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MOLECULAR PATHOGENESIS OF T LYMPHOCYTE-INDUCED LIVER INJURY IN ALCOHOLIC HEPATITIS

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1. ABSTRACT

The development of alcohol-induced liver injury is, in part, a consequence of the immunological/inflammatory response that alcohol stimulates. The abnormalities of immune function in heavy drinkers have been documented well. Cytokines, especially TNF α, produced from macrophages/Kupffer cells, play a role in the induction of liver cell necrosis and apoptosis. TNF alpha can cause liver cell apoptosis through the TNF alpha receptor or Fas/CD95 which is expressed by liver cells. Furthermore, chronic ethanol consumption may damage the liver by inhibiting the hepatotrophic and hepatoprotective actions of TNF α and other cytokines. There exists an intrinsic lymphocyte population in the normal liver. Intrahepatic T lymphocytes consist of a heterogeneous population of cells that has many and varied functional characteristics in addition to classical T cell activity. The population of intrahepatic T lymphocytes may arise via a thymus-independent pathway. Our recent work has demonstrated the role of liver-associated T lymphocytes in the pathogenesis of alcohol related liver injury initiated by a variety of stimuli such as endotoxin (lipopolysaccharide, LPS) or concanavalin A (Con A). Our studies have, for the first time, suggested that alcohol consumption alone does not lead to the development of marked liver necrosis (at least in the rat), but rather that a second insult is required for this to occur. Liver-associated T lymphocytes in rats spontaneously secrete interleukin-1 alpha, interleukin-6 and TNF α *in vitro* culture. There is a significant decline in the amounts of interleukin-1 α and TNF α secreted in ethanol-consuming rats compared with non-ethanol consuming rats. The numbers of T cells, NK cells and Kupffer cells in liver perfusates remains stable over a prolonged period of ethanol consumption. However, following Con A injection, there was an inappropriate increase in the amounts of interleukin-6 and TNF α secreted in *in vitro* culture of liver-associated T lymphocytes and a significant increase in the percentage of CD^{4+} T cells and CD^{25+} T cells in liver perfusates compared with non-ethanol consuming rats. It suggested that liver-associated T lymphocytes are involved in the inflammatory

process associated with alcohol related liver injury through increased cytokine secretion (TNF α).

2. INTRODUCTION

Alcohol (ethanol) related liver disease (ALD) is an increasingly frequent cause of morbidity and mortality in developed countries. Ethanol toxicity, nutritional deficiency, and intercurrent noxious factors each contribute to its development in a susceptible person. The demonstration that cultured lymphocytes from peripheral blood of alcoholics were stimulated by mitogens, such as ethanol introduced the concept that immunological reactivity may also contribute to its pathogenesis. Subsequent investigations have emphasized the importance of lymphokines, cytokines, and humoral factors in perpetuating alcoholic liver disease (1,2). Clinical evidence of ethanol-related depression of lymphocyte-mediated immunity includes a high incidence of both tuberculosis and head and neck cancers among heavy drinkers. A large number of lymphoid alterations have been described in heavy drinkers. The ability of liver and circulating lymphocytes to undergo blast transformation under mitogenic activation is impaired in alcohol liver disease (ALD), especially when either malnutrition or long-term drinking have already produced some degree of liver damage (3,4,5,6,7). Other changes include peripheral blood lymphopenia (perhaps secondary to hepatic sequestration of activated cytotoxic CD^{8+} lymphocytes), reduced *in vitro* response of circulating PBMC to both phytohemagglutinin (PHA) and concanavalin A, and reduction in *in vitro* IL-2 production, as well as impaired natural killer and/or lymphokine-activated killer cell activity. Recent studies have identified that liver associated CD^{4+} T lymphocytes (LAL) play an important role in the development of hepatocyte injury in alcohol-consuming rats (8,9,10,11,12). CD^{4+} cells in culture secrete high levels of tumor necrosis factor (TNF a) and interleukin-6 (IL-6) in ethanol-fed rats injected with Con A. This paper reviews recent literature dealing with immunological mechanisms underlying ALD. We focus on the role of intrahepatic T lymphocytes in evolving alcohol related liver disease in order to illustrate the role of T lymphocyte in the pathogenesis of ALD.

3. PATHOGENESIS OF ALCOHOL RELATED LIVER DISEASE AND THE ROLE OF CYTOKINES

3.1. Pathogenesis of alcohol related liver disease

Among the many mechanisms of alcohol-induced liver injury, the most notable is a change in the phenotype of effector cells and their tissue matrix in response to a variety of stimuli. Underlying these processes are the intercellular and intracellular signals between a large number of cell types including endothelial cells, hepatocytes, Kupffer cells, stellate cells, neutrophils, NK cells and lymphocytes (2,9,13,14). Prominent among the events are the production of reactive oxygen species (ROS) and hydroxyethyl radicals by cytochrome p 450 2E1 (CYP 2E1) which, in turn, leads to lipid peroxidation (15,16,17). One other mechanism of injury is the oxidative stress on mitochondrial DNA linked to impaired mitochondrial oxidation of fatty acids and ATP production (18).

Cell death by necrosis in ALD generally has been attributed to the consequences of alcohol metabolism which include: acetaldehyde generation, mitochondrial dysfunction, and an infiltrate of neutrophils which are all linked to an increase in the oxidative stress experienced by hepatocytes. Recent studies have shown that hepatocyte apoptosis is an additional and vital mechanism of alcohol-induced liver injury (19). Fragmentation of DNA is a biochemical feature of apoptosis with internucleosomal-sized fragments ranging from 180-200 base pairs. Kawahara *et al* (20) applied histological indicators of apoptosis such as the DNA nick-end labeling (TUNEL) assay to liver biopsies from patients with ALD and reported significantly greater apoptosis in ALD compared with non-diseased livers, In this study, apoptosis was especially frequent near hepatocytes containing Mallory bodies. By using the same technology, Zhao *et al*.(21) also noted increased liver cell apoptosis in ALD, especially in area of fibrosis or more severe injury.

3.2. Role of cytokines

3.2.1. Cytokines and their regulation in alcohol related liver disease

It has become clear that cytokines may cause liver cell injury indirectly through the actions of other cells or by directly binding to specific cytokine receptors on the hepatocyte to generate toxic intracellular signals. Cytokines are low-molecular-weight mediators of cellular communication that are produced and released by numerous cell types such as monocytes, macrophages, and, of particular relevance to liver disease, Kupffer cells and intrahepatic T lymphocytes.

Pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF a, are consistently found to be elevated in the blood of subjects with ALD (22,23,24,25,26). For example, serum levels of IL-6, IL-8 and TNF a are significantly higher in subjects with alcohol hepatitis than in subjects with inactive cirrhosis or without liver disease. In addition, peripheral blood mononuclear cells from subjects with alcohol cirrhosis secrete higher levels of all three cytokines than blood mononuclear cells from healthy controls. This is not surprising since endotoxemia is frequently present in alcohol hepatitis (AH) subjects. Endotoxemia is reported to be the initiator of pro-inflammatory cytokine release by mononuclear cells which express CD14. CD 14 interacts with endotoxin in the presence of so-called LPS-binding protein (LBP) derived from hepatocytes (27). Since LBP is synergistically up-regulated by IL-6 and TNF α, a vicious cycle of inflammation driven by a feedback loop is established to produce more IL-6 and TNF a in the liver. In alcohol hepatitis, no correlation is found between endotoxin, IL-6, IL-8 and TNF a levels (28). This suggests that either other factors apart from endotoxin are involved or that the measurement of serum or liver homogenate cytokine levels are not providing an accurate picture of events occurring in the liver.

Lipid peroxidation and oxidative products are known to participate in the regulation of pro-inflammatory cytokine levels. Endotoxin and lipid peroxidation activate the

transcription factor NF-kB which then influences gene expression for pro-inflammatory cytokines such as IL-6 and TNF a (29,30). High levels of NF-kB and increased TNF a expression are detected in rats fed ethanol with polyunsaturated fatty acids. In addition, both cyclo-oxygenase 2 (Cox-2) and TNF a production are also up-regulated (31), suggesting that Cox-2 may interact with TNF α to produce liver injury. This view is supported by studies in which mice with disrupted Cox-2 gene expression did not develop hepatocellular injury following administration of LPS (32,33). However, since TNF a under certain conditions is toxic to the liver, the relative contribution of hepatotoxic lipid peroxidation is not known. It is likely that in ALD, Cox-2 induces liver injury via pro-inflammatory cytokine and eicosanoid production (34).

Chronic alcohol consumption impairs mitochondrial transport of glutathione (GSH) from the cytosol, resulting in the depletion of GSH (35,36). GSH constitutes an important regulator of cell function, particularly the availability of reactive oxygen species (ROS), in response to inflammatory cytokines including TNF a that cause oxidative stress in hepatocytes. Depletion of mitochondrial GSH in hepatocytes by actinomycin results in enhanced activation of NK-kB compared with hepatocytes depleted of cytosolic GSH (37). Restoration of mitochondrial GSH in hepatocytes from ethanol-fed rats confers resistance to hepatocytes treated with TNF α (35,36) , suggesting that by indirectly regulating cytokine gene expression, mitochondrial GSH is vital to hepatocyte survival .

In both humans and experimental animals, chronic alcohol consumption affects cell-mediated immunity (CMI) and humoral immunity. Cell-mediated and humoral immunity are regulated by type 1 (Th1) and type 2 (Th2) CD^{4+} T helper cells, respectively. Th1 responses are characterized by IL-12 and interferon-gamma (IFN ?) production associated with delayed-type hypersensitivity (DTH) whereas Th2 responses are characterized by the production of IL-4 and IL-10 cytokines and the induction of an antibody response. Ethanol consumption impairs antigen presenting cell function by spleen cells through its ability to downregulate IL-12 (38). In heavy drinkers the defective secretion of IL-10 or IL-12 in relation to the regulation of pro-inflammatory cytokine production may be able to be linked to the development of alcohol hepatitis.

3.2.2. TNF a mediation in alcohol related liver disease

Cytokines play a pivotal role in the pathogenesis of ALD. The role of TNF a has been studied extensively and it has been postulated that two factors lead to the susceptibility of hepatocytes to this cytokine. Firstly, hepatocytes become "sensitized" to TNF a and secondly, the cells capable of secreting TNF a become "primed" (39). In experimental liver injury, hepatocyte apoptosis induced by TNF a involves an early event associated with either the TNF a receptor or the CD 95 (Fas/Apol) receptor. The activation of the death receptors, tumor necrosis factor-receptor-1 (TNF-R1) or CD95, is a hallmark of inflammatory or alcoholic liver disease. Activation of either the 55 kD TNF-R1 or CD95 causes apoptosis of cells and liver failure in mice (40,41). *In vivo* or *in vitro* stimulation of CD95 caused apoptosis of murine hepatocytes even in the absence of TNF-R1, suggesting that the Fas/Apo l receptor may be involved in liver injury. Indeed, hepatocytes lacking the functional expression of CD95 are not affected by apoptosis in the presence of endogenous or exogenous TNF α, suggesting TNF-R1 and CD95 are independent and differentially regulated triggers of hepatocyte apoptosis (42,43). Hepatic expression of TNF a and TNF-R1, as well as the activation of TNF-TNF-R1 targets during liver regeneration in chronic alcohol consuming rats have been studied (44), TNF mRNA levels and TNF-R1 levels are increased in liver and extrahepatic tissues, such as white fat. This finding suggested that in ALD, Fas ligand may be expressed as a membrane-bound form and might cause apoptosis as an act of "fratricide" by interacting with the Fas receptor on a neighboring cell (45,46). Another possibility would be Fas-induced apoptosis occuring in an autocrine or paracrine fashion due to the release of a soluble ligand (47). Kupffer cells have been found to mediate hepatocyte apoptosis through intercellular mediators including TNF a and p55 TNFR-1 receptor (47,48). LPS stimulation of kupffer cells and sinusoidal endothelial cells (SECs) leads to a three-fivefold increase in Fas ligand mRNA within 6 hours (47).

Oxidant injury (especially mitochondrial injury) is one putative direct mechanism of TNF a mediated cytotoxicity (36,49). TNF a causes morphological abnormalities in the mitochondria and inhibits hepatic mitochondrial respiration. Mitochondrial apoptosis and mitochondrial glutathione depletion are thought to play key roles in TNF-induced liver cell death (36,50). Mitochondrial cytochrome c release plays a critical role in apoptotic signal cascade after the TNF activation of cell surface death receptors (51). In cells that are vulnerable to TNF lethality, mitochondrial ROS release occurs within 5 minutes, followed by increased NF-kB DNA binding activity within 15 minutes and apoptosis within 1 hour of TNF exposure (52). Kim and his colleagues investigated the role played by nitric oxide (NO) in mitochondrial apoptotic signaling in TNF α plus actinomycin D (TNF α/ActD)-induced apoptosis. They found that one mechanism by which NO protects hepatocytes from TNF α/ActD-induced apoptosis is via the interruption of mitochondrial apoptotic signaling through S-nitrosylation of caspase-8 (53).

NF-kB is a redox-sensitive transcription factor for several cytokines such as TNF a, IL-6, IL-8, as well as several potentially protective factors such as nitric oxide and polyamines (54,55). NF-kB activation was increased by ethanol and associated with up-regulation of TNF α. These increases were blunted significantly up to 2 weeks by I-kB SR (I-kB superrepressor), suggesting that NF-kB inhibition prevents early alcohol-induced liver injury (56). Also, in another study, Kim reported that ethanol activates hepatic NF-kB via its metabolism and that HBX or HCV core protein activates hepatic NF-kB via TNFR1. With the essential role of TNFR1 in alcoholic liver injury, targeting TNFR1 by hepatitis viral proteins could contribute to the cooperative effects of alcohol consumption and viral

hepatitis on liver disease (57). Transcriptional activation by NF-kB is thought to correlate reasonably well with its DNA binding activity in nuclear extracts. In many types of cells, TNF a is known to increase the DNA binding activity of NF-kB. Acute consumption of ethanol accelerates this process, such that NF-kB binding activity is significantly increased as early as 15 min after hepatectomy. However, chronic ethanol consumption significantly inhibits induction of NF-kB binding activity in the regenerating liver (58). Hepatocyte apoptosis can be initiated by inhibition of NF-kB, at least in part by allowing unregulated signaling via the TNF a " death domain".(55) Studies inhibiting NF-kB activation in hepatocytes *in vitro* and in mice *in vivo* were consistent with the concept that this transcription factor has an important anti-apoptotic function in hepatocytes (59). Thus, in ALD, there may be a paradoxical situation of increased NF-kB activation in the Kupffer cell (with increased TNF a production) and inhibited NF-kB activation in the hepatocyte (with increased sensitivity to TNF a killing) (55).

3.2.3. IL-6 - its role in alcohol related liver disease

The role of IL-6 in alcohol hepatitis is still unclear although it may play a protective role in regulating TNF a cytotoxicity. Because the latter depends on protease activity for its effect, the release of C-reactive proteins and protease inhibitors by the liver in response to IL-6 may contribute to the down-regulation of the inflammatory response induced by TNF a (60). Indeed, recent studies have shown that IL-6 has a prophylactic effect against Con A -induced liver injury in mice but is ineffective after disease induction (60). In our previous studies, however, increases in IL-6 production appeared to have no beneficial effect on liver injury. The reason for this is unclear. It is possible that, in addition to its capacity to down-regulate TNF a cytotoxicity, IL-6 may aggravate liver injury by enhancing TNF a receptor expression on hepatocytes which become more sensitive to TNF a cytotoxicity, particularly in the presence of sustained release of TNF a. The dual role of IL-6 in down-regulating TNF a cytotoxicity as well as enhancing TNF a receptor expression could have major implications in alcoholic hepatitis where impaired ability to make acute phase proteins may increase hepatocyte susceptibility to cytokine-induced liver injury.

4. IMMUNE EFFECTS OF CHRONIC ALCOHOL CONSUMPTION ON LYMPHOCYTES IN HUMAN AND ANIMAL EXPERIMENTATION

4.1. Immune abnormalities of peripheral T lymphocytes

Alcohol consumption significantly alters the cell numbers extractable from the thymus and spleen. Spleen cell numbers are reduced after 20% (V/V) alcohol consumption in female mice, as are thymus cell numbers in 18-day old fetuses whose mothers are fed a 25% ethanol diet. In ALD, abnormalities in lymphocyte cell numbers, cellular function, and serum factors are well documented (61). A decrease in CD^{8+} T cells concomitant with an increase in CD^{4+} T cells has been reported whereas other studies show a significant decrease in both CD^{4+} and CD^{8+} cells. This discrepancy is probably due to varying degrees of liver disease severity in the study populations (61,62). It has been shown that in earlier stages, there is more often a mild reduction in the percentage of lymphocyte numbers in alcohol hepatitis, but with a return to normal levels after several weeks of recovery. There are altered expressions of various molecules on the surface of the T lymphocytes in alcoholics, e.g. an increase in the percentage displaying the histocompatibility molecule MHC-II, and alteration of adhesion molecules (61,63,64**).**

Immunosuppression, associated with chronic alcohol use, is characterized by a reduced antigen-specific T-cell response and impaired delayed type hypersensitivity. Exposure of peripheral blood CD+3 T cells in culture to alcohol results in decreased production of interleukin-2 and an impaired proliferative response to mitogen stimulation (4). FAE tends to accelerate the decline of double-positive CD8-TcR and CD8-CD45RC cells (65). Furthermore, chronic alcohol consumption is shown to alter the distribution and possibly the migration of circulating CD^{4+} and CD^{8+} T cells expressing L-selectin (CD62L, peripheral lymph node homing receptor) (4,7). However, in rats fed alcohol, an increased blood CD^{4+}/CD^{8+} ratio and serum immunoglobulin levels are observed, suggesting that alcohol consumption results in hypergammaglobulinemia due to a decrease in T cell suppression (7). Also, Santos-Perez et al (64) reported that lymphocytes from ALD patients showed increased basal and stimulated expression of CD4, CD25, LFA-1, ICAM-1 and LFA-3 markers, the over-expression of activation markers and TNF α production was similar to that obtained with mitogens. In contrast, a predominant suppressive effect of ethanol was observed in lymphocytes from controls. This implied that the originally inhibitory effect of ethanol on lymphocytes may be reversed, becoming stimulatory in the situation of chronic excessive alcohol intake. Ethanol might act as a hapten by binding to other structures.

The allo-specific mixed lymphocyte response (MLR) of the responder cells from alcohol-consuming mice was significantly reduced , and the addition of exogenous interleukin 2 (IL-2) could not reverse the suppression of MLR induced by ethanol (66). Alcohol intake reduces allostimulatory T-cell activation via decreasing accessory cell function. Increased IL-10 and IL-13 plus the reduced IFN γ production after acute alcohol use are likely to contribute to both the reduced T-cell proliferation and monocyte accessory cell function (67).

Studying the pattern of cytokine secretion by the major CD^{4+} and CD8 strong+ peripheral blood (PB) T-cell subsets in patients with chronic alcoholism, Laso *et al* recently found that in alcohol liver cirrhosis patients, the pattern of expression of intracellular cytokines by peripheral blood T cells was different depending on the status of alcohol intake at the moment of entering this study. Accordingly, as in AWLD (alcoholics without liver disease) patients, alcohol cirrhosis individuals who were actively drinking also displayed increased numbers of both CD^{4+} and CD8 strong+ T cells expressing Th-1-associated cytokines, suggesting that active alcohol intake is associated with a Th-1 pattern of cytokine production by PB T cells (6).

4.2. Signal-transduction abnormalities of peripheral T lymphocytes

To study the transmembrane signal-transduction pathway on T lymphocytes in ALD, Spinozzi *et al* (68) found that peripheral lymphocytes from ALD patients failed to respond to anti-CD3 and anti-CD2 after *in vitro* stimulation. They display decreased intracellular Ca2+ mobilization and IP3 generation but show a normal proliferative response to phytohemagglutinin. *In vitro* ethanol incubation of normal T lymphocytes results in rearrangement of the membrane CD45 antigen, favoring the expression of high-molecular-weight isoforms, these cells show a poor blastogenic response to anti-CD3 and anti-CD2 with a decrease in intracellular Ca2+ mobilization and IP3 production. After a 6 month period of ethanol withdrawal, some patients demonstrate a normalization of the phenotypic and functional alterations reported above (5). The addition of small amounts of phorbol 12-myristate, 13-acetate was able to overcome the defect of faulty signal transduction in the CD2 pathway, suggesting a direct PKC involvement and PKC impairment (68). It is suggested that ethanol-specific impairment of T -lymphocyte activation and function is caused by a transitory block in signal-transduction pathways. A recent report (69) indicated that, ethanol-mediated enhancement of T cells to apoptosis involves increased activation of Caspase-3 and can be abrogated by treatment with a specific inhibitor of Caspase-3.

4.3. Immune abnormalities of peripheral B lymphocytes

B cells (antibody-producing lymphocytes) in the AWLD tend to be normal in numbers (70), or slightly reduced (63), but are often significantly decreased in number in ALD (71), despite the fact that they produce abnormally large amounts of immunoglobulins. B cells also display changes in their subset patterns (70), but these changes appear to be shorter lived than the T-cell changes. Together, the T and B-cell changes suggest the likelihood that there are alterations in the interactions between T and B cells that are important for understanding the inappropriate immunoglobulin production and other defects of immune regulation in alcoholics (61).

4.4. Immune abnormalities of peripheral NK lymphocytes

Long- term alcohol consumption is reported to significantly depress the functions of NK cells in various animal models including mice and rats (72,73,74). Furthermore, changes in NK subpopulations have been reported to occur in mouse fetuses exposed to alcohol (75) with significantly reduced expression of the L3T4+(CD4+), and Lyt-2+(CD8+) cells, but normal expression is observed by 6 days after birth. In human studies, some ALD have greatly reduced NK cell numbers and reduced NK activity (76). However, a significant increase in both NK cells(CD3-CD56+) and NK activity as well as T cells coexpressing the CD3+ and CD56+ molecules, is detected in peripheral blood from subjects with AH (7). An unusually large NK cell population is detected in the liver of primary sclerosing cholangitis, chronic active hepatitis, and non-A, non-B (NANB) hepatitis, but not in advanced or severe ALD (3,75). The reason for the depressed NK activity in ALD is not clear. It is suggested that alcohol may not alter NK cell function per se but rather the capacity to interact with tumor cells in the *in vitro* assay. It is likely that alcohol causes the relocation of NK cells into peripheral lymphoid tissues thus reducing the numbers in the circulation. Because NK cell activity is regulated by IFN ? and IL-2 released from T lymphocytes, it allows/follows that changing the ratio of NK cells to lymphocytes in circulation may alter NK cell function.

4.5. Immune abnormalities of liver-associated T lymphocytes

Most studies on the effects of alcohol on T cells are carried out with cell populations derived from peripheral blood or lymphoid tissues. Morphological and phenotypical data indicate that the liver contains not only circulating lymphocytes but also liver associated T cells (77). The function of liver associated T cells in alcoholics and patients with ALD is not clearly defined partly due to the difficulties in isolating sufficient viable cells for functional studies. Liver associated T cells consist of CD^{4+},CD^{8+} and CD4-CD8- T cells, and T cell receptor (TCR) IL-2R a+ T cells (77,78). In ALD, LAL are characterized by an increase in the cytotoxic suppressor T cell subset (CD^{8+}) and a decrease in the helper T cell subset (CD^{4+}) (8,77). In both mice and humans, T cells were found in abundance in liver sinusoids, indicating that the liver could be a site of extrathymic differentiation of these cells (77). In contrast, T cells in humans and mice constitute a minority population among PBL (77). In histological studies, sinusoid lymphocytes are mostly in contact with Kupffer and endothelial cells. The capacity of LAL to adhere to normal liver sections is higher than that of PBL, suggesting an increased expression of adhesion molecules by LAL compared with PBL (79). Quantitative immunohistochemical analysis of liver associated lymphocyte subsets in liver biopsies from patients with ALD reveals that increased numbers of CD^{4+} and CD^{8+} T cells correlate with regenerating nodules, intralobular inflammation, central sclerosis, and abnormalities of Kupffer cells (3,80). B cells and NK cells are rare or absent in healthy liver tissue. Enhanced MHC class I expression correlates significantly with portal inflammation, limiting plate erosion , vascular abnormalities and hemosiderosis while MHC class II expression correlates significantly with necrosis, bile stasis, and Mallory bodies (3). Recently, our laboratory reported that LAL in ethanol consuming rats spontaneously secrete IL-1a , IL-6 and TNF a after *in vitro* culture; There was a significant decline in the amounts of IL-1 a and TNF a secreted over a 26 week period of exposure to ethanol compared with non-ethanol consuming rats. The numbers of T cells, NK cells and Kupffer cells in liver perfusate remained stable over a prolonged period of ethanol consumption (see Table 1) (8). However, following Con A injection, there was a significant increase in the percentage of CD^{4+} T cells and CD5+ T cells in liver perfusates as well as blood (see table 2) (9,10,12). There was an inappropriate increase in the amounts of TNF a and IL-6 secreted compared with non-ethanol consuming rats (10,11). It suggested that ethanol has a selective effect on the constitutive production of cytokines by liver-associated T cells. Therefore, it suggested that liver associated T cells may be involved in the inflammatory process associated with ALD.

Table 1. Effect of Chronic Alcohol Consumption on the Distribution of Mononuclear Cells Types in Liver Perfusate

Groups	Cell yield(x10^6)	T cells	NK cells	Kupffer cells
Pre-feed	24.0±2.2	64.0±0.7*	16.5±2.1*	15.5±3.5*
40% Ethanol				
• 4 weeks	24.5±4.9	61.0±1.4	16.5±3.5	16.0±1.4
• 8 weeks	25.5±3.6	66.5±0.71	18.0±0.0	17.0±1.5
• 12 weeks	23.5±2.1	64.5±0.70	16.0±1.4	15.5±2.1
2% Sucrose				
• 4 weeks	21.5±4.9	66.5±3.6	14.5±0.71	18.6±2.1
• 8 weeks	27.0±2.8	63.5±0.71	17.5±2.1	16.5±4.9
• 12 weeks	27.5±4.9	64.0±1.4	17.0±1.4	20.5±0.71
Isocalroic				
• 4 weeks	30.0±3.5	63.0±1.4	17.0±1.4	14.5±2.1
• 8 weeks	26.5±6.5	66.5±3.5	13.0±2.8	16.0±1.4
• 12 weeks	25.5±4.9	65.5±2.1	15.4±3.5	13.0±3.5

Female Wistar rats were fed 40% ethanol, 2% sucrose, or isocalroic sucrose for 4,8,or 12 weeks. Intrahepatic mononuclear cells were obtained from liver perfusate. *Cell percentages of T cells, NK cells, and Kupffer cells determined by flow cytometry(8). Data represent the mean±SD for three rats per group.

Table 2. T Cell Subpopulations in Peripheral Blood Lymphocytes and Liver Perfusate Lymphocytes in Chronicin Ethanol-Consuming Rats Administered Con A

Groups	CD5+ %	CD8+ %	CD4+ %	CD4+/CD8+
		Peripheral Blood Lymphocytes		
40% ethanol				
• 0 hr	67.70±4.75	21.65±2.31	44.50±2.20	2.06
• 4 hr	88.50±6.30∗	39.50±2.85∗	54.35±3.00∗	1.38∗
• 24 hr	79.30±6.00∗	33.00±2.55∗	50.50±3.10∗	1.53∗
Isocaloric				
• 0 hr	65.50±4.70	20.09±2.00	41.20±2.50	2.05
• 4 hr	80.50±5.00∗	35.10±2.10∗	51.00±3.00∗	1.45∗
• 24 hr	77.30±4.56∗	31.60±2.15∗	49.35±2.90∗	1.56∗
2% sucrose				
• 0hr	67.40±3.85	20.50±2.30	44.50±2.50	2.18
• 4hr	81.30±5.00∗	34.32±3.00∗	52.00±3.74∗	1.52∗
• 24hr	79.43±4.75∗	32.50±2.50∗	50.00±3.31∗	1.60∗
		Liver Perfusate Lymphocytes		
40% ethanol				
• 0hr	65.47±3.50	36.35±2.50	34.10±2.50	0.94
• 4hr	89.60±4.67∗	41.50±3.00	50.40±3.54∗	1.21∗
• 24hr	82.70±4.00∗	40.60±2.13	48.30±3.67∗	1.19∗
Isocaloric				
• 0hr	67.51±3.30	34.20±2.83	32.27±2.50	0.95
• 4hr	80.30±5.42∗	37.30±3.21	45.90±3.10∗	1.23∗
• 24hr	78.85±4.30∗	38.56±3.01	43.68±2.91∗	1.13∗
2%sucrose				
• 0hr	68.05±3.40	35.24±2.50	33.45±2.50	0.95
• 4hr	81.25±4.78∗	38.55±3.30	47.58±3.67∗	1.23∗
• 24hr	79.58±3.67∗	39.80±2.50	46.70±3.23∗	1.17∗

Female Wistar rats were fed 40% ethanol, isocaloric sucrose, or 2%sucrose for 8 weeks. Peripheral blood mononuclear cells and intrahepatic mononuclear cells were obtained from rats at various times after Con A (20mg/kg weight) injection(11). Data points represent the mean±SE for 6-10 rats. ∗ $P<0.05$, compared with controls that represent animals sacrificed at time 0.

Table 3. Hepatic Immunological Cell Populations [1]

Cell type	Distribution of subpopulations	Peripheral blood
Intrahepatic lymphocytes(IHL)	25%of nonparenchymal cells	20%-40% lymphocytes
Total T cells	23% of IHL	80%
B cells	5% of IHL	20%
CD^{3+} αβ T cells	20% of IHL	
CD^{3+} γδ T cells	3% of IHL	
CD^{4+} T cells	20% of αβ T cells	
CD^{8+} T cells	80% of αβ T cells	
CD^{4-} CD8- T cells	14% of CD3+ cells	
CD $^{4+}$ CD^{8+} T cells	5.5% of CD3+ cells	
CD^{8+}α+β- T cells	15% of total T cells	
CD^{4+}/CD^{8+} ratio	1:3.5	2-3:1
NK cells	20%	
NKT cells(Pit cells)	>30% of CD3+ cells and 50% of IHL	0.2%
Nonlymphoid Sinusodial Endothelial cells	50% of nonlymphoid cells	
Kupffer cells	25% of cells in liver	
Hepatic stellate cells	Proportion unknown	
Biliary epithelium	3%-5% of cells in liver	

[1] From: Reference 78

5. INTRAHEPATIC T LYMPHOCYTE INVOLVEMENT IN THE PATHOGENESIS OF ALCOHOL RELATED LIVER INJURY

5.1. Phenotypes, natural immunity and origin of intrahepatic lymphocytes in non-diseased liver

5.1.1. Human intrahepatic lymphocyte populations, numbers and proportions

The human liver has an intrinsic lymphocyte population normally resident in the portal tract with CD3+ T lymphocytes predominating, and B lymphocytes comprising only 5%. T cell numbers were shown to comprise 16-20% of the nonparenchymal cell pool in initial studies, and this has subsequently been confirmed (81). The absolute number of lymphocytes in liver tissue is in the range of 10-20 million cells per gram of tissue. The high concentration of lymphocytes and the size of the liver results in a total hepatic lymphocyte number of 15%-20% of the lymphoid cells in the spleen. While the liver associated lymphocyte population is a very diverse, studies have shown that that the liver associated lymphocyte population is a. remarkably consistent population across species such as mice and humans. Among the conventional T cells, the usual ration of CD^{4+}: CD^{8+} T cells is usually reversed in the liver with more CD^{8+} cells than CD^{4+} cells (77). The liver lymphocytes contains a high frequency of classical natural killer (NK) cells, identified by the CD56 marker in humans and by asialo-GM1 ganglioside and the NK-1.1 marker in mice (82). There is a population of cells that express a level of T- cell receptor (TCR) aß that is lower than the level on circulating T cells (83,84). This "TCR-intermediate" cell population appears to consist of at least two distinct subsets. These are: (a) conventional T cells engaged in apoptosis, many of which express the B220 marker and (b) a distinctive population of NK- T cells found at very low frequency in the blood and in most lymphoid organs, but are very abundant among the T cells of the liver. They express both a TCRaß and the NK-cell marker NK-1.1 (85). Human hepatic T cell numbers and proportions are shown in Table 3 (78).

5.1.2. Natural immune functions of intrahepatic T lymphocytes

The majority of intrahepatic T cells can rapidly secrete a number of cytokines of the inflammatory (Th1/Tc1) type upon pharmacological stimulation or CD3/TCR cross-linking(86,87).These include IFN ?, TNF α and IL-2. Additionally, the same cytokines may be released in response to pro-inflammatory cytokines that are produced by hepatocytes and kupffer cells (88,89). About 5% of human intrahepatic T cells produce a cytokine of the Th2/Tc2 (helper) type, IL-4, but these cells do not produce IL-5 (89). Interestingly, while cytokine production is generally polarized to the mutually inhibitory Th1/Tc1 or Th2/Tc2 profiles, most intrahepatic T cells that produce IL-4 also produce IFN ?, indicating they are of the Th0 type, rather than conventional Th2 cells(87,90). Th0 cells are thought to be either undifferentiated or regulatory cells, and there is evidence that both of these T-cell types are present in the liver. Further evidence of the unique functional potential of intrahepatic T cells is the presence of a population of potent LAK cells that can be induced by stimulation in the presence of cytokines to lyse a large range of tumor cells types (91). Thus, intrahepatic T lymphocytes consist of a heterogeneous population of cells that has many and varied functional characteristics in addition to classical T-cell activity(85).

In addition, intrahepatic T cells recognize some self-antigens in the context with monorphic MHC class I antigens(87).

5.1.3. Origin and development of intrahepatic T lymphocytes

The studies found that all T cells in congenitally athymic nude mice are intermediate TCR, it is obvious that these TCR cells are generated extrathymically. In both athymic mice and normal euthymic mice, the major site appears to be the liver. Because some of the unusual T-cell population exist in the liver, particularly the TCR aß-low population, it has been proposed that these T-cell

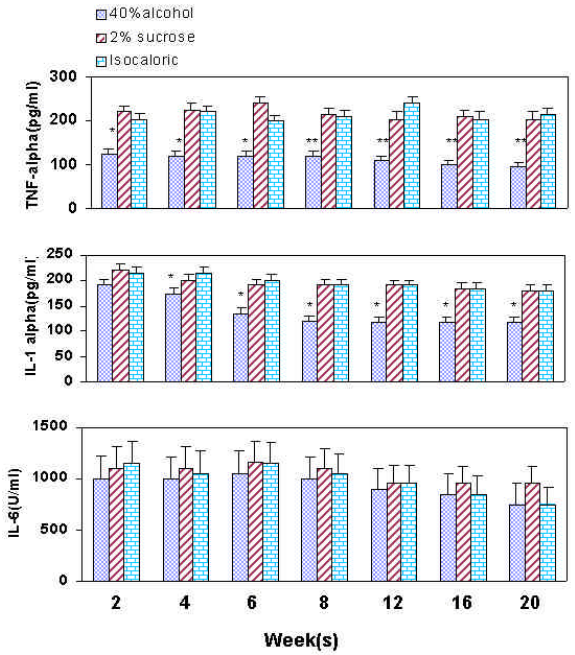

Figure 1. Decreased IL-1α, IL-6, and TNF-α production by intrahepatic mononuclear cells aftrer chronic ethanol consumption(8). Intrahepatic mononuclear cells were collected and cultured from chronic alcohol-consuming rats. Data represent the mean±SE for six rats. * $p<0.05$ and ** $p<0.01$vs.controls.

populations may arise via a thymus-independent pathway (87). c-kit+ stem cells exist in the parenchymal space of the liver and thus cells give rise to extrathymic T cells, forming clusters of many lymphoid cells. Also, these c-kit+ cells have the potential to give rise not only to lymphoid cells but also to other hematopoietic cells such as granulocytes and erythrocytes (92,93). Transfer of hepatic c-kit+ cells to irradiated severe combined immunodeficient (SCID) mice resulted in the development of virtually all lineages in the liver and periphery as well as intraepithelial T cells in the intestine (92,94). Direct demonstration of T-cell development from liver cells populations has been possible by *in vitro* culture of fetal liver precursors (95).

Intrahepatic T cells might mature and differentiate locally and be supplemented by infiltrating lymphocytes from the PB(85). Evidence shown that populations of local T cells in mice that express CD4-CD8-, and are homodimeric for the CD8 α chain or express ?d TCRs, develop in the liver, uterus, intestine and omentum as well as the thymus (96,97,98). According to their limited receptor variability, their ability to respond immediately to stimulation, and their roles in innate immune responses, these cells are thought of as primitive T cells and are thought to be an evolutionary bridge between the innate and adaptive immune systems (85,96).

Local immune systems in organs such as the liver and the intestine are thought to predate the central, adaptive immune system, and the thymus may have evolved from primitive local immune systems as a site for classical T-cell differentiation (85). In certain phylogenic periods, extrathymic T cells in the liver and small intestine, develop in a similar way, but then develop independently. Similarities and differences between primitive and classical pathways of T-cell maturation are only currently being delineated (85). CD8aa+?d T cells and $CD^{4+}CD^{8+}$ aß T cells are uniquely present in the small intestine and can complete their development program subject to profoundly different influences from those found in the thymus (99). Large numbers of CD8aa+ ?d T cells are found in the human liver, suggesting that a similar T-cell selection process may occur in this organ (100). In normal human liver, hematopoietic stem cells have been found as well as molecular evidence of T-cell maturation (101). These cells not only expressed CD34 and CD45, but also expressed HLA-DR on their surfaces, suggesting that they were activated and undergoing differentiation. In humans, RNA transcripts specific for recombinase activating gene-1 (RAG-1) and RAG-2, the cell-specific components required for lymphoid development, have been detected in hepatic lymphocytes(CD2+/CD7+) isolated from normal liver tissue. In addition, pre-TCR-α, a T-cell specific chaperone expressed at any early stage of αβ T-cell development, has been demonstrated in the same cell populations (102). In the liver, IL-7 functions as a cytokine for the initial maturation of extrathymic T cells (103), but IL-12, IL-15 and IL-18 may function as cytokines for functional maturation (104,105). Alternatively, the bile duct epithelium perhaps provides an appropriate environment for local T-cell maturation(85).

5.2. Intrahepatic T Lymphocytes mediate liver injury of ALD

Studies in rodent models of alcoholic liver disease, which examine the evolution of the disease (106), have been able to demonstrate progression through fatty liver to necrosis, cell infiltration, and fibrosis under certain circumstances. Marked necrosis, however, will only occur in animals additionally exposed to endotoxin, LPS, or to Con A (8,11,17,107). LPS is known to activate Kupffer cells which release IL-6 and TNF α but Con A does not induce such a change leading to our own focus on the T cell population as an important part of the pathogenesis of ALD. As both agents, LPS and Con A, are polyclonal mitogenic activators of lymphocytes, studies have been undertaken to define intrahepatic T cell function in evolving ALD. A series of studies from our laboratory have generated the hypothesis that T lymphocytes in the liver or liver associated T lymphocytes involved in the pathogenesis of ALD. Our studies have generated a series of findings that can be summarized as follows:

- First, alcohol exposure alone is associated with a downregulation in the production of TNF a and IL-6 by intrahepatic T cells (see Figure 1). Data are consistent with other reports which show that alcohol depresses TNF a and IL-6 release by macrophages and T cells in the rodent and in humans. These findings also demonstrate that alcohol does not induce injury directly by causing the release of cytokines.

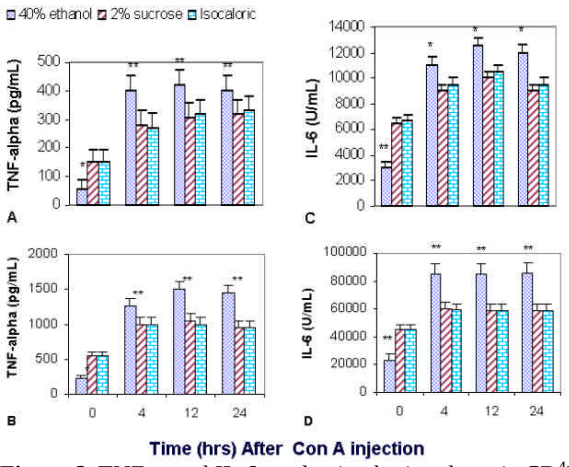

Figure 2. TNF-α and IL-6 production by intrahepatic CD^{4+} T cells following Con A injection(12). Intrahepatic CD^{4+} T cells isolated from chronic ethanol-consuming rats at various times after Con A injection were cultured in the absense or presence of Con A(5 μg/ml). After 24 hr,TNF-α and IL-6 were measured in culture supernatants.(A) and (C): CD^{4+} T cells alone. (B) and (D): CD4+ T cells cultured with Con A(5 μg/ml). Each point represents the mean±SE for six rats. * $p<0.05$ and ** $p<0.01$, compared with controls.

- Second, there is a rapid and marked increase in the production of TNF a and IL-6 by intrahepatic T cells and Kupffer cells in alcohol-exposed animals injected with LPS or ConA (see Figure 2).

- Third, liver cell necrosis and release of the enzyme alanine aminotransferase(ALT) occurs within 4 hours of cytokine secretion by T cells and Kupffer cells. The time course of the injury suggests that damage is induced by cytokines.

- Fourth, studies that involve the transfer of intrahepatic lymphocytes, from alcohol-consuming rats to non-alcohol-consuming rats, demonstrate a critical role of T cells in this pathological process. Animals infused with alcohol-primed T cells respond to the injection of LPS and ConA in a fashion similar to animals that had been consuming alcohol.

- Fifth, co-culture studies involving hepatocytes with T cells or Kupffer cells highlighted the importance of T cells (especially CD^{4+} cells) in the genesis of the elevated cytokine levels. TNF α was an important mediator of cell damage in the coculture model. In this model, neutrophil infiltration of the liver is not a major feature, whereas in human alcoholic hepatitis these cells are prominent. Their presence reflects a significant release of IL-8 from intrahepatic cells in response to injury, but their role in the overall pathogenesis of ALD remains incompletely defined.

- From our studies involving the co-culture of liver associated T cell and hepatocytes, we have shown that alcohol can result in the dysregulation of liver CD^{4+} function. The ethanol-modified liver CD^{4+} cells could induce significant hepatocyte apoptosis after exposure to a second stimulus and liver CD^{4+} -mediated liver injury resulted from increased production of IFN-? and TNF a which could be mediated by NF-kB activation and calcium mobilization. The origin of cytokines was mainly from activated liver CD^{4+}, but nitric oxide produced by hepatocytes could be in part involved in the process (108).

Individual susceptibility to the injurious effects of alcohol in humans is not well explained. Data from a rat model suggest an explanation based on a genetic variability in T cell responsiveness to mitogens after alcohol ingestion. Humans who drink regularly and who experience an additional" insult," may release increased amounts of cytokines such as TNF a , IL-6, and IL-8. Examples of such an insult would be a severe infection, hypoxia, or endotoxemia after an alcoholic binge or a gastrointestinal hemorrhage (109) . Those with chronic alcohol ingestion, who have appropriately primed lymphocytes, may respond with a burst of cytokine release after exposure to a secondary stimulus such as endotoxin, and this, in turn, could induce hepatocellular necrosis.

6. PERSPECTIVE

The studies from our laboratory have, for the first time, demonstrated that alcohol consumption alone does not lead to the development of significant liver necrosis in the rat. Rather, a second stimulus of T lymphocytes is necessary to trigger the process of cell death. They also suggest that alcohol consumption induces a dysregulated liver-associated T-cell population that mediates the development of liver necrosis through increased secretion of cytokines such as IL-6, TNF a and IFN ? following activation by polyclonal mitogens (e.g. endotoxin, Con A). The origin of cytokines was mainly from activated liver-associated T cells, but Kupffer cells could be in part involved in the process. It is highly likely that in humans a similar activation of T cells could occur, and associated insults such as sepsis and hypoxia could act as a stimulant leading to more severe liver injury in these patients. Molecular pathogenesis of T lymphocyte-injury in alcoholic liver disease can be explained as excessive cytokine release from primed-T lymphocytes after exposure to secondary stimuli, especially from liver-associated T lymphocytes. We hypothesize that T-lymphocyte-involved liver injury plays a role in the pathogenesis of alcoholic liver fibrosis (see Figure 3). Further investigations are being conducting to clarify the related mechanisms. Genetic variation in the outcome of T cell damage after chronic alcohol ingestion is the key link to explain the confusing data base found in humans. Genetic or signal-transduction variation in T cell responsiveness to polymitogenic activators can be related to levels of exposure to alcohol. The alternative to the above variation in susceptibility of hepatocyes to damage by cytokines, can also be assessed experimentally. Other questions that we need to answer include.

- Do T cell recirculation characteristics change with chronic alcohol ingestion? What is the biochemical, biological or structural "lesion" induced in T cells that, on

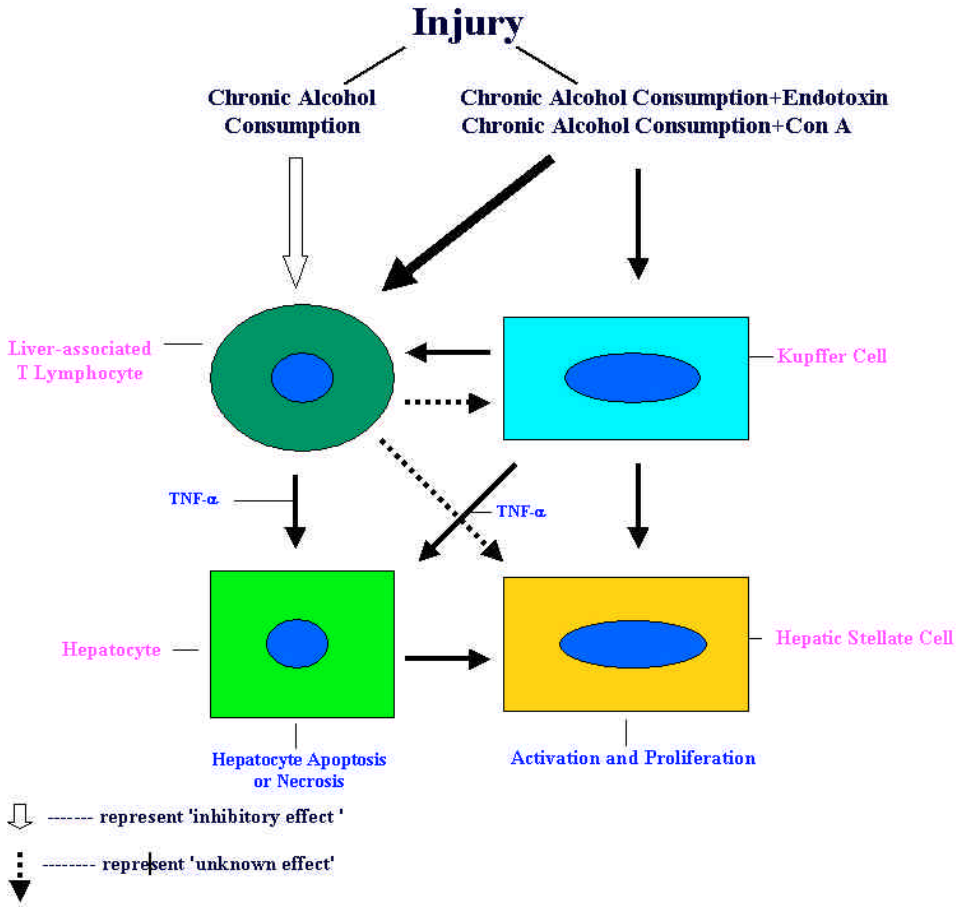

Figure 3. Liver Associated T Lymphocytes Mediate Alcohol Related Liver Injury.

one hand, leads to reduced cytokine secretion in resting T cells, while on the other , determines an excessive secretory cytokine response when activated by exposure to secondary stimuli?

- In humans, what is the nature of the secondary factors that activate cytokine secretion?

- To what extent do non-T cell populations, particularly the Kupffer cell population, contribute to damaging cytokine levels within the liver?

- How do the different cell populations communicate with each other and alter cell function?

- To what extent do T cells in alcoholic liver contribute to the development of liver fibrosis?

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