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## Kupffer Cell Metabolism and Function

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### Abstract

Kupffer cells are resident liver macrophages and play a critical role in maintaining liver functions. Under physiological conditions, they are the first innate immune cells and protect the liver from bacterial infections. Under pathological conditions, they are activated by different components and can differentiate into M1-like (classical) or M2-like (alternative) macrophages. The metabolism of classical or alternative activated Kupffer cells will determine their functions in liver damage. Special functions and metabolism of Kupffer cells suggest that they are an attractive target for therapy of liver inflammation and related diseases, including cancer and infectious diseases. Here we review the different types of Kupffer cells and their metabolism and functions in physiological and pathological conditions.

### Keywords

Kupffer cells; Liver; Metabolism; Macrophages

### Introduction

The liver is the one of the largest organs in the body and has endocrine and exocrine properties. It is composed of 60% parenchymal cells, *i.e.*, hepatocytes, and 30% to 35% non-parenchymal cells, *i.e.*, Kupffer cells (KCs), hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs) [1]. Kupffer cells were first identified by Karl Wilhelm von Kupffer in 1876 using a gold chloride-staining method and were named “Sternzelle” (stellate cells) [2]. Initially, KCs were associated to the family of perivascular cells of the connective tissues or to the adventitial cells (pericytes). Finally, after fundamental research by Tadeusz Browicz, KCs were identified as macrophages [3]. Kupffer cells are liver resident macrophages that localize within the lumen of the liver sinusoids and are adherent to the endothelial cells that compose the blood vessel walls. KCs are the first immune cells in the liver that come in contact with the gut bacteria and gut bacterial endotoxins and

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### Competing Interests Statement

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microbial debris derived from the gastrointestinal tract that have been transported to the liver *via* the portal vein [4]. They also play an essential role in the host defense [5,6] and participate in the metabolism of multiple compounds such as protein complexes, small particles, and lipids, and in removing apoptotic cells from the circulation [7,8]. Consequently, modifications or alterations of KC functions are associated with various liver diseases: viral hepatitis, steatohepatitis, alcoholic liver disease, intrahepatic cholestasis, activation or rejection of the liver during liver transplantation [9] and liver fibrosis [10]. Here we review the different type of KCs and their metabolism and functions in physiological and pathological conditions.

## Ontogeny and Different Populations of Kupffer Cells

### Ontogeny of Kupffer cells

KCs are liver resident macrophages and appear for the first time in the yolk sac during embryonic development in mammals [11]. Macrophages first migrate into the fetal liver *via* the umbilical veins and the left vitelline vein. The F4/80-positive macrophages are detected in the hepatic sinusoid at 11 days of gestation in mouse embryos, and their number increases with fetal age. At day 17, F4/80-positive macrophages exhibit peroxidase activity in the nuclear envelope and rough endoplasmic reticulum as observed in mouse adult liver KCs [12]. They proliferate quickly and differentiate into KCs in the late stage of embryonic development and after birth [13].

### Life span and renewal of Kupffer cells in liver

Little is known concerning the life span and the renewal mechanisms of KCs. The calculated life span of mammalian KCs was determined to be 3.8 days [14]; however, experimental data showed a longer life span. Bouwens and collaborators [15] have shown in rats that the life span of KCs stretched from several weeks to 14 months. Moreover, in transplanted human livers, donor KCs persisted for up to one year [16].

The mechanisms of KC renewal have still remained elusive. Two hypotheses were put forward: The classical dogma assumes that KCs are not able to self-renew and come from bone marrow-derived monocytes [17,18], whereas the second hypothesis supports that KCs are a self-renewing population and can proliferate as mature cells, or they come from local intrahepatic progenitors [19–23]. To support this second hypothesis, Varol's group treated mice with acetaminophen after an adoptive transfer experiment. Their data showed that monocytes characterized as Ly6C<sup>high</sup>CD11b<sup>high</sup>MHCII<sup>neg</sup> were massively recruited and infiltrated into the damaged liver after 24 hours of treatment; at the same time, the number of KCs in the injured liver was decreased. These infiltrating monocytes differentiated into Ly6C<sup>low</sup>F4/80<sup>high</sup> macrophages in the injured liver and became the predominant population at 72 hours following acetaminophen treatment before disappearing completely after 96 hours. These macrophages negatively regulated the recruitment of neutrophils in the injured liver. After 120 hours of treatment, KCs became the major macrophage population in the liver, and this repopulation of KCs was due to the self-renewal of differentiated KCs present in the liver [22]. Compared to bone marrow-derived macrophages, KCs exhibited a positive function on the recruitment of neutrophils and also protected hepatocytes from bacterial

infection [24]. In order to maintain the constant number of KCs in liver, some data showed that KCs are able to migrate from the liver to the portal areas and into hepatic lymph nodes [25]. However, other hypotheses suggest that KCs can undergo apoptosis, and the apoptotic cells are recognized and phagocytized by adjacent KCs [14].

### Subsets of mouse Kupffer cells

KCs are derived from monocytes and differentiate into liver resident macrophages. Because of their origin, macrophage surface markers were used for their identification; for example, F4/80, CD11b and CD68 are commonly used in mice [26]. F4/80 is a stable antigen of mononuclear phagocytes and does not present in other types of leukocytes [27,28]. CD11b antigen is present on the monocyte/macrophage, granulocyte and natural killer cytoplasmic surface [29], and CD68 antigen is usually used as a surface marker of macrophages and activated KCs [30]. Based on these surface markers, four populations of KCs were identified by Seki's group [31]: F4/80<sup>+</sup>CD11b<sup>-</sup>, F4/80<sup>+</sup>CD11b<sup>+</sup>, F4/80<sup>+</sup>CD68<sup>-</sup> and F4/80<sup>+</sup>CD68<sup>+</sup>. F4/80<sup>+</sup>CD68<sup>+</sup> and F4/80<sup>+</sup>CD11b<sup>-</sup> cells presented a higher phagocytic activity and showed significant reactive oxygen species (ROS) production after lipopolysaccharide (LPS) or adenosine triphosphate (ATP) stimulation, whereas the F4/80<sup>+</sup>CD11b<sup>+</sup> and F4/80<sup>+</sup>CD68<sup>-</sup> cells showed a strong intensity of intracellular tumor necrosis factor (TNF) and interleukin 12 (IL-12) after LPS stimulation.

At the same time, Klein and collaborators irradiated mice and performed an adoptive transfer experiment. They identified two populations of KCs: the first one derived from the bone marrow and the second one was sessile KCs [21]. These two populations of KCs shared the same morphology and phagocytic activity. However, only the first one was implicated in inflammatory responses since they were recruited to the inflammatory foci after the generation of a liver inflammatory environment. Varol's group used other surface markers to identify different populations of macrophages present in the injured liver caused by acetaminophen treatment [22]. As described above, they identified Ly6C<sup>high</sup>CD11b<sup>high</sup>MHCII<sup>neg</sup> monocytes, which were able to differentiate into Ly6C<sup>low</sup>F4/80<sup>high</sup> macrophages, and resident KCs. Furthermore, they analyzed the molecular signature of these three populations of liver macrophages and observed that: 1) KCs present in liver after 72 hours of acetaminophen treatment expressed the same gene profile as KCs in normal liver; 2) Ly6C<sup>high</sup> monocytes shared only one gene with KCs in normal liver and 667 genes with Ly6C<sup>low</sup>F4/80 macrophages; and 3) the latter did not express any common gene with the KCs in the normal liver.

Together, data obtained from different groups show the presence of different populations of macrophages in injured liver, and moreover they express distinct gene expression profiles and are associated with specific functions to repair liver damage.

### Metabolism and Functions of Activated Kupffer Cells

In the normal liver, KCs and other non-parenchymal cells represent from 30% to 35% of total liver cells. Thanks to their strategic position in the liver, KCs are the first ones that are in contact with materials absorbed from the gastrointestinal tract. The liver can be damaged by different injuries such as bacterial LPS [32], chemical substances, toxins and

pharmacological agents [33,34] such as carbon tetrachloride (CCl<sub>4</sub>) [35], endotoxin [36], galactosamine [37], acetaminophen [38] and diethylnitrosamine (DEN) [39]. The immediate resulting effects of liver injuries are increased hepatocellular necrosis, which is one of the principal sources of KC activators [10]. Once they are activated, KCs display the ability to differentiate into M1-like macrophages (classical) or M2-like macrophages (alternative) depending on the signals they receive from their environment [40]. The term macrophage activation (classical activation) was used for the first time by Mackness in the 1960s in an infection context to describe the antigen-dependent microbicidal activity of macrophages towards bacillus Calmette-Guerin and Listeria [41]. It was only in the 1990s, that Stein, Doyle and co-workers demonstrated the existence of an alternative activation phenotype of macrophages induced by IL-4 and IL-13 [42,43]. M1 and M2 macrophage populations differ from their capacity to respond to different stimuli and the repertoire of chemokines/ cytokines and receptors they express after their activation [44]. However, both of them become active macrophages with high synthesis and secretion of inflammatory mediators including cytokines, superoxide, nitric oxide, eicosanoids, chemokines, and lysosomal and proteolytic enzymes [10]. Moreover, they exhibit high phagocytic and secretory activities.

### M1-like macrophages

Interferon gamma (IFN- $\gamma$ ), alone or with microbial products such as LPS or inflammatory cytokines, such as TNF, can induce macrophage differentiation to the M1 population [45]. Interaction between IFN- $\gamma$  and its receptor on macrophages activates STAT1 (signal transducers and activators of transcription1) and interferon regulatory factors (IRF) [46]. These classical macrophages are characterized by a high capacity to present antigen, high expression and production of IL-12, IL-23 [47] and IRF-5 [45], high production of nitric oxide (NO), and production of ROS [44,48]. Classical macrophages are IL-12<sup>high</sup> and IL-10<sup>low</sup>. The activated M1 macrophages express opsonic receptors such as Fc $\gamma$ RIII and exhibit a high level of arginine metabolism. This metabolism consists of the transformation of arginine to nitric oxide and citrulline by nitric oxide synthase (iNOS; NOS2) [49]. Furthermore, they display an important glycolytic activity by inducing the expression of the pro- glycolytic PFKFB3 isoform (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), which gives them an energetic advantage in hypoxic regions. In response to different stimuli, classical M1 macrophages up-regulate the expression and secretion of IL-1 $\beta$ , and they increase the succinylation of metabolic proteins and the expression of immunoresponsive genes (IRGs) such as IRG1, which exerts an anti-microbial activity during bacterial infections. Consequently, M1 macrophages are associated with antimicrobial killing and skewing T cell responses toward T helper cells type 1 (TH1) [50].

### M2-like macrophages

Activation of macrophages to the M2 population can be induced by IL-4, IL-10, IL-13, IL-33, transforming growth factor (TGF- $\beta$ ), and granulocyte colony-stimulating factor (G-CSF). These activated macrophages are also divided by Mantovani and colleagues into different subtypes [41], because of the broad range of activities they perform: M2a (where "a" stands for alternative), induced by IL4 or IL-13; M2b, induced by exposure to immune complex and agonists of Toll-like receptors (TLRs) or IL-1R (Interleukine-1 Receptor); and M2c, induced by IL-10 and glucocorticoid hormones. M2a macrophages display the

alternatively activated phenotype typically attributed to M2 cells, *i.e.* stimulation by IL-4 and IL-13 leads to, STAT6 activation and translocation, and activation of other proteins, such as c-Myc, IRF-4, transglutaminase 2 (TGM2), mannose receptor C type 1 (MRC1), cholesterol 25-hydroxylase (CH25H) and the prostaglandin-endoperoxide, synthase 1 (PTGS1), Krüppel-like factor 4 (KLF4) and the signaling modulators CISH (cytokine inducible SH2-containing protein) and SOCS1 (suppressor of cytokine signaling 1) [51]. M2b macrophages exhibit a high production and secretion of IL-10, and their activation turns off IL-12. They express both pro- and anti-inflammatory cytokines (TNF- $\alpha$ , IL-1, IL-6, IL-10<sup>high</sup> and IL-12<sup>low</sup>). Moreover they up-regulate antigen presentation and recruit TH2 (T helper cells type 2) responses [52,53]. Finally, M2c macrophages are responsible for the production of IL-10, TGF- $\beta$  and extracellular matrix components [44]. In general, the M2 population is characterized by IL-10<sup>high</sup> and IL-12<sup>low</sup>, and low production of pro-inflammatory cytokines (IL-1, TNF and IL-6), except for M2b, which expresses high levels of inflammatory cytokine production in contact with immune complexes and LPS. On their cell surface, they exhibit non-opsonic receptors such as mannose receptor. Opposite to the classical M1 macrophages, the alternative M2 macrophages use oxidative metabolism. They express a high level of fatty acid  $\beta$ -oxidation (FAO) and oxidative phosphorylation (OXPHOS) *via* the IL-4 signaling pathway. Usually, mannose receptor (MRC1 or CD206), TMG2 and the chitinase-like secretory protein YM-1 have been used to identify them. The roles associated to alternative M2 macrophages are sometimes confusing. Song and collaborators [54] demonstrated *in vitro* that these cells produced pro-fibrogenic factors such as platelet-derived growth factor BB (PDGFBB) and TGF- $\beta$ . Consequently, they induced proliferation of myofibroblasts (for example, activated HSCs), facilitated collagen production by differentiated HSCs and encouraged fibrogenesis, tissue remodeling and angiogenesis. In contrast, results from other studies seemed to associate alternative M2 macrophages to the resolution of fibrosis by phagocytizing apoptotic cells and matrix components *via* mannose and scavenger receptors [45]. In some studies, data showed that activated macrophages participated in the suppression or promotion of cancer. Two populations of macrophages distinguished by their metabolism were identified in cancer: M1 tumor-associated macrophages (TAMs) and M2 TAMs. M1 TAMs were able to suppress tumor growth, whereas M2 TAMs played a role in cancer progression. M1 TAM metabolism is especially based on iNOS, which converts arginine to NO, which is toxic for cancer cells. However, M2 TAMs express the enzyme arginase I, which metabolizes arginine to ornithine and also promotes M2 polarization and impairs the anti-tumor activity of T cells [30].

## Kupffer Cells in Physiological Condition

### Tolerogenic function of Kupffer cells

The liver is located at a strategic position that allows it to carry out its metabolic functions in lipid, carbohydrate and protein generation and in the degradation of toxic and waste products. Kupffer cells, the largest population of tissue-resident macrophages, are found in the sinusoidal lumen and display an important tolerogenic function to avoid the induction of immunity against innocuous antigens, such as gut-derived nutrients and antigens from aged or dead cells that have been cleared from the bloodstream. Along with dendritic cells (DCs)

and liver sinusoidal endothelial cells (LSECs), KCs constitute the reticulo-endothelial system, whose functions are to clear antigens and pathogen-associated molecular patterns (PAMPs) and to degrade products and toxins from sinusoidal blood. Because Kupffer cells are positioned in the liver, they have the ability to encounter T cells, NK (Natural Killer) cells and NKT (Natural Killer T) cells [55,56]. Under normal conditions, KCs express low levels of MHC (Major Histocompatibility Complex) class II and co-stimulatory molecules and can inhibit DC-induced antigen-specific T cell activation *via* the production of prostaglandin E (PGE) and 15-deoxy-delta12, 14-PGJ2 [57]. Moreover, KCs can induce the suppressive activity of Treg cells by interacting with the Treg cells and stimulating the production of IL-10, which is crucial for the induction of tolerance to hepatocyte-expressed antigens [58]. To accomplish their tolerogenic function, KCs have the ability to express and secrete IL-10 [59] and TGF- $\beta$  [60] and then suppress T cells. This tolerogenic property of KCs is essential to prevent undesired immune responses under the physiological conditions.

### **Kupffer cells and clearance function**

The complement system exhibits an important function in the clearance of pathogens, immune complexes and apoptotic cells present in the bloodstream. The complement component C3 is an important protein complex involved in complement activation; C3 is able to bind to bacterial surfaces *via* a thioester bond after cleavage to C3b, a subunit of active C3 convertase. Active C3 products present on the surface of pathogens are then recognized by complement receptors expressed on phagocytic cells [61]. Complement C3 contains four fragment receptors: CR1, CR2, CR3 and CR4. In 2005, Helmy and co-workers identified a new receptor, CRig, belonging to complement C3, the majority of which is expressed on the KC surface [62]. CRig binds to C3b and iC3b, cleavage products of C3, and induces the opsonisation of pathogens by KCs.

Other complement receptors are found on the KC surface, such as anaphylatoxin C3a receptor, C5a receptor [63,64] and complement receptors 1 and 4 [65]. These properties give KCs a key role in the clearance of pathogens and dead or dying erythrocytes from the blood circulation by their phagocytosis activity [14]. Moreover, KCs express the enzyme heme oxygenase 1 (HO-1) in their endoplasmic reticulum and peri-nuclear envelope. This enzyme functions in oxidative degradation of the heme molecules contained inside senescent erythrocytes and controls heme metabolism by generating carbon monoxide and bile pigments.

### **Kupffer cells and hepatocyte metabolism**

Chawla's group [66] identified peroxisome proliferator activated receptor  $\delta$  and  $\gamma$  (PPAR $\delta$  and PPAR $\gamma$ ) expression on the cell membrane of KCs. Activation of PPAR $\gamma$  signaling regulates metabolic programs in M2-like activated KCs, and that induces the expression of arginase I, an essential enzyme that activates KCs into alternative M2 macrophages. Moreover, PPAR $\delta$  is required for the full expression of the immune phenotype of activated KCs. This activation consists of the expression of recognition receptors, such as Mrc1 and Clec7a, but also of co-stimulatory molecules, such as Pcd11g2, and of suppression of macrophage-inflammatory responses. The investigators observed first that chimeric PPAR $\delta^{-/-}$ -depleted mice exhibited a reduced expression of  $\beta$ -oxidation and OXPHOS genes

in liver, suggesting that PPAR $\delta^{-/-}$  KCs could modulate hepatocyte metabolism. To confirm this hypothesis, they performed an in vitro experiment that consisted of co-culturing wild-type hepatocytes in the presence of PPAR $\delta^{-/-}$  macrophages or wild-type macrophages. They observed that hepatocytes co-cultured with PPAR $\delta^{-/-}$ -depleted macrophages exhibited an approximately 25% decrease in the rate of fatty acid oxidation compared to controls. Moreover, histological analysis revealed that mouse livers with PPAR $\delta^{-/-}$  KCs showed the presence of hepatic steatosis and an approximately 50% increase in extractable liver triglycerides. These data suggest that PPAR $\delta^{-/-}$  KCs synthesized and secreted factors that could directly modulate oxidative metabolism in parenchymal cells.

## Kupffer Cells in Pathological Conditions

### Kupffer cells and anti-bacterial defense

KCs, as macrophages, have an important function in the innate immune response in liver. Liver expresses in normal conditions low levels of mRNAs encoding TLRs and their downstream signaling pathways, such as myeloid differentiation primary response gene-88 (MyD88) [67,68]. KCs exhibit TLR2, TLR3, TLR9 and TLR4, which are responsive to LPS, the Gram-negative bacteria cell wall component [69,70]. Recognition of LPS from intestinal microbiota by TLRs present on the KC surface induces both immune activation and tolerance under specific conditions. For example, increased exposure of TLR4 to LPS and/or increased expression or sensibility of TLR4 could remove the tolerogenic phenotype of KCs. The binding of LPS on their TLR4 initiates specific signaling pathways that activate transcription factors such as nuclear factor NF- $\kappa$ B, activator protein 1 (AP-1) and IRFs. These activated transcription factors induce the transcription of specific genes involved in pro-inflammatory, anti-viral and anti-bacterial responses, and genes involved in the control of cell survival and apoptosis [71]. Data have shown that mice depleted of KCs died with a sublethal dose of *Listeria monocytogenes* [72]. The absence of KCs facilitated bacterial growth in the liver, especially in apoptotic hepatocytes and in the spleen. Moreover, Brenner's group observed that in KC-depleted mice, there was less neutrophil accumulation and infiltration into the liver. KCs seemed to participate in the recruitment of neutrophils to protect hepatocytes from bacterial infection. Activation of KCs during the bacterial infection involves the recognition between bacterial surface sugars and lectins and receptors expressed on the KC surface [10]. These ligand-receptor interactions activated the synthesis of inflammatory mediators such as IL-6, IL-12, IL-1 $\beta$ , TNF- $\alpha$  and NO. Thus, these chemokines inhibited the proliferation of microorganisms [73,74]. NO produced by KCs in hepatic injury had a double role [10]; it can protect hepatocytes *via* inhibition of caspases and apoptosis in endotoxemia or CCl<sub>4</sub>-induced damage, but in other conditions such as ischemia/ reperfusion injury, shock or galactosamine-induced liver injury, NO can increase oxidative stress or the expression of inflammatory mediators [75]. Furthermore, KCs produced cytokines such as MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1 and MCP-2 to recruit monocytes and neutrophils into the liver to control bacterial infection [76].

### Kupffer cells and non-alcoholic liver diseases

Non-alcoholic fatty liver diseases (NAFLDs) are a spectrum of disorders that include non-alcoholic fatty liver (NAFL), steatosis with inflammation, non-alcoholic steatohepatitis

(NASH) and NASH with fibrosis. 10% to 29% of patients with NASH will progress to cirrhosis within 10 years, and of these, 4% to 27% are expected to develop hepatocellular carcinomas [77]. The shift from NFL to NASH is characterized first by various metabolic syndromes and insulin resistance, which induce accumulation of free fatty acids and lipids in peripheral blood and hepatocytes, and secondly, by a series of innate immune responses that result from the stimulation of lipotoxins and LPS [78]. KCs, liver-resident macrophages, display a critical mediator in the development of NAFLD, and specifically in the second step of this disease progression [79]. Results obtained by different research groups showed that: 1) chemical depletion of KCs prevents the release of pro-inflammatory cytokines and alleviates hepatocellular damage [80] and 2) ablation of KCs protects against the development of hepatic insulin resistance in response to high-fat diets [79] and hepatic steatosis after longer feeding of high-fat diets [81]. One of the signaling pathways that appear to be involved in this disorder is LPS/TLR4, and TLR4 is widely present on the KC surface. Yang, et al [82] demonstrated that genetically obese ob/ob (leptin-deficient) mice were more sensitive to low-dose LPS compared to wild-type mice. Rivera and co-workers showed that TLR4<sup>-/-</sup> mice fed with a methionine/choline-deficient diet, a model system for NASH, exhibited less severe hepatic injury and less accumulation of intrahepatic lipids compared to TLR4<sup>+/+</sup> mice [83]. More recently, it was shown that over-expression of CD14, a co-receptor of TLR4, in KCs of mice with high-fat diet (HFD)-induced steatosis increased the hypersensitivity against low-dose LPS [84]. Together these data support the implication of TLR4 signaling pathway in NAFLD.

High-glucose and high-fat diets increase the gut permeability and trigger the accumulation of LPS. Binding of LPS to their receptors on the KC surface promotes the production and secretion of pro-inflammatory cytokines that recruit T and B lymphocytes and other leukocytes [85,86]. The aggregation of innate immune system cells in liver encourages steatohepatitis and inflammatory necrosis in hepatocytes, followed by NASH progression [87]. Activation of the TLR4 signaling pathway induces MYD88- dependent and MYD88-independent pathway responses. The first one involved the participation of interleukin receptor associative kinases (IRAKs) and tumor necrosis receptor associated factor 6 (TRAF-6) [88,89]. This MYD88-dependent pathway activates first the complexes JNK/MAPK and IKK that stimulate the synthesis and secretion of cytokines such as TNF- $\alpha$ , IL-6 and IL-10. On the other hand, LPS/TLR4 can also activate MYD88-independent pathway, which is mediated by the Toll/IL-1 receptor, and promotes the expression of IFN-1 $\beta$  and IL-2 [90]. Thus, activated KCs secrete many cytokines that encourage the infiltration of neutrophils, NK cells, NK T cells, T cells, and monocytes. Experiments conducted by Ma et al showed the presence of KCs containing a significant accumulation of intracellular toxic lipids in a NAFLD mouse model [91]. This high accumulation of lipids in KCs may affect the function of mitochondria and induced oxidative and endoplasmic reticulum (ER) stress [92]. Oxidative stress resulted from insufficient free fatty acid (FFA)  $\beta$ -oxidation and dysfunction of mitochondria and leads to the activation of the NF- $\kappa$ B/JNK pathway, high mobility group box 1 (HMGB1)/TLRs, cytokines and chemokines [91,93,94]. ER stress in KCs promotes activation of the JNK/NF- $\kappa$ B/(C/EBP) pathway, which results in insulin resistance and apoptosis.



### Kupffer cells and alcoholic liver diseases

Alcoholic liver diseases (ALD) and NAFLDs are the major liver-associated causes of morbidity and mortality in Western countries [95,96]. In the United States of America, 18 million people are affected by alcohol abuse [97], and fatty liver was observed in up to 90% of alcoholics [98]. Alcohol consumption encourages intestinal permeability and increases plasma and liver endotoxin and LPS levels [99]. The presence of a high level of LPS in the alcoholic liver activates KCs, and they release active mediators such as pro-inflammatory cytokines, eicosanoids and ROS. It has been shown that ALD affect more women than men [100], even though the underlying mechanisms remain unclear. Iimuro et al [101] observed that after exposure to ethanol, female rats exhibited higher plasma endotoxin levels than males; this could be due to the presence of estradiol in female rats, which increases intestinal permeability and endotoxin accumulation in the portal vein [102]. Indeed, KCs isolated from rats treated with estradiol and injected with a sublethal dose of LPS exhibited higher expression of TNF- $\alpha$ , and CD14 compared to control rats [103]. Activation of TLR4 signaling pathway was widely described in the previous paragraph. Here, we will focus on the function of TNF- $\alpha$  and ROS produced by KCs in ALD.

TNF- $\alpha$  was known to be strongly involved in ALD and can induce hepatocellular damage *via* the generation of superoxide anions by hepatocytes and can increase the synthesis and secretion of IL-8, which recruits neutrophils [104]. Mice deleted of TNF- $\alpha$  receptors are more resistant to ethanol-induced liver damage [105]. Activated KCs are the major source of TNF- $\alpha$  production and secretion after activation of the LPS/TLR4 signaling pathway. Stabilization of TNF- $\alpha$  mRNA in KCs seems to play a crucial role in the high TNF- $\alpha$  level in ALD. Data obtained by Saklatava et al showed that after chronic ethanol exposure, p38 mitogen-activated protein, an important regulator of TNF- $\alpha$  mRNA stability in macrophages, exhibited a higher phosphorylation level [106]. McMullen and co-workers observed that rats chronically exposed to ethanol translocated HuR (Human antigen R), a TNF- $\alpha$  mRNA-binding protein, from the nucleus to the cytoplasm and showed a higher binding capacity of HuR on TNF- $\alpha$  mRNA in KCs, which allowed stabilization of TNF- $\alpha$  mRNA [107]. Thus, stabilization of TNF- $\alpha$  mRNA in KCs could be an important mechanism for ALD progression. Oxidative stress was observed during chronic alcohol consumption, suggesting its involvement in ALD. ROS can be generated by various enzymes in liver: CYP2E1, NADH/NADPH oxidase, xanthine oxidase and arachidonic pathway enzymes such as lipoxygenase (LOX) and cyclooxygenase (COX). KCs express superoxide dismutase (SOD), which uses superoxide anion to produce hydrogen peroxide. Superoxide and hydrogen peroxide can interact and achieve more cytotoxic radicals such as hydroxyl radical [108]. Under normal conditions, hydrogen peroxide is very quickly metabolized by glutathione peroxidase to produce H<sub>2</sub>O and O<sub>2</sub>. Under alcohol abuse conditions, enhanced ROS release [109] and reduced glutathione [110] were observed in KCs, as well as lipid peroxidation and mitochondrial dysfunction [111,112].

### Kupffer cells and hepatitis

Hepatitis B virus (HBV) and Hepatitis C virus (HCV) have infected more than 500 million people worldwide. These viruses cause liver inflammation, fibrosis, cirrhosis and hepatocellular carcinoma. The modes of transmission of HBV and HCV are percutaneous

and sexual exposure, albeit perinatal exposure is often observed for HBV [113,114]. HBV is a 3.2 kb partially double-strand DNA- envelope virus that replicates *via* RNA intermediates. HBV is composed of two particles: HBcAg (Hepatitis B core protein)-encapsulated viral DNA and HBsAg (Hepatitis B surface antigen). HBsAg and a truncated form of HBcAg, HBeAg (Hepatitis B extracellular form of HBcAg), are secreted by infected hepatocytes and can be detected in HBV patient sera [115,116].

HCV contains a 9.6 kb positive-strand RNA genome that translates into the structural E1 core protein and E2 envelope protein, and the non-structural proteins NS1-NS5 [117]. HBV and HCV only infect and replicate in humans and non-human primates, and immunocompetent small animal models for viral hepatitis are not yet available [118]. Several mouse models infected with lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus (MCMV), mouse hepatitis virus (MHV) and adenovirus were used to understand the function of KCs in viral infection. However, unlike HBV and HCV, which infect only hepatocytes, these viruses infect not only hepatocytes but also other cells and organs.

Little is known about the interaction between KCs and HBV/ HCV, and how HBV/HCV can activate KC responses. Studies using THP-1 monocytic cells suggested that HBcAg could bind to TLR2 and HSPG (heparan sulfate proteoglycan) present on the THP-1 cell surface. These ligand/receptor interactions activate THP-1, which in turn produced IL-6, IL-12p40 and TNF [119]. Other studies with different systems showed that HBV can bind to receptors expressed on the KC surface, such as HSPG, CD14, and mannose receptor, and induce production and secretion of IL-1 $\beta$ , IL-6, TNF, TGF- $\beta$ , CXCL8, PD-L2, galectin-9, TRAIL, FasL, granzyme B, perforin, and ROS [120,121]. More is known concerning the interaction of HCV on hepatocyte receptors. Some of these receptors are present on the KC surface, such as HSPG, SR-B1, LDL-receptor, DC-SIGN, TLR2, TLR4, CD40, CD80 and MHC class II, and activated KCs can then express IL-1 $\beta$ , TNF, IL-10, PD-L1, galectin-9, TRAIL, granzyme B and perforin [121].

The activation of KCs and resulting secretion of pro-inflammatory cytokines positively promotes the NF- $\kappa$ B pathway to inhibit HBV replication in primary hepatocytes [122]. During HCV infection, the number of KCs increases in liver and produce TNF, which encourages the further susceptibility of hepatocytes to HCV infection [123]. On the other hand, HCV can promote production and secretion of IL-6, IL-1 $\beta$ , and IFN- $\beta$ , which may inhibit HCV replication [124–126]. Cytokines secreted by activated KCs encourage differentiation of neighboring cells, such as HSC to promote fibrogenesis, and recruit and activate other immune cells from bone marrow, which will further increase the anti-viral and inflammatory response.

### **Kupffer cells and liver fibrosis**

Liver fibrosis is caused by chronic damage of the liver and is one of the resulting syndromes of chronic HCV infection, alcohol abuse and NAFLDs. In healthy liver, HSCs are quiescent, and initiation of fibrogenesis occurs with activation and differentiation of the HSCs. These activated HSCs synthesize and secrete different types of collagens into the extracellular matrix (ECM). Some studies suggest that KCs have a significant role in initiation of

fibrogenesis whereas other studies suggest a more minimal role [127]. The group of D. Brenner [72] identified the importance of the CCR2 receptor, which is expressed on the KC surface in liver fibrosis. They performed bile duct ligation (BDL) on wild-type and CCR2<sup>-/-</sup> mice or treated the mice with 12 injections of CCl<sub>4</sub>. They observed that CCR2<sup>-/-</sup> mice presented significantly less hepatic fibrosis, reduced collagen deposition and HSC activation. At the same time, other groups showed the involvement of KCs in liver fibrosis by inducing the decreased synthesis of metalloproteinases (MMPs) and the increased production of specific tissue inhibitors of metalloproteinases (TIMPs) or non-specific metalloproteinase inhibitors [128,129]. KCs appeared to synthesize and secrete different molecules that facilitate HSC proliferation and activation, such as TGF-β1 and growth factors [130]. Moreover, KCs induced the expression of platelet-derived growth factor (PDGF) receptors on activated HSCs, thus enhancing HSC proliferation [131], and/or they synthesized and secreted TNF-α, IL-1 and MCP-1, which are mitogenic and chemoattractant molecules for HSCs [132,133]. In addition, activated HSCs initiated production of specific collagens and proteoglycans. Furthermore, Benyon and collaborators demonstrated that gelatinases produced by KCs could induce the phenotypic change of HSCs, since these enzymes could degrade collagen type IV, essential for the maintenance of normal function of quiescent HSCs, and facilitate the synthesis of collagen type I, which triggers the phenotypic change of HSCs [134]. Other data showed that activated KCs in NASH expressed chitotriosidase enzyme, which can influence HSC activation [135]. Activated and differentiated HSCs are not the only source of myofibroblasts, which are involved in liver fibrosis. Other cells come from bone marrow, and portal fibroblasts could be differentiated into myofibroblasts [136,137]. In 1994, Bucala and colleagues [138] discovered the role of bone marrow-derived fibrocytes in fibrosis. They are CD45<sup>+</sup> and express collagen-α1, α-SMA and other cytoskeletal proteins. Under normal conditions, fibrocytes exhibit both fibroblast markers (fibronectin, vimentin, collagen type I) [139] and hematopoietic markers (CD45, CD34, MHCII, CD11b, Gr1, Ly6C, and CD54) [140]. In response to liver injury, and specifically to TGF-β produced and secreted by activated KCs, these bone marrow-derived fibrocytes are recruited to liver and initiate their activation and differentiation to fibroblasts by reducing the expression of hematopoietic markers and by activating the expression of α-SMA, and collagen type I [141]. A higher number of CD68<sup>+</sup> cells was found in fibrotic livers after CCl<sub>4</sub>-induced liver damage, and these macrophages are concentrated in scars during advanced fibrosis [45]. Moreover, the position of these CD68<sup>+</sup> cells in fibrotic liver was overlaid with the expression of YM-1 protein (chitinase). During fibrotic liver resolution, the number of macrophages expressing CD68 surface marker was significantly decreased, and the expression of YM-1, a marker of M2-like macrophages, almost completely disappeared, whereas the amount of M1-like macrophages did not change. These data suggest that alternative M2 macrophages played a pro-fibrogenesis role and were resorbed as soon as the liver fibrosis resolution started.

### **Kupffer cells and liver cancer**

It was shown previously that KCs could be activated directly or indirectly by various components. The results of this activation involve production and secretion of multiple inflammatory cytokines, ROS and growth control mediators. More recently, some experiments demonstrated the implication of activated KCs in the process of hepatic

carcinogenesis. More specifically, they are involved in the enhancement of clonal expansion of preneoplastic cells leading to neoplasia. Neoplasia induction requires two processes: DNA damage and alteration in cell growth control. Genotoxic agents or non-genotoxic agents are known to modulate cell growth and cell death, with changes of gene expression, by increasing DNA replication with accumulation of DNA damage. Also, they encourage the clonal expansion of preneoplastic hepatocytes [142].

Injured liver, and specifically apoptotic hepatocytes, induces activation of KCs and production of cytokines, in particular TNF- $\alpha$  [143], interleukines and ROS, which modulate the hepatocellular growth [144]. Indeed, inactivation, but not depletion, of KCs by injecting glycine or methyl palmitate in mice, has been shown to decrease hepatocellular growth after treatment with genotoxic and non-genotoxic agents [145,146]. Klaunig's group utilized clodronate-encapsulated liposomes to delete KCs [147] in male B6C3F1 mice, and they injected LPS into control and KC-depleted mice. They observed that LPS induced increased DNA synthesis in control mice, whereas in KC-depleted mice, DNA synthesis was decreased 80% compared to control. These data demonstrated the implication of KCs in the induction of cell growth. They also wondered whether KCs might play a role in the modulation of preneoplastic lesion growth. To answer this question, control or KC-depleted B6C3F1 mice pretreated with DEN, a DNA-damaging compound that promotes preneoplastic foci, were treated with LPS. In normal mice, LPS increased the relative volume of hepatic focal lesions 4 fold compared to KC-depleted mice. Moreover, LPS induced enhanced DNA synthesis (3 fold) within focal lesions in normal mice, while DNA synthesis was decreased in KC-depleted mice [148]. To better understand the mechanisms implicating KCs in hepatocyte proliferation and tumor modulation, experiments with peroxisome proliferators, a non-genotoxic agent, were performed. Peroxisome proliferators, a class of rodent liver carcinogens, display an important role in activation of the PPAR- $\alpha$  signaling pathway [149]. Activation of PPAR- $\alpha$  is essential for liver carcinogenesis induction in animals fed with peroxisome proliferators. Peroxisome proliferators are known to increase proliferation of rodent hepatocytes both in vitro and in vivo. Interestingly, proliferation of hepatocytes induced by peroxisome proliferators in vivo is increased 8–10 fold, while it was increased only 2 fold in vitro. Moreover, purified primary hepatocytes cultured in the presence of peroxisome proliferators failed to increase the level of DNA synthesis [148]. After treatment with peroxisome proliferators, TNF- $\alpha$  mRNA and protein levels were increased in whole liver and in serum. Neutralizing antibody of TNF- $\alpha$  prevented liver cell proliferation in rats [150], and inactivation of KCs with glycine or methyl palmitate prevented enhanced TNF- $\alpha$  mRNA and protein levels, as well as cell proliferation [145] after peroxisome proliferator treatment. These data suggested that KCs are crucial to induce hepatocyte proliferation. Peroxisome proliferators act directly on KCs and induce activation of NADPH, and consequently increase superoxide anion [151].

Studies performed in our laboratory demonstrated that DEN- induced liver injury increases the number of mouse necrotic hepatocytes. Hepatocytes undergoing cell death released high-mobility group protein B1 (HMGB1) proteins, which activated KCs by binding to the triggering receptor expressed on myeloid cells 1 (TREM-1). Activation of KCs showed increased transcriptional and translational expression of TREM-1 and induced inflammatory responses that drive hepatocarcinogenesis [152]. Furthermore, activated KCs produced high

levels of chemokines, such as CCL2 and CXCL10, which are important for the regulation of inflammatory and immune cell migration, differentiation and function. Another study showed that KCs are attracted to liver tumor cells and have the ability to phagocytize them [153,154]. Moreover, activated KCs can produce NO, which is an effective weapon of the KC machinery against tumor cells. Another indirect mechanism of KC action against tumor cells is the secretion of IL-12, which recruits and induces NK cell cytotoxicity [155].

### Kupffer cells in liver metastases

KCs play an essential function in the host tumoral surveillance system. Their strategic position in liver allows to them discriminate and remove neoplastic cells that rich to liver. During metastasis, metastatic cells migrate *via* the bloodstream to colonize other organs. Liver is the main site of metastatic disease for many gastrointestinal and extra-gastrointestinal cancers, as melanoma, breast, pancreatic and renal cancer [156]. Four different stages of liver metastasis have been identified: 1) the microvascular phase, which implicates tumor cell arrest in the sinusoidal vessels, tumor cell death or extravasation; 2) the extravascular, preangiogenic phase, during which host stromal cells are recruited into avascular micrometastases; 3) the angiogenic phase, the stage which recruits endothelial cells and tumors become vascularized; and 4) the growth phase, which leads to establishment of clinical metastases [157]. Obstruction of sinusoidal vessels by tumor cells promote local release of NO and ROS by KCs and by LSECs [158]. In addition, KCs may recruit inflammatory cells, and together they may arrest metastatic cells by inhibiting their growth and eliminating them [151]. Experiments performed on rat showed that in early states of colorectal cancer liver metastasis, KCs display tumoricidal activity in cooperation with NK cells [56]. NK cells were recruited by activated KCs and, in turn, they secreted pro-inflammatory cytokines such as GM-CSF and IFN- $\gamma$ , that activate KCs, enhance KC phagocytosis capacity or sensitize tumor cells to cytotoxic effects [159]. To defend against innate immune cells, tumors cells can produce and secrete HMGB1, which triggers macrophage and monocyte apoptosis; when the HMGB1 level increases, the KC number decreases, and HMGB1 promotes liver metastases [160].

Recently, interesting studies performed by Wen and collaborators showed a dual role of Kupffer cells during colorectal cancer liver metastasis [161]. Their results showed that KC depletion by gadolinium chloride (GdCl<sub>3</sub>) before tumor induction and during the early time points (day 0, 10 and 14) of tumor induction increased tumor growth. Whereas late KC depletion (after day 14) has a completely inverse effect: it decreased tumor growth. These data suggest that KCs exhibit an anti-tumor function during the early stages of tumor progression and they display a pro-tumor effect during the later stages. To promote liver metastasis, tumor cells can initiate by distance a pre-metastatic niche formation *via* activation of KCs by exosomes secretion in the bloodstream [162]. Costa-Silva et al used a model of pancreatic ductal adenocarcinoma (PDAC) to understand how pancreatic tumor cell can colonize liver and induce metastasis. Pancreatic cancers are one of the most lethal cancers with five years survival rate of about 6% and PDAC represents 90% of cases [163]. In their study, they showed that these PDAC primary tumor cells secrete exosomes, which migrate to liver *via* the bloodstream. Mass spectrometry analysis reveals that these PDAC-derived exosomes highly contain migration inhibitory factor (MIF). Furthermore, they demonstrated

that PDAC-derived exosomes have been taken up by KCs in liver leading to KC activation. Activated KC produce MIF-dependent cytokines (TGF- $\beta$ ), which activate HSCs to produce fibronectin to create a fibrotic microenvironment leading to recruit bone marrow-derived cells, as macrophages and neutrophils. The pre-metastatic niche formation is essential for the tumor cells to establish in the liver and induce liver metastasis. Additionally, KCs produce many growth factors such as HGF, which encourages tumor cell proliferation [5], and MMPs, especially MMP-9 and MMP-14, which facilitate angiogenesis and tumor invasion, *via* ECM alterations [129].

## Summary

Kupffer cells assume various functions under physiological conditions and controversial functions in liver injury and repair. Under normal conditions, KCs are the major immune cells that are permanently present in the liver. Their strategic position gives them the key role in dead or dying erythrocyte clearance and in the fight against bacterial infections. At the same time, they interact with other hepatic cells, and parenchymal or non-parenchymal cells, to maintain their metabolism homeostasis. Under pathological conditions (such as ALD/NAFLD) chemical reagent-induced injury (such as DEN, CCl<sub>4</sub>, or acetaminophen), exposure to LPS from bacterial degradation, and/or the tumor process, necrotic hepatocytes release many signaling molecules that activate KCs. Activation of KCs results in differentiation of these cells into classical M1 or alternative M2 macrophages. Macrophage differentiation induces metabolism modifications and specialized gene expression patterns.

Activated KCs synthesize and secrete many inflammatory chemokines and cytokines in order to recruit innate immune cells to the injured site and induce their differentiation. At the same time, they are able to induce HSCs from a quiescent state to an activated state and recruit bone marrow-derived fibrocytes/fibroblasts or portal fibroblasts, although the signaling molecules and pathways involved in this process remain to be elucidated. Activation of HSCs, bone marrow-derived fibroblast and portal fibroblasts to myofibroblasts allows the production of different collagens and initiation of liver fibrogenesis.

Some studies reported that Kupffer cells play a role in tumor cell phagocytosis in hepatic carcinomas. However, they also produce, at the same time, some cytokines and chemokines that promote hepatocyte proliferation (Figure 1). Still, functions of KCs during fibrogenesis and its resolution, as well as its role in hepatic carcinogenesis remain elusive. Some studies reported that classical M1 macrophages are anti-inflammatory, whereas alternative M2 macrophages are pro-inflammatory and pro-fibrogenic. However, little is known about the signaling pathways and molecules required for KC differentiation and about the mechanisms that modulate and regulate these processes. As we know, activation of KCs involves PPAR- $\gamma$  signaling pathway, and inhibition of this pathway may modulate KC functions. Thalidomide ( $\alpha$ -N- phthalimidoglutarimide) and pioglitazone are ligands for PPAR- $\gamma$ . Thalidomide was recognized to suppress TNF- $\alpha$  production by macrophages and other cell types, such as activated T cells [164]. Furthermore, thalidomide prevents the LPS-induced increase in CD14 expression [165]. Unfortunately, thalidomide also presents teratogenic effects. The possibility to synthesize thalidomide analogs lacking these teratogenic effects could be a next step to modulate KC functions in pathological conditions. Further analyses

of the regulatory mechanisms in KC differentiation and function should allow the development of a new range of therapeutics.

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## Abbreviations

<b>ALD</b>	alcoholic liver diseases
<b>AP-1</b>	activator protein 1
<b>ATP</b>	adenosine triphosphate
<b>BDL</b>	bile duct ligation
<b>CCl<sub>4</sub></b>	carbon tetrachloride
<b>CH25H</b>	cholesterol 25-hydroxylase
<b>CISH</b>	cytokine inducible SH2-containing protein
<b>COX</b>	cyclooxygenase
<b>DCs</b>	dendritic Cells
<b>DEN</b>	diethylnitrosamine
<b>ECM</b>	extracellular matrix
<b>FAO</b>	fatty acid $\beta$ -oxidation
<b>G-CSF</b>	granulocyte colony-stimulating factor
<b>HBV</b>	hepatitis B virus
<b>HCV</b>	hepatitis C virus
<b>HMGB1</b>	high mobility group box 1
<b>HO-1</b>	heme oxygenase
<b>HSCs</b>	hepatic stellate cells
<b>INF-<math>\gamma</math></b>	interferon gamma
<b>IL</b>	interleukin
<b>iNOS</b>	induced nitric oxide synthase
<b>IRF</b>	interferon regulatory factor
<b>KCs</b>	Kupffer Cells
<b>KLF</b>	Krüppel-like factor
<b>LCMV</b>	lymphocytic choriomeningitis virus
<b>LOX</b>	lipoxygenase

<b>LPS</b>	lipopolysaccharide
<b>LSECs</b>	liver sinusoidal endothelial cells
<b>MCMV</b>	murine cytomegalovirus
<b>MHV</b>	mouse hepatitis virus
<b>MHC</b>	major histocompatibility complex
<b>MMPs</b>	matrix metalloproteinases
<b>MRC</b>	mannose receptor C
<b>MyD88</b>	myeloid differentiation primary response gene-88
<b>NAFLD</b>	non-alcoholic fatty liver diseases
<b>NAFL</b>	non-alcoholic fatty liver
<b>NASH</b>	non-alcoholic steatohepatitis
<b>NF-<math>\kappa</math>B</b>	nuclear factor kappa B
<b>NK</b>	natural killer
<b>NKT</b>	natural killer T cells
<b>NO</b>	nitric oxide
<b>NOS</b>	nitric oxide synthase
<b>OXPHOS</b>	oxidative phosphorylation
<b>PPAR</b>	peroxisome proliferator activated receptor
<b>PFKFB</b>	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase
<b>PGE</b>	prostaglandin E
<b>PTGS1</b>	prostaglandin-endoperoxide synthase 1
<b>ROS</b>	reactive oxygen species
<b>SOCS</b>	suppressor of cytokine signaling
<b>SOD</b>	superoxide dismutase
<b>STAT</b>	signal transducers and activators of transcription
<b>TAMs</b>	tumor-associated macrophages
<b>TGF-<math>\beta</math></b>	transforming growth factor beta
<b>TGM</b>	transglutaminase
<b>TH1</b>	T helper cells type 1
<b>TH2</b>	T helper cells type 2
<b>TIMPs</b>	tissue inhibitors of metalloproteinases
<b>TLRs</b>	Toll-like receptors



<b>TNF</b>	tumor necrosis factor
<b>TREM-1</b>	triggering receptor expressed on myeloid cells 1

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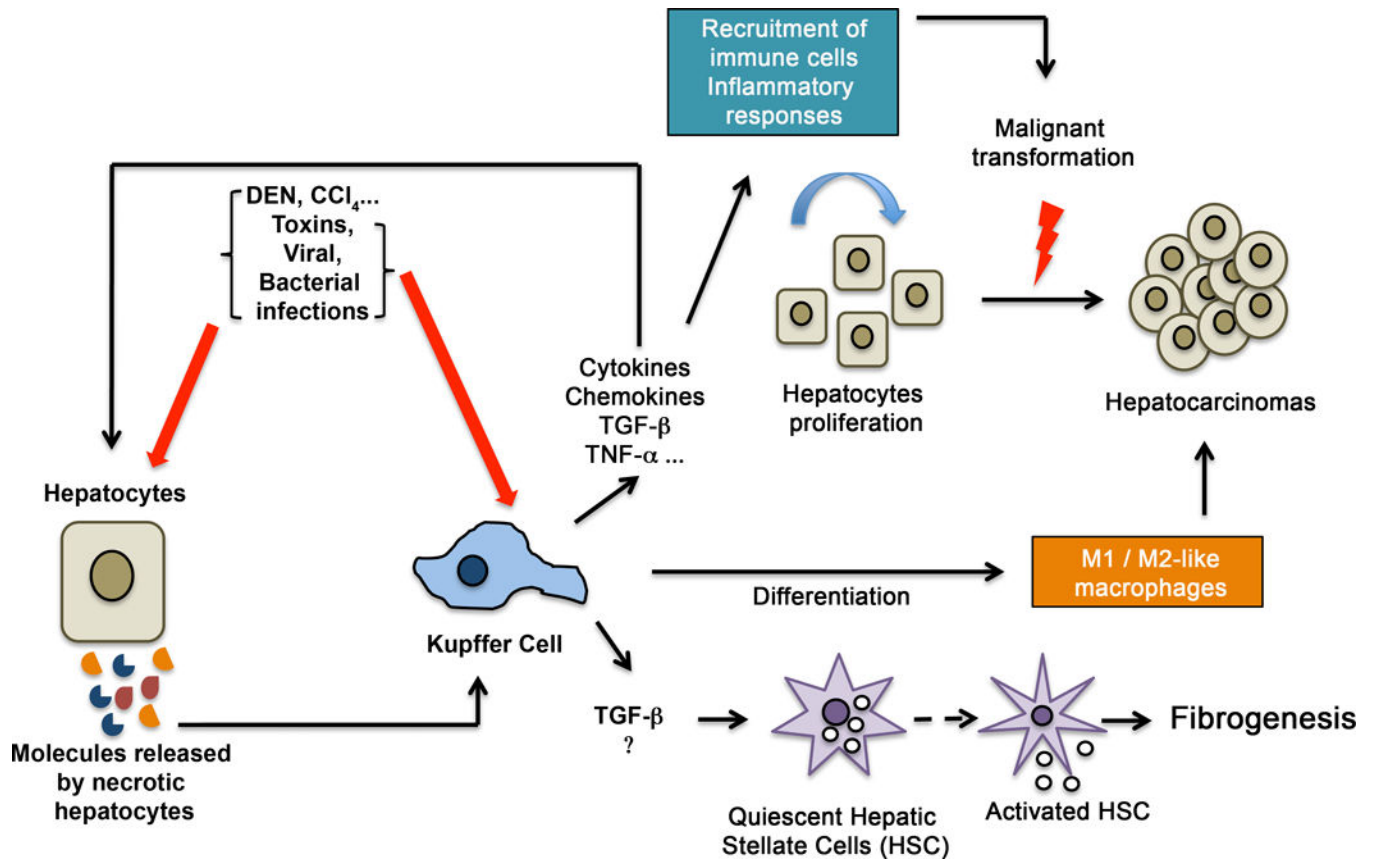
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**Figure 1. Implication of Kupffer cells in liver injury**

Toxic components, bacterial/viral infections and other injuries target hepatocytes. Necrotic hepatocytes release various molecules that activate Kupffer cells. These latter synthesize and secrete important cytokines, chemokines, TNF- $\alpha$ , and TGF- $\beta$ , allowing the recruitment of inflammatory responses into the injured liver and the activation of HSCs. The inflammatory responses induce chronic inflammation, which induces hepatocyte proliferation and transformation to generate hepatocellular carcinoma. Activation of HSCs drives the synthesis of collagens and specific proteins facilitating fibrogenesis. Activated KCs differentiate into classical M1 or alternative M2 macrophages, which participate in fibrosis or tumor progression or fibrosis resolution and elimination of tumor cells.