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Inflammatory Pathways in Alcoholic Steatohepatitis

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Abstract

Inflammatory processes are primary contributors to the development and progression of alcoholic steatohepatitis (ASH), with severe alcoholic hepatitis (AH) characterized by non- resolving inflammation. Inflammation in the progression of ASH is a complex response to microbial dysbiosis, loss of barrier integrity in the intestine, hepatocellular stress and death, as well as inter-organ cross talk. Here we review the roles of multiple cell types in the liver involved in inflammation in ASH, including resident macrophages and infiltrating monocytes, as well as other cell types in the innate and adaptive immune system. In response to chronic, heavy alcohol exposure, hepatocytes themselves also contribute to the inflammatory process; hepatocytes express a large number of chemokines and inflammatory mediators and can also release damage associated molecular patterns during injury and death. These cellular responses are mediated and accompanied by changes in the expression of pro- and anti- inflammatory cytokines and chemokines, as well as by signals which orchestrate the recruitment of immune cells and activation of the inflammatory process. Additional mechanisms for cell-cell and inter-organ communication in ASH are also reviewed, including the roles of extracellular vesicles and microRNAs, as well as the inter-organ cross talk between the liver and gut, adipose and nervous system. We highlight the concept that inflammation also plays an important role in promoting liver

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repair and controlling bacterial infection. Understanding of the complex regulatory processes that are disrupted during the progression of ASH will likely lead to better targeted strategies for therapeutic interventions.

Keywords

Alcoholic hepatitis; Kupffer cells; infiltrating monocytes; intestinal dysbiosis; DAMPS; PAMPs; gut barrier; neutrophils

INTRODUCTION

Alcoholic liver disease (ALD) is a metabolic liver disease in which pathologic progression is largely predicated by inflammatory responses. In general, infection and cell death are the two most common reasons for inflammation, and the evidence to date also supports this concept for ALD. Pathogen associated molecular patterns (PAMPs) derived from gut microbes, which translocate to mesenteric lymphatic system and portal circulation, constitute a central mechanism in the former pathway (infection) as exemplified by, but not limited to, the role of endotoxin in hepatic macrophage proinflammatory activation [1]. In contrast, sterile inflammation is initiated by the latter pathway (cell death), resulting in the release of damage associated molecular patterns (DAMPs) which trigger inflammation via TLRs or inflammasome. Both PAMPs and DAMPs activate multiple cell types, including immune cells, hepatocytes, and liver non-parenchymal cells, to release chemokines, cytokines, acute phase response proteins, and extracellular vesicles etc that play an important role in regulating inflammatory responses in ALD. Further, inter-organ crosstalk involving gut, liver, adipose, muscle, lung, and neuroendocrine system, likely also contributes to inflammation development in ALD. From a clinical perspective, inflammation is an obvious therapeutic focus for the treatment of alcoholic hepatitis (AH) characterized by acute neutrophilic infiltration superimposed on chronic liver failure and high mortality.

Emerging data from both preclinical and clinical studies suggest some of the inflammatory pathways and mediators identified may serve as potential therapeutic targets; however, we must also recognize alcohol-mediated immunosuppression likely is an underlying cause of microbial infection and consequent inflammatory responses in alcoholic steatohepatitis (ASH). This review outlines the current state of understanding on the pathogenic mechanisms and implications of inflammation in ASH by categorically dividing discussions into multiple cell types that contribute to inflammation, major inflammatory pathways to ASH, and specific inflammatory mediators involved. The review also highlights outstanding questions concerning how best therapeutic interventions can be designed to prevent a transition from mild and chronic ASH to AH by carefully unravelling the complexities of multifaceted homeostatic functions of inflammatory signals, mediators, and cells.

I. Multiple cell populations contribute to ASH

Resident Kupffer cells and infiltrating monocytes—In mild and chronic ASH, the number of hepatic macrophages increases; infiltrating monocyte- derived macrophages are believed to contribute to this expansion and the pathogenesis of ASH (Table 1).[2] Both

resident and infiltrating immune cells exhibit a tremendous plasticity, modulating the function in response to signals within their microenvironment [3]. For example, Kupffer cells become sensitized to TLR4-induced signalling after chronic ethanol exposure, at least in part due to redox-dependent modulation of key signalling events down-stream of TLR4[4]. These proinflammatory macrophages together with infiltrating macrophages dictated by LPS, interferon-γ and granulocyte-macrophage colony stimulatory factor (GM-CSF) signalling are commonly classified as M1 macrophages as opposed to M2 macrophages which usually arise in Th2 responses in allergy, granuloma formation, and wound healing. It is generally believed that activated M1 macrophages produce high amounts of cytokines such as IL-1β, TNFα, IL-12, IL-18 and IL-23, which help to induce antigen specific Th1 and Th17 cell inflammatory responses, thereby promoting inflammation. In contrast, activated M2 macrophages secret large amounts of IL-10, IL-1R antagonist, TGF-β, and subsequently suppress inflammation and promote tissue repair. However, this polarization definition is vague and controversial, and macrophages are plastic enough to respond to multiple and divergent signals during the evolution of pathology. Infiltrating monocytes develop into M1-like hepatic macrophages via Notch-1 dependent mitochondrial reprogramming in ASH. [5] Whether and how these monocyte- derived M1 macrophages persist or reprogram in the course of ALD is unknown. Infiltrating monocytes are characterized by the expression of Ly6C in mice; Ly6Clow monocytes function by patrolling endothelial surfaces for injury [6], while $Ly6C^{high}$ monocytes are recruited to sites of inflammation [6]. In CCl_4-induced fibrosis, $Ly6C^{high}$ monocytes are initially recruited to the liver and then transform into a restorative $Ly6C^{low}$ phenotype that promotes the resolution of fibrosis7. However, it is not well understood how ethanol exposure may modify this wound healing response. A recent publication implicates the role of $gp91^{phox}$, a catalytic subunit of NADPX oxidase 2 dominantly expressed in phagocytes, in supporting tissue-restorative M2-like macrophages in experimental ALD as the mice deficient in $gp91^{phox}$ developed liver pathology with intensified inflammation and increased accumulation of apoptotic cells [8].

In models of sterile liver injury and inflammation, peritoneal macrophages can also directly enter into the liver across its mesothelium in a process requiring CD44 and the DAMP molecule ATP [9]. These macrophages replicate rapidly and switch themselves to the M2 alternatively activated phenotype to perform reparative functions[9]. It is yet to be studied whether peritoneal macrophages contribute to ALD via a similar mechanism. Regulation of cell fate of M1 and M2 macrophages in the evolution and progression of ALD is an obvious area of research interest.

Neutrophils—More than 90% heavy drinkers develop fatty liver; however, only some of them develop AH with significant hepatic neutrophil infiltration. The underlying mechanism for this predisposition remains unclear. Recent data revealed binge alcohol feeding markedly elevated hepatic and circulating neutrophils in chronically ethanol-fed mice[10] and in human alcoholics,[11] and shifted chronic ASH with macrophage inflammation to AH with marked neutrophil infiltration,[12] suggesting binge drinking facilitates hepatic neutrophil infiltration in ASH.

There is an outstanding question with regard to the pathogenetic role of neutrophil infiltration in AH, a salient feature of this unique pathologic spectrum. It is generally

believed that neutrophils infiltrating into the liver are damaging hepatocytes in AH. Indeed, in AH, the expression of cytokines/chemokines (IL-1, IL-8, IL-17, chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL5) known to promote neutrophil infiltration is markedly upregulated and correlates with the disease severity, supporting the notion of the detrimental roles of neutrophils in AH. However, a recent clinical study shows that infiltration of neutrophils is associated with better prognosis in AH, indicating that neutrophilic inflammation may be beneficial in promoting wound healing by secreting growth factors and/or controlling bacterial infection in these patients [13]. In AH with gut microbial translocation, it is not surprising that neutrophils are migrating into the liver to fight against microbes, as discussed below in relation to pyroptosis in AH. Further, neutrophils in AH patients are often shown to be defective in their phagocytic and bactericidal activities, [14] causing a failure in infection control and sustained upregulation of inflammatory cytokines/chemokines. This may rationalize the use of G-CSF for treatment of severe AH [15] because it is believed that G-CSF stimulates the generation and activity of neutrophils while promoting liver regeneration but preclinical data to support this hypothesis are still lacking.

T lymphocytes, NKT, MAIT cells—Patients with ALD have significant infiltration and activation of $CD3^+$ T cells including both $CD4^+$ and $CD8^+$ T cells in the liver[16] and increased expression of activation markers (CD69, CD38) in circulating T cells.[17] However, it was not clear until a recent study by Liaskou et al[18] whether these intrahepatic T cells in ALD patients merely reflect bystander activation or a consequence of antigen-specific activation. By using high-throughput T-cell receptor sequencing analysis, Liaskou and colleagues identified a pronounced oligoclonal nature of T cells in ALD, suggesting the presence of neoantigen-specific T-cell responses in ALD. These neoantigens are likely derived from protein adducts formed with acetaldehyde generated from alcohol metabolism or/and lipid peroxidation aldehydic products. In addition, bystander activation of T cells is induced in the absence of specific T-cell receptor stimulation by a large number of cytokines, DAMP, and PAMP that are generated in ALD. Thus, both bystander and antigen-specific activation of T cells likely contribute to the pathogenesis of ALD. But their exact roles remain unclear and are probably complex. Infiltration of T cells correlated with liver inflammation, necrosis, and regenerating activities in ALD patients,[16, 18] suggesting T cells not only promote disease progression by releasing inflammatory mediators (e.g. TNF-α, IL-1, IL-17 etc.) and directly killing hepatocytes via cytotoxic CD8+ T lymphocytes,[16, 18] but may also play beneficial roles in ALD by promoting liver regeneration and anti-bacterial immunity.[19] More specifically, the $CD4^+$ helper T cells are subdivided into at least Th1, Th2, Th9, Th17, Th22, and T regulatory (Treg) groups, and each subset plays different roles in the pathogenesis of ALD by producing a characteristic profile of cytokines.[20] For example, greater proinflammatory Th1 responses were found in AH than in alcoholic cirrhosis, which promotes disease progression by producing IFN-γ.[21] Th17 cells not only promote liver inflammation and fibrosis in ALD by producing IL-17 [22] but may also help liver repair by stimulating IL-22 production.[23] Although activated T cells are often detected in ALD and may contribute to disease pathogenesis, excessive alcohol drinking also caused broad immunosuppression [24] including inhibition of T cells via acetaldehyde and glucocorticoids, [25] thereby resulting in an increased risk of bacterial infection in ALD patients.

Natural killer T (NKT) cells, the most abundant lymphocytes in mouse livers, is another subset of T cells characterized with a highly restricted T cell receptor (TCR) that recognizes lipid antigens. Data from experimental models of ALD suggest that NKT cells promote alcoholic liver injury via the activation of Kupffer cells and macrophages.[26], [27] However, NKT cells are low in human livers and likely play a less important role in the pathogenesis of ALD in patients. In contrast, human livers contain abundant mucosa-associated invariant T cells (MAIT) cells, representing 20%–50% of intrahepatic T cells. MAIT cells express invariant TCR that recognizes microbial riboflavin/vitamin B2 metabolites presented by the major histocompatibility complex class I-related protein 1, playing a key role in controlling bacterial infection.[28] Patients with ALD are associated with marked reduction of MAIT cells, which may contribute to the increased risk of bacterial infection in these patients.[29], [30]

Hepatocytes—Hepatocytes are also important contributors to modulating the immune environment in ASH. Damaged hepatocytes produce several chemokines [12, 31, 32] and DAMPs such as mitochondrial DNA and high mobility group protein-1 (HMGB1)[33], [34] that promote neutrophil infiltration in ALD. CXCL1 and IL-8 are two major chemokines for neutrophil recruitment released by hepatocytes and are highly elevated in patients with AH, [17], [35] and correlate with disease severity.[35] Elevation of hepatic CXCL1 was also reported in several mouse models of steatohepatitis especially in those which utilized high-fat diet and binge ethanol challenges.[12, 31, 32] Free fatty acid and cytokines (e.g. TNFα, IL-17) can strongly upregulate CXCL1 and IL-8 expression in hepatocytes, and likely contribute to elevation of both chemokines in ASH. [22, 32, 36.] Hepatocytes are also an important source of macrophage migration inhibitory factor (MIF), a pluripotent cytokine/chemokine that contributes to the progression of ALD[37, 38]. Monocyte chemoattractant protein-1 (MCP-1)/ CCL2, another chemokine often termed a “steatokine”, is involved in the development of steatosis[39].Interestingly, MIF regulates the expression of MCP-1 by hepatocytes[40], thereby affecting ALD. Ethanol also increases the expression of a number of acute phase proteins and complement factors4, likely regulating inflammatory responses and/or promoting inflammation in ALD.

II. Pathways to inflammation in ASH

Loss of intestinal integrity and dysbiosis—Intestinal dysbiosis and impaired intestinal barrier are important contributors to the pathogenesis of ASH (Figure 1). The gut connects to the liver by the biliary tract and portal vein, allowing for direct transfer of gut-derived components that impact liver pathophysiology[41]. Chronic alcohol consumption significantly increases gut permeability to endotoxin/lipopolysaccharide (LPS), elevating the concentration of LPS in the portal and systemic circulation[41]. While the mechanisms for increased permeability are not completely understood, nitration and oxidation of tubulin, damage to the microtubule cytoskeleton, as well as activation of iNOS and NF-κB signaling by acetaldehyde have been implicated in the disruption of tight and adherens junctions of intestinal epithelium [42, 43]. Shifts in the ratios of short-chain fatty acids in favor of acetate, the enzymatic product of acetaldehyde, rather than butyrate, the primary fuel source for colonocytes, also contribute to impaired barrier function[44].

Enteric dysbiosis can be considered as an upstream causal event in organ crosstalk-based pathogenesis of ALD[41,45] Small intestinal bacterial overgrowth is evident even during the stage of alcoholic fatty liver. Imbalanced growth of bacterial phyla is associated with decreased expression of anti-microbial molecules such as *Reg3b* and *Reg3g* lectin [41, 45], increased mucosal-associated bacteria, and bacterial translocation to the mesenteric lymph nodes and liver. Importantly, a correction of REG3 deficiency prevents all these changes and alcoholic liver injury [41, 45]. Bacterial metabolomic changes are also important. ALD is associated with reduced gut synthesis of long-chain fatty acids (LCFA) that support the growth of commensal *Lactobacillus* and the integrity of gut epithelium. LCFA supplementation, thus restores eubiosis and ameliorates alcoholic liver injury[41, 45]. Another metabolomic consequence of alcohol-induced gut dysbiosis is an increased intestinal concentration of unconjugated bile acids by overexpressed bacterial choloylglycine hydrolase [41, 45]. This leads to reduced farnesoid X receptor (FXR) activity and fibroblast growth factor (FGF-15) expression by enterocytes, causing upregulated hepatic CYP7A1 expression and increased bile acid concentrations in blood. Treatment with the intestine-restricted FXR agonist fexaramine or overexpression of a human FGF-15 orthologue, restores the intestinal barrier and reduces ASH[41, 45.] In contrast, gastric acid suppression worsens ALD by promoting overgrowth of Enterococcus[41, 45]. *Akkermansia Municiphila*, a gram-negative commensal gut bacterium, is reduced in ALD and when supplemented, promotes gut barrier function in part by enhancing mucus production, prevents the development of ALD and ameliorates pre-existing ALD in mice [41, 45]. Intestinal fungi are also involved in intestinal microbial overgrowth and fungal β-glucan translocates to the liver to cause inflammation in ALD[46.] Thus, both fungal and bacterial diversity are affected by alcohol, contributing to translocation of various microbial products and PAMP-mediated inflammation in the liver.

Hepatocyte death and inflammation—Hepatocytes can undergo cell death via a number of regulated and non-regulated pathways, including apoptosis, necrosis, necroptosis, pyroptosis and ferroptosis. The type of hepatocyte cell death is an important determinant of inflammation in the liver and most likely in the different spectra of ALD. While apoptosis of hepatocytes is generally considered to be non-inflammatory, death via necroptosis or pyroptosis is inflammatory, due to the lytic nature of cell death. During chronic ethanol consumption, hepatocytes undergo apoptosis, triggered by activation of intrinsic or extrinsic proapoptotic pathways mediated by organelle stress or cytokines[47, 48]. Hepatocytes also undergo necroptosis mediated by receptor-interacting protein kinase (RIP) 1, recruiting RIP3 to form necrosome which in turn phosphorylates, oligomerizes, and activates Mixed Lineage Kinase Domain Like Pseudokinase (MLKL). MLKL is recruited to the plasma membrane, where it forms ion selective channels, thus inducing necroptosis, distinct from the non-ion selective pores made by GSDMD during pyroptosis[49]. While it is clear that ethanol-induced necroptosis involves RIP3, it is not yet known if MLKL mediates cell death or if MLKL-independent pathways are involved, as has been reported in models of autoimmune arthritis[50].

There is cross-regulation by the effectors of different cell death pathways which most likely influences liver inflammation. For example, pro-apoptotic caspase 8 (CASP8)

depolymerizes the RIP1-RIP3 complex, prevents necrosome formation, and suppresses proinflammatory necroptosis. CASP1 activates CASP 3 and 7 to induce apoptosis while CASP 3 and 7 may cleave GSDMD at a distinct site to inactivate this pyroptosis effector protein[51]. In chemotherapy- induced pyroptosis, it is CASP3 that cleaves GSDME, but not GSDMD to render the cell death[52]. Thus, apoptosis may be present at early steatotic stage of ALD followed by necroptosis in early ASH. After transition to AH, pyroptosis may become predominant as a form of cell death, which mechanistically links to neutrophilic inflammation and consequentially leads to endotoxemia and septicemia, the common cause of death from AH. Cleaved GSDMD also activates nucleotide- binding domain, leucine-rich-containing family, pyrin domain-containing (NLRP)3- dependent CASP1 activation via a cell-intrinsic pathway[53]. In fact, CASP1 can still induce plasma membrane damage in GSDMD-deficient cells, but pyroptosis is delayed [54]. Gram- negative bacteria also secrete LPS-laden outer membrane vesicles (20–150nm) to deliver their contents including LPS to host cells [55]. Thus, bacteria do not need to invade cells to allow LPS access to cytosol where CASP11/4 become activated to execute GSDMD-mediated pyroptosis.

Inter-organ crosstalk—Inter-organ crosstalk contributes to inflammation, metabolic alternations, and cell death in ALD (Figure 1). The gut-liver axis, discussed above, is part of this inter-organ crosstalk that involves extensive interactions among multiple organs such as adipose, muscle, lung and nervous system. Adipose tissue is an important organ in integrating metabolism and immunity; ethanol impacts both the metabolic and immune functions of adipose tissue [56]. Ethanol consumption dysregulates lipid metabolism in adipose tissue, contributing to hepatic steatosis through increased transport of fatty acids to the liver[56, 57]. Adipose tissue modifies target cells via autocrine, paracrine and endocrine activity primarily through the secretion of adipokines and extracellular vesicles (EVs)[58]. Adipose tissue becomes inflamed in response to chronic ethanol, increasing expression of inflammatory cytokines, which in turn inhibit release of adiponectin, an anti-inflammatory adipokine. Decreased plasma levels of adiponectin and/or adiponectin resistance also impairs lipid metabolism in the liver and may lead to the development of hepatic steatosis and injury [59]. Ethanol also modifies the adipokine cargo of EVs released by adipocytes [60]. These ethanol-induced changes in adipose metabolic and immune function all contribute to inflammation and injury in the liver.

Other organs also likely contribute to interorgan cross-talk promoting a pro-inflammatory environment in ASH. One understudied area of interorgan cross-talk in ASH is the protective role of the vagus-α7nAChR (α7 nicotinic acetylcholine receptor coded by *Chrna7*) axis that has been explored in other models of liver disease [61]. Activation of the α7nAChR on Kupffer cells suppresses inflammation[62]. However, this anti-inflammatory effect of vagal innervation may be compromised by the autonomic dysfunction that is a common complication of cirrhosis [61]. A second organ that may interact with liver is the lung[63], lung-liver interactions may indeed contribute to the interactions between cigarette smoking and ASH/ALD.

III. Mediators of inflammation in ASH PAMPs and DAMPs

PAMPs and DAMPs—PAMPs entering the liver activate pro-inflammatory signaling. The best studied of these pathways is activation of TLRs (e.g., TLR1, 2, 4, 5, 9) by microbial products, with recent data also implicating fungal activation of the C-type lectin receptor C-type lectin domain family 7 (CLEC7A)/dectin-1 [46]. DAMPs such as HMGB1, DNA, ATP, adenosine, uric acid, fragments of heparan sulfate or hyaluronic acid, heat shock proteins, and fibrinogen, are also recognized by some of these TLRs (TLR2, 4, 9) and NOD-like receptors such as NLRPs. TLRs activated by PAMPs or DAMPs usually cause transcriptional activation of proinflammatory mediators, such as cytokines, chemokines, and adhesion molecules and this pathway serves to prime the cells for inflammation. This priming is followed or accompanied by post-translational activation of proinflammatory cytokines such as pro-IL-1β and pro-IL-18 by canonical inflammasome, causing release of active forms of these cytokines. Proinflammatory TLR activation also occurs in non-immune cells. TLR2 and TLR9 activation mediates the release of CXCL1 by hepatocytes and hepatic stellate cells (HSCs) to promote transient neutrophilic infiltration after alcohol binge[64]. TLR4 activation may also take place in HSCs which not only represses the TGF-β pseudoreceptor Bambi to activate the fibrogenic TGF-β pathway [65] but also leads to NF-κB activation, upregulation of chemokines and adhesion molecules, and recruitment of inflammatory cells [66, 67].

Canonical and non-canonical inflammasome—The inflammasome is a multiprotein oligomer composed of pro-CASP-1, PYCARD, and NLRP, which mediates pro-CASP1 activation and subsequent processing of pro-IL-1β and pro-IL-18 by active CASP1. The role of the canonical NLRP3 inflammasome in DAMP-mediated activation of pro-IL-1β in alcoholic fatty liver has been suggested[68]. IL-1β is directly pro-inflammatory but also activates HSCs via upregulation and activation of pro-MMP9, an event essential for early matrix remodeling and pro-inflammatory HSC activation[69].

The non-canonical inflammasome CASP4/11-GSDMD pathway for programmed, lytic cell death “pyroptosis”, has recently been disclosed. This pathway links infection to cell death and DAMPs to incite intense inflammation and possibly to systemic inflammatory response syndrome (SIRS) as seen in AH[70]. This pathway is activated by increased intracellular, not extracellular levels of LPS as seen in infection of gram-negative bacteria. Intracellular LPS oligomerizes and activates CASP4/11 (4 in man and 11 in mouse). This leads to proteolytic activation of pro-GSDMD, releasing N-terminal 30kD GSDMD, which is recruited to the plasma membrane to form ~20nm pores[53, 71]. This lytic death releases intracellular bacteria, PAMPs, DAMPs, and cytokines (IL- 1/IL-18) and may be protective for infected intestinal epithelial cells as bacteria and these inflammatory mediators are expelled into the gut lumen. However, if hepatic macrophages or hepatocytes undergo pyroptosis, this process locally or systemically disseminates bacteria and PAMPs/DAMPs, and the latter may cause endotoxemia, sepsis, and SIRS. In fact, the mice lacking CASP11 or GSDMD are protected from lethality caused by a high dose of LPS[53]. Activation of CASP11 and GSDMD are not present in chronic mild ASH in an experimental model but become evident when liver histology transitions to AH by weekly alcohol binge, concomitant with increased bacterial load and neutrophilic infiltration in the liver[70]. Defic iency of CASP1/11 abrogates GSDMD

activation, bacterial load, and neutrophil infiltration. Conversely, the deficiency of IL-18, an important anti-microbial cytokine, aggravates CASP11-GSDMD activation, liver bacterial load and neutrophilic inflammation[70]. AAV-mediated expression of active GSDMD in hepatocytes causes submassive hepatocyte necrosis accompanied by intense neutrophilic infiltration in the AH model. More importantly, CASP4 and GSDMD activation are robust in explant livers of AH patients but not evident in normal human livers[70]. These results collectively establish pyroptosis as the novel and unique type of cell death triggering neutrophilic inflammation in AH.

Cytokines and chemokines—A wide variety of cytokines are highly upregulated in the liver and serum from patients with severe AH and many of them are probably also elevated in mild and moderate ALD.[72] Most of these cytokines (such as TNF-α, IL-6) play dual roles in the pathogenesis of ALD by not only promoting inflammation and injury but may also promoting liver regeneration; whereas some cytokines may have more specific functions, such as IL-1β and IL-22, both of them are currently being tested as therapeutic targets in clinical trials for the treatment of AH.[73] Preclinical studies demonstrated that IL-1β plays an important role in inducing liver inflammation and injury but may play a minor role in promoting liver repair, thus blockage of IL-1β may ameliorate ALD without reducing liver repair.[74] IL-22 plays a key role in preventing liver injury, promoting liver regeneration, and suppressing bacterial infection by specifically targeting hepatocytes without affecting inflammatory cells.[23] Administration of recombinant IL-22 protein generated minor side effects in healthy human subjects [75] and will likely have some beneficial effects for the treatment of patients with severe AH.[23] IL-17, which is highly elevated in ALD, likely plays a complex and detrimental role in promoting ALD disease progression by acting on many cell types including hepatocytes, nonparenchymal cells, and inflammatory cells in the liver. [76]

IL-8 and CXCL1 are two of the most highly elevated chemokines in AH patients and likely promote liver inflammation and injury by stimulating neutrophil infiltration.[35] Blockade of IL-8 receptor or CXCL1 ameliorated mouse ASH.[32, 77] CCL20 is also one of the most upregulated chemokines and correlates with disease severity in patients with AH. The data from experimental studies suggest that CCL20 promotes liver inflammation and fibrosis by targeting hepatic stellate cells.[78] Although many chemokines and their receptors are implicated in ALD based on animal model studies, none of them have been tested as therapeutic targets for AH. In blocking chemokines and their receptors as AH therapy, the target redundancy (one chemokine interacting with multiple receptors and vice versa) presents a major challenge.[79] Thus, therapeutics utilizing specific antagonists for an individual chemokine receptor may have poor clinical efficacy in AH. Promiscuous chemokine receptor antagonists, possessing broad specificity for several G-protein-coupled chemokine receptors, may be considered for the treatment of AH.

Complement—Complement is an intrinsic part of the innate immune system that provides links to adaptive immune function. Complement is activated within the hepatic sinusoids in response to ethanol exposure and contributes to hepatic inflammation and injury; the classical pathway of activation is particularly critical to this process[80, 81]. Further, it is clear

that both the anaphylatoxin receptors, C3aR and C5aR1, are important to progress the ethanol-induced activation of complement to subsequent liver inflammation and injury [60]. As with many innate immune functions, while complement activation is pro-inflammatory, it is also required for resolution of injury. For example, complement activation via the alternative pathway is critical to the removal of injury and dying hepatocytes in models of both fibrosis and early ASH[82, 83].

MicroRNA (miRNA), Extracellular vesicles (EVs)—MiRNAs, small non-coding RNA molecules with 19–25 nucleotides, can induce RNA silencing and regulate gene expression at post-transcriptional levels, playing important roles in a variety of cellular functions. Recent studies have demonstrated that many miRNAs are involved in regulating directly or indirectly the inflammatory pathways in ASH[84, 85]. For example, miRNA-155, a miRNA enriched in macrophages/Kupffer cells, is upregulated in ALD and promotes liver inflammation in ALD.[86] In contrast, miR-181b-3p, a critical negative regulator for TLR4 signaling in Kupffer cells, is downregulated in ALD; thus such downregulation results in Kupffer cell activation and liver inflammation via the upregulation of importin-α5 and NF-κB activation.[87] In addition, miR-223, a neutrophil-specific miRNA, is upregulated in neutrophils in experimental models and patients with ALD, acting as an important negative regulator to prevent neutrophil over activation in ALD.[11] MiR-122, a hepatocyte-specific miRNA, protects against liver inflammation and injury in ALD by inhibiting hypoxia-inducible factor 1α.[88] However, hepatic expression of miR-122 is markedly downregulated in ALD, thereby further exacerbating liver inflammation in ALD.[88] Interestingly, miRNAs not only can regulate gene expression within the cells that generate these miRNAs but can also be transferred into other target cells via EVs including exosomes, playing an important role in cell-cell communication. The concentration of many miRNAs is increased in hepatocyte EVs isolated from a mouse model of ASH compared to these from pair-fed mice, and these miRNAs target a large number of genes that are involved in inflammatory response in ASH.[89] However, how EVs transfer these miRNA and exactly regulate the inflammatory pathways in ALD by affecting targeting cells remains unknown.

In addition to transfer of miRNA, EVs can also transfer RNA, DNA, lipids, proteins etc., into target cells and subsequently regulate inflammation in ALD. For example, EVs can carry hepatocyte-derived mitochondrial DNAs (mtDNAs) and transferred them into neutrophils, and consequently activate neutrophils in ALD by binding TLR9.[34] EVs can also transfer proteins such as heat shock protein-90 and CD40L into macrophages, thereby activating macrophages and liver inflammation in ALD.[90, 91] Interestingly, recent data suggest an interaction between complement C5aR1 and chronic ethanol in determining the adipokine cargo of EVs released from adipocytes [60]. Adipocyte-derived EVs are likely a mechanism for the cross-talk between adipose and liver in ALD.

Because they may contain disease-specific cargos, EVs have been actively investigated as biomarkers for liver diseases including ALD.[84, 85] For example, three miRNAs, let7f, miR-29a, and miR-340, were elevated in blood EVs from ethanol-fed mice, but not in those from other liver injury models; these miRNA-enriched EVs are also elevated in patients with mild ALD.[89] Future studies are needed to confirm whether these three miRNA-enriched EVs are good biomarkers for ALD diagnosis and identify new EV biomarkers for ALD.

The cells infected with bacteria release exosomes containing PAMPs which may incite inflammatory signaling in recipient cells [92], a situation relevant to AH because of increased bacterial translocation. Constitutively active $MyD88^{L265P}$ in patients with diffuse large B-cell lymphoma, can be released via EVs to propagate inflammation and promote tumor growth [93];this exemplifies a possible and important transmission mode of a cellular signaling component via EVs. EVs are also released by prokaryotes (bacterial microvesicles)[94], and this opens up potential crosstalk between gut bacteria with intestinal epithelial cells or liver cells if bacterial translocation occurs. What regulates EV release from different liver cell types and how differentially the cells are targeted by EVs in different spectra of ALD, are important outstanding questions.

Pro- and anti-inflammatory signaling in ASH—In addition to increased exposure to PAMPs and DAMPs in the progression of ASH, the sensitivity of immune cells to these signals can be impacted in the context of ASH. Hepatic macrophages are more sensitive to activation of TLR2 and TLR4 [4] characterized by increased activation of both Myeloid differentiation primary response gene 88 (MyD88) and TIR-domain- containing adapter-inducing interferon (TRIF) mediated down-stream signals including Mitogen- activated protein kinases (MAPK) family members and NFκB [4]. Signaling via the complement anaphylatoxins C3aR and C5aR1[95], as well as C-type lectin receptors, such as Mincle[96], is also exacerbated by chronic ethanol exposure. While there is a good understanding of the consequences of chronic ethanol on macrophage signaling, the precise mechanisms for these effects are not well understood. There are likely interactions of ethanol with redox-dependent signaling and NADPH oxidase [4, 97], as well as expression of heat shock protein 90[39]. Recent studies have identifed changes in the expression of specific microRNAs regulating TLR4 signaling, including miR 181b3p, up-regulating importin-α5 and p65 nuclear translocation, miR291b down-regulating Tollip, a negative regulator of TLR4 signaling and miR155 controlling the stability of TNFα mRNA[87, 98, 99]. Interestingly, chronic ethanol also regulates Slu7, an mRNA splicing factor, to increase inflammation[100].

Chronic ethanol also impairs anti-inflammatory signaling. For example, increases in the expression of PDE4 during chronic ethanol lower the production of cAMP, a potent anti-inflammatory signal[101]. Importantly, many anti-inflammatory pathways can still be effectively activated in ASH, suggesting potential therapeutic avenues for normalizing inflammation in ASH. For instance, phosphodiesterase 4 (PDE4) inhibitors are being tested for their ability to enhance cAMP production in ASH[101]. Adiponectin treatment leads to the production of IL-10 by macrophages and reduces inflammatory responses[4]. Nutraceuticals also offer potential anti- inflammatory therapies. For example, S-adenosyl methionine down-regulates PDE4 and increases cAMP [102]and 35kD hyaluronic acid normalizes TLR4 signaling in hepatic macrophages by impacting the expression of specific miRNAs [87, 98]

SUMMARY:

Inflammation induces the progression of ALD from simple steatosis to steatohepatitis and severe forms. Infiltration of neutrophils is a hallmark of severe ASH, however, ALD is also associated with infiltration of many other types of inflammatory cells including macrophages, T cells, NKT cells etc. (Table 1). These inflammatory cells, together with

hepatocytes and nonparenchymal cells (e.g. Kupffer cells, HSCs) in the liver, promote and control inflammation in ALD by producing a wide variety of inflammatory mediators (Table 1). In addition, both DAMPs produced by damaged/stressed cells (e.g. hepatocytes) and PAMPs derived from gut bacteria, are two important factors to activate inflammatory cells, causing inflammation in ALD. Recent studies show that miRNAs and EVs also play a critical role in controlling liver inflammation in ALD by regulating the expression of a variety of inflammatory genes and promoting cell-cell communication, respectively. In contrast to inducing liver injury, inflammation also plays a key role in promoting liver repair and anti-bacterial immunity in ALD. For example, most of inflammatory mediators (e.g. TNF-α) have both detrimental (e.g. inducing liver injury and fibrosis) and beneficial (e.g. promoting liver regeneration and suppressing bacterial infection) roles in the pathogenesis of ALD, which likely accounts for some ALD therapy failure by using these mediators (e.g. TNF-α) as targets. Thus, there is an urgent need to identify more specific inflammatory mediators that have either beneficial or detrimental functions, but not both. However, severe AH is associated with elevation of a wide variety of inflammatory mediators that synergistically promote disease progression, using a single mediator as a therapeutic target may not be effective and combination therapy is likely required for the treatment of this deadly malady.

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Abbreviations:

ALD	alcoholic liver disease
AH	alcoholic hepatitis
ASH	alcoholic steatohepatitis
CASP	caspase
CCL	Chemokine (C-C motif) ligand
CLEC7	C-type lectin domain family 7
CXCL	chemokine (C-X-C motif) ligand
DAMPs	damage associated molecular patterns
EV	extracellular vesicle
FGF-15	fibroblast growth factor-15
FXR	farnesoid X receptor
GSDMD	gasdermin-D
GSDME	gasdermin-E

HMGB1	high mobility group box-1
LCFA	long-chain fatty acids
LPS	lipopolysaccharide
MAIT	mucosa-associated invariant T cells
MAPK	Mitogen- activated protein kinases
MCP-1	monocyte chemoattractant protein-1
MIF	macrophage migration inhibitory factor
miRNA	microRNA
MLKL	mixed lineage kinase domain like pseudokinase
MyD88	Myeloid differentiation primary response gene 88
NKT	natural killer T cells
NLRP	nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing
PAMPs	pathogen associated molecular patterns
PDE4	phosphodiesterase 4
RIP	receptor- interacting protein kinase
SIRS	systemic inflammatory response syndrome
TLR	toll-like receptor
TRIF	TIR-domain-containing adapter-inducing interferon

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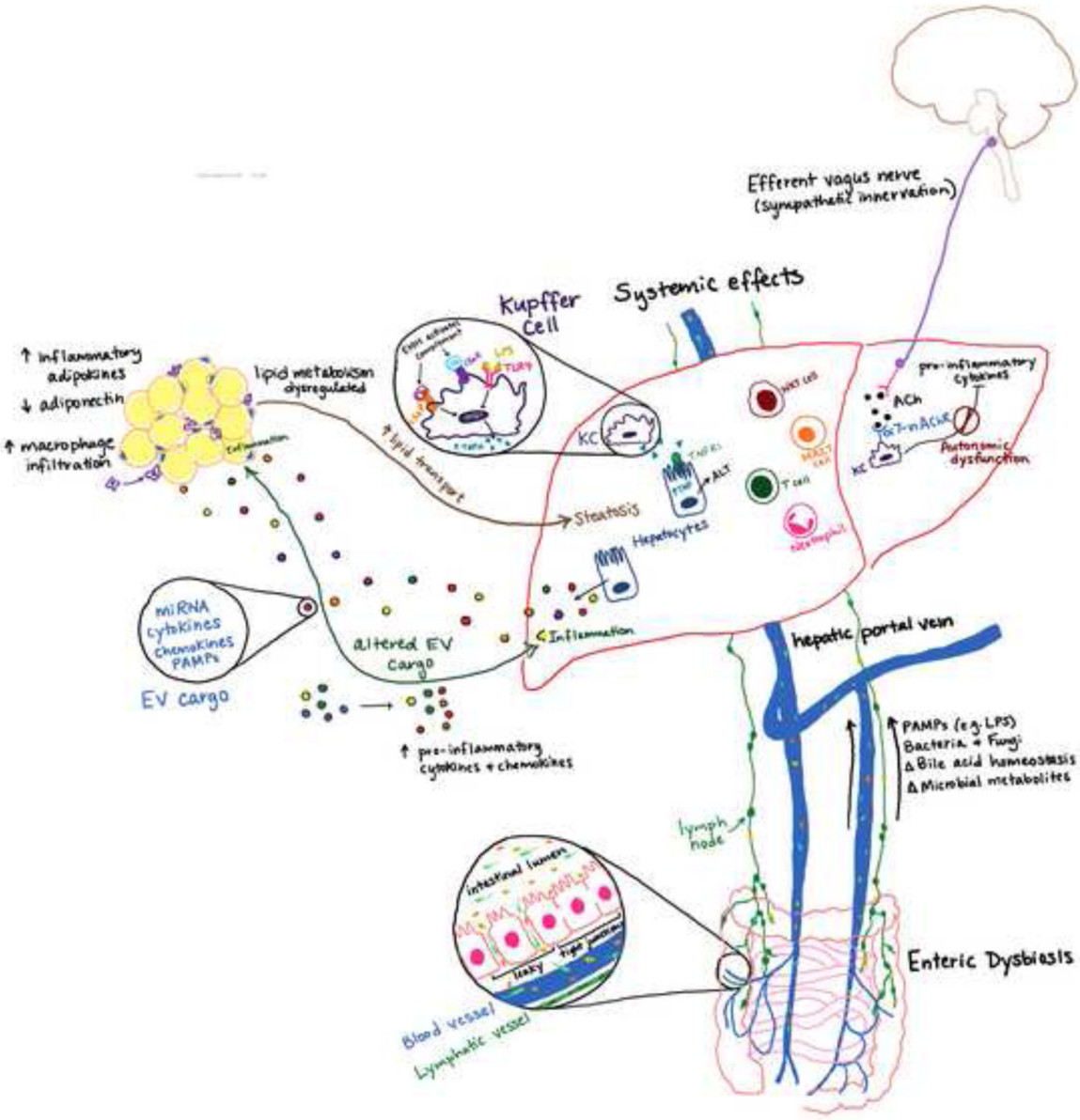

Figure 1: Inter-organ cross-talk contributes to the progression of ALD.

Inter-organ crosstalk contributes to inflammation, metabolic alternations, and cell death in ALD. The gut- liver axis involves enteric dysbiosis, a loss of barrier function leading to translocation of microbes and microbial products to the portal circulation. Loss of bile acid homeostasis also contributes to liver injury. Adipose tissue is an important organ in integrating metabolism and immunity; ethanol impacts both the metabolic and immune functions of adipose tissue. Sympathetic innervation to the liver via the vagus nerve can also regulate inflammatory responses. Organ-organ cross talk is mediated by the release of mediators, including neurotransmitters, cytokines, chemokines, adipokines, miRNAs and metabolites. These mediators can either be present in the circulation and/or carrier in extracellular vesicles.

Table 1:

Potential roles of different cell types in regulating inflammatory pathways in ALD

Cell types	Subsets	Functions in inflammation in ALD	References
Kupffer cells	Kupffer cells	Liver resident, sessile (*Markers:* F4/80^{hi}, CD11b^{low}, CD68, CD11c^{int}, TLR4, TLR9). Induce liver injury and inflammation by producing pro-inflammatory cytokines (TNF, IL-1β).	4, 103
Infiltrating macrophages	Inflammatory macrophages	Promote inflammation and fibrosis via the activation of TLRs by producing inflammatory cytokines (TNF, IL1β), chemokines (CCL2), iNOS, and pro-fibrogenic (via TGFβ).	5, 8, 103
	Restorative macrophages	Promote resolution of inflammation and fibrosis by producing anti-inflammatory cytokines (IL10), MMPs, Arg-1. Also post-phagocytic.	8, 103
Circulating monocytes	*Ly6C^{high}*	Promote inflammation, rapid recruitment to sites of inflammation (*Markers:* CCR2, CD11b^{hi})	6, 8, 103
	Ly6C^{low}	Mature monocytes, patrol for injury (*Markers:* CX3CR1, CD11b^{low})	6, 8, 103
Neutrophils	Neutrophils	Neutrophils not only promote hepatocyte injury by producing ROS, but may also promote liver repair by removing dead hepatocytes and producing growth factors. Neutrophils play a key role in controlling bacterial infection in ALD but severe ALD is associated with impaired phagocytic and bactericidal activities.	10-14
CD4+T cells	Th1	Produce IFN-γ, IL-2, TNF-α, etc. which activate macrophages, inducing liver injury, inflammation, and anti-bacterial immunity in ALD.	19, 21
	Th2	Produce IL-4, IL-5, IL-10, and IL-13. The role of Th2 in ALD remains unclear.	
	Th17	Promote liver injury, inflammation, and fibrosis via the production of IL-17. Promote liver repair via the production of IL-22.	22, 76, 104
	Th22	Produce IL-22 to protect against liver injury, but Th22 cells in ALD have not been studied.	23
	T reg	Chronic ethanol increases T regulator cells, causing immunosuppression.	105
CD8+ T cells	CD8	Directly kill hepatocytes.	16, 18
NKT cells	Type I NKT	Activate Kupffer cells and neutrophils, exacerbating ALD in mice.	26, 27, 106
	Type II NKT	Protect against ALD in mice via the inhibition of type I NKT.	106
MAIT cells	MAIT	MAIT cells, which play a key in suppressing bacterial infection, are downregulated in ALD.	29, 30
Hepatocytes	Hepatocytes	Stressed hepatocytes promote inflammation by producing chemokines (CXCL1, IL-8, MIF, MCP-1), DAMPs (mtDNA, HMGB1), acute phase proteins etc. Hepatocytes also play a key role in inhibiting bacterial growth by producing innate immunity proteins (acute phase proteins, complement, lipocalin-2, etc.).	12, 31-34, 40, 107, 108
HSCs	HSCs	Promote inflammation by producing chemokines and supporting neutrophil survival.	109, 110