Recent Advances in Alcoholic Liver Disease

III. Role of the innate immune response in alcoholic hepatitis

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The innate immune response serves as the first line of defense against invading pathogens, acting nonspecifically and with no memory toward a few highly conserved antigens present on microorganisms including LPS. Phagocytic cells (macrophages, neutrophils) and, to a lesser extent, certain nonspecific cytotoxic T and natural killer (NK) cells are saddled with the responsibility of carrying out this rapid immune reaction to invading pathogens (Table 1). Within the liver, this response is particularly important, because it is the first to encounter substances including bacteria and bacterial cell wall components absorbed from the intestines.

THE INNATE IMMUNE RESPONSE IS ACTIVATED IN ACUTE ETHANOL-INDUCED LIVER INJURY

The Kupffer cell serves as the primary effector cell of the innate immune response within the liver. A large body of experimental data would suggest that this cell population plays a key role in the early pathogenesis of alcohol-induced liver injury. First, it was demonstrated that Kupffer cell depletion using gadolinium chloride significantly blunted increases in serum transaminase levels [aspartate aminotransferase (AST), alanine aminotransferase (ALT)], fatty changes, inflammation, and necrosis caused by chronic ethanol (18). Second, destruction of Kupffer cells blocks formation of ethanol-derived free radicals (oxidants) after chronic enteral ethanol treatment, implicating these cells as potential sources of damaging oxidants (18).

The mechanism(s) by which Kupffer cells are activated following ethanol consumption have only recently been investigated. Considerable evidence supports the hypothesis that endotoxin, which is known to activate isolated Kupffer cells, participates in this mechanism (18). Endotoxin (LPS) is one of the components of the outer wall of gram-negative bacteria that has been implicated in sepsis, organ failure, and lethal shock. Elevated levels of circulating endotoxin delivered to the liver via portal blood are known to cause hepatocellular injury. Moreover, sterilization of the gut with nonabsorbable antibiotics, which bind LPS and kill gram-negative bacteria, blocks alcohol-induced liver injury (18). The mechanism(s) by which ethanol increases Kupffer cell activation by endotoxin are not known with certainty but could involve alteration of the gut permeability to endotoxin, modification of the gut flora, or changes in the rates of endotoxin clearance.

KUPFFER CELL-DERIVED MEDIATORS AND ALD

What then is the consequence of Kupffer cell activation by endotoxin? It is apparent from intensive investigation that Kupffer cells are stimulated to produce a number of inflammatory mediators including TNF-α, a proinflammatory cytokine known to cause hepatocellular inflammation and cell death, and reactive metabolites of oxygen, superoxide specifically. Early investigation (19) revealed that serum TNF-α increased in patients with ALD and correlated with mortality, supporting the idea that TNF-α may be important in ALD. It was hypothesized that endotoxin activates Kupffer cells that are the primary source of oxidants contributing to TNF-α production and early injury due to alcohol. To address this critical question, the rat enteral ethanol-feeding model developed by Drs. Tsukamoto and French was adapted to mice by

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Components of the innate immune response and their role in alcohol-induced liver injury

<table>
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<th>Immune Cell Type</th>
<th>Role in Innate Immune Response</th>
<th>Role in Alcoholic Liver Disease</th>
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<tr>
<td>Macrophage</td>
<td>- Primary response to bacterial cell wall antigens</td>
<td>- Activated by LPS derived from intestinal barrier dysfunction through ligation of Toll-like Receptor 4</td>
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<tr>
<td></td>
<td>- Activation of oxidant and cytokine production</td>
<td>- Produces large amounts of superoxide and TNF-α</td>
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<tr>
<td></td>
<td>- Phagocytosis of bacterial antigens, clearance from circulation</td>
<td>- Recruited to site of oxidant and cytokine generation by chemotactic gradients and up-regulated adhesion molecule expression</td>
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<tr>
<td></td>
<td>- Production of additional oxidants and chemotactic mediators to perpetuate inflammation</td>
<td>- Phagocytosis of bacterial antigens and dead or dying hepatocytes</td>
</tr>
<tr>
<td></td>
<td>- Recruit to site of oxidant and cytokine secretion</td>
<td>- Production of additional oxidants and chemotactic mediators leading to sustained inflammation</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>- Activated by cytokines and mitogenic mediators produced during inflammation</td>
<td>- Activated by TNF-α and IFN-γ produced within the inflamed liver</td>
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<td></td>
<td>- Immune surveillance for potential tumor cells</td>
<td>- Production of additional inflammatory mediators</td>
</tr>
<tr>
<td>NK cell</td>
<td>- Activated by cytokines and mitogenic mediators produced during inflammation</td>
<td>- Direct effect on liver injury and hepatocellular regeneration not known</td>
</tr>
<tr>
<td>T cell/NK T cell</td>
<td>- Activated by cytokines and mitogenic mediators produced during inflammation</td>
<td>- Including TNF-α and IFN-γ</td>
</tr>
<tr>
<td></td>
<td>- Accessory function to augment phagocytic cell activity/recruitment, cytokine secretion</td>
<td>- Perpetuates inflammation, close association with alcoholic cirrhosis</td>
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Several components of the innate immune response appear to be activated and play significant roles in the initiation and propagation of alcoholic hepatitis including Kupffer cells. NK, natural killer.

Our group. We first addressed the role of TNF-α in ethanol-induced liver injury using mice deficient in TNF-α receptor 1 (TNFR1−/−) (18). Compared with wild-type control mice, TNFR1 knockout mice exhibited essentially no pathological changes due to ethanol and no increase in serum transaminases, consistent with the hypothesis that TNF-α is essential for ethanol-induced pathology in mice. Importantly, this study represented a major advance in alcoholic liver research, because it provided a relevant model in which genetic factors can be manipulated to address critical questions related to alcoholic pathogenesis.

In addition to cytokines, such as TNF-α, Kupffer cells are also capable of producing large amounts of potentially damaging free radicals including superoxide. Indeed, products of oxidative stress can be measured in rodent livers treated chronically with ethanol (18). One potential source of superoxide within macrophages including Kupffer cells is the phagocytic NADPH oxidase system. This multisubunit enzyme complex is assembled on activation by a variety of stimuli and serves an important function in immune-mediated pathogen destruction. The role of NADPH oxidase in alcohol-induced liver injury was tested using mice deficient in p47phox, a regulatory subunit of the oxidase (18). After 4 wk of enteral ethanol feeding, these mice presented with significantly reduced liver injury as assessed by serum transaminase levels and histopathological assessment of tissue damage. Interestingly, it was also shown that in addition to free radical generation, cytokine production was significantly blunted. This provided strong evidence for a link between oxidant production and cytokine generation in this model of ALD. Further evidence demonstrating a connection between oxidant production and cytokine generation was provided by studies using adenoviral overexpression of mitochondrial as well as cytosolic superoxide dismutases. In these studies (18), scavenging of superoxide from both the cytosol as well as the mitochondria significantly reduced hepatocellular injury and TNF-α production. It was further demonstrated that isolated Kupffer cells adenovirally transfected with SOD had reduced NF-κB activation and TNF-α production in response to LPS. These data demonstrate the importance of endotoxin-induced Kupffer cell activation and the subsequent oxidant and cytokine production in the pathogenesis of early alcohol-induced liver injury.

KUPFFER CELLS AND LPS SIGNALING PATHWAYS: ROLE OF CD14

It is now clear that Kupffer cells are activated during early alcohol-induced liver injury and that this process involves gut-derived endotoxin. LPS is known to bind CD14, a cell surface protein found on mononuclear cells, including Kupffer cells. CD14, without a transmembrane-spanning domain, requires an association with toll-like receptors (TLR) to initiate an intracellular signal. It is studies involving these membrane proteins that provide the best argument for a role for endotoxin in ALD. Recent experimental evidence (18) has demonstrated that mice deficient in CD14 endotoxin receptor or TLRs are resistant to ethanol-induced liver injury. Because treatment of rats with non-absorbable antibiotics blunted ethanol-induced liver injury, it is reasonable to hypothesize that chronic ethanol-induced pathogenesis is at least triggered by gut-derived endotoxins that activate Kupffer cells. Further evidence can be gathered from studies demonstrating an enhanced sensitivity of female rats to enteral ethanol feeding. In a recent study (22), it was shown that Kupffer cells from female rats fed ethanol had a significantly greater response to LPS than Kupffer cells from male rats. Moreover, it was shown that females produce more TNF-α in response to chronic ethanol than males, suggesting that Kupffer cells in females are more active both in vivo and in vitro following ethanol exposure. Interestingly, CD14 expression and NF-κB activation are higher in Kupffer cells isolated from female mice. These and other studies demonstrate the importance of CD14 in the activation of Kupffer cells during chronic alcohol consumption.

How this translates to the human condition is a critical question. It was recently reported (18) that the severity of ALD in humans correlated with a polymorphism in the CD14 pro-
motor that conferred an increase in CD14 levels. Then, it was reported (15) that serum from human patients with severe alcoholic hepatitis contained higher levels of soluble CD14 than healthy controls. These data pose some critical questions in our understanding of ethanol-induced liver injury. Specifically, what role does the increase in CD14 expression due to acute ethanol have in chronic ethanol toxicity? Certainly, understanding the regulation of CD14, considering its association as a risk factor in humans for severe ALD, is potentially important for the development of therapies for ALD.

REGULATION OF CD14: A POTENTIAL THERAPEUTIC TARGET

Exactly how CD14 is regulated is unclear. Several groups have recently reported that CD14 expression is increased after long-term ethanol, and the implications for the increase in CD14 in the pathogenesis have not been fully examined. Because of its role in inflammation, endotoxin clearance, and even apoptosis of immune cells, CD14 expression requires a considerable amount of regulation. However, until recently, little has been known about the regulation of this important receptor. As discussed, activation of CD14 is necessary for early ethanol-induced liver injury in mice. We recently demonstrated that acute ethanol upregulated the expression of CD14 in liver. Moreover, CD14 upregulation was dependent on activation of redox-sensitive transcription factor AP-1 (20). Thus targeting signal-transduction pathways initiated through CD14 or inhibiting upregulation of CD14 will likely be important in the development of therapeutic approaches for the treatment of alcohol-related liver disease. However, a significant increase in our understanding of these mechanisms is required for these approaches to be developed.

ACUTE ETHANOL TOXICITY AND INFLAMMATORY CELL SIGNAL TRANSDUCTION

Ethanol as a toxicant has been studied in a wide variety of signaling pathways and cellular processes. Most notably is ethanol’s influence on kinase signal-transduction either through altering phosphorylation status, enzymatic activity, localization, or association. In particular, cAMP-dependent kinase (PKA) and PKC have received considerable attention as targets of ethanol toxicity (12, 17).

The effects of ethanol on inflammatory responses have also been investigated. However, the effect of ethanol on Kupffer cell function is still a critical gap in our understanding. Interestingly, ethanol can induce both tolerance and sensitization to LPS in Kupffer cells (4). This apparent paradox is most likely explained by ethanol’s effects on several signal-transduction molecules involved in LPS-induced signaling. For example, Mandrekar et al. (10) showed that acute ethanol inactivates Kupffer cells by inducing the formation of p50 homodimers of the NF-κB complex, which is transcriptionally inactive. The “tolerance” effect as well as the “priming” effect of ethanol may also be explained by temporal changes in signaling molecules following ethanol administration. It was hypothesized that temporal alterations in IL-1 receptor-associated kinase (IRAK), an early kinase involved in LPS signaling, are associated with ethanol-induced tolerance and sensitization (21). Yamashina et al. (21) recently demonstrated a decrease in the activity and expression of IRAK 1 h after ethanol exposure and a subsequent increase after 24 h. Also, an increase in CD14 expression on Kupffer cells has been observed following chronic ethanol exposure, suggesting heightened sensitivity to circulating endotoxin (6). Collectively, it is reasonable that altered signaling may play an important role in the pathogenesis of ALD.

THE INNATE IMMUNE RESPONSE AND HEPATIC FIBROSIS

Hepatic fibrosis is a consequence of long-term ethanol consumption in ~20% of humans. Liver fibrosis is regarded as the turning point in the pathological evolution of ALD because it can lead to cirrhosis, the irreversible stage of liver fibrosis that is a leading cause of morbidity and mortality associated with chronic alcohol consumption. It has been hypothesized that steatosis and inflammatory cell activation and infiltration promote the subsequent activation of hepatic stellate cells, a population of fibroblast-like cells capable of producing large amounts of collagen when activated. Oxidants (O2·−) and cytokines (TNF-α, IFN-γ, IL-2) produced in massive quantities in early ethanol-induced liver disease are known activators of stellate cells. Recently, it was reported (11) that hepatic stellate cells themselves can be activated directly by endotoxin through the CD14/TLR4 pathway. Signaling was reported to occur in a similar mechanism as that reported for Kupffer cells that have been activated by LPS, suggesting that stellate cells themselves may participate in the innate immune response. The overall involvement of this phenomenon in alcohol-induced hepatic fibrosis represents a new and exciting area of investigation.

T CELLS AND ALD

It is apparent from the preceding discussion that hepatic macrophages play an integral role in alcohol-induced liver injury through LPS-induced oxidant and cytokine-dependent mechanisms. The role that hepatic as well as peripheral lymphocytes play in this process has only recently been examined. Initial investigation demonstrated a significant reduction in T cell reactivity and proliferation in chronic alcohol consumers, suggesting an immunosuppressive role for alcohol (1). A growing body of experimental and clinical data would, however, suggest that both intrahepatic as well as extrahepatic T cells do contribute to the pathology associated with ALD (1). Studies have identified altered T cell composition within the livers of chronic alcohol consumers, alterations in their reactivity to specific and broad spectrum activators, and their close association and correlation with the severity of ALD (14).

The liver contains a significant number of lymphocytes, including NK T cells, T cells, and B cells. In fact, nearly 3–5% of the cells are lymphocytes. The overall number of B cells in liver is usually very small (i.e., <5% of the hepatic lymphocyte populations). The largest subset of lymphocytes in liver is the NK T cell, and their role in alcohol-induced liver disease is questioned. After NK T cells, the predominant hepatic lymphocytes are CD8+ T cells, unlike the predominance of CD4+ T cells in spleen or peripheral blood. After chronic alcohol consumption, significant changes in hepatic T cell function and numbers can be observed. Cao et al. (2) demonstrated the ability of ethanol to prime liver-associated T cells to show an exaggerated response to the polyclonal mitogen activator concanavalin A. Additional studies revealed that T cells isolated from ethanol-treated rodents and transplanted to control recip-
ient rodents could serve to damage the ethanol-naive livers (2). Further support for a role for lymphocytes in early alcohol-induced liver injury came from studies by Kono et al. (5). With the use of mice deficient in ICAM-1, an adhesion molecule capable of causing firm adhesion of activated lymphocytes, they demonstrated the importance of lymphocyte infiltration in the ethanol-induced liver injury. Indeed, ICAM-1-deficient mice presented with reduced serum transaminase levels in conjunction with reduced inflammatory cell infiltrate, the majority of which were lymphocytes.

Correlating well with these experimental studies are clinical findings suggesting activation of hepatic T cell populations. T cells isolated from alcohols are shown to express markers associated with activation and memory phenotypes including CD45RO, CD57, and CD11b (16). Not surprisingly, these cells are also capable of rapid production of cytokines, including IFN-γ and TNF-α, again demonstrating the ability of ethanol to prime hepatic T cells (16). Activation of peripheral T cells has also been reported in cirrhotic patients. Santos-Perez et al. (14) demonstrate the enhanced expression of CD25 on the surface of peripheral T cells when compared with controls. Furthermore, their investigation revealed enhanced reactivity of these cells in vitro to ethanol stimulation, whereas T cells from normal patients showed depressed reactivity. Interestingly, the immediate level of alcohol consumption, in addition to the severity of ALD, also positively correlated with the numbers of peripheral CD4+ and CD8+ T cells expressing inflammatory mediators such as TNF-α and IL-4 (7). Taken together, these and other studies demonstrate the ability of ethanol to activate hepatic and peripheral T cells toward that of a more inflammatory phenotype.

**T CELLS AND HEPATIC FIBROSIS, IS THERE A LINK?**

Quantitative immunohistochemical analysis of lymphocytes in liver biopsies of patients with ALD revealed increased numbers of both CD4+ and CD8+ T cells (13). Moreover, enhanced major histocompatibility complex I expression, which is specific for CD8+ T cells, correlates with regenerating nodules, intralobular inflammation, and fibrosis, possibly implicating these cells in the proinflammatory and profibrotic process of ALD (13). Unfortunately, animal models of chronic ALD fail to result in significant fibrosis and cirrhosis. Interestingly, the numbers of CD8+ T cells present in the alcohol-treated livers are relatively low compared with those of humans. However, other models, including repeated carbon tetrachloride administration, are capable of inducing hepatocellular fibrosis in rodents. In this model, massive infiltration of inflammatory cells composed primarily of CD8+ T cells can be observed in close proximity to areas of hepatic fibrosis (R. J. Milton and M. D. Wheeler, unpublished observation). Interestingly, activated CD8+ T cells rapidly accumulate in the normal liver and subsequently undergo depletion, presumably by a number of potential mechanisms including Fas-mediated apoptosis (3). It could be hypothesized that changes in the hepatic microenvironment, including hepatocellular fat accumulation and inflammation, alter the normal signaling pathways responsible for clearance of these cells, thereby increasing their longevity and potential for hepatocellular cytotoxicity and stellate cell activation. Unfortunately, critical gaps in our understanding of the roles of these cell types in ALD still remain. Figure 1 describes a working hypothesis whereby intrahepatic T cell accumulation, in addition to Kupffer cell activation, results in the release of proinflammatory and potentially profibrogenic mediators serving to perpetually activate hepatic stellate cells leading to collagen production and fibrotic changes within the liver.

**NK CELLS AND ALD**

In addition to T cells, the liver contains a significant number of resident NK cells that play an important role in the innate immune response. Hepatic NK cells are similar in structure and function to peripheral NK cells, being CD3- and CD56+, and exhibiting powerful antitumor cell activity while possessing little or no phagocytic capacity and being responsive to several proinflammatory mediators including IFN-γ and IL-12. In addition, studies (9) have suggested that these cells may be reactive to some bacterial antigens including LPS. In the context of ALD, little is known about the role of hepatic NK cells. Studies have demonstrated enhanced peripheral NK cell activity and numbers after chronic alcohol administration in humans, whereas others have reported reduced NK cell numbers and cytotoxic activity (8). The reasons for these differences most likely center around the populations tested and the severity of ALD. Examination of the role of NK cell activity in mouse models of ALD has not been reported. It is likely, given the dependence of NK cells on KC-derived signals and potentially LPS directly, that NK cells are also activated through the consequences of this activation are not known.

**FUTURE PERSPECTIVES**

Open questions related to the role of both hepatic and peripheral innate immune cells remain. Considerable interest in their role in ALD has been stirred following the major advances in the field by the development of the intragastric feeding model in mice and the implementation of transgenic and knockout mice by the Thurman group. Notable discoveries such as the critical role on TNF-α and superoxide free radicals derived by Kupffer cells underlie the fundamental hypothesis that the innate immune system is important in the pathogenesis...
of ethanol-induced liver injury. However, recent ideas including T cell recruitment, effector cell-mediated apoptosis, and natural killer cell subsets represent exciting new areas of research and potential therapeutic targets.

REFERENCES


