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MYOFIBROBLAST (TATIANA KISSELEVA, SECTION EDITOR)

New Approaches for Studying Alcoholic Liver Disease

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Abstract Alcoholic liver disease (ALD) is major cause of chronic liver injury which results in liver fibrosis and cirrhosis. According to the surveillance report published by the National Institute on Alcohol Abuse and Alcoholism, liver cirrhosis is the 12th leading cause of death in the United States with 48 % of these deaths being attributed to excessive alcohol consumption. ALD includes a spectrum of disorders from simple steatosis to steatohepatitis, fibrosis, and hepatocellular carcinoma. Several mechanisms play a critical role in the pathogenesis of ALD. These include ethanol–induced oxidative stress and depletion of glutathione, pathological methionine metabolism, increased gut permeability and release of endotoxins into the portal blood, recruitment and activation of inflammatory cells including bone marrow-derived and liver resident macrophages (Kupffer cells). Chronic alcohol consumption results in liver damage and activation of hepatic stellate cells (HSCs) and myofibroblasts, leading to liver fibrosis. Here we discuss the current view on factors that are specific for different stages of ALD and those that regulate its progression, including cytokines and chemokines, alcohol-responsive intracellular signaling pathways, and transcriptional factors. We also review recent studies demonstrating that alcohol-mediated changes can be regulated on an epigenetic level, including microRNAs. Finally, we discuss the reversibility of liver fibrosis and inactivation of HSCs as a potential strategy for treating alcohol-induced liver damage.

Keywords Alcoholic liver disease · Activated myofibroblasts · Hepatocellular carcinoma · Innate immunity · Adaptive immunity

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Abbreviations

Col	Collagen α1(I)
α-SMA	α-Smooth muscle actin
qHSCs	Quiescent hepatic stellate cells
aHSCs	Activated hepatic stellate cells
iHSCs	Inactivated hepatic stellate cells

Introduction

Alcoholic Liver Disease (ALD)

Chronic alcohol use is a major cause of cirrhosis and liver failure and is the 12th leading cause of death in adult

patients in the United States [1••]. ALD progresses from a healthy liver, to steatosis, alcoholic steatohepatitis, fibrosis, and finally cirrhosis, and hepatocellular carcinoma (HCC) [2]. Histologically it is manifested by hepatocyte steatosis, ballooning and apoptosis, lobular inflammation, deposition of extracellular matrix (ECM) and cirrhosis with formation of regenerative nodules [2, 3]. Development of alcohol-induced liver cirrhosis is associated with high levels of proinflammatory and profibrogenic cytokines, increased portal hypertension [2–4], hepatocyte dysplasia, HCC, and hepatic failure. However, only 35 % of alcoholics develop advanced ALD, suggesting that other co-factors are also important for ALD progression. These risk factors include sex, obesity, drinking patterns, genetic and metabolic factors, and cigarette smoking [1••]. Females are more susceptible to alcohol-induced liver damage. Obesity is an important risk factor that synergistically facilitates alcohol-induced damage of hepatocytes, modulating the response of the endoplasmic reticulum and mitochondria to injury and stress, promoting activation of pro-inflammatory macrophages, and causing resistance to insulin and adiponectin [1••, 5••, 6]. Recently identified genetic factors include genetic polymorphism of patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), whose expression affects the development of alcoholic cirrhosis in patients with ALD [1••, 7–9]. This PNPLA3 polymorphism is also a risk for NAFLD, suggesting a connection between NAFLD and ALD. Finally, environment and dietary habits can also affect ALD progression in alcoholics. Several injury-triggered events play a critical role in the pathogenesis of ALD, which are discussed below.

Stages of ALD

Alcoholic Fatty Liver

Alcoholic fatty liver is an early response to alcohol consumption, develops in more than 90 % of heavy drinkers. Early-mild steatosis occurs in zone 3 (perivenular) hepatocytes; it can also affect zone 2 and even zone 1 (periportal) hepatocytes during ALD progression [1••]. Fatty liver is characterized by the accumulation of fat droplets (mainly triglycerides and phospholipids) in hepatocytes. Alcoholic fatty liver results from a direct (via acetaldehyde) or indirect (via up-regulation of cytochrome P450 2E1) effect of ethanol on up-regulation of SREBP-1c and down-regulation of PPAR-α expression, leading to the induction of fatty acid synthesis and inhibition of β-oxidation [1••, 10]. Alcohol consumption could directly increase transcription of SREBP-1c gene via its metabolite acetaldehyde [11] or indirectly up-regulate SREBP-1c expression and processing, by activating processes and factors that stimulate SREBP-1c expression, such as the endoplasmic reticulum response to cell stress [12, 13], adenosine [14], endocannabinoids [15, 16], LPS signaling via Toll-like receptor (TLR) 4, and its downstream mediators, including IRF-3, Egr-1, or tumor necrosis factor (TNF)-α [17–19, 20••] (reviewed in [1••]). Alcohol also down-regulates factors that reduce SREBP-1c expression, such as AMP-activated protein kinase (AMPK) and Sirtuin1 [21, 22], adiponectin [23, 24], and signal transducer and activator of transcription 3 (STAT3) [20••, 25••, 26, 27] Consistent with this notion, *SREBP-1c* knockout mice are protected from alcohol-induced fatty liver [27, 28]. In addition several other genes have been identified to play a critical role in regulation of fatty liver disease; and the following knockout mice: HIF-$1^{-/-}$ [29], $C3^{-/-}$ [30], $C1qa^{-/-}$ [31], $PKC\varepsilon^{-/-}$ [32], and $iNOS,^{-/-}$ [33] develop less steatosis, demonstrating the contribution of these molecules to the pathogenesis of alcoholic fatty liver [1••]. Finally, autophagy has an important role in regulation of steatosis in hepatocytes, and is inhibited during chronic alcohol consumption, resulting in inability to remove lipid droplets from damaged hepatocytes, but is activated during acute alcohol consumption, which may play a compensatory role in preventing acute alcohol-induced liver damage [1••, 20••, 34–36].

Pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) represent the products of intestinal bacteria and activate inflammasome signaling pathways that promote migration of innate immune and vascular cells to sites of injury. In contrast, the process by which injured cells release endogenous danger-associated molecular patterns (DAMPs) that recruit inflammatory cells in absence of infection has been referred to as "sterile inflammation" [37–40]. Recent studies have outlined the synergistic role of sterile inflammation in angiogenesis and HSC activation. High-mobility group box 1 (HMGB1) [41] is a pro-inflammatory mediator released from the nucleus of ethanol-damaged liver parenchymal cells that stimulates recruitment of HSC and liver endothelial cells (LEC) to the site of injury. HMGB1 is an intracellular DNA-binding protein expressed by all mammalian cells that in response to ethanol exposure is translocated from the nucleus of injured hepatocytes into the extracellular space. It interacts with various receptors including TLR2, TLR4, TLR9, and RAGE (receptor for advanced glycation end products) [40], but mediates its pro-fibrogenic signals via TLR4. HSCs and LEC are immediate responders to hepatocyte apoptosis that play a critical role in initiation a cascade of events leading to liver fibrosis in association with angiogenesis [42].

Alcoholic Steatohepatitis (ASH)

ASH is a syndrome characterized by infiltration of inflammatory cells and by hepatocellular injury. ASH, which develops in 10–35 % of alcoholic patients with steatosis, is

usually associated with progressive fibrosis, and is manifested by steatosis, centrilobular ballooning of hepatocytes, neutrophilic infiltration, and Mallory–Denk hyaline inclusions [1••, 43]. In response to chronic alcohol consumption, lipid peroxidation and mitochondrial damage cause apoptosis of hepatocytes, which triggers recruitment of inflammatory cells to the fatty liver, activation of bone marrow-derived and liver resident macrophages (Kupffer cells) and release of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α). Infiltrating neutrophils kill sensitized hepatocytes and further exacerbate alcohol-induced liver injury, which is a prominent feature of ASH. Up-regulation of IL-8, CXCL1 (Gro-α), and IL-17 in the liver contributes to neutrophil infiltration and correlates with the severity of ASH [43, 44]. IL-17 not only acts as a neutrophil chemoattractant, but also stimulates HSCs to produce IL-8 and CXCL1 to facilitate neutrophil recruitment [45, 46]. A rodent model of ASH has demonstrated a pivotal role of neutrophils in pathogenesis of ALD [47, 48••]. Besides neutrophils, T and B lymphocytes are also recruited to the damaged liver, suggesting that the adaptive immune response contributes to alcohol-induced liver damage and activation of myofibroblasts. In addition to a direct hepatotoxic effect, alcohol induces changes in intestinal microflora (dysbiosis), which results in increased intestinal permeability and translocation of bacterial products (LPS) into blood, facilitating activation of myofibroblasts [49••, 50].

Intestinal bacterial overgrowth is common in patients with ALD. Sequencing of bacterial 16S rRNA genes revealed a relative abundance of Bacteroidetes and Verrucomicrobia bacteria in mice fed alcohol compared with a relative predominance of Firmicutes bacteria in control mice. This was associated with downregulation of host gene and protein expression of bactericidal c-type lectins Reg3b and Reg3g in the small intestines [49••]. Alcohol not only causes enteric dysbiosis and bacterial over-growth, but also increases gut permeability and the translocation of bacteria-derived LPS from the gut to the liver [51]. Consistently, increased levels of LPS are observed in patients with ALD [50]. One mechanism by which LPS exacerbates fibrosis is by activation of the innate immune system in the liver. In Kupffer cells, LPS binds to TLR4 to activate the MyD88-dependent and independent (TRIF/IRF-3) signaling pathway, leading to production of oxidative stress and proinflammatory cytokines, including TNF-α, that cause liver inflammation and hepatocellular damage [5••, 52–54]. TLR4 and complement factors also cause Kupffer cells/recruited macrophage to produce cytokines such as interleukin (IL)-6 and IL-10 [2]. Other TLRs expressed by innate immune cells can recognize distinct PAMPs, including bacterial peptidoglycan, double-stranded RNA, and unmethylated DNA, and may also play a role in promoting liver inflammation in ALD.

Alcoholic Liver Fibrosis/Cirrhosis

Alcoholic liver fibrosis/cirrhosis is characterized by excessive accumulation of collagen and other ECM proteins, steatosis, and fibrosis, and is accompanied by release of the major pro-fibrogenic cytokines TGF-β1, mostly produced by bone marrow-derived macrophages and resident Kupffer cells [2, 55–58]. HSCs are the major source of myofibroblasts in hepatotoxic liver injury [59••]. Under physiological conditions, HSCs store Vitamin A and exhibit a quiescent phenotype (qHSCs), but in response to alcohol-induced injury, α-smooth muscle actin (α-SMA) and collagen Type I are upregulated [2]. LPS not only stimulates Kupffer cells/macrophages to produce ROS and TGF-β1, but also promotes HSC activation via direct binding to TLR4 [60]. Acetaldehyde is produced mainly by hepatocytes and acts on HSCs by directly increasing expression of collagen I [61, 62]. Activated natural killer cells inhibit liver fibrosis by causing apoptosis of activated HSCs [63] or producing interferon (IFN)-γ, which induces HSC growth arrest [64].

Regression of Liver Fibrosis

When the cause of liver injury is removed (e.g., alcohol cessation), hepatic fibrosis is reversible in both patients and rodents. Regression of fibrosis is associated with decreased cytokine and ECM production, increased collagenase activity, disappearance of the myofibroblast population and dissolution of the fibrous scar [2, 65]. Only recently has the fate of myofibroblasts during regression been revealed. The previous concept was that myofibroblasts undergo apoptosis on the basis of documented senescence and apoptosis of some aHSCs during reversal of fibrosis. We [66••] and subsequently others [67] have used genetic marking to demonstrate an alternative pathway in which myofibroblasts revert to a quiescent-like phenotype in CCl_4-induced liver injury. Of more relevance, we also demonstrated that experimental ALD regresses with the discontinuation of alcohol and that the aHSCs/myofibroblasts revert to an inactivated phenotype [66••].

Inactivation of HSCs (iHSCs) into a Quiescent-Like State During Regression of Liver Fibrosis

The Cre-*loxP*-system [68] provides a critical tool to study the fate of HSCs and their progeny in mice during fibrosis progression and/or regression. Genetic labeling of aHSCs/myofibroblasts results from crossing mice expressing recombinant Cre under control of collagen-α1(I) promoter ($Col\alpha1(I)^{Cre}$ mice) with reporter mice ($Rosa26^{f/f\text{-}YFP}$ mice, ubiquitously expressing a *yfp* gene in which transcription is blocked by a *floxed* Stop cassette) [66••]. In the offspring

mice (Colα1(I)$^{Cre\text{-}YFP}$ mice), genetic Cre-*loxP* recombination causes excision of the *floxP*-Stop-*floxP* sequence from genomic DNA and activation of YFP transcription in cells expressing type I collagen. Following induction of liver injury in these mice, aHSCs and their progeny are permanently labeled by YFP expression [66••]. Phenotypic changes of aHSCs and the mechanism of their inactivation can be now studied during regression of liver fibrosis.

Using this genetic labeling methodology, the fate of HSCs/myofibroblasts during recovery from alcohol-induced liver fibrosis has been elucidated. For the first time, it was demonstrated that half of the myofibroblasts escape apoptosis during regression of liver fibrosis, downregulate fibrogenic genes and acquire a phenotype similar to, but distinct from, quiescent (q)HSCs. Inactivated (i)HSCs more rapidly reactivate into myofibroblasts in response to fibrogenic stimuli and more effectively contribute to liver fibrosis [66••]. Inactivation of HSCs is associated with re-expression of lipogenic genes peroxisome proliferator-activated receptor gamma (PPAR-γ), Insig1 [66••], and CREBP [69]. The role of PPAR-γ in regulation of HSC activation in culture has been proposed. Ectopic over-expression of PPAR-γ results in the phenotypic reversal of activated HSCs to qHSC in culture, and upregulation of adipogenic transcription factors causing morphologic and biochemical reversal of activated HSCs to quiescent-like cells [69–72, 73•]. Findings from mouse models also demonstrate the importance of PPAR-γ for the maintenance of qHSCs, and inactivation of iHSCs during regression of liver fibrosis [66••].

Hepatocellular Carcinoma (HCC)

HCC is a malignant tumor made of cells resembling hepatocytes [74]. It usually arises in a cirrhotic liver [75, 76], and is identified by expression of alpha-fetoprotein (AFP), CD90, CD133, and EpCAM [77]. Several mechanisms have been suggested to contribute to HCC development in patients with alcoholic cirrhosis, including sustained inflammation, immunosuppressive effect of alcohol, impaired hepatocyte proliferation, loss of cell cycle checkpoints, increased tumor cell survival, telomere shortening and chromosomal instability. Accumulating evidence suggests that HCC originates from dedifferentiation and transformation of mature hepatocytes [76, 77]. Progression of HCC is associated with upregulation of IL-6 [78••, 79••], IL-17 [80, 81] and IL-22 [82] and constitutive activation of STAT32 [6, 83••]. Consistent with this, IL-6$^{-/-}$ and IL-22$^{-/-}$ mice are less susceptible to diethylnitrosamine (DEN)-induced HCC than wt mice. In addition to STAT3 [84], the NF-κB, Wnt/β-catenin, and Hedgehog signaling pathways are also implicated in HCC development [80, 85, 86••, 87••, 88]. There are some unique mechanisms that contribute to the development of HCC in patients with ALD [89–92]. These include the formation of acetaldehyde, which is a carcinogen with mutagenic properties, ethanol-stimulated induction of CYP2E1 which metabolizes alcohol, and the immunosuppressive effect of alcohol. LPS and chronic damage to hepatocytes can synergistically promote liver tumorigenesis via up-regulation of cancer stem cells as a mechanism of liver regeneration, indicated by analysis of stem cell markers [93••].

Mouse Models of ALD

A significant attempt to understand the mechanism of ALD development has been made over the past 20 years. However, this work has been hampered by the absence of suitable animal models. First, there are differences in time courses (weeks in mice vs years in human patients). Second, most models of chronic alcohol feeding in mice (such as the Lieber–Decarli diet) do not mirror the stages of ALD in patients. Dr. Gao's group developed a chronic-plus-binge ethanol feeding model, which induces significant liver inflammation and neutrophil infiltration [48••, 94••] (but no fibrosis), and was highly successful in analyzing the ALD stages of steatosis and steatohepatitis. Serum levels of about 250 IU/L ALT and 420 IU/L AST were reached 9 h post gavage, and correlated with increased expression of hepatic and serum inflammatory cytokines and hepatic oxidative stress. In addition, this model is improved in the intragastric model of ethanol feeding [95••], and achieved a significant level of liver fibrosis in mice after 2 months of injury [66••]. Therefore, the Tsukamoto-French model of alcoholic liver injury can be used to study alcoholic fibrosis [95••]. Using this model, specific components of the gastrointestinal tract and liver were identified as causative in alcohol-induced liver injury. The levels of endotoxin in the blood begin to rise after about two weeks of continuous intragastric administration of ethanol and correlate with histology of alcohol-induced liver injury [96]. Development of alcohol-induced liver injury causes profound changes in gut enteric microflora; and altering the enteric flora with lactobacillus or antibiotics reduces liver injury [96]. Inactivation of Kupffer cells/macrophage in vivo by gadolinium chloride or by a calcium channel blocker markedly reduces inflammatory response in the liver and suppresses the extent of alcoholic liver injury [1••]. Meanwhile, little is known about the effects of ethanol on HCC progression. Only recently, a model of alcohol-induced HCC has been reported in which the addition of ethanol to drinking water increased tumor incidence in DEN-injected male mice [90, 92], suggesting that alcohol consumption promotes hepatic tumorigenesis [90].

Factors Affecting ALD Progression

Hepatoprotective Versus Oncogenic Functions of STAT3

STAT3 [97] plays an important role in pathogenesis of fibrogenic liver injury, ALD and progression to HCC by mediating different functions in T cells, macrophages, hepatocytes, and HSCs. T cell-specific STAT3-deficient mice are resistant to Con A-induced liver inflammation and exhibit reduced IL-17 production [27, 98]. STAT3 also regulates the expression of the RORγt and RORα transcription factors and promotes differentiation of Th17 cells [27]. IL-17A can directly activate STAT3 signaling in macrophages [98, 99••]. Anti-inflammatory cytokine IL-10 also activates STAT3 signaling in macrophages [100, 101], and myeloid-specific STAT3-deficient mice are prone to a higher degree of liver inflammation in liver injury induced by several hepatic toxins [98, 102, 103] including alcohol [25••]. In concordance, the studies from hepatocyte-specific STAT3 knockout mice demonstrate that IL-6, IL-10, and IL-22 produce hepatoprotective and anti-fibrogenic effects via the activation of STAT3 in hepatocytes, and STAT3 in hepatocytes promotes an anti-inflammatory signal to suppress liver inflammation under most conditions [104–107].

For the past decade, the role of STAT3 in the pathogenesis of liver fibrosis was only investigated in inflammatory cells and hepatocytes [27, 47, 103, 108–110]. Only recently it was shown that STAT3 is activated by IL-6 and modulates collagen production in aHSC/myofibroblasts [99••, 111], and mice deficient of STAT3 in HSCs are less susceptible to liver fibrosis, implying that IL-17A, leptin, IL-6, and HGH signaling in HSCs may promote the development of liver fibrosis by activation of STAT3 in HSCs. Interestingly, IL-22 also activates STAT3 in HSCs but induces HSC senescence, thereby inhibiting liver fibrosis [112]. It is unclear why IL-6 and IL-22 have such different effects on HSCs.

At present, the mechanisms underlying the anti-inflammatory functions of STAT3 are not well understood. Even less is known about the tumorigenic effects of STAT3 on progression of ALD to HCC. However, it has been demonstrated that constitutive activation of STAT3 at the end-stage of liver cirrhosis promotes tumor cell survival leading to HCC [27]. Furthermore, deletion of STAT3 in hepatocytes reduced DEN-induced HCC development in mice [27, 113]. Similarly, deletion of STAT3-inhibitory proteins SHP-2 or SOCS3 in hepatocytes resulted in constitutive STAT3 activation, and increased DEN-induced HCC development [83••, 114–116]. In human HCC, increased STAT3 activation is likely due to persistent stimulation from cytokines such as IL-6 and IL-22 [82, 114]. In concordance, overexpression of IL-22 in hepatocytes caused STAT3-dependent activation of a variety of anti-apoptotic genes (Cyclin D1, Bcl-XL, Bcl-2), making these IL-22TG mice more susceptible to DEN-induced liver cancer [114]. Therefore, STAT3 inhibitors may have therapeutic potential for the treatment of HCC.

IL-6 is a cytokine involved in the regulation of several cellular processes including proliferation and differentiation and plays a pivotal role in acute phase response and in the control of the balance between pro-inflammatory and anti-inflammatory pathways. Increased levels of IL-6 are linked to development of HCC, and activation of IL-6 signaling plays a critical role in hepatocyte survival, and activation of pathways responsible for their transformation (outlined in [47, 113, 117, 118]). IL-6 binding to IL-6R and its signal transducing chain, gp130, induces gp130 dimerization and the subsequent activation and dimerization of gp130-associated Janus kinases (JAKs), which leads to JAK phosphorylation, followed by STAT3 activation [97]. Other signaling pathways activated by IL-6 include PI3 kinase, p38 MAP kinase, c-Jun NH2-terminal kinase (JNK), and TORC1-S6K, which ultimately lead to cell proliferation, protection from apoptosis and increased metastatic potential. The human *IL-6* gene is located on chromosome 7p21 [119, 120]. A number of studies indicated that the presence of a G/C single nucleotide polymorphism (SNP) at the promoter −174 of the *IL-6* gene is related to the *IL-6* gene transcription and, that significantly affects production of IL-61 [21]. Two phenotypes for this polymorphism were identified: the high-producer phenotype, including the −174 G/G and −174 G/C genotypes, characterized by higher circulating IL-6 levels; and the low-producer phenotype, including the −174 C/C genotype [120, 121]. The GG *IL-6* genotype showed the strongest influence on HCC risk among all the cytokine polymorphisms studied [122] and was associated with high the incidents of HCC in patients with liver cirrhosis [123, 124]. Meanwhile, *IL-6* polymorphisms with the low-producer genotype (−174 CC) correlated with the absence of HCC in the same patient population [120, 123, 124].

Interleukin 22 (IL-22)

IL-22 is a member of the IL-10 family of cytokines. Expression of IL-22 is restricted to hematopoietic cells. Th17 cells selectively synthesize both IL-17 and IL-22 [125–128]. Expression of IL-22 is also dependent on IL-23, as demonstrated by use of IL-23p19$^{-/-}$ mice [127, 129]. IL-22 can be also produced by subsets of γδT cells, NK and NKT cells [129]. The IL-22 receptor is composed of IL-22R1 and IL-10R2 subunits, and receptor ligation results in STAT3 phosphorylation and activation of the p38 mitogen-activated protein kinase pathway [130, 131]. Expression of IL-22R1 is found only on cells of non-hematopoietic

origin, which include hepatocytes and pancreatic acinar cells [129]. IL-22 is implicated in inflammation, immune surveillance and mucosal host defense. In the liver, IL-22 promotes hepatic production of acute phase proteins and plays protective roles against liver injury [129]. IL-22 possesses strong hepatoprotective properties and protects mice from ConA- and alcohol-induced hepatitis [10, 48••, 105]. IL-22$^{-/-}$ mice are more susceptible to CCl_4-induced liver fibrosis [99••], while treatment with IL-22 attenuates development of CCl_4-induced liver fibrosis in mice [99••]. Therefore, IL-22 might be used to treat patients with ALD because of its antioxidant, antiapoptotic, anti-steatotic, proliferative, and antimicrobial effects [48••, 132]. While the exact action of IL-22 still remains to be resolved, progress has been made using transgenic mice over-expressing IL-22 in hepatocytes [114]. These IL-22TG mice are protected from CCl_4-induced liver fibrosis, but are more susceptible to tumorigenesis [114], suggesting that IL-22 may have opposing short-term and long-term effects on the liver. Thus, overexpression of IL-22 drives proliferation and hyperactivation of liver progenitor cells [82, 133]. IL-22$^{-/-}$ mice exhibit reduced tumorigenesis [20••, 84]. It remains unclear if IL-22 promotes survival of tumor cells or facilitates transformation of hepatic progenitors via constitutive activation of STAT3, which drives tumor progression [84].

Interleukin 17 (IL-17)

Interleukin-17 (IL-17)-producing effector CD4$^+$ T (Th17) cells [134, 135] originate from naïve T cells via activation of lineage specific transcription factors [136, 137], regulated by TGF-β1 and IL-6, and other cytokines [138, 139]. IL-17 cytokines are mainly produced by CD4$^+$ Th17 cells, but also by a variety of cells, including γδ T cells, CD8$^+$ T cells, NKT cells, NK cells, innate lymphoid cells, eosinophils, neutrophils, and monocytes [140]. Th17 cells secrete a family of cytokines comprised of IL-17A, IL-17F, IL-17B, IL-17C, and IL-17E [141]. IL-17A homodimers (also known as IL-17) are the most abundant product of Th17 cells, exhibit higher biological activity than other family members, and signal through their cognate receptors IL-17RA and IL-17RC [140]. IL-17RA is ubiquitously expressed, but is strongly induced in hematopoietic cells [142] and fibroblasts [143] in response to stress. IL-17A signaling activates inflammatory signaling in target cells, including STAT3, TRAF6, Act1, JNK, ERK, NF-κB [142, 144]. IL-17 mediates autoimmunity, and the autoimmune inflammatory diseases psoriasis and rheumatoid arthritis respond to anti-IL-17 biological therapies [145]. Most recently, IL-17 has been implicated in liver, lung, and skin fibrosis, and in tumorigenesis [44, 140, 141, 146–151]. Although anti-TNF-α therapy has been ineffective in patients with ALD [1••, 152], anti-IL-17 drugs are a potential novel therapy for ALD.

The role of IL-17 in ALD progression is not understood. However, recent studies implicated IL-17 in regulation of IL-8 production, a chemokine which plays a critical role in neutrophil recruitment into alcohol-damaged liver at the stage of steatohepatitis. In concordance, exposure of hepatic stellate cells to IL-17 in vitro induced production of IL-8 and GRO, the factors that have a strong chemoattractive effect on neutrophils, suggesting that IL-17 plays a role in promoting both liver inflammation and fibrogenesis of ALD [151, 153, 154].

Most of the data implicating IL-17 in the pathogenesis of ALD are based on clinical studies. Elevated levels of serum IL-17 were detected in patients with ALD [154]. The cellular origins of IL-17 in this study were monocytes and T cells in the circulation and infiltrating neutrophils and T cells in the liver [155]. Another study based on immunocolocalization staining with fluorescently-labeled antibodies, demonstrated that liver infiltrating IL-17-expressing cells were mainly composed of neutrophils and T lymphocytes [20••, 151]. The number of hepatic IL-17-producing cells correlated with the severity of ALD-induced fibrosis [20••], suggesting that IL-17 may serve as a marker for ALD progression. Moreover, expression of the IL-17 receptor (IL-17RA) was detected on activated HSCs (aHSCs) in the liver biopsies of ALD patients [151].

Regulation of Th17 Differentiation

TGF-β1, IL-6 and IL-21 drive differentiation of Th17 cells from naïve Th0 cells [134, 138, 139] via activation of retinoid-related orphan receptor γ*t* (ROR γ*t*) [136]. IL-23 is required for Th17 proliferation [156–159]. IL-27 antagonizes expansion of Th17 directly via inhibition of IL-23-producing cells. IL-25 also blocks Th17 responses via release of IL-13 which, in turn, suppresses IL-23, IL-1β1 and IL-6 secretion by dendritic (and other) cells [160, 161]. In response to alcohol-induced liver injury, Th17 cells release IL-17, which causes induction of IL-18, CXC and recruitment of neutrophils into the liver. Neutrophils facilitate hepatocyte injury and activation of BM-derived and liver resident Kupffer cells [148–151, 162, 163]. IL-17 stimulates Kupffer cells to express inflammatory cytokines IL-6, IL-1β, and TNF-α, as well as the major fibrogenic cytokine TGF-β1 via activation of STAT3 and NFκB signaling pathways. Using BM chimeric mice, we determined that deletion of either IL-17A or IL-17RA in inflammatory and Kupffer cells decreases liver fibrosis by 50–55 %, and this effect is mediated via regulation of TGF-β1 production, while deletion of IL-17RA in non-immune liver resident cells decreased liver fibrosis by 25 % [99••]. Furthermore, we demonstrated that IL-17A

stimulates activation of HSCs in a STAT3-dependent manner. In turn, murine Th17 cells are believed to produce IL-22, which mediates hepatoprotective functions [105] and facilitates oval cells proliferation, suggesting that murine Th17 may also contribute to tumorigenesis.

Epigenetic Regulation

Understanding the mechanisms of HSC inactivation during regression of liver fibrosis is critical for identifying new targets for therapy. Inactivation of HSCs is most likely regulated at an epigenetic level (vs genetic mutations) [164, 165]. Epigenetics are heritable changes in gene function that occur without a change in the DNA sequence [166]. These changes, including nucleosome dynamics and histone modifications, cause structural alterations in the chromatin structure, and regulate gene expression. Post-translational modifications of the core histone subunits of nucleosomes by methylation, acetylation, and phosphorylation [167, 168, 169••] are a fundamental mechanism by which the transcriptional activity of an associated gene locus can be regulated.

Epigenetic Regulation of HSCs, the Role of PPARγ in HSC Activation

Extensive studies have demonstrated that ethanol affects metabolism of methionine and DNA methylation. Methionine metabolism occurs primarily in the liver, where homocysteine is methylated to methionine and then *S* adenosylmethionine (SAMe) in a transmethylation reaction catalyzed by methionine adenosyltransferase [170]. SAMe is a methyl donor in methylation and has an important role in inducing DNA and histone methylation. Long-term ethanol consumption reduces hepatic levels of SAMe and DNA and histone methylation, increasing expression of genes that regulate stress response and alcoholic liver injury [1••, 12]. PPARγ expression is associated with the adipogenic features of qHSC and must be silenced for the cell to activate into a myofibroblast [69, 171•, 172••, 173]. A multi-step epigenetic network that controls activation of HSCs has been described, and involves activation of MeCP2 which causes alterations at the H3K27 methylation sites and generates transcriptionally repressed chromatin structure in the PPARγ promoter [69, 164]. Consistent with this observation, over-expression of PPARγ in cultured aHSCs results in reversion of HSC activation, and reacquisition of their adipogenic characteristics [173, 174].

Epigenetic Regulation of HSC Activation Via miRNAs

MicroRNAs (miRNAs) are short, noncoding RNAs that are average 22–23 nucleotides long. They control expression of genes involved in cell growth, differentiation, and apoptosis and are believed to be involved in the pathogenesis of liver disease, especially cancer [175]. They regulate gene expression by interacting with the 3′ untranslated region (3′-UTR) of target gene mRNA to repress translation or enhance mRNA cleavage. Several studies have demonstrated the role of miRNAs in ALD [176–178]. Ethanol exposure up-regulates miRNA-155 in macrophages, which increases TNF-α production (via increased mRNA stability) [177]; short-term ethanol exposure up-regulates miRNA-212 in intestinal epithelial cells, which down-regulates zonula occludens-1 protein [179], a factor that maintains intestinal permeability. Expression of liver miRNAs has also been shown to be significantly altered in ethanol-fed mice [180], but the functions of these miRNAs in the pathogenesis of ALD are not clear. Recent study has delineated an important role of miRNA-214 in regulation of CCN2 (CTGF) [181], the second member of the cystein-rich-61/connective tissue growth factor/nephroblastoma-overexpressed protein that plays a critical role in hepatic fibrogenesis due to the ability to directly activate HSCs into myofibroblasts [182]. miRNA-214 inhibits CCN2 expression in HSCs by binding to the CCN2 3′-UTR [183]. Thus, quiescent HSCs expressed high level of miRNA-214, while increased expression of CCN2 mRNA in ethanol-activated HSCs was associated with reciprocal downregulation of miRNA-214 [182, 183]. Furthermore, HSC can produce nano-size exosomes (small vesicles released extracellularly that arise by inward budding from the limiting membranes of multivesicular bodies) which transfer miRNA-214 to neighboring HSC or hepatocytes to inhibit CCN2 3′-UTR activity and suppress CCN2 expression. Exosomes from HSCs were a conduit for uptake of miRNA-214 by HSCs and hepatocytes [183], suggesting that exosomal transfer of miRNA-214 is a paradigm for the regulation of CCN2-dependent fibrogenesis and identifying intercellular regulation of exosomal miRNA as a target for anti-fibrotic therapy [176, 183].

Ethanol Metabolism

Evidence suggests that alcohol metabolites can aggravate liver fibrosis by direct activation of HSCs. During ethanol metabolism, ethanol is converted into acetaldehyde, then to acetate. Acetate rapidly releases into circulation and is eventually metabolized to CO2 in heart, skeletal muscle, and brain cells. Acetaldehyde (the first metabolite of ethanol) upregulates expression of collagen Type I in HSCs. Several mechanisms were identified to mediate acetaldehyde effects in HSCs. Acetaldehyde was shown to induce expression of Col1a2 gene [184] via a de novo protein synthesis-independent, PI3K-dependent mechanism. Acetaldehyde induces phosphorylation and activation of Smad2 and 4, and downregulation of Smad7 [185]. In turn, adenosine is a regulatory nucleoside that is generated in

response to cellular stress, damage, tissue hypoxia, inflammation, and alcohol exposure. Hepatic level of adenosine is strongly increased in animal models of alcohol-induced liver injury, and ethanol and its metabolite acetaldehyde were shown to stimulate accumulation of extracellular adenosine through its action on nucleoside transporter [186, 187]. Extracellular adenosine is generated by ecto-5′-nucleotidase (CD73), and mediates its functions via the adenosine A2A receptor (A2AR). A2AR is functionally present on HSCs, and acetaldehyde-induced production and accumulation of extracellular adenosine results in adenosine binding to adenosine A2AR receptor and its subsequent activation [188, 189]. Adenosine-A2AR signaling in HSCs triggers Gs-cAMP-PKA-SRC-ERK1/2-MAPK signaling cascade which mediates collagen Type I production and ECM production in HSC activation and ECM production [190]. These findings explain why antagonists of A2AR signaling, such as caffeine (1, 3, 7-trimethylxanthine), a non-selective adenosine receptor antagonist, could reduce ALD, and provide a novel pathway for anti-fibrotic therapy [191].

Conclusions

Despite extensive studies, the available therapy for ALD remains limited. Recent improvements in mouse models of ALD are providing new insights into the pathogenesis of the different stages of ALD, which may provide new targets for therapy.

Compliance with Ethics Guidelines

Conflict of Interest Jun Xu, Xiao Liu, Bin Gao, Michael Karin, Hidekazu Tsukamoto, David Brenner and Tatiana Kisseleva declare no conflicts of interest. This work is supported by National Institutes of Health (DK088837, U01AA022614, P50 AA011999-10, P50 AA011999-16).

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- •• Of major importance

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