Review

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Inflammatory pathways in alcoholic steatohepatitis

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Summary

Inflammatory processes are primary contributors to the development and progression of alcoholic steatohepatitis (ASH), with severe alcoholic hepatitis characterised 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 crosstalk. Herein, we review the roles of multiple cell types that are 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 inter-organ crosstalk. We highlight the concept that inflammation also plays an important role in promoting liver repair and controlling bacterial infection. Understanding 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.

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Introduction

Alcohol-related liver disease (ALD) is a metabolic liver disease in which pathologic progression is largely driven by inflammatory responses. In general, infection and cell death are the 2 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 the 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 pro-inflammatory activation of hepatic macrophages.[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 toll-like receptors (TLRs) or the 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 (EVs) that play an important role in regulating inflammatory responses in ALD. Further, inter-organ crosstalk involving the gut, the liver, adipose tissue, muscle, the lungs, and the neuroendocrine system, is also likely to contribute to the development of inflammation in ALD. From a clinical perspective, inflammation is an obvious therapeutic focus for the treatment of alcoholic hepatitis (AH) characterised 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 recognise alcohol-mediated immunosuppression is likely to be an underlying cause of microbial infection and consequent inflammatory responses in alcoholic steatohepatitis (ASH). This review outlines the current state of understanding surrounding the pathogenic mechanisms and implications of inflammation in ASH by categorically dividing discussions into the multiple cell types, major inflammatory pathways, and specific inflammatory mediators that contribute to inflammation in ASH. The review also highlights outstanding questions concerning how best to design therapeutic interventions 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.

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

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Key points

A number of resident and infiltrating immune cells are involved in the inflammatory response, which is a hallmark of ASH pathogenesis.

their function in response to signals within their microenvironment.[3] For example, Kupffer cells become sensitised to TLR4-induced signalling after chronic ethanol exposure, at least in part due to redox-dependent modulation of key signalling events downstream of TLR4.[4] These pro-inflammatory macrophages, together with infiltrating macrophages activated by lipopolysaccharide (LPS), interferon-γ and granulocyte-macrophage colony stimulatory factor (GM-CSF [CSF2]) 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 levels of cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF), 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 secrete large amounts of IL-10, IL-1R antagonist and transforming growth factor beta (TGFβ), subsequently suppressing inflammation and promoting tissue repair. However, this definition of polarisation 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 during ALD is unknown. Infiltrating monocytes are characterised by the expression of Ly6C in mice; Ly6C^{low} monocytes function by patrolling endothelial surfaces for injury,[6] while Ly6C^{high} monocytes are recruited to sites of inflammation.[6] In carbon tetrachloride-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 fibrosis.[7] However, it is not well understood how ethanol exposure may modify this wound healing response. In a recent publication, a catalytic subunit of NADPX oxidase 2 that is dominantly expressed in phagocytes, gp91^{phox}, was shown to support tissue-restorative M2-like macrophages in experimental ALD, as 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 the 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] Whether peritoneal macrophages contribute to ALD via a similar mechanism is yet to be studied. The regulation of cell fate determination of M1 and M2 macrophages in the evolution and progression of ALD is an obvious area of research interest.

Neutrophils

More than 90% of 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 unresolved question regarding the pathogenetic role of neutrophil infiltration in AH, a salient feature of this unique pathologic spectrum. It is generally believed that neutrophils that infiltrate the liver damage hepatocytes in AH. Indeed, in AH, the expression of cytokines/chemokines (IL-1, IL-8, IL-17, C-X-C motif chemokine ligand [CXCL]1, 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 showed that infiltration of neutrophils was 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 migrate to the liver to fight against microbes, as discussed below in relation to pyroptosis in AH. Further, neutrophils in patients with AH are often defective in their phagocytic and bactericidal activities,[14] causing a failure in infection control and sustained upregulation of inflammatory cytokines/chemokines. This may provide the rationale for treatment of severe AH with granulocyte colony-stimulating factor (G-CSF [CSF3]),[15] because it is believed that G-CSF stimulates the generation and activity of neutrophils while promoting liver regeneration. However, preclinical data to support this hypothesis are still lacking.

T lymphocytes, NKT cells, 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 patients with ALD reflected bystander activation or were a consequence of antigen-specific activation. By using high-throughput T-cell receptor (TCR) sequencing analysis, Liaskou *et al.* identified the pronounced oligoclonal nature of T cells in ALD, suggesting the presence of

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Table 1. Potential roles of different cell types in regulating inflammatory pathways in ALD.

Cell types	Subsets	Role in inflammation in ALD	Refs
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
Infiltrating macrophages	Inflammatory macrophages	Promote inflammation and fibrosis via the activation of TLRs by producing inflammatory cytokines (TNF, IL-1β), chemokines (CCL2), iNOS, and pro-fibrogenic (via TGFβ)	5,8,103
	Restorative macrophages	Promote resolution of inflammation and fibrosis by producing anti-inflammatory cytokines (IL-10), MMPs, Arg1. 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, *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
	Treg	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

ALD, alcohol-related liver disease; Arg1, arginase 1; CCL2, C-C motif chemokine ligand 2; CX3XR1, C-X3-C motif chemokine receptor 1; DAMPs, damage-associated molecular patterns; HMGB1, high mobility group box 1; HSC, hepatic stellate cells; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; MAIT, mucosa-associated invariant T; MCP-1, monocyte chemoattractant protein-1; MIF, migration inhibitory factor; MMPs, matrix metallopeptidases; mtDNA, mitochondrial DNA; NKT, natural killer T; ROS, reactive oxygen species; TGFβ, transforming growth factor beta; TLR, toll-like receptor; Treg, regulatory T cells.

neoantigen-specific T cell responses. These neoantigens are likely derived from protein adducts formed with acetaldehyde generated from alcohol metabolism and/or the aldehydic products of lipid peroxidation. In addition, bystander activation of T cells is induced in the absence of specific TCR stimulation by a large number of cytokines, DAMPs, and PAMPs 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 patients with ALD,[16,18] suggesting T cells not only promote disease progression by releasing inflammatory mediators (*e.g.* TNF, 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 groups, and each subset plays different roles in the pathogenesis of ALD by producing a characteristic profile of cytokines.[20] For example, greater pro-inflammatory Th1 responses, which promote disease progression by producing interferon-γ, were found in AH than in alcoholic cirrhosis.[21] Th17 cells not only promote liver inflammation and fibrosis in ALD by producing IL-17,[22] but may also help in 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 patients with ALD.

Natural killer T (NKT) cells, the most abundant lymphocytes in mouse livers, are a subset of T cells characterised by a highly restricted TCR that recognises 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 cell numbers 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 (MAIT) cells, representing 20%–50% of intrahepatic T cells. MAIT cells express invariant TCR that recognises 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] A marked reduction of MAIT cells has been observed in patients with ALD, which may contribute to the increased risk of bacterial infection in these patients.[29,30]

Hepatocytes

Hepatocytes also play an important role in 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 2 major chemokines involved in neutrophil recruitment that are released by hepatocytes and are highly elevated in patients with AH,[17,35] as well as correlating with disease severity.[35] Elevation of hepatic CXCL1 was also reported in several mouse models of steatohepatitis, especially in those which utilised high-fat diet and binge ethanol challenges.[12,31,32] Free fatty acids and cytokines (*e.g.* TNF, 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 factors.[4] likely regulating inflammatory responses and/or promoting inflammation in ALD.

Pathways to inflammation in ASH

Loss of intestinal integrity and dysbiosis

Intestinal dysbiosis and impaired intestinal barrier integrity are important contributors to the pathogenesis of ASH (Fig. 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/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 inducible nitric oxide synthase (iNOS [NOS2]) and NF-κB signalling by acetaldehyde have been implicated in the disruption of tight and adherens junctions in the intestinal epithelium.[42,43] Shifts in the ratios of short-chain fatty acids in favour 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 an upstream causal event and example of organ crosstalk in the 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 regenerating family member 3 beta and gamma (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 (LCFAs) 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 cholylglycine 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 muciniphila*, a gram-negative commensal gut bacterium, is reduced in ALD; when supplemented, it promotes gut barrier function 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, with translocation of fungal β-glucan to the liver causing 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.

Key points

Chronic alcohol consumption increases gut permeability, leading to translocation of microbial products to the liver and the induction of PAMP-mediated inflammation.

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, because of 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 serine/threonine kinase (RIPK)1, recruiting RIPK3 to form the necrosome which in turn phosphorylates, oligomerises, and activates mixed lineage kinase domain like pseu-

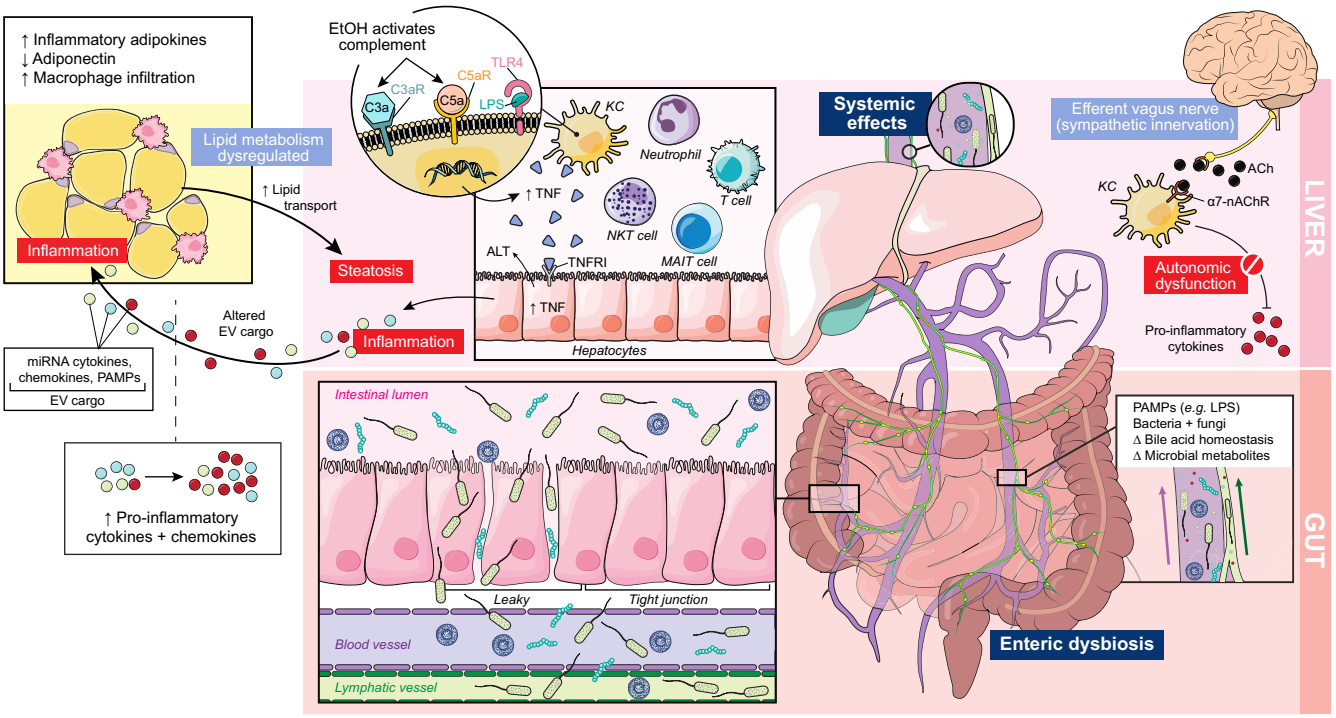

Fig. 1. Inter-organ crosstalk contributes to the progression of ALD. Inter-organ crosstalk contributes to inflammation, metabolic alternations, and cell death in ALD. The gut-liver axis involves enteric dysbiosis, a loss of barrier function leading to translocation of microbes and microbial products 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 crosstalk 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. ALD, alcohol-related liver disease.

dokinase (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 gasdermin D (GSDMD) during pyroptosis.[49] While it is clear that ethanol-induced necroptosis involves RIPK3, 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) depolymerises the RIPK1-RIPK3 complex, prevents necrosome formation, and suppresses pro-inflammatory necroptosis. CASP1 activates CASP3 and CASP7 to induce apoptosis while CASP3 and CASP7 may cleave GSDMD at a distinct site to inactivate this pyroptosis effector protein.[51] In chemotherapy-induced pyroptosis, CASP3 cleaves GSDME, but not GSDMD, to cause cell death.[52] Thus, apoptosis may occur at early steatotic stages of ALD, followed by necroptosis in early ASH. After the transition to AH, pyroptosis may become the predominant form of cell death, which mechanistically links to neutrophilic inflammation and consequentially leads to endotoxemia and septicaemia, the most common cause of death from AH. Cleaved GSDMD also activates NLR family pyrin domain containing 3 (NLRP3)-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 (20–150 nm) to deliver their contents including LPS to host cells.[55] Thus, bacteria do not need to invade cells for LPS to enter the cytosol and activate CASP11/4, which subsequently execute GSDMD-mediated pyroptosis.

Inter-organ crosstalk

Inter-organ crosstalk contributes to inflammation, metabolic alterations, and cell death in ALD (Fig. 1). The aforementioned gut-liver axis is part of this inter-organ crosstalk that involves extensive interactions between multiple organs, such as adipose tissue, muscle, the lungs and the nervous system. Adipose tissue has an important role 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 EVs.[58] Adipose tissue becomes inflamed in response to chronic ethanol, increasing the expression of inflammatory

cytokines, which in turn inhibit the release of adiponectin, an anti-inflammatory adipokine. Decreased plasma levels of adiponectin and/or adiponectin resistance also impair 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 the metabolic and immune function of adipose tissue all contribute to inflammation and injury in the liver.

Other organs are also likely to contribute to inter-organ crosstalk, promoting a pro-inflammatory environment in ASH. One understudied area of inter-organ crosstalk 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 the liver is the lung,[63] lung-liver interactions may indeed contribute to the interactions between cigarette smoking and ASH/ALD.

Mediators of inflammation in ASH

PAMPs and DAMPs

PAMPs entering the liver activate pro-inflammatory signalling. 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 C-type lectin domain containing 7A (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 recognised 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 pro-inflammatory 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 pro-inflammatory cytokines such as pro-IL-1β and pro-IL-18 by the canonical inflammasome, causing release of active forms of these cytokines. Pro-inflammatory TLR activation also occurs in non-immune cells. TLR2 and TLR9 activation mediates the release of CXCL1 by hepatocytes and hepatic stellate cells (HSCs), promoting transient neutrophilic infiltration after alcohol binge.[64] TLR4 activation may also take place in HSCs, repressing the TGFβ pseudoreceptor Bambi and thereby activating the fibrogenic TGFβ pathway,[65] as well as leading to NF-κB activation, upregulation of chemokines and adhesion molecules, and recruitment of inflammatory cells.[66,67]

Key points

Pyroptosis is a form of inflammatory cell death, with important implications in the pathogenesis and complications of ALD.

Canonical and non-canonical inflammasome

The inflammasome is a multiprotein oligomer composed of pro-CASP1, PYCARD, and NLRP, which mediates pro-CASP1 activation and subsequent processing of pro-IL-1β and pro-IL-18 by active CASP1. It has been suggested that the canonical NLRP3 inflammasome has a role in DAMP-mediated activation of pro-IL-1β in alcoholic fatty liver.[68] IL-1β is directly pro-inflammatory but also activates HSCs via upregulation and activation of pro-matrix metallopeptidase 9 (MMP9), an event essential for early matrix remodelling and pro-inflammatory HSC activation.[69]

The non-canonical inflammasome CASP4/11-GSDMD pathway for programmed, lytic cell death "pyroptosis", has recently been elucidated. This pathway links infection to cell death and DAMPs, which incite intense inflammation and possibly 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 gram-negative bacterial infections. Intracellular LPS oligomerises and activates CASP4/11 (4 in man and 11 in mouse). This leads to proteolytic activation of pro-GSDMD, releasing N-terminal 30 kD GSDMD, which is recruited to the plasma membrane to form ~20 nm 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, mice lacking Casp11 or Gsdmd are protected from lethality caused by a high dose of LPS.[53] In an experimental model, CASP11 and GSDMD are not activated in chronic mild ASH, but become evident, concomitant with increased bacterial load and neutrophilic infiltration in the liver, when liver histology transitions to AH following weekly alcohol binge.[70] Deficiency 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] Adeno-associated virus-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 patients with AH but not evident in normal human livers.[70] These results collectively establish pyroptosis as the novel and unique type of cell death that triggers neutrophilic inflammation in AH.

Cytokines and chemokines

A wide variety of cytokines are highly upregulated in the liver and serum of 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, IL-6) play dual roles in the pathogenesis of ALD by not only promoting inflammation and injury but also promoting liver regeneration; whereas some cytokines may have more specific functions, such as IL-1β and IL-22, both of which 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 humans[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, non-parenchymal cells, and inflammatory cells in the liver.[76]

IL-8 and CXCL1 are 2 of the most highly elevated chemokines in patients with AH and likely promote liver inflammation and injury by stimulating neutrophil infiltration.[35] Blockade of IL-8 receptor or CXCL1 ameliorated mouse ASH.[32,77] C–C motif chemokine ligand 20 (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 HSCs.[78] Although many chemokines and their receptors are implicated in ALD based on studies in animal models, none of them have been tested as therapeutic targets for AH. When aiming to inhibit chemokines and their receptors in AH therapy, target redundancy (one chemokine interacting with multiple receptors and vice versa) presents a major challenge.[79] Thus, therapeutics utilising 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, complement C3a receptor 1 (C3AR1) and complement C5a receptor 1 (C5AR1), are important for ethanol-induced complement activation to progress to 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 injured and dying hepatocytes in models of both fibrosis and early ASH.[82,83]

MicroRNA, EVs

MicroRNAs (miRNAs), small non-coding RNA molecules of 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 directly or indirectly regulating inflammatory pathways in ASH.[84,85] For example, miRNA-155, an 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 of TLR4 signalling 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 overactivation 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.[88] Interestingly, miRNAs not only regulate gene expression within the cells in which they are generated 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 EVs from pair-fed mice, and these miRNAs target a large number of genes that are involved in the inflammatory response in ASH.[89] However, how EVs transfer these miRNAs and exactly how these miRNAs regulate the inflammatory pathways in ALD by affecting targeted cells remains unknown.

In addition to the transfer of miRNA, EVs can also transfer RNA, DNA, lipids, proteins *etc.*, into target cells, where they subsequently regulate inflammation in ALD. For example, EVs can carry hepatocyte-derived mitochondrial DNAs, transferring them into neutrophils, and consequently activating 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 crosstalk between adipose tissue and the 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, 3 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 3 miRNA-enriched EVs are good biomarkers for ALD diagnosis and to identify new EV biomarkers for ALD.

Key points

The importance of miRNAs and extracellular vesicles in ALD pathogenesis is an area of increasing research.

The cells infected with bacteria release exosomes containing PAMPs which may incite inflammatory signalling in recipient cells,[92] a situation relevant to AH because of increased bacterial translocation. In patients with diffuse large B-cell lymphoma, constitutively active myeloid differentiation primary response gene 88 (MyD88)^{L265P} can be released via EVs to propagate inflammation and promote tumour growth,[93] exemplifying the transmission of a cellular signalling component via EVs. EVs are also released by prokaryotes (bacterial microvesicles),[94] opening up potential crosstalk between gut bacteria and intestinal epithelial cells, or liver cells if bacterial translocation occurs. What regulates EV release from different liver cell types and how cells are differentially targeted by EVs in different spectra of ALD are important outstanding questions.

Pro- and anti-inflammatory signalling 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 altered in the context of ASH. Hepatic macrophages are more sensitive to activation of TLR2 and TLR4[4] characterised by increased activation of both MyD88 and TIR-domain-containing adapter-inducing interferon (TRIF)-mediated downstream signals, including mitogen-activated protein kinase (MAPK) family members and NF-κB.[4] Signalling via the complement anaphylatoxins C3AR1 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 signalling, the precise mechanisms for these effects are not well understood. Ethanol is likely to interact with redox-dependent signalling 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 miRNAs regulating TLR4 signalling, including miR-181b3p upregulating importin-α5 and p65 nuclear translocation, miR-291b downregulating Tollip (a negative regulator of TLR4 signalling) and miR-155 controlling the stability of *TNF* mRNA.[87,98,99] Interestingly, chronic ethanol also regulates Slu7, an mRNA splicing factor, to increase inflammation.[100]

Chronic ethanol also impairs anti-inflammatory signalling. For example, increases in the expression of phosphodiesterase 4 (PDE4) during chronic ethanol exposure 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 normalising inflammation in ASH. For instance, 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 downregulates PDE4 and increases cAMP[102] and 35 kD hyaluronic acid normalises TLR4 signalling in hepatic macrophages by impacting the expression of specific miRNAs.[87,98]

Conclusion

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 non-parenchymal 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 important factors for activation of 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 inflammatory mediators (*e.g.* TNF) 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 when using these mediators (*e.g.* TNF) 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. Therefore, 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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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Review concept and design: B Gao, LE Nagy, H Tsukamoto. Drafting of the manuscript: B Gao, MF Ahmad, LE Nagy, H Tsukamoto. Critical revision of the manuscript for important intellectual content: B Gao, MF Ahmad, LE Nagy, H Tsukamoto.

Supplementary data

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Author names in bold designate shared co-first authorship

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