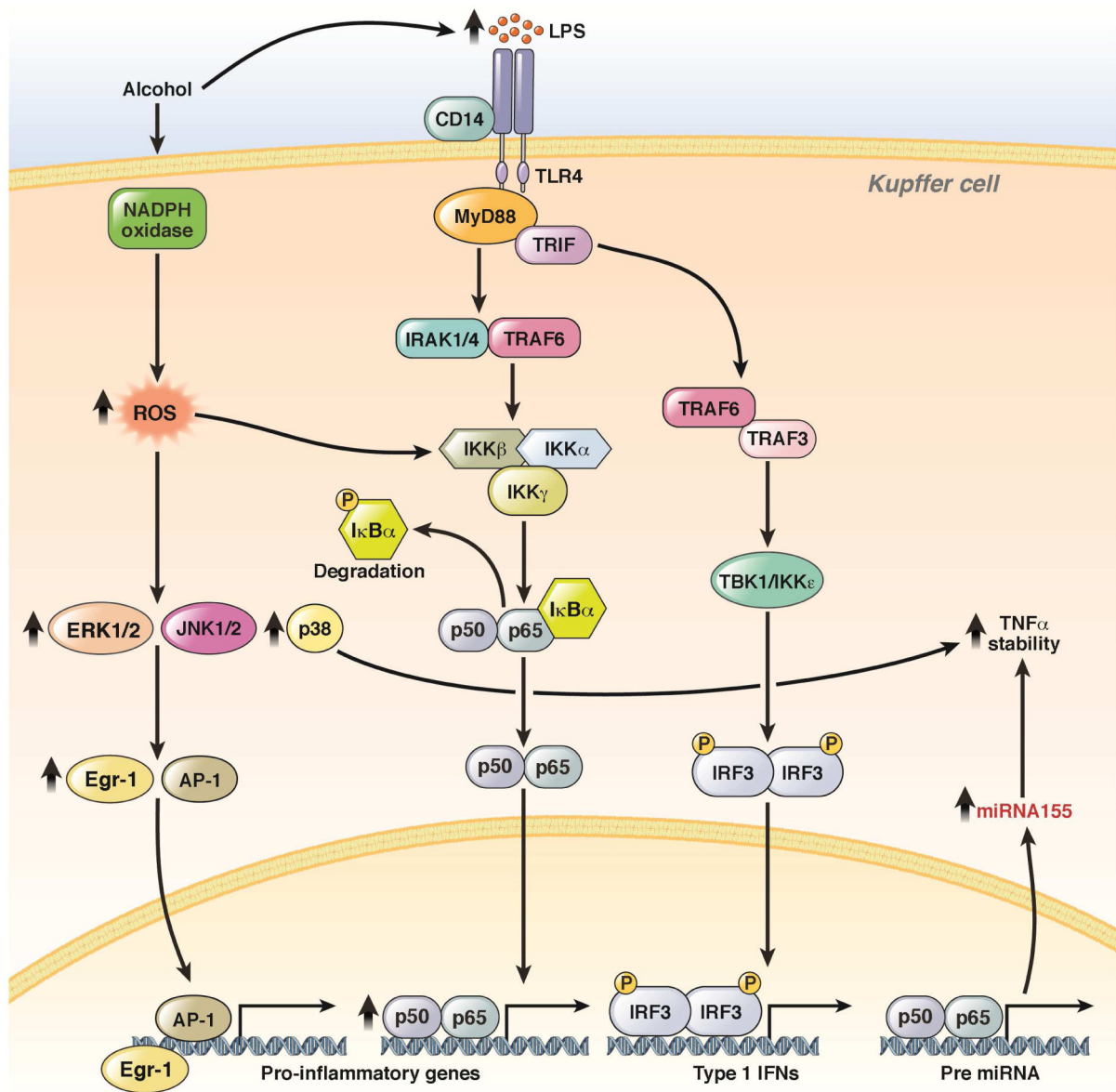
Figure 4. Sensitization of TLR4-mediated signal transduction in hepatic macrophages
Chronic ethanol feeding not only increases the amount of circulating gut-derived endotoxin (LPS), but also sensitizes Kupffer cells to TLR4-mediated signaling via MyD88-dependent and –independent pathways. These processes increase expression of TLR4 and increase free radical formation by NADPH oxidase and CYP2E1. Increased ROS then increases TLR4-mediated signaling via MyD88-dependent activation of MAPKs and NF-κB. These signaling pathways integrate to increase transcription of cytokines, as well as the mRNA stability of TNF. This sensitization depends on ethanol-induced changes expression of microRNAs—particularly related to the stabilization of TNF mRNA. MyD8-independent signaling pathways activate STAT transcription factors and promote interferon production to regulate other immune cells in the liver.