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Review

Kupffer cells in non-alcoholic fatty liver disease: The emerging view☆

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Non-alcoholic fatty liver disease (NAFLD) has become the most common liver disorder of our times. Simple steatosis, a seemingly innocent manifestation of NAFLD, may progress into steatohepatitis and cirrhosis, but this process is not well understood. Since NAFLD is associated with obesity and insulin resistance, mechanisms that link lipid metabolism to inflammation offer insights into the pathogenesis. An important parallel between obesity-related pathology of adipose tissue and liver pertains to the emerging role of macrophages and evidence is growing that Kupffer cells critically contribute to progression of NAFLD. Toll-like receptors, in particular TLR4, represent a major conduit for danger recognition linked to Kupffer cell activation and this process may be perturbed at multiple steps in NAFLD. Steatosis may interfere with sinusoid microcirculation and hepatocellular clearance of microbial and host-derived danger signals, enhancing responsiveness of Kupffer cells. Altered lipid homeostasis in NAFLD may unfavourably affect TLR4 receptor complex assembly and sorting, interfere with signalling flux redistribution, promote amplification loops, and impair negative regulation including alternative activation of Kupffer cells. These events are further promoted by altered adipokine secretion and reactive oxygen species production. Specific targeting of these interactions may provide more effective strategies in the treatment of NAFLD.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common liver disorder in the United States and other developed countries, affecting over one-third of the population [1]. This remarkable increase in NAFLD prevalence coincides with the obesity epidemic. NAFLD is a spectrum of disorders, beginning as simple steatosis that is mostly considered an innocent condition. Being both the source and the result of insulin resistance, however, steatosis may be associated with increased risk for cardiovascular morbidity [2]. Steatosis may also alter the natural history of other liver diseases such as chronic viral hepatitis [3]. Most importantly, in about 15% of all NAFLD cases steatosis may evolve into steatohepatitis, a medley of inflammation, hepatocellular injury, and fibrosis, often resulting in cirrhosis and even hepatocellular cancer [4]. Although this full sequence of progression is relatively rare, the overwhelming prevalence of NAFLD predicts a major healthcare burden.

NAFLD was originally defined by Ludwig and colleagues as a condition indistinguishable by histology from alcoholic steatohepatitis, although most patients carried the hallmarks of obesity and the metabolic syndrome [5]. Subsequently, the term 'NAFLD' was introduced to denote the entire spectrum of obesity-related

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Abbreviations: DAMP, damage-associated molecular pattern; LPS, lipopolysaccharide; LXR, liver X receptor; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PAMP, pathogen-associated molecular pattern; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TLR, Toll-like receptor; UCP2, uncoupling protein-2.

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fatty liver disease [6]. The pathogenesis of NAFLD is often interpreted by the 'double-hit' hypothesis [7]. Accordingly, hepatocellular lipid accumulation presents the 'first hit', followed by a 'second hit' in which pro-inflammatory mediators and reactive oxygen species (ROS) induce inflammation, hepatocellular injury, and fibrosis [8]. While this is a useful conceptual framework, our understanding of the cellular and molecular mechanisms that define NAFLD and guide therapeutic approaches remains insufficient. Liver disease is often characterized by complex interactions between resident and recruited cells that may determine the form and severity of pathologic changes and this principle certainly applies to NAFLD.

As our knowledge is expanding on the role of macrophages in danger recognition, immune tolerance, and lipid homeostasis, the significance of these cellular pathways as they pertain to liver macrophages in NAFLD is increasingly appreciated. This review summarizes evidence and considerations for the involvement of Kupffer cells in the pathogenesis of NAFLD, while the reader is referred to current literature on other emerging aspects of the disease. Since isolation, culture, and transfection of Kupffer cells is challenging, some conclusions originate from observations on other macrophage populations. Moreover, species-specific differences require caution when extrapolating experimental findings to human disease. These limitations notwithstanding, rapidly growing insights into this exciting field invite a review of the most pertinent advances.

2. Kupffer cells in health and disease: general considerations

Kupffer cells constitute the largest component of the reticuloendothelial system, representing 80–90% of all tissue macrophages in the body [9]. Central to innate immunity, Kupffer cells are responsible for swift containment and clearance of exogenous particulate and immunoreactive material that is perceived as foreign and harmful [10]. Similar to other macrophages, Kupffer cells also sense endogenous molecular signals that may result from perturbed homeostasis of the host. Kupffer cells rapidly recognize potential danger from both sources and undergo activation. Through a series of co-ordinated cellular events, activated Kupffer cells are enabled to (1) launch biochemical attack and initiate interactions with hepatocytes and other liver cells by releasing a variety of biologically active mediators including cytokines, chemokines, eicosanoids, proteolytic enzymes, ROS, and nitric oxide; (2) recruit and retain non-resident cellular players to the liver such as neutrophils, natural killer (NK) T lymphocytes, NK cells, and blood monocyte-derived macrophages by expressing adhesion molecules and secreting chemokines; (3) engulf, ingest, and eliminate solid particles, including microorganisms, apoptotic cells, and cell debris; and (4) process and present antigens to attract cytotoxic and regulatory T cells and therefore also contribute to adaptive immunity [10–15]. These functions of Kupffer cells need rigorous control to avoid escalation of the inflammatory response. Liver damage may either result from inability of Kupffer cells to properly recognize and eliminate danger molecules or from excessive mobilization of cytotoxic mechanisms and failure to halt inflammation. Accordingly, Kupffer cells are predisposed to modulate the pathogenesis of NAFLD in many ways.

3. Topologic and functional heterogeneity of Kupffer cells

Kupffer cells represent about 10% of the resting total liver cell population and are strategically located in the liver sinusoids, which provide the anatomical structure for capillary-level confluence of portal vein and hepatic artery tributaries [10,12]. Thus, Kupffer cells come in contact with a variety of molecular substances such as nutrients, microorganisms, cell debris, immune complexes, and toxic agents carried by hepatic circulation. While initially described as 'fixed tissue macrophages', Kupffer cells migrate between the sinusoids and the space of Disse [16] and orchestrate a cross-talk between various resident and recruited cells of the liver.

Specific to their position within the liver acinus, Kupffer cells differ in their population density, morphological characteristics, and physiological functions [17,18]. This distribution correlates with the acinar concentration gradient of immunoreactive substrates and regulatory factors. Large Kupffer cells are located in the periportal zone with exposure to incoming molecular signals. Accordingly, large Kupffer cells exhibit higher phagocytosis, lysosomal protease activity, and output of biologically active mediators than smaller Kupffer cells in mid-zonal and perivenous areas [17,18]. Large Kupffer cells can be identified by cell surface expression of the scavenger receptor CD163, also described as ED2 antigen in the rat [19]. By contrast, glycosylated transmembrane protein CD68 (ED1) is located in lysosomes and can be detected in all Kupffer cells regardless of their acinar location [20].

While changing abundance and distribution of Kupffer cells may reflect anomalous gain or loss of function, the importance of zonal heterogeneity remains to be seen in NAFLD. Increased presence of CD68-positive Kupffer cells correlates with the histological severity of human NAFLD [21]. Moreover, aggregates of enlarged Kupffer cells are present in perivenular regions of the liver of NASH patients as compared to diffuse distribution seen in simple steatosis [22]. Selective depletion of large Kupffer cells by administration of gadolinium

chloride ($GdCl_3$), presumably as a result of higher uptake and increased toxicity of the rare-earth metal compound in these cells [23,24], markedly attenuates liver injury induced by thioacetamide [25], carbon tetrachloride [26], alcohol [27] and ischemia/reperfusion [28], indicating that ED2-positive Kupffer cells critically contribute to liver damage in these conditions. Similarly, administration of liposome-encapsulated dichloromethylene bisphosphonate (clodronate), which eliminates 90% of large Kupffer cells and 50% of small Kupffer cells [29], reduces hepatotoxicity in response to concanavalin [30], alcohol [31], and acetaminophen [32]. In experimental NAFLD induced by methionine/choline deficient diet, clodronate effectively blunts all histological evidence of steatohepatitis [33]. These observations indicate that activation of Kupffer cells positioned at the 'frontline' is an essential element in the pathogenesis of NAFLD similar to other types of liver injury.

An increasing pool of macrophages is characteristic to many pathologic conditions of the liver including steatohepatitis [22]. Contribution of blood monocyte-derived macrophages to this pool and to the heterogeneity of Kupffer cells in steatohepatitis remains unclear since there is currently no reliable marker to distinguish resident macrophages from recruited macrophages in the liver. The finding that resident macrophages in lean adipose tissue originate from a $CCR2^{-}CX3CR1^{hi}$ monocyte pool whereas recruited adipose tissue macrophages originate from a pool of $CCR2^{+}CX3CR1^{low}$ circulating monocytes [34] may prove helpful in this effort.

Weakened or absent Kupffer cells may also associate with deleterious effects. Thus, impaired clearance of LPS and other danger molecules by Kupffer cells may result in accelerated liver injury and this mechanism needs to be considered in NAFLD. In support of this concept, imaging studies by contrast-enhanced ultrasound [35] or super-paramagnetic iron oxide (SPIO)-magnetic resonance imaging [36] suggest impaired phagocytic function of Kupffer cells in NAFLD. Moreover, depletion of Kupffer cells by $GdCl_3$ or clodronate may shift the acinar distribution of phagocytosis, alter the balance between pro- and anti-inflammatory cytokines, and interfere with liver regeneration, reflecting the functional complexity and phenotypic plasticity of Kupffer cells [37–39].

4. Disease-specific pathways of molecular pattern recognition in NAFLD

The molecular signals received by Kupffer cells such as structural motifs of proteins, lipids, and nucleic acids that originate from invading microorganisms are commonly referred to as pathogen-associated molecular patterns (PAMPs) [14,40]. Kupffer cells also detect components released from host cells that are injured, dying, or undergoing malignant transformation. These endogenous protein and non-protein ligands belong to damage-associated molecular patterns (DAMPs) and are alternatively termed alarmins [41]. Endogenous sources of DAMPs include heat shock proteins, high mobility group box 1 protein, breakdown products of the extracellular matrix (e.g., hyaluronan, fibrinogen, and fibronectin), and non-protein substrates (e.g., uric acid) [41].

Exogenous and endogenous DAMPs are identified by a large variety of pattern recognition receptors (PRPs) [14,40,41]. Toll-like receptors (TLRs) comprise a family of highly conserved PRPs that recognize bacterial, viral, and fungal components [40,42]. Of these, TLR4 has a central role in Kupffer cell activation. TLR4 responds to lipopolysaccharide (LPS) or endotoxin, the prototypical PAMP located in the outer wall of Gram-negative bacteria [40,43]. Recognition of LPS initiates assembly of the plasma membrane-tethered TLR4 signalling complex [41]. Downstream targets of TLR4 signalling are determined by selective recruitment of cytosolic sorting and signalling adaptor proteins via interactions between Toll-IL-1 receptor (TIR) domains [44–46]. Thus, TLR4 activation may engage myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor protein or MyD88 adaptor-like (TIRAP/Mal), leading to the activation of nuclear factor (NF)-κB and AP-1 transcription factors [40,41,43]. By contrast, TLR4 may signal through TIR domain-containing adaptor inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM) primarily to activate interferon regulatory factor 3 (IRF3) and promote the transcription of interferon-β [40,41,43].

There is substantial evidence that TLR4-mediated cellular events escalate liver injury in steatosis [40,43]. Recent studies indicate that TLR4 sorting specificity may reflect the etiology of fatty liver disease. Thus, the protective effect of TLR4 deficiency against alcohol-induced liver injury is replicated in IRF3−/− mice, but not in MyD88−/− mice [47,48]. Preferential TLR4 sorting to the IRF3 cascade has also been reported in mouse livers after warm hepatic ischemia/reperfusion injury [49]. Conversely, altered MyD88 signalling may associate with NAFLD. Thus, the C558T single-nucleotide polymorphism variant of TIRAP gene impairs MyD88-mediated TLR signalling and correlates with lack of liver fibrosis in a cohort of patients with biopsy-proven NAFLD, while confers no protection from alcohol-induced liver injury [50]. These preliminary observations suggest that TRAM/TRIF-dependent interferon responses are the primary target of alcohol in the liver, while altered activation of TIRAP/Myd88-dependent pathways may dominate the progression of NAFLD. Notably, proper balance between TIRAP/Myd88-mediated cytokine production and TRAM/TRIF-mediated interferon responses may be necessary

to avoid immunopathology. In support of this concept, *in silico* simulation predicts signalling flux redistribution between alternative Myd88 and TRAM activation and this concept has been validated in mouse macrophages [51]. Due to the rather ubiquitous presence of TLR4 among various types of liver cells, the specific role of Kupffer cells in differential activation of TLR4 pathways remains to be seen. It must also be noted that endogenous ligands such as certain fatty acids and other alarmins may also be linked to TLR4 sorting specificity, a question particularly relevant to NAFLD.

5. Amplification of danger signals in NAFLD

LPS is considered a pivotal exogenous danger molecule in the pathogenesis of fatty liver disease. Circulating LPS levels are elevated and the liver exhibits remarkable sensitivity to LPS in most experimental models of NAFLD [52–54]. Whereas translocation of LPS from the gut lumen to portal circulation in alcoholic liver injury may result from direct alcohol toxicity disrupting the barrier function of intestinal epithelium [55], increased exposure to intestinal LPS has also been considered in NAFLD pathogenesis. Thus, dietary factors (e.g., increased fructose ingestion) may contribute to altered intestinal motility, bacterial overgrowth, and increased epithelial permeability in both experimental and human NAFLD [54,56]. Improvement of liver disease by administering probiotics in these conditions provides indirect support to this concept [57,58].

Disturbed hepatic clearance is a major mechanism that may contribute to increased LPS levels in NAFLD, in particular when facing higher loads via the portal circulation. Scavenger receptors are transmembrane proteins located in the lipid raft domains (caveolae), which bind lipoproteins and are able to remove and detoxify foreign substances including LPS [59]. Kupffer cells express high levels of class A scavenger receptors (SR-A), which have affinity to modified (e.g., oxidized, acetylated, or glycated) low density lipoprotein (LDL), but do not bind native lipoproteins [59]. In addition, SR-A receptors are capable of LPS uptake [60]. Importantly, binding of modified LDL and LPS to SR-A may trigger an inflammatory response by Kupffer cells. Since SR-A promotes cell adhesion, it may also contribute to recruitment and retention of various cells at the site of inflammation [59]. In contrast, hepatocytes express class B scavenger receptors such as SR-B1, which binds both native and modified lipoproteins in addition to mediating LPS uptake [61]. Recent studies on SR-B1-null mice indicate that hepatocellular SR-B1 activity may considerably lower the LPS burden [62]. Others found that β2-integrin (CD11b/CD18) and TIRAP also mediate hepatocellular LPS uptake [63]. As a result, physiologic hepatocellular activity may 'mop up' LPS and thwart inflammatory signalling cascades in Kupffer cells [62]. This concept may be extended to modified LDL and other danger signals and it is reasonable to speculate that impaired clearance of DAMP molecules by fatty hepatocytes may enhance activation of Kupffer cells and contribute to the pathogenesis of NAFLD.

Insufficient control of danger recognition may also lead to increased LPS sensitivity. Since TLR4 is the primary conduit for cellular effects of LPS, stringent regulation of TLR4 signalling is essential to avoid excessive inflammatory response [64]. LPS tolerance, or hyporesponsiveness to repeated LPS exposure is a consequence of these anti-inflammatory feedback circuits [65,66]. TLR4-mediated responses are controlled at multiple levels, most proximally by inhibition of the TLR4 signalling complex via homotypic TIR–TIR interactions [44,67,68]. Tyrosine phosphatases provide yet another way of inhibitory regulation. Protein tyrosine phosphatase-1B (PTP1B), SH2-containing protein tyrosine phosphatase 1 (SHP1), and the dual specificity (tyrosine/threonine) MAPK phosphatase MKP-1 appear essential in balancing TLR-mediated responses [69–71]. Finally, suppressor of cytokine signalling protein SOCS-1 is a versatile inhibitor induced by elevated cytokine levels and targeting multiple steps of Myd88-dependent pathways [72]. SOCS-1 may directly interact with phosphorylated TIRAP/Mal and initiate proteasomal degradation [73]. How these mechanisms specifically pertain to the function of Kupffer cells need further elucidation.

6. Kupffer cell functions in altered lipid homeostasis

Hepatocellular accumulation of lipids is a key morphologic feature of NAFLD. Lipidomic analysis of human liver tissue is a promising novel approach to associate abnormal fat composition with various stages of NAFLD. Thus, total and damaged phospholipids are more abundant in simple steatosis at the expense of triglycerides [74], while increased ratio of stearic to arachidonic acid in NASH may correlate with fibrosis [75]. Altered abundance and composition of liver tissue lipids may modulate the biological activity of Kupffer cells in NAFLD through a number of mechanisms. First, the space-occupying effect of fat-laden hepatocytes may lead to impaired sinusoidal perfusion [76]. Leukocytes trapped in narrowed sinusoids may increasingly engage Kupffer cells in the microvascular inflammatory response [76]. Second, excessive exposure of Kupffer cells to fatty acids may modulate pathways of inflammation and insulin resistance through interaction with cell surface receptors and intracellular mediators [77]. Third, anomalous deposition of lipids in the plasma membrane may alter the structure of lipid raft domains and interfere with clustering and function of cell surface receptors

[78]. Altered lipid composition may also affect proper functioning of intracellular membranes as seen with free cholesterol loading of mitochondria [79]. Finally, abundant or abnormal lipids may confound recognition of fatty hepatocytes as dangerous and promote adverse interactions with Kupffer cells [15]. Nevertheless, existence of a lipid-derived quintessential alarmin expressed or released by steatotic hepatocytes remains speculative.

Recent findings indicate that TLR-mediated recognition of fatty acid moieties is an important mechanism by which lipids regulate pathways of inflammation and innate immunity [78]. Depending on fatty acid composition, the outcome of this effect may be highly variable. Saturated fatty acids, implicated in the development of chronic conditions such as atherosclerosis, have been shown to activate TLR4 signalling in adipocytes and macrophages through both Myd88-dependent and TRIF-dependent pathways [80,81]. By contrast, polyunsaturated fatty acids inhibit these events in several cell types including macrophages [81]. Consequently, TLR4 is a sensor of endogenous fatty acid levels and composition, and Kupffer cells most likely benefit from this ability.

Emerging evidence indicates that altered cholesterol metabolism may also contribute to the pathogenesis of NAFLD. Rats fed choline-deficient (but methionine-sufficient) diet supplemented with high amounts (2%) of cholesterol develop impaired mitochondrial function, characterized by accumulation of free cholesterol, glutathione depletion, and increased susceptibility to TNF-α and Fas-mediated liver injury [79]. Moreover, cholesterol metabolism may directly affect the function of Kupffer cells. Thus, high-fat diet fed to LDL receptor-deficient mice rapidly results in significant hepatic inflammation, but only if the diet contains cholesterol [82]. Presence of 'foamy' Kupffer cells suggests that scavenging of modified lipoproteins may induce this early inflammatory response [82]. While these findings need to be extrapolated to human NAFLD with caution, they point to the importance of altered cholesterol metabolism. In addition, some of these observations challenge the 'second-hit' concept since steatosis is not necessarily a forerunner of hepatic inflammation as these events may develop simultaneously [82,83].

7. Alternative macrophage activation in NAFLD

Alternatively activated macrophages (also termed M2 as opposed to the classical M1 or pro-inflammatory phenotype) represent another critical pathway for resolution of the inflammatory response [84]. The coordinated program of alternative activation is primarily stimulated by Th2 cytokines IL-4 and IL-13, and characterized by cell surface expression of M2 signature genes such as the mannose receptor, arginase-1, and dectin-1 [84]. There is evidence that steatosis promotes Th1 polarization of the cytokine balance favouring innate or classic activation of macrophages in NAFLD. Thus, in experimental and human NAFLD alike, the pool of hepatic NKT cells is reduced and liver tissue level of Th1 cytokines, such as TNF-α, IL-12, IL-18, and interferon-γ, is elevated [85–88].

Peroxisome proliferator-activated receptors PPARα, PPARγ, and PPARδ and liver X receptors LXR-α and LXR-β are members of the nuclear hormone receptor superfamily of transcription factors that coordinate complex genetic programs of metabolism [89,90]. Therapeutic use of synthetic ligands to target these receptors and exploit their biological functions is increasing. Beneficial effects of PPARγ in hepatocellular lipid homeostasis have prompted large clinical trials to assess impact on NAFLD and these efforts have been recently reviewed elsewhere [91]. However, the recognition that nuclear hormone receptors link lipid metabolism to alternative activation of macrophages adds a new dimension to their potential use in the treatment of NAFLD [84,92].

While PPARγ promotes alternative activation of macrophages that contribute to valuable metabolic changes such as improved insulin sensitivity [93,94], recent research indicates that PPARδ is specifically required for a similar program in Kupffer cells [95,96]. Thus, signature gene expression of PPARδ-deficient Kupffer cells is greatly reduced in livers of obese mice and in response to IL-4 stimulation [95,96]. Moreover, PPARδ ablation results in severe steatosis and insulin resistance [95,96]. Notably, the effect of PPARδ in Kupffer cells is modulated by fatty acids [95] and may fail due to altered lipid homeostasis and hepatic microenvironment in NAFLD. Thus, hepatocytes as a previously unsuspected source of Th2 cytokines stimulate M2 gene expression in Kupffer cells and this important regulatory circuit may be altered in steatosis [96]. These findings raise the intriguing possibility that specific targeting of PPARδ in Kupffer cells to induce alternative activation may improve both inflammation and steatosis in NAFLD. One important caveat is that the M2 phenotype includes stimulation of the extracellular matrix that may contribute to hepatic fibrosis [97].

8. Adipokines and the liver inflammatory response

Adipose tissue produces a large variety of humoral factors, collectively termed adipokines, which include pro-inflammatory cytokines, e.g., TNF-α and IL-6, and polypeptide hormones, e.g., leptin, resistin, visfatin, and adiponectin [98,99]. These substances have important regulatory roles in cellular and biochemical events that define the pathogenesis of obesity and

associated chronic conditions, including NAFLD [100]. Leptin, the archetypal adipokine, primarily acts by suppressing food intake and promoting energy expenditure [101]. In addition, leptin has marked effects on the innate immune response by promoting activation and phagocytosis of macrophages, presumably through JAK/STAT signalling [102]. Pro-inflammatory and pro-fibrogenic effects of leptin have also been observed in Kupffer cells and stellate cells [98,103,104]. Accordingly, hyperleptinemia associated with obesity may contribute to progression of NAFLD, although this issue remains controversial [105]. Recent studies indicate that resistin may cause lipid accumulation in macrophages by up-regulating the SR-A scavenger receptor [106]. Visfatin, the characteristic adipokine of mesenteric adipose tissue, also has pro-inflammatory properties by inducing TNF-α and IL-6 in monocytes [107]. Further studies are needed to fully understand the effect of these newer adipokines in Kupffer cells.

While many adipokines are associated with adverse biological functions, adiponectin, the most abundant adipose-derived hormone, seems to have a protective effect in NAFLD. Full-length adiponectin (Acrp30) and its cleavage derivative, globular adiponectin (gAcrp), have been credited with anti-diabetic, anti-inflammatory, and anti-atherogenic properties [108]. Accordingly, adiponectin and its receptors, the ubiquitous AdipoR1 and the predominantly hepatocellular AdipoR2, are expressed at reduced levels in patients with obesity, insulin resistance, type-2 diabetes, and NAFLD [109,110]. Moreover, serum levels of adiponectin fall further in NASH compared to uncomplicated steatosis [111], suggesting that adiponectin may prevent 'second-hit' events in the pathogenesis of NAFLD.

Adiponectin stimulates hepatic fatty acid oxidation and ketogenesis, while it inhibits cholesterol and triglyceride synthesis [108]. Whereas these metabolic activities primarily occur in hepatocytes, adiponectin has potent anti-inflammatory effects in macrophages. Thus, adiponectin is able to suppress the effects of LPS in macrophages, including activation of NF-κB and ERK1/2 [112–114]. Similarly, adiponectin prevents LPS-mediated inflammatory signalling in Kupffer cells [115]. These anti-inflammatory effects of adiponectin may involve IL-10 signalling pathways [116]. Interestingly, NADPH oxidase is a major IL-10 target in various cell systems including macrophages [117]. Moreover, adiponectin controls hepatic ROS levels through inhibition of NADPH oxidase in experimental alcohol-induced liver injury (Laura E. Nagy, personal communication). These findings suggest that the beneficial effects of adiponectin in NAFLD may also occur through controlling intracellular ROS production and activation of Kupffer cells.

9. ROS biology of Kupffer cells

Cellular reactive oxygen species (ROS) are byproducts of normal aerobic metabolism [118,119]. ROS may cause macromolecular toxicity, necessitating an elaborate antioxidant defense system. The toxic effects of ROS, however, also protect the host from invading microorganisms as observed in the cellular events of innate immunity [120,121]. Moreover, ROS regulate signalling cascades in a large variety of physiologic cellular responses including pathways of danger recognition [122]. This dual biological function of ROS has been termed antagonistic pleiotropy [123]. Accordingly, ROS contribute to the function of Kupffer cells and other macrophages at multiple levels.

A pivotal source of ROS in inflammatory cells is the NADPH oxidase or NOX [124]. Rapid release of ROS in response to LPS and other microbial stimuli in Kupffer cells and other macrophages occurs through the specialized phagocyte oxidase gp91phox or NOX2 [121]. Activated NOX2 produces superoxide, a major form of ROS that assists microbial killing and signals to redox-sensitive targets such as thioredoxin, protein kinase C, ERK family members, and NF-κB [120,125]. Oxidative injury in livers of NOX2-deficient mice treated with alcohol [126] or with the genotoxic carcinogen diethylnitrosamine [127] is greatly reduced, indicating that NOX2 is essential to the pathogenesis. By contrast, acetaminophen-induced hepatotoxicity still occurs in gp91phox−/− mice along with increased levels of mitochondrial oxidized glutathione to the same extent as in wild type mice, suggesting that NOX2 is not the source of ROS in this setting [128]. Similarly, NOX2 seems irrelevant in methionine/choline-deficient diet-induced experimental NAFLD, since NOX2 deficiency has no effect on liver tissue lipid peroxidation, steatosis, and fibrosis [129].

ROS are also produced at other intracellular sites (e.g., mitochondria, peroxisomes, microsomal cytochrome P450 system) that may affect redox-sensitive effector pathways in Kupffer cells. Mitochondria are the largest source of metabolically derived ROS [130,131]. Substrate oxidation by respiring mitochondria generates a proton gradient that maintains the electrochemical potential ($\Delta\psi_m$) across the mitochondrial inner membrane [132]. The energy of $\Delta\psi_m$ can be either used for ATP synthesis (oxidative phosphorylation) or dissipated as heat via proton leak in a process termed uncoupling [132]. The respiratory chain also produces superoxide due to electron spin-off and incomplete reduction of molecular oxygen, which is more likely to occur at higher $\Delta\psi_m$ [130,133]. Thus, regulation of $\Delta\psi_m$ may control mitochondrial ROS, a function recently associated with uncoupling proteins [134,135]. Of all uncoupling proteins, UCP2 has the broadest tissue distribution with abundance in cells of the immune

system [134,136]. As further discussed below, UCP2 overexpression suppresses ROS production and the activation of Kupffer cells and other macrophages [137–139], while UCP2 inhibition or ablation results in increased ROS, release of pro-inflammatory cytokines, and persistent activation of NF-κB [140–142].

Little is known about the interaction of mitochondrial and non-mitochondrial ROS-generating systems, although these functions may overlap. Thus, antigen-presenting ability of Kupffer cells is impaired to similar degree when ROS production is inhibited at different intracellular sites such as NADPH oxidase, mitochondria, or cytosolic xanthine oxidase [143]. Furthermore, anti-microbial and pro-inflammatory activity is greatly augmented in ucp2−/− macrophages as a result of uncontrolled mitochondrial ROS production [141]. Plausibly, ROS from any cellular source may similarly affect antioxidant defense and redox-sensitive signalling pathways in Kupffer cells. Notably, Kupffer cells have lower antioxidant capacity than hepatocytes, assuming therefore a higher impact of ROS-mediated regulatory mechanisms [144].

10. Uncoupling protein-2 and activation of Kupffer cells

UCP2 has been considered in the pathogenesis of NAFLD since its identification [145]. Although hepatic UCP2 primarily resides in Kupffer cells and its presence in hepatocytes is negligible under normal conditions [146], UCP2 becomes markedly abundant in hepatocytes of genetically obese (*ob*/*ob*) mice and following high-fat diet [147,148], while Kupffer cells and other macrophages have diminished UCP2 in these conditions [148,149]. The clinical significance of cell-specific alterations of UCP2 expression in experimental NAFLD is not entirely understood. Since large amounts of UCP2 interfere with ATP synthesis [147], fatty hepatocytes with up-regulated UCP2 may have an energetic disadvantage as demonstrated during acute challenges by Fas-mediated hepatotoxicity [149] and ischemia/reperfusion injury [150,151]. By contrast, down-regulation of UCP2 may enhance the responsiveness of Kupffer cells in fatty liver, consistent with increased activity of macrophages in which UCP2 is inhibited or ablated [139–141].

LPS is a powerful inhibitor of UCP2 expression in macrophages [139,141,148], suggesting that TLR4-mediated signalling may utilize mitochondrial ROS in amplifying circuits. Indeed, a positive feedback loop has recently been identified in LPS-mediated TLR4 signalling that involves augmented activation of JNK and p38 by mitochondrial ROS in peritoneal macrophages of ucp2−/− mice [152]. These findings indicate that UCP2 may act as a physiological break to ROS-sensitive components of TLR4 signalling such as JNK, p38, and NF-κB. Increased susceptibility to even small amounts of LPS has been considered in the pathogenesis of NAFLD [85,153] and ROS-mediated amplification of TLR4 signalling may contribute to this phenomenon as a result of insufficient UCP2 action in Kupffer cells.

Recently, Zhou and co-workers suggested an interesting mechanism that may further clarify the role of UCP2 in NAFLD [154]. These authors describe profound structural abnormalities and impaired respiratory activity of liver mitochondria in adiponectin-deficient mice with pre-existing steatosis [154]. Protection from LPS-induced hepatocellular injury by adenovirus-mediated replenishment of adiponectin is abolished if these mice are also made UCP2-deficient, indicating that in this model UCP2 is critical to the beneficial effects of adiponectin. In the absence of liver cell-specific studies, however, it remains unclear if UCP2 deficiency primarily prevents adiponectin from regulating hepatocellular lipid metabolism or interferes with anti-inflammatory effects of adiponectin in Kupffer cells. Nonetheless, evidence is mounting that dysregulation of UCP2 alters the balance of pro- and anti-inflammatory mechanisms and NAFLD may benefit from restoration of mitochondrial ROS control in Kupffer cells.

Based on above considerations, use of antioxidants to prevent and treat advanced NAFLD appears warranted. Interestingly, however, many trials have failed to show significant benefits from antioxidant therapy in NAFLD as most recently reviewed by Younossi [91]. Mitochondrially targeted antioxidants may represent a novel strategy for limiting ROS-mediated pathology in NAFLD. Thus, mitoQ, a synthetic analog of coenzyme Q10 (ubiquinol/ubiquinone), selectively accumulates in the mitochondrial matrix and eliminates ROS by continual redox cycling [155], while SS-31 is a cell-permeable aromatic-cationic peptide targeted to the inner mitochondrial membrane where it acts as a potent local antioxidant [156,157]. These compounds have proved helpful in early trials for neurodegenerative disorders associated with mitochondrial dysfunction [158]. In addition, mitoQ protects against organ damage in a LPS-peptidoglycan model of sepsis [159]. It will be interesting to see the impact of this approach on controlling ROS-dependent Kupffer cell responses in NAFLD.

11. Concluding remarks

One of the unmet challenges of NAFLD is to satisfactorily predict its progression from simple steatosis into steatohepatitis. This transition represents a milestone in the natural history with a considerable probability for developing end-stage liver disease. Elucidation of molecular and cellular events that may lead to this outcome is therefore critically important. Fortunately, the past few years have brought remark-

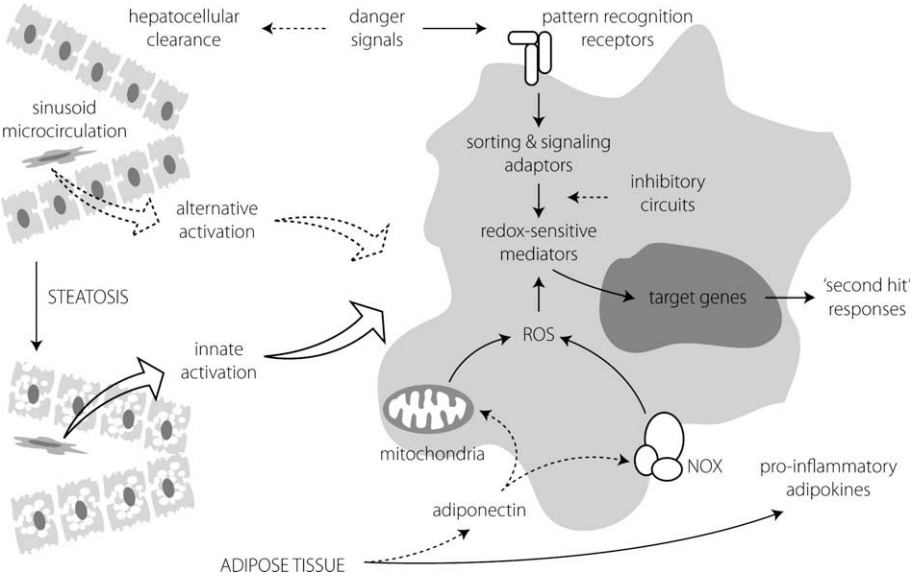

Fig. 1. Scheme for dysfunctional activation of Kupffer cells in NAFLD. Pattern recognition receptors of Kupffer cells such as TLR4 may be increasingly exposed to exogenous and endogenous danger signals (e.g., LPS, excess fatty acids, modified lipoproteins) via the portal circulation, enhanced by lack of hepatocellular clearance. Pattern recognition pathways may intensify due to altered sorting and signalling, impaired inhibitory circuits, or amplification of redox-sensitive signalling loops. Adipokine imbalance may contribute to these events including low adiponectin levels that fail to suppress intracellular ROS generation. Fat-laden hepatocytes may compromise sinusoid microcirculation leading to entrapment of inflammatory cells. Finally, steatosis may shift away Kupffer cells from alternative activation. Please see details in the text. Solid lines, pro-inflammatory effects; dotted lines, anti-inflammatory mechanisms. Malfunction at one or more steps may promote 'second hit' responses, while cellular targeting of these checkpoints has the potential for identifying novel treatment strategies in NAFLD.

able advances in our understanding of NAFLD pathogenesis, often by extension of research in adipose tissue biology, obesity, and insulin resistance. These efforts point to the intricate relationship of innate immune system and lipid homeostasis in NAFLD with a prominent role for Kupffer cells and a number of biochemical and cellular mechanisms involved (Fig. 1). The mist continues to clear and it is now time to take advantage of what we already know and develop new ways of predicting, preventing, and treating advanced NAFLD.

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