MINIREVIEW

Recent Insights into the Role of the Innate Immune System in the Development of Alcoholic Liver Disease

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The innate immune system is responsible for the rapid, initial response of the organism to potentially dangerous stresses, including pathogens, tissue injury, and malignancy. Pattern-recognition receptors of the toll-like receptor (TLR) family expressed by macrophages provide a first line of defense against microbial invasion. Activation of these receptors results in a stimulus-specific expression of genes required to control the infection, including the production of inflammatory cytokines and chemokines, followed by the recruitment of neutrophils to the site of infection. The early stages in the development of alcoholic liver disease (ALD) follow a pattern characteristic of an innate immune response. Kupffer cells, the resident macrophages in the liver, are activated in response to bacterial endotoxins (lipopolysaccharide, LPS), leading to the production of inflammatory and fibrogenic cytokines, reactive oxygen species, as well as the recruitment of neutrophils to the liver. One mechanism by which chronic ethanol can turn the highly regulated innate immune response into a pathway of disease is by disrupting the signal transduction cascades mediating the innate immune response. Recent studies have identified specific modules in the TLR-4 signaling cascade that are disrupted after chronic ethanol exposure, including CD14 and the mitogen-activated protein kinase family members, ERK1/2 and p38. Enhanced activation of these TLR-4 dependent signaling pathways after chronic ethanol likely contributes to the development of alcoholic liver disease. Exp Biol Med 228:882–890, 2003

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Innate Immune Response/Wound Healing Response

The innate immune system is the most ancient part of an animal's defense mechanisms. It provides surveillance for invading pathogens via evolutionarily conserved pattern recognition receptors, the Toll-like receptor (TLR) family (reviewed in Ref. 1). The germ line-encoded TLRs recognize pathogen-associated molecular patterns (PAMP). The most well-studied interaction is that between lipopolysaccharide (LPS), a component of gram-negative bacterial cell wall, with the TLR-4 receptor. Additional microbial ligands activating the innate immune response via the TLR family include peptidoglycan, zymosan (a component of yeast cell walls), flagellin, and unmethylated CpG DNA (1). In addition to cell surface receptors that recognize PAMPs, cytosolic pattern recognition sensors are also active in the innate immune systems. For example, dsRNA can activate two antiviral pathways, the protein kinase PKR pathway, which acts to decrease viral and cellular protein synthesis, and the 2′-5′-oligoadenylate synthase (OAS)/RNase L pathway, which results in the destruction of both viral and cellular RNAs (1).

Surveillance for potential pathogens by the innate immune system is particularly critical at sites of potential pathogen entry, including the skin and mucosal barrier (1, 2). The liver also plays an important role in the innate immune response, providing a first line of defense against intestinal microbes and toxins crossing the intestinal barrier. Kupffer cells, the resident macrophages in the liver, are critical to the rapid clearance of bacteria from the bloodstream. Although Kupffer cells themselves are highly phagocytic and contribute directly to the removal of bacteria (2), they also act to orchestrate a proinflammatory response, resulting in the rapid recruitment of neutrophils to the liver, which are also highly active in microbicidal activity (2).

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Macrophages produce a variety of soluble mediators in response to activation of TLRs by microbial ligands (3, 4). These mediators can act locally to recruit other immune cells to the site of activation, or can act systemically to mediate a systemic immune response. Important soluble mediators include the inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin (IL) 1 and IL-6, chemokines and reactive oxygen species (ROS), including superoxide and nitric oxide, as well as the anti-inflammatory cytokine IL-10 (3). Recent evidence indicates that TLR-dependent activation of nuclear factor κB (NFκB) stimulates the expression of a central core array of inflammatory and chemotactic mediators. In addition, activation by PAMPs stimulates the expression of a distinctive subset of microbial defense mediators that are specific for the particular TLR activated (5, 6). Activation of macrophages by PAMPs also enables the macrophage to carry out effective antigen processing and presentation (6). This response provides an important link between the innate and adaptive immune systems (1).

Although PAMPs are the most well-studied activators of the innate immune response, additional factors, such as tissue damage or damaged proteins, are emerging as potent activators of innate inflammatory responses. For example, cells dying by necrosis release their intracellular contents upon loss of membrane integrity and can induce an inflammatory response (7). In contrast, although apoptotic cells are rapidly phagocytosed by macrophages, they do not induce an inflammatory response (7). Activation of NFκB via signaling components of the TLR pathway in macrophages by necrotic cells, but not apoptotic cells, is essential to the initiation of an inflammatory response (8). Oxidized low-density lipoproteins (oxLDL) are a second example of non-microbial products that activate inflammatory responses. OxLDL is taken up via scavenger receptors on macrophages, resulting in the production of a variety of inflammatory mediators (1).

Ethanol and Liver Disease

Alcoholic liver disease (ALD) develops in approximately 20% of all alcoholics, with a higher prevalence in females (9). The development of fibrosis and cirrhosis is a complex process involving both parenchymal and nonparenchymal cells resident in the liver, as well as the recruitment of other cell types to the liver in response to damage and inflammation (10). The progression of the alcohol-induced liver injury follows a pattern characteristic to all types of liver fibrosis, regardless of the causative agent. This progression is marked by the appearance of fatty liver, hepatocyte necrosis, inflammation, regenerating nodules, fibrosis, and cirrhosis (11). Fibrosis is thought to be initiated in response to hepatocellular damage, with inflammatory processes contributing to the progression of the disease (10). Activation of hepatic stellate cells, the principal fibrogenic cell type in the liver, is a prerequisite for increased deposition of extracellular matrix during liver injury (12). The precise mechanisms involved in the activation of hepatic stellate cells from their resting state to myofibroblasts, which actively synthesize extracellular matrix, are not completely understood. However, it is clear that local production of reactive oxygen intermediates, as well as inflammatory cytokines, by hepatic macrophages is an important contributor to the activation of hepatic stellate cells during liver injury (12). Interestingly, many of the events involved in the development of fibrosis are typical of other tissue responses to injury, such as wound healing in the skin and soft tissues (13). Continued ethanol exposure may disorder the highly regulated wound healing response, resulting in continued hepatocellular damage, inflammation, and fibrosis. It is our hypothesis that abnormal regulation of the innate immune system during long-term exposure to alcohol exacerbates the development of alcoholic liver injury.

Activation of the Innate Immune System in the Liver by Chronic Ethanol Exposure

Impaired Gastrointestinal Epithelial Barrier-Function. Chronic alcohol consumption disrupts the function of organ systems involved in the innate immune response. The gastrointestinal tract may be the initial target of ethanol, leading to activation of the innate immune response. Alcohol consumption is associated with impaired barrier function of the intestinal mucosa in patients with various stages of alcoholic liver injury, as well as rodents exposed to short-term or long-term ethanol (14, 15). LPS concentration is increased in the blood of alcoholics (16, 17) and rats exposed to ethanol via gastric infusion (18). Moreover, LPS clearance from the blood is decreased in patients with various degrees of alcoholic liver disease (19), as well as in the presence of an acute alcohol dose in rats chronically fed alcohol in their diet (20). In rats exposed to ethanol via intragastric infusion, antibiotic treatment decreases TNFα expression and ethanol-induced liver injury (21), suggesting that increased TNF-α after ethanol exposure is due, at least in part, to increased exposure to LPS. However, although antibiotic treatment trials of patients with alcoholic liver disease demonstrate a reduction in endotoxemia, improvement in liver function is not observed in many individuals (22).

Activation of Kupffer Cells, the Resident Macrophages in the Liver. Kupffer cells, the resident macrophages in the liver, are critical to the onset of ethanol-induced liver injury, at least in part because of their function as the first site of exposure to gut-derived endotoxin. Thurman (21) developed a working model for the progression of alcoholic liver disease, proposing that increased exposure of Kupffer cells to LPS during chronic ethanol consumption results in increased production of inflammatory mediators, in particular TNF-α and ROS, leading to the progression of fatty liver, inflammation, and fibrosis. Ablation of Kupffer cells prevents the development of fatty liver and inflammation in rats chronically exposed to ethanol via intragastric feeding (23, 24). Work from my laboratory and others in-

dicates that in addition to the increased exposure of Kupffer cells to LPS in response to ethanol exposure, chronic ethanol also sensitizes Kupffer cell responses to LPS-mediated activation (25–28) (see below), likely contributing to the progression of liver injury.

Recruitment of Leukocytes to the Liver. Activation of Kupffer cells in the liver leads to the production of a variety of soluble mediators, including inflammatory and fibrogenic cytokines, as well as ROS. Another aspect of the Kupffer cell response is the production of chemokines in both the CXC and CC subfamilies. CXC chemokines act as chemoattractants for the recruitment of polymorphonuclear leukocytes (neutrophils), whereas CC family chemokines recruit mononuclear cells to the liver. The recruitment of neutrophils and mononuclear cells to the liver serves to exacerbate the response to LPS, as neutrophils and mononuclear cells are very responsive to TNF-α. IL-8, a chemokine in the CXC family, is increased in the circulation of patients with alcoholic hepatitis (29). Furthermore, protein and mRNA for a number of chemokines, including RANTES, IL-8, and MCP-1, are increased in liver in patients with alcoholic hepatitis (30), with neutrophil infiltration observed during severe alcoholic hepatitis (31). Bautista and colleagues (32, 33) have reported that chronic ethanol feeding to rats for 16 weeks and mice for 12 weeks is associated with increased chemokine expression and neutrophil infiltration in the liver. Although Kupffer cells are essential for chemokine production and neutrophil recruitment in ischemia/reperfusion injury to liver (34), as well as in response to acute ethanol injury (35), studies demonstrating a required role for Kupffer cells in mediating neutrophil recruitment during chronic ethanol liver injury have not been carried out. Furthermore, it is not yet clear whether increased neutrophil numbers in the liver during chronic ethanol liver injury are due to enhanced recruitment via chemokines and adhesion factors and/or reduced clearance of the neutrophils from the liver.

Production of Inflammatory Mediators by Kupffer Cells During Chronic Ethanol Exposure

As described above, the Kupffer cell plays a central role in the development of alcoholic liver disease. The Kupffer cell is programmed to respond quickly and efficiently to stimulation by gut-derived endotoxins and tissue debris resulting from hepatocellular injury. In most situations, activation of the Kupffer cell is quickly resolved. Mechanisms controlling the resolution of the innate immune response in the liver ensure that uncontrolled inflammatory responses do not occur. However, in the context of chronic ethanol exposure, the activation of the innate immune response does not resolve, leading to chronic inflammation and continued hepatocellular damage. Understanding the molecular mechanisms by which chronic ethanol exposure prevents the resolution of Kupffer cell activation, either due to sustained/enhanced responses of the Kupffer cell to activation and/or impaired activation of anti-inflammatory mechanisms, is the focus of current research in a number of laboratories. Recent studies suggest one important mechanism by which chronic ethanol impairs the regulation of the innate immune response in the liver is by disrupting LPS-stimulated signal transduction via TLR-4.

Chronic ethanol consumption is characterized by an increase in the expression of a number of inflammatory cytokines. The increased production of TNF-α by Kupffer cells is thought to be of particular importance in the pathogenesis of ALD. TNFα is one of the principal mediators of the inflammatory response in mammals, transducing differential signals that regulate cellular activation and proliferation, cytotoxicity, and apoptosis (36, 37). In addition to its role in acute septic shock, TNF-α has been implicated in the pathogenesis of a wide variety of chronic inflammatory diseases (37–40), as well as in the progression of ALD (21, 41). The role of TNF-α in the development of ethanol-induced liver injury has been well characterized in animal models (21, 41).

Production of TNF-α is one of the earliest responses of the liver to injury (41). Circulating TNF-α is increased in the blood of alcoholics and in animals chronically exposed to ethanol (42, 43). Treatment of rats exposed to ethanol via intragastric feeding with antibiotics decreases TNF-α expression and ethanol-induced liver injury (21), suggesting that increased TNF-α after ethanol exposure is due, at least in part, to increased exposure to LPS. In addition to increasing LPS exposure, chronic ethanol also increases sensitivity to LPS. For example, long-term ethanol consumption increases the susceptibility of rats to endotoxin-induced liver injury (44, 45). Moreover, we have shown that LPS-stimulated TNF-α secretion by Kupffer cells is increased in rats fed ethanol in their diet for 4 weeks compared with pair-fed controls (25–27). If we can understand the specific mechanisms for this increased sensitivity of Kupffer cells to LPS after chronic ethanol exposure, very specific therapeutic strategies can be developed that target the molecular site(s) of ethanol action. First, studies are needed to identify intermediates in the TLR-4 mediated signaling cascade that are required for alcohol-induced liver injury. Then, more importantly, studies are also need to identify the specific signaling intermediates that are directly targeted by ethanol, i.e., to identify the TLR-4 signaling intermediates whose activity is disrupted by chronic ethanol exposure.

CD14-TLR4 Receptor Complex. Activation of TLR4 signaling in macrophages involves the interaction of a number of membrane and soluble proteins. LPS, bound to LPS-binding protein (LBP), interacts with CD14, a glycosyl-phosphatidylinositol-anchored membrane protein, at the surface of the macrophage. This complex then activates TLR-4. This interaction likely involves additional secreted proteins, such as MD-2 (46). Although studies have shown that both the quantity of TLR-4 (47) and CD14 (48) regulates the intensity of the LPS signal, the direct relationship between CD14-TLR-4 expression *in vivo* is not well understood (49). Quantitative relationships between expression of

CD14-TLR4 and LPS sensitivity *in vivo* are complicated by potential contributions of soluble CD14 and LBP, expressed and secreted by hepatocytes (49).

Both CD14 and TLR-4 appear to be involved in the development of alcohol-induced liver injury. In human populations, polymorphisms in the promoter of the CD14 gene, which lead to enhanced expression of CD14, are associated with increased severity of alcoholic liver disease (50). CD-14 (51) and LBP-deficient (52) mouse lines, generated by gene-deletion technology, exhibit resistance to alcohol-induced liver injury. Chronic ethanol exposure has been found to increase the expression of CD14 mRNA and protein in rodent models of ethanol exposure (27, 53, 54) The CD14 promoter contains AP1 and Sp regulatory sites (55). Recent data suggests that upregulation of CD14 mRNA in response to acute ethanol is dependent on oxidant production and subsequent activation of the AP-1 transcription factor (56).

C3H/HeJ mice, a naturally occurring mutant line that does not express TLR-4, show decreased sensitivity to endotoxin (47, 57), as well as less severe liver injury in response to chronic ethanol exposure via gastric infusion (58). These studies demonstrate that TLR-4 is essential for the development of liver injury in mice; however, no information is available as to whether chronic ethanol regulates the expression and/or activity of TLR-4.

IL Receptor-Activated Kinase (IRAK). Upon activation of TLR-4, IRAK is recruited to the TLR-4 complex via interaction with MyD88. This signaling response is analogous to the activation of the IL-1 receptor (59). Upon association with TLR-4, IRAK becomes highly phosphorylated and interacts with TNF receptor-associated factor 6 (TRAF6) to activate downstream signaling events. Interestingly, the kinase activity of IRAK is not essential for signaling, as kinase-deficient IRAK mutants can stimulate NFκB activation (60, 61). Although IRAK-1 is the most well-studied isoform, other IRAK family members, including IRAK-M and IRAK-4, may contribute unique signaling functions in TLR-4-dependent responses (62). In response to acute ethanol exposure, IRAK-1 phosphorylation is increased (63), suggesting enhanced signaling through this complex. However, it is not known whether increased IRAK phosphorylation in response to LPS is maintained during chronic ethanol exposure or whether chronic ethanol effects the expression and/or activity of additional IRAK family members.

NFκB. Activation of NFκB and the stimulation of NFκB-dependent gene transcription mediate "core" TLR-dependent responses, events common to activation of TLR-4, TLR-2, and TLR-6 (5). Activation of NFκB is also required for the activation of the innate immune system in response to necrotic cells (8). In monocytes from patients with alcoholic hepatitis, NFκB is activated compared with monocytes from controls (64, 65). Activation of DNA-binding activity of NFκB in liver has also been reported in experimental models of ALD. In rats chronically exposed to ethanol via gastric infusion, NFκB DNA-binding activity is increased in liver (66, 67). This response is thought to occur primarily in Kupffer cells (66) and precedes the appearance of pathological liver injury (68). The greatest increases in NFκB activation are observed in animals fed ethanol within a diet high in fat and/or containing fish oils (66, 69). The functional significance of this activation in the pathophysiology of alcoholic liver injury has been demonstrated by inhibiting NFκB activity in liver via transduction with the IκB super-repressor (70). Suppression of NFκB activation with the IκB super-repressor prevents the appearance of ethanol-induced liver injury (70).

Thus, although data from several laboratories has convincingly demonstrated an increase in NFκB activation during chronic exposure to ethanol in rodent liver, as well as increases in monocytes isolated from human alcoholics, the mechanisms for this activation are not clear. Several lines of evidence suggest that *in vivo* activation of NFκB after chronic ethanol exposure is not due to a direct effect of LPS on Kupffer cells, but may involve other activators of NFκB, such as ROS and/or other cytokines. A recent study investigating the mechanism by which chronic ethanol sensitizes mice to LPS exposure *in vivo* is consistent with this hypothesis (71). In that report, treatment of control mice with LPS increased hepatic NFκB DNA-binding activity. However, after chronic ethanol feeding, LPS did not activate NFκB DNA-binding activity (71). A similar loss of LPS-stimulated NFκB activity was reported in Kupffer cells isolated from rats allowed free access to ethanol-containing diets for 4 weeks and then cultured overnight. In this model, LPS treatment increased NFκB activity in Kupffer cells from control rats, but not in Kupffer cells from ethanol-fed rats (27). Similarly, although LPS increases NFκB DNA-binding activity in RAW 264.7 macrophages, this response is suppressed after chronic exposure to ethanol in culture (72).

Several studies provide indirect evidence that ROS, rather than LPS per se, may be critical to the *in vivo* activation of NFκB in livers from ethanol-fed animals. For example, administration of the antioxidant curcurmin during chronic intragastric infusion with ethanol not only prevents the activation of NFκB, but also reduces the pathological changes in liver observed during ethanol feeding (73). Similarly, dilinoleoylphosphatidylcholine, a compound with antioxidant properties that protects against alcohol-induced liver injury, also prevents increased NFκB activation in Kupffer cells after chronic ethanol feeding (28). Because oxidant stress can activate a number of signaling pathways involved in inflammatory responses (74), further investigations will be necessary to determine if the primary effect of antioxidants in these studies in preventing alcohol-induced liver injury is due to reduced NFκB activation.

Mitogen-Activated Protein Kinase (MAPK). MAPK family members are important mediators of innate immune responses (75). Stimulation of macrophages with

LPS activates several members of the MAPK family, including extracellular receptor activated kinases 1/2 (ERK1/2), p38; and c-jun N-terminal kinase (JNK) (3, 4, 76, 77). The role of LPS-stimulated MAPK activation in the innate immune response has been best studied in relation to TNF-α production. Transcriptional activation of TNF-α by LPS requires the activation of a distinct set of transcription factors binding to at least two regions of the TNF-α promoter, which include NFκB, Egr-1, and AP-1 binding sites (78, 79). Activation of ERK1/2 and subsequent transcription of Egr-1, an immediate early gene transcription factor, contributes to LPS-stimulated TNF-α expression in macrophages (76, 77). Similarly, LPS stimulation of JNK leads to phosphorylation of c-jun and subsequent binding of c-jun to the CRE/AP-1 site on the TNF-α promoter (78, 79). Finally, activation of p38 MAPK by LPS is involved in regulation of TNF-α mRNA stability (80), possibly via an interaction with tristetraprolin (TTP), a protein that modulates the stability of TNF-α mRNA (81).

Recent data indicates that chronic ethanol exposure has differential effects on the ability of LPS to activate individual MAPK family members. In Kupffer cells isolated from rats fed ethanol containing diets for 4 weeks, LPS stimulation of ERK1/2 and p38 activity is increased compared with cells from pair-fed rats (26–28). Similarly, chronic exposure of RAW 264.7 macrophages to ethanol during culture increased LPS-stimulated ERK1/2 and p38 activation (26, 72). Increased ERK1/2 activation is associated with increased Egr-1 expression (27, 72), suggesting that the ERK1/2-Egr-1 pathway may be involved in increased TNF-α expression after chronic ethanol. LPS administration to mice after chronic ethanol feeding results in enhanced ERK1/2 activation at early time points, as well as altered kinetics of activation, compared with pair-fed controls (71). In contrast, chronic ethanol feeding decreased LPS-stimulated JNK activity compared with pair-fed mice (71).

Increased TNF-α Expression after Chronic Ethanol Exposure. Increased production of TNF-α is associated with the development of ALD. Given the complex disruption of LPS-stimulated signal transduction observed in macrophages after chronic ethanol exposure, it is likely that these changes contribute to increased expression of TNF-α. Although the regulation of TNF-α expression is complex, occurring at the level of transcription, translation, mRNA stability, and cleavage/secretion, the initial site of regulation in response to LPS is increased transcription (82).

Role of Increased ERK1/2 in TNF-α Transcription. After chronic ethanol feeding, treatment of isolated Kupffer cells with LPS results in enhanced expression of TNF-α mRNA and peptide compared with controls (26). This response is also observed in RAW 264.7 macrophages chronically exposed to ethanol during culture (72). Because increases in ERK1/2 and Egr-1 are required for LPS-stimulated TNF-α expression (76, 77), and chronic ethanol increased LPS-stimulated ERK1/2-Egr-1 activity (27, 72), we investigated the role of chronic ethanol-induced changes in LPS-stimulated ERK1/2 activity in increased TNF-α expression.

In Kupffer cells isolated from rats fed ethanol for 4 weeks, pretreatment with PD98059, an inhibitor of ERK1/2 activation, decreases LPS-stimulated ERK1/2 activation, Egr-1 binding to the TNFα promoter, as well as TNF-α mRNA and peptide secretion (27). Similarly, overexpression of dominant negative ERK1/2 constructs in RAW 264.7 macrophages prevented the chronic ethanol-induced increase in TNF-α mRNA accumulation (72). Inhibition of ERK1/2 activation also prevented increased expression and DNA-binding activity of Egr-1 (72), suggesting that ERK1/2-dependent activation of Egr-1 expression was essential for chronic ethanol-induced increases in TNF-α expression.

Interestingly, inhibition of ERK1/2 by treatment with PD98059 or overexpression of dominant-negative ERK1/2 has a greater inhibitory effect on TNF-α expression after chronic ethanol compared with pair-fed controls (27, 28, 72). These results led us to hypothesize that ERK1/2-Egr-1 pathways make a greater contribution to TNF-α expression after chronic ethanol exposure than in control macrophages. Using TNF-α promoter-luciferase reporter constructs transfected into RAW 264.7 macrophages, we compared the contribution of the Egr-1-binding site with LPS-stimulated luciferase activity in cells cultured for 48 hr in the presence or absence of ethanol (72). LPS-stimulated reporter activity driven by the full-length TNF-α promoter did not differ between control and ethanol-treated cells. This is consistent with the observation that chronic ethanol has no net effect on total LPS-stimulated TNF-α transcription, measured by nuclear run-on assays, in Kupffer cells isolated from ethanol-fed rats (26). However, deletion of the Egr-1-binding site in the TNF-α promoter, either due to a truncation or point mutation in the promoter, decreased LPS-stimulated promoter activity to a much greater degree in ethanol-treated cells compared with control (72). In contrast, although deletion of the κB3 site in the TNF-α promoter decreased LPS-stimulated luciferase by 50% in control cells, there was no effect after chronic ethanol exposure (72). Thus, increased Egr-1 activity was critical to the maintenance of TNF-α transcription after chronic ethanol exposure, acting to compensate for the decrease in NFκB-binding and promoter activity (72). Taken together, these results demonstrate that enhanced LPS-dependent activation of ERK1/2-Egr-1 contributes to increased LPS-stimulated TNF-α production after chronic ethanol exposure.

Role of p38 MAPK in TNF-α mRNA Stability. Recent evidence also suggests that chronic ethanol exposure increases the stability of TNF-α mRNA. Chronic ethanol consumption stabilized LPS-induced TNF-α mRNA in Kupffer cells isolated from ethanol-fed rats ($t_{1/2}$ >100 min) compared with those isolated from pair-fed rats ($t_{1/2}$ < 40 min). A similar effect of chronic ethanol was observed on the TNF-α mRNA stability in RAW 264.7 macrophages. In control cells, LPS-induced TNF-α mRNA decayed with an

approximate half-life of 35 min. However, treatment of cells with 25 m*M* ethanol for 48 hr not only increased the accumulation of TNF-α mRNA, but also substantially stabilized the TNF-α transcript ($t_{1/2}$ >100 min) (26). These data demonstrate that exposure to chronic ethanol both *in vivo* and *in vitro* results in a marked stabilization of LPS-induced TNF-α mRNA. A similar stabilization of TNF-α mRNA is also observed in Kupffer cells isolated from rats chronically exposed to ethanol via intragastric infusion even when they are not stimulated with exogenous LPS (83). This stabilization was associated with a depletion of retinoic acid during *in vivo* ethanol exposure (83).

Activation of p38 and ERK1/2 MAPKs has been linked to mRNA stabilization of otherwise short-lived cytokine and other immediate early response genes (84–89). Because chronic ethanol exposure, both *in vivo* and during culture, increases both LPS-stimulated ERK1/2 and p38 activation (26, 27, 72), we asked whether ethanol-induced potentiation of p38 or ERK1/2 MAPKs was involved in the stabilization of TNF-α mRNA observed after chronic ethanol exposure. Kupffer cells isolated from ethanol- and pair-fed rats were pretreated with either SB203580, an inhibitor of p38 MAPK activation, or PD98059 followed by stimulation with LPS. Inhibition of p38 activation completely abrogated ethanol-mediated stabilization of TNF-α mRNA (26). In contrast, inhibition of ERK1/2 activation by PD98059 had no effect on ethanol-mediated stabilization of TNFα mRNA (26).

Anti-Inflammatory Responses/Resolution of Inflammation. Although a number of recent studies have advanced our understanding of the specific mechanisms by which chronic ethanol can increase activation of the innate immune response in the liver, very little information is available as to the impact on chronic ethanol on the resolution of the inflammatory response. The resolution of an inflammatory response involves suppression of inflammatory gene expression, inhibition of neutrophil recruitment, as well as removal of inflammatory cells by apoptosis and phagocytosis (2, 90). Important anti-inflammatory mediators include IL-10, glucocorticoids, cAMP, and prostaglandins in the PGJ_2 and $PGA_{1/2}$ families (90).

Chronic exposure of rodents to ethanol has consistently been found to increase expression of IL-10 (71, 91, 92), suggesting that a lack of IL-10 expression does not contribute to uncontrolled inflammatory responses during ethanol exposure. In contrast, chronic ethanol feeding to rats decreases adenosine and PGE_2-stimulated cAMP production in isolated Kupffer cells (25). However, despite decreased cAMP production after chronic ethanol exposure, adenosine and PGE_2 still effectively inhibited LPS-stimulated TNF-α production (25). Clearly, future studies investigating the effects of chronic ethanol on specific mechanisms required to resolve an inflammatory response will be required to understand whether, in addition to enhancing the activation of TLR-4 dependent signals, chronic ethanol also impairs the ability of the organism to extinguish the innate immune response.

Ethanol and ROS. Production of reactive oxygen by macrophages is an important aspect of the bactericidal activity of the innate immune response. ROS are involved in many aspects of immunoregulation, including regulation of cytokine production, apoptosis, and signal transduction pathways (74). Kupffer cells are an important source of ROS in the liver, produced during ethanol exposure, as well as in response to LPS/endotoxin (93). It is widely accepted that ROS play a critical role in the development of alcoholic liver injury (94, 95). However, the targets of ROS during ethanol exposure have not been completely elucidated. One potential mechanism involves activation of autoimmunity in response to oxidative damage to hepatic proteins and phospholipids (96, 97). Although oxidized macromolecules, such as oxidized LDL, are known to activate the innate immune response via scavenger receptors (1), it is not known whether oxidized macromolecules observed after chronic ethanol exposure (96) can also activate the innate immune system in the liver. In addition, ROS generated in response to ethanol exposure may also modulate the activity of signal transduction pathways involved in the innate immune response. The most well studied example is the activation of NFκB by ROS. Many studies have described a potential role of ROS in activation of NFκB during ethanol exposure (for examples, see Refs. 66 and 98). Additional signaling pathways involved in the innate immune response are also targeted by ROS. For example, LPS-stimulated ROS production enhances IL-1 gene expression in an MAPK-dependent mechanism (99). ROS can contribute to the activation of both ERK1/2 and Egr-1 (100, 101). In addition to ROS activation of MAPK family members, recent data also indicates that H_2O_2 inhibits protein tyrosine phosphatases (102), which could lead to delayed inactivation of kinases. A recent report from by Lieber's group (28) finds that treatment of Kupffer cells with dilinoleoylphosphtidylcholine (DPC), which protects against liver injury and acts as an antioxidant, prevents enhanced LPS stimulated TNF-α production after chronic ethanol feeding. Moreover, DPC also prevents the increase in LPS-stimulated NFκB and ERK1/2 activation observed in Kupffer cells isolated from rats chronically fed ethanol (28). Therefore, it is possible that ethanol-induced ROS production contributes to enhanced activation of a number of signaling pathways regulating the innate immune system and thus contributes to the dysregulation of this system during the development of alcoholic liver injury.

Summary and Future Directions

The innate immune response is a rapid, highly regulated response to potentially dangerous stresses, including pathogens and tissue damage. Recent work from a number of laboratories has demonstrated that many of the signal transduction intermediates involved in the innate immune response are required for the development of alcoholic liver injury. In particular, activation of the TLR-4 receptor by LPS appears to be required for the development of fatty

liver, inflammation, and necrosis in rodent models of chronic ethanol exposure. Increased exposure to LPS, due to impaired barrier function of the intestinal epithelium in response to ethanol, is an important contributor to activation of the innate immune response in liver. However, in addition to increasing the exposure of the liver to LPS, chronic ethanol also exacerbates the response of Kupffer cells to LPS, resulting in increased production of inflammatory cytokines. Recent studies have identified a number of intermediates in the TLR-4 signaling cascade that are affected by chronic ethanol, including increased expression of CD14, as well as enhanced activation of NFκB and the MAPK family members, ERK1/2 and p38. Understanding the molecular mechanisms by which chronic ethanol exposure results in a dysregulation of the highly regulated innate immune response in the liver will facilitate the development of treatment strategies to prevent and/or delay the progression of alcoholic liver injury.

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