<div style="text-align:center">**POINT OF VIEW**</div>

Kupffer cells and alcoholic liver disease

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ABSTRACT

Liver disease is a major cause of illness and death worldwide. A central component in the complex network leading to the development of alcoholic liver disease is the activation of Kupffer cells by endotoxin and other soluble mediators. Alcohol consumption induces a state of 'leaky gut' increasing plasma and liver endotoxin levels. When Kupffer cells become activated, they interact with a complex of proteins located on the extracellular membrane signaling to produce a wide array of soluble factors, including cytokines, chemokines, growth factors, cyclooxygenase and lipoxygenase metabolites, and reactive oxygen species such as superoxide anion, hydrogen peroxide, and nitric oxide, all of which provide physiologically diverse and pivotal paracrine effects on all other liver cell types and, ultimately, liver injury. Kupffer cells are also central to the liver homeostatic response to injury as upon cellular degenerative changes, they immediately respond to the insult and release mediators to orchestrate inflammatory and reparative responses. Thus, the homeostatic responses are initiated by Kupffer cell-derived mediators at the cellular level and underlie the liver's defense and reparative mechanisms against injury. In order to understand better the role of Kupffer cells in the onset of liver injury, animal models in which Kupffer cells are inactivated, and cell culture settings (e.g. co-cultures) are being used with promising results that advance our understanding of alcoholic liver disease.

Key words: Kupffer cells. Alcoholic liver disease. Reactive oxygen species. Lipopolysaccharide. Hepatic stellate cells.

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ABBREVIATIONS (IN ALPHABETICAL ORDER)

Alcoholic liver disease (ALD); arachidonic acid (AA); carcinoembryonic antigen (CEA); c-Jun kinase (JNK); Cyclooxygenase (COX); dilinoleoylphosphatidylcholine (DLPC); early-immediate gene-1 (Egr-1); endotoxin or lipopolysaccharide (LPS); ethylenediamine tetra acetic acid (EDTA); extracellular membrane (ECM); extracellular signal-regulated protein kinase 1/2 (ERK1/2); glucose-6-phosphate dehydrogenase (G6PDH); glutathione (GSH); glutathione disulfide (GSSG); glutathione-S-transferase (GST); hepatic stellate cells (HSC); hydroxyl radical (OH·); leukotrienes (LT); lipoxygenase (LOX); low-density lipoprotein (LDL); LPS-binding protein (LBP); major histocompatibility complex (MHC); nitrate (NO_3^-); nitric oxide (NO); nitric oxide synthase (NOS); nitrite (NO_2^-); nuclear factor-kappa beta (NF-κB); P_{450} cytochrome 2E1 (CYP2El); peroxynitrite (ONOO-); phospholipase A_2 (PLA_2); platelet activation factor (PAF); platelet-derived growth factor (PDGF); propylthiouracil (PTU); prostaglandin D_2 (PGD_2); prostaglandin E_2 (PGE_2); prostaglandin H_2 (PGH_2); reactive oxygen species (ROS); superoxide anion ($O_2^{·-}$); superoxide dismutase (SOD); transforming growth factor-beta (TGF-β); transmembrane toll-like receptor (TLR); tumor necrosis factor-alpha (TNF-α); very-low-density lipoprotein (VLDL).

GENERAL ARCHITECTURE OF THE LIVER

There are five different cell types in the liver which occupy about 80% of the hepatic volume. The remaining 20% of the hepatic volume comprises the extracellular space and extracellular matrix. Among the liver cells, hepatocytes are the largest in size and the most abundant as they occupy close to 50 to 60% of the total liver volume and account for two-thirds of total liver cells. The other four cell types are referred to as non-parenchymal or liver

sinusoidal cells. They are smaller in size and lesser in number than the parenchymal cells (1).

The liver sinusoidal cells play a critical role in the maintenance of liver function, under both physiological and pathological conditions (2). The hepatic sinusoidal cells are endothelial cells, Kupffer cells, stellate cells (fat-storing cells or Ito cells), and pit cells (NK cells). This review will focus on the role of Kupffer cells.

KUPFFER CELLS

Kupffer cells are liver-specific macrophages which were first identified in the liver by von Kupffer (1876) (3). Kupffer cells are amoeboid in shape and adhere to the surface of fenestrated sinusoidal endothelial cells. In the cytosol of Kupffer cells, there are a number of dense bodies and electron-lucent vacuoles of various sizes including lysosomes. Golgi apparatus, coated vesicles, pinocytic vesicles, ribosomes, centrioles, microfilaments, and microtubules are also present in the cytosol (4). The nucleus of Kupffer cells is ovoid or indented, and occasionally lobulated (5). Wormlike structures, fuzzy coat, microvilli, and pseudopodia at the cell surface are characteristic structural components of Kupffer cells involved in endocytic mechanisms (6). Kupffer cells incorporate large particles such as erythrocytes and bacteria by phagocytosis and take up small particles and molecules via pinocytic vesicles (7-12).

Peroxidase activity is observed in the rough endoplasmic reticulum, nuclear envelope, and annulate lamellae (13,14). The majority of Kupffer cells display an endogenous peroxidase pattern typical of resident tissue macrophages and show positive staining for macrophage markers such as ED1, ED2, and Ki-M2R in rats and F4/80 in mice (6,15). Other markers are the presence of inducibility of major histocompatibility complex (MHC) class II antigens (16), receptors for fructose and galactose-exposing glycoproteins (17,18), peroxidase activity, and the capacity to phagocytose fluorescent labeled latex particles (19). Kupffer cells display high glucose-6-phosphate dehydrogenase (G6PDH) activity, which plays an important role in the metabolic response to phagocytosis. A key enzyme present in Kupffer cells is NADPH oxidase which plays a decisive role in the development of alcohol-induced liver injury (20). The cytochrome P_{450} enzymes, and concretely P_{450} 2E1 (CYP2E1), are also expressed in Kupffer cells. In addition, several glutathione-S-transferase (GST) isoenzymes and glutathione peroxidase may enable the Kupffer cell to detoxify potentially hepatoxic substances (21-23).

FUNCTION OF KUPFFER CELLS

Kupffer cells have many specific functions that are essential for the preservation of homeostasis in the liver un-

der several conditions. Endocytosis, pivotal for maintaining homeostasis, is not only essential for the removal of several (plasma) proteins and other material from the blood, but the receptor-mediated uptake of these substances is directly coupled with a metabolic response, leading to the production of cytokines and eicosanoids. Both cytokines and eicosanoids may act subsequently in an autocrine and/or paracrine fashion.

Clearance of galactose-terminated particles from the circulation is performed by a galactose-specific receptor in Kupffer cells (24). The Mannose/N-acetylglucosamine receptor is probably involved in the clearance of larger mannose-exposing particles, like potentially hazardous microorganisms such as bacteria, yeast, and parasites (25). Low-density lipoproteins (LDL) are almost exclusively taken up by Kupffer cells (26). Kupffer cells possess an additional receptor which binds oxidatively modified LDL, very low-density lipoprotein (VLDL), and lipoprotein A (27-29).

Like endothelial cells, Kupffer cells bind IgG immune complexes, which may contribute significantly to the normal host defense (30,31). The uptake of IgA immune complexes is mediated by a specific receptor present in Kupffer cells, which recognizes the Fc part of IgA (32, 33). Under several pathological conditions, like primary biliary cirrhosis, obstructive jaundice, and ethanol consumption, the capacity of Kupffer cells to take up immunoglobulin immune complexes is reduced, which may lead to a reduction of the host defense capacity. Kupffer cells possess receptors for the complement components C1q and C3b (30,34). Complement factors adhere to immunoglobulins (35), DNA, bacteria, and platelets (36-38). Human Kupffer cells also express several complement receptors, and the presence of CR1, CR3, and CR4 receptors provides Kupffer cells with an optimal capacity to remove complexes coated with complement from the circulation (39).

The effects of platelet activation factor (PAF) on the liver are mediated by Kupffer cells that possess specific binding sites for PAF (40). Carcinoembryonic antigen (CEA) is a gut-derived glycoprotein that is cleared from the circulation by Kupffer cells (41). These binding sites may be important for the development of liver metastases (42). Several cellular components, like DNA and cell-derived enzymes, are released during cell death in several clinical situations. DNA can be removed from the circulation by binding to a receptor on the Kupffer cells without opsonization (43).

KUPFFER CELLS ISOLATION, PURIFICATION AND CULTURE

One of the first methods to isolate Kupffer cells was based in the *in vivo* uptake of iron particles by Kupffer cells, washing the sinusoidal cells out of the liver, and purifying the Kupffer cells using magnets (44). Later, chela-

tors such as citrate, tetraphenylboron, or ethylenediamine tetra acetic acid (EDTA) were used to prepare liver cell suspensions, and iron-loaded Kupffer cells were isolated using magnets (45-48). These methods provided only low numbers of viable sinusoidal cells and contamination with other cell populations remained an issue of concern. Alternative isolation procedures described by Berry and Friend (49) and Seglen and Gjessing (50), and modified by Arahuetes et al. (51), utilized collagenase for the perfusion and dispersion of the liver, and were used to isolate parenchymal and non-parenchymal cells simultaneously from the same liver. Pronase, which preferentially destroys parenchymal liver cells, can be used directly for digestion of the liver or can be used in addition to collagenase digestion.

When centrifugal elutriation was introduced in the protocol, the yield was much higher as contamination of the endothelial and Kupffer cell fractions by blood lymphocytes and so-called blebs was minimized (52). Percoll density gradients, followed by selective adherence of both Kupffer and endothelial cells, have been described in order to obtain pure cell fractions from the crude nonparenchymal liver cell suspensions (53). Centrifugal elutriation after an initial gradient in Histodenz or Nycodenz to separate Kupffer cells and endothelial cells from sellate cells is generally the method of choice (54).

Finally, separation by velocity sedimentation, which is based on the same physical properties as elutriation, normally gives less satisfactory results unless high-resolution equipment is used and diminishes the viability of both fractions as longer times are required (55). These cultured cells are still capable of phagocytosis of colloidal carbon and latex particles and may preserve their functions, with regard to endocytosis, for at least two weeks in culture.

ALCOHOLIC LIVER DISEASE (ALD) AND KUPFFER CELLS

Endotoxin

Alcoholic liver disease (ALD) involves several stages of liver injury: steatosis, alcoholic steatohepatitis, alcoholic hepatitis, and cirrhosis (56). Kupffer cells play an important role in alcohol-induced liver damage. Alcohol increases the gut permeability to endotoxin or lipopolysaccharide (LPS), a major constituent of the outer membrane of gram-negative bacteria, which triggers a variety of inflammatory reactions, including the release of proinflammatory cytokines and other soluble factors (11). Adachi and colleagues showed that the inactivation of Kupffer cells using gadolinium chloride prevents early alcohol-induced liver injury, and that a concurrent reduction of the ethanol-induced CYP2El induction was observed (57). They also showed that intestinal sterilization with antibiotics (polymyxin B and neomycin) could pre-

vent alcohol-induced liver injury by reducing intestinal bacteria and lowering the risk for endotoxemia (58).

Multiple mammalian receptors for LPS have been identified, including two glycoproteins: LBP and CD14. CD14 binds to the LPS-binding protein (LPS-LBP) complex but is not, by itself, capable of initiating a transmembrane activation signal (6). It has been postulated that LPS/CD14 complexes interact with a transmembrane toll-like receptor (TLR) responsible for signal transduction (59).

Both *in vivo* and *in vitro* data suggest that chronic ethanol sensitizes Kupffer cells to at least some LPS-mediated responses. For example, chronic ethanol consumption increases the susceptibility of rats to LPS-induced liver injury (60). Hijioka et al. showed that binge ethanol drinking rapidly inactivated voltage-dependent Ca^{2+} channels in Kupffer cells becoming activated to release other critical mediators (61). Thus, inactivation of these channels may be involved in mechanisms of rapid tolerance to ethanol (62).

Shibayama et al. showed that acute administration of ethanol enhanced endotoxin hepatotoxicity. In his work, Kupffer cells became sensitized to LPS in cells isolated 24 hours after ethanol administration as reflected by increased intracellular Ca^{2+}, tumor necrosis factor-alpha (TNF-α) production, and large increases in CD14. These effects were all blocked by antibiotics, indicating that sensitization of Kupffer cells by ethanol is also mediated by gut-derived LPS (63).

Oxidative stress

Oxidative stress caused by generation of reactive oxygen species (ROS) during alcohol consumption is suggested as one of the major mechanisms of alcohol-induced liver injury (56,61-71). ROS can be generated by a variety of enzymes including, but not limited to CYP2E1, NADH/NADPH oxidase, xanthine oxidase, and arachidonic pathways, such as lipoxygenase (LOX) and cyclooxygenase (COX). Induction of ROS can be also triggered by damaged mitochondria as it happens in ALD (72-76).

Kupffer cells contain superoxide dismutase (SOD) that dismutates $O_2^{\cdot-}$ (superoxide anion) to yield H_2O_2. H_2O_2 and $O_2^{\cdot-}$ can interact via the Fenton reaction to produce more powerful and cytotoxic radicals, such as $OH^{\cdot}$ (hydroxyl radical) (77). However, under non-pathologic conditions intracellular accumulation of $OH^{\cdot}$ is also limited because H_2O_2 is metabolized by glutathione peroxidase and/or catalase to form H_2O and O_2. Thus, together with glutathione (GSH), SOD, glutathione peroxidase, and catalase are the major endogenous antioxidant enzyme systems responsible for limiting intracellular accumulation of $O_2^{\cdot-}$ and H_2O_2 during normal aerobic metabolism (78).

Nitric oxide synthase (NOS) has been shown to modulate levels of ROS in a variety of cells (79,80). There are

three isoforms of NOS: two are constitutively expressed (endothelial and neuronal; eNOS and nNOS, respectively) and one is an inducible isoform (iNOS). Nitric oxide (NO) generated by NOS can interact with $O_2^{\cdot-}$. This pathway for detoxification of $O_2^{\cdot-}$ does not result in the formation of H_2O_2, making it a more benign decomposition pathway than SOD. However, one of the potential products of this reaction that has received a great deal of attention is peroxynitrite ($ONOO^-$), a potent oxidizing agent. $ONOO^-$ has been implicated as the causative agent in a variety of pathologies (81-84). Alternatively, $ONOO^-$ has been implicated as a protective agent (85-87). The latter can either spontaneously rearrange to form nitrate (NO_3^-) or undergo cleavage to generate OH-like radicals and nitrite (NO_2^-).

In ethanol binge drinking, Kupffer cells are primed for enhanced ROS release, due in part, to complement activation (71). Acute alcohol administration causes accumulation of reactive oxygen species, including $O_2^{\cdot-}$, $OH^{\cdot}$, and H_2O_2 (88). Lipid peroxidation and mitochondrial dysfunction have been detected in both animal models and humans receiving acute doses of alcohol (72-76,89,90). Acute ethanol administration also enhanced iNOS mRNA in hepatocytes and Kupffer cells (68). There is also a decrease in GSH levels along with an increase in glutathione disulfide (GSSG) levels (68,91). However, the effect of chronic ethanol consumption on hepatic GSH levels is complex, with reports of no change, decreases, or even increases (64,67,68,70,72-76,89,90,92, 93).

Chronic ethanol consumption increases the cytosolic activity of iNOS and may increase $O_2^{\cdot-}$ production via activation or increasing the levels of CYP2E1, xanthine oxidase, NADPH oxidase, or damaged mitochondria, resulting in enhanced production of both NO and $O_2^{\cdot-}$. Dietary supplementation with phosphatidylcholine has been shown to attenuate ethanol-induced fibrosis acting as a 'sink' for free radicals (94).

Role of eicosanoids

Kupffer cells synthesize eicosanoids and are responsible for about 65% of the total amount of eicosanoids produced in the liver (95). Arachidonic acid (AA) can be released from the cell membrane by the action of phospholipase A_2 (PLA_2). This enzyme is activated by an increase in the intracellular Ca^{2+} concentration, whereas higher intracellular cAMP levels inhibit PLA_2 (96). COX_2 catalyzes the conversion of arachidonic acid into prostaglandin H_2 (PGH_2), which is subsequently converted to other prostaglandins and thromboxanes, the so called *cyclooxigenase pathway* (97). COX_2 is induced by endotoxin, cytokines, and oxidative stress (98). Conversion of AA by LOX and subsequent enzymes leads to the production of leukotrienes, the so called *lipoxygenase pathway* (59).

Leukotrienes and prostaglandins are collectively called eicosanoids. The main product of Kupffer cells is prostaglandin D_2 (PGD_2), representing 55% of the total amount of eicosanoids produced by them (96). Kupffer cells lack the capacity to produce very potent leukotrienes (LT), like LTB_4 or LTC_4 (99). LPS is removed primarily by Kupffer cells that are activated, leading to rapid increases in COX_2 and intracellular Ca^{2+}, the latter of which, in turn, activates PLA_2 (96). Increased prostaglandin E_2 (PGE_2) causes triglycerides' accumulation in hepatocytes, and therefore, a state of steatosis (97). This pathway is one of many physiological processes involved in mechanisms of fatty liver caused by ethanol (100,101). Alterations of the hepatic metabolic state during binge drinking has been also attributed to the release of mediators such as prostaglandins from activated Kupffer cells, and this effect can be attenuated by the anti-thyroid drug, propylthiouracil (PTU) (94).

Role of cytokines and nuclear factor-kappa B (NF-κB)

Production of inflammatory cytokines is a highly regulated process; regulation has been reported at the levels of transcription, translation, and secretion. Mechanistic studies have demonstrated that endotoxin binds to the LPS $CD14/TLR_4$ complex on Kupffer cells causing nuclear factor-kappa beta (NF-κB) activation, which in turn leads to TNF-α production and liver injury (102-104). Increasing experimental evidence supports that TNF-α signaling, generates an increase in mitochondrial ROS generation in the hepatocte through ubiquinone cycling via the electron transport chain (105-107).

Recent studies in rodents have confirmed a role of Kupffer cell-derived TNF-α in alcoholic liver injury (102) demonstrating increased immunostaining for TNF-α, IL-1, IL-6, and IL-8 on bile duct epithelial cells and Kupffer cells (108).

In unstimulated cells, NF-κB, a ubiquitous transcription factor, is sequestered in the cytoplasm with the Iκβ family of inhibitors (59). On stimulation, Iκβ is phosphorylated with subsequent release of NF-κB. NF-κB then translocates to the nucleus, in which it binds cis-acting elements in the promoter of target genes such as TNF-α and other proinflammatory cytokines (109).

Stimulation of macrophages with LPS activates tyrosine kinases, protein kinase C, NF-κB, as well as members of the mitogen-activated protein kinase family including extracellular signal-regulated protein kinase 1/2 (ERK1/2), p38, and c-Jun kinase (JNK) (110). Chronic ethanol feeding differentially regulates the expression of TNF-α and IL-1 in Kupffer cells. These differential responses are associated with impaired LPS activation of NF-κB counteracted by enhanced activation of ERK1/2 and early-immediate gene-1 (Egr-1) (111).

Dilinoleoylphosphatidylcholine (DLPC) decreases lipopolysaccharide-induced TNF-α generation by Kupffer cells of ethanol-fed rats by blocking p38, ERK1/2, and NF-κB activation (112). DLPC also decreases TNF-α induction by acetaldehyde, a toxic metabolite released by ethanol oxidation (57). Iron has long been implicated in the pathogenesis of chronic liver disease, including ALD. It is believed that iron accumulates in chronic liver inflammation and catalyzes hydroxyl radical-mediated oxidative injury, activating the NF-κB pathway (102).

Paracrine effects on hepatic stellate cells

Activated Kupffer cells release a number of soluble agents, including cytokines, such as transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), and TNF-α, ROS, and other factors (113). These factors act on hepatic stellate cells (HSC), which are localized in the parasinusoidal space, and store most of the vitamin A in the body (114).

In normal liver, the space of Disse contains a non electron-dense basement membrane-like matrix which is essential for maintaining the differentiated function of all resident liver cells. However, as the liver becomes fibrotic, the total content of collagens and non-collagenous components greatly increases and it is accompanied by a shift in the type of extracellular matrix in the subendothelial space from the normal low density basement membrane-like matrix to interstitial type matrix containing fibril-forming collagens.

Under normal conditions, HSC remain quiescent and produce small amounts of extracellular membrane (ECM), such as laminin and collagen type IV which are essential components of basement membrane (115). Upon exposure to soluble factors from damaged hepatocytes and from activated Kupffer cells, HSC will lose their lipid content (retinyl palmitate), and undergo morphological transition to myofibroblast-like cells (70,98, 116-121).

Activated HSC produce then large amounts of extracellular matrix components e.g. collagen I in an accelerated fashion, triggering a fibrogenic response (70,98, 116-121). During the cross-talk of both liver cell types, mediated by different cytokines, reactive oxygen species, and other soluble factors, hepatocellular damage is initiated, and subsequent establishment of liver fibrosis occurs.

In summary, a conceptual issue that has emerged in the field of ALD is the importance of cell-type specific research. It is well-known that Kupffer cells actively participate in the pathogenesis of ALD. The role of Kupffer cells in both the autocrine and paracrine mode of proinflammatory and cytotoxic actions has been supported by different experimental strategies. Among them, the development of co-culture models of Kupffer cells and HSCs for investigating the effects of various fibrogenic

mediators represents a promising tool for the study of ALD aiming at finding treatment strategies for this disease.

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