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Innate Immunity in Alcohol Liver Disease

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1. Introduction

Excessive ingestion of alcohol is one of the major causes of chronic liver disease worldwide. Alcoholic liver disease (ALD) encompasses a broad spectrum of diseases ranging from steatosis (fatty liver), steatohepatitis, fibrosis, cirrhosis to hepatocarcinoma. Almost all heavy drinkers develop fatty liver; however, only up to 30% of heavy drinkers may develop more severe forms of chronic liver injury such as alcoholic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (O'Shea et al., 2010). Despite extensive research, cellular and molecular mechanisms contributing to the pathogenesis of ALD remain to be fully elucidated. Classically, direct hepatotoxicity and production of reactive oxygen species (ROS) induced by alcohol and its metabolites (e.g. acetaldehyde, acetate) are considered the major causative factors. Nevertheless, growing evidence suggests innate immunity also plays an important role in the pathogenesis of ALD (Byun & Jeong, 2010; Gao et al., 2011; Miller et al., 2011).

In this chapter we discuss the association between innate immunity and ALD. Specifically, we discuss the following topics: i) role of liver in innate immunity; ii) mechanisms of alcohol-induced dysregulation of innate immunity; iii) role of dysregulation of innate immunity in the pathogenesis of ALD; iv) modulation of innate immunity in the treatment of ALD. Additionally, we also discuss the role of innate immunity impairment in ALD-associated infection risk. In this topic, we detail the data of our recent study on Toll-like receptor (TLR)2- and 4-mediated immune response in patients with alcoholic cirrhosis.

2. Role of liver in innate immunity

Innate immunity is an important first line of defense against infection, quickly responding to potential attacks by pathogens. It consists of anatomic barriers (e.g., skin, epidermis, dermis, and mucous membranes), physiologic barriers (e.g., temperature, low pH, oxygen), humoral factors (e.g., pepsin, lysozyme, anti-microbial substances, interferons, complement), phagocytic cells (e.g., neutrophils and macrophages), and lymphocyte cells (e.g., natural killer [NK] and NKT cells). Many of these barriers and factors can prevent or destroy the invading pathogens nonspecifically. However, recent evidence suggests that innate immunity can also specifically detect infection through pattern-recognition receptors (PRRs) that recognize specific structures, called pathogen-associated molecular patterns (PAMPs), that are expressed by invading pathogens. Many PAMPs have been identified, including bacterial carbohydrates (e.g., lipopolysaccharide or LPS, mannose), bacterial peptides
(flagellin), peptidoglycans and lipoteichoic acids (from Gram-positive bacteria), N-formylmethionine, lipoproteins and fungal glucans, and nucleic acids (e.g., bacterial or viral DNA or RNA). The PRRs can be divided into 3 categories: secreted PRRs, membrane-bound PRRs, and phagocytic PRRs. Secreted PRRs are a group of proteins that kill pathogens through complement activation and opsonization of microbial cells for phagocytosis. Secreted PRRs include complements, pentraxins, and peptidoglycan-recognition proteins, which are mainly produced by hepatocytes and secreted into the blood stream. Membrane-bound or intracellular PRRs include TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-induced gene I-like helicases. Phagocytic (or endocytic) PRRs which are expressed on the surface of macrophages, neutrophils, and dendritic cells can bind directly to pathogens, and this is followed by phagocytosis into lysosomal compartments and elimination. These phagocytic PRRs include scavenger receptors, macrophage mannose receptors, and β-glucan receptors (Janeway & Medzhitov, 2002).

Blood circulating from the intestines to the liver is rich in bacterial products, environmental toxins, and food antigens. To effectively and quickly defend against potentially toxic agents without launching harmful immune responses, the liver relies on its strong immune system. Interestingly, increasing evidence has suggested that the immune system in the liver consists of predominantly innate immunity (Gao et al., 2008). First, the liver is responsible for the biosynthesis of 80–90% innate proteins including complements, secreted PRRs and acute phase proteins. Second, the liver contains a large number of Kupffer cells (KCs), which account for 80-90% of the total population of fixed tissue macrophages in the body. KCs, in combination with liver sinusoidal cells, are responsible for clearance of soluble macromolecules and insoluble waste in the body. Third, liver lymphocytes are enriched in innate immune cells including NK and NKT cells. Human intrahepatic lymphocyte population contain about 30% to 50% NK cells and up to 10% NKT cells. Fourth, liver non-parenchymal cells also express high levels of membrane-bound PRRs, such as TLRs. Finally and interestingly, the adaptive immunity in the liver seems less active because the liver is a major site to induce T cell apoptosis.

3. Dysregulation of innate immunity in ALD

Growing evidence suggests alcohol induces dysregulation of innate immunity through three main mechanisms: i) activation of LPS/TLR4 signalling pathway; ii) activation of complement system; iii) inhibition of innate immunity cells, namely NK cells.

3.1 Activation of LPS/TLR4 signalling pathway

LPS is a component of Gram-negative bacteria cell wall. It consists of hydrophilic polysaccharides of the core and O-antigen and a hydrophobic lipid A component. This hydrophobic component corresponds to the conserved molecular pattern of LPS and is the main inducer of biological responses to LPS. TLR4, a member of human TLR family, is the receptor of LPS. Stimulation of TLR4 by LPS involves the participation of several molecules [LPS binding protein (LBP), cluster of differentiation-14 (CD14) and myeloid differentiation-2 (MD-2)] (figure 1). LBP (a soluble protein) extracts LPS from the bacterial membrane and shuttles it to CD14 (a glycosylphosphatidylinositol-anchored protein, which also exists in a soluble form). CD14 then transfers the LPS to MD-2 (a soluble protein that non-covalently
associates with the extracellular domain of TLR4). Binding of LPS to MD-2 induces a conformational change in MD-2 which then allows the complex MD-2-TLR4 to bind to a second TLR4 receptor thus achieving TLR4 homo-dimerisation and signalling.

Fig. 1. Overview of LPS/TLR4 signalling pathway. Stimulation of TLR4 by LPS involves the participation of several molecules (LBP, CD14 and MD-2). Activation of TLR4 induces two downstream signalling pathways. First, the MyD88-dependent pathway is initiated by recruitment of TIRAP and MyD88 to the TLR4 complex which leads to early-phase activation of NF-κB and subsequent induction of the expression of NF-κB-controlled genes including pro-inflammatory cytokines (TNF-α). The MyD88-dependent pathway can also activate JNK, leading to transcription of several genes including TNF-α, via activation of AP-1. Second, the MyD88-independent pathway is initiated by recruitment of TRAM and TRIF to the TLR4 complex, followed by late activation of NF-κB complex and activation of IRF3 which leads to the transcription of IFN-β as well as other interferon-induced genes. See text for abbreviations.

Activation of TLR4 induces two downstream signalling pathways (figure 1). First, the MyD88-dependent pathway is initiated by recruitment of TIR domain-containing adaptor protein (TIRAP) and myeloid differentiation factor 88 (MyD88) to the TLR4 complex which
leads to early-phase activation of nuclear factor-κB (NF-κB) and subsequent induction of the expression of NF-κB-controlled genes including pro-inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6)] and chemokines [monocyte chemotactic protein-1 (MCP-1)] genes. The MyD88-dependent pathway can also activate c-Jun N-terminal kinase (JNK), leading to activator protein-1 (AP-1) activation that initiates the transcription of genes involved in regulation of cell proliferation, morphogenesis, apoptosis, and differentiation. Second, the MyD88-independent pathway is initiated by recruitment of TIR-domain containing adaptor inducing interferon-ǃ (TRIF) and TRIF-related adaptor molecule (TRAM) to the TLR4 complex, followed by late activation of NF-κB complex and activation of interferon regulatory factor 3 (IRF3) which leads to the transcription of interferon-ǃ (IFN-ǃ) as well as other interferon-induced genes (Lu et al., 2008).

Most parenchymal and non-parenchymal liver cells express TLR4. Nonetheless, with the exception of KCs, the amount of TLR4 expression and the level of responsiveness to LPS in most liver cells appear to be low in non-inflamed liver (Su et al., 2000; Zarember & Godowski, 2002).

Several studies suggest that chronic alcohol ingestion can enhance hepatic LPS/TLR4 signalling through increase of portal and systemic levels of LPS and upregulation and sensitization of hepatic TLR4 (Soares et al., 2010). Chronic ingestion of alcohol leads to a strong elevation of portal and systemic levels of LPS in animal models and humans (Mathurin et al., 2000; Parlesak et al., 2000). The elevation of LPS levels appears to be predominantly caused by two mechanisms. First, alcohol exposure can promote the growth of Gram-negative bacteria in the intestine, which leads to enhanced production of LPS (Hauge et al., 1997). Second, alcohol metabolism by Gram-negative bacteria and intestinal epithelial cells can result in accumulation of acetaldehyde, which in turn can increase intestinal permeability by opening intestinal tight junctions. Increased intestinal permeability can lead to increased transfer of LPS from the intestine to portal and systemic circulation (Purohit et al., 2008). Furthermore, chronic alcohol consumption upregulates hepatic TLR4 and sensitizes it to LPS to enhance TNF-ǂ production, a process known as priming (Gustot et al., 2006).

Besides LPS, TLR4 also senses endogenous ligands initiating danger signals, such as high mobility group box 1 (HMGB1), hyaluronan, heat shock protein 60 and free fatty acids (C12:0, C14:0, C16:0, and C18:0) (Erridge, 2010). In particular, HMGB1 has been shown to be released from damaged hepatocytes and contribute to liver injury (Tsung et al., 2005). Due to the association of many endogenous ligands with tissue injury, they are termed damage-associated molecular patterns (DAMPs). Interestingly, recent studies show that many of the proposed endogenous TLR4 ligands may also have the capacity to bind and transport LPS and/or enhance the sensitivity of cells to LPS, suggesting that many of these molecules may be more accurately described as PAMP-binding molecules or PAMP-sensitizing molecules, rather than genuine ligands of TLR4 (Erridge, 2010). Therefore, these endogenous ligands, namely HMGB1, may enhance TLR4 signalling in ALD.

3.2 Activation of complement system

The complement system is a component of innate immunity that consists of multiple plasma proteins which act to fight infection by opsonizing pathogens, inducing inflammatory
responses, enhancing antibody responses, and attacking some pathogens directly. Activation of the complement cascade relies on cleavage of a zymogen to yield an active enzyme that in turns cleaves and activates the next zymogen in the cascade. Through this series of cleavage and enzyme activation, the immune system is able to produce a wide-reaching response to few stimulation events (Gasque, 2004). The complement system is activated by three different pathways: classical, lectin and alternative pathways (figure 2). The classical pathway is activated by IgM- or IgG-containing immune complexes. The lectin pathway is activated when mannose-binding lectin binds its receptor, mannose, which is expressed by microbial pathogens. The alternative pathway is activated by C3b-coated pathogens. The three pathways converge at the generation of a C3 convertase that cleaves C3 to C3a and C3b. C3b is an opsonizing protein that coats pathogen surfaces to facilitate their uptake and destruction by phagocytes. C3b can also activate alternative pathway or associate to C3 convertase forming C5 convertase that cleaves C5 to C5a and C5b. C3a and C5a lead to increased migration of phagocytes to the site of infection and induce mast cells to release histamine and TNF-α, which contribute to the enhancement of inflammatory response. C5b forms with C6, C7, C8, and C9 the membrane-attack complex that destroys certain pathogens by disrupting their membrane integrity (Gasque, 2004).

The liver (primarily hepatocytes) is a major site that biosynthesizes complement components found in plasma. Hepatocytes are also primarily responsible for the biosynthesis of several complement regulator proteins found in plasma, such as factor I, factor H, and the C1 inhibitor. Additionally, cells in the liver also express complement factor receptors, as well as intrinsic regulatory proteins (Qin & Gao, 2006).

A growing body of evidence in mouse models suggests that alcohol exposure results in activation of the complement system and inhibition of regulatory proteins. Chronic alcohol feeding to mice for 4-6 weeks increases activation of C3, as evidenced by increased C3a in the circulation (Pritchard et al., 2007), as well as increased accumulation of C3 or its proteolytic end product C3b/iC3b/C3c in liver (Jarvelainen et al., 2002; Roychowdhury et al., 2009). In rats, chronic alcohol exposure increases C3 activity and decreases expression of Crry, the rat homologue of the complement inhibitory protein CD55/DAF (decay-accelerating factor), and CD59 in the liver (Jarvelainen et al., 2002).

Complement is activated early in the progression of alcohol-induced liver injury, prior to detectable increases in ALT/AST or accumulation of hepatic triglycerides (Roychowdhury et al., 2009). Early activation of complement contributes to increased inflammatory cytokine expression, mediated via the activation of the anaphylatoxin receptors, C3aR (C3a receptor) and C5aR (C5a receptor), on KCs (Roychowdhury et al., 2009). The contribution of each pathway of complement activation in response to alcohol exposure is still unclear. It has been suggested that alcohol-induced increase in LPS levels may contribute to activation of complement via the alternative pathway (Jarvelainen et al., 2002). Recent evidence shows that alcohol feeding activates the classical complement pathway via C1q binding to apoptotic cells in the liver, suggesting that the classical complement pathway also contributes to complement activation in the pathogenesis of ALD (Cohen et al., 2010). It is also likely that activation of complement by any mechanism will initiate the alternative pathway-mediated feedback loop (Gasque, 2004). Further studies are still needed to elucidate the specific role of each pathway of complement activation in response to alcohol exposure.
Fig. 2. Overview of complement system. The complement system is activated by three different pathways: the classical, lectin and alternative pathways. The classical pathway is activated by IgM- or IgG-containing immune complexes. The lectin pathway is activated when mannose-binding lectin bind its receptor, mannose, which is expressed by microbial pathogens. The alternative pathway is activated by C3b-coated pathogens. The three pathways converge at the generation of a C3 convertase that cleaves C3 to C3a and C3b. C3b is an opsonizing protein that coats pathogen surfaces to facilitate their uptake and destruction by phagocytes. C3b can also activate alternative pathway or associate to C3 convertase forming C5 convertase that cleaves C5 to C5a and C5b. C3a and C5a lead to increased migration of phagocytes to the site of infection and induce mast cells to release histamine and TNF-α, which contribute to the enhancement of inflammatory response. C5b forms with C6, C7, C8 and C9 the membrane-attack complex that destroys certain pathogens by disrupting their membrane integrity.

3.3 Inhibition of NK cells

Over last several decades, many studies have shown that liver lymphocytes are rich in NK cells and that these cells play an important role in innate immune response against tumors and microbial pathogens including viruses, bacteria and parasites (Gao et al., 2009). NK cells can kill virus-infected cells and tumor cells via releasing granules containing granzyme and perforin, death ligand as TNF-related apoptosis-inducing ligand (TRAIL) and a variety of proinflammatory cytokines such as IFN-γ and TNF-α (Gao et al., 2009). Increasing evidence suggests that NK cells may also be involved in the pathogenesis of liver injury, fibrosis, and regeneration. For example, it has been shown that activation of NK cells inhibits liver fibrosis in vivo (Melhem et al., 2006).
Chronic alcohol consumption inhibits NK cells and such inhibition likely contributes to the pathogenesis of ALD. The inhibitory effect of chronic alcohol consumption on NK cells function has been observed for many years in alcoholic patients and rodents fed alcohol diets (Cook et al., 1997). This inhibitory effect is mediated by multiple mechanisms. First, chronic alcohol consumption directly attenuates NK cell cytotoxicity against activated hepatic stellate cells (HSCs) via down regulation of NK cell-associated molecules such as TRAIL, Natural killer group 2, member D (NKG2D) and interferon-γ (IFN-γ) (Jeong et al., 2008). Second, chronic alcohol consumption indirectly attenuates NK cell killing activity by stimulating HSCs to produce transforming growth factor-β (TGF-β), an inhibitor of NK cells (Jeong et al., 2008), by elevating serum levels of corticosterone, which inhibits NK cells functions (Arjona et al., 2004), and by reducing central and peripheral levels of opioid peptide β-endorphin that can induce NK cells activation (Boyadjieva et al., 2004). Third, chronic alcohol exposure renders activated HSCs resistant to NK cell killing, because it induces higher expression of suppressor of cytokine signaling 1 (SOCS1) and ROS that inhibit IFN-γ activation of signal transducer and activator of transcription 1 (STAT1) (Jeong et al., 2008). Lastly, alcohol consumption blocks NK cells release from the bone marrow and enhances splenic NK cell apoptosis (Zhang & Meadows, 2009).

4. Role of dysregulation of innate immunity in ALD

Recent studies have revealed how alcohol-induced dysregulation of innate immunity may contribute to the pathogenesis of ALD (figure 3).

4.1 Alcoholic liver steatosis

Alcoholic liver steatosis corresponds to fat accumulation in hepatocytes, which is the result of unbalanced fat metabolism characterized by decreased mitochondrial lipid oxidation and enhanced synthesis of triglycerides. This unbalancing may be related with increased nicotinamide adenine dinucleotide (NADH)/NAD⁺ ratio (Fromenty et al., 1997), increased sterol regulatory element-binding protein-1 (SREBP-1) activity (You et al., 2004), decreased peroxisome proliferator-activated receptor-α (PPAR-α) activity (Ip et al., 2003) and decreased AMP-activated protein kinase (AMPK) activity (You et al., 2004).

In addition to these mechanisms, growing evidence suggests alcohol-induced dysregulation of innate immunity may also contribute to alcohol-induced liver steatosis, mainly through increased TNF-α production by KCs in response to LPS. Increased expression of TNF-α has been observed in alcoholic liver steatosis of mice (Pritchard et al., 2007) and absence of its receptor (TNF-α R1) activity inhibits the development of alcoholic liver steatosis (Yin et al., 1999). In addition, it has been reported that TNF-α has a potential to increase mRNA expression of SREBP-1, a potent transcription factor of fat synthesis, in the liver of mice and to stimulate the maturation of SREBP-1 in human hepatocytes, respectively (Endo et al., 2007). In contrast, IL-6 produced by KCs in response to LPS has been shown to protect against alcoholic liver steatosis via activation of signal transducer and activator of transcription 3 (STAT3), consequently inhibiting of SREBP-1 gene expression in hepatocytes (El-Assal et al., 2004). Interestingly, chronic alcohol exposure inhibits IL-6 activation of STAT3 in hepatocytes and thus can counterbalance the protective effective of IL-6 (Weng et al., 2008).
In addition to alcohol-induced activation of LPS/TLR4 signalling pathway, alcohol-induced inhibition of NK cell cytotoxicity against HSCs can also contribute liver steatosis as HSCs have been shown to stimulate accumulation of fat in hepatocyte (Jeong et al., 2008). It has been shown that chronic alcohol drinking activates HSCs to produce 2-arachidonoylglycerol (2-AG), one of endocannabinoids, which, activating its receptor, cannabinoid receptor 1 (CB1R) on hepatocytes increases the expression of SREPB-1 and fatty acid synthase (FAS) but decreased AMPK activation, consequently leading to accumulation of fat in hepatocytes. These data, however, are provided by a single study and require further studies.

### 4.2 Alcoholic liver steatohepatitis

Alcoholic steatohepatitis (ASH) refers to infiltration of liver by inflammatory cells, mainly granulocytes, in addition to fat accumulation. The recruitment of inflammatory cells seems to be related with the production of cytokines, chemokines and ROS. Although this production was historically linked to direct hepatotoxicity of alcohol and its metabolites, recent evidence suggest that alcohol-induced LPS/TLR4 signalling can also contribute to
this production and be a key player in the pathogenesis of ASH. Exposure to LPS during chronic alcohol consumption results in increased production of inflammatory mediators (TNF-α, IL-1, IL-6 and IL-8) as well as in induction of ROS, which subsequently aggravate steatohepatitis (Arteel, 2003). The role of LPS/TLR4 signalling pathway in the pathogenesis of ASH is further supported by studies showing that inhibition of LPS/TLR4 signalling, by altering intestinal microbiota and LPS production (through the use of antibiotics or probiotics) or suppressing TLR4, LBP or CD14 genes expression, protects against ASH. Indeed treatment with antibiotics or probiotics suppresses alcohol-induced liver injury by reducing LPS circulating levels (Adachi et al., 1995; Nanji et al., 1994). Studies in knockout mouse models have shown that chronic alcohol feeding in mice deficient of TLR4, LBP or CD14 results in attenuation of alcohol-induced liver injury despite elevated LPS circulating levels (Uesugi et al., 2001; Uesugi et al., 2002; Yin et al., 2001).

Recent studies have clarified the cellular and molecular pathways by which LPS/TLR4 signalling promotes ASH. KCs have been established as a crucial cellular target of LPS in ASH as demonstrated by a strong reduction of alcoholic liver injury following depletion of KCs with gadolium chloride (Adachi et al., 1994). Moreover, it was shown that disruption of the TLR4 downstream signaling molecule MyD88 in mice failed to prevent ASH (Hritz et al., 2008), while disruption of the MyD88-independent signaling molecule TRIF in mice abolished ASH (Zhao et al., 2008), suggesting that the MyD88-independent pathway contributes to TLR4-mediated alcoholic liver injury. Further studies suggest that TRIF/IRF-3 plays a critical role in alcohol-induced transactivation of the TNF-α gene in KCs/macrophages in vitro and in vivo, thereby initiating alcoholic liver injury (Zhao et al., 2008). Furthermore, it was also shown that TLR4 deficiency prevented hepatic alcohol-induced production of inflammatory mediators (TNF-α and IL-6), TLR4 coreceptors (CD14 and MD2) and ROS by cytochrome P450 and the nicotinamide adenine dinucleotide phosphate (NADPH) complexes (Hritz et al., 2008). These data suggest that TLR4-mediated alcoholic liver injury is carried out by increased inflammatory mediators (TNF-α and IL-6) and ROS production and that there is a crosstalk between oxidative stress and TLR4 pathways in ALD. This is further supported by studies showing that mice deficient in p47phox, the main cytosolic component of NADPH complex, show an absence of free-radical production, NF-κB activation, TNF-α mRNA induction and liver pathology after alcohol treatment (Kono et al., 2000) and that inhibition of NADPH complex prevents upregulation of TLR4 and sensitization to LPS-induced liver injury (Gustot et al., 2006). Taken together these data suggest that activation of TLR4 in KCs by LPS is a key pathogenic mediator of ASH, through production of inflammatory cytokines and ROS.

In addition to alcohol-induced activation of LPS/TLR4 signalling pathway, alcohol-induced activation of complement can also contribute to ASH. This is mainly supported by studies showing that mice deficient in C3 and C5 are protected against alcohol-induced increases in hepatic triglycerides and circulating ALT, respectively (Pritchard et al., 2007) and that chronic alcohol-induced liver injury is exacerbated in mice lacking CD55/DAF, a complement regulatory protein, compared to wild-type controls (Pritchard et al., 2007). At present, the molecular mechanisms by which C3 and C5 contribute to ASH are not fully understood and require further studies.
4.3 Alcoholic liver fibrosis

Alcoholic liver fibrosis is characterized by excessive deposition of extracellular matrix components due to increased matrix production and decreased matrix degradation (Henderson & Iredale, 2007). Several studies have highlighted the central role of HSCs in the production of extracellular matrix and the promotion of liver fibrosis.

Alcohol contributes to activation of HSCs by several mechanisms, including upregulation of collagen transcription in HSCs by acetaldehyde or ROS from alcohol-exposed hepatocytes. Recently, alcohol-induced innate immunity dysregulation has also been shown to contribute to liver fibrosis, mainly through activation of LPS/TLR4 signalling in HSCs and inhibition of NK cells, as discussed below (figure 4).

![Diagram of the role of alcohol-induced innate immunity dysregulation in liver fibrosis](image)

The crucial role of LPS/TLR4 signalling in liver fibrosis is supported by studies showing that inhibition of LPS/TLR4 signalling by altering intestinal microbiota and LPS production (through use of antibiotics or probiotics) or suppressing TLR4, LBP or CD14 genes expression protects against liver fibrosis. It has been shown that antibiotics prevent fibrosis induced by CCl4 treatment or a choline-deficient diet (MCDD), and that LPS enhances hepatic fibrosis induced by a MCDD (Luckey et al., 1954; Rutenburg et al., 1957). Treatment of mice with nonabsorbable broad-spectrum antibiotics also resulted in a clear reduction in
the fibrotic response of mice, upon bile duct ligation (Seki et al., 2007). Recently, Velayudham et al showed that VSL#3 (a probiotic) protects against MCDD-induced liver fibrosis, through modulation of collagen expression and inhibition of TGF-β expression and signalling (Velayudham et al., 2009). TLR4-, LBP- and CD14-deficient mice also have demonstrated the crucial role for the LPS–TLR4 pathway in hepatic fibrogenesis (Isayama et al., 2006; Seki et al., 2007). TLR4-mutant mice display a profound reduction in hepatic fibrogenesis in three different experimental models of biliary and toxic fibrosis (Seki et al., 2007). LBP- and CD14-deficient mice also have a marked reduction of hepatic fibrosis upon bile duct ligation (Isayama et al., 2006).

In a recent study, Seki et al analyzed the cell-specific molecular mechanism underlying the role of LPS/TLR4 on liver fibrosis (Seki et al., 2007). They showed that chimeric mice that contain TLR4-mutant KCs and TLR4-intact HSCs developed significant fibrosis and the mice that contain TLR4-intact KCs and TLR4-mutant HSCs developed minimal fibrosis after bile duct ligation, indicating that TLR4 on HSCs, but not on KCs, is crucial for hepatic fibrosis. Notably, KCs are essential for fibrosis by producing TGF-β independent of TLR4. TLR4-activated HSCs produce CC-chemokines [chemokine ligand (CCL)2, CCL3, and CCL4] and express adhesion molecules [inter-cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)] that recruit KCs to the site of injury. Simultaneously, TLR4 signalling downregulates the TGF-β decoy receptor (Bambi) to boost TGF-β signalling and allow for unrestricted activation of HSCs by KCs, leading to hepatic fibrosis. Finally, by using adenoviral vectors expressing an inhibitor of NF-κB kinase (IκB)-superrepressor and knockout mice for MyD88 and the adapter molecule TRIF, the authors demonstrated that TLR4-dependent down-regulation of Bambi is mediated via a pathway involving MyD88 and NF-κB, but not TRIF. In summary, they demonstrated that LPS/TLR4 signalling acts in a profibrogenic manner via two independent mechanisms: it induces the secretion of chemokines from HSCs and chemotaxis of KCs which secrete the profibrogenic cytokine TGF-β (in a TLR4-independent manner); additionally, TLR4-dependent signals augment TGF-β signalling on HSCs via down-regulation of the TGF-β pseudoreceptor Bambi.

The strong association of the LPS/TLR4 signalling pathway and liver fibrosis has been recently confirmed in patients with chronic hepatitis C virus (HCV) infection by studying TLR4 single nucleotide polymorphisms (SNPs). Huang et al conducted a gene centric functional genome scan in patients with chronic HCV infection, which yielded a Cirrhosis Risk Score signature consisting of seven SNPs that may predict the risk of developing cirrhosis (Huang et al., 2007). Among these, a major CC allele of TLR4 encoding a threonine at amino acid 399 (p.T399I) was the second most predictive SNP among the seven, indicating a protective role in fibrosis progression of its c.1196C>T (rs4986791) variant at this location (p.T399I), along with another highly cosegregated c.896A>G (rs4986790) SNP located at coding position 299 (p.D299G). In a subsequent study the same group examined the functional linkage of these SNPs to HSCs responses (Guo et al., 2009). They showed that both HSCs from TLR4-deficient mice and a human HSC line (LX-2) reconstituted with either TLR4 D299G and/or T399I complementary DNAs were hyporesponsive to LPS stimulation compared to those expressing wild-type TLR4, as assessed by the expression and secretion of LPS-induced inflammatory and chemotactic cytokines (i.e., MCP-1, IL-6), downregulation of Bambi expression and activation of NF-κB-responsive luciferase reporter. In addition, spontaneous apoptosis, as well as apoptosis induced by pathway inhibitors of NF-κB,
extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase were greatly increased in HSCs from either TLR4-deficient or Myd88-deficient mice, as well as in murine HSCs expressing D299G and/or T399I SNPs (Guo et al., 2009). Thus, the protective effect of the TLR4 SNP (c.1196C>T [rs4986791, p.T399I]) is explained at least in part by its ability to increase apoptosis and decrease fibrogenic signalling in HSCs. Recently, Li et al expanded the list of TLR4 SNPs that are independently associated with the risk of liver fibrosis progression and the development of cirrhosis (Li et al., 2009). Taken together these data suggest LPS/TLR4 signalling in HSCs is essential for liver fibrosis development, by stimulating production chemokines that recruit KCs and at the same time allowing for unrestricted activation of HSCs by KCs-derived TGF-β.

**Fig. 5.** Mechanisms of killing of activated stellate cells by NK cells. NK cells kill early activated HSCs but not quiescent HSCs. This is because early activated HSCs express increased RAE-1 via retinol metabolism, a NK cell-activating ligand of NKG2D, but express MHC-I, a NK cell-inhibitory ligand of iKIR, thus activating NK cells. After activation, NK cells initiate killing of activated HSCs through releasing of TRAIL, which targets TRAILR that is upregulated on activated HSCs, and IFN-γ, which targets IFN-γR on HSCs to induce cell cycle arrest and apoptosis in a STAT1-dependent manner. ↓, decrease; ↑, increase. See text for abbreviations.

In addition to alcohol-induced activation of LPS/TLR4 signalling pathway, alcohol-induced NK cells inhibition, can also lead liver fibrosis as these cells have been shown to have anti-fibrotic effects via multiple mechanisms (figure 5). Interestingly, NK cells directly kill early activated HSCs but not quiescent HSCs (Melhem et al., 2006; Radaeva et al., 2006; Radaeva et al., 2007). This is because early activated HSCs express increased retinoic acid early inducible gene 1 (RAE-1) via retinol metabolism, a NK cell-activating ligand of NKG2D but express decreased class I major histocompatibility complex (MHC-I), a NK cell-inhibitory ligand of inhibitory killer immunoglobulin-related receptor (iKIR), thus activating NK cells (Radaeva et al., 2007; Taimr et al., 2003). After activation, NK cells initiate killing of activated HSCs through releasing of TRAIL, which targets TRAIL receptor (TRAILR) that is upregulated on activated HSCs, and IFN-γ, which targets IFN-γ receptor (IFN-γR) on HSCs.
to induce cell cycle arrest and apoptosis in a STAT1-dependent manner (Baroni et al., 1996; Jeong et al., 2006). The crucial role of alcohol-induced NK cells inhibition on alcoholic liver fibrosis has been suggested by the finding that attenuated NK cell cytotoxicity against HSCs in alcohol-fed mice contributed to acceleration of liver fibrosis associated with CCl(4) treatment (Jeong et al., 2008).

Few studies have evaluated the role of complement in alcoholic liver fibrosis. By using intercross studies in animal models of liver fibrosis, Hillebrandt et al. (Hillebrandt et al., 2005) demonstrated that C5 plays an important role in promoting liver fibrogenesis via targeting C5aR on activated HSCs and KC in mice, because C5 deficiency resulted in lowered liver fibrosis, whereas overexpression of the C5 gene resulted in increased liver fibrosis. Thus, C5 activation during alcohol consumption, as discussed above, likely also contributes to the development of alcoholic liver fibrosis. In addition, Hillebrandt et al. (Hillebrandt et al., 2005) also reported that two C5 hSNPs (rs 2300929 and rs17611) are associated with the high risk for developing advanced fibrosis in patients with chronic HCV infection. At present, the molecular mechanisms by which the C5 contributes to liver fibrosis are not fully understood and require further studies.

4.4 Hepatocarcinoma

Hepatocarcinoma is a complication of ALD, which always develops in a cirrhotic liver. Thus, alcoholic liver cirrhosis is a premalignant condition with approximately fourfold increase in the risk of hepatocarcinoma. The five-year cumulative incidence of hepatocarcinoma reaches 8%. In addition, clinical and epidemiological evidence implicates long-term alcohol consumption in accelerating HCV-mediated tumorigenesis (Hassan et al., 2002). A recent study provided evidence that TLR4 mediates the synergism between alcohol and HCV in hepatic oncogenesis (Machida et al., 2009). Machida et al. studied the molecular mechanism of synergism between alcohol and HCV, using mice with hepatocyte-specific transgenic expression of the HCV nonstructural protein NS5A, which is known to have a cryptic trans-acting activity for cellular gene promoters. They demonstrated that NS5A and alcohol synergistically induce hepatocellular damage and transformation via accentuated and/or sustained activation of TLR4 signalling, which results from HCV NS5A-induced hepatic TLR4 expression and alcohol-induced endotoxaemia. Additionally, Nanog, a stem cell marker, was identified as a novel downstream gene transcriptionally induced by activated TLR4 signalling, that is largely responsible for TLR4-mediated liver tumor development.

Taken together these data suggest TLR4 signalling in hepatocytes may constitute the link between alcoholic liver cirrhosis and hepatocarcinoma.

5. Role of innate immunity impairment in ALD infection risk

Patients with ALD are particularly susceptible to infections, with increased morbidity and mortality from sepsis, mainly in the presence of cirrhosis (Linderoth et al., 2006; Navasa et al., 1999). We and others have shown that in advanced stages of alcoholic liver disease the alcohol-induced pro-inflammatory state is replaced by a state of immune paralysis that can greatly decrease the innate immune response of immunological cells (Lin et al., 2007; Pimentel-Nunes et al., 2010; Wasmuth et al., 2005). These data support the hypothesis that
Patients with alcoholic cirrhosis are likely to have underlying immune dysfunction, particularly innate immunity dysfunction that makes them susceptible to increased risk of infections.

Homman et al have shown that acquired C3 deficiency and decreased haemolytic complement function predisposes to infection and increased mortality in patients with alcoholic cirrhosis (Homann et al., 1997).

Recently we evaluated ex vivo TLR2- and TLR4-mediated innate immune response in patients with stable well-compensated alcoholic cirrhosis (Pimentel-Nunes et al., 2010). Namely, we evaluated TNF-α production by peripheral blood monocytes (PM) primary cultures after stimulation with the TLR2/TLR6 ligand zymosan and the TLR2/TLR1 ligand lipopeptide, as well as with the TLR4 ligand LPS. We found an attenuated TLR2 response to zymosan and lipopeptide whereas the TLR4-mediated response to LPS was not significantly different to controls. We also studied a subset of patients with decompensated liver disease, where in addition to the blunted TLR2 response, the TLR4 response to LPS was also defective. Interestingly, we could not find any changes in protein or mRNA expression of TLRs between PM of patients and controls, which suggest that this blunted TLR2- and TLR4-response probably implies dysfunction in intracellular signalling pathways. To further clarify the molecular mechanisms underlying the selective attenuation of TLR2-mediated innate immune response in patients with stable compensated alcoholic cirrhosis, the differential effect of zymosan and LPS in PM stimulation on TLR2 and TLR4 gene expression was analyzed. In fact, zymosan and LPS stimulation has distinct effects on TLR2 and TLR4 expression levels. Whereas zymosan-mediated TLR2 stimulation induced a downregulation of both TLR2 and TLR4, LPS-mediated TLR4 stimulation was accompanied by a selective upregulation of TLR2 and a downregulation of TLR4. These differences could be related to distinct intracellular pathway activation. In fact, although TLR2 and TLR4 share most of its intracellular pathways, TLR4 also activates MyD88-independent pathways.

Other authors also found a decrease TLR2- and TLR4- response in immune cells, particularly in advanced stages of disease, that was associated with decreased, normal or increased levels of TLRs, depending on the study (Riordan et al., 2003; Stadlbauer et al., 2008; Stadlbauer et al., 2009; Tazi et al., 2006; Testro et al., 2009; Wasmuth et al., 2005). The data of these studies are compared in table 1. Analyzing all these studies, we conclude that decreased TLRs levels are insufficient to alter TLR function. Instead blunted TLRs response probably implies dysfunction in intracellular signalling pathways. Actually, in our study, we found blunted TLR2 activation that was independent of TLR2 levels (Pimentel-Nunes et al., 2010). Furthermore, we have shown in vitro that TLR2 and/or TLR4 agonists change the expression levels of these receptors (Pimentel-Nunes et al., 2010). Hence, we believe that the frequent episodes of bacteraemia that occur in cirrhosis, by changing TLR expression on immune cells, can help explain these discrepancies concerning TLR expression. This also might be the reason why Stadlbauer et al (Stadlbauer et al., 2008), using probiotics, promoted the decrease, and Testro et al (Testro et al., 2009), using antibiotics, the increase in TLR4 levels, both trending towards normal levels of expression. Possibly, these two different therapeutic agents decrease episodes of bacteraemia, consequently with less fluctuation of TLR levels. Why they restored TLR4 function remains unclear because expression levels cannot explain the results from these two studies.
Taking in consideration data from all these studies several conclusions can be made. Firstly, the data point to an important role of bacterial translocation, endotoxaemia and alteration of TLR2 and TLR4 signalling providing potential biomarkers to identify patients at risk of infection and potential targets for intervention. Secondly, our study (Pimentel-Nunes et al., 2010) and others (Riordan et al., 2003) clearly suggest a blunted TLR2 function even in the early stages of cirrhosis, which may help explain the growing risk of Gram-positive bacteria infection in these patients. Thirdly, at least in advanced cirrhosis, TLR4 impairment is also present (Pimentel-Nunes et al., 2011). Fourthly, taking together the discrepancies in the expression levels of TLRs, it appears that other factors, probably intracellular, are fundamental to this immunodeficiency. Finally, this process may be reversible with antibiotics and/or probiotics (Stadlbauer et al., 2008; Testro et al., 2009). However, further studies are needed before generalization since Riordan et al. (Riordan et al., 2003) showed that the use of a symbiotic (mixture of probiotic and probiotic) further compromised TLR2 function, in contrast to the positive immunological effects obtained by Stadlbauer et al. (Stadlbauer et al., 2008) and Testro et al. (Testro et al., 2009).

<table>
<thead>
<tr>
<th>Study</th>
<th>Cirrhotic population</th>
<th>Cell</th>
<th>TLR2 expression</th>
<th>TLR4 expression</th>
<th>TLR function</th>
<th>Therapeutic intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riordan et al., 2003†</td>
<td>Stable (n=36) several etiologies</td>
<td>PBMC</td>
<td>↑</td>
<td>=</td>
<td>TLR4 = TLR2 ↓</td>
<td>Symbiotic ↑ TLR2 levels and ↓ function</td>
</tr>
<tr>
<td>Wasmuth et al., 2005</td>
<td>Advanced (n=27) alcohol</td>
<td>PM</td>
<td>NE</td>
<td>NE</td>
<td>TLR4 ↓</td>
<td>NE</td>
</tr>
<tr>
<td>Tazi et al., 2006‡</td>
<td>Advanced (n=48) alcohol</td>
<td>PM</td>
<td>NE</td>
<td>↓</td>
<td>TLR4 ↑</td>
<td>NE</td>
</tr>
<tr>
<td>Lin et al., 2007</td>
<td>Stable (n=64) several etiologies</td>
<td>PM</td>
<td>NE</td>
<td>NE</td>
<td>TLR4 ↓ only in Child C</td>
<td>NE</td>
</tr>
<tr>
<td>Stadlbauer et al., 2008†</td>
<td>Stable (n=12) alcohol</td>
<td>PN</td>
<td>↑</td>
<td>↑</td>
<td>TLR4 =§</td>
<td>Probiotic decreased TLR4 levels to normal§</td>
</tr>
<tr>
<td>Pimentel-Nunes et al., 2010*†</td>
<td>Stable (n=26) and advanced (n=5) alcohol</td>
<td>PM</td>
<td>=</td>
<td>=</td>
<td>TLR4 = ↓ only in unstable; TLR2 ↓</td>
<td>NE</td>
</tr>
<tr>
<td>Testro et al., 2009†</td>
<td>Advanced (n=41) alcohol</td>
<td>PBMC</td>
<td>=</td>
<td>TLR4 ↓ in patients without ATB</td>
<td>TLR4 apparently ↓ in patients without ATB; TLR2 =</td>
<td>ATB increased TLR4 levels to normal with increase of function</td>
</tr>
</tbody>
</table>

*TLRs quantified by RNA.
†TLRs quantified by flow cytometry.
‡TLR4 quantified by Western blotting.
§Despite presenting decrease phagocytic capacity, stimulated TNF-α in culture was not different to controls and probiotic restored phagocytic capacity.
ATB, antibiotics; PBMC, peripheral blood mononuclear cell; PM, peripheral monocytes; PN, peripheral neutrophils; NE, not evaluated; =, equal to controls; ↓, decrease when compared with controls; ↑, increase when compared with controls. Adapted with permission from Liver Int 2011;31:140-1.

Table 1. Review of the studies about the role of TLR2 and TLR4 in cirrhotic patients according to TLR expression and function (considered as TNF-α production in culture).
6. Modulation of innate immunity in the treatment of ALD

Recently, a number of different approaches that modulate innate immunity, mainly LPS/TLR4 signalling pathway, have been developed and studied in the treatment of ALD (Petrasek et al., 2010). Among these approaches, two of them, modulation of LPS release by probiotics or antibiotics and interference with cytokines induced by TLR4 signalling, have progressed into clinical trials in patients with ALD.

Modulation of intestinal microbiota using probiotics has been shown to reduce bacterial translocation, circulating LPS levels in animal models, and bacterial infection, a marker for bacterial translocation, in patients with liver cirrhosis (Petrasek et al., 2010). In liver cirrhosis, probiotics have shown positive effects on several parameters including the improvement of liver function, prevention of infection, improvement of the hyperdynamic circulation and prevention of hepatic encephalopathy (Liu et al., 2004). Beneficial effects of probiotics have been reported in an animal model of alcohol-induced liver injury (Nanji et al., 1994) and of LPS-induced liver injury (Ewaschuk et al., 2007; Osman et al., 2007). Patients with alcoholic liver cirrhosis treated with Lactobacillus casei Shirota three times daily for 4 weeks showed restoration of deranged neutrophil phagocytic capacity, compared to controls (Stadlbauer et al., 2008). A recent open-label pilot trial showed that a 5-day administration of Bifidobacterium bifidum and Lactobacillus plantarum in alcohol-addicted psychiatric patients with mild alcoholic hepatitis ameliorated serum markers of liver injury to a significantly higher extent compared to control group treated with abstinence only (Kirpich et al., 2008). However, not all studies associate probiotics with improvement, since in the study from Riordan et al, the use of symbiotic (mixture of probiotic and prebiotic) further compromised TLR2 function (Riordan et al., 2003). Other problem with probiotics is that the number of studies is relatively small and many of these are uncontrolled studies. The large number of probiotic strains and combinations of strains represents other important problem, and it will require additional studies to confirm and ideally compare the efficacy of these probiotic strains.

A second approach to reduce TLR4 ligand is the treatment with antibiotics to achieve selective intestinal decontamination of Gram-negative bacteria, the predominant source of LPS. Selective intestinal decontamination has been shown to reduce bacterial translocation in many studies performed in rats (Runyon et al., 1995). Importantly, norfloxacin administration reduced the 1 year probability of developing spontaneous bacterial peritonitis (SBP), hepatorenal syndrome, and improved the 3 month and 1 year probability of survival compared with placebo (Fernandez et al., 2007). While the reduction of SBP in norfloxacin treated patients is a direct consequence of reducing bacterial strains in the microbiota responsible for spontaneous peritonitis, some of the positive effect on mortality are likely SBP-independent and related to reducing bacterial translocation and circulating levels of LPS (Fernandez et al., 2007). One problem with antibiotics is the severe consequences of long-term antibiotics treatment. Rifaxmin may help to solve this problem (Butterworth, 2011). Rifaximin is a minimally absorbed oral antimicrobial agent that is concentrated in the gastrointestinal tract, has broad-spectrum in vitro activity against gram-positive and gram-negative aerobic and anaerobic enteric bacteria, and has a low risk of inducing bacterial resistance. In randomized studies, rifaximin was more effective than nonabsorbable disaccharides and had efficacy that was equivalent to or greater than that of other antibiotics used in the treatment of acute hepatic encephalopathy. Furthermore, with minimal systemic bioavailability, rifaximin may be more conducive to long-term use than other, more bioavailable antibiotics with detrimental side effects.
These data suggest that modulation of the bowel flora may play a role in the pathogenesis and treatment of ALD and indicate a need for larger and rigorously designed clinical trials to support the use of probiotics or antibiotics in treatment of ALD.

While the role of TNF-α in the development of ALD has been well characterized, clinical investigations of the therapeutic efficacy of antibodies to TNF-α (e.g., infliximab) to treat patients with acute alcoholic hepatitis have generated variable results (Naveau et al., 2004; Tilg et al., 2003). There is particular concern about off-target effects of completely inhibiting TNF-α function. For example, since TNF-α is a critical component of immunity, infectious disease is a primary concern during TNF-α therapy (Naveau et al., 2004). Moreover, TNF-α is required for normal liver regeneration as hepatocyte proliferation in response to injury is impaired in mice lacking TNF-α receptors (Yamada et al., 1997). Etanercept, a TNF-α neutralizing antibody, appeared to increase short-term survival of patients with alcoholic hepatitis in a small pilot study (Menon et al., 2004), although a subsequent randomized, placebo-controlled trial conducted by the same investigators showed a worse 6-month survival rate in the group treated with etanercept than in the placebo group (Boetticher et al., 2008). Thus, it seems very unlikely that inhibition of TNF-α may become a therapeutic target in ALD, especially at the long-term.

7. Conclusion

In summary, the liver is an organ with predominant innate immunity function. Dysregulation of many components of innate immunity in the liver due to chronic alcohol consumption likely contributes additively or synergistically to alcohol-induced liver disease. Chronic alcohol consumption activates LPS/TLR4 signalling pathway on KCs, which produce large amounts of pro-inflammatory cytokines, including TNF-α, leading to liver steatosis and inflammation. Alcohol-induced LPS/TLR4 signalling pathway activation also contributes to alcoholic liver fibrosis via two independent mechanisms: it induces the secretion of chemokines from HSCs and chemotaxis of KCs which secrete the profibrogenic cytokine TGF-β; additionally, TLR4-dependent signals augment TGF-β signalling on HSCs via down-regulation of the TGF-β pseudoreceptor Bambi. Inhibition of NK cells during chronic alcohol consumption also seem to contribute to alcoholic liver fibrosis, since NK cells have anti-fibrotic effects through suppression of HSCs. Activation of LPS/TLR4 signalling pathway on hepatocytes may also contribute to hepatocarcinoma development, through activation of Nanog gene. In contrast to activation LPS/TLR4 signalling pathway and inhibition of NK cells, the role of complement activation in the pathogenesis of ALD remains largely obscure. Few studies suggest that alcohol-induced complement activation may contribute to liver steatosis, inflammation and fibrosis, but more studies are needed to clarify the underlying mechanisms.

Alcohol-induced dysregulation of innate immunity also seem to contribute to the increased risk of infections of patients with alcoholic cirrhosis, as we and others have demonstrated a blunted response of immune cells to TLR2/4 ligands, probably associated with compromised intracellular signalling, in these patients.

Modulation of innate immunity, mainly of LPS/TLR4 signalling through the use of probiotics or antibiotics, may play a role in the treatment of ALD, but we need for larger and rigorously designed clinical trials to support the use of probiotics or antibiotics in treatment.
of ALD. Inhibition of TNF-α has produced variable results in the treatment of ALD and may be associated with serious off-target effects.

Although, in the last decade, we have gained significant insight over the role of alcohol-induced innate immunity dysregulation in ALD, further research is still needed to further clarify and identify the interrelationships between innate immunity components involved in ALD. Examples of questions for future studies are:

1. Which is the role of other TLRs than TLR2 and TLR4 in the development of ALD?
2. Which is the role of DAMPs (HMGB1, heat shock proteins) and Myd88-independent pathway in ALD progression?
3. Which are the molecular mechanisms by which complement system contribute to alcohol-induced liver steatosis, inflammation and fibrosis?
4. Which are the molecular mechanisms underlying blunted response of immune cells to TLR2/4 ligands in patients with alcoholic cirrhosis?
5. Is there any correlation between TLR4 SNPs and the progression of ALD?
6. Which is the effect in ALD of neutralization of LPS or LPS-signalling through the use of TLR4 antagonists (e.g., Cyp, CRX-526, Eritoran), or LPS signalling interfering molecules (e.g., TAK-242, besifloxacin, compound K)?

The answers to these questions may help us identify novel therapeutic targets to treat ALD.

8. Acknowledgment

The authors greatly appreciate Dr. Raquel Gonçalves and Prof. Mário-Dinis Ribeiro for critical reading of the chapter.

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Alcoholic liver disease occurs after prolonged heavy drinking. Not everyone who drinks alcohol in excess develops serious forms of alcoholic liver disease. It is likely that genetic factors determine this individual susceptibility, and a family history of chronic liver disease may indicate a higher risk. Other factors include being overweight and iron overload. This book presents state-of-the-art information summarizing the current understanding of a range of alcoholic liver diseases. It is hoped that the target readers - hepatologists, clinicians, researchers and academicians - will be afforded new ideas and exposed to subjects well beyond their own scientific disciplines. Additionally, students and those who wish to increase their knowledge will find this book a valuable source of information.

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