MACROPHAGES IN LIVER DISEASE

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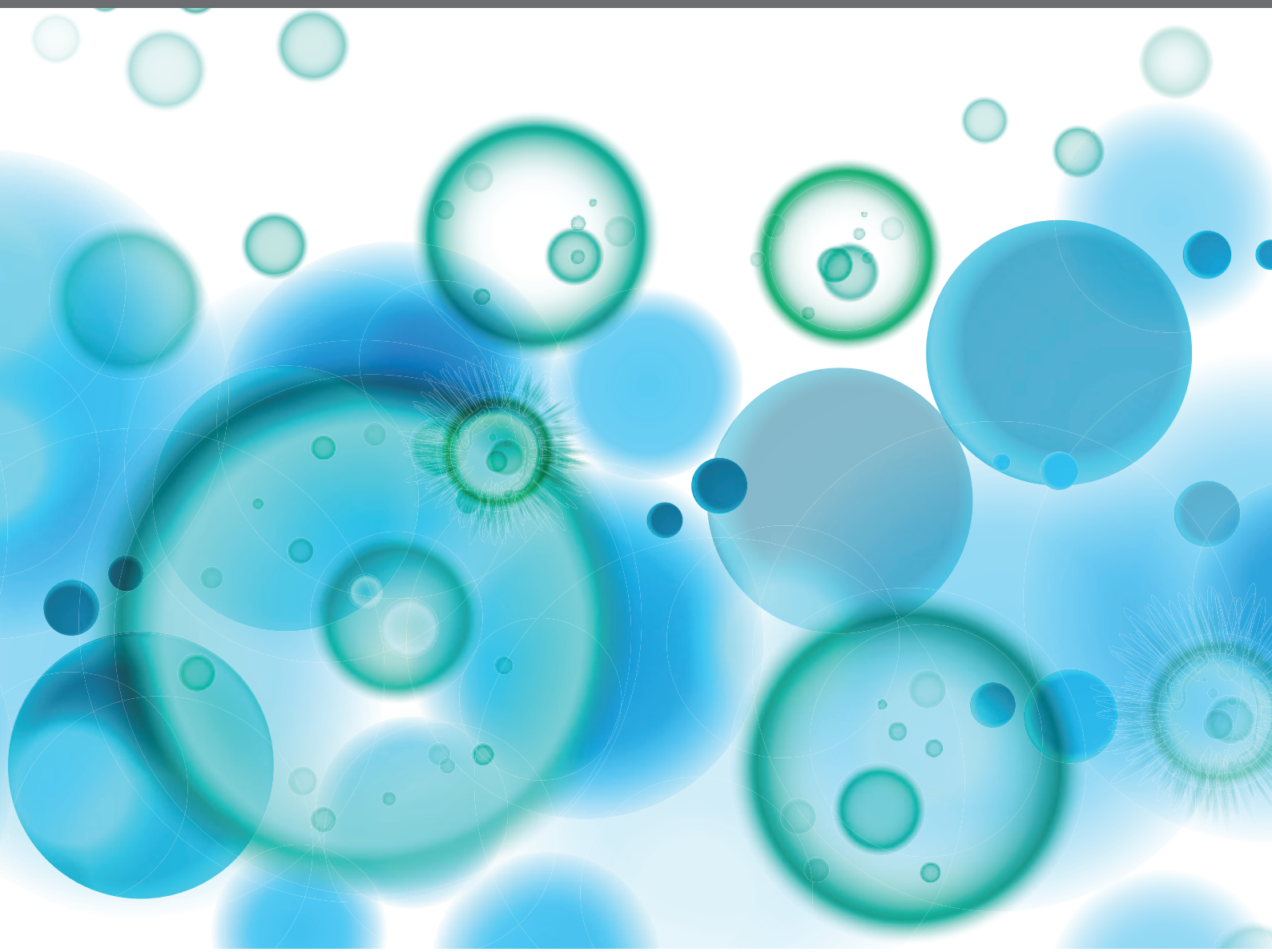

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MACROPHAGES IN LIVER DISEASE

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Table of Contents

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EDITORIAL
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Editorial: Macrophages in Liver Disease

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Editorial on the Research Topic

Macrophages in Liver Disease

Macrophages constitute a key component of our immune system and play an important role in immune surveillance. Hepatic macrophages are a heterogeneous population of immune cells that mainly comprises of embryonically-derived resident Kupffer cells (KCs), and circulating monocyte-derived macrophages (MoMFs). They play a critical role in disease initiation and progression as well as contribute to disease resolution. Traditionally, macrophages were defined by two broad subsets: classically-activated pro-inflammatory M1 or alternatively-activated anti-inflammatory M2 macrophages. However, it has been recognized that macrophages can differentiate into multiple phenotypes with distinct functions based on the tissue microenvironment.

A review by van der Heide et al. summarizes the current understanding of the hepatic macrophages, their diverse origins and phenotypes, and their role in maintaining homeostasis, progression, as well as in the resolution of liver diseases. Furthermore, the review provides a comprehensive overview of the therapeutic targeting strategies against hepatic macrophages developed for the treatment of liver diseases. Blériot and Ginhoux further elaborated on understanding the heterogeneity of hepatic macrophages. The review briefly discuss the intrinsic and extrinsic factors including metabolic zonation and how they impact cellular phenotypes, functions, and liver physiology. It further provides insights into the recent advances of single-cell transcriptomic approaches, and how they contribute to decipher the liver macrophage heterogeneity and biology.

Liver injury triggers the recruitment of extrahepatic monocytes that replenish the pool of hepatic macrophages upon resident KCs depletion or damage. This heterogeneous population of macrophages are involved in the pathogenesis of alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), hepatitis B virus/hepatitis C virus (HBV/HCV), and hepatocellular carcinoma (HCC). Dou et al. summarize current knowledge about the role of tissue-resident and recruited macrophages in the pathogenesis of different etiological liver diseases. The review further describes the existence of multiple macrophage origins and phenotypes, their identification markers and roles in disease pathogenesis, and how this knowledge can be translated into future therapies. Oates et al. highlight the landscape of mechanisms underlying macrophage dynamics, macrophage interplay with other cells/tissues, and immunometabolism that collectively contribute to NAFLD progression. Since macrophage-driven inflammation is intricately linked to various metabolic pathways, the potential benefits to be gained from understanding the interplay between metabolic and inflammatory pathways in macrophages are immense.

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In an original research article, Schierwagen et al. investigated the involvement of macrophage-inducible C-type lectin (mincle), expressed on macrophages, in different stages of chronic liver disease (CLD). The authors showed increased mincle expression that correlated with disease severity as examined in rodent models of cirrhosis, non-alcoholic steatohepatitis (NASH) and acute-on-chronic liver failure (ACLF), and in patients with NASH and cirrhosis, and that undergoing bariatric surgery. They further showed that mincle activation using mincle agonist, trehalose-6,6-dibehenate, significantly increased collagen production in ApoE-deficient high-fat Western diet-induced NASH mouse model and further confirmed in cirrhosis and ACLF animal models. These findings suggested that mincle expressed on macrophages contribute to inflammation and fibrosis, when the intestinal barrier becomes leaky, during advanced stages of CLD. Macrophages (and innate immunity) also play an important role in the pathogenesis of biliary atresia (BA), a devastating cholangiopathy of infancy progressing to end-stage liver disease often requiring liver transplantation. A review by Ortiz-Perez et al. provides a comprehensive overview of BA immunopathogenesis and the intricate mechanisms involved in the disease pathogenesis. The authors further highlighted the challenges such as lack of suitable experimental models that hinder the deeper understanding of the disease, etiology, and development of new therapies.

Hepatic macrophages interact with multiple cell types in the liver including hepatocytes, cholangiocytes, hepatic stellate cells (HSCs), liver sinusoidal endothelial cells (LSECs), platelets, and other immune cells. A review by Shan and Ju describes the impact of tissue microenvironmental factors that determine the phenotype and function of hepatic macrophages, and the crosstalk between hepatic macrophages and other hepatic cells, in regulating the extents of liver injury, repair and disease progression. For instance, macrophages secrete factors, can physically interact with vasculature to assist the formation of complex vascular networks, and activate HSCs and LSECs, and promote fibrogenesis and angiogenesis, respectively. Conversely, HSCs and LSECs (and other hepatic cells) secrete chemotactic factors increasing intra-hepatic macrophage infiltration. Ramirez-Pedraza and Fernández reviewed the molecular and cellular crosstalk between macrophages and angiogenesis, and provides detailed insights into the contribution of macrophages to liver steatosis, fibrosis, cirrhosis, HCC, and extrahepatic complications. Targeting angiogenesis-inflammation axis therefore can be an interesting approach for the treatment of liver diseases.

Macrophages also play a central role in keeping the balance of immunity and tolerance. During early HCC development, fibro-inflammation driven by pro-inflammatory macrophages (and HSCs) provide a microenvironment permissive for tumor initiation, while at advanced HCC, macrophage switching to immunosuppressive tumor-associated macrophages (TAMs) support tumor progression and malignancy. The crosstalk between tumor cells (the "seeds") and microenvironment (the "soil") play an important role in tumor development and metastasis. A review by Sällberg and Pasetto focuses on macrophages and other key cellular components of the liver and tumor microenvironment, their role in controlling the balance between tolerance and activation, and the potential therapeutic interventions to tilt the balance against liver cancer progression. Hepatic macrophages, particularly KCs, are essential for maintaining homeostasis by scavenging bacteria and cellular debris, and thereby induce immunological tolerance. An inefficient efferocytosis mechanism impairing the clearance of cellular debris results in subsequent loss of tissue homeostasis. Horst et al. describe how efferocytosis in hepatic macrophages regulate tissue homeostasis and regeneration in liver diseases. It is essential to understand the tempo-spatial contribution of macrophages and efferocytosis mechanisms in different etiological liver diseases, for the development of potential interventions against liver diseases.

Besides efferocytosis, macrophages facilitate resolution of liver fibrosis [characterized by excessive accumulation of extracellular matrix (ECM) proteins] by producing matrix-degrading enzymes (matrix metalloproteinases, MMPs) that degrade fibrotic ECM. Upon liver damage, macrophages induce HSCs trans-differentiation into proliferative, fibrogenic myofibroblasts that secrete large amounts of ECM proteins, predominantly fibrillar collagens, and ECM crosslinking enzymes, lysyl oxidase-like 2 (LOXL2), that stabilize ECM components. Klepfish et al. demonstrated that a novel anti-LOXL2 monoclonal antibody (GS341) ameliorated liver fibrosis *in vivo*. Mechanistically, anti-LOXL2 antibody inhibited LOXL2-mediated collagen crosslinking and facilitated the recruitment of so-called scar-associated MoMFs (SAMs) expressing a unique repertoire of collagenolytic MMPs (in particular MMP-14) to the proximity of collagenous fibrotic fibers. These findings suggest that therapies augmenting the recruitment of collagenolytic macrophages and/or polarization of macrophages into collagenolytic macrophages might be an interesting approach to reverse fibrosis and facilitate endogenous liver regeneration.

MoMFs recruitment is regulated by monocyte chemotactic protein 1 (MCP-1 or CCL2). Queck et al. investigated intrahepatic expression and circulating levels of MCP-1, and its correlation with monocyte infiltration and severity of liver diseases. Using rodent models of liver cirrhosis and ACLF, authors showed an elevated hepatic expression of CCL2 along with increased F4/80-positive macrophages in the liver. In human liver explants and ACLF patients, hepatic transcription levels of CCL2 correlated with the MELD score, and higher portal and hepatic vein levels of MCP-1 correlated with Child-Pugh score, respectively. This study concluded that MCP-1 circulating levels, derived from the injured liver, reflect the intra-hepatic macrophages and correlate with severity of liver disease.

An interesting review by Shwartz et al. describes an emergence of the teleost zebrafish, an attractive new vertebrate model to study liver macrophages. Authors summarize the origin and functions of macrophages in the livers of zebrafish models of ALD, NAFLD, HCC, and liver regeneration. The review discusses how macrophages in zebrafish models can be compared with that described in mammals and highlights the advantages and challenges of using zebrafish models to study liver macrophages.

Colino et al. summarizes the different types of passive (driven by anatomical and physiological features) and

active (using specific ligands) targeted nanoparticle (NP) systems for macrophage recognition and drug targeting. To design NPs, NPs biocompatibility, degradability, toxicity, *in vivo* pharmacokinetics and drug release should be contemplated. Furthermore, recognition mechanisms by macrophages must be investigated considering the changes in the microenvironment that can influence the macrophage phenotype and impact NPs uptake. The authors further present the physiological-based pharmacokinetic (PBPK) model to characterize the biodistribution of NPs.

Altogether, this special issue presents a series of 9 reviews, 2 mini-reviews, and 3 original articles focusing on the understanding of macrophages and/or innate immune system in liver diseases. It also highlights the intricacies of distinct macrophage phenotypes at different stages of the diverse etiological liver diseases and provides a comprehensive overview of the therapeutic targets and macrophages targeting approaches for the treatment of liver diseases.

AUTHOR CONTRIBUTIONS

RB drafted and all the other authors read and revised the editorial. All the authors contributed equally to the review and editorial process for this collection.

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REVIEW
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Contribution of Macrophage Efferocytosis to Liver Homeostasis and Disease

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The clearance of apoptotic cells is pivotal for both maintaining tissue homeostasis and returning to homeostasis after tissue injury as part of the regenerative resolution response. The liver is known for its capacity to remove aged and damaged cells from the circulation and can serve as a graveyard for effector T cells. In particular Kupffer cells are active phagocytic cells, but during hepatic inflammatory responses incoming neutrophils and monocytes may contribute to pro-inflammatory damage. To stimulate resolution of such inflammation, myeloid cell function can change, via sensing of environmental changes in the inflammatory milieu. Also, the removal of apoptotic cells via efferocytosis and the signaling pathways that are activated in macrophages/phagocytes upon their engulfment of apoptotic cells are important for a return to tissue homeostasis. Here, we will discuss, how efferocytosis mechanisms in hepatic macrophages/phagocytes may regulate tissue homeostasis and be involved in tissue regeneration in liver disease.

Keywords: liver injury, macrophage, efferocytosis, inflammation, resolution, apoptosis, phagocytosis, cytokines

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INTRODUCTION

Under homeostatic conditions, new cells are produced and old cells die constantly within in the body. These aged and damaged cells need to be efficiently removed from the surroundings without inducing inflammation or tissue damage, which is achieved by a process called *efferocytosis*. Here, dying/dead cells are specifically recognized by phagocytes and subsequently engulfed. Inefficient removal of apoptotic cells is associated with the development of (auto) immune-mediated diseases (1–3) highlighting the importance of efferocytosis for homeostasis within the immune system. The liver has long been recognized as a major system for removal of aging/dying/activated cells. Under homeostatic conditions, aging erythrocytes and neutrophils, but also effector $CD8^+$ T cells can be eliminated in the liver (4–7). This is mostly attributed to the activity of the organ-resident macrophages in the liver, the Kupffer cells. However, also during liver disease, both in experimental models as well as in patients, large amounts of dying cells are generated directly due to liver injury and/or following inflammatory responses. Various forms of insults leading to liver damage, such as infections, toxins or inflammation, often leads to rapid death of intrahepatic cell populations (mostly hepatocytes), making the efficient removal of such damaged cells extremely important to prevent unwarranted tissue damage and excessive inflammatory responses and support the restitution of tissue integrity. As the liver is known for its extreme and rapid capacity for regeneration upon liver injury, such as after the experimental procedure of partial hepatectomy, it must harbor efficient mechanisms and pathways to deal with large amounts of dying cells. Although cell death may occur through different mechanisms, such as apoptosis, necrosis or necroptosis (8), in this review we will focus on the role of mechanisms related to the efferocytic clearance

of apoptotic cells in the context of liver immune tolerance and liver disease. Most hepatic cell types, including hepatocytes, hepatic stellate cells and liver sinusoidal endothelial cells have been reported to be able to take up apoptotic cells in some way (9, 10). However, the most important hepatic efferocytes are thought to be Kupffer cells together with other myeloid phagocytic cells, like neutrophils and monocytes, which are attracted into the liver after injury for the removal of apoptotic cells. Here, we will discuss the mechanisms and pathways involved in the process of efferocytosis in general, the role for these pathways in hepatic myeloid cells (tissue resident and migrating) and their relevance for both the induction and resolution of liver disease.

FINDING, SENSING, AND CLEARING APOPTOTIC CELLS

The process of efferocytosis is essential for the clearance of apoptotic cells dying from physiological and pathological causes without arousing inflammation. Soluble "find me" signals are released during the onset of apoptosis to attract the phagocytically active myeloid subsets. The most well-known "find me" signals are lyso-phosphatidyl choline (LPC), sphingosine-1-phosphate (S1P), fractalkine (CX_3CL1), and the nucleotides ATP and UTP. LPC is generated via the caspase-3-dependent activation of the inflammatory phospholipase A2 (iPLA2), which hydrolyses phosphatidyl choline (PC) from the cell membrane (11, 12). LPC then functions as an attractant for macrophages via the ligation of the G-protein coupled receptor G2A (13). Additionally, during apoptosis, the activity of sphingosine kinase leads to the caspase-3 dependent generation of S1P from sphingosine. Binding of S1P to sphingosine 1 phosphate receptors (S1PR1-5), expressed on macrophages and other phagocytes, leads to their attraction toward the apoptotic cells (11). Furthermore, membrane-bound or soluble CX_3CL1 from either micro-vesicles of apoptotic cells (12), or cleaved by caspase-3 activity, acts as an attractant for restorative $CX3CR1^+$ phagocytes. Finally, the release of the nucleotides ATP and UTP that function as extracellular alarmins, attract phagocytes via signaling through purinergic P2Y receptors and secretion via pannexin-1 channels.

The main recognition of apoptotic cells by phagocytes occurs via caspase-dependent expulsion of phosphatidyl serine (PS) onto the outer leaflet of the plasma membrane. This characteristic "eat-me" signal can be recognized directly via PS binding receptors on macrophages or indirectly by "bridging molecules" that bind to PS on the one hand and to their cognate receptors on the other hand to trigger engulfment of apoptotic cells. Most prominent are the bridging molecules milk fat globule epidermal growth factor VIII (MFG-E8; also lactadherin), and the vitamin K-dependent proteins growth arrest specific 6 (Gas6) and protein S (11, 14). Macrophages and dendritic cells can secrete MFG-E8, which then interacts with PS and can be recognized via the integrins αvβ3 or αvβ5. Both Gas6 and Protein S act as bridging molecules for the TAM receptor family, consisting of Tyro3, Axl, and MerTK (15). These interactions induce efficient clearance of apoptotic cells. Similarly, the binding of the complement factor C1q as an opsonin to PS serves as a bridging molecule for recognition by the scavenger receptor class F member 1 (SCARF1) (16) on phagocytes and endothelial cells, but may also as part of a protein complex containing calreticulin be involved in the initiation of efferocytosis (17). Recently, soluble CD93 was identified to act as an efferocytic opsonin by bridging PS on apoptotic cells with the complement receptor integrin αxβ2 (CD11c/CD18) (18). Experimentally, the detection of efferocytosis by phagocytes is most often probed with fluorescently labeled apoptotic cells, like dexamethasone-treated thymocytes or neutrophils that die via apoptosis upon *in vitro* culture. Ingestion of such apoptotic cells by phagocytes is then analyzed by microscopy or flow cytometry.

PS receptors are not ubiquitously expressed but are rather tissue and/or cell type-specific: For instance, the T cell immunoglobulin and mucin-domain-containing molecule (Tim) family of receptors act as PS receptors. In particular, Tim3 and Tim4 are expressed on phagocytes, such as macrophages and dendritic cells (DC) (11). Although Tim4 can bind directly to PS, actual clearance of apoptotic cells via phagocytosis requires its cooperation with other receptors, such as MerTK and/or integrin β1 (19, 20). The receptors stabilin-1 and-2 both recognize PS on apoptotic cells for engulfment and are expressed by macrophages, but are most prominently known for their role in the capture and elimination of PS-exposed damaged and/or aged erythrocytes by LSEC (9). Besides the uptake of aged erythrocytes by the asialo-glycoprotein receptors (ASGPR) by hepatocytes, this is the only liver cell-specific mechanism for efferocytosis described so far (10).

Like Tim4, stabilin-2 also requires cofactors/receptors [engulfment adapter phospho-tyrosine binding domain-containing protein 1 (GULP1) and thymosin4] for the initiation of engulfment (12). Additionally, some members of the CD300 family of type I transmembrane proteins (CD300a, CD300f, and CD300b) are capable of recognizing phosphatidyl-serine (PS) and -ethanolamine (PE), which are both exposed on the outer leaflet of the plasma membrane early during apoptosis (21–24). Knock-down or knock-out of CD300b and CD300f, respectively, results in impaired efferocytosis by macrophages (23, 24). Also, binding of the receptor for advanced glycation end products (RAGE) to PS increases the potential of macrophages to take up apoptotic cells (25). Contrary, secretion of the pro-inflammatory high mobility group box 1 (HMGB1) during inflammation interferes with RAGE-mediated efferocytosis by binding to PS (26). Similarly, secreted soluble RAGE itself can also inhibit efferocytosis by binding to and masking exposed PS for recognition by other PS receptors. Additional receptors, such as the scavenger receptors SR-A1 and SR-B1 and CD36 (27–29), are also implicated to play a role in (oxidized) PS recognition during efferocytosis, but their definite role has not been determined so far.

Not only do apoptotic cells signal phagocytes in various ways to enhance efferocytosis, healthy viable non-apoptotic cells express surface molecules that prevent efferocytosis. The transmembrane CD47 molecule constitutes such a "don't eat-me" signal via interaction with the ITIM-containing receptor Signal regulatory protein 1 alpha (SIRP1α; CD172a). This leads

to the inhibition of actin cytoskeleton rearrangements necessary for phagocytosis (30). Recently, also the sialoglycoprotein CD24 (heat stable antigen) was shown to inhibit phagocytosis via interacting with and signaling via Sialic acid-binding Ig like lectin 10 (Siglec-10) on macrophages (31). In addition to the regulation of apoptotic cell clearance via "find-me," "eat-me," and "don't eat-me" signals for the phagocytotic activity of macrophages cross talk between these signaling pathways can modulate the efferocytosis process. For instance, signaling via the "find-me" receptors S1PR, CX_3CR1 and "eat-me" receptor SCARF1 can enhance the release of the bridging molecules Gas6 and/or MFG-E8, but also upregulate their receptors, e.g., MerTK (11). This feed forward loop further enhances the capability of phagocytes after the engulfment of apoptotic cells has already been initiated.

MODULATION OF PHAGOCYTE FUNCTION DUE TO EFFEROCYTOSIS

The process of efferocytosis and the subsequent signaling events are critical for the upkeep of homeostasis and even more important for the return to tissue homeostasis after tissue damage due to inflammation and/or disease [reviewed in (32) and (33)]. Cellular signaling and metabolic adaptation initiated by efferocytosis enables the return to tissue homeostasis by anti-inflammatory reprogramming of the formerly pro-inflammatory leukocytes: Signaling via the CX_3CR1 receptor, recognizing CX_3CL1 released from apoptotic cells, induces pro-survival signals and expression/generation of antioxidant factors. Signaling via S1PR invokes an anti-inflammatory gene expression program, including reduction of pro-inflammatory Interleukin (IL)-12 and tumor necrosis factor alpha (TNFα) whilst promoting production of anti-inflammatory mediators such as IL-10, vascular endothelial growth factor (VEGF) and prostaglandin E2 (PGE_2) (32). Indeed, after efferocytosis, macrophages can produce several anti-inflammatory and pro-resolving lipid mediators that promote macrophage conversion toward phenotypes associated with resolution of inflammation (34). Also, the ATP released from apoptotic cells can be converted to adenosine by sequentially acting nucleotidases, and subsequent signaling through adenosine receptors suppresses production of proinflammatory mediators and chemokines (35).

In addition to the modulation of cellular signaling, efferocytosis leads to the accumulation of high amounts of cellular material in phagocytes that needs to be processed and digested. The metabolic adaptation to this high ingestion of cargo leads to the activation of several lipid sensing nuclear receptors, such as liver X receptor (LXR) and members of the peroxisome proliferator-activated receptor (PPAR) family (33, 36). The activation of these pathways in macrophages promotes anti-inflammatory reprogramming and can reinforce their efferocytic capacity by upregulation of phagocytic receptors, like MerTK, and inducing the synthesis of precursors for pro-resolving lipid mediators (33, 37). The uptake of apoptotic material is also closely linked to a non-canonical rubicon-dependent autophagy pathway, called light chain (LC)3-associated phagocytosis (LAP) (38, 39). Specific signaling via the TAM receptor Axl induces autophagy, which is not only involved in the physical clearance of apoptotic material, but also prevents NOD, LRR-, and pyrin-domain containing 3 (NLRP3) inflammasome activation and concomitantly reduces IL-1β and IL-18 production (40). Similarly, Tim4-mediated apoptotic cell engulfment connects to LAP after recognition of PS on apoptotic cells (41) and incomplete cargo digestion via defective LAP is associated with development of autoinflammation (42).

Classical activation or polarization of macrophages into M1 (pro-inflammatory, mostly induced by LPS/IFNγ) vs. M2 (alternative or anti-inflammatory, induced by the cytokines IL-4/IL-13) shows that also the cytokine/chemokine microenvironment in which phagocytes encounter apoptotic cells can modulate their cognate response. Classically, Th2 cells are major sources for the cytokines IL-4 and IL-13, and the simultaneous recognition of apoptotic cells (neutrophils) and IL-4/IL-13 stimulation of macrophages is necessary for the induction of an anti-inflammatory, pro-resolving tissue repair gene signature (43). Congruently, expulsion of the helminth *N. Brasiliensis* was unsuccessful in mice lacking the TAM receptors Axl and MerTK even though the capacity to produce IL-4 and IL-13 was not affected (43). IL-13 production is also essential for the induction of efferocytosis in macrophages by regulatory T cells (Treg) during resolution of a zymosan-induced model of peritonitis (44). Treg-derived IL-13 promotes autocrine IL-10 induction in macrophages leading to Vav1-Rac1-dependent phagosome formation thus promoting their capacity to phagocytose apoptotic cells (44). Although anti-inflammatory mediators, like IL-13 and IL-10, can augment efferocytosis in macrophages, not all pro-inflammatory signals reduce efferocytosis. For instance, both interferon (IFN)α, which is produced as a result of signaling via toll-like receptor (TLR)3, or direct TLR3 signaling using Poly:IC down regulates MerTK expression (as do LPS and IFNγ), but at the same time induces Axl expression in human macrophages, which may facilitate efferocytosis during viral infections (45). Signaling via the TLR-dependent upregulated TAM receptors may then interfere with TLR signaling via the induction of suppressor of cytokine signaling proteins (SOCS) 1 and 3, thus scaling down the proinflammatory response induced by the initial TLR signals (46).

RELEVANCE FOR EFFEROCYTOSIS MECHANISMS IN PHAGOCYTES IN THE LIVER

Macrophage populations in adult tissues arise from different origins. Several tissue-resident macrophage populations, like Langerhans cells, microglia and Kupffer cells, are seeded in distinct waves during embryogenesis and are self-sustaining without input from the bone marrow during adulthood (47). The bone marrow also contributes to the myeloid/macrophage cell pool found in different tissues under homeostatic conditions and include $Ly6C^+$ monocyte derived cells and dendritic cells. In particular in the liver, mas cytometry combined with gene expression analysis (48), shows that within the non-B,

non-T CD45$^+$ population the most abundant myeloid cells are dendritic cells, Kupffer cells and Ly6C$^+$ monocytes (48). Distinct but small populations of plasmacytoid DC, neutrophils and basophils are also present. F4/80$^+$MHCII$^+$CD11b$^+$ positive Kupffer cells comprise two populations additionally expressing CD11c or CD206, CD317 (plasmacytoid dendritic cell antigen-1, PCDA-1) and CD1d, pointing to a possible functional difference. Within Ly6C$^+$ infiltrating monocytes, an F4/80 positive and F4/80 negative population are present. By intravital microscopy, the expression of F4/80 and/or CX_3CR1, which is not expressed on Kupffer cells under homeostasis (49), shows that F4/80$^+$ cells are exclusively to be found intravascularly (which may include both F4/80$^+$ Kupffer cells and F4/80 expressing monocytes) and CX_3CR1^+ cells extravascularly in the parenchyma, suggesting that in addition to gene signatures another level of functionality is established via localization. Other markers that are used to distinguish tissue-resident Kupffer cells from recruited monocytes/neutrophils include the C-type lectin receptor Clec4f and the V-set and immunoglobulin domain-containing 4 (Vsig4) (50, 51). Interestingly, also the PS-receptor Tim4 is used for identification of liver resident Kupffer cells, indicating that these cells are set for efferocytosis under homeostatic conditions (50, 51). Thus, when studying the contribution of myeloid subsets to different liver diseases and/or injuries, the definition and identification of these different highly plastic myeloid subsets is challenging. Moreover, due to the large influx of pro-inflammatory myeloid subsets [neutrophils and inflammatory monocytes, but possibly also eosinophils (52)] the composition of the myeloid compartment at any given time during inflammatory liver disease can vary dynamically. We will focus on how local and recruited phagocytes are involved in the safeguarding of and return to immunological homeostasis in the Iiver and how their capacity for efferocytosis plays a role in the pathogenesis and resolution of liver diseases. Due to the highly divergent etiologies of liver disease, being either infectious, toxic or nutritional in origin, and depending on whether liver (immunological) function is being compromised acutely or in a chronic fashion, it is very well-possible that similar mechanisms controlling macrophage efferocytic function may have unexpected opposite effects.

First reports that intrahepatic phagocytes are capable of recognizing PS to trigger phagocytosis relate to the elimination of aged/damage erythrocytes from the circulation (4). The PS receptors Stabilin-1 and -2 expressed on LSEC are important for erythrocyte sequestration in this process (9). However, the removal of erythrocytes is blocked by clodronate-containing liposomes, indicating that Kupffer cells mediate actual phagocytosis. Also, aged erythrocytes that are taken up by Ly6C$^+$ monocytes elsewhere in the circulation are carried to the liver as cargo to recover the iron from hemoglobin (53). Kupffer cells can express several receptors involved in PS recognition, such as MerTK (54) and Tim4 (53), or phagocytic receptors thought to assist in the uptake of PS exposed apoptotic cells like CD36 and scavenger receptors (55). The relevance for recognition of apoptotic cells and its downstream signaling events for potential treatment options in inflammatory diseases of the liver can be taken from experiments using apoptotic cells to treat inflammation and damage in D-galactosamine (D-GalN)/LPS induced liver damage (56). Here, adoptively transferred UV-irradiated splenocytes that are taken up by Kupffer cells prevent mortality in the D-GalN/LPS model, induces expression of IL-4, IL-10, and transforming growth factor beta (TGFβ) and suppresses expression of TNFα, IL-6, and IL-1β (56). Moreover, in a more controlled fashion liposomes expressing phosphatidyl serine may mimic apoptotic cell effects on phagocyte function in immune cells and hepatic disease (57, 58).

INVOLVEMENT OF THE EFFEROCYTOSIS MACHINERY IN LIVER DISEASE

Contribution of Efferocytosis Inducing Signals From Soluble Mediators Released by Apoptotic Cells

Early in the process of apoptosis, soluble mediators, such as ATP and UTP, act as damage-associated molecular patterns (DAMP) and "find-me" signals for efferocytes. In the liver, extracellular nucleotides trigger dichotomous responses; on the one hand, release of nucleotides by apoptotic cells after tissue injury initiates inflammation, and on the other hand, activation of non-parenchymal, liver-resident, and infiltrating efferocytes is a prerequisite for the activation of phagocytosis and the clearance of dead cells. Kupffer cells can detect and respond to the soluble mediator ATP released by apoptotic cells via expression of several P2Y receptors (59) and increase IL-6 production in response. Interestingly, however, Kupffer cells themselves also release ATP in response to LPS, which in an autocrine fashion promotes pro-inflammatory IL-6 release $PY2_{13}$-dependently (59). Thus, attraction to apoptotic cells via ATP may lead to pro-inflammatory cytokine release by Kupffer cells. *In vivo* the recognition of and response to ATP in liver disease is more complex. After partial hepatectomy, both Kupffer cells and hepatocytes release ATP, which is important for the regenerative response in hepatocytes via direct signaling through the $P2Y_2$ receptor (60), indicating that ATP release may have pro-inflammatory effects but at the same time may be indispensable for the initiation of hepatocyte proliferation necessary for liver regeneration. Depending on the longevity of ATP release and the expression of different purinergic receptors capable of recognizing ATP, the outcome of ATP release can be modulated. For instance, in the model of partial hepatectomy ATP release peaks within 15 min (59). In other models, where ATP is likely released over a prolonged time period, effects on intrahepatic phagocytes and disease progression may be different. In acetaminophen (APAP)-mediated liver injury, ATP (and also NAD) signaling via the purinergic $P2X_7$ receptor mediates hepatotoxicity to a large extent as $P2X_7$-deficient mice, and the use of selective $P2X_7$ antagonists decreases APAP-dependent liver toxicity (61). Although direct ATP signaling induces proinflammatory signaling, such as IL-1β and IL-6 production by Kupffer cells (59, 61), ATP released during liver injury and inflammation *in vivo* can be further modulated to attenuate liver inflammation. The cell surface ectonucleotidase CD39 counteracts proinflammatory effects of ATP release by catalyzing the hydrolysis of ATP/ADP to AMP, which can then be further metabolized to adenosine

that can results in A_{2A} receptor stimulation. In APAP liver injury, in both a caecal ligation and puncture (CLP) liver dysfunction model and a chemically induced biliary fibrosis model (by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DCC)-feeding), CD39-deficiency exacerbates disease (61–63). In DCC-dependent biliary fibrosis, CD39 deficiency in myeloid cells, via LysM-Cre-mediated excision of the CD39 gene, was sufficient for exacerbation of disease (63). As ATP signaling via the $P2X_7$ receptor not only induces a proinflammatory phenotype in macrophages but also increases CD39 expression and CD39 activity in macrophages (62), this represents a mechanism to induce an anti-inflammatory pro-resolving response after initial pro-inflammatory signaling in macrophages during inflammation and injury in the liver.

The release of LPC from apoptotic cells and it signaling via the G-protein coupled receptor G2A (13) can function as a "find-me" signal for efferocytes. Additionally, LPC signaling via the G2A receptor may also enhance phagocytic activity by promoting phagosome maturation (64, 65) and induce an anti-inflammatory (M2-like) mRNA expression profile [arginase 1 (Arg1), chitinase-like 3 (Chil3), CD206] and cytokine/mediator production (IL-10, PGE_2) (66). Preventing G2A signaling in CLP leads to higher mortality. However, inhibition Kupffer cell activity by gadolinium chloride treatment prevents this higher mortality in $G2A^{-/-}$ mice (67), indicating that G2A signaling in Kupffer cells reduces mortality in this model. Unexpectedly, G2A deficiency leads to overproduction of not only pro-inflammatory IL-6 and TNFα but also anti-inflammatory IL-10 by Kupffer cells. In CLP, the increased production of IL-10 by $G2A^{-/-}$ Kupffer cells is pivotal for the lack of bacterial clearance (and higher mortality) as this is reversed by either Kupffer cell depletion or IL-10 blockade (67).

The involvement of S1P as an efferocytic "find-me" signal regulating myeloid cells in liver disease has not been studied extensively. S1P is a major factor regulating immune cell migration to and from bone marrow and lymphoid organs and can signal through five S1PR. Reduced S1P serum levels in patients with liver cirrhosis and hepatocellular carcinoma (HCC) are associated with worse outcome and HBV co-infection (68, 69). However, in the experimental model of bile duct ligation, specific inhibition of $S1PR_{2/3}$ diminishes liver injury and fibrosis due to the inhibition of monocyte migration from the bone marrow into the liver (70). Similarly, treatment with the S1P antagonist FTY in mice fed a NASH diet also prevents infiltration of inflammatory monocytes and reduces liver inflammation and injury (71). The disparity between low serum S1P in patients being a predictor for higher mortality and a decreased inflammatory liver injury due to inhibition of S1P signaling in experimental models, may be the result of different timing (prevention and onset in experimental settings vs. end-stage disease in patients) but may also have to with comorbidities in patients. For instance, hyperglycemia, associated with type II diabetes, directly affects intrahepatic myeloid cell function. In mice treated with the pancreatic β cell toxin streptozotocin, that induces hyperglycemia, Kupffer cells produce more IL-6 and TNFα and less IL-10 in response to APAP-mediated injury compared to normoglycaemic animals (72). In these hyperglycemic mice, an imbalance between AMPK and Akt signaling leads to enhanced reactive oxygen species (ROS) production promoting an M1 type instead of M2 type gene signature in Kupffer cells. Hyperglycemia also increases intrahepatic S1P and S1PR3 levels both in patients and experimental animals (73). Targeting ROS production with *N*-acetyl cysteine (72) or treatment with a S1PR3 antagonist (73) both reduces the proinflammatory polarization of Kupffer cells and is associated with less inflammatory liver damage, which suggests that the combination of these treatments might bring additional advantage to counteract pro-inflammatory skewing of intrahepatic macrophages due to hyperglycemia.

The CX_3CR1 chemokine receptor is widely used to identify different populations of myeloid cells with the help of GFP-reporter mice (74). In the non-inflamed murine liver, CX_3CR1 expression identifies a myeloid cell population residing outside of the hepatic sinusoids, whereas $F4/80^+$ Kupffer cells are to be found intravascularly (48). Interestingly, single cell sequencing of healthy human hepatic immune cells did not reveal a similar division based on CX_3CR1 expression, but rather identified two populations of $CD68^+$ myeloid cells according to the expression of the macrophage receptor with collagenous structure (MARCO) receptor, with $MARCO^{pos}$ cells transcriptionally resembling murine Kupffer cells and $MARCO^{neg}$ having a rather inflammatory gene signature (75). It cannot, however, be excluded that CX_3CR1 may play a role in human intrahepatic inflammatory responses, as human peripheral blood monocytes express high levels of CX_3CR1 also in patients with liver disease (76). Although in the murine liver, CX_3CR1 positive cells are present under homeostatic conditions, it is widely accepted that during inflammatory responses, the up-regulation of CX_3CR1 on macrophages signifies their change from largely pro-inflammatory toward an anti-inflammatory, wound healing type of cell. Their anti-inflammatory properties could be enhanced by recognition of CX_3CL1 that is released by apoptotic cells. The change of $CCR2^{high}CX_3CR1^{low}$ inflammatory monocytes locally into $CCR2^{low}CX_3CR1^{high}$ macrophages in a sterile liver injury model leads to optimal repair and is dependent on local production of IL-4 and IL-10 (77). However, in liver disease, also endothelial-related expression of CX_3CL1 may attract $CD16^+$ monocytes CX_3CR1-dependently into areas with active inflammation (78) to dampen inflammation and promote resolution of injury. Fittingly, CX_3CR1-deficient mice develop greater liver fibrosis in both chronic CCl_4 and bile duct ligation models due to development of high amounts of TNFα- and NO-producing macrophages (79). Additionally, in the absence of CX_3CR1 signaling, the pro-inflammatory circle is perpetuated, as expression of the anti-apoptotic B cell lymphoma associated oncogene 2 protein (Bcl-2) is not induced in CX_3CR1-deficient macrophages.

Relevance of Indirect Phosphatidyl Serine Recognizing Receptors for Efferocytosis in the Liver

Recognition of apoptotic cells is mediated by binding to phosphatidyl serine exposed on the outer leaflet cell membrane. PS receptors either engage directly with PS (e.g., Tim family,

CD300 molecules, stabilins) or via bridging molecules that bind to PS (Gas6, Protein S and MGF-E8) that can then be recognized by certain integrins or receptors of the TAM family. Although Protein S is abundant in the circulation, Gas6 expression is more restricted. In acute CCl_4-mediated liver injury, Gas6 expression is induced in liver macrophages, indicating a role for detection of apoptotic cells via Gas6 and TAM receptors in this model. However, Gas6-deficiency does not change acute liver damage after CCl_4, but in the regenerative phase after CCl_4 induced liver injury, hepatocyte proliferation is stunted, and a reduction in Kupffer cell numbers and infiltrating monocytes are observed, with concomitant reduction of pro-inflammatory cytokine production (80). Mechanistically, Gas6 can signal via Axl, which is also induced in the liver after acute CCl_4 damage, and leads to autophagy induction, inhibition of NLRP3 inflammasome activation and subsequent reduction in IL-1β and IL-18 secretion, which is required to reinstall homeostasis (40).

The TAM (Tyro3-Axl-MerTK) family of receptor tyrosine kinases connects the recognition and removal of apoptotic cells to the induction of anti-inflammatory signaling in macrophages. General features of TAM-induced signaling include enhancement of phagocytosis, reduction of Type I IFN induced inflammation, inhibition of NLRP3 inflammasome activation via induction of autophagy and the promotion of anti-inflammatory cytokine production (34). The importance for these receptors in liver immune homeostasis becomes apparent in animals lacking all three TAM receptors. These mice develop an auto-immune hepatitis like disease, with high ALT, auto-antibodies and large immune cell infiltrates (81), which is dependent on bone-marrow derived cells. Individual TAM receptors can also modulate inflammatory response in the liver. In patients with acute liver failure MerTK-expressing macrophages expand, and migrate to the necrotic areas in the liver. Fittingly, in MerTK-deficient mice acute liver failure (ALF) due to APAP administration is aggravated and associated with a reduced number of hepatic macrophages and increased MPO^+ neutrophils (54). This suggests that MerTK may be involved in the removal of apoptotic neutrophils in the course of ALF. By increasing the amounts of MerTK-expressing macrophages, through *in vivo* application of secretory leukocyte protease inhibitor (SLPI), the amount of MPO^+TUNEL^+ neutrophils after APAP administration are reduced and this may constitute a therapeutic approach in acute liver failure after accidental or deliberate paracetamol intoxication (54). However, in patients with decompensated cirrhosis and acute-on-chronic liver failure (ACLF) the opposite may hold true. In ACLF patients, high susceptibility to infections and accompanying monocyte dysfunction correlates with disease severity (82). Here, MerTK expressing monocytes and macrophages are highly increased and are associated with decreased TNFα and IL-6 production. Interestingly, the treatment of $MerTK^+$ monocytes with a MerTK inhibitor increased proinflammatory cytokine production (82). Thus, either inhibiting or promoting expansion of MerTK expressing myeloid cells may be used to prevent immune paralysis in ACLF patients or enhance the clearance of necrotic material and apoptotic neutrophils in ALF patients, respectively.

Other non-TAM PS receptors may influence liver disease as well. The integrins αvβ3 and αvβ5, which recognize apoptotic cells via binding to the bridging molecule MFG-E8, can contribute to limiting fibrosis and the inhibition of proinflammatory mediator production in the chronic liver inflammation models of bile duct ligation and thioacetamine (TAA)-induced fibrosis (83). As the infiltration of macrophages with or without Cilengitide® (an αvβ3 and αvβ5 inhibitor mimicking the RDG-peptide ligand for integrins) treatment remains the same in these models, it is not clear whether or not the effects of αvβ3 and αvβ5 inhibition targets efferocytosis mechanisms in macrophages.

Relevance of Receptors Directly Recognizing Phosphatidyl Serine by Hepatic Macrophages for Liver Disease

The receptor stabilin-1 is well-known for mediating phosphatidyl serine-dependent removal of aged erythrocytes in the liver (9). In chronic CCl_4 liver injury the absence of stabilin-1 aggravates fibrosis and delays resolution after termination of CCl_4 administration. Furthermore, the phenotypic switch of pro-inflammatory macrophages to the pro-resolving $Ly6C^{low}$ macrophage is impaired in stabilin-1-deficient mice (84). Interestingly, stabilin-1 expression in non-injured animals is restricted to endothelial cells, but in inflamed/injured livers an $F4/80^+$stablilin-1^+ macrophage population can be detected (84). In addition to stabilin-1 binding to PS to promote efferocytosis, fibrosis development can additionally be prevented via stabilin-1. Pro-fibrogenic chemokine production can be inhibited via binding of malondialdehyde (MDA)-LDL, generated from lipid peroxidation, to stabilin-1. Furthermore, during resolution of liver injury the absence of stabilin-1 prevents the change to $Ly6C^{low}$ macrophages, which are thought to be pro-resolving macrophages (84). Besides stabilin-1, Tim4 can also bind directly to PS. Tim4 is over-expressed in hepatic macrophages of mice fed both a high fat diet (HFD) and methionine-choline deficient (MCD) diet (85) and its absence leads to increased inflammation and severe steatosis. Tim4 strongly binds to PS, but does not induce efferocytosis independently; it collaborates with TAM receptors to elicit efferocytosis (19, 86). Strikingly, signal transduction via the Tim4 cytoplasmic domain upon PS binding induces activation of AMPKα via LKB1, which is critical to initiate autophagy (85). What is more, the levels of Tim4 expression determine the degree of inhibition of NLRP3 protein complex expression. Tim4 deficiency, therefore, leads to high levels of IL-1β and IL-18 and proinflammatory damage in both NASH diets (85).

Phagocytosis and Autophagy of Apoptotic Cells and Its Relevance for Liver Disease

The sensing and physical uptake of apoptotic cells by macrophages can enhance phagocytosis. The cargo that is taken up needs to be digested and processed appropriately and several intermediates that are generated during this process may have signaling functions to modulate macrophage function

toward a pro-resolving phenotype. For instance, the TAM-receptors and also the Tim4 receptor connect the uptake of apoptotic cells to autophagy, which in its turn prevents inflammasome activation and ultimately the production of pro-inflammatory IL-1β and IL-18. This means that macrophages that are incapable of properly digesting their apoptotic cargo may display aberrant inflammatory functions or phagocytosis. For instance, macrophages that can engulf apoptotic cells but are defective in processing their apoptotic cargo, due to the lack of LC3 interacting glycoprotein non-metastatic melanoma protein B (Gpnmb) expression (87) are neither capable to increase pSTAT3-dependent IL-6 transcription and IL-10 release nor sustain efficient efferocytosis by increasing phagocytosis. Addition of exogenous IL-6 and IL-10 increased phagocytosis in Gpnmb-deficient macrophages, indicating that secondary cytokine production is an important mechanism induced by proper cargo digestion to regulate phagocyte function. Indeed, *in vivo* absence of Gpnmb reduces the amount of $Ly6C^{low}$ restorative macrophages in acute APAP-mediated damage and also in chronic CCl_4 induced fibrosis (87) which can be reversed by treatment with IL-6. Different secondary signaling events that influence macrophage efferocytosis and anti-inflammatory function may also depend on the appropriate digestion of apoptotic material. Chemical inhibition of the lysosomal acid lipase (LIPA) reduces cholesterol hydrolysis in macrophages, and leads to mitochondrial stress and pro-inflammatory NLRP3 and caspase-1 activation. Furthermore, anti-inflammatory LXR activation is reduced due to the limited production of oxysterols from cholesterol (88). *In vivo,* the inhibition of LIPA activity reduces efferocytosis of both stressed erythrocytes and apoptotic lymphocytes. Also, under conditions of hypercholesterolemia, in which efferocytosis is constrained (89), additional LIPA inhibition exacerbated this effect and this consequently leads to increased liver inflammation. Interestingly, LIPA inhibition does not only increases the production of *iNos* and *Il1b* mRNA, it is accompanied by an almost complete reduction of *Mertk*, *Axl,* and *Gas6* mRNA expression (88). The efferocytic uptake of apoptotic cells connects to autophagy, which is achieved via a process called LC3-complex associated phagocytosis (LAP) (38). This non-canonical autophagy pathway requires a so-called class III PI3K complex that comprises the core proteins Beclin1, UV radiation resistance-associated gene protein (UVRAG), and RUN domain containing, Beclin1-interacting protein (Rubicon) (90). During LAP, rubicon localizes to the single membrane LAPosome, which is decorated with LC3-II, and promotes PI(3)P production necessary to recruit downstream autophagic machinery, including the ubiquitin-conjugating enzymes autophagy related proteins ATG5, 7 and 12 (38, 90). As a results, alterations in LAP lead to stalled degradation of apoptotic cells, which is observed in autoimmune disorders such as systemic lupus erythematosus (SLE) (42). Furthermore, LAP that is active during efferocytosis instructs phagocytes to secrete the anti-inflammatory cytokines TGFβ and IL-10, and conversely to repress secretion of the pro-inflammatory cytokines IL-1β, IL-12, and TNFα (37, 91, 92).

In HFD livers, Kupffer cells show defective LC3-I and LC3-II induction after LPS treatment, which leads to overproduction of TNFα. This phenotypically overlaps with ATG7-deficiency in Kupffer cells, which leads to defective induction of autophagy and provokes increased TNFα release (93). Furthermore, ATG5-deficiency restricted to myeloid cells leads to higher inflammatory responses in liver injury models (CCl_4, D-GalN/LPS) (94, 95). The lack of ATG5 expression results most prominently in NLRP3 and caspase-1 dependent IL-1β production, which promotes hepatic stellate cells to synthesize fibrotic factors and augments infiltration of $Ly6C^{high}$ inflammatory monocytes and MPO^{+} neutrophils (94). Interestingly, the application of an IL-1R antagonist almost completely reverses injury and inflammatory phenotype due to ATG5 deficiency in mice, and offers a promising potential therapeutic approach in the prevention of liver fibrosis and limitation of acute toxic liver injury (94, 95). Thus, the way in which apoptotic cells are processed and digested in macrophages determines the signaling cascades that impose immunologically quiet anti-inflammatory responses on the liver and could be exploited to protect from liver inflammatory diseases.

Interactions Between Phagocytes Can Affect Efferocytosis Efficiency in the Liver

In infections and inflammatory diseases, neutrophilic granulocytes are first responders and infiltrate into the inflamed injured tissue. Pro-inflammatory cytokine release, protease release and the formation of extracellular nuclear traps (NETs) during the process of NETosis are deployed by neutrophils to combat the infection/injury (96, 97). Neutrophils die partially after NETosis or spontaneously via apoptosis and are cleared by tissue-resident or infiltrating macrophages via efferocytosis and the ordered clearance of apoptotic neutrophils by hepatic macrophages is essential for resolution of liver injury. Expectedly, CCR2-antibody depletion of pro-inflammatory $Ly6C^{high}$ monocytes during acute APAP injury increases the numbers of infiltrating neutrophils. However, these neutrophils are functionally changed and produce less ROS due to decreased NADPH2 expression and seem more long-lived as they upregulate pro survival factors (98). Reversely, changes in neutrophil activity may also influence efferocytic activity in macrophages. Mediators released by neutrophils during NETosis, may promote inflammatory signaling in macrophages but can also directly corrupt recognition of apoptotic cells by phagocytes: HMGB1, for example, can either bind to the PS-recognizing the RAGE receptor or directly binds to PS which interferes with recognition by other PS-binding proteins (26). Alcoholic liver disease (ALD) patients have elevated blood serum levels of HMGB1, which is secreted by neutrophils during NETosis. Interestingly, alcohol can directly increase NETosis by neutrophils (99) and thus in ALD patients the consumption of alcohol may directly affect pro- and anti-inflammatory pathways via HMGB1 in a dichotomous way: first, HMGB1 may act as a damage-associated molecular pattern protein (DAMP) that initiates pro-inflammatory TLR and NLRP3 signaling whilst simultaneously preventing efferocytosis via obscuring PS recognition. Furthermore,

TABLE 1 | Efferocytosis mechanisms in liver disease.

Molecules directly or indirectly involved in efferocytosis	Cell-associated or soluble	Model of gastrointestinal disease, function in the liver, and/or function in efferocytosis	References
IMMUNE RECEPTORS			
Vsig4	KC	Homeostasis	(51)
Tim4	KC	Homeostasis; in HFD and MCD, Tim4 deficiency: increase in inflammation and steatosis	(50, 53, 85)
Tim3	Liver mph	Hepatocellular carcinoma	(114)
SCAVENGER RECEPTORS			
Stabilin-1	LSEC, F4/80$^+$ stabilin1$^+$ mph	Removal of aged erythrocytes; CCl4; absence of Stabilin 1 aggravates fibrosis, delays in resolution	(9, 84)
Stabilin-2	LSEC	Removal of aged erythrocytes	(9)
CD36	KC, other phagocytes		(55)
PURINERGIC G-PROTEIN COUPLED RECEPTORS RECEPTORS			
P2Y receptors, P2Y13	KC	Expression in response to extracellular ATP, attraction of apoptotic cells, partial hepatectomy	(59)
P2Y2	HC	Partial hepatectomy, ATP release, and attraction of apoptotic cells	(60)
P2X7	KC	APAP, ATP release and attraction of apoptotic cells	(61)
G-PROTEIN COUPLED RECEPTORS AND LIGANDS			
G2A	KC	Enhancing phagosome maturation, CLP (cecal ligation and puncture)	(66, 67)
S1PR2/3		Bile duct ligation	(70)
S1P antagonist		NASH, S1P antagonist reduces monocyte infiltration, and reduces inflammation and injury	(71)
S1PR serum levels	Soluble, serum	Reduction of serum levels S1P, worse outcome in human liver cirrhosis, HCC, HBV	
S1P and S1PR3	Intrahepatic levels	Ischemia/reperfusion, hyperglycemia mice, and humans	(73)
S1PR3 antagonist		Reduction of inflammatory KC polarization	(73)
CHEMOKINE RECEPTORS AND LIGANDS			
Potenital: CX3CR1	KC, mph	recognition of CX3CL1 released by apoptotic cells, sterile liver injury	(77)
CX3CL1	LSEC	Potential attraction of CX3CR1 monocytes into areas of inflammation	(78)
CX3CR1		absence in deficient mice: greater liver fibrosis,CCl4 and BDL	(79)
TAM FAMILY RECEPTORS AND LIGANDS			
Gas6, Axl	Liver mph	CCl4, induction of Gas6/Axl expression, induction of autophagy, inhibition NLRP3 inflammasome	(80)
MertTK, Axl, Tyro3		MerTK$^{-/-}$, Axl$^{-/-}$ Tyro3$^{-/-}$ mice, development of auto-immune hepatitis like disease, autoantibodies, immune cell infiltrates	(81)
MerTK	KC	MerTK deficient mice, ALF after APAP	(54)
MerTK		Human ACLF, decompensated cirrhosis, increase in MerTK expressing myeloid cells, immune paralysis, Hepatocellular carcinoma	(82, 107)
INTEGRINS			
avb3 and avb5		binding to MFG-E8, BDL, TAA-induced fibrosis	(83)
INTRACELLULAR MOLECULES			
Gpnmb	mph	Lack of Gpnmb impairs cytokine release and sustaining efferocytosis; in APAP, CCl4: reduction of restorative macrophages	(87)
Lysosomal acid lipase		Reduction Mertk, Axl, Gas6 expression, reduction of LXR activation, reduction of efferocytosis; in hypercholesteremia, increase in liver inflammation	(88, 89)
LC3-I and LC3-II deficiency	KC	HFD induces deficiency in LC3-I and LC3-II, overproduction of inflammatory cytokines	(93)

(Continued)

TABLE 1 | Continued

Molecules directly or indirectly involved in efferocytosis	Cell-associated or soluble	Model of gastrointestinal disease, function in the liver, and/or function in efferocytosis	References
ATG7	KC	ATG7-deficiency in KC, increased release of inflammatory cytokines, defective autophagy	(93)
ATG5	Myeloid cells	Myeloid-restricted ATG5-deletion, hyperinfammation in CCl4/D-GalN/LPS, Hepatocellular carcinoma	(94, 95, 117)
HMGB1		PS or RAGE binding, impairs efferocytosis in ALD	(99)
miR-223	Neutrophils	CCl4, LPS-mediated liver injury: miR-223-releasing neutrophils promote generation of resolving macrophages; miR-223 downregulated in human NASH	(100, 102)
OTHER			
CD47	Non-phagocytes	Hepatocellular carcinoma	(118, 119)

neutrophils can act as anti-inflammatory modulators that support the formation of pro-resolving macrophages via the release of micro-vesicles carrying microRNAs (100). MicroRNA-223, which inhibits NLRP3 inflammasome activation (101), is highly expressed by neutrophils. As such, miR-223-deficient mice are highly susceptible to liver inflammation in response to low-dose LPS injection or CCl_4-mediated liver injury. During the resolution phase of inflammation after 1 week of CCl_4 treatment or during the resolving phase after MCD diet feeding, depletion of $Ly6G^+$ neutrophils impairs spontaneous resolution of liver injury and leads to the prevalence of pro-inflammatory $Ly6C^{high}$ monocytes (100). Due to the lack of neutrophils that release miR-223 to be taken up by macrophages, NLPR3 inflammasome protein expression is exacerbated and liver inflammation persists, as pro-inflammatory macrophages cannot be converted into the $CD163^{pos}$ anti-inflammatory, resolving type. Strikingly, this phenotype can be reversed by application of a miR-223 mimetic or the infusion of miR-223-compentent neutrophils. (100). In line with these observations, miR-223 is amongst the most prominent down regulated microRNAs in NASH patients (100, 102).

Involvement of Efferocytosis Mechanisms in Liver Cancer

The tumor microenvironment (TME) is rich in immune cells of which macrophages and other myeloid cells are the most abundant (103, 104) and the presence of high amounts of CD68 and CD163 positive tumor-associated macrophages is associated with poor prognosis in a number of human cancers (105), including hepatocellular carcinoma (HCC) (106). The presence of tumor-associated macrophages (TAM) is also associated with the expression of efferocytosis receptors like MerTK, and the uptake of apoptotic tumor cells may promote further anti-inflammatory, tumor promoting polarization of TAMs (107). In hepatocellular carcinoma, molecules involved in efferocytosis are clinically relevant. The presence of soluble Axl in the serum of patients, for instance, is used as a biomarker for liver cirrhosis and HCC development (108, 109) and receptor tyrosine kinase inhibitors that can inhibit Axl function are currently being tested in therapeutic settings (110). However, here it is not clear whether Axl function in tumor-associated macrophages plays a role, as hepatocellular cancer cells themselves overexpress Axl, which provides survival advantages via activation of downstream signal transduction promoting epithelial to mesenchymal transition (EMT) (111). Similarly, overexpression of the bridging molecule Gas6 also promotes HCC metastasis via direct signaling through the Axl receptor in carcinoma cells (112). Contrary to this, several studies in preclinical cancer models have provided evidence that efferocytosis, mostly, but not exclusively, dependent on the TAM receptor MerTK is involved in promoting tumor progression, and several Axl, Tyro3, and MerTK targeting small molecular inhibitors are being tested in clinical trials (113). Tim3 expression on TAMs also correlates negatively with HCC patient survival, most likely via the promotion of pro-inflammatory IL-6 production by Tim3 expressing macrophages (114), which is thought to be essential for the development of hepatocellular carcinoma (115). Although the process of efferocytosis is associated with the development of an M2-like pro-resolving anti-inflammatory phenotype in tumor associated macrophages, which supports tumor progression, and oppositely the induction of pro-inflammatory M1-like macrophages promotes anti-tumor immunity (116), boosting the pro-inflammatory milieu in the absence of efferocytosis may contrarily promote tumorigenesis in the preneoplastic stage of carcinogenesis in the liver (117). Lastly, tumors may also evade immunosurveillance, by preventing recognition for efferocytosis. The "don't eat me" ligand CD47 is highly overexpressed in HCC samples and preventing interaction with SIRP1α by blocking anti-CD47 antibodies augments macrophage efferocytosis of HCC cells *in vitro* and diminishes HCC tumor growth in *in vivo* xenograft models (118, 119).

CONCLUSION

The removal of dead cells poses a challenge to the maintenance of tolerance and the return to tissue integrity after inflammation or injury. Also in the liver, the maintenance of and return to immune homeostasis relies on functional efferocytosis (as summarized in **Table 1**), as demonstrated by the development of hepatic autoimmune disease in TAM receptor deficient

mice. It has not been elucidated whether specific efferocytosis mechanisms exist in the liver. As during liver disease the most abundant cells involved in efferocytosis are infiltrating myeloid cells, general efferocytosis mechanisms known to be operative in macrophages have been investigated and found to play a role. However, under homeostatic conditions the most abundant myeloid cells in the liver are Kupffer cells. Due to their different ontogeny and expression of specific phagocytic receptors, such as the PS-receptor Tim4, complement receptor Vsig4 and the C-type lectin Clec4f, Kupffer cells may well-utilize additional pathways for efferocytosis under homeostasis. In liver disease, the use of global and cell specific knock-out mouse models for molecules involved in various aspects of efferocytosis has shown that efferocytosis mechanisms are heavily deployed in virtually all settings and stages of disease. The absence of functional efferocytosis in these genetic models can on the one hand lead to exacerbation of liver inflammation and injury or on the other hand to a lack and/or delay of resolution of inflammation and a insufficient return to tissue integrity. These opposite effects can be partly explained by the type of liver injury/inflammatory response (e.g., due to toxins or diet induced), but also by the length of the disease course and the timing of interference with efferocytosis. For instance, the inhibition of sufficient pro-inflammatory signaling can lead to defects in resolution, due to the fact that already early inflammatory responses set the stage for the induction of anti-inflammatory, pro-resolving pathways. The following inefficient induction of efferocytosis mechanisms impairs the clearance of damaged and apoptotic cells and subsequent loss of tissue homeostasis. In different etiologies of liver disease, it will be essential to understand the tempo-spatial contribution of pro- and anti-inflammatory and efferocytosis mechanisms in order to utilize these for treatment and prevention strategies in liver disease.

AUTHOR CONTRIBUTIONS

AH, GT, and LD contributed to analysis of publications, drafting of the manuscript, and critical revision of the content.

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REFERENCES

1. Wium M, Paccez JD, Zerbini LF. The dual role of TAM receptors in autoimmune diseases and cancer: an overview. *Cells*. (2018) 7:166. doi: 10.3390/cells7100166
2. Bäck M, Yurdagul A, Tabas I, Öörni K, Kovanen PT. Inflammation and its resolution in atherosclerosis: mediators and therapeutic opportunities. *Nat Rev Cardiol*. (2019) 16:389–406. doi: 10.1038/s41569-019-0169-2
3. Grabiec AM, Hussell T. The role of airway macrophages in apoptotic cell clearance following acute and chronic lung inflammation. *Semin Immunopathol*. (2016) 38:409–23. doi: 10.1007/s00281-016-0555-3
4. Terpstra V, van Berkel TJ. Scavenger receptors on liver Kupffer cells mediate the *in vivo* uptake of oxidatively damaged red blood cells in mice. *Blood*. (2000) 95:2157–63. doi: 10.1182/blood.V95.6.2157
5. Shi J, Fujieda H, Kokubo Y, Wake K. Apoptosis of neutrophils and their elimination by Kupffer cells in rat liver. *Hepatology*. (1996) 24:1256–63. doi: 10.1002/hep.510240545
6. Shi J, Gilbert GE, Kokubo Y, Ohashi T. Role of the liver in regulating numbers of circulating neutrophils. *Blood*. (2001) 98:1226–30. doi: 10.1182/blood.V98.4.1226
7. Crispe IN, Dao T, Klugewitz K, Mehal WZ, Metz DP. The liver as a site of T-cell apoptosis: graveyard, or killing field? *Immunol Rev*. (2000) 174:47–62. doi: 10.1034/j.1600-0528.2002.017412.x
8. Tang D, Kang R, Berghe TV, Vandenabeele P, Kroemer G. The molecular machinery of regulated cell death. *Cell Res*. (2019) 29:347–64. doi: 10.1038/s41422-019-0164-5
9. Lee S-J, Park S-Y, Jung M-Y, Bae SM, Kim I-S. Mechanism for phosphatidylserine-dependent erythrophagocytosis in mouse liver. *Blood*. (2011) 117:5215–23. doi: 10.1182/blood-2010-10-313239
10. Davies SP, Reynolds GM, Stamataki Z. Clearance of apoptotic cells by tissue epithelia: a putative role for hepatocytes in liver efferocytosis. *Front Immunol*. (2018) 9:44. doi: 10.3389/fimmu.2018.00044
11. Park S-Y, Kim I-S. Engulfment signals and the phagocytic machinery for apoptotic cell clearance. *Exp Mol Med*. (2017) 49:e331. doi: 10.1038/emm.2017.52
12. Medina CB, Ravichandran KS. Do not let death do us part: "find-me" signals in communication between dying cells and the phagocytes. *Cell Death Differ*. (2016) 23:979–89. doi: 10.1038/cdd.2016.13
13. Kabarowski JH. G2A and LPC: regulatory functions in immunity. *Prostaglandins Other Lipid Mediat*. (2009) 89:73–81. doi: 10.1016/j.prostaglandins.2009.04.007
14. Bellido-Martín L, de Frutos PG. Vitamin K-dependent actions of Gas6. *Vitam Horm*. (2008) 78:185–209. doi: 10.1016/S0083-6729(07)00009-X
15. Rothlin CV, Lemke G. TAM receptor signaling and autoimmune disease. *Curr Opin Immunol*. (2010) 22:740–6. doi: 10.1016/j.coi.2010.10.001
16. Ramirez-Ortiz ZG, Pendergraft WF, Prasad A, Byrne MH, Iram T, Blanchette CJ, et al. The scavenger receptor SCARF1 mediates the clearance of apoptotic cells and prevents autoimmunity. *Nat Immunol*. (2013) 14:917–26. doi: 10.1038/ni.2670
17. Païdassi H, Tacnet-Delorme P, Verneret M, Gaboriaud C, Houen G, Duus K, et al. Investigations on the C1q-calreticulin-phosphatidylserine interactions yield new insights into apoptotic cell recognition. *J Mol Biol*. (2011) 408:277–90. doi: 10.1016/j.jmb.2011.02.029
18. Blackburn JWD, Lau DHC, Liu EY, Ellins J, Vrieze AM, Pawlak EN, et al. Soluble CD93 is an apoptotic cell opsonin recognized by α xβ 2. *Eur J Immunol*. (2019) 49:600–10. doi: 10.1002/eji.201847801
19. Nishi C, Yanagihashi Y, Segawa K, Nagata S. MERTK tyrosine kinase receptor together with TIM4 phosphatidylserine receptor mediates distinct signal transduction pathways for efferocytosis and cell proliferation. *J Biol Chem*. (2019) 294:7221–30. doi: 10.1074/jbc.RA118.006628
20. Flannagan RS, Canton J, Furuya W, Glogauer M, Grinstein S. The phosphatidylserine receptor TIM4 utilizes integrins as coreceptors to effect phagocytosis. *Mol Biol Cell*. (2014) 25:1511–22. doi: 10.1091/mbc.e13-04-0212
21. Borrego F. The CD300 molecules: an emerging family of regulators of the immune system. *Blood*. (2013) 121:1951–60. doi: 10.1182/blood-2012-09-435057
22. Nakahashi-Oda C, Tahara-Hanaoka S, Honda S-I, Shibuya K, Shibuya A. Identification of phosphatidylserine as a ligand for the CD300a immunoreceptor. *Biochem Biophys Res Commun*. (2012) 417:646–50. doi: 10.1016/j.bbrc.2011.12.025

23. Tian L, Choi S-C, Murakami Y, Allen J, Morse HC, Qi C-F, et al. p85α recruitment by the CD300f phosphatidylserine receptor mediates apoptotic cell clearance required for autoimmunity suppression. *Nat Comms*. (2014) 5:3146. doi: 10.1038/ncomms4146
24. Murakami Y, Tian L, Voss OH, Margulies DH, Krzewski K, Coligan JE. CD300b regulates the phagocytosis of apoptotic cells via phosphatidylserine recognition. *Cell Death Differ*. (2014) 21:1746–57. doi: 10.1038/cdd.2014.86
25. Friggeri A, Banerjee S, Biswas S, de Freitas A, Liu G, Bierhaus A, et al. Participation of the receptor for advanced glycation end products in efferocytosis. *J Immunol*. (2011) 186:6191–8. doi: 10.4049/jimmunol.1004134
26. Davis K, Banerjee S, Friggeri A, Bell C, Abraham E, Zerfaoui M. Poly(ADP-ribosyl)ation of high mobility group box 1 (HMGB1) protein enhances inhibition of efferocytosis. *Mol Med*. (2012) 18:359–69. doi: 10.2119/molmed.2011.00203
27. Zhang Z, Jiang Y, Zhou Z, Huang J, Chen S, Zhou W, et al. Scavenger receptor A1 attenuates aortic dissection via promoting efferocytosis in macrophages. *Biochem Pharmacol*. (2019) 168:392–403. doi: 10.1016/j.bcp.2019.07.027
28. Tao H, Yancey PG, Babaev VR, Blakemore JL, Zhang Y, Ding L, et al. Macrophage SR-BI mediates efferocytosis via Src/PI3K/Rac1 signaling and reduces atherosclerotic lesion necrosis. *J Lipid Res*. (2015) 56:1449–60. doi: 10.1194/jlr.M056689
29. Driscoll WS, Vaisar T, Tang J, Wilson CL, Raines EW. Macrophage ADAM17 deficiency augments CD36-dependent apoptotic cell uptake and the linked anti-inflammatory phenotype. *Circ Res*. (2013) 113:52–61. doi: 10.1161/CIRCRESAHA.112.300683
30. Matozaki T, Murata Y, Okazawa H, Ohnishi H. Functions and molecular mechanisms of the CD47-SIRPalpha signalling pathway. *Trends Cell Biol*. (2009) 19:72–80. doi: 10.1016/j.tcb.2008.12.001
31. Barkal AA, Brewer RE, Markovic M, Kowarsky M, Barkal SA, Zaro BW, et al. CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy. *Nature*. (2019) 572:392–6. doi: 10.1038/s41586-019-1456-0
32. Elliott MR, Koster KM, Murphy PS. Efferocytosis signaling in the regulation of macrophage inflammatory responses. *J Immunol*. (2017) 198:1387–94. doi: 10.4049/jimmunol.1601520
33. Szondy Z, Sarang Z, Kiss B, Garabuczi É, Köröskényi K. Anti-inflammatory mechanisms triggered by apoptotic cells during their clearance. *Front Immunol*. (2017) 8:909. doi: 10.3389/fimmu.2017.00909
34. Lee C-H, Chun T. Anti-inflammatory role of TAM family of receptor tyrosine kinases via modulating macrophage function. *Mol Cells*. (2019) 42:1–7. doi: 10.14348/molcells.2018.0419
35. Haskó G, Pacher P. Regulation of macrophage function by adenosine. *Arterioscler Thromb Vasc Biol*. (2012) 32:865–9. doi: 10.1161/ATVBAHA.111.226852
36. Röszer T. Transcriptional control of apoptotic cell clearance by macrophage nuclear receptors. *Apoptosis*. (2017) 22:284–94. doi: 10.1007/s10495-016-1310-x
37. A-Gonzalez N, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, et al. Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. *Immunity*. (2009) 31:245–8. doi: 10.1016/j.immuni.2009.06.018
38. Heckmann BL, Boada-Romero E, Cunha LD, Magne J, Green DR. LC3-associated phagocytosis and inflammation. *J Mol Biol*. (2017) 429:3561–76. doi: 10.1016/j.jmb.2017.08.012
39. Wong SW, Sil P, Martinez J. Rubicon: LC3-associated phagocytosis and beyond. *FEBS J*. (2017) 285:1379–88. doi: 10.1111/febs.14354
40. Han J, Bae J, Choi C-Y, Choi S-P, Kang H-S, Jo E-K, et al. Autophagy induced by AXL receptor tyrosine kinase alleviates acute liver injury via inhibition of NLRP3 inflammasome activation in mice. *Autophagy*. (2016) 12:2326–43. doi: 10.1080/15548627.2016.1235124
41. Martinez J, Almendinger J, Oberst A, Ness R, Dillon CP, Fitzgerald P, et al. Microtubule-associated protein 1 light chain 3 alpha. (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proc Natl Acad Sci USA*. (2011) 108:17396–401. doi: 10.1073/pnas.1113421108
42. Martinez J, Cunha LD, Park S, Yang M, Lu Q, Orchard R, et al. Non-canonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. *Nature*. (2016) 533:115–9. doi: 10.1038/nature17950
43. Bosurgi L, Cao YG, Cabeza-Cabrerizo M, Tucci A, Hughes LD, Kong Y, et al. Macrophage function in tissue repair and remodeling requires IL-4 or IL-13 with apoptotic cells. *Science*. (2017) 356:1072–6. doi: 10.1126/science.aai8132
44. Proto JD, Doran AC, Gusarova G, Yurdagul A, Sozen E, Subramanian M, et al. Regulatory T cells promote macrophage efferocytosis during inflammation resolution. *Immunity*. (2018) 49:666–77.e6. doi: 10.1016/j.immuni.2018.07.015
45. Grabiec AM, Goenka A, Fife ME, Fujimori T, Hussell T. Axl and MerTK receptor tyrosine kinases maintain human macrophage efferocytic capacity in the presence of viral triggers. *Eur J Immunol*. (2018) 48:855–60. doi: 10.1002/eji.201747283
46. Rothlin CV, Carrera-Silva EA, Bosurgi L, Ghosh S. TAM receptor signaling in immune homeostasis. *Annu Rev Immunol*. (2015) 33:355–91. doi: 10.1146/annurev-immunol-032414-112103
47. Ginhoux F, Guilliams M. Tissue-resident macrophage ontogeny and homeostasis. *Immunity*. (2016) 44:439–49. doi: 10.1016/j.immuni.2016.02.024
48. David BA, Rezende RM, Antunes MM, Santos MM, Freitas Lopes MA, Diniz AB, et al. Combination of mass cytometry and imaging analysis reveals origin, location, and functional repopulation of liver myeloid cells in mice. *Gastroenterology*. (2016) 151:1176–91. doi: 10.1053/j.gastro.2016.08.024
49. Yona S, Kim K-W, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*. (2013) 38:79–91. doi: 10.1016/j.immuni.2012.12.001
50. Devisscher L, Scott CL, Lefere S, Raevens S, Bogaerts E, Paridaens A, et al. Non-alcoholic steatohepatitis induces transient changes within the liver macrophage pool. *Cell Immunol*. (2017) 322:74–83. doi: 10.1016/j.cellimm.2017.10.006
51. van der Tuin SJL, Li Z, Berbée JFP, Verkouter I, Ringnalda LE, Neele AE, et al. Lipopolysaccharide lowers cholesteryl ester transfer protein by activating F4/80$^+$Clec4f$^+$Vsig4$^+$Ly6C$^-$ kupffer cell subsets. *J Am Heart Assoc*. (2018) 7:395. doi: 10.1161/JAHA.117.008105
52. Goh YPS, Henderson NC, Heredia JE, Red Eagle A, Odegaard JI, Lehwald N, et al. Eosinophils secrete IL-4 to facilitate liver regeneration. *Proc Natl Acad Sci USA*. (2013) 110:9914–9. doi: 10.1073/pnas.1304046110
53. Theurl I, Hilgendorf I, Nairz M, Tymoszuk P, Haschka D, Asshoff M, et al. On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. *Nat Med*. (2016) 22:945–51. doi: 10.1038/nm.4146
54. Triantafyllou E, Pop OT, Possamai LA, Wilhelm A, Liaskou E, Singanayagam A, et al. MerTK expressing hepatic macrophages promote the resolution of inflammation in acute liver failure. *Gut*. (2018) 67:333–47. doi: 10.1136/gutjnl-2016-313615
55. Trahtemberg U, Mevorach D. Apoptotic cells induced signaling for immune homeostasis in macrophages and dendritic cells. *Front Immunol*. (2017) 8:1356. doi: 10.3389/fimmu.2017.01356
56. Zhang M, Xu S, Han Y, Cao X. Apoptotic cells attenuate fulminant hepatitis by priming Kupffer cells to produce interleukin-10 through membrane-bound TGF-β. *Hepatology*. (2010) 53:306–16. doi: 10.1002/hep.24029
57. Rodriguez-Fernandez S, Pujol-Autonell I, Brianso F, Perna-Barrull D, Cano-Sarabia M, Garcia-Jimeno S, et al. Phosphatidylserine-liposomes promote tolerogenic features on dendritic cells in human type 1 diabetes by apoptotic mimicry. *Front Immunol*. (2018) 9:253. doi: 10.3389/fimmu.2018.00253
58. Yousefi-Manesh H, Shirooie S, Partoazar A, Nikoui V, Estakhri MRA, Bakhtiarian A. Hepatoprotective effects of phosphatidylserine liposomes on carbon tetrachloride-induced hepatotoxicity in rats. *J Cell Biochem*. (2019) 120:11853–8. doi: 10.1002/jcb.28464
59. Ishimaru M, Yusuke N, Tsukimoto M, Harada H, Takenouchi T, Kitani H, et al. Purinergic signaling via P2Y receptors up-mediates IL-6 production by liver macrophages/Kupffer cells. *J Toxicol Sci*. (2014) 39:413–23. doi: 10.2131/jts.39.413
60. Gonzales E, Julien B, Serrière-Lanneau V, Nicou A, Doignon I, Lagoudakis L, et al. ATP release after partial hepatectomy regulates liver regeneration in the rat. *J Hepatol*. (2010) 52:54–62. doi: 10.1016/j.jhep.2009.10.005
61. Hoque R, Sohail MA, Salhanick S, Malik AF, Ghani A, Robson SC, et al. P2X7 receptor-mediated purinergic signaling promotes liver injury in acetaminophen hepatotoxicity in mice. *AJP*. (2012) 302:G1171–9. doi: 10.1152/ajpgi.00352.2011

62. Savio LEB, de Andrade Mello P, Figliuolo VR, de Avelar Almeida TF, Santana PT, Oliveira SDS, et al. CD39 limits P2X7 receptor inflammatory signaling and attenuates sepsis-induced liver injury. *J Hepatol*. (2017) 67:716–26. doi: 10.1016/j.jhep.2017.05.021
63. Rothweiler S, Feldbrügge L, Jiang ZG, Csizmadia E, Longhi MS, Vaid K, et al. Selective deletion of ENTPD1/CD39 in macrophages exacerbates biliary fibrosis in a mouse model of sclerosing cholangitis. *Purinergic Signal*. (2019) 15:375–85. doi: 10.1007/s11302-019-09664-3
64. Frasch SC, Fernandez-Boyanapalli RF, Berry KZ, Leslie CC, Bonventre JV, Murphy RC, et al. Signaling via macrophage G2A enhances efferocytosis of dying neutrophils by augmentation of Rac activity. *J Biol Chem*. (2011) 286:12108–22. doi: 10.1074/jbc.M110.181800
65. Lee H-J, Ko H-J, Song D-K, Jung Y-J. Lysophosphatidylcholine promotes phagosome maturation and regulates inflammatory mediator production through the protein kinase A-phosphatidylinositol 3 kinase-p38 mitogen-activated protein kinase signaling pathway during *Mycobacterium tuberculosis* infection in mouse macrophages. *Front Immunol*. (2018) 9:920. doi: 10.3389/fimmu.2018.00920
66. Assunção LS, Magalhães KG, Carneiro AB, Molinaro R, Almeida PE, Atella GC, et al. Schistosomal-derived lysophosphatidylcholine triggers M2 polarization of macrophages through PPARγ dependent mechanisms. *Biochim Biophys Acta Mol Cell Biol Lipids*. (2017) 1862:246–54. doi: 10.1016/j.bbalip.2016.11.006
67. Li H-M, Jang JH, Jung J-S, Shin J, Park CO, Kim Y-J, et al. G2A protects mice against sepsis by modulating kupffer cell activation: cooperativity with adenosine receptor 2b. *J Immunol*. (2019) 202:527–38. doi: 10.4049/jimmunol.1700783
68. Becker S, Kinny-Köster B, Bartels M, Scholz M, Seehofer D, Berg T, et al. Low sphingosine-1-phosphate plasma levels are predictive for increased mortality in patients with liver cirrhosis. *PLoS ONE*. (2017) 12:e0174424. doi: 10.1371/journal.pone.0174424
69. Dong H, Xiao J, Zhu R, Liu B, Dong M, Luo D, et al. Serum sphingosine 1-phosphate in hepatocellular carcinoma patients is related to HBV infection. *J BUON*. (2018) 23:1711–6.
70. Yang L, Han Z, Tian L, Mai P, Zhang Y, Wang L, et al. Sphingosine 1-phosphate receptor 2 and 3 mediate bone marrow-derived monocyte/macrophage motility in cholestatic liver injury in mice. *Sci Rep*. (2015) 5:13423. doi: 10.1038/srep13423
71. Mauer AS, Hirsova P, Maiers JL, Shah VH, Malhi H. Inhibition of sphingosine 1-phosphate signaling ameliorates murine non-alcoholic steatohepatitis. *AJP: Gastrointest Liver Physiol*. (2017) 312:G300–13. doi: 10.1152/ajpgi.00222.2016
72. Wang Q, Wei S, Zhou H, Shen G, Gan X, Zhou S, et al. Hyperglycemia exacerbates acetaminophen-induced acute liver injury by promoting liver-resident macrophage proinflammatory response via AMPK/PI3K/AKT-mediated oxidative stress. *Cell Death Discov*. (2019) 5:119–12. doi: 10.1038/s41420-019-0198-y
73. Hu Y, Yang C, Shen G, Yang S, Cheng X, Cheng F, et al. Hyperglycemia-triggered sphingosine-1-phosphate and sphingosine-1-phosphate receptor 3 signaling worsens liver ischemia/reperfusion injury by regulating M1/M2 polarization. *Liver Transpl*. (2019) 25:1074–90. doi: 10.1002/lt.25470
74. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol*. (2000) 20:4106–14. doi: 10.1128/MCB.20.11.4106-4114.2000
75. MacParland SA, Liu JC, Ma X-Z, Innes BT, Bartczak AM, Gage BK, et al. Single cell RNA sequencing of human liver reveals distinct intrahepatic macrophage populations. *Nat Comms*. (2018) 9:4383–21. doi: 10.1038/s41467-018-06318-7
76. Gadd VL, Patel PJ, Jose S, Horsfall L, Powell EE, Irvine KM. Altered peripheral blood monocyte phenotype and function in chronic liver disease: implications for hepatic recruitment and systemic inflammation. *PLoS ONE*. (2016) 11:e0157771. doi: 10.1371/journal.pone.0157771
77. Dal-Secco D, Wang J, Zeng Z, Kolaczkowska E, Wong CHY, Petri B, et al. A dynamic spectrum of monocytes arising from the in situ reprogramming of $CCR2^+$ monocytes at a site of sterile injury. *J Exp Med*. (2015) 212:447–56. doi: 10.1084/jem.20141539
78. Aspinall AI, Curbishley SM, Lalor PF, Weston CJ, Blahova M, Liaskou E, et al. CX_3CR1 and vascular adhesion protein-1-dependent recruitment of $CD16^+$ monocytes across human liver sinusoidal endothelium. *Hepatology*. (2010) 51:2030–9. doi: 10.1002/hep.23591
79. Karlmark KR, Zimmermann HW, Roderburg C, Gassler N, Wasmuth HE, Luedde T, et al. The fractalkine receptor CX3CR1 protects against liver fibrosis by controlling differentiation and survival of infiltrating hepatic monocytes. *Hepatology*. (2010) 52:1769–82. doi: 10.1002/hep.23894
80. Lafdil F, Chobert M-N, Deveaux V, Zafrani E-S, Mavier P, Nakano T, et al. Growth arrest-specific protein 6 deficiency impairs liver tissue repair after acute toxic hepatitis in mice. *J Hepatol*. (2009) 51:55–66. doi: 10.1016/j.jhep.2009.02.030
81. Qi N, Liu P, Zhang Y, Wu H, Chen Y, Han D. Development of a spontaneous liver disease resembling autoimmune hepatitis in mice lacking tyro3, axl and mer receptor tyrosine kinases. *PLoS ONE*. (2013) 8:e66604. doi: 10.1371/journal.pone.0066604
82. Bernsmeier C, Pop OT, Singanayagam A, Triantafyllou E, Patel VC, Weston CJ, et al. Patients with acute-on-chronic liver failure have increased numbers of regulatory immune cells expressing the receptor tyrosine kinase MERTK. *Gastroenterology*. (2015) 148:603–15.e14. doi: 10.1053/j.gastro.2014.11.045
83. Patsenker E, Popov Y, Stickel F, Schneider V, Ledermann M, Sägesser H, et al. Pharmacological inhibition of integrin alphavbeta3 aggravates experimental liver fibrosis and suppresses hepatic angiogenesis. *Hepatology*. (2009) 50:1501–11. doi: 10.1002/hep.23144
84. Rantakari P, Patten DA, Valtonen J, Karikoski M, Gerke H, Dawes H, et al. Stabilin-1 expression defines a subset of macrophages that mediate tissue homeostasis and prevent fibrosis in chronic liver injury. *Proc Natl Acad Sci USA*. (2016) 113:9298–303. doi: 10.1073/pnas.1604780113
85. Liu W, Bai F, Wang H, Liang Y, Du X, Liu C, et al. Tim-4 inhibits NLRP3 inflammasome via the LKB1/AMPKα pathway in macrophages. *J Immunol*. (2019) 203:990–1000. doi: 10.4049/jimmunol.1900117
86. Nishi C, Toda S, Segawa K, Nagata S. Tim4- and MerTK-mediated engulfment of apoptotic cells by mouse resident peritoneal macrophages. *Mol Cell Biol*. (2014) 34:1512–20. doi: 10.1128/MCB.01394-13
87. Campana L, Starkey Lewis PJ, Pellicoro A, Aucott RL, Man J, O'Duibhir E, et al. The STAT3-IL-10-IL-6 pathway is a novel regulator of macrophage efferocytosis and phenotypic conversion in sterile liver injury. *J Immunol*. (2018) 200:1169–87. doi: 10.4049/jimmunol.1701247
88. Viaud M, Ivanov S, Vujic N, Duta-Mare M, Aira L-E, Barouillet T, et al. Lysosomal cholesterol hydrolysis couples efferocytosis to anti-inflammatory oxysterol production. *Circ Res*. (2018) 122:1369–84. doi: 10.1161/CIRCRESAHA.117.312333
89. Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. *Nat Rev Immunol*. (2015) 15:104–16. doi: 10.1038/nri3793
90. Green DR, Oguin TH, Martinez J. The clearance of dying cells: table for two. *Cell Death Differ*. (2016) 23:915–26. doi: 10.1038/cdd.2015.172
91. Fadok VA, Bratton DL, Guthrie L, Henson PM. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *J Immunol*. (2001) 166:6847–54. doi: 10.4049/jimmunol.166.11.6847
92. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest*. (1998) 101:890–8. doi: 10.1172/JCI1112
93. Fukada H, Yamashina S, Izumi K, Komatsu M, Tanaka K, Ikejima K, et al. Suppression of autophagy sensitizes Kupffer cells to endotoxin. *Hepatol Res*. (2012) 42:1112–8. doi: 10.1111/j.1872-034X.2012.01024.x
94. Lodder J, Denaës T, Chobert M-N, Wan J, El-Benna J, Pawlotsky J-M, et al. Macrophage autophagy protects against liver fibrosis in mice. *Autophagy*. (2015) 11:1280–92. doi: 10.1080/15548627.2015.1058473
95. Ilyas G, Zhao E, Liu K, Lin Y, Tesfa L, Tanaka KE, et al. Macrophage autophagy limits acute toxic liver injury in mice through down regulation of interleukin-1β. *J Hepatol*. (2016) 64:118–27. doi: 10.1016/j.jhep.2015.08.019
96. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. (2013) 13:159–75. doi: 10.1038/nri3399

97. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol*. (2014) 15:135–47. doi: 10.1038/nrm3737
98. Graubardt N, Vugman M, Mouhadeb O, Caliari G, Pasmanik-Chor M, Reuveni D, et al. Ly6Chi monocytes and their macrophage descendants regulate neutrophil function and clearance in acetaminophen-induced liver injury. *Front Immunol*. (2017) 8:626. doi: 10.3389/fimmu.2017.00626
99. Bukong TN, Cho Y, Iracheta-Vellve A, Saha B, Lowe P, Adejumo A, et al. Abnormal neutrophil traps and impaired efferocytosis contribute to liver injury and sepsis severity after binge alcohol use. *J Hepatol*. (2018) 69:1145–54. doi: 10.1016/j.jhep.2018.07.005
100. Calvente CJ, Tameda M, Johnson CD, Del Pilar H, Lin YC, Adronikou N, et al. Neutrophils contribute to spontaneous resolution of liver inflammation and fibrosis via microRNA-223. *J Clin Invest*. (2019) 130:187. doi: 10.1172/JCI122258
101. Bauernfeind F, Rieger A, Schildberg FA, Knolle PA, Schmid-Burgk JL, Hornung V. NLRP3 inflammasome activity is negatively controlled by miR-223. *J Immunol*. (2012) 189:4175–81. doi: 10.4049/jimmunol.1201516
102. Cheung O, Puri P, Eicken C, Contos MJ, Mirshahi F, Maher JW, et al. Non-alcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. *Hepatology*. (2008) 48:1810–20. doi: 10.1002/hep.22569
103. Biswas SK, Allavena P, Mantovani A. Tumor-associated macrophages: functional diversity, clinical significance, and open questions. *Semin Immunopathol*. (2013) 35:585–600. doi: 10.1007/s00281-013-0367-7
104. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol*. (2012) 12:253–68. doi: 10.1038/nri3175
105. Engblom C, Pfirschke C, Pittet MJ. The role of myeloid cells in cancer therapies. *Nat Rev Cancer*. (2016) 16:447–62. doi: 10.1038/nrc.2016.54
106. Wu K, Kryczek I, Chen L, Zou W, Welling TH. Kupffer cell suppression of $CD8^+$ T cells in human hepatocellular carcinoma is mediated by B7-H1/programmed death-1 interactions. *Cancer Res*. (2009) 69:8067–75. doi: 10.1158/0008-5472.CAN-09-0901
107. Akalu YT, Rothlin CV, Ghosh S. TAM receptor tyrosine kinases as emerging targets of innate immune checkpoint blockade for cancer therapy. *Immunol Rev*. (2017) 276:165–77. doi: 10.1111/imr.12522
108. Dengler M, Staufer K, Huber H, Stauber R, Bantel H, Weiss KH, et al. Soluble Axl is an accurate biomarker of cirrhosis and hepatocellular carcinoma development: results from a large scale multicenter analysis. *Oncotarget*. (2017) 8:46234–48. doi: 10.18632/oncotarget.17598
109. Staufer K, Dengler M, Huber H, Marculescu R, Stauber R, Lackner C, et al. The non-invasive serum biomarker soluble Axl accurately detects advanced liver fibrosis and cirrhosis. *Cell Death Dis*. (2017) 8:e3135. doi: 10.1038/cddis.2017.554
110. Personeni N, Rimassa L, Pressiani T, Smiroldo V, Santoro A. Cabozantinib for the treatment of hepatocellular carcinoma. *Expert Rev Anticancer Ther*. (2019) 19:847–55. doi: 10.1080/14737140.2019.1674141
111. Pinato DJ, Brown MW, Trousil S, Aboagye EO, Beaumont J, Zhang H, et al. Integrated analysis of multiple receptor tyrosine kinases identifies Axl as a therapeutic target and mediator of resistance to sorafenib in hepatocellular carcinoma. *Br J Cancer*. (2019) 120:512–21. doi: 10.1038/s41416-018-0373-6
112. Lee H-J, Jeng Y-M, Chen Y-L, Chung L, Yuan R-H. Gas6/Axl pathway promotes tumor invasion through the transcriptional activation of Slug in hepatocellular carcinoma. *Carcinogenesis*. (2014) 35:769–75. doi: 10.1093/carcin/bgt372
113. Myers KV, Amend SR, Pienta KJ. Targeting Tyro3, Axl and MerTK (TAM receptors): implications for macrophages in the tumor microenvironment. *Mol Cancer*. (2019) 18:94–14. doi: 10.1186/s12943-019-1022-2
114. Yan W, Liu X, Ma H, Zhang H, Song X, Gao L, et al. Tim-3 fosters HCC development by enhancing TGF-β-mediated alternative activation of macrophages. *Gut*. (2015) 64:1593–604. doi: 10.1136/gutjnl-2014-307671
115. Bergmann J, Müller M, Baumann N, Reichert M, Heneweer C, Bolik J, et al. IL-6 trans-signaling is essential for the development of hepatocellular carcinoma in mice. *Hepatology*. (2017) 65:89–103. doi: 10.1002/hep.28874
116. Capece D, Fischietti M, Verzella D, Gaggiano A, Cicciarelli G, Tessitore A, et al. The inflammatory microenvironment in hepatocellular carcinoma: a pivotal role for tumor-associated macrophages. *BioMed Res Int*. (2013) 2013:187204–15. doi: 10.1155/2013/187204
117. Sun K, Xu L, Jing Y, Han Z, Chen X, Cai C, et al. Autophagy-deficient Kupffer cells promote tumorigenesis by enhancing mtROS-NF-κB-IL1α/β-dependent inflammation and fibrosis during the preneoplastic stage of hepatocarcinogenesis. *Cancer Lett*. (2017) 388:198–207. doi: 10.1016/j.canlet.2016.12.004
118. Lo J, Lau EYT, So FTY, Lu P, Chan VSF, Cheung VCH, et al. Anti-CD47 antibody suppresses tumour growth and augments the effect of chemotherapy treatment in hepatocellular carcinoma. *Liver Int*. (2016) 36:737–45. doi: 10.1111/liv.12963
119. Xiao Z, Chung H, Banan B, Manning PT, Ott KC, Lin S, et al. Antibody mediated therapy targeting CD47 inhibits tumor progression of hepatocellular carcinoma. *Cancer Lett*. (2015) 360:302–9. doi: 10.1016/j.canlet.2015.02.036

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Understanding the Heterogeneity of Resident Liver Macrophages

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Resident tissue macrophages (RTMs) are cells with a high functional plasticity assuming pleiotropic roles in their tissue of residence, from clearance of dead cells and metabolic sensing in steady state to cytokine production and tissue repair during inflammation. The liver has long been considered as only populated by Kupffer cells (KCs), a macrophage population assumed to be in charge of all of these functions. However, we know now that KCs are not the only macrophage population in the liver, that recently was shown to contain also capsular macrophages, monocyte-derived macrophages as well as recruited peritoneal macrophages inherited from previous inflammatory events. These macrophages exhibit different origins, time of establishing residence and locations in the liver, with both ontogenical and environmental factors shaping their identity and functions. Furthermore, liver macrophages reside in a complex environment with a pronounced metabolic zonation. Here, we briefly discuss how these intrinsic and extrinsic factors influence macrophage biology and liver physiology in general. We notably focus on how the recent advances of single cell transcriptomic approaches are changing our understanding of liver macrophages and diseases.

Keywords: liver, macrophage, heterogeneity, single cell RNA sequencing, monocyte

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INTRODUCTION

The liver is known to assume a large repertoire of diverse functions such as detoxication of numerous metabolites, synthesis of essential proteins, or recycling of iron-containing red blood cells (1). Such versatility renders this organ indispensable for a healthy physiological state knowing that the only treatment of liver failure remains limited to organ transplantation. Liver and notably the hepatocytes, its fundamental metabolic units, can be affected by numerous pathologies among which are hepatitis, steatosis, cirrhosis, or hepatocarcinoma (1). Etiology of these distinct diseases is complex and involves genetic and environmental factors yet difficult to stratify in a comprehensive manner. Furthermore, at a mechanistic level, development of a liver pathology such as fibrosis for example not only implies hepatocytes but also the other liver cell populations in the forefront of which are macrophages, but also stellate and endothelial cells (2). So, understanding the relationships that are established between these different essential cellular liver components appear necessary to better understand liver functions and pathologies.

DIVERSITY OF LIVER MACROPHAGES: ORIGIN AND LOCATION MATTER

Macrophages represent by far the most abundant immune cells in the liver. Hepatic macrophages are still often referred as Kupffer cells (KCs) that indeed represent the major fraction of liver macrophages. KCs were first described more than one century ago by Kupffer who initially described them as endothelial cells, components of liver vascular walls (3). Then, a few years later, they were correctly reassigned as macrophages by Browicz (4). Indeed, even if KCs are located in the liver, they do not reside in the parenchyma and are not in direct contact with the hepatocytes, as they are located within the liver sinusoids where they are in contact with the blood compartment (**Figure 1**). KCs were then included in the mononuclear phagocyte system by Van Furth et al. (5) and considered thereafter as the liver-resident monocyte-derived macrophages. But numerous recent studies using notably powerful fate-mapping models have completely revisited the dogma of the monocytic origin of many resident macrophages, including KCs (6–11). It is now clearly established that KCs do not derive from adult circulating monocytes but rather from fetal liver monocytic precursors that expand and maintain themselves during the entire life of the organism (8–10). This renders KC renewal almost independent of bone-marrow derived cells at steady state.

Besides of the embryonically-derived KC population that represents the vast majority of liver macrophages, at least at steady-state, another population of macrophages residing in the hepatic capsule has been recently described (12). These liver capsular macrophages (LCMs) are phenotypically and developmentally different from KCs. Indeed, although expressing typical macrophage markers such as CD64 and F4/80, LCMs are negative for the canonical KC markers Tim4 and Clec4F, and express rather markers traditionally expressed by dendritic cells such as MHCII and CD11c (12, 13). Moreover, LCMs do not derive from embryonic precursors but arise from adult circulating monocytes. Whether such LCMs represent an homogeneous population or comprised subsets of macrophages and dendritic cells remain to be established.

In addition to these two main macrophage populations, the liver may contain a variable amount of recruited blood monocyte-derived macrophages. Indeed, in several inflammatory conditions and notably when KC depletion occurs, replacement by monocyte-derived macrophages can be observed (14, 15). Some of the newly-recruited cells will acquire a similar transcriptomic pattern with time and resolution of inflammation and will establish residence in the liver, assuming similar functions than the original macrophages (15). Interestingly, even if adult monocyte-derived cells represent a very minor fraction of liver macrophages in mice grown in pathogen-free facilities with controlled diets, the situation could be very different in humans which are exposed to a more challenging and diverse environment with notably a plethora of foodborne entero-pathogens and various diets. Each infection, even minor and without triggering any detectable symptoms, could induce monocyte recruitment in the liver with few of them differentiating in monocyte-derived macrophages, as observed in the lung (16). This process could be regarded as an immune scar in the liver, each individual having his own immune history shaped by his past infections but also his genetic identity. So in this context, the notion of steady-state appears very restricted to laboratory mice and hardly transposable to healthy humans. In addition, as human fate-mapping models are lacking, although attempt in the single cell genomic era might soon provide answers (17), the origin (embryonic vs. monocyte-derived) of human macrophages is less understood.

Finally, it has also been shown that murine mature peritoneal macrophages could rapidly invade the liver after an injury (18). By using a model of sterile inflammation induced by thermal injury in the liver, the authors have shown that fully differentiated F4/80^{hi} GATA6^{+} peritoneal macrophages migrated to the site of injury. This non-vascular recruitment was mediated by ATP released from dead cells acting as a damage-associated molecular pattern and involved also the Hyaluronan-CD44 interaction. Recruited peritoneal macrophages are responsible for disassembling the necrotic nuclei of dead cells and authors have shown that depletion of peritoneal macrophages significantly delayed the wound healing process (18). Whether these macrophages can maintain themselves in a long-term manner and can become fully integrated in the liver macrophage network as well as the relevance of this phenomenon in human diseases remain to be determined. A table summarizing the phenotypes of these different liver macrophage populations is provided (**Table 1**) as a comparison with other known liver myeloid populations.

UNDERSTANDING HEPATIC MACROPHAGES THROUGH THEIR NICHE OF RESIDENCE

Different populations of liver macrophages reside in distinct hepatic niches and are therefore exposed to a different microenvironment. It has now been demonstrated that macrophage homeostasis is tightly controlled by tissue-specific and niche-specific signals (19–22). Macrophages are known to be sessile and self-renewing cells (23) implying that they are solely in direct and intimate interactions with only few tissue cells, allowing profound relationship to be established from the first stages of development (24). An exciting question is to understand in the most exhaustive manner how these cells interact together and with the other components of the liver tissue to shape macrophage identity and functions.

Functionally, the liver is organized in metabolic units called acini. The bloodstream flows from the portal vein and hepatic artery, and circulates through the sinusoids toward the central vein. Hepatocytes represent between 60 and 70% of liver cells. It is known for decades that hepatocytes are heterogeneous with a differential production of enzymes along the portal-central axis resulting in a metabolic zonation (25, 26). Therefore, it has been proposed that oxidative energy metabolism, β-oxidation, amino acid catabolism were mostly performed in the portal zone whereas glycolysis and lipogenesis took place

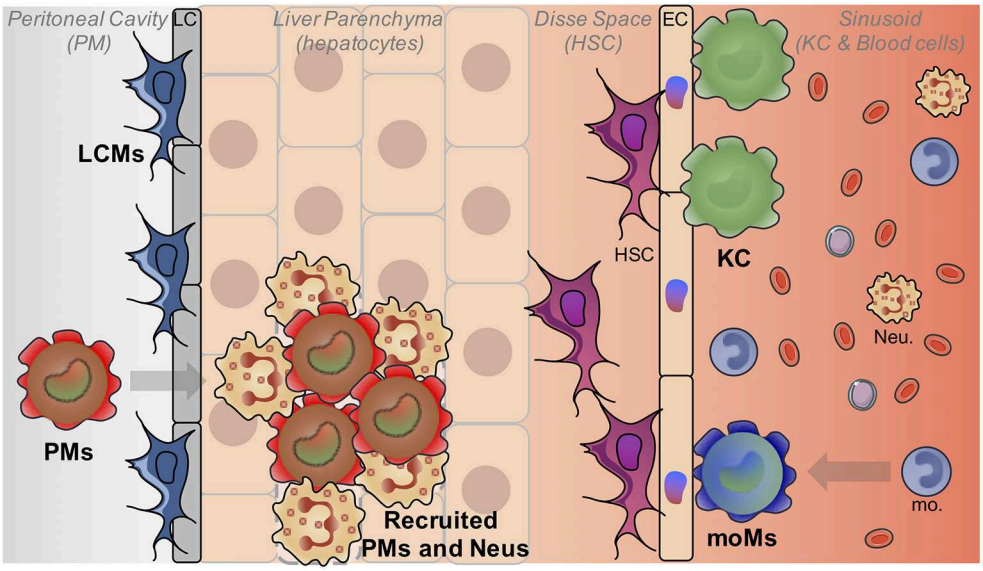

FIGURE 1 | Liver macrophage heterogeneity. The liver is populated by different macrophage populations. The most abundant one is composed by embryonically-derived Kupffer cells (KC) which reside in liver sinusoids and interact mainly with HSC and EC. Monocyte-derived macrophages (MoM) can also acquire a KC-like phenotype after inflammation. Liver capsular macrophages (LCM) are present at the level of the liver capsule (LC). Finally, mature peritoneal macrophages (PMs) can also be recruited in the liver notably in case of injuries in the parenchyma. (Neu, Neutrophils).

TABLE 1 | Phenotype of liver phagocyte populations.

Markers (mouse)	Macrophages				Monocytes	Dendritic cells	Neutrophils
	Kupffer cells	Monocyte-derived liver macs	Capsular macs	Peritoneal macs			
CD11b	+	++ to +	+	**++**	**++**	+	**++**
CD11c	–	–	+	–	–	**++**	–
CD64	+	++ to +	+	+	**++**	+	+
Clec4F	**++**	**– to ++**	–	–	–	–	–
CX3CR1	–	+ to –	**++**	–	+	–	–
F4/80	**++**	**– to ++**	**++**	**++**	–	–	–
Ly6C	+	++ to +	+	+	**++**	+	**++**
Ly6G	–	–	–	–	–	–	**++**
MHCII	+	+	**++**	+	+	**++**	–
Tim4	**++**	**– to ++ (slowly)**	–	+	–	–	–

The different populations of liver phagocytes can be resolved by using a panel of common myeloid cell markers. This list of murine markers is not exhaustive but allows enough resolution to identify these populations. Of note, this separation is not accurate for human samples, as mouse-restricted markers such as F4/80 and Clec4F cannot be used, ontogeny remains unclear and the existence of all the subpopulations described herein has not yet been confirmed. Bold values indicate the positive markers that can be used for an efficient gating strategy.

predominantly in the central zone (25). The decreasing oxygen gradient that is established between the blood arriving in the periportal area and leaving by the central vein is obviously one of the key determining factors of the metabolic zonation (27). A genome-wide description of this phenomenon has recently been established by measuring the transcriptomes of thousands of hepatocytes (28) and confirmed at the protein level (29). The same group has also identified zonation at the level of endothelial cells by using paired-cell RNA sequencing, an innovative strategy allowing the profiling of endothelial cells attached to hepatocytes (30). This co-zonation between genes and functions across hepatocytes and endothelial cell was also confirmed recently in humans (31). Whether such zonation impacts KC phenotype, gene expression profile, and functions remains to be investigated.

The other main population of liver-resident cells, even often overlooked by immunologists are the hepatic stellate cells (HSC).

These pericytes are localized in the space of Disse between hepatocytes and endothelial cells (**Figure 1**). They are mainly known for their vitamin A storage function at resting state (32) but they are also known to drive fibrosis by the production of extracellular matrix once activated and trans-differentiated into myofibroblasts (33). Even if HSCs have been less characterized so far, a zonation of these cells has been reported in porcine livers based essentially on morphological criteria (34, 35). Furthermore, a certain level of heterogeneity of HSCs has been observed in an healthy, non-injured mouse liver (36). A most recent contribution has focused on the heterogeneity of HSCs, quiescent at steady-state or activated in a chemically induced model of fibrosis (37). Interestingly, this study mainly reveals heterogeneity for myofibroblasts in fibrotic contexts. Finally, recent studies have elegantly deciphered how these different cells, hepatocytes, endothelial cells and HSCs efficiently collaborate to drive recruited monocyte transition to macrophages in a specific KC depletion model (38, 39). Authors have notably revealed the crucial role of the DLL4-Notch pathway for the programing of recruited monocytes by liver endothelial cells. This activation results in the production of LXRα, a crucial transcription factor involved in the induction and maintenance of KC identity. In parallel of this, HSCs were shown to produce the macrophage colony stimulating factor and hepatocytes to induce expression by monocytes of ID3 (38), a key transcription factor shown to drive fetal liver monocyte to mature KC transition (11). These studies revealed also that such transition from monocyte to monocyte-derived KC was unexpectedly fast, with the different cells producing complementary signals within hours after recruitment allowing monocytes to acquire their new tissue resident identity.

Altogether, even if at a macroscopic level, liver lobules appear homogeneous with a relatively simple tridimensional architecture, they hide a complexity shaped by many factors that should be taken into account to decipher liver macrophage biology and their potential heterogeneity.

NEW APPROACHES TO IMPROVE OUR UNDERSTANDING OF LIVER MACROPHAGES

So far, most of the studies dealing with liver macrophages have used very few but specific markers such as CD45 (pan-leukocyte marker), CD11b (pan-myeloid marker) and F4/80 (pan-macrophage marker in mice) to study macrophages, most often assimilated to KCs. While it has been very useful to extend our knowledge on KC biology, this conventional approach consisting in defining populations of interest based on the expression of limited markers by flow cytometry appears more and more outdated nowadays. Discoveries of the distinct ontogeny of KCs and monocyte-derived macrophages and the complexity of the liver niche have challenged the view of liver macrophages as a uniform F4/80+ cell population.

The very recent burst in single cell transcriptomics is indeed profoundly reshaping our approaches to solve key questions in immunology (40). This technology offers the obvious advantage to get access to the expression of thousands of genes at the single cell level instead of a handful of markers which may be highly selective, but which nevertheless remain limited. But the most valuable feature of single cell transcriptomics is the unbiased approach that it is offering. Herein, the most meaningful parameters are not the ones previously anticipated but may be completely unexpected ones, designated in an objective manner by unbiased algorithms. Accordingly, so far, most of the cell populations that have been deeply analyzed turn out to be much more heterogeneous than previously anticipated in every organs, with the existence of overlooked clusters with their own identity and functions (22, 41).

In the liver, the idea of the coexistence of different subsets of KCs has been already proposed (42, 43). Of note, these observations were made by using bone marrow chimeras, an irradiation murine model in which there is a huge recruitment of inflammatory monocytes in the liver giving rise to monocyte-derived macrophages. So as in the studies using the specific KC depletion model aforementioned (38, 39), the irradiated liver undergoes a damage resulting in an inflammatory reaction and this context should therefore be considered different than the steady-state. Interestingly, it has been very recently shown that a subset of the embryonically-derived KCs resists to lethal irradiation, through *cdkn1a* upregulation. This radio-resistance property is lost when native KCs are replaced by their monocyte-derived counterparts, showing clearly that ontogeny contribute to macrophage functional heterogeneity (44). Others have also successfully used the mass cytometry to analyze liver macrophages and notably described two subsets of KCs different from infiltrating monocytes (13). Whether these populations represent ontogenetically independent subsets or distinct activation stages residing in specific locations remains to be established.

Single cell transcriptomic approaches now offer unprecedented sensitivity to investigate liver macrophage biology. It also remains to be established if KC subpopulations exist at the transcriptomic single cell level. Interestingly, there are already few databases publically available such as *tabula muris* (45) or the mouse cell atlas (46) for mouse studies, but also human liver databases (31, 47). These databases offer the possibility for everyone, even without being equipped to perform single cell transcriptomics, to ask such questions on liver macrophages or others immune cell heterogeneity and screen for their own potential genes of interest. However, we cannot exclude the possibility that the strategies and technologies used in these studies did not allow for KC heterogeneity to be discovered: the number of cells sampled may have been too low, the sequencing depth may have been too superficial to reveal subtle and deeply hidden transcriptomic signatures, or the techniques allowing the isolation of KC could have introduced a bias of selection of a particular subset. A massive, single-cell study of KC heterogeneity at steady-state that includes a large enough cell population and a pipeline that permits deep sequencing and detects high numbers of genes is urgently needed.

Nevertheless, two recent studies discussed thereafter have exemplified how single cell transcriptomics can be used to gain insights into macrophage biology. The first one has clarified the

crosstalk between endothelial cells, HSC and liver macrophages during the development of non-alcoholic steatohepatitis (NASH) (48). Authors used mainly a mouse model of NASH but also validated their observations in humans. Briefly, by doing single cell sequencing of liver non-parenchymal cells, they have observed that vascular signaling was dysregulated during NASH. They have also observed the emergence of a NASH-specific population of KC, expressing notably Trem2 and CD9. Very interestingly, it was also observed that these effects on endothelial cells and liver macrophages were orchestrated by HSCs via the expression of key secreted factors called "stellakines" (48). The second study, is focused on human liver cirrhosis (49). Authors have sequenced around 100,000 single cells, observed an heterogeneity in endothelial cells and the appearance of a Trem2+ CD9+ fibrotic macrophage population. They have also reconstructed the interactions between endothelial cells, macrophages and HSCs (49). These studies are interesting in many ways but focusing on macrophages, they are in line with another recent study describing a population of adipose tissue Trem2+ CD9+ macrophages that emerge during obesity and that regulate adipocyte hypertrophy and body fat accumulation (50). It argues for a pan-organ role of Trem2 signaling in tissue macrophages that is beyond the limits of hepatology but that is definitively interesting considering what is known on the central role of Trem2 in Alzheimer disease for example (51).

CONCLUDING REMARKS

Herein, we discussed liver macrophage heterogeneity and how the most recent advances in single cell transcriptomics could be used to decipher liver macrophage biology. Clearly, last years of research have revealed an unexpected heterogeneity of liver macrophages, both at ontogeny and environmental levels. We now need to take into account this diversity in future studies focusing on liver diseases, and the use of the most recent and still evolving technologies such as the single cell transcriptomics will be crucial for this.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Sanyal A, Boyer T, Terrault N, Lindor K. *Zakim and Boyer's Hepatology: A Textbook of Liver Disease.* Philadelphia, PA: Elsevier (2017).
2. Pradere JP, Kluwe J, De Minicis S, Jiao JJ, Gwak GY, Dapito DH, et al. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology.* (2013) 58:1461–73. doi: 10.1002/hep.26429
3. Kupffer KV. Ueber Sternzellen der leber. *Archiv fur mikroskopische Anatomie.* (1876) 12:353–8. doi: 10.1007/BF02933897
4. Browicz T. Ueber intravasculare zellen in den blutcapillaren der leberacini. *Separat aus dem anzeiger der akademie der wissenschaften in Krakau.* (1899) 55:420. doi: 10.1007/BF02977740
5. Van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bull World Health Organ.* (1972) 46:845–52.
6. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science.* (2010) 330:841–5. doi: 10.1126/science.1194637
7. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science.* (2012) 336:86–90. doi: 10.1126/science.1219179
8. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity.* (2013) 38:792–804. doi: 10.1016/j.immuni.2013.04.004
9. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity.* (2013) 38:79–91. doi: 10.1016/j.immuni.2012.12.001
10. Hoeffel G, Chen J, Lavin Y, Low D, Almeida FF, See P, et al. C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity.* (2015) 42:665–78. doi: 10.1016/j.immuni.2015.03.011
11. Mass E, Ballesteros I, Farlik M, Halbritter F, Gunther P, Crozet L, et al. Specification of tissue-resident macrophages during organogenesis. *Science.* (2016) 353:aaf4238. doi: 10.1126/science.aaf4238
12. Sierro F, Evrard M, Rizzetto S, Melino M, Mitchell AJ, Florido M, et al. A liver capsular network of monocyte-derived macrophages restricts hepatic dissemination of intraperitoneal bacteria by neutrophil recruitment. *Immunity.* (2017) 47:374–88.e6. doi: 10.1016/j.immuni.2017.07.018
13. David BA, Rezende RM, Antunes MM, Santos MM, Freitas Lopes MA, Diniz AB, et al. Combination of mass cytometry and imaging analysis reveals origin, location, and functional repopulation of liver myeloid cells in mice. *Gastroenterology.* (2016) 151:1176–91. doi: 10.1053/j.gastro.2016.08.024
14. Bleriot C, Dupuis T, Jouvion G, Eberl G, Disson O, Lecuit M. Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection. *Immunity.* (2015) 42:145–58. doi: 10.1016/j.immuni.2014.12.020
15. Scott CL, Zheng F, De Baetselier P, Martens L, Saeys Y, De Prijck S, et al. Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Commun.* (2016) 7:10321. doi: 10.1038/ncomms10321
16. Machiels B, Dourcy M, Xiao X, Javaux J, Mesnil C, Sabatel C, et al. A gammaherpesvirus provides protection against allergic asthma by inducing the replacement of resident alveolar macrophages with regulatory monocytes. *Nat Immunol.* (2017) 18:1310–20. doi: 10.1038/ni.3857
17. Ludwig LS, Lareau CA, Ulirsch JC, Christian E, Muus C, Li LH, et al. Lineage tracing in humans enabled by mitochondrial mutations and single-cell genomics. *Cell.* (2019) 176:1325–39.e22. doi: 10.1016/j.cell.2019.01.022
18. Wang J, Kubes P. A reservoir of mature cavity macrophages that can rapidly invade visceral organs to affect tissue repair. *Cell.* (2016) 165:668–78. doi: 10.1016/j.cell.2016.03.009
19. Okabe Y, Medzhitov R. Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell.* (2014) 157:832–44. doi: 10.1016/j.cell.2014.04.016
20. Guilliams M, Scott CL. Does niche competition determine the origin of tissue-resident macrophages? *Nat Rev Immunol.* (2017) 17:451–60. doi: 10.1038/nri.2017.42
21. Zhou X, Franklin RA, Adler M, Jacox JB, Bailis W, Shyer JA, et al. Circuit design features of a stable two-cell system. *Cell.* (2018) 172:744–57.e17. doi: 10.1016/j.cell.2018.01.015
22. Chakarov S, Lim HY, Tan L, Lim SY, See P, Lum J, et al. Two distinct interstitial macrophage populations coexist across tissues in specific subtissular niches. *Science.* (2019) 363:eaau0964. doi: 10.1126/science.aau0964
23. Baranska A, Shawket A, Jouve M, Baratin M, Malosse C, Voluzan O, et al. Unveiling skin macrophage dynamics explains both tattoo

persistence and strenuous removal. *J Exp Med.* (2018) 215:1115–33. doi: 10.1084/jem.20171608
24. Thion MS, Ginhoux F, Garel S. Microglia and early brain development: an intimate journey. *Science.* (2018) 362:185–9. doi: 10.1126/science.aat0474
25. Jungermann K, Saase D. Heterogeneity of liver parenchymal cells. *Trends Biochem Sci.* (1978) 3:198–202. doi: 10.1016/S0968-0004(78)91764-4
26. Gebhardt R. Metabolic zonation of the liver: regulation and implications for liver function. *Pharmacol Ther.* (1992) 53:275–354. doi: 10.1016/0163-7258(92)90055-5
27. Bhatia SN, Toner M, Foy BD, Rotem A, O'neil KM, Tompkins RG, et al. Zonal liver cell heterogeneity: effects of oxygen on metabolic functions of hepatocytes. *Cell Eng.* (1996) 1:125–35.
28. Halpern KB, Shenhav R, Matcovitch-Natan O, Toth B, Lemze D, Golan M, et al. Single-cell spatial reconstruction reveals global division of labour in the mammalian liver. *Nature.* (2017) 542:352–6. doi: 10.1038/nature21065
29. Ben-Moshe S, Shapira Y, Moor AE, Manco R, Veg T, Halpern KB, et al. Spatial sorting enables comprehensive characterization of liver zonation. *Nat Metab.* (2019) 1:899–911. doi: 10.1038/s42255-019-0109-9
30. Halpern KB, Shenhav R, Massalha H, Toth B, Egozi A, Massasa EE, et al. Paired-cell sequencing enables spatial gene expression mapping of liver endothelial cells. *Nat Biotechnol.* (2018) 36:962–70. doi: 10.1038/nbt.4231
31. Aizarani N, Saviano A, Sagar, M. L, Durand S, Herman JS, Pessaux P, et al. A human liver cell atlas reveals heterogeneity and epithelial progenitors. *Nature.* (2019) 572:199–204. doi: 10.1038/s41586-019-1373-2
32. Geerts A. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis.* (2001) 21:311–35. doi: 10.1055/s-2001-17550
33. Puche JE, Saiman Y, Friedman SL. Hepatic stellate cells and liver fibrosis. *Compr Physiol.* (2013) 3:1473–92. doi: 10.1002/cphy.c120035
34. Wake K, Sato T. Intralobular heterogeneity of perisinusoidal stellate cells in porcine liver. *Cell Tissue Res.* (1993) 273:227–37. doi: 10.1007/BF00312824
35. Zou Z, Ekataksin W, Wake K. Zonal and regional differences identified from precision mapping of vitamin A-storing lipid droplets of the hepatic stellate cells in pig liver: a novel concept of addressing the intralobular area of heterogeneity. *Hepatology.* (1998) 27:1098–108. doi: 10.1002/hep.510270427
36. D'ambrosio DN, Walewski JL, Clugston RD, Berk PD, Rippe RA, Blaner WS. Distinct populations of hepatic stellate cells in the mouse liver have different capacities for retinoid and lipid storage. *PLoS ONE.* (2011) 6:e24993. doi: 10.1371/journal.pone.0024993
37. Krenkel O, Hundertmark J, Ritz TP, Weiskirchen R, Tacke F. Single cell RNA sequencing identifies subsets of hepatic stellate cells and myofibroblasts in liver fibrosis. *Cells.* (2019). 8:503. doi: 10.3390/cells8050503
38. Bonnardel J, T'jonck W, Gaublomme D, Browaeys R, Scott CL, Martens L, et al. Stellate cells, hepatocytes, and endothelial cells imprint the kupffer cell identity on monocytes colonizing the liver macrophage niche. *Immunity.* (2019) 51:638–54.e9. doi: 10.1016/j.immuni.2019.08.017
39. Sakai M, Troutman TD, Seidman JS, Ouyang Z, Spann NJ, Abe Y, et al. Liver-derived signals sequentially reprogram myeloid enhancers to initiate and maintain Kupffer cell identity. *Immunity.* (2019) 51:655–70.e8. doi: 10.1016/j.immuni.2019.09.002
40. See P, Lum J, Chen J, Ginhoux F. A single-cell sequencing guide for immunologists. *Front Immunol.* (2018) 9:2425. doi: 10.3389/fimmu.2018.02425
41. Giladi A, Amit I. Single-cell genomics: a stepping stone for future immunology discoveries. *Cell.* (2018) 172:14–21. doi: 10.1016/j.cell.2017.11.011
42. Klein I, Cornejo JC, Polakos NK, John B, Wuensch SA, Topham DJ, et al. Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages. *Blood.* (2007) 110:4077–85. doi: 10.1182/blood-2007-02-073841
43. Beattie L, Sawtell A, Mann J, Frame TCM, Teal B, De Labastida Rivera F, et al. Bone marrow-derived and resident liver macrophages display unique transcriptomic signatures but similar biological functions. *J Hepatol.* (2016) 65:758–68. doi: 10.1016/j.jhep.2016.05.037
44. Soysa R, Lampert S, Yuen S, Douglass AN, Li W, Pfeffer K, et al. Fetal origin confers radioresistance on liver macrophages via p21(cip1/WAF1). *J Hepatol.* (2019) 71:553–62. doi: 10.1016/j.jhep.2019.04.015
45. Tabula Muris Consortium. Single-cell transcriptomics of 20 mouse organs creates a *Tabula Muris*. *Nature.* (2018) 562:367–72. doi: 10.1038/s41586-018-0590-4
46. Han X, Wang R, Zhou Y, Fei L, Sun H, Lai S, et al. Mapping the mouse cell atlas by microwell-seq. *Cell.* (2018) 172:1091–107.e17. doi: 10.1016/j.cell.2018.02.001
47. Macparland SA, Liu JC, Ma XZ, Innes BT, Bartczak AM, Gage BK, et al. Single cell RNA sequencing of human liver reveals distinct intrahepatic macrophage populations. *Nat Commun.* (2018) 9:4383. doi: 10.1038/s41467-018-06318-7
48. Xiong X, Kuang H, Ansari S, Liu T, Gong J, Wang S, et al. Landscape of intercellular crosstalk in healthy and NASH liver revealed by single-cell secretome gene analysis. *Mol Cell.* (2019) 75:644–60.e5. doi: 10.1016/j.molcel.2019.07.028
49. Ramachandran P, Dobie R, Wilson-Kanamori JR, Dora EF, Henderson BEP, Luu NT, et al. Resolving the fibrotic niche of human liver cirrhosis at single-cell level. *Nature.* (2019) doi: 10.1038/s41586-019-1631-3
50. Jaitin DA, Adlung L, Thaiss CA, Weiner A, Li B, Descamps H, et al. Lipid-associated macrophages control metabolic homeostasis in a Trem2-dependent manner. *Cell.* (2019) 178:686–98.e14. doi: 10.1016/j.cell.2019.05.054
51. Ulland TK, Song WM, Huang SC, Ulrich JD, Sergushichev A, Beatty WL, et al. TREM2 maintains microglial metabolic fitness in alzheimer's disease. *Cell.* (2017) 170:649–63.e13. doi: 10.1016/j.cell.2017.07.023

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REVIEW
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Therapeutic Targeting of Hepatic Macrophages for the Treatment of Liver Diseases

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Hepatic macrophages play a central role in maintaining homeostasis in the liver, as well as in the initiation and progression of liver diseases. Hepatic macrophages are mainly derived from resident hepatic macrophages called Kupffer cells or circulating bone marrow-derived monocytes. Kupffer cells are self-renewing and typically non-migrating macrophages in the liver and are stationed in the liver sinusoids in contrast to macrophages originating from circulating monocytes. Kupffer cells regulate liver homeostasis by mediating immunity against non-pathogenic blood-borne molecules, while participating in coordinated immune responses leading to pathogen clearance, leukocyte recruitment and antigen presentation to lymphocytes present in the vasculature. Monocyte-derived macrophages infiltrate into the liver tissue when metabolic or toxic damage instigates and are likely dispensable for replenishing the macrophage population in homeostasis. In recent years, different populations of hepatic macrophages have been identified with distinct phenotypes with discrete functions, far beyond the central dogma of M1 and M2 macrophages. Hepatic macrophages play a central role in the pathogenesis of acute and chronic liver failure, liver fibrosis, non-alcoholic fatty liver disease, alcoholic liver disease, viral hepatitis, and hepatocellular carcinoma, as well as in disease resolution. The understanding of the role of hepatic macrophages in liver diseases provides opportunities for the development of targeted therapeutics for respective malignancies. This review will summarize the current knowledge of the hepatic macrophages, their origin, functions, their critical role in maintaining homeostasis and in the progression or resolution of liver diseases. Furthermore, we will provide a comprehensive overview of the therapeutic targeting strategies against hepatic macrophages developed for the treatment of liver diseases.

Keywords: hepatic macrophages, targeted therapeutics, liver diseases, monocytes, Kupffer cells

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INTRODUCTION

The liver is the largest gland in the human body, weighing about 1.5 kg in an adult. It plays an important role in metabolism and mediate several functions including glycogen storage, plasma protein synthesis, and drug detoxification. Liver tissue is highly vascularized with a continuous blood flow and contain extremely permeable fenestrated endothelia facilitating the interaction between the bloodstream and liver cells. The liver is also a central immunological organ in the

human body that is exposed to large amounts of circulating antigens and endotoxins from gut microbiota. To maintain liver homeostasis, the liver employs multiple mechanisms to suppress immune responses and create tolerance (1). Liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs) and dendritic cells are essential players in initiating and shaping liver immune responses, through antigen presentation, and cytokine and chemokine excretion along with neutrophils, B and T lymphocytes, and natural killer (NK) cells that circulate in the hepatic sinusoids (2).

In this review, we present the insights on hepatic macrophages [collectively referred to as resident KCs and monocyte-derived macrophages (MoMFs)], a heterogeneous population of immune cells that originate from different sources (3). Traditionally, macrophages are classified into a classical M1 pro-inflammatory phenotype, and a dichotomic M2 pro-resolving phenotype (3). However, in past years, tremendous heterogeneity in hepatic macrophages, with distinct functions and gene signatures, have been revealed highlighting their cruciality in liver diseases. While KCs represent the main hepatic macrophages during steady state involved in homeostasis, hepatic metabolic or toxic damage results in massive infiltration of MoMFs into the injured liver. Mice with injured livers showed that the infiltration of MoMFs resulted in antigen redistribution between myeloid cell populations and loss of specific markers for tolerogenic phenotype by KCs, due to amplification of the hepatic phagocytic compartment (4, 5). Hepatic macrophages generally maintain homeostasis, but when imbalance ensues this can result in liver inflammation and fibrosis. The imbalance in hepatic macrophage functioning can result in different liver diseases consequently liver inflammation that has been shown to be associated with the poor disease prognosis in patients.

During liver damage, hepatic macrophages interacts and communicates with hepatic stellate cells that are also known as the liver pericytes located in the space of Disse between parenchymal cells and LSECs of the hepatic lobule. Hepatic stellate cells have numerous functions like vitamin A storage, hemodynamic functions, immuno-regulation and extracellular matrix (ECM) remodeling (6). Upon liver injury, these cells transdifferentiate into activated proliferative, migratory and contractile myofibroblasts, and secrete multiple pro-inflammatory and pro-fibrotic factors (7, 8). In addition, these hepatic myofibroblasts promote the differentiation of liver macrophages with pro-inflammatory and pro-fibrotic functions (9), hence play a central role in the development of liver fibrosis and hepatocellular carcinoma (HCC) in combination with liver macrophages.

In the past decades, it has become apparent that macrophages play a central role in initiation, perpetuation and restoration of liver inflammation and damage. Tremendous progress has been made in the understanding of their origin, heterogeneity and functions that has provided with new therapeutics that have been—or currently being—explored in preclinical models and clinical trials. These increasing developments in understanding hepatic macrophage biology will provide new perspectives toward the effective treatment of liver diseases.

ORIGIN OF HEPATIC MACROPHAGES

Ninety percent of all macrophages in the human body reside in the liver. These macrophages can be derived from different cells. The diverse origins of the macrophages result in cellular heterogeneity in the liver, which is reflected in a high diversity in released cytokines, cell surface markers and transcriptional profiles (3, 10). Hepatic macrophages can be derived from either resident hepatic macrophages, called KCs and from distinct populations of infiltrating macrophages i.e., circulating bone marrow (BM)-derived macrophages, avascular peritoneal macrophages (PMs) that reside in subcapsular regions of the liver or splenic monocytes (3, 11–13) (**Figure 1**). In response to microenvironmental signals, macrophages can migrate and polarize toward different phenotypes with pro-inflammatory and/or anti-inflammatory responses (14).

Kupffer Cells

KCs are the resident, self-renewing and non-migrating macrophages, localized at the luminal side of the liver sinusoids (3, 10). KCs are the largest tissue-resident macrophage population that maintain liver integrity, restore tissue after injury and infection, and initiate the innate and adaptive immune responses (15). KCs form a highly dynamic and complex network in this defense and mediate tolerance, mostly via the interaction with hepatic regulatory T cells (Tregs) (2). KCs provide an anti-inflammatory microenvironment, during homeostasis, by secreting an anti-inflammatory cytokine interleukin (IL)-10 (4, 12). Antigen presentation to KCs induces CD4 T-cell arrest and secretion of immunosuppressive cytokine IL-10 producing antigen-specific Tregs, which results in promoting the immune tolerance. To maintain liver homeostasis, KCs do not only interact with T cells, but also with another macrophage population, circulating MoMFs and hepatic stellate cells (liver fibroblasts) (**Figure 1**).

The resident macrophages are developed asynchronously in three waves at multiple anatomical locations (**Figure 1**). The first wave, primitive hematopoiesis, starts at embryonic day 7.5. In the first wave, primitive erythroid progenitors are detected in the yolk sac (YS) and give rise to primitive erythroblasts (16, 17). In the second wave, transient hematopoiesis, fate-mapping studies in mice showed that resident macrophages descend from a $Tie2^{+}$, also called Tek receptor tyrosine kinase through a cellular pathway generating colony-stimulating factor 1 receptor (CSF1R) positive erythro-myeloid progenitors (EMPs). The EMPs are developed in the YS at embryonic day 8.5, they migrate *via* the bloodstream and colonize to the nascent fetal liver in a chemokine-receptor-dependent manner before embryonic day 10.5 and give rise to the pre-macrophages until embryonic day 16.5. KCs are marginally replaced by hematopoietic stem cells derived macrophages in 1-year-old mice, hereby generating macrophage diversity observed in postnatal tissues (18–20). Finally, the third wave, definitive hematopoiesis, hematopoietic stem cells can be distinguished from other hematopoietic progenitors by their self-renewal capacity, presence in adults and repopulation potential after

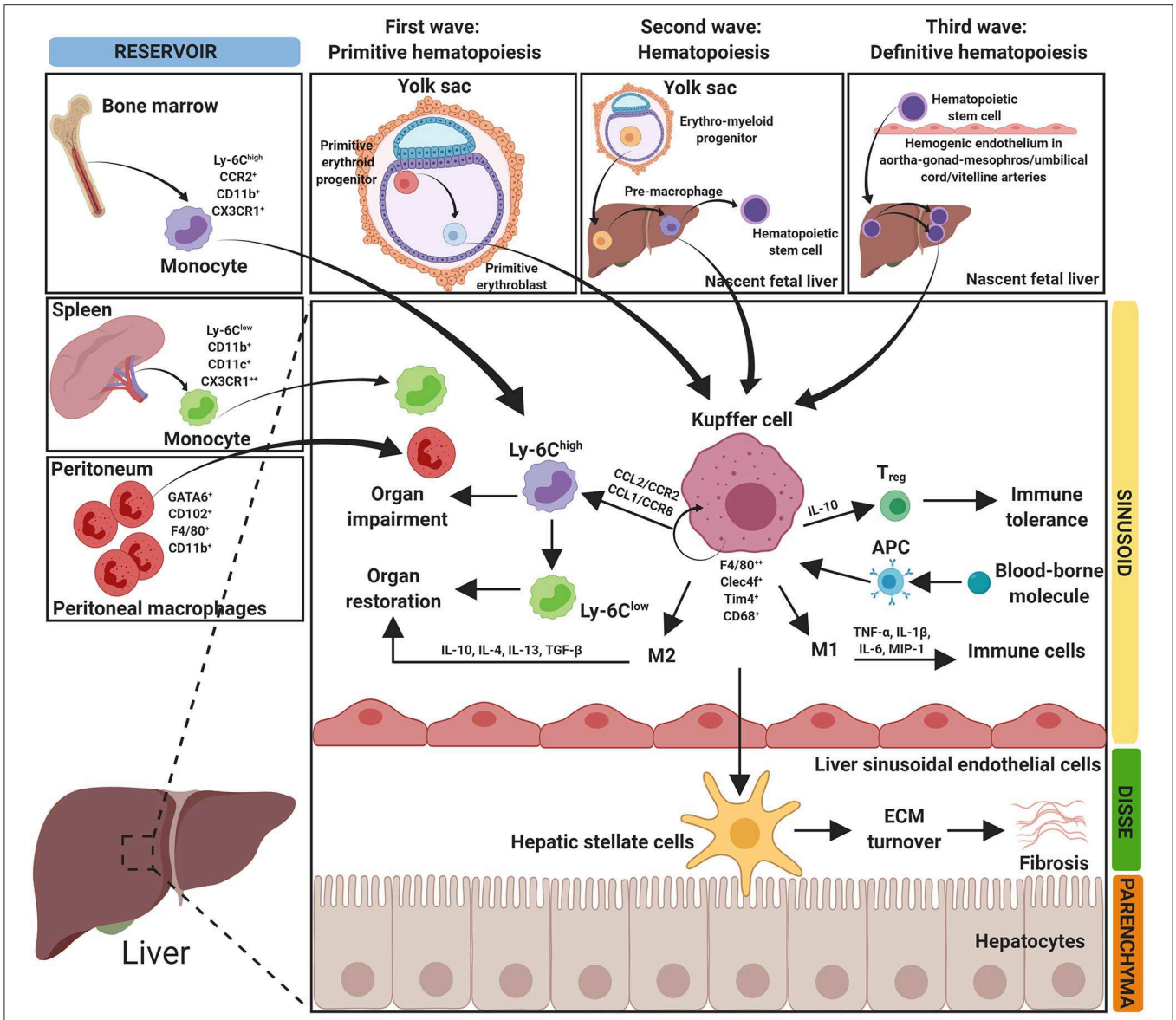

FIGURE 1 | Origin of hepatic macrophages. The figure depicts the diverse origin of hepatic macrophages. Hepatic macrophages can arise from bone marrow derived monocytes, spleen derived monocytes and peritoneum-derived macrophages. Resident hepatic macrophages (Kupffer cells, KCs) develop in three waves at multiple anatomical locations. In the first wave (primitive hematopoiesis), primitive erythroid progenitors originate and differentiate into primitive erythroblasts in the yolk sac. In the second wave (transient hematopoiesis), erythro-myeloid progenitors develop in the yolk sac and migrate into the nascent fetal liver. In the third wave (definitive hematopoiesis), hematopoietic stem cells arise intra-embryonically from hemogenic endothelium in the aorta-gonad-mesonephros region and in the umbilical and vitelline arteries. Hematopoietic stem cells migrate into the fetal liver and expand/differentiate into resident KCs. Hepatic macrophages mediate different functions in organ homeostasis, impairment, restoration, and in fibrosis. APC, antigen presenting cells; CCL, chemokine (C-C) motif ligand; CCR, chemokine (C-C) motif receptor; CD, cluster of differentiation; Clec4f, C-type lectin domain family 4 member F; CX3CR1, C-X3-C motif chemokine receptor 1; ECM, extracellular matrix; F4/80, EGF-like module-containing mucin-like hormone receptor-like 1; GATA6, GATA-binding factor 6; IL, interleukin; MIP-1, macrophage inflammatory protein 1; Tim4, T-cell membrane protein 4; TGFβ, transforming growth factor beta; TNF-α, tumor necrosis factor-α; Treg, regulatory T cells; Ly-6C, lymphocyte antigen 6 complex locus C1.

transplantation (21). Hematopoietic stem cells arise intra-embryonically from hemogenic endothelium in the aorta-gonad-mesonephros region and in the umbilical and vitelline arteries at embryonic days 10.5. The hematopoietic stem cells migrate to the fetal liver, expand and differentiate into resident macrophages (17, 22).

KCs are primarily identified as $CD45^+$ $F4/80^+$ $CD11b^{intermediate/int}$ cells expressing C-type lectin 4F (*Clec4f*) as a specific KC marker. Based on intravital microscopy, morphometric analysis and gene expression profiling, two distinct intrahepatic KCs subsets i.e., BM-derived KCs and YS-derived KCs, highlighting KCs heterogeneity (23).

YS-derived KCs express Macrophage Receptor with Collagenous Structure (MARCO) and T-cell immunoglobulin and mucin domain containing 4 (Tim4) and possess pro-inflammatory functions. On the other hand, BM-derived KCs display an immunoregulatory gene signature possessing protective functions in the maintenance of liver homeostasis (23). Interestingly, recent studies have highlighted a rapid KCs loss upon infection (24, 25), and in HCC (26), thereby questioning why and how KCs loss is established. Several hypotheses have been proposed e.g., KCs loss as a self-inflicted brake of immune system to prevent excessive inflammation, as supported by studies whereby KCs depletion attenuated chronic inflammatory diseases such as non-alcoholic steatohepatitis (NASH) (27, 28). However, in contrast, studies have revealed that tissue-resident macrophages inhibit inflammation after injury and maintain homeostasis (29, 30). With these contradicting findings, it is highly critical to understand if KCs loss is an essential to control liver inflammation or is a consequence of the inflammation. Considering the distinct KC populations (BM-derived and YS-derived) with different functions might explain these contradictions, however further studies are required to deeply understand the underlying mechanisms. These insights will improve our current understanding about liver diseases and will help in developing novel therapeutic strategies targeting specific KC phenotype for the efficient treatment of liver diseases.

Monocyte-Derived Macrophages

Circulating MoMFs mainly infiltrate into the liver when hepatic metabolic or toxic damage occurs and are likely dispensable for replenishing the macrophage population in homeostasis (**Figure 1**). MoMFs are mainly derived from a chemokine C-X3-C motif receptor 1 $(CX3CR1)^{+}$ $CD117^{+}$ Lin^{-} (lineage-negative) BM progenitors and express CX3CR1, lymphocyte antigen 6 complex locus C1 (Ly-6C), CD11b and chemokine (C-C motif) receptor 2 (CCR2) (31). MoMFs recruitment is primarily initiated by activated toll-like receptor (TLR) signaling in KCs or hepatic stellate cells that results in increased secretion of chemokine (C-C motif) ligand 2 (CCL2) or monocyte chemoattractant protein 1 (MCP-1) (32–34).

Upon recruitment, MoMFs differentiate into a plethora of phenotypes with discrete functions depending on the microenvironmental cues. Studies in mice have shown that inflammatory lymphocyte antigen 6 complex, locus C1 (Ly-6C)^{high} expressing monocytes (analogous to $CD14^{hi}$ $CD16^{lo}$ human monocytes) are attracted and accumulated in the injured liver tissue depending on chemokine ligand/receptor interactions of CCL2/CCR2 or CCL1/CCR8 (3, 34–37). In acute liver injury mouse model, Ly-6C^{high} monocytes have been shown to express the increasing levels of T cell Ig Mucin 3 (*Havcr2*), toll-like receptors (*Tlr2*), C-type lectins (*Clec4d*, *Clec4e*, and *Clec5a*), CD209a and CD93 (38). Ly-6C^{high} monocytes provoke organ impairment, but can locally undergo a functional switch into restorative Ly-6C^{low} monocytes (analogous to $CD14^{low}$ $CD16^{high}$ human monocytes) that can restore liver damage. Therefore, this study showed a Ly-6C^{high}/Ly-6C^{low} phenotype beyond the traditional M1/M2 classification (39).

Ly-6C^{high} monocytes express high levels of CCR2 and activated hepatic stellate cells have been to secrete increasing levels of CCR2 ligand CCL2. It has been further suggested using CCR2 deficient mouse model, that CCR2 critically controls intrahepatic Ly-6C^{high} monocytes recruitment during liver injury via CCR2-dependent BM egress and promote the progression of liver fibrosis (39, 40). The CCL2/CCR2 signaling therefore critically mediates hepatic macrophage recruitment upon liver injury, leading to shaping of the inflammatory response in the injured liver. Furthermore, other CCRs such as CCR1 and CCR5, via CCL3/MIP1α, CCL4/MIP1β and CCL5/RANTES interaction have been implicated in liver fibrosis attributed to Ly-6C^{high} monocytes recruitment or stellate cells activation, respectively (39–41). CCR8 that interacts with CCL1 was also shown to be involved in the directed infiltration of infiltrating monocytes into injured liver (37). In the respective study, CCR8-deficient mice having reduced intrahepatic monocytes/macrophages showed significantly attenuated hepatic fibrosis that could be restored by adoptive transfer of CCR8-expressing Ly-6C^{high} BM-derived monocytes (37). These studies highlight the importance of MoMFs recruitment in disease progression and therefore the therapeutic potential of CCRs e.g., CCR2, CCR5, and CCR8 antagonists to inhibit MoMFs infiltration thereby limiting liver inflammation and fibrosis.

Peritoneal and Splenic Macrophages

Wang and Kubes have identified a distinct avascular population of macrophages, PMs, that are recruited through the visceral endothelium upon liver injury and contribute to liver regeneration (13). PMs, that reside in the peritoneal cavity with self-renewal abilities (42), exists as two distinct PM subsets i.e., large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs). LPMs originate from embryonic precursors and represent the most abundant subset under steady conditions that display F4/80^{high} CD11b^{high} MHCII^{low} phenotype (**Figure 1**). While SPMs are the minor subset with F4/80^{low} CD11b^{low} MHCII^{high} phenotype and originate from BM-derived myeloid precursors and predominantly appear during infection (42). Flow cytometry studies confirmed the recruitment of subpopulation of mature PMs expressing CD102 and GATA-binding protein 6 (GATA6) transcription factor within 1 h at the site of thermal injury at the liver surface (13, 43). Furthermore, depletion of PMs prevented the early $F4/80^{+}$ macrophage influx and finally, macrophage recruitment and tissue regeneration was reported to be impaired in GATA6-deficient mice, suggesting an important role of PMs during resolution of liver diseases (13, 43).

Besides KCs, MoMFs and PMs, splenic derived monocytes may also contribute to hepatic macrophages during liver injury (11, 44) (**Figure 1**). Studies have unraveled spleen as a site for storage and deployment of monocytes, and have identified the contribution of splenic monocytes in regulating immune response during injury (44). Furthermore, splenic macrophages have been shown to promote monocytes infiltration and supports M1 dominant phenotype *via* secretion of CCL2, and regulate KCs activation and hepatic inflammation by

releasing of factors such as lipocalin-2 in the portal vein (45, 46). However, more studies are vital to gain insights into distinct phenotypes and functions of splenic macrophages during liver diseases.

MACROPHAGE HETEROGENEITY: BEYOND M1 AND M2 POLARIZATION DOGMA

Within hepatic macrophage populations, there is a substantial heterogeneity characterized by a broad spectrum of released cytokines, cell surface markers and transcriptional profiles. Within the simplistic M1/M2 terminology, classically activated M1 macrophages—activated by interferon gamma (IFN-γ) and lipopolysaccharides (LPS)—are pro-inflammatory, microbicidal, tumoricidal, and release numerous inflammatory cytokines e.g., tumor necrosis factor (TNF)-α, IL-1, IL-6, IL-12, IL-15, and IL-18. While alternatively activated M2 macrophages downregulate inflammatory responses and facilitate tissue repair by secreting IL-10, IL-4/IL-13, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF)-α. Due to the complex biological characteristics, M2 macrophages can be further sub-categorized into distinct phenotypes based on the stimuli: M2a (induced by IL-4 and IL-13), M2b (elicited by immune complexes), M2c (stimulated by IL-10, TGF-β and glucocorticoids) and M2d (activated by IL-6, TLR ligands and adenosine) (47, 48). M2a macrophages are wound healing macrophages that express high levels of mannose receptor (MR, also called CD206), secrete pro-fibrotic factors such as TGF-β, insulin-like growth factor (IGF), and fibronectin, and contribute to tissue repair. M2b macrophages possess both protective and pathogenic roles, and secrete both pro- and anti-inflammatory cytokines. M2c phenotype display regulatory phenotype, can repress inflammation and fibrosis, and promote tissue repair. In addition, M2c macrophages have the ability to induce regulatory T cells and are involved in the phagocytosis of apoptotic cells. M2d macrophages have phenotypic and functional attributes similar to tumor-associated macrophages (TAMs), and are distinct from M2a-c. M2d constitute the major inflammatory component in tumor, contributing to angiogenesis and metastasis (47, 48).

Strikingly, recent studies have unraveled a complex and spectrum of macrophage polarization states beyond the ancient dogma of M1 and M2 macrophages (11, 49). A recent study, using single-cell RNA sequencing, has provided a comprehensive map of the human liver at a single-cell resolution and revealed distinct intrahepatic monocyte/macrophage populations with unique functional pathways. Furthermore, this study highlighted the disparity between different macrophage populations and biological differences between livers from mice and humans. This recent study describing a transcriptional map of the human liver microenvironment provides a framework for understanding the human liver and the role of different cellular phenotypes that will provide a benchmark for the development of novel immunomodulatory therapies (49).

FUNCTION OF HEPATIC MACROPHAGES IN LIVER DISEASES

Homeostasis of the liver is important for tolerating and regulating immune responses. Hepatic immune tolerance is mostly dependent on the interaction between KCs and Tregs to create a local suppressive microenvironment, while the recruitment of MoMFs and crosstalk between hepatic stellate cells and macrophages are important determinant for the disease progression/pathogenesis, and tissue regeneration following liver injury. For the development of targeting therapies for liver diseases, especially targeting liver inflammation, it is highly crucial to gain insights into the origin, phenotypic heterogeneity and functions of hepatic macrophages. Here, the role of hepatic macrophages in different liver diseases has been described, including acute liver failure (ALF), liver fibrosis, non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD), viral hepatitis and HCC.

As mentioned earlier, hepatic macrophages (KCs and MoMFs) are the key players in various types of liver disease. The understanding of their roles in different liver diseases defines them as promising targets to develop new therapies for different liver diseases. Several commendable reviews have detailed the divergent roles and mechanisms of hepatic macrophages in liver diseases (3, 10). The function of hepatic macrophages in these different liver diseases is briefly summarized below and depicted in **Figure 2**.

Acute Liver Failure

ALF is a syndrome that is characterized by peripheral vasodilation, encephalopathy and coagulopathy resulting in multiple organ dysfunction and death (50). Patients with ALF, without previously recognized liver disease, sustain a liver injury that results in a rapid loss of hepatic function. Hepatic insult activates KCs and MoMFs to release large amounts of pro-inflammatory cytokines and chemokines, such as TNF-α, IFN-γ, MCP-1/CCL2, and IL-8 (**Figure 2**). They also express death ligands resulting in hepatocyte apoptosis (50). KCs release wide range of cytokines that are critical in determining the subsequent reactions of other immune cells, hepatocytes and the degree of organ damage (51). MoMFs have been shown to be actively recruited and secrete large amounts of pro-inflammatory cytokines, present antigens *via* their surface HLA class II molecules and trigger the adaptive immune response. In ALF, a counter-regulatory process ensues that is intended to offset the damaging effects of unhindered pro-inflammatory KCs and MoMFs activation. This counter-regulatory process consists of KCs secreting cytokines, such as IL-6, IL-10, and IL-18, to compensate for the deleterious effects of the pro-inflammatory response (50, 52).

Liver Fibrosis

Liver fibrosis is the final common pathway of chronic liver diseases caused by toxic damage, viral infections, autoimmune conditions, and metabolic and genetic diseases (53). The advanced stage of liver fibrosis is called cirrhosis, which is characterized by a loss of architecture, function of the liver

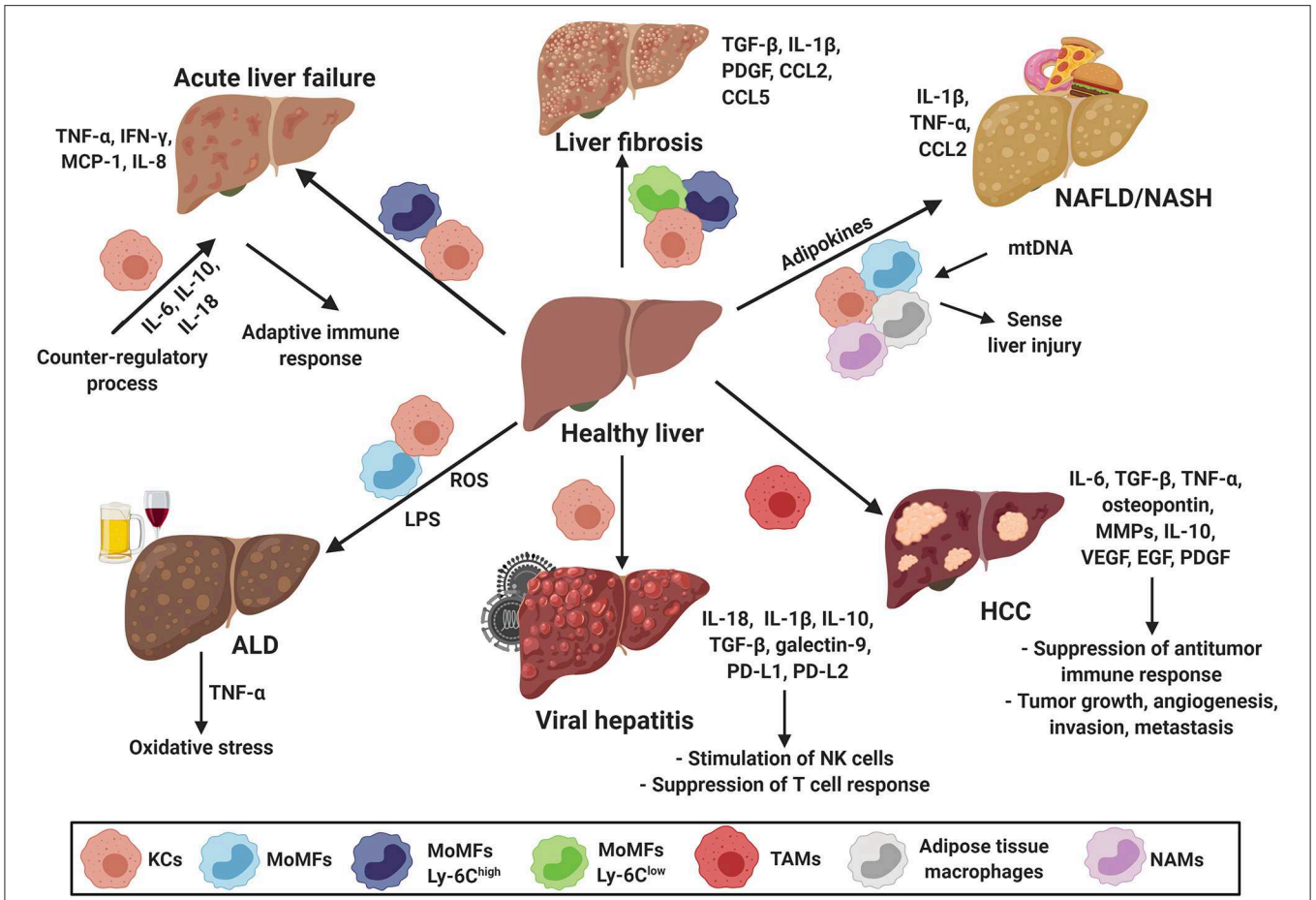

FIGURE 2 | Macrophages in hepatic disease. Hepatic macrophages, KCs and MoMFs, are the key players in various types of liver disease including acute liver failure (ALF), liver fibrosis, non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD), viral hepatitis and hepatocellular carcinoma (HCC). They modulate adaptive immune responses, fibrosis progression/resolution, sense disease severity, contribute to the establishment of a tumorigenic environment, promote tumor growth, angiogenesis, invasion, and metastasis. CCL, chemokine (C-C) motif ligand; EGF, epidermal growth factor; HCC, hepatocellular carcinoma; IL, interleukin; IFN-γ, interferon-γ; KCs, Kupffer cells; LPS, lipopolysaccharides; Ly-6C, lymphocyte antigen 6 complex locus C1; MCP-1, monocyte chemoattractant protein 1; MMPs, matrix metalloproteinases; MoMFs, monocyte derived macrophages; mtDNA, mitochondrial DNA; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NAMs, NASH-associated macrophages; PD-L, programmed death-ligand; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; TAMs, tumor-associated macrophages; TGFβ, transforming growth factor beta; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

and development of life-threatening complications. Hepatocytes, cholangiocytes, hepatic stellate cells, LSECs, immune cells and especially macrophages have been identified in the pathogenesis of liver fibrosis (54). KCs are important in the initial response to injury, produce cytokines and chemokines, and recruit monocytes *via* secretion of CCL2 and CCL5 chemokines (39, 55). Studies have shown that that infiltration of Ly-6C^{high} MoMFs contributing to the expansion of hepatic macrophages to 3–5-fold. MoMFs could promote fibrosis by releasing factors like TGF-β, IL-1β, platelet-derived growth factor (PDGF) and CCL2, which activates hematopoietic stem cells (HSCs) and progresses the inflammation. Ly-6C^{high} MoMFs are pro-fibrogenic and pro-inflammatory (34) and can be switched to Ly-6C^{low} macrophages that are pro-restorative, anti-fibrotic and anti-inflammatory based on microenvironmental cues (39) (**Figure 2**). Importantly, when the injury in the liver is removed, macrophages are also responsible for the reversal of the liver fibrosis (55).

Non-alcoholic Fatty Liver Disease

NAFLD refers to a wide range of liver damage, from simple steatosis to NASH, advanced fibrosis, cirrhosis and liver failure (56). Pathological NAFLD resembles alcohol-induced liver disease but occurs in patients who do not abuse alcohol (57). NAFLD is a metabolic syndrome associated with unhealthy lifestyle and several risk factors, obesity, dyslipidemia and insulin resistance (58). Besides liver macrophages, adipose tissue macrophages are also shown to contribute to NAFLD and secretes adipokines and cytokines (59). Hepatic macrophages play the central role in NAFLD, the activation and polarization of macrophages affect the NAFLD progression. Dysregulation in macrophage polarization assist in progression to steatosis, the early stage of NAFLD (60). During chronic liver injury, KCs become activated and release inflammatory cytokines and chemokines. Endotoxins and bacterial components released due to increased intestinal permeability, (lipo)apoptotic hepatocytes,

gut microbiota, free fatty acids, cholesterol etc. are the factors that mediate macrophage activation during NAFLD (61). Upon activation, KCs and MoMFs secrete inflammatory cytokines IL-1β, TNF-α and CCL2, that are involved in the development of steatosis, serve as lipogenic factors and promote the inflammatory progression from NAFLD to NASH (62–65) (**Figure 2**). NASH patients have increased levels of mitochondrial DNA (mtDNA) in serum, as damaged hepatocytes release exosomes/extracellular vesicles containing mitochondrial DNA. KCs recognizes this mitochondrial DNA *via* TLR9 and can therefore sense the severity of the liver injury (66, 67).

More recently, Krenkel et al., has reported the distinct fate of myeloid cells in liver and BM during obesity-related NASH. The study showed a unique, however common and functionally relevant, inflammatory signature in liver myeloid compartment and the BM precursors during NAFLD progression (68). This study suggests that myeloid cells, especially macrophages, adapt their phenotype in response to metabolic microenvironment, indicating the metabolic reprogramming of macrophages in NASH. Interestingly, using single-cell secretome gene analysis, Xiong et al., has identified NASH-associated macrophages (NAMs). NAMs markedly express high levels of triggering receptors expressed on myeloid cells 2 (Trem2) as a feature of mouse and human NASH correlating with disease severity, and responsive to pharmacological and dietary interventions. This study further provide insights into reprogramming of macrophages (and other non-parenchymal cells) in NASH (69).

Alcoholic Liver Disease

ALD includes different disease stages from simple steatosis to cirrhosis and HCC. Liver injury in ALD can be caused by the following factors: dose, duration and type of alcohol consumption, drinking patterns, sex, ethnicity, obesity, iron overload, viral hepatitis and genetic factors (70). Alcohol ingestion can cause alcohol-induced liver injury through activation of the innate and adaptive immune responses by cytotoxic and reactive oxygen species (ROS)-mediated effects of alcohol and its metabolite, acetaldehyde, on hepatocytes (71). Hepatic macrophages (both M1 and M2 phenotype) increases significantly during disease progression with increased intrahepatic inflammation e.g., increased expression of inflammatory genes, M1 and M2 markers, cytokines and chemokines (70–75).

KCs play a key role in ALD and alcohol consumption can lead to an increase in gut permeability resulting in endotoxemia. KCs can bind to endotoxin *via* CD14 receptor in combination with TLR4, leading to oxidative stress and release of pro-inflammatory cytokines such as TNF-α, causing alcohol-induced liver injury (72–75) (**Figure 2**). LPS levels are increased in ALD patients, which activates TLR4 signaling in KCs, HSCs and LSECs, and contributes to the regulation of angiogenesis and fibrogenesis, leading to fibrosis. Complement activation, TLR pathways and LPS-mediated pathways, including inflammasome activation could be potential therapeutic targets to develop new therapies for the treatment of ALD (74, 76).

Viral Hepatitis

Acute viral hepatitis is the most common cause of chronic liver disease worldwide. Chronic hepatitis may progress to cirrhosis, liver failure or HCC (77). A major obstacle in studying viral hepatitis is that there only exist a few immunocompetent animal models for chronic viral hepatitis (10). Hepatic macrophages are recognized as important cells in viral hepatitis. Hepatic macrophages can provide an efficient antiviral response but can also contribute to adverse effects as liver fibrosis or suppression of antiviral immunity (78). KCs play a pivotal role in both the hepatitis B virus (HBV) and the hepatitis C virus (HCV) infection. HBV can infect KCs, leading to the release of inflammatory cytokines, such as IL-18, and stimulation of NK cells (79, 80). The HCV can activate KCs through TLR2, which results in secretion of inflammatory molecules such as IL-1β and IL-18 (81, 82). KCs secrete IL-10, TGF-β, galectin-9 and induces expression of programmed death-ligands 1 and 2 (PD-L1 and PD-L2) during both HBV and HCV infection, resulting in suppression of T cell response (12) (**Figure 2**).

Hepatocellular Carcinoma

HCC is one of the most aggressive form of human cancer and a growing cause of cancer-related deaths worldwide (83, 84). Chronic inflammation seems to be essential in the initiation and development of HCC but idem for fibrosis and cirrhosis, which finally result in HCC. HBV and HCV as well as chronic alcohol abuse, biliary disease, metabolic disorders, drugs, toxins and genetic alterations are the major risk factors for the development of HCC (85, 86). TAMs are the key players in cancer-related inflammation (86, 87). TAMs originate from circulating monocytes that are recruited to the tumor microenvironment by CCL2, macrophage colony-stimulating factor (M-CSF), VEGF, and TGF-β, where they differentiate to mature hepatic macrophages (88). In the HCC tumor microenvironment, TAMs are mostly polarized into the M2 phenotype (87, 89) and promote HCC growth, angiogenesis, invasion and metastasis. They are also shown to suppress an antitumor immune response through the interaction with stromal and cancer cells in the tumor microenvironment. In the tumor microenvironment, TAMs release many cytokines, chemokines and growth factors. While IL-6 and TGF-β favor tumor growth, TNF-α, osteopontin, matrix metalloproteases (MMPs), and IL-6 supports invasion and metastasis. TGF-β in combination with IL-10 favors suppression of an antitumor immune response, and VEGF, epidermal growth factor (EGF), PDGF and TGF-β induces angiogenesis (86) (**Figure 2**).

As described above, hepatic macrophages play an highly essential role in liver diseases like ALF, liver fibrosis, NAFLD, ALD, viral hepatitis, and HCC. Therefore, hepatic macrophages represent the potential targets for therapeutic targeting for the treatment of liver diseases.

THERAPEUTIC TARGETING OF HEPATIC MACROPHAGES

Based on our increasing understanding about macrophages, several pathways have been identified that regulate their

recruitment, differentiation/polarization and activation, based on which, a number of drugs have been designed and investigated in different preclinical murine models. Furthermore, it has been increasingly recognized that macrophages possess high scavenging ability thereby allowing their preferential targeting using nanoparticles (NPs).

However, there are several challenges that are hampering the drug development: (1) disparity in macrophage phenotypes in humans and in animal models resulting in poor translation of therapeutics studied in animal models to human patients; (2) greater macrophage heterogeneity in humans as compared to inbred mouse strains due to several intrinsic (genetics, ethnicity, sex, and age) and extrinsic factors (microbiota, infections, medications); (3) limited in-depth knowledge about human macrophage subsets as compared to mouse models. Importantly, macrophages display incredible heterogeneity with distinct functions in disease initiation and progression as well as protective role and maintain homeostasis. Therefore, it is crucial to target the pathogenic phenotypes of macrophages therapeutically without hindering the functions of so-called restorative or homeostatic macrophages.

Here, we summarize different approaches that have been explored for targeting of hepatic macrophages and are sub-categorized into three major categories: (a) modulation of macrophage polarization/reprogramming, (b) inhibition of KCs activation and (c) dampening of monocyte recruitment. These strategies have been investigated in experimental animal models, while some have been translated in the clinical settings (**Table 1** and **Figure 3**).

Modulation of Macrophage Polarization/Reprogramming

Macrophages phenotypes exert contrasting functions, therefore a therapeutic approach that promotes a switch from pathogenic phenotype to restorative phenotype is an interesting approach to accelerate disease resolution and promote liver regeneration. This can be achieved by using therapeutics that promote macrophage polarization and/or using nanoparticles that can selectively reprogram macrophages to restorative phenotype. Steroids (e.g., Dexamethasone), IL-4, IL-10, secretory leukocyte protease inhibitor (SLPI), prostaglandin E2 (PGE2) and colony-stimulating factor 1 receptor (CSF-1R) agonists are the promising therapeutics targeting different immuno-modulatory pathways that have been explored for macrophage reprogramming in liver diseases (90).

NPs are materials with dimensions between 10^{-9} and 10^{-7} m. Such particles can be used as nanocarriers for diagnosis and targeted delivery of therapeutic agents for liver diseases (109). There are several nanocarriers that can be used for diagnosis and therapy referred to as nano-theranostics. The NPs for drug and gene delivery systems include polymeric NPs, lipid NPs, organic and inorganic NPs (110). NPs provide multiple new properties, which can be exploited to improve the ability to detect, treat, monitor and prevent diseases. Moreover, the interactions between these nanomaterials and comparably sized physiological structures in the human body e.g., DNA, proteins and organelles, can be used in combination with existing medical diagnostic and treatment strategies to develop more efficacious approaches (111). These NPs could be loaded with small drug molecules, proteins, DNA or RNA. The drug release can be favorably tuned in different NPs, and in some cases, the release of the therapeutic agents could also be initiated by internal stimuli such as pH (112), or external stimuli such as light (113). Various surface modifications can be applied to NPs including small drug molecules, antibodies, fluorescent dyes, peptides, proteins, polyethylene glycol (PEG), DNA or RNA.

The NPs can be delivered at the targeted site *via* active or passive targeting. With active targeting, the NP surface can be modified with targeting ligands, e.g., targeting peptides, antibodies, which leads to specific binding to the targeted cells. Unlike active targeting, passive targeting does not use any targeting ligands, but uses the physiological properties e.g., enhanced permeability and retention (EPR) in tumors, due to leaky vasculature, to deliver the NPs to the target cells (114). To prolong the circulation time of the NPs in the circulatory system, with both active and passive targeting, the NPs can be surface modified with PEG.

NPs can be made out of different nanomaterials and are generally non-toxic. Gold NPs (AuNPs), which are similar to other inorganic NPs, are mostly non-toxic, but when used in small sizes of 1.4 nm they showed an increased toxicity. Silica NPs induces toxicity due to activation of macrophages. However, at higher doses, many nanotherapeutics have been shown to be toxic. To reduce non-specific uptake by macrophages and alter the response of immune cells, NPs can be modified with PEG or with peptides (115). Liver inflammation and fibrosis can be targeted by nanomedicine. This could be done by the therapeutic targeting of macrophages and especially KCs, because these macrophages have an inherent ability of efficient and non-specific uptake of most nanomaterials and these macrophages play a critical function during inflammation and fibrogenesis. KCs can be targeted by the mannose receptor in liver disease or become activated by specific nanomaterials like peptide-modified gold nanorods (AuNRs) to polarize them into the pro-inflammatory phenotype (115).

Different strategies have been proposed for hepatic macrophage targeting with nanomedicine in the preclinical setting (**Table 1** and **Figure 3**). A system studied by He et al., was to inhibit TNF-α using small interfering RNA (siRNA) delivered *via* mannose-modified trimethyl chitosan-cysteine (MTC) conjugated NPs that are mostly internalized by macrophages, due to macrophages-specific delivery route using the mannose receptor. With this strategy, inflammation-driven liver damage and lethality induced by acute lipopolysaccharide/D-galactosamine administration *in vivo* in mice was prevented. This system offers possibility for oral delivery, which is advantageous for clinical application (94).

Bartneck and colleagues showed that dexamethasone-loaded liposomes were efficient *in vivo* by ameliorating inflammatory liver diseases in a model of acute hepatitis and in chronic carbon tetrachloride (CCl_4)-based chronic toxic liver injury. This approach resulted in a M2 activation profile of macrophages and with a significant reduction in the number of T cells in the liver

TABLE 1 | Therapeutic targeting strategies of hepatic macrophages.

Strategy	(nano) Therapeutics	Mechanism/outcome	References
Modulation of macrophage polarization/macrophage reprogramming	Steroids e.g., glucocorticoids	Anti-inflammatory, anti-fibrotic	(90, 91)
	CSF-1R agonists CSF1-Fc	Proliferation of resident macrophages and recruitment of monocytes	(92)
	SLPI	Anti-inflammatory responses through modulation of monocyte/macrophage function	(93)
	MTC-TNF-α siRNA NPs	Inhibition of TNF-α production, reduction in liver inflammation	(94)
	Dexamethasone liposomes	Induction of T cells apoptosis	(95)
	COOH-micelles	Improvement, restoration of tolerance autoimmune disease and chronic inflammation	(96)
	Galectin-3	Inhibits inflammatory macrophage functions	(97)
	SYK pathway inhibitor R406	Anti-inflammatory, anti-fibrotic	(76)
	R406-PLGA NPs	Anti-inflammatory, anti-fibrotic	(98)
Inhibition of KCs activation	ASK1 inhibitor selonsertib	Anti-inflammatory, anti-fibrotic	(99)
	DAMPs (e.g., HMGB1) antagonists	Defected TLR9 signaling, decreased tumor cell proliferation Inhibits acute liver injury and bacterial translocation	(90, 100)
	PRR antagonists	Attenuates DAMPs/PAMPs mediated liver injury	(101)
	Curcumin and calcitriol liposomes	Immuno-modulatory	(102)
Dampening of monocyte recruitment	CCR2 antagonists Cenicriviroc, propagermanium	Anti-inflammatory, anti-fibrotic	(103–106)
	CCL2 antagonist mNOX-E36	Anti-inflammatory, anti-fibrotic	(33, 107)
	CCR5 antagonist Miraviroc	Anti-inflammatory, anti-fibrotic	(108)

ASK-1, apoptosis signal-regulating kinase 1; CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C) motif receptor; COOH-micelles, carboxy-modified micelles; CSF-1R, colony stimulating factor 1 receptor; CVC, cenicriviroc; KCs, Kupffer cells; DAMPs,/PAMPs damage-associated/pathogen-associated molecular patterns; HMGB1, High mobility group box 1 protein; SLPI, secretory leukocyte protease inhibitor; SYK, spleen tyrosine kinase; mNOX-E36, emapticap pegol; MTC, mannose-modified trimethyl chitosan-cysteine; NPs, nanoparticles; PLGA, poly(lactic-co-glycolic acid); siRNA, small interfering RNA.

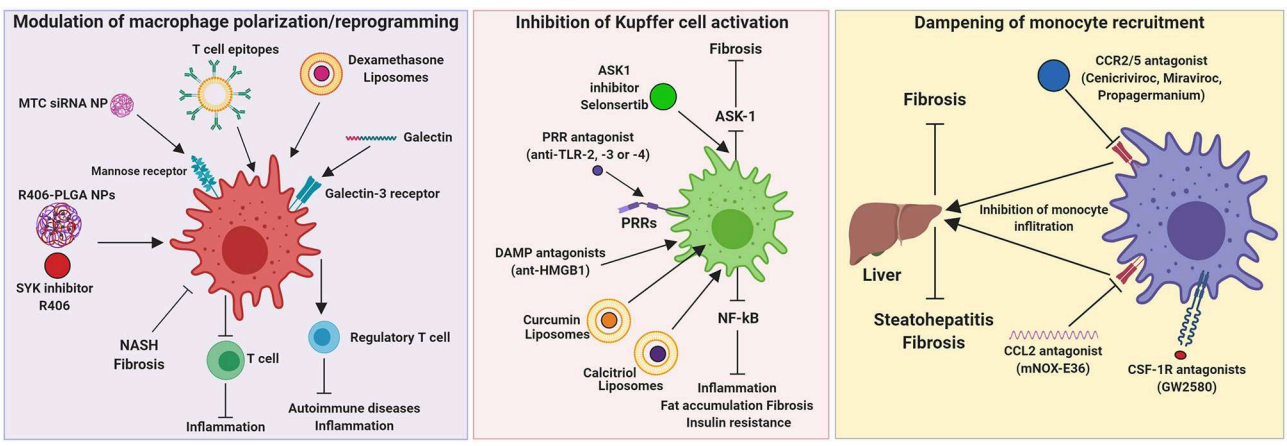

FIGURE 3 | Therapeutic targeting of hepatic macrophages. Modulation of macrophage polarization and function, inhibition of Kupffer cell activation, and dampening of monocyte recruitment into the inflamed liver are the three strategies that have been investigated for the resolution of hepatic inflammation and fibrogenesis. ASK1, Apoptosis signal-regulating kinase 1; CCL, chemokine (C-C) motif ligand; CCR, chemokine (C-C) motif receptor; CSF-1R, colony stimulating factor 1 receptor; DAMP, damage-associated molecular patterns; HMGB1, High mobility group box 1 protein; mNOX-E36, emapticap pegol; MTC, Mannose-modified trimethyl chitosan-cysteine; NASH, non-alcoholic steatohepatitis; NP, nanoparticle; PLGA, poly(lactic-co-glycolic acid); PRR, pattern recognition receptors; TLR, Toll like receptors.

(95). Herkel and co-workers published a patent based on a study of the induction of tolerance in liver by influencing Tregs by LSEC- and KC-directed carboxy-modified micelles. The micelles have been modified with T cell epitopes on their surface and were targeted to LSECs and KCs. These micelles can deliver antigens and induce the generation of Tregs to suppress autoimmunity.

These NPs are intended to induce tolerance against autoantigens and therefore ameliorating autoimmune diseases and chronic inflammation (96). Traber and Zomer used galactin-3 inhibitors to target inflammatory macrophage functions in liver diseases in mice. The treatment with galectin-3 resulted in regression of NASH and fibrosis. Therefore, they suggested that this galectin-targeting drugs have potential in treatment in human for NASH and fibrosis (97).

Furthermore, Bukong et al., demonstrated the central role of spleen tyrosine kinase (SYK) in multiple proinflammatory pathways involved in the ALD pathogenesis. Furthermore, SYK inhibitor R406 abrogated immune cell infiltration, macrophage and inflammasome activation, thereby ameliorated liver injury, liver inflammation, and reduced hepatic steatosis induced by alcohol (76). In another recent study, Kurniawan and colleagues reported an efficient delivery of small molecule SYK kinase inhibitor R406 using Poly(lactic-co-glycolic acid) (PLGA) NPs for the treatment of NASH in Methionine-Choline-deficient (MCD)-diet induced NASH mouse model (98).

Inhibition of Kupffer Cell Activation

When liver injury ensues, KCs initiate inflammatory cascades in the liver *via* different mechanisms. For instance, the early communication of cellular distress or hepatocyte damage is mediated by KCs through damage-associated molecular patterns (DAMPs)/pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) and NF-κB signaling and inflammasome activation etc. Therefore, inhibition of PRRs using TLR2, TLR3, and TLR4 antagonists have been shown to ameliorate liver inflammation in murine models (101). Another interesting strategy that has been explored is targeting of released DAMPs such as high mobility group box 1 (HMGB1) proteins and histones. Intriguingly, HMGB1 neutralizing antibodies are shown to attenuate liver injury and reduce bacterial translocation *in vivo* in murine models (90). A possible targeting strategy to treat liver diseases is to influence KCs activation. There are several approaches for influencing KCs activation, such as reducing bacterial translocation and inhibition of TLR4-dependent macrophage activation using a broad spectrum of antibiotics. This could improve steatohepatitis, liver fibrosis and HCC (10).

Modifying KCs activation has been explored (**Table 1** and **Figure 3**). Hepatic macrophages and hepatocytes share some of the intracellular inflammatory signaling pathways like NF-κB, ASK1, JNK, or p38 (116). Loomba et al., developed selonsertib, an inhibitor of the inflammatory signaling pathway ASK1 (apoptosis signal-regulating kinase 1). Selonsertib treatment has been shown to have an effect on hepatocyte metabolism as well as macrophage activation. In a randomized phase 2 trial, selonsertib showed an improvement in fibrosis, lobular inflammation and serum biomarkers of apoptosis and necrosis in patients with NASH and fibrosis (99). However, in phase 3 randomized double-blind placebo-controlled STELLAR-3 and STELLAR-4 studies in patients with F3 fibrosis and compensated F4 cirrhosis respectively, due to NASH, selonsertib did not show a histologic improvement in fibrosis however well-tolerated safety results. In the study of Maradana et al., liposome-encapsulated lipophilic curcumin or 1,25-dihydroxy-vitamin D3, also called calcitriol, was investigated in mice with diet-induced NASH. Curcumin and calcitriol are both NF-κB inhibitors and were shown to be taken up by hepatic macrophages and dendritic cells, leading to the suppression of hepatic inflammation, fat accumulation, fibrosis and insulin resistance (102).

Dampening of Monocyte Recruitment

MoMFs are recruited by KCs to the liver, where they amplify and maintain liver inflammation. The recruitment of monocytes by KCs is driven by chemokine receptor interactions of CCL2/CCR2 or CCL1/CCR8 (16–19). A strategy to reduce the number of monocytes recruited into the liver include an interference with chemokine signaling. Interference with chemokine signaling can be achieved with monoclonal antibodies against chemokines or receptors, receptor antagonists, inhibition of chemokines by aptamer molecules or small molecule inhibitors blocking chemokine-induced intracellular signaling (10).

Chemokine interference as a targeting therapy for treating liver diseases has been intensively studied (**Table 1** and **Figure 3**). Lefebvre and co-workers as well as Krenkel and colleagues showed that the dual CCR2/CCR5 inhibitor, called cenicriviroc (CVC) that efficiently blocks CCL2-mediated monocyte recruitment to the liver and has an anti-fibrotic effect in mouse models of liver and kidney fibrosis (103, 104). In a randomized controlled trial, Friedman et al., showed $\geq$2-point improvement in NAS with no worsening of fibrosis after 1 year of CVC treatment in patients with NASH (NAS $\geq$ 4) and stage 1–3 liver fibrosis (105). Baeck et al., investigated CCL2 inhibitor, RNA-aptamer molecule mNOX-E36 in CCl_4 fibrosis model and MCD-diet induced NASH model. mNOX-E36 inhibited early influx of $Ly6C^+$ monocytes thereby shifting macrophage equilibrium to $Ly6C^-$-restorative monocytes hence favoring fibrosis resolution (33, 107). Furthermore, Mulder et al., showed that CCR2 has a crucial role in the recruitment of immune cells to white adipose tissue and the liver, and a CCR2 inhibitor, propagermanium, attenuated liver inflammation and NASH development (106). Interestingly, chemokine CCL5/RANTES has been documented to play an important role in the progression of hepatic inflammation and fibrosis. Maraviroc, a CCL5/RANTES inhibitor, ameliorated hepatic steatosis in a high-fat diet (HFD)-induced model of NAFLD (108). These studies suggest that significant involvement of CCL/CCR pathways in macrophage recruitment and that the inhibition of these pathways showed potential therapeutic effects. However, monocyte recruitment to the liver when injury ensues does not only have negative consequences, but can also have positive implications, as shown in a study in which CSF1-Fc fragment promoted KC proliferation and monocyte infiltration and differentiation, restored innate immunity (92). However, as described previously, monocytes can differentiate into a plethora of phenotypes with discrete functions depending on the microenvironmental cues. They can also differentiate into a phenotype that is involved in the restoration of organ damage depending on the liver disease and stage of the disease.

CONCLUSION AND FUTURE PROSPECTIVE

Research about the insights in the initiation and progression of liver diseases has given the opportunity to develop novel therapeutic targeting strategies for the treatment of different liver diseases. The liver consists of a heterogenic population of hepatic macrophages, called KCs and MoMFs. Multiple studies have shown that hepatic macrophages play a pivotal role in liver homeostasis and liver diseases. Hepatic macrophages have central functions in the progression and regression of liver inflammation, ALF, liver fibrosis, NAFLD, ALD, viral hepatitis, and HCC. Different approaches have been developed to target hepatic macrophages to treat these different liver diseases. Due to the rapid advancement in nanomedicine attributable to its versatile application from drug delivery to diagnosis and imaging, few nanotechnology-enabled therapeutic modalities such as liposomes and polymeric micelles have been successfully approved for cancer treatment while others are under clinical investigation. Liposome and micelles based nanomedicines cause low toxicity and have a cost-efficient production. These are recent advantages that could help in the clinical translation of these nanomedicines. However, most of them have not been extensively tested yet in context to liver (and macrophage) targeting. Another promising nanocarrier could be solid lipid nanoparticles (SLN), which are based on solid components and are stable at room- and body temperature, resulting in a prolonged drug release. SLN consist of a lipid core that can be functionalized and stabilized with polymers to reduce non-specific cellular uptake (115).

Targeting therapies based on reducing the activation of KCs have been investigated. These therapies are mostly based on inhibiting intracellular inflammatory signaling pathways. KCs activation could also be dampened by restoring the normal gut microbiome, with probiotics, antibiotics, fecal microbiota transfer and sequestration of bile acids (12). In another study, cadherin-11 (CDH11) was increased during liver fibrosis suggesting this protein as an important regulator during liver fibrosis (117). The expression of CDH11 in injured cells, such as HSCs and macrophages, has been shown to regulate myofibroblasts activation and ECM production during the development of fibrosis. Therefore, CDH11 could be a potential therapeutic target of macrophages for the treatment of liver fibrosis. Furthermore, therapies have been focused on reducing monocyte recruitment to the liver. These therapies are mostly based on interfering with the chemokine signaling for monocytes. But as mentioned earlier, MoMFs can be categorized as Ly-6C^{high} monocytes, that cause organ impairment, and Ly-6C^{low} monocytes, which are organ restorative (39). Another potential strategy is to restore normal liver function by switching Ly6C^{high} monocytes into Ly6C^{low} monocytes. With this switch the function of the MoMFs could be changed into restorative instead of destructive.

Furthermore, therapies have been focused on reducing monocyte recruitment to the liver. These therapies are mostly based on interfering with the chemokine signaling for monocytes. But as mentioned earlier, MoMFs can be categorized as Ly-6C^{high} monocytes, that cause organ impairment, and Ly-6C^{low} monocytes, which are organ restorative (39). Another potential strategy is to restore normal liver function by switching Ly-6C^{high} monocytes into Ly-6C^{low} monocytes. With this switch the function of the MoMFs could be changed into restorative instead of destructive.

The recent studies have unraveled the large spectrum of macrophage phenotypes suggesting the heterogeneity and immunomodulatory functions of macrophages in liver diseases. Inspired by the recent developments, the questions that remain unanswered are: what is the significance and function of different macrophage phenotypes, can we reprogram the selective phenotypic macrophages for disease resolution and, finally how can we induce disease regression without affecting functions of other macrophage phenotypes and other cell types thereby reducing adverse effects? Taken together, the understanding of macrophage heterogeneity and their role in liver diseases gives the opportunity to translate this knowledge into developing targeted therapies to treat these diseases in the clinic.

AUTHOR CONTRIBUTIONS

DH and RB drafted the manuscript and designed the figures. RB and RW made the final corrections. All authors corrected and approved the manuscript.

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REFERENCES

1. Jenne CN, Kubes P. Immune surveillance by the liver. *Nat Immunol.* (2013) 14:996–1006. doi: 10.1038/ni.2691
2. Heymann F, Tacke F. Immunology in the liver-from homeostasis to disease. *Nat Rev Gastroenterol Hepatol.* (2016) 13:88–110. doi: 10.1038/nrgastro.2015.200
3. Tacke F, Zimmermann HW. Macrophage heterogeneity in liver injury and fibrosis. *J Hepatol.* (2014) 60:1090–6. doi: 10.1016/j.jhep.2013.12.025
4. Heymann F, Peusquens J, Ludwig-Portugall I, Kohlhepp M, Ergen C, Niemietz P, et al. Liver inflammation abrogates immunological tolerance induced by Kupffer cells. *Hepatology.* (2015) 62:279–91. doi: 10.1002/hep.27793
5. Breous E, Somanathan S, Vandenberghe LH, Wilson JM. Hepatic regulatory T cells and Kupffer cells are crucial mediators of systemic T cell tolerance to antigens targeting murine liver. *Hepatology.* (2009) 50:612–21. doi: 10.1002/hep.23043

6. Geerts A. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis.* (2001) 21:311–35. doi: 10.1055/s-2001-17550
7. Hellerbrand C. Hepatic stellate cells–the pericytes in the liver. *Pflugers Arch.* (2013) 465:775–8. doi: 10.1007/s00424-012-1209-5
8. Weiskirchen R, Weiskirchen S, Tacke F. Recent advances in understanding liver fibrosis: bridging basic science and individualized treatment concepts. *F1000Res.* (2018) 7:F1000. doi: 10.12688/f1000research.14841.1
9. Chang J, Hisamatsu T, Shimamura K, Yoneno K, Adachi M, Naruse H, et al. Activated hepatic stellate cells mediate the differentiation of macrophages. *Hepatol Res.* (2007) 43:658–69. doi: 10.1111/j.1872-034X.2012.01111.x
10. Tacke F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol.* (2017) 66:1300–12. doi: 10.1016/j.jhep.2017.02.026
11. Guillot A, Tacke F. Liver macrophages: old dogmas and new insights. *Hepatol Commun.* (2019) 3:730–43. doi: 10.1002/hep4.1356
12. Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cell Mol Immunol.* (2016) 13:316–27. doi: 10.1038/cmi.2015.104
13. Wang J, Kubes P. A reservoir of mature cavity macrophages that can rapidly invade visceral organs to affect tissue repair. *Cell.* (2016) 165:668–78. doi: 10.1016/j.cell.2016.03.009
14. Sica A, Invernizzi P, Mantovani A. Macrophage plasticity and polarization in liver homeostasis and pathology. *Hepatology.* (2014) 59:2034–42. doi: 10.1002/hep.26754
15. Abdullah Z, Knolle PA. Liver macrophages in healthy and diseased liver. *Pflugers Arch.* (2017) 469:553–60. doi: 10.1007/s00424-017-1954-6
16. Palis J, Robertson S, Kennedy M, Wall C, Keller G. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development.* (1999) 126:5073–84.
17. Mass E. Delineating the origins, developmental programs and homeostatic functions of tissue-resident macrophages. *Int Immunol.* (2018) 30:493–501. doi: 10.1093/intimm/dxy044
18. Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, et al. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature.* (2015) 518:547–51. doi: 10.1038/nature13989
19. Mass E, Ballesteros I, Farlik M, Halbritter F, Günther P, Crozet L, et al. Specification of tissue-resident macrophages during organogenesis. *Science.* (2016) 353:eaaf4238. doi: 10.1126/science.aaf4238
20. Hoeffel G, Chen J, Lavin Y, Low D, Almeida FF, See P, et al. C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity.* (2015) 42:665–78. doi: 10.1016/j.immuni.2015.03.011
21. Cumano A, Godin I. Ontogeny of the hematopoietic system. *Annu Rev Immunol.* (2007) 25:745–85. doi: 10.1146/annurev.immunol.25.022106.141538
22. Bertrand JY, Jalil A, Klaine M, Jung S, Cumano A, Godin I. Three pathways to mature macrophages in the early mouse yolk sac. *Blood.* (2005) 106:3004–11. doi: 10.1182/blood-2005-02-0461
23. Beattie L, Sawtell A, Mann J, Frame TCM, Teal B, de Labastida Rivera F, et al. Bone marrow-derived and resident liver macrophages display unique transcriptomic signatures but similar biological functions. *J Hepatol.* (2016) 65:758–68. doi: 10.1016/j.jhep.2016.05.037
24. Borst K, Frenz T, Spanier J, Tegtmeyer PK, Chhatbar C, Skerra J, et al. Type I interferon receptor signaling delays Kupffer cell replenishment during acute fulminant viral hepatitis. *J Hepatol.* (2018) 68:682–90. doi: 10.1016/j.jhep.2017.11.029
25. Blériot C, Dupuis T, Jouvion G, Eberl G, Disson O, Lecuit M. Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection. *Immunity.* (2015) 42:145–58. doi: 10.1016/j.immuni.2014.12.020
26. Lefere S, Degroote H, Van Vlierberghe H, Devisscher L. Unveiling the depletion of Kupffer cells in experimental hepatocarcinogenesis through liver macrophage subtype-specific markers. *J Hepatol.* (2019) 71:631–3. doi: 10.1016/j.jhep.2019.03.016
27. Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, Dube JJ, et al. Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. *Diabetes.* (2010) 59:347–57. doi: 10.2337/db09-0016
28. Reid DT, Reyes JL, McDonald BA, Vo T, Reimer RA, Eksteen B. Kupffer cells undergo fundamental changes during the development of experimental NASH and are critical in initiating liver damage and inflammation. *PLoS ONE.* (2016) 11:e0159524. doi: 10.1371/journal.pone.0159524
29. Roberts AW, Lee BL, Deguine J, John S, Shlomchik MJ, Barton GM. Tissue-resident macrophages are locally programmed for silent clearance of apoptotic cells. *Immunity.* (2017) 47:913–927.e6. doi: 10.1016/j.immuni.2017.10.006
30. Uderhardt S, Martins AJ, Tsang JS, Lämmermann T, Germain RN. Resident macrophages cloak tissue microlesions to prevent neutrophil-driven inflammatory damage. *Cell.* (2019) 177:541–55.e17. doi: 10.1016/j.cell.2019.02.028
31. Fogg DK., Sibon C, Miled C, Jung S, Aucouturier P, Littman DR., et al. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science.* (2006) 311:83–7. doi: 10.1126/science.1117729
32. Miura K, Namba N, Fujiwara M, Ohata Y, Ishida H, Kitaoka T, et al. An overgrowth disorder associated with excessive production of cGMP due to a gain-of-function mutation of the natriuretic peptide receptor 2 gene. *PLoS ONE.* (2012) 7:e42180. doi: 10.1371/journal.pone.0042180
33. Baeck C, Wehr A, Karlmark KR, Heymann F, Vucur M, Gassler N, et al. Pharmacological inhibition of the chemokine CCL2 (MCP-1) diminishes liver macrophage infiltration and steatohepatitis in chronic hepatic injury. *Gut.* (2012) 61:416–26. doi: 10.1136/gutjnl-2011-300304
34. Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, et al. Hepatic recruitment of the inflammatory $Gr1^+$ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology.* (2009) 50:261–74. doi: 10.1002/hep.22950
35. Miura K, Yang L, van Rooijen N, Ohnishi H, Seki E. Hepatic recruitment of macrophages promotes non-alcoholic steatohepatitis through CCR2. *Am J Physiol Gastrointest Liver Physiol.* (2012) 302:G1310–21. doi: 10.1152/ajpgi.00365.2011
36. Galastri S, Zamara E, Milani S, Novo E, Provenzano A, Delogu W, et al. Lack of CC chemokine ligand 2 differentially affects inflammation and fibrosis according to the genetic background in a murine model of steatohepatitis. *Clin Sci.* (2012) 123:459–71. doi: 10.1042/CS20110515
37. Heymann F, Hammerich L, Storch D, Bartneck M, Huss S, Rüsseler V, et al. Hepatic macrophage migration and differentiation critical for liver fibrosis is mediated by the chemokine receptor C-C motif chemokine receptor 8 in mice. *Hepatology.* (2012) 55:898–909. doi: 10.1002/hep.24764
38. Zigmond E, Samia-Grinberg S, Pasmanik-Chor M, Brazowski E, Shibolet O, Halpern Z, et al. Infiltrating monocyte-derived macrophages and resident Kupffer cells display different ontogeny and functions in acute liver injury. *J Immunol.* (2014) 193:344–53. doi: 10.4049/jimmunol.1400574
39. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci USA.* (2012) 109:E3186–95. doi: 10.1073/pnas.1119964109
40. Tacke F. Functional role of intrahepatic monocyte subsets for the progression of liver inflammation and liver fibrosis *in vivo*. *Fibrogenesis Tissue Repair.* (2012) 5(Suppl. 1):S27. doi: 10.1186/1755-1536-5-S1-S27
41. Seki E, De Minicis S, Gwak GY, Kluwe J, Inokuchi S, Bursill CA, et al. CCR1 and CCR5 promote hepatic fibrosis in mice. *J Clin Invest.* (2009) 119:1858–70. doi: 10.1172/jci37444
42. Cassado Ados A, D'Império Lima MR, Bortoluci KR. Revisiting mouse peritoneal macrophages: heterogeneity, development, and function. *Front Immunol.* (2015) 6:225. doi: 10.3389/fimmu.2015.00225
43. Rehermann B Mature peritoneal macrophages take an avascular route into the injured liver and promote tissue repair. *Hepatology.* (2017) 65:376–9. doi: 10.1002/hep.28883
44. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science.* (2009) 325:612–6. doi: 10.1126/science.1175202
45. Li L, Wei W, Li Z, Chen H, Li Y, Jiang W, et al. The spleen promotes the secretion of CCL2 and supports an M1 dominant phenotype in hepatic macrophages during liver fibrosis. *Cell Physiol Biochem.* (2018) 51:557–74. doi: 10.1159/000495276

46. Aoyama T, Kuwahara-Arai K, Uchiyama A, Kon K, Okubo H, Yamashina S, et al. Spleen-derived lipocalin-2 in the portal vein regulates Kupffer cells activation and attenuates the development of liver fibrosis in mice. *Lab Invest.* (2017) 97:890–902. doi: 10.1038/labinvest.2017.44
47. Roszer T. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediators Inflamm.* (2015) 2015:816460. doi: 10.1155/2015/816460
48. Wang LX, Zhang SX, Wu HJ, Rong XL, Guo J. M2b macrophage polarization and its roles in diseases. *J Leukoc Biol.* (2019) 106:345–58. doi: 10.1002/JLB.3RU1018-378RR
49. MacParland SA, Liu JC, Ma XZ, Innes BT, Bartczak AM, Gage BK, et al. Single cell RNA sequencing of human liver reveals distinct intrahepatic macrophage populations. *Nat Commun.* (2018) 9:4383. doi: 10.1038/s41467-018-06318-7
50. Antoniades CG, Berry PA, Wendon JA, Vergani D. The importance of immune dysfunction in determining outcome in acute liver failure. *J Hepatol.* (2008) 49:845–61. doi: 10.1016/j.jhep.2008.08.009
51. Wu Z, Han M, Chen T, Yan W, Ning Q. Acute liver failure: mechanisms of immune-mediated liver injury. *Liver Int.* (2010) 30:782–94. doi: 10.1111/j.1478-3231.2010.02262.x
52. Ju C, Reilly TP, Bourdi M, Radonovich MF, Brady JN, George JW, et al. Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem Res Toxicol.* (2002) 15:1504–13. doi: 10.1021/tx0255976
53. Pellicoro A, Ramachandran P, Iredale JP, Fallowfield JA. Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat Rev Immunol.* (2014) 14:181–94. doi: 10.1038/nri3623
54. Weiskirchen R, Weiskirchen S, Tacke F. Organ and tissue fibrosis: Molecular signals, cellular mechanisms and translational implications. *Mol Aspects Med.* (2019) 65:2–15. doi: 10.1016/j.mam.2018.06.003
55. Campana L, Iredale JP. Regression of Liver Fibrosis. *Semin Liver Dis.* (2017) 37:1–10. doi: 10.1055/s-0036-1597816
56. Drescher HK, Weiskirchen S, Weiskirchen R. Current status in testing for non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). *Cells.* (2019) 8:845. doi: 10.3390/cells8080845
57. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med.* (2002) 346:1221–31. doi: 10.1056/NEJMra011775
58. Adams LA, Anstee QM, Tilg H, Targher G. Non-alcoholic fatty liver disease and its relationship with cardiovascular disease and other extrahepatic diseases. *Gut.* (2017) 66:1138–53. doi: 10.1136/gutjnl-2017-313884
59. Boutens L, Stienstra R. Adipose tissue macrophages: going off track during obesity. *Diabetologia.* (2016) 59:879–94. doi: 10.1007/s00125-016-3904-9
60. Krenkel O, Tacke F. Macrophages in non-alcoholic fatty liver disease: a role model of pathogenic immunometabolism. *Semin Liver Dis.* (2017) 37:189–97. doi: 10.1055/s-0037-1604480
61. Kazankov K, Jørgensen SMD, Thomsen KL, Møller HJ, Vilstrup H, George J, et al. The role of macrophages in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. *Nat Rev Gastroenterol Hepatol.* (2019) 16:145–59. doi: 10.1038/s41575-018-0082-x
62. Stienstra R, Saudale F, Duval C, Keshtkar S, Groener JE, van Rooijen N, et al. Kupffer cells promote hepatic steatosis via interleukin-1β-dependent suppression of peroxisome proliferator-activated receptor α activity. *Hepatology.* (2010) 51:511–22. doi: 10.1002/hep.23337
63. Miura K, Yang L, van Rooijen N, Brenner DA, Ohnishi H, Seki E. Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of non-alcoholic steatohepatitis through inflammasome activation in mice. *Hepatology.* (2013) 57:577–89. doi: 10.1002/hep.26081
64. Negrin KA, Roth Flach RJ, DiStefano MT, Matevossian A, Friedline RH, Jung D, et al. IL-1 signaling in obesity-induced hepatic lipogenesis and steatosis. *PLoS ONE.* (2014) 9:e107265. doi: 10.1371/journal.pone.0107265
65. Clément S, Juge-Aubry C, Sgroi A, Conzelmann S, Pazienza V, Pittet-Cuenod B, et al. Monocyte chemoattractant protein-1 secreted by adipose tissue induces direct lipid accumulation in hepatocytes. *Hepatology.* (2008) 48:799–807. doi: 10.1002/hep.22404
66. Garcia-Martinez I, Santoro N, Chen Y, Hoque R, Ouyang X, Caprio S, et al. Hepatocyte mitochondrial DNA drives non-alcoholic steatohepatitis by activation of TLR9. *J Clin Invest.* (2016) 126:859–64. doi: 10.1172/JCI83885
67. Miura K, Ohnishi H. Role of gut microbiota and Toll-like receptors in non-alcoholic fatty liver disease. *World J Gastroenterol.* (2014) 20:7381–91. doi: 10.3748/wjg.v20.i23.7381
68. Krenkel O, Hundertmark J, Abdallah AT, Kohlhepp M, Puengel T, Roth T, et al. Myeloid cells in liver and bone marrow acquire a functionally distinct inflammatory phenotype during obesity-related steatohepatitis. *Gut.* (2019). doi: 10.1136/gutjnl-2019-318382. [Epub ahead of print].
69. Xiong X, Kuang H, Ansari S, Liu T, Gong J, Wang S, et al. Landscape of intercellular crosstalk in healthy and NASH liver revealed by single-cell secretome gene analysis. *Mol Cell.* (2019) 75:644–60.e5. doi: 10.1016/j.molcel.2019.07.028
70. O'Shea RS, Dasarathy S, McCullough AJ, Practice Guideline Committee of the American Association for the Study of Liver Diseases, Practice Parameters Committee of the American College of Gastroenterology. Alcoholic liver disease. *Hepatology.* (2010) 51:307–28. doi: 10.1002/hep.23258
71. Ju C, Liangpunsakul S. Role of hepatic macrophages in alcoholic liver disease. *J Investig Med.* (2016) 64:1075–7. doi: 10.1136/jim-2016-000210
72. Bala S, Marcos M, Gattu A, Catalano D, Szabo G. Acute binge drinking increases serum endotoxin and bacterial DNA levels in healthy individuals. *PLoS ONE.* (2014) 9:e96864. doi: 10.1371/journal.pone.0096864
73. Chen P, Stärkel P, Turner JR, Ho SB, Schnabl B. Dysbiosis-induced intestinal inflammation activates tumor necrosis factor receptor I and mediates alcoholic liver disease in mice. *Hepatology.* (2015) 61:883–94. doi: 10.1002/hep.27489
74. Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology.* (2011) 141:1572–85. doi: 10.1053/j.gastro.2011.09.002
75. Gao B, Seki E, Brenner DA, Friedman S, Cohen JI, Nagy L, et al. Innate immunity in alcoholic liver disease. *Am J Physiol Gastrointest Liver Physiol.* (2011) 300:G516–25. doi: 10.1152/ajpgi.00537.2010
76. Bukong TN, Iracheta-Vellve A, Saha B, Ambade A, Satishchandran A, Gyongyosi B, et al. Inhibition of spleen tyrosine kinase activation ameliorates inflammation, cell death, and steatosis in alcoholic liver disease. *Hepatology.* (2016) 64:1057–71. doi: 10.1002/hep.28680
77. Reiss G, Keeffe EB. Review article: hepatitis vaccination in patients with chronic liver disease. *Aliment Pharmacol Ther.* (2004) 19:715–27. doi: 10.1111/j.1365-2036.2004.01906.x
78. Boltjes A, Movita D, Boonstra A, Woltman AM. The role of Kupffer cells in hepatitis B and hepatitis C virus infections. *J Hepatol.* (2014) 61:660–71. doi: 10.1016/j.jhep.2014.04.026
79. Tu Z, Bozorgzadeh A, Pierce RH, Kurtis J, Crispe IN, Orloff MS. TLR-dependent cross talk between human Kupffer cells and NK cells. *J Exp Med.* (2008) 205:233–44. doi: 10.1084/jem.20072195
80. Boltjes A, van Montfoort N, Biesta PJ, Op den Brouw ML, Kwekkeboom J, van der Laan LJ, et al. Kupffer cells interact with hepatitis B surface antigen *in vivo* and *in vitro*, leading to proinflammatory cytokine production and natural killer cell function. *J Infect Dis.* (2015) 211:1268–78. doi: 10.1093/infdis/jiu599
81. Shrivastava S, Mukherjee A, Ray R, Ray RB. Hepatitis C virus induces interleukin-1β (IL-1β)/IL-18 in circulatory and resident liver macrophages. *J Virol.* (2013). 87:12284–90. doi: 10.1128/JVI.01962-13
82. Chang S, Dolganiuc A, Szabo G. Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins. *J Leukoc Biol.* (2007) 82:479–87. doi: 10.1189/jlb.0207128
83. Yang JD, Nakamura I, Roberts LR. The tumor microenvironment in hepatocellular carcinoma: current status and therapeutic targets. *Semin Cancer Biol.* (2011) 21:35–43. doi: 10.1016/j.semcancer.2010.10.007
84. Bruix J, Boix L, Sala M, Llovet JM. Focus on hepatocellular carcinoma. *Cancer Cell.* (2004) 5:215–9. doi: 10.1016/S1535-6108(04)00058-3
85. Berasain C, Castillo J, Perugorria MJ, Latasa MU, Prieto J, Avila MA. Inflammation and liver cancer: new molecular links. *Ann N Y Acad Sci.* (2009) 1155:206–21. doi: 10.1111/j.1749-6632.2009.03704.x
86. Capece D, Fischietti M, Verzella D, Gaggiano A, Cicciarelli G, Tessitore A, et al. The inflammatory microenvironment in hepatocellular carcinoma: a pivotal role for tumor-associated macrophages. *Biomed Res Int.* (2013) 2013:187204. doi: 10.1155/2013/187204
87. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *J Leukoc Biol.* (2009) 86:1065–73. doi: 10.1189/jlb.0609385

88. Porta C, Larghi P, Rimoldi M, Totaro MG, Allavena P, Mantovani A, et al. Cellular and molecular pathways linking inflammation and cancer. *Immunobiology.* (2009) 214:761–77. doi: 10.1016/j.imbio.2009.06.014
89. Qian BZ, Pollard J W. Macrophage diversity enhances tumor progression and metastasis. *Cell.* (2010) 141:39–51. doi: 10.1016/j.cell.2010.03.014
90. Triantafyllou E, Woollard KJ, McPhail MJW, Antoniades CG, Possamai LA. The role of monocytes and macrophages in acute and acute-on-chronic liver failure. *Front Immunol.* (2018) 9:2948. doi: 10.3389/fimmu.2018.02948
91. LiverTox: *Clinical and Research Information on Drug-Induced Liver Injury*. Bethesda, MD: National Institute of Diabetes and Digestive and Kidney Diseases (2012). Available online at: http://www.ncbi.nlm.nih.gov/books/NBK548400/
92. Stutchfield BM, Antoine DJ, Mackinnon AC, Gow DJ, Bain CC, Hawley CA, et al. CSF1 restores innate immunity after liver injury in mice and serum levels indicate outcomes of patients with acute liver failure. *Gastroenterology.* (2015) 149:1896–909.e14. doi: 10.1053/j.gastro.2015.08.053
93. Antoniades CG, Khamri W, Abeles RD, Taams LS, Triantafyllou E, Possamai LA, et al. Secretory leukocyte protease inhibitor: a pivotal mediator of anti-inflammatory responses in acetaminophen-induced acute liver failure. *Hepatology.* (2014) 59:1564–76. doi: 10.1002/hep.26933
94. He C, Yin L, Tang C, Yin C. Multifunctional polymeric nanoparticles for oral delivery of TNF-α siRNA to macrophages. *Biomaterials.* (2013) 34:2843–54. doi: 10.1016/j.biomaterials.2013.01.033
95. Bartneck M, Scheyda KM, Warzecha KT, Rizzo LY, Hittatiya K, Luedde T, et al. Fluorescent cell-traceable dexamethasone-loaded liposomes for the treatment of inflammatory liver diseases. *Biomaterials.* (2015) 37:367–82. doi: 10.1016/j.biomaterials.2014.10.030
96. Freund B, Heeren J, Nielsen P, Carambia A, Herkel J, Bruns O, et al. *Nanoparticle Compositions for Generation of Regulatory T Cells and Treatment of Autoimmune Diseases and Other Chronic Inflammatory Conditions. United States Patent Application 20180325821*. Available online at: http://www.freepatentsonline.com/y2018/0325821.html (accessed August 22, 2019). (2018).
97. Traber PG, Zomer E. Therapy of experimental NASH and fibrosis with galectin inhibitors. *PLoS ONE.* (2013) 8:e83481. doi: 10.1371/journal.pone.0083481
98. Kurniawan DW, Jajoriya AK, Dhawan G, Mishra D, Argemi J, Bataller R, et al. Therapeutic inhibition of spleen tyrosine kinase in inflammatory macrophages using PLGA nanoparticles for the treatment of non-alcoholic steatohepatitis. *J Control Release.* (2018) 288:227–38. doi: 10.1016/j.jconrel.2018.09.004
99. Loomba R, Lawitz E, Mantry PS, Jayakumar S, Caldwell SH, Arnold H, et al. The ASK1 inhibitor selonsertib in patients with non-alcoholic steatohepatitis: a randomized, phase 2 trial. *Hepatology.* (2018) 67:549–59. doi: 10.1002/hep.29514
100. Liu Y, Yan W, Tohme S, Chen M, Fu Y, Tian D, et al. Hypoxia induced HMGB1 and mitochondrial DNA interactions mediate tumor growth in hepatocellular carcinoma through Toll-like receptor 9. *J Hepatol.* (2015) 63:1114–21. doi: 10.1016/j.jhep.2015.02.009
101. Brenner C, Galluzzi L, Kepp O, Kroemer G. Decoding cell death signals in liver inflammation. *J Hepatol.* (2013) 59:583–94. doi: 10.1016/j.jhep.2013.03.033
102. Maradana MR, Yekollu SK, Zeng B, Ellis J, Clouston A, Miller G, et al. Immunomodulatory liposomes targeting liver macrophages arrest progression of non-alcoholic steatohepatitis. *Metab Clin Exp.* (2018) 78:80–94. doi: 10.1016/j.metabol.2017.09.002
103. Lefebvre E, Moyle G, Reshef R, Richman LP, Thompson M, Hong F, et al. Antifibrotic effects of the dual CCR2/CCR5 antagonist cenicriviroc in animal models of liver and kidney fibrosis. *PLoS ONE.* (2016) 11:e0158156. doi: 10.1371/journal.pone.0158156
104. Krenkel O, Puengel T, Govaere O, Abdallah AT, Mossanen JC, Kohlhepp M, et al. Therapeutic inhibition of inflammatory monocyte recruitment reduces steatohepatitis and liver fibrosis. *Hepatology.* (2018) 67:1270–83. doi: 10.1002/hep.29544
105. Friedman SL, Ratziu V, Harrison SA, Abdelmalek MF, Aithal GP, Caballeria J, et al. A randomized, placebo-controlled trial of cenicriviroc for treatment of non-alcoholic steatohepatitis with fibrosis. *Hepatology.* (2018) 67:1754–67. doi: 10.1002/hep.29477
106. Mulder P, van den Hoek AM, Kleemann R. The CCR2 inhibitor propagermanium attenuates diet-induced insulin resistance, adipose tissue inflammation and non-alcoholic steatohepatitis. *PLoS ONE.* (2017) 12:e0169740. doi: 10.1371/journal.pone.0169740
107. Baeck C, Wie X, Bartneck M, Fech V, Heymann F, Gassler N, et al. Pharmacological inhibition of the chemokine C-C motif chemokine ligand 2 (monocyte chemoattractant protein 1) accelerates liver fibrosis regression by suppressing Ly-6C^{+} macrophage infiltration in mice. *Hepatology.* (2014) 59:1060–72. doi: 10.1002/hep.26783
108. Pérez-Martínez L, Pérez-Matute P, Aguilera-Lizarraga J, Rubio-Mediavilla S, Narro J, Recio E, et al. Maraviroc, a CCR5 antagonist, ameliorates the development of hepatic steatosis in a mouse model of non-alcoholic fatty liver disease (NAFLD). *J Antimicrob Chemother.* (2014) 69:1903–10. doi: 10.1093/jac/dku071
109. Ferrari M. Cancer nanotechnology: opportunities and challenges. *Nat Rev Cancer.* (2005) 5:161–71. doi: 10.1038/nrc1566
110. Ranghar S, Sirohi P, Verma P, Agarwal V. Nanoparticle-based drug delivery systems: promising approaches against infections. *Brazilian Arch Biol Technol.* (2013) 57:209–22. doi: 10.1590/S1516-89132013005000011
111. Dreaden EC, Austin LA, Mackey MA, El-Sayed MA. Size matters: gold nanoparticles in targeted cancer drug delivery. *Ther Deliv.* (2012) 3:457–78. doi: 10.4155/tde.12.21
112. Polizzi MA, Stasko NA, Schoenfisch MH. Water-soluble nitric oxide-releasing gold nanoparticles. *Langmuir.* (2007) 23:4938–43. doi: 10.1021/la0633841
113. Han G, You CC, Kim BJ, Turingan RS, Forbes NS, Martin CT, et al. Light-regulated release of DNA and its delivery to nuclei by means of photolabile gold nanoparticles. *Angew Chem Int Ed Engl.* (2006) 45:3165–9. doi: 10.1002/anie.200600214
114. Maeda H. Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. *Bioconjug Chem.* (2010) 21:797–802. doi: 10.1021/bc100070g
115. Bartneck M, Warzecha KT, Tacke F. Therapeutic targeting of liver inflammation and fibrosis by nanomedicine. *Hepatobiliary Surg Nutr.* (2014) 3:364–76. doi: 10.3978/j.issn.2304-3881.2014.11.02
116. Weiskirchen R, Tacke F. Liver fibrosis: from pathogenesis to novel therapies. *Dig Dis.* (2016) 34:410–22. doi: 10.1159/000444556
117. Pedroza M, To S, Smith J, Agarwal SK. Cadherin-11 contributes to liver fibrosis induced by carbon tetrachloride. *PLoS ONE.* (2019) 14:e0218971. doi: 10.1371/journal.pone.0218971

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REVIEW
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Macrophages in Zebrafish Models of Liver Diseases

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Hepatic macrophages are key components of the liver immunity and consist of two main populations. Liver resident macrophages, known as Kupffer cells in mammals, are crucial for maintaining normal liver homeostasis. Upon injury, they become activated to release proinflammatory cytokines and chemokines and recruit a large population of inflammatory monocyte-derived macrophages to the liver. During the progression of liver diseases, macrophages are highly plastic and have opposing functions depending on the signaling cues that they receive from the microenvironment. A comprehensive understanding of liver macrophages is essential for developing therapeutic interventions that target these cells in acute and chronic liver diseases. Mouse studies have provided the bulk of our current knowledge of liver macrophages. The emergence of various liver disease models and availability of transgenic tools to visualize and manipulate macrophages have made the teleost zebrafish (*Danio rerio*) an attractive new vertebrate model to study liver macrophages. In this review, we summarize the origin and behaviors of macrophages in healthy and injured livers in zebrafish. We highlight the roles of macrophages in zebrafish models of alcoholic and non-alcoholic liver diseases, hepatocellular carcinoma, and liver regeneration, and how they compare with the roles that have been described in mammals. We also discuss the advantages and challenges of using zebrafish to study liver macrophages.

Keywords: kupffer cells, monocytes, regeneration, NAFLD, ALD, hepatocellular carcinoma

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INTRODUCTION

The liver is the largest internal organ in the body and exerts vital metabolic and immunological functions. Liver disease is a major health burden and accounts for ∼2 million deaths per year worldwide (1). Liver transplantation is often the only curative option for patients with liver failure due to acute or chronic liver injury, and thus there is an urgent unmet need for alternative treatment.

The liver contains the largest number of tissue-resident macrophages that account for 80–90% of all macrophages in the body (2). These so-called Kupffer cells are considered to be self-renewing and non-migratory. During homeostasis, they exert phagocytic function to clear pathogens that reach the liver through the circulating blood. This macrophage population also maintains immunological tolerance in the liver to reduce accidental immune responses. During injury, Kupffer cells become activated and secrete pro-inflammatory cytokines and chemokines to recruit bone marrow-derived monocytes to the liver (3). Extensive research in samples from patients with liver diseases and rodent models of liver injury has revealed that both Kupffer cells and monocyte-derived liver macrophages play critical roles in hepatic steatosis, inflammation, fibrosis, and cancer, making them appealing therapeutic targets. Developing macrophage-based therapy, however, is challenging because it is a highly heterogeneous population. In fact, a recent study using single-cell RNAseq has identified 10 subpopulations of macrophages in human control and cirrhotic livers (4). Furthermore, macrophages are very plastic and often have multiple and sometime opposing functions in promoting liver disease progression vs. repairing injured liver (5).

The teleost zebrafish, an increasingly popular vertebrate model for studying development and genetics, has shown promise in bringing new insights into our understanding of the ontogeny of liver macrophages and their responses to injury. Zebrafish form a functional liver by just 4 days post fertilization (6). Despite some architectural differences, the zebrafish liver contains a highly similar parenchymal and non-parenchymal cell inventory as the mammalian liver. Taking advantages of the transparent larva and the accessibility to genetic manipulation, researchers have generated transgenic fluorescent reporter strains to mark individual liver cell types, enabling real-time tracking of their morphology and behaviors during development and injury (6). Zebrafish have been used in translational research modeling various liver diseases such as drug-induced acute liver failure, cholestasis, non-alcoholic liver disease, alcoholic liver disease, and cancer (6–8). These studies have demonstrated that the signaling pathways governing liver injury responses are highly conserved between zebrafish and mammals. Zebrafish are also an excellent *in vivo* model system for studying the innate immune system. The embryos have functional macrophages at 1 day post fertilization and neutrophils by 2 days (9). The zebrafish macrophages have conserved marker gene expression and functions as their mammalian counterparts. They can be easily visualized during homeostasis and inflammatory processes using the fluorescent reporter lines (9). **Table 1** summarizes the tools for observing and manipulating macrophages in zebrafish.

Recent studies have confirmed the presence of macrophages in the livers of larval and adult zebrafish in physiological and pathological conditions. In this review, we provide an overview of the origin and development of hepatic macrophages in zebrafish. We highlight the recent advances where zebrafish transgenesis and imaging approaches reveal new aspects of macrophage functions in liver diseases. In particular, we focus on their roles in non-alcoholic and alcoholic liver disease, hepatocellular carcinoma, and liver regeneration. The capabilities and potential

TABLE 1 | Tools to study macrophages in Zebrafish.

Markers for macrophages		
Dye		
Neutral Red	Marks live macrophages	(10)
Riboprobes for *in situ* hybridization		
csf1ra	Also labels neural crests	(11)
mfap4		(12)
cxcr3.2		(12)
mpeg1		(12, 13)
mpeg1.2		(14)
ptpn6		(12)
Antibody		
L-plastin	Pan-leukocyte marker	(15, 16)
Mpeg		(15)
WCL15	Antigen unknown	(11, 17, 18)
Transgenic Reporter Line		
Tg(mpeg1:GFP); Tg(mpeg1:mCherry); Tg(mpeg1:Gal4-VP16)		(13)
Tg(mpeg1:Dendra2)	Photoconvertible protein	(19)
Tg(mpeg1:Kaede)	Photoconvertible protein	(20)
Tg(mpeg1:Cre)	Applications include lineage tracing and tracking macrophage-dependent cytoplasmic transfer.	(21)
TgBAC(csf1ra:Gal4-VP16) *TgBAC(csf1ra:EGFP)*	Marks mononuclear phagocytes	(22) (23)
Tg(mfap4:quoise); Tg(mfap4:GFP)		(24, 25)
Tg(tnfα:EGFP-F)	Marks activated macrophage	(26)
Tg(irg1:EGFP)	Marks activated macrophage	(27)
TG(CORONIN1A:GFP)	Marks myeloid cells and lymphocytes	(28)
MACROPHAGE-SPECIFIC ABLATION MODELS		
Chemicals		
Clodronate liposomes		(29, 30)
Carrageenan		(31)
Macrophage-deficient mutants and morphants (morpholino-injected animals)		
Panther/csfr1a mutant	Reduced primitive macrophages	(11, 20, 32)
irf8 mutant	Reduced macrophages and increased neutrophils	(33, 34)
irf8 morphant	Reduced macrophages and increased neutrophils	(33)
Pu.1 morphant	Lacks macrophage up to 3 days post fertilization; shows mortality after day 7.	(35, 36)
Nitroreductase-based macrophage ablation		
Tg(mpeg1:NTR-eYFP)		(37)
Tg(mpeg1:Gal4-VP16; UAS:NTR-mCherry);		(38)

(Continued)

TABLE 1 | Continued

Markers for macrophages	
MODELS WITH IMPAIRMENT IN MACROPHAGE MIGRATION/CHEMOTAXIS	
cxcr3.2 mutant	(39)
cxcr3.2 morphant	(12)
Thymosin β4 sulfoxide treatment	(40)

of the zebrafish model in studying liver macrophages are also discussed (summarized in **Figure 1**).

THE ORIGIN OF HEPATIC MACROPHAGES IN ZEBRAFISH

Overview of Zebrafish Hematopoiesis

Similar to mammals, the development of the zebrafish hematopoietic system is characterized by several distinct waves (41, 42). The first wave, referred as primitive, occurs during early somitogenesis in the ventral lateral mesoderm and rostral blood island (RBI) at ∼11 h post fertilization (hpf). The progenitors converge to the midline to form the intermediate cell mass, which is the primary site for primitive hematopoiesis and functionally equivalent to mammalian yolk sac blood islands. The process continues at ∼24 hpf in the RBI during which the transient erythro-myeloid precursors (EMPs) are formed. The EMPs have limited lineage differentiation potential and lack the self-renewal capacity (43). The second or definitive wave of hematopoiesis starts at ∼36 hpf when the first hematopoietic stem cells (HSCs) emerge from the ventral wall of the dorsal aorta (VDA) in the aorta-gonad mesonephros (AGM) region. This process is conserved among vertebrate species and gives rise to a multipotent cell type that can contribute to the entire hematopoietic lineage (44, 45). Another conserved feature between mammals and zebrafish is the migratory ability of the HSCs as they seed in different anatomical niches in order to differentiate and proliferate. Subsequently, hematopoiesis proceeds in the distal region of the tail, which is known as the caudal hematopoietic tissue (CHT) and represents the equivalent of the mammalian fetal liver (46). At about 96 hpf, the HSCs migrate either from the CHT or directly from the AGM to colonize the pronephros (47). There they will constitute the kidney, which corresponds to the mammalian bone marrow, to provide the adult zebrafish with hematopoiesis throughout their lifespan.

Tissue Resident Macrophages Arise From the HSC Origin, a Lesson From Fish

The zebrafish innate immune system is mainly composed of macrophages and neutrophils. Both are derived from the myeloid lineage that emerges during the primitive hematopoietic wave from the cells in the lateral plate mesoderm expressing *Spi-1 proto-oncogene* (*spi1*) and *lymphocyte cytosolic protein 1 (lcp1)* also known as *L-plastin* (48, 49). Definitive hematopoiesis continues to contribute to the myeloid lineage and sustains its functionality throughout the lifespan. The innate immune system solely provides zebrafish with immune defense during the first month of life until the adaptive immune system fully develops (50).

The macrophage population consists of tissue-resident macrophages, bone marrow-derived recruited macrophages, and peritoneal macrophages. Resident macrophages are present in most tissues across the body and fulfill vital functions in homeostasis (51). It has been shown that the early EMPs populate different organs during development to form most of the resident macrophages in mice. This macrophage population acquires specialized, tissue-resident properties, and harbors self-renewing potential to maintain the adult population (52–55). One exception is the gut, where the macrophage population is continually replenished by circulating monocytes that differentiate into the mature resident macrophages (56).

Studies of resident macrophages in zebrafish have provided novel assessments of their origin. Recent work identified the age-dependent origin of microglia (57). While the primitive macrophages give rise to a transient population of microglia during the early larval stage, the adult microglia originate from the *cmyb*-dependent HSCs. Similar observations were made in adult zebrafish Langerhans cells and several other resident-macrophage populations (58), challenging the current model of the erythro-myeloid origin of tissue macrophages. The zebrafish results are supported by a recent mammalian study (59), although there is still much controversy in the field (54).

Origin of Liver Resident Macrophages in Zebrafish

The liver is continuously exposed to antigens, microbial products, and xenobiotics. To adapt to such an environment, the liver harbors the largest population of macrophages among the solid organs and is constantly patrolled by circulating monocytes. Based on the ontogeny studies conducted mainly in mice, Kupffer cells originate from the yolk sac-derived erythro-myeloid progenitors that express macrophage colony stimulating factor 1 receptor (CSF1R) and are self-renewing (54). Recently, HSCs and some common circulating precursors have also been implicated in the development of Kupffer cells (53, 59, 60).

Resident macrophages have been observed in the adult zebrafish liver (58, 61–63). In elegant work, He and colleague utilized a laser-mediated temporal-spatially resolved cell labeling IR-LEGO-CreER-loxP system to mark cells within different hematopoietic compartments during distinctive waves of hematopoiesis and trace the destination of the labeled cells in adults (58). Followed by fine fate-mapping analysis, they showed that most of the primary tissue-resident macrophages in adult zebrafish, including those in the liver, are derived from the VDA, suggesting their HSC origin. This work illustrates how zebrafish can offer unique tools to elucidate the ontogeny of hepatic macrophages, which is a challenging topic in hepatology.

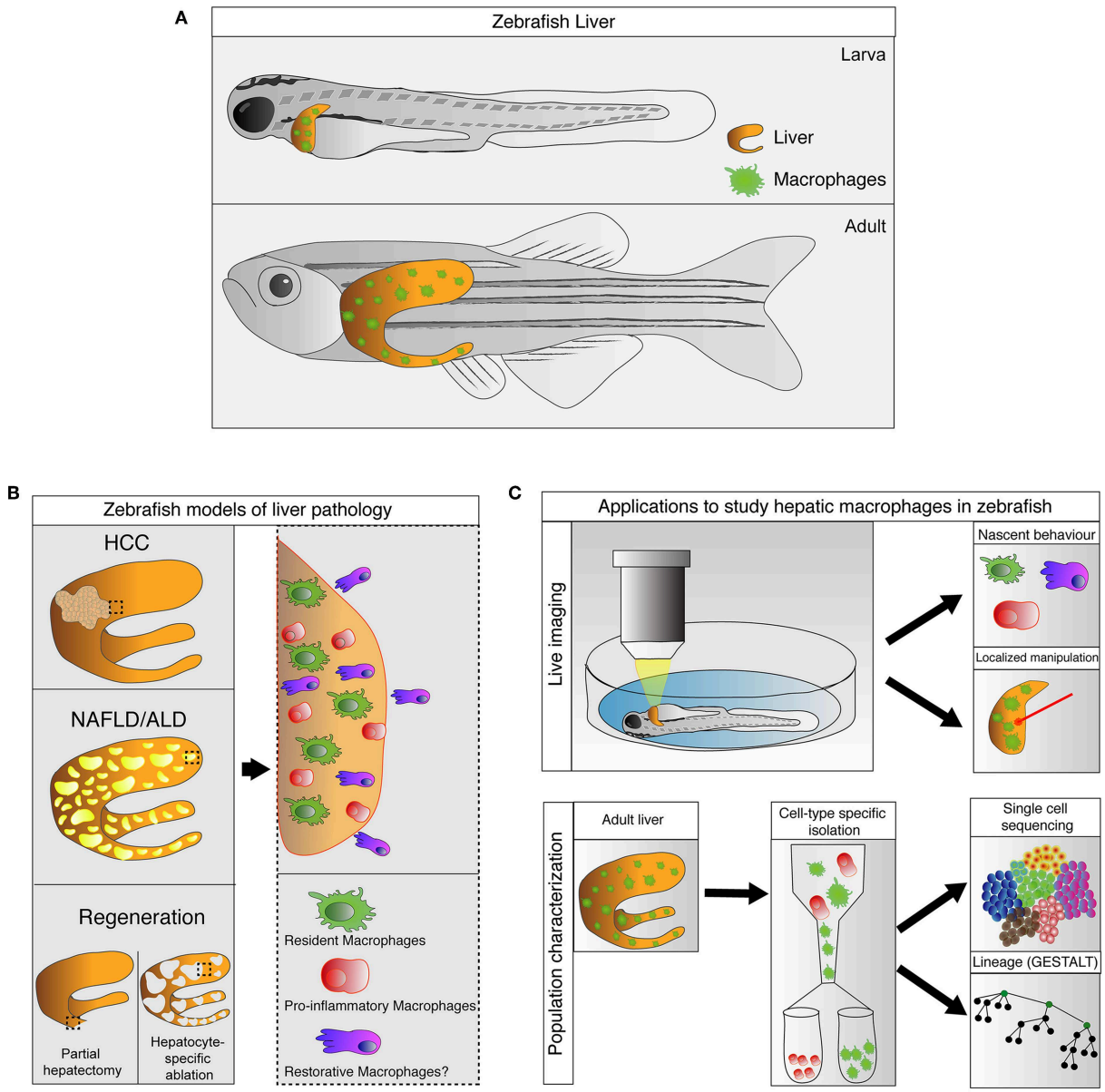

FIGURE 1 | Zebrafish, an emerging model for study hepatic macrophages. **(A)** Hepatic macrophages are present in the zebrafish liver at both larval and adult stages. **(B)** Increases in macrophage numbers have been observed in zebrafish models of liver pathology include non-alcoholic liver disease (NAFLD), alcoholic liver disease (ALD), and hepatocellular carcinoma (HCC), as well as in liver regeneration after partial hepatectomy and hepatocyte-specific ablation (left). Involvement of heterogeneous macrophage populations has been implicated in these models (right). **(C)** Current and potential applications available in zebrafish to study hepatic macrophages. Zebrafish larva is accessible for live imaging, allowing characterization of macrophage behaviors during early stages of immune responses. The live imaging platform in larva can also be utilized for laser-mediated localized manipulations of gene expression and cell ablation. Technologies such as GESTALT (genome editing of synthetic target arrays for lineage tracing) and single cell RNA-sequencing can be utilized to study the ontology and plasticity of macrophages in healthy and injured livers at a population level.

MACROPHAGES IN ZEBRAFISH MODELS OF LIVER DISEASES

Non-alcoholic and Alcoholic Liver Disease

Non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) are among the leading causes of liver-related morbidity and mortality and primary indications for liver transplantation. In both diseases, extensive hepatic lipid accumulation caused by metabolic stress or alcohol consumption induces hepatocyte cell death (64). Damaged hepatocytes release danger-associated molecular patterns (DAMPs) to trigger activation of Kupffer cells and infiltration of circulating monocytes (3). Macrophages play divergent roles in NAFLD and ALD: they exhibit a pro-inflammatory phenotype during disease progression (65, 66) and become anti-inflammatory and tissue-protective during disease regression (66, 67). Feeding zebrafish

larvae with a high cholesterol diet (5% cholesterol w/w) for a week can cause elevated triglyceride and total cholesterol levels and lipid accumulation in the body. The animals develop macrovesicular steatosis in the liver by 1-week of feeding and display ballooning degeneration by 3 weeks (68). De Oliveira et al. showed that short-term feeding with a high fat diet (HFD) results in clustering of macrophages in the zebrafish larval liver (61). Whereas, the macrophages in the control livers constantly patrol the environment, the macrophages in the HFD-fed liver are more stationary and adopt a rounder morphology. They start to express TNFα, a consensus marker of M1 macrophages, consistent with activation and polarization of these cells (26).

It has been reported by multiple groups that acute and chronic ethanol treatment can induce hepatic steatosis in larval and adult zebrafish, respectively (69–73). In human and mouse, after alcohol consumption, ethanol enters the blood circulation through the gastrointestinal tract and reaches the liver via the portal vein (74). Ethanol is metabolized in the liver mainly by alcohol dehydrogenase ADH1 and cytochrome P450 2E1/CYP2E1 enzymes. Zebrafish have analogs of ADH1 and CYP2E1 that are capable of metabolizing ethanol (75). Treatment with pharmacological inhibitors of ADH1 and CYP2E1 blocks ethanol-induced hepatic steatosis in zebrafish larvae, indicating that steatosis is caused by ethanol metabolism (76). Zebrafish alcoholic injury models are achieved by aqueous exposure of the animals to ethanol. Thus, ethanol exposure can go through multiple routes, including the gastrointestinal tract, gill, and skin. Since the expression of ethanol-metabolizing enzymes has not been characterized at the tissue level in zebrafish, it is not clear whether tissues other than the liver participate in ethanol metabolism.

In the acute alcoholic liver injury model, exposing 4-day-old zebrafish larvae to 2% ethanol for 24 h causes hepatic steatosis (**Figure 2**) (71, 76). At this stage, the yolk provides the animal all the nutrients and is likely the source of fat in steatosis (76). Mammalian studies indicate that alcohol exposure increases the ratio of reduced nicotinamide adenine dinucleotide/oxidized nicotinamide adenine dinucleotide and subsequently impairs mitochondrial β-oxidation of fatty acids (77). Alcohol exposure also promotes lipogenesis and inhibits fatty acid oxidation by regulating the transcription factors of lipid metabolism. In the zebrafish acute alcoholic liver injury model, alcohol-induced lipogenesis requires activation of the sterol regulatory element binding protein (SREBP) transcription factors and involves the unfolded protein response pathway (71, 76, 78). In zebrafish larvae, acute ethanol exposure also prompts hepatic stellate cells to express extracellular matrix proteins and causes dilatation of the hepatic blood vessels (73). One day after ethanol is removed, there is an increase in the number of macrophages in the treated liver (**Figure 2**), accompanied with increased hepatic angiogenesis and hepatic stellate cell proliferation (73). In mammalian models of chronic liver injury, macrophages are the source of vascular endothelial growth factor that promotes angiogenesis (79). They also have dual function in fibrosis: both Kupffer cells and monocyte-derived macrophages are profibrogenic during fibrosis progression as they secrete TGFβ1 and PDGF to activate hepatic stellate cells and mediate the survival of myofibroblasts (80). When the insults are removed, monocyte-derived macrophages become antifibrotic to aid in the resolution of fibrosis. In our opinion, the zebrafish acute alcoholic liver injury model is useful for studying the initial responses of macrophages, endothelial cells, and hepatic stellate cells upon the addition and removal of ethanol. Such responses trigger the subsequent cascades of events underlying disease progression and regression.

It is important to note that NAFLD and ALD are chronic diseases progressing from hepatic steatosis to steatohepatitis, and further to fibrosis and cirrhosis, increasing the risk for hepatocellular carcinoma. The zebrafish NAFLD and ALD models described above are mainly based on short-term treatment at larval stages and do not recapitulate the full spectrum of the disorders in human. Fibrosis has not been observed in the larval NAFLD and ALD models, which could be due to the fact that the zebrafish liver does not have the portal-central arrangement as the mammalian liver. The duration of the experiments may not be long enough for fibrosis to develop. Therefore, it is important to validate the findings in adult chronic injury models and mammalian systems.

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most prevalent primary malignancy of the liver and results in ∼800,000 deaths globally per year (81). It is the fastest growing cancer in the US. Accumulation of tumor-associated macrophages is commonly seen in the livers of patients with HCC and the number of macrophages correlates with HCC progression and poor prognosis (82, 83). As key components of the tumor microenvironment, macrophages are thought to be pro-inflammatory and pro-tumorgenic during HCC progression, but may switch to become anti-tumorgenic during HCC regression (79). In zebrafish, HCC can be induced by carcinogen and mutagen treatment, genetic mutations of tumor suppressor genes and oncogenes, and transgenic overexpression of oncogenes (7, 84). Zebrafish and human HCCs share similar histological features and gene signatures (85, 86). Increases in macrophage numbers have been observed in zebrafish HCC models with different tumorigenic triggers (35, 61, 87, 88).

By live imaging, De Oliverira et al. showed that HFD feeding induces changes in macrophage morphology and polarization in a transgenic zebrafish HCC model expressing activated β-cateninin in the hepatocytes (61). Ablating macrophages prior to HFD feeding suppresses the exacerbated liver enlargement in HCC fish that is caused by HFD. Treatment with anti-diabetic agent metformin has similar inhibitory effects on HCC progression associated with HFD. Whereas, metformin has previously been proposed as a promising treatment for HCC, the zebrafish study provides direct *in vivo* evidence to show that it suppresses NAFLD-associated HCC progression by decreasing the number of pro-inflammatory macrophages and increasing T cell infiltration.

One challenge for investigating the roles of liver macrophages in HCC is that tumor formation often occurs in parallel with the progression of chronic liver disease. It is difficult to

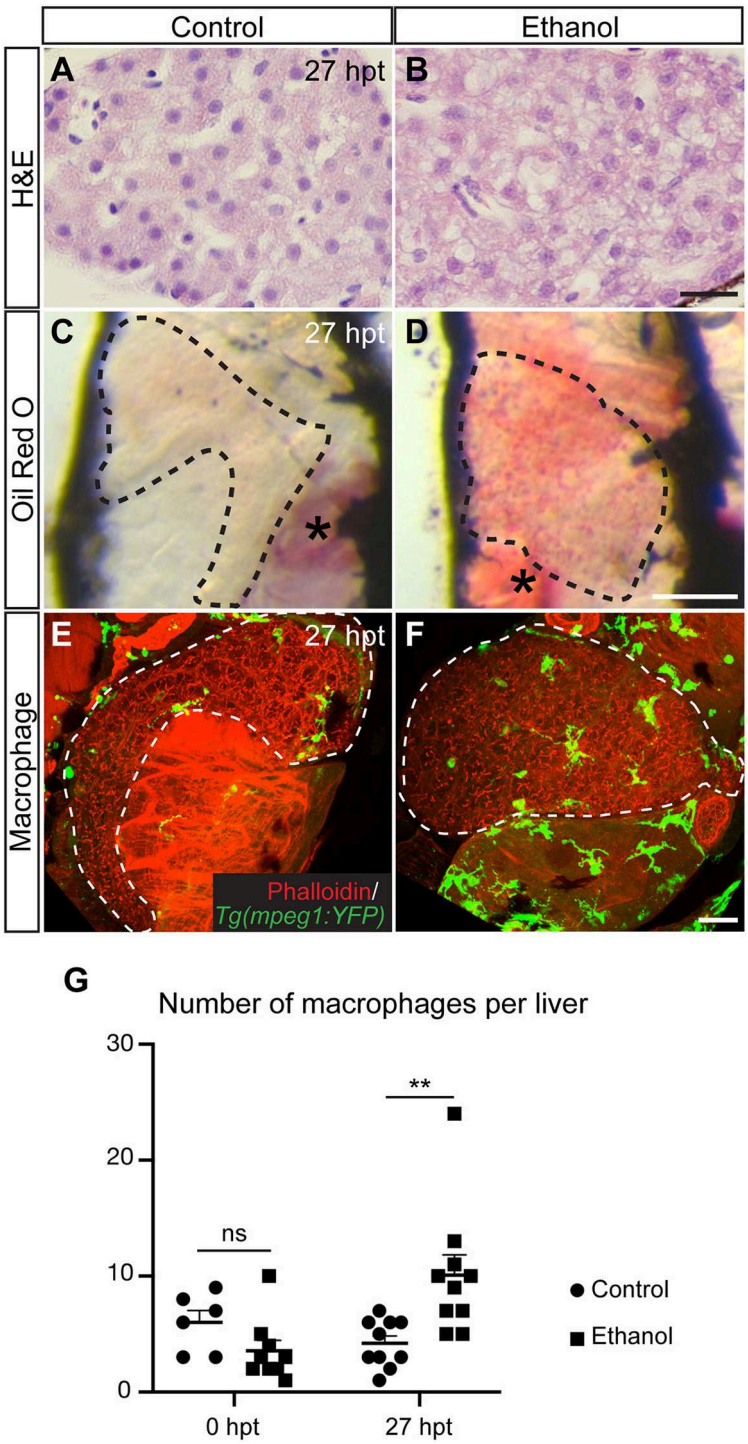

FIGURE 2 | Acute ethanol treatment causes hepatic steatosis and increases macrophage numbers in larval zebrafish. **(A,B)** Hematoxylin and eosin (H&E) staining of the paraffin sections showing the livers in a control larva **(A)** and a larva treated with 2% ethanol from 96 to 120 h post fertilization **(B)**. The livers were harvested at 27 h post treatment (hpt). Scale bar, 20 μm. **(C,D)** Representative images of the whole-mount Oil Red O staining in the control **(C)** and ethanol-treated larvae **(D)**. Dashed line outlines the liver. Lateral view, anterior is to the top. Oil Red O also stains the swim bladder (asterisk in C) and the residual yolk tissue (asterisk in D). Scale bar, 250 μm. **(E,F)** Confocal three-dimensional projections showing *Tg(mpeg1:YFP)*-expressing macrophages (green) in the whole liver at 27 hpt. Phalloidin staining (red) that labels cell cortex is used for recognizing various organs. Ventral views, anterior is to the top. Dashed line outlines the liver. Scale bar, 30 μm. **(G)** Numbers (mean±s.e.m.) of macrophages per liver at 0 hpt (left) and 27 hpt (right). Statistical significance was calculated by one-way ANOVA and Tukey's *post-hoc* test. $^{**}p < 0.01$, ns, not significant. This figure is reproduced with permission from Zhang et al. (73) and *Disease Models & Mechanisms*.

segregate the roles of macrophages in maintaining a prone-tumor inflammatory microenvironment vs. promoting HCC in response to tumor-derived signals (3). Multiple transgenic zebrafish lines utilizing chemically inducible expression systems (Tet-on, Tet-Off, and Mifepristone) have been generated to overexpress different oncogenes specifically in the hepatocytes (89). These models exclude the impact of chronic liver disease on HCC formation. Moreover, HCC can be induced in a temporally controlled manner and is regressed after removal of the chemicals, allowing investigation of macrophages at different stages of HCC progression and regression. In a transgenic zebrafish HCC model with inducible expression of oncogene *Xmrk* that encodes a hyperactive epidermal growth factor receptor (EGFR) homolog, the number of macrophages is increased during both HCC formation and regression (87). Interestingly, the macrophages are randomly distributed during HCC formation, and gradually show prominent blood vessel association as HCC regresses, implying that they have different functions at these two stages.

In vivo live imaging of the interactions between oncogenic hepatocytes and their microenvironment can be technically difficult in rodent models. Such analyses are readily feasible in zebrafish larvae due to their transparent body and availability of cell type-specific transgenic fluorescent reporter lines. Yang et al. investigated the responses of hepatocytes, innate immune cells and hepatic stellate cells during early stage of liver tumorigenesis in a $kras^{v12}$-induced HCC model (63). Upon hepatocyte-specific $kras^{v12}$ overexpression, there is sequential infiltration of neutrophils and macrophages, followed by proliferation and activation of hepatic stellate cells. Whereas, decreasing macrophage numbers by knocking down *irf8* or *pu.1* impairs both survival and activation of hepatic stellate cells, reducing neutrophils only affects their activation. The study further revealed reciprocal interactions between hepatic stellate cells and immune cells in HCC. Upon HCC induction, hepatocytes and macrophages increase expression levels of serotonin to regulate hepatic stellate cell survival and activation. In return, activated hepatic stellate cells secrete TGFβ1 to promote the pro-tumorigenesis function of neutrophils and macrophages. This work demonstrates the dynamic intercellular crosstalks within the tumor microenvironment that are crucial for liver tumorigenesis.

HCC is a male-biased disease with a male-to-female ratio of 2.4 worldwide (90). It is more aggressive and has worse prognosis in men than in women. The gender disparity also exists in rodent and zebrafish HCC models (91–93). In a series of reports from the Gong laboratory, the mechanisms of male-biased HCC carcinogenesis were explored in the transgenic zebrafish with inducible expression of oncogenes. In HCC models induced by $kras^{v12}$ and *xmrk* expression, there is an enhancement of hepatocarcinogenesis in male zebrafish compared to females (35, 94). Male HCC livers express higher levels of serotonin. It is accompanied with higher numbers of total hepatic stellate cells and activated hepatic stellate cells, as well as more severe infiltration of macrophages and neutrophils. The sex disproportion of HCC is thought to be not only due to varying risk factors in men and women, but also associated with the regulation of inflammatory responses in the tumor microenvironment by sex hormones (95, 96). Yet, the results of estrogen- and androgen-related clinical trials are inconclusive (97–99), suggesting the possible involvement of other hormones. One candidate is cortisol that is predominantly expressed in the male livers (35, 100). In the zebrafish $kras^{v12}$ and *xmrk* HCC models, cortisol induces expression of TGFβ1, which subsequently promotes infiltration of macrophages and neutrophils to accelerate hepatocarcinogenesis. The positive correlation between cortisol, TGFβ1, and macrophage/neutrophil infiltration has also been observed in patients with HCC (35).

MACROPHAGES IN ZEBRAFISH MODELS OF LIVER REGENERATION

Aligned with their involvement in liver diseases, macrophages are key participants in liver regeneration (101–103). Upon injury, liver macrophages infiltrate to the wound site to remove the dead hepatocytes. They also produce cytokines IL6 and TNFα that prompt hepatocytes to enter the mitotic cycle. Depletion of Kupffer cells in rodents by clodronate liposomes delays liver regeneration and exaggerates liver damage after partial hepatectomy (104, 105). Three liver regeneration models have been characterized in depth in zebrafish and the contribution of macrophages has been investigated. Following one-third partial hepatectomy, the adult zebrafish liver regains its original volume within 14 days via compensatory growth of the remnant hepatocytes (106–108). Macrophages accumulate at the amputation site within 48 h after the surgery to clear up neutrophils and resolve local inflammation (109). Digestive-organ-expansion-factor (Def) is a nucleolar protein that mediates p53 degradation in the nucleolus. In zebrafish with haploinsufficiency of Def, aberrant expression of cytokines halts the timely migration of macrophages to the amputation site. The resulting delay in neutrophil clearance and prolonged inflammation cause fibrotic scar formation.

Two hepatocyte-specific ablation models have been established in zebrafish. In one model, the transgenic zebrafish expressing the oxygen-insensitive NAD(P)H nitroreducatse (NTR) in hepatocytes are treated with the antiprotozoal metronidazole. This prodrug is metabolized into a cytotoxin by NTR to induce rapid death of the hepatocytes (110, 111). Treatment with metronidazole from 3.5 to 5 days post fertilization results in nearly complete hepatocyte ablation. The liver size fully recovers just 5 days after removal of the drug (112). In a second model, temporary knockdown of mitochondrial importer gene *tomm22* by morpholino oligonucleotide leads to hepatocyte degeneration. The liver in the morpholino-injected animal is smaller at 4 days post fertilization, but starts to regenerate as the morpholino effect expires and *tomm22* expression is restored to the wild-type level (17). By 8 days post fertilization, the liver displays the size and structure that resemble the uninjected control. Unlike partial hepatectomy in which liver regeneration is driven by proliferation of existing hepatocytes, in both hepatocyte-NTR and *tomm22*-knockdown

models, extensive hepatocyte loss triggers dedifferentiation of biliary epithelial cells into liver progenitor cells to form new hepatocytes (112, 113). Robust recruitment of macrophages and engulfment of hepatocyte debris by macrophages are seen in both models (17, 114). In *tomm22*-knockdown model, the surviving hepatocytes turn on biliary markers to become hybrid cells that express both hepatocyte and biliary markers (38). Ablation of macrophages suppresses the formation of hydrid cells, which coincides with the reduction of Wnt/β-catenin signaling activity. This is consistent with the mammalian findings that macrophages produce Wnt3a to promote liver progenitor cell differentiation toward the hepatocyte fate during regeneration (115).

By combining the liver regeneration models with the transgenic macrophage reporter lines, it is feasible to monitor macrophage recruitment, efferocytosis, and their interactions with other hepatic cells *in vivo* throughout the course of liver regeneration. The liver macrophages in *tomm22*-knockdown model exhibit a shift in morphology during the regeneration phase (38), suggesting that they undergo activation and polarization similar to their mammalian counterparts. In rodents, Kupffer cells and blood monocyte-derived macrophages play different roles in liver regeneration depending on the type of the original injury (2, 5). It will be interesting to utilize the zebrafish partial hepatectomy and hepatocyte depletion models to compare the source of macrophages and their functions in hepatocyte- and biliary-driven liver regeneration, respectively.

CONCLUSION AND FUTURE PERSPECTIVES

Several possible strategies can be used to design macrophage-based treatment for acute and chronic liver diseases: (1) Suppressing Kupffer cell activation; (2) blocking monocyte recruitment; (3) rendering macrophages toward a more restorative phenotype; and (4) macrophage cell therapy (3, 116). Not every aspect of liver macrophage biology can be easily investigated using *in vitro* systems and rodent models and including complementary animal models will be beneficial. Zebrafish has the complexity of a vertebrate system, established models of acute and chronic liver injury, conserved innate immune cells, and superior genetic and live-imaging capabilities, making it an attractive alternative animal model for studying macrophages in liver homeostasis and diseases. In this review, we have discussed the strengths of using zebrafish to visualize macrophages and monitor their interactions with other hepatic cells, and to manipulate these cells using genetic approaches.

The characterization of zebrafish liver macrophages is only at the beginning stage and much remains to be learned. Transcriptomic analysis of zebrafish macrophages has been performed in the context of Mycobacterial infection (117). However, the macrophage transcriptome has not been investigated in healthy and injured liver in zebrafish and to what degree this is comparable to humans is not clear. Few macrophage-specific antibodies are available in zebrafish. In particular, cell surface markers labeling macrophages at different polarization states have yet to be identified. Most of the zebrafish liver studies are conducted on larvae, as live imaging becomes less feasible beyond the larval stage when they are no longer transparent. Fibrosis and cirrhosis, however, are chronic processes and the duration of the larvae studies may not be long enough for fibrosis to develop. Another caveat of studying larvae is that the zebrafish immune system is primarily innate during the first month of life and the adaptive system only becomes fully functional afterwards (50). On one hand, the temporal separation of innate and adaptive immune systems permits exclusive interrogation of innate immune cell function without having significant influence from adaptive immunity. On the other hand, pathogenesis of human liver diseases does involve both innate and adaptive immunity and it is necessary to validate the larval findings in adult liver disease models. Comparative studies on human vs. zebrafish liver macrophages in physiological and disease conditions are very limited, and thus the human relevance of zebrafish findings should be evaluated.

Liver macrophages are highly polymorphic. The lack of tools to distinguish macrophages from different origins and at different activation states has prevented the assignment of specific functionalities to each subgroup, making it difficult to develop treatment that only targets the macrophage subgroups with detrimental effect. Some emerging technologies in zebrafish may open exciting revenues for interrogating the ontogeny, activation, heterogeneity, and plasticity of liver macrophages. The zebrafish model possesses an excellent toolbox for lineage-tracing and fate-mapping analyses to understand the ontogeny of different liver macrophage subgroups in normal and diseased livers. For instance, the Zebrabow system allows tracing of the clonal origin of different liver macrophage subtypes (118, 119). Distinct clones can be sorted and sequenced separately to uncover the transcriptional states of different subpopulations. The multicolor labeling can also be utilized in adult zebrafish to assess the maintenance of liver macrophages population and distinguish between self-renewal and monocyte-based replenishment. It is possible to partially ablate the labeled macrophages by using clodronate liposomes (120). Subsequently, the clonal composition can be assessed to identify the source of the recovering cells. Moreover, the labeled clones can be analyzed to determine how different subpopulations of macrophages react to various insults. The GESTALT system, which stands for genome editing of synthetic target arrays for lineage tracing, is another tool to add more depth to the understanding of liver macrophage ontogeny (121). It utilizes CRISPR genome editing to progressively introduce and accumulate distinct mutations in a DNA barcode over multiple rounds of cell division. The barcode can be used to dissect lineage relationship among liver macrophages via the mutation patterns shared between them. With the use of a heat shock inducible Cas9, it is also possible to laser-activate the GESTALT system in a spatio-temporally restricted manner to restrict the labeling to a specific site of hematopoiesis and study the lineage relationship within this particular group. The GESTALT method can be combined with single-cell RNA sequencing to not only provide the identity of the subpopulations but also link each of them to a specific

hematopoietic lineage and site (122). One may apply the GESTALT method in different liver pathologies to evaluate the liver macrophages plasticity at a population level. Lastly, a recent study from Paul and colleagues describes successful transplantation of primary human monocytes/macrophages into larval zebrafish, both directly into circulation and in an organ-specific manner (123). The human monocytes differentiate into functional macrophages at the physiological temperature of zebrafish, and survive for at least 2weeks in the presence of zebrafish immunity. This methodology may permit *in vivo* characterization of human macrophages in zebrafish models of liver pathology at a cellular level. The new lines of experiments described above have the potential to advance our understanding of liver macrophage biology and contribute to the design of novel macrophage-targeted therapeutic strategies to treat liver diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Asrani SK, Devarbhavi H, Eaton J, Kamath PS. Burden of liver diseases in the world. *J Hepatol*. (2019) 70:151–71. doi: 10.1016/j.jhep.2018.09.014
2. Elchaninov AV, Fatkhudinov TK, Vishnyakova PA, Lokhonina AV, Sukhikh GT. Phenotypical and functional polymorphism of liver resident macrophages. *Cells*. (2019) 8:E1032. doi: 10.3390/cells8091032
3. Tacke F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol*. (2017) 66:1300–12. doi: 10.1016/j.jhep.2017.02.026
4. Ramachandran P, Dobie R, Wilson-Kanamori JR, Dora EF, Henderson BEP, Luu NT, et al. Resolving the fibrotic niche of human liver cirrhosis at single-cell level. *Nature*. (2019) 575:512–8. doi: 10.1101/766113
5. Guillot A, Tacke F. Liver macrophages: old dogmas and new insights. *Hepatol Commun*. (2019) 3:730–43. doi: 10.1002/hep4.1356
6. Pham DH, Zhang C, Yin C. Using zebrafish to model liver diseases-Where do we stand? *Curr Pathobiol Rep*. (2017) 5:207–21. doi: 10.1007/s40139-017-0141-y
7. Goessling W, Sadler KC. Zebrafish: an important tool for liver disease research. *Gastroenterology*. (2015) 149:1361–77. doi: 10.1053/j.gastro.2015.08.034
8. Wang S, Miller SR, Ober EA, Sadler KC. Making It new again: insight into liver development, regeneration, and disease from zebrafish research. *Curr Top Dev Biol*. (2017) 124:161–95. doi: 10.1016/bs.ctdb.2016.11.012
9. Astin JW, Keerthisinghe P, Du L, Sanderson LE, Crosier KE, Crosier PS, et al. Innate immune cells and bacterial infection in zebrafish. *Methods Cell Biol*. (2017) 138:31–60. doi: 10.1016/bs.mcb.2016.08.002
10. Herbomel P, Thisse B, Thisse C. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. *Dev Biol*. (2001) 238:274–88. doi: 10.1006/dbio.2001.0393
11. Parichy DM, Ransom DG, Paw B, Zon LI, Johnson SL. An orthologue of the kit-related gene fms is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, Danio rerio. *Development*. (2000) 127:3031–44.
12. Zakrzewska A, Cui C, Stockhammer OW, Benard EL, Spaink HP, Meijer AH. Macrophage-specific gene functions in Spi1-directed innate immunity. *Blood*. (2010) 116:e1–11. doi: 10.1182/blood-2010-01-262873
13. Ellett F, Pase L, Hayman JW, Andrianopoulos A, Lieschke GJ. mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. *Blood*. (2011) 117:e49–56. doi: 10.1182/blood-2010-10-314120
14. Benard EL, Racz PI, Rougeot J, Nezhinsky AE, Verbeek FJ, Spaink HP, et al. Macrophage-expressed perforins mpeg1 and mpeg1.2 have an anti-bacterial function in zebrafish. *J Innate Immun*. (2015) 7:136–52. doi: 10.1159/000366103
15. Martins RR, Ellis PS, MacDonald RB, Richardson RJ, Henriques CM. Resident immunity in tissue repair and maintenance: the Zebrafish model coming of age. *Front Cell Dev Biol*. (2019) 7:12. doi: 10.3389/fcell.2019.00012
16. Redd MJ, Kelly G, Dunn G, Way M, Martin P. Imaging macrophage chemotaxis in vivo: studies of microtubule function in zebrafish wound inflammation. *Cell Motil Cytoskeleton*. (2006) 63:415–22. doi: 10.1002/cm.20133
17. Curado S, Ober EA, Walsh S, Cortes-Hernandez P, Verkade H, Koehler CM, et al. The mitochondrial import gene tomm22 is specifically required for hepatocyte survival and provides a liver regeneration model. *Dis Model Mech*. (2010) 3:486–95. doi: 10.1242/dmm.004390
18. van der Sar AM, Appelmelk BJ, Vandenbroucke-Grauls CM, Bitter W. A star with stripes: zebrafish as an infection model. *Trends Microbiol*. (2004) 12:451–7. doi: 10.1016/j.tim.2004.08.001
19. Harvie EA, Green JM, Neely MN, Huttenlocher A. Innate immune response to Streptococcus iniae infection in zebrafish larvae. *Infect Immun*. (2013) 81:110–21. doi: 10.1128/IAI.00642-12
20. Wu S, Xue R, Hassan S, Nguyen TML, Wang T, Pan H, et al. Il34-Csf1r pathway regulates the migration and colonization of microglial precursors. *Dev Cell*. (2018) 46:552–63 e4. doi: 10.1016/j.devcel.2018.08.005
21. Roh-Johnson M, Shah AN, Stonick JA, Poudel KR, Kargl J, Yang GH, et al. Macrophage-dependent cytoplasmic transfer during melanoma invasion *in vivo*. *Dev Cell*. (2017) 43:549–62 e6. doi: 10.1016/j.devcel.2017.11.003
22. Gray C, Loynes CA, Whyte MK, Crossman DC, Renshaw SA, Chico TJ. Simultaneous intravital imaging of macrophage and neutrophil behaviour during inflammation using a novel transgenic zebrafish. *Thromb Haemost*. (2011) 105:811–9. doi: 10.1160/TH10-08-0525
23. Dee CT, Nagaraju RT, Athanasiadis EI, Gray C, Fernandez Del Ama L, Johnston SA, et al. CD4-transgenic Zebrafish reveal tissue-resident Th2- and regulatory T cell-like populations and diverse mononuclear phagocytes. *J Immunol*. (2016) 197:3520–30. doi: 10.4049/jimmunol.1600959
24. Oehlers SH, Cronan MR, Scott NR, Thomas MI, Okuda KS, Walton EM, et al. Interception of host angiogenic signalling limits mycobacterial growth. *Nature*. (2015) 517:612–5. doi: 10.1038/nature13967
25. Walton EM, Cronan MR, Beerman RW, Tobin DM. The Macrophage-specific promoter mfap4 allows live, long-term analysis of macrophage behavior during mycobacterial infection in Zebrafish. *PLoS ONE*. (2015) 10:e0138949. doi: 10.1371/journal.pone.0138949
26. Nguyen-Chi M, Laplace-Builhe B, Travnickova J, Luz-Crawford P, Tejedor G, Phan QT, et al. Identification of polarized macrophage subsets in zebrafish. *Elife*. (2015) 4:e07288. doi: 10.7554/eLife.07288
27. Sanderson LE, Chien AT, Astin JW, Crosier KE, Crosier PS, Hall CJ. An inducible transgene reports activation of macrophages in live zebrafish larvae. *Dev Comp Immunol*. (2015) 53:63–9. doi: 10.1016/j.dci.2015.06.013
28. Li L, Yan B, Shi YQ, Zhang WQ, Wen ZL. Live imaging reveals differing roles of macrophages and neutrophils during zebrafish tail fin regeneration. *J Biol Chem*. (2012) 287:25353–60. doi: 10.1074/jbc.M112.349126
29. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods*. (1994) 174:83–93. doi: 10.1016/0022-1759(94)90012-4

30. Bernut A, Herrmann JL, Kissa K, Dubremetz JF, Gaillard JL, Lutfalla G, et al. *Mycobacterium abscessus* cording prevents phagocytosis and promotes abscess formation. *Proc Natl Acad Sci USA*. (2014) 111:E943–52. doi: 10.1073/pnas.1321390111
31. Phelps HA, Neely MN. SalY of the Streptococcus pyogenes lantibiotic locus is required for full virulence and intracellular survival in macrophages. *Infect Immun*. (2007) 75:4541–51. doi: 10.1128/IAI.00518-07
32. Chatani M, Takano Y, Kudo A. Osteoclasts in bone modeling, as revealed by in vivo imaging, are essential for organogenesis in fish. *Dev Biol*. (2011) 360:96–109. doi: 10.1016/j.ydbio.2011.09.013
33. Yan C, Huo X, Wang S, Feng Y, Gong Z. Stimulation of hepatocarcinogenesis by neutrophils upon induction of oncogenic kras expression in transgenic zebrafish. *J Hepatol*. (2015) 63:420–8. doi: 10.1016/j.jhep.2015.03.024
34. Shiau CE, Kaufman Z, Meireles AM, Talbot WS. Differential requirement for irf8 in formation of embryonic and adult macrophages in zebrafish. *PLoS ONE*. (2015) 10:e0117513. doi: 10.1371/journal.pone.0117513
35. Yan C, Yang Q, Gong Z. Tumor-associated neutrophils and macrophages promote gender disparity in hepatocellular carcinoma in Zebrafish. *Cancer Res*. (2017) 77:1395–407. doi: 10.1158/0008-5472.CAN-16-2200
36. Su F, Juarez MA, Cooke CL, Lapointe L, Shavit JA, Yamaoka JS, et al. Differential regulation of primitive myelopoiesis in the zebrafish by Spi-1/Pu.1 and C/ebp1. *Zebrafish*. (2007) 4:187–99. doi: 10.1089/zeb.2007.0505
37. Petrie TA, Strand NS, Yang CT, Rabinowitz JS, Moon RT. Macrophages modulate adult zebrafish tail fin regeneration. *Development*. (2014) 141:2581–91. doi: 10.1242/dev.098459
38. Wu J, Choi TY, Shin D. tomm22 knockdown-mediated hepatocyte damages elicit both the formation of hybrid hepatocytes and biliary conversion to hepatocytes in Zebrafish Larvae. *Gene Expr*. (2017) 17:237–49. doi: 10.3727/105221617X695195
39. Torraca V, Cui C, Boland R, Bebelman JP, van der Sar AM, Smit MJ, et al. The CXCR3-CXCL11 signaling axis mediates macrophage recruitment and dissemination of mycobacterial infection. *Dis Model Mech*. (2015) 8:253–69. doi: 10.1242/dmm.017756
40. Evans MA, Smart N, Dube KN, Bollini S, Clark JE, Evans HG, et al. Thymosin beta4-sulfoxide attenuates inflammatory cell infiltration and promotes cardiac wound healing. *Nat Commun*. (2013) 4:2081. doi: 10.1038/ncomms3081
41. Jagannathan-Bogdan M, Zon LI. Hematopoiesis. *Development*. (2013) 140:2463–7. doi: 10.1242/dev.083147
42. Stachura DL, Traver D. Cellular dissection of zebrafish hematopoiesis. *Methods Cell Biol*. (2011) 101:75–110. doi: 10.1016/B978-0-12-387036-0.00004-9
43. Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D. Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. *Development*. (2007) 134:4147–56. doi: 10.1242/dev.012385
44. Goessling W, North TE. Hematopoietic stem cell development: using the zebrafish to identify the signaling networks and physical forces regulating hematopoiesis. *Methods Cell Biol*. (2011) 105:117–36. doi: 10.1016/B978-0-12-381320-6.00005-9
45. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell*. (2008) 132:631–44. doi: 10.1016/j.cell.2008.01.025
46. Murayama E, Kissa K, Zapata A, Mordelet E, Briolat V, Lin HF, et al. Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. *Immunity*. (2006) 25:963–75. doi: 10.1016/j.immuni.2006.10.015
47. Bertrand JY, Kim AD, Teng S, Traver D. CD41+ cmyb+ precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis. *Development*. (2008) 135:1853–62. doi: 10.1242/dev.015297
48. Bennett CM, Kanki JP, Rhodes J, Liu TX, Paw BH, Kieran MW, et al. Myelopoiesis in the zebrafish, Danio rerio. *Blood*. (2001) 98:643–51. doi: 10.1182/blood.V98.3.643
49. Hsu K, Traver D, Kutok JL, Hagen A, Liu TX, Paw BH, et al. The pu.1 promoter drives myeloid gene expression in zebrafish. *Blood*. (2004) 104:1291–7. doi: 10.1182/blood-2003-09-3105
50. Lam SH, Chua HL, Gong Z, Lam TJ, Sin YM. Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. *Dev Comp Immunol*. (2004) 28:9–28. doi: 10.1016/S0145-305X(03)00103-4
51. Varol C, Mildner A, Jung S. Macrophages: development and tissue specialization. *Annu Rev Immunol*. (2015) 33:643–75. doi: 10.1146/annurev-immunol-032414-112220
52. Ginhoux F, Guilliams M. Tissue-resident macrophage ontogeny and homeostasis. *Immunity*. (2016) 44:439–49. doi: 10.1016/j.immuni.2016.02.024
53. Hoeffel G, Ginhoux F. Ontogeny of tissue-resident macrophages. *Front Immunol*. (2015) 6:486. doi: 10.3389/fimmu.2015.00486
54. Perdiguero EG, Klapproth K, Schulz C, Busch K, de Bruijn M, Rodewald HR, et al. The origin of tissue-resident macrophages: when an erythro-myeloid progenitor is an erythro-myeloid progenitor. *Immunity*. (2015) 43:1023–4. doi: 10.1016/j.immuni.2015.11.022
55. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science*. (2012) 336:86–90. doi: 10.1126/science.1219179
56. Bain CC, Bravo-Blas A, Scott CL, Perdiguero EG, Geissmann F, Henri S, et al. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat Immunol*. (2014) 15:929–37. doi: 10.1038/ni.2967
57. Ferrero G, Mahony CB, Dupuis E, Yvernogeau L, Di Ruggiero E, Miserocchi M, et al. Embryonic microglia derive from primitive macrophages and are replaced by cmyb-dependent definitive microglia in Zebrafish. *Cell Rep*. (2018) 24:130–41. doi: 10.1016/j.celrep.2018.05.066
58. He S, Chen J, Jiang Y, Wu Y, Zhu L, Jin W, et al. Adult zebrafish Langerhans cells arise from hematopoietic stem/progenitor cells. *Elife*. (2018) 7:e36131. doi: 10.7554/eLife.36131
59. Sheng J, Ruedl C, Karjalainen K. Most Tissue-resident macrophages except microglia are derived from fetal hematopoietic stem cells. *Immunity*. (2015) 43:382–93. doi: 10.1016/j.immuni.2015.07.016
60. Mass E, Ballesteros I, Farlik M, Halbritter F, Gunther P, Crozet L, et al. Specification of tissue-resident macrophages during organogenesis. *Science*. (2016) 353:aaf4238. doi: 10.1126/science.aaf4238
61. de Oliveira S, Houseright RA, Graves AL, Golenberg N, Korte BG, Miskolci V, et al. Metformin modulates innate immune-mediated inflammation and early progression of NAFLD-associated hepatocellular carcinoma in zebrafish. *J Hepatol*. (2019) 70:710–21. doi: 10.1016/j.jhep.2018.11.034
62. Wittamer V, Bertrand JY, Gutschow PW, Traver D. Characterization of the mononuclear phagocyte system in zebrafish. *Blood*. (2011) 117:7126–35. doi: 10.1182/blood-2010-11-321448
63. Yang Q, Yan C, Gong Z. Interaction of hepatic stellate cells with neutrophils and macrophages in the liver following oncogenic kras activation in transgenic zebrafish. *Sci Rep*. (2018) 8:8495. doi: 10.1038/s41598-018-26612-0
64. Vonghia L, Van Herck MA, Weyler J, Francque S. Targeting myeloid-derived cells: new frontiers in the treatment of non-alcoholic and alcoholic liver disease. *Front Immunol*. (2019) 10:563. doi: 10.3389/fimmu.2019.00563
65. Bartneck M, Fech V, Ehling J, Govaere O, Warzecha KT, Hittatiya K, et al. Histidine-rich glycoprotein promotes macrophage activation and inflammation in chronic liver disease. *Hepatology*. (2016) 63:1310–24. doi: 10.1002/hep.28418
66. Wan J, Benkdane M, Teixeira-Clerc F, Bonnafous S, Louvet A, Lafdil F, et al. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. *Hepatology*. (2014) 59:130–42. doi: 10.1002/hep.26607
67. Wan J, Benkdane M, Alons E, Lotersztajn S, Pavoine C. M2 kupffer cells promote hepatocyte senescence: an IL-6-dependent protective mechanism against alcoholic liver disease. *Am J Pathol*. (2014) 184:1763–72. doi: 10.1016/j.ajpath.2014.02.014
68. Ma J, Yin H, Li M, Deng Y, Ahmad O, Qin G, et al. A Comprehensive study of high cholesterol diet-induced larval zebrafish model: a short-time *in vivo* screening method for non-alcoholic fatty liver disease drugs. *Int J Biol Sci*. (2019) 15:973–83. doi: 10.7150/ijbs.30013
69. Lin JN, Chang LL, Lai CH, Lin KJ, Lin MF, Yang CH, et al. Development of an animal model for alcoholic liver disease in Zebrafish. *Zebrafish*. (2015) 12:271–80. doi: 10.1089/zeb.2014.1054

70. Park KH, Kim SH. Low dose of chronic ethanol exposure in adult zebrafish induces hepatic steatosis and injury. *Biomed Pharmacother*. (2019) 117:109179. doi: 10.1016/j.biopha.2019.109179
71. Passeri MJ, Cinaroglu A, Gao C, Sadler KC. Hepatic steatosis in response to acute alcohol exposure in zebrafish requires sterol regulatory element binding protein activation. *Hepatology*. (2009) 49:443–52. doi: 10.1002/hep.22667
72. Schneider AC, Gregorio C, Uribe-Cruz C, Guizzo R, Malysz T, Faccioni-Heuser MC, et al. Chronic exposure to ethanol causes steatosis and inflammation in zebrafish liver. *World J Hepatol*. (2017) 9:418–26. doi: 10.4254/wjh.v9.i8.418
73. Zhang C, Ellis JL, Yin C. Inhibition of vascular endothelial growth factor signaling facilitates liver repair from acute ethanol-induced injury in zebrafish. *Dis Model Mech*. (2016) 9:1383–96. doi: 10.1242/dmm.024950
74. Cederbaum AI. Alcohol metabolism. *Clin Liver Dis*. (2012) 16:667–85. doi: 10.1016/j.cld.2012.08.002
75. Tran S, Nowicki M, Chatterjee D, Gerlai R. Acute and chronic ethanol exposure differentially alters alcohol dehydrogenase and aldehyde dehydrogenase activity in the zebrafish liver. *Prog Neuropsychopharmacol Biol Psychiatry*. (2015) 56:221–6. doi: 10.1016/j.pnpbp.2014.09.011
76. Tsedensodnom O, Vacaru AM, Howarth DL, Yin C, Sadler KC. Ethanol metabolism and oxidative stress are required for unfolded protein response activation and steatosis in zebrafish with alcoholic liver disease. *Dis Model Mech*. (2013) 6:1213–26. doi: 10.1242/dmm.012195
77. Dunn W, Shah VH. Pathogenesis of alcoholic liver disease. *Clin Liver Dis*. (2016) 20:445–56. doi: 10.1016/j.cld.2016.02.004
78. Howarth DL, Passeri M, Sadler KC. Drinks like a fish: using zebrafish to understand alcoholic liver disease. *Alcohol Clin Exp Res*. (2011) 35:826–9. doi: 10.1111/j.1530-0277.2010.01407.x
79. Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol*. (2017) 17:306–21. doi: 10.1038/nri.2017.11
80. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest*. (2005) 115:56–65. doi: 10.1172/JCI22675
81. Global Burden of Disease Liver Cancer C, Akinyemiju T, Abera S, Ahmed M, Alam N, Alemayohu MA, et al. The burden of primary liver cancer and underlying etiologies from 1990 to 2015 at the global, regional, and national level: results from the global burden of disease study 2015. *JAMA Oncol*. (2017) 3:1683–91. doi: 10.1001/jamaoncol.2017.3055
82. Ding T, Xu J, Wang F, Shi M, Zhang Y, Li SP, et al. High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection. *Hum Pathol*. (2009) 40:381–9. doi: 10.1016/j.humpath.2008.08.011
83. Yeung OW, Lo CM, Ling CC, Qi X, Geng W, Li CX, et al. Alternatively activated (M2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. *J Hepatol*. (2015) 62:607–16. doi: 10.1016/j.jhep.2014.10.029
84. Wrighton PJ, Oderberg IM, Goessling W. There is something fishy about liver cancer: zebrafish models of hepatocellular carcinoma. *Cell Mol Gastroenterol Hepatol*. (2019) 8:347–63. doi: 10.1016/j.jcmgh.2019.05.002
85. Lam SH, Wu YL, Vega VB, Miller LD, Spitsbergen J, Tong Y, et al. Conservation of gene expression signatures between zebrafish and human liver tumors and tumor progression. *Nat Biotechnol*. (2006) 24:73–5. doi: 10.1038/nbt1169
86. Zheng W, Li Z, Nguyen AT, Li C, Emelyanov A, Gong Z. Xmrk, kras and myc transgenic zebrafish liver cancer models share molecular signatures with subsets of human hepatocellular carcinoma. *PLoS ONE*. (2014) 9:e91179. doi: 10.1371/journal.pone.0091179
87. Li Z, Luo H, Li C, Huo X, Yan C, Huang X, et al. Transcriptomic analysis of a transgenic zebrafish hepatocellular carcinoma model reveals a prominent role of immune responses in tumour progression and regression. *Int J Cancer*. (2014) 135:1564–73. doi: 10.1002/ijc.28794
88. Yan C, Yang Q, Gong Z. Transgenic expression of tgfb1a induces hepatic inflammation, fibrosis and metastasis in zebrafish. *Biochem Biophys Res Commun*. (2019) 509:175–81. doi: 10.1016/j.bbrc.2018.12.098
89. Lu JW, Ho YJ, Yang YJ, Liao HA, Ciou SC, Lin LI, et al. Zebrafish as a disease model for studying human hepatocellular carcinoma. *World J Gastroenterol*. (2015) 21:12042–58. doi: 10.3748/wjg.v21.i42.12042
90. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin*. (2005) 55:74–108. doi: 10.3322/canjclin.55.2.74
91. Li H, Lu JW, Huo X, Li Y, Li Z, Gong Z. Effects of sex hormones on liver tumor progression and regression in Myc/xmrk double oncogene transgenic zebrafish. *Gen Comp Endocrinol*. (2019) 277:112–21. doi: 10.1016/j.ygcen.2019.03.018
92. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science*. (2007) 317:121–4. doi: 10.1126/science.1140485
93. Wolf MJ, Adili A, Piotrowitz K, Abdullah Z, Boege Y, Stemmer K, et al. Metabolic activation of intrahepatic CD8+ T cells and NKT cells causes nonalcoholic steatohepatitis and liver cancer via cross-talk with hepatocytes. *Cancer Cell*. (2014) 26:549–64. doi: 10.1016/j.ccell.2014.09.003
94. Yang Q, Yan C, Gong Z. Activation of liver stromal cells is associated with male-biased liver tumor initiation in xmrk and Myc transgenic zebrafish. *Sci Rep*. (2017) 7:10315. doi: 10.1038/s41598-017-10529-1
95. Iyer JK, Kalra M, Kaul A, Payton ME, Kaul R. Estrogen receptor expression in chronic hepatitis C and hepatocellular carcinoma pathogenesis. *World J Gastroenterol*. (2017) 23:6802–16. doi: 10.3748/wjg.v23.i37.6802
96. Ma WL, Lai HC, Yeh S, Cai X, Chang C. Androgen receptor roles in hepatocellular carcinoma, fatty liver, cirrhosis and hepatitis. *Endocr Relat Cancer*. (2014) 21:R165–82. doi: 10.1530/ERC-13-0283
97. Chow PK, Machin D, Chen Y, Zhang X, Win KM, Hoang HH, et al. Randomised double-blind trial of megestrol acetate vs placebo in treatment-naive advanced hepatocellular carcinoma. *Br J Cancer*. (2011) 105:945–52. doi: 10.1038/bjc.2011.333
98. Chow PK, Tai BC, Tan CK, Machin D, Win KM, Johnson PJ, et al. High-dose tamoxifen in the treatment of inoperable hepatocellular carcinoma: a multicenter randomized controlled trial. *Hepatology*. (2002) 36:1221–6. doi: 10.1053/jhep.2002.36824
99. Manesis EK, Giannoulis G, Zoumboulis P, Vafiadou I, Hadziyannis SJ. Treatment of hepatocellular carcinoma with combined suppression and inhibition of sex hormones: a randomized, controlled trial. *Hepatology*. (1995) 21:1535–42. doi: 10.1002/hep.1840210610
100. Van Cauter E, Leproult R, Kupfer DJ. Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol. *J Clin Endocrinol Metab*. (1996) 81:2468–73. doi: 10.1210/jc.81.7.2468
101. Cressman DE, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, Poli V, et al. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science*. (1996) 274:1379–83. doi: 10.1126/science.274.5291.1379
102. Michalopoulos GK. Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am J Pathol*. (2010) 176:2–13. doi: 10.2353/ajpath.2010.090675
103. Webber EM, Bruix J, Pierce RH, Fausto N. Tumor necrosis factor primes hepatocytes for DNA replication in the rat. *Hepatology*. (1998) 28:1226–34. doi: 10.1002/hep.510280509
104. Abshagen K, Eipel C, Kalff JC, Menger MD, Vollmar B. Loss of NF-kappaB activation in Kupffer cell-depleted mice impairs liver regeneration after partial hepatectomy. *Am J Physiol Gastrointest Liver Physiol*. (2007) 292:G1570–7. doi: 10.1152/ajpgi.00399.2006
105. Meijer C, Wiezer MJ, Diehl AM, Schouten HJ, Schouten HJ, Meijer S, et al. Kupffer cell depletion by CI2MDP-liposomes alters hepatic cytokine expression and delays liver regeneration after partial hepatectomy. *Liver*. (2000) 20:66–77. doi: 10.1034/j.1600-0676.2000.020001066.x
106. Goessling W, North TE, Lord AM, Ceol C, Lee S, Weidinger G, et al. APC mutant zebrafish uncover a changing temporal requirement for wnt signaling in liver development. *Dev Biol*. (2008) 320:161–74. doi: 10.1016/j.ydbio.2008.05.526
107. Kan NG, Junghans D, Izpisua Belmonte JC. Compensatory growth mechanisms regulated by BMP and FGF signaling mediate liver regeneration in zebrafish after partial hepatectomy. *FASEB J*. (2009) 23:3516–25. doi: 10.1096/fj.09-131730
108. Sadler KC, Krahn KN, Gaur NA, Ukomadu C. Liver growth in the embryo and during liver regeneration in zebrafish requires the cell cycle regulator, uhrf1. *Proc Natl Acad Sci USA*. (2007) 104:1570–5. doi: 10.1073/pnas.0610774104

109. Zhu Z, Chen J, Xiong JW, Peng J. Haploinsufficiency of Def activates p53-dependent TGFbeta signalling and causes scar formation after partial hepatectomy. *PLoS ONE*. (2014) 9:e96576. doi: 10.1371/journal.pone.0096576
110. Curado S, Anderson RM, Jungblut B, Mumm J, Schroeter E, Stainier DY. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. *Dev Dyn*. (2007) 236:1025–35. doi: 10.1002/dvdy.21100
111. Curado S, Stainier DY, Anderson RM. Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies. *Nat Protoc*. (2008) 3:948–54. doi: 10.1038/nprot.2008.58
112. Choi TY, Ninov N, Stainier DY, Shin D. Extensive conversion of hepatic biliary epithelial cells to hepatocytes after near total loss of hepatocytes in zebrafish. *Gastroenterology*. (2014) 146:776–88. doi: 10.1053/j.gastro.2013.10.019
113. He J, Lu H, Zou Q, Luo L. Regeneration of liver after extreme hepatocyte loss occurs mainly via biliary transdifferentiation in zebrafish. *Gastroenterology*. (2014) 146:789–800 e8. doi: 10.1053/j.gastro.2013.11.045
114. Stoddard M, Huang C, Enyedi B, Niethammer P. Live imaging of leukocyte recruitment in a zebrafish model of chemical liver injury. *Sci Rep*. (2019) 9:28. doi: 10.1038/s41598-018-36771-9
115. Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, et al. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. *Nat Med*. (2012) 18:572–9. doi: 10.1038/nm.2667
116. Starkey Lewis PJ, Moroni F, Forbes SJ. Macrophages as a cell-based therapy for liver disease. *Semin Liver Dis*. (2019) 39:442–51. doi: 10.1055/s-0039-1688502
117. Rougeot J, Torraca V, Zakrzewska A, Kanwal Z, Jansen HJ, Sommer F, et al. RNAseq profiling of leukocyte populations in zebrafish larvae reveals a cxcl11 chemokine gene as a marker of macrophage polarization during mycobacterial infection. *Front Immunol*. (2019) 10:832. doi: 10.3389/fimmu.2019.00832
118. Henninger J, Santoso B, Hans S, Durand E, Moore J, Mosimann C, et al. Clonal fate mapping quantifies the number of haematopoietic stem cells that arise during development. *Nat Cell Biol*. (2017) 19:17–27. doi: 10.1038/ncb3444
119. Pan YA, Freundlich T, Weissman TA, Schoppik D, Wang XC, Zimmerman S, et al. Zebrabow: multispectral cell labeling for cell tracing and lineage analysis in zebrafish. *Development*. (2013) 140:2835–46. doi: 10.1242/dev.094631
120. Wu Z, Koh B, Lawrence LM, Kanamala M, Pool B, Svirskis D, et al. Liposome-mediated drug delivery in larval Zebrafish to manipulate macrophage function. *Zebrafish*. (2019) 16:171–81. doi: 10.1089/zeb.2018.1681
121. McKenna A, Findlay GM, Gagnon JA, Horwitz MS, Schier AF, Shendure J. Whole-organism lineage tracing by combinatorial and cumulative genome editing. *Science*. (2016) 353:aaf7907. doi: 10.1126/science.aaf7907
122. Raj B, Wagner DE, McKenna A, Pandey S, Klein AM, Shendure J, et al. Simultaneous single-cell profiling of lineages and cell types in the vertebrate brain. *Nat Biotechnol*. (2018) 36:442–50. doi: 10.1038/nbt.4103
123. Paul CD, Devine A, Bishop K, Xu Q, Wulftange WJ, Burr H, et al. Human macrophages survive and adopt activated genotypes in living zebrafish. *Sci Rep*. (2019) 9:1759. doi: 10.1038/s41598-018-38186-y

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REVIEW
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Macrophage Function in the Pathogenesis of Non-alcoholic Fatty Liver Disease: The Mac Attack

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Obesity is a prevalent predisposing factor to non-alcoholic fatty liver disease (NAFLD), the most common chronic liver disease in the developed world. NAFLD spectrum of disease involves progression from steatosis (NAFL), to steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (HCC). Despite clinical and public health significance, current FDA approved therapies for NAFLD are lacking in part due to insufficient understanding of pathogenic mechanisms driving disease progression. The etiology of NAFLD is multifactorial. The induction of both systemic and tissue inflammation consequential of skewed immune cell metabolic state, polarization, tissue recruitment, and activation are central to NAFLD progression. Here, we review the current understanding of the above stated cellular and molecular processes that govern macrophage contribution to NAFLD pathogenesis and how adipose tissue and liver crosstalk modulates macrophage function. Notably, the manipulation of such events may lead to the development of new therapies for NAFLD.

Keywords: macrophage, metabolism, NAFLD, inflammation, cytokines

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INTRODUCTION

The unabated obesity pandemic is directly linked with the incidence of non-alcoholic fatty liver disease (NAFLD). NAFLD afflicts ∼35% of obese individuals worldwide (1, 2). Current epidemiological estimates suggest that NAFLD will soon surpass chronic hepatitis C infection as the leading cause of liver transplantation. Given the lack of effective therapies for NAFLD, costs of care and management of associated symptoms come with a considerable economic burden (3).

NAFLD spectrum of disease progresses from non-alcoholic fatty liver (NAFL) or hepatic steatosis, to non-alcoholic steatohepatitis (NASH), to cirrhosis and hepatocellular carcinoma (HCC). Steatosis is characterized by increased macrovesicular and microvesicular lipid droplet accumulation that occurs in more than 5% of hepatocytes (1, 2, 4). Approximately 25% of individuals afflicted with NAFL progress to NASH (1). NASH is characterized by hepatocellular ballooning, in part due to increased immune cell infiltration, activation, and proinflammatory cytokine production (4–6). These mechanisms coupled with others such as adipokine production

and activation of endoplasmic reticulum stress and reactive oxygen species (ROS) promote hepatic fibrosis and progression to cirrhosis and HCC (7, 8).

The contribution of various immune cells in hepatic inflammation and the mechanisms that govern their migration to the liver, polarization, and inflammatory capabilities, in NAFLD progression represent an intense area of investigation. Here, we specifically focus on the contribution of macrophages to NAFLD pathogenesis. We review the landscape of underlying mechanisms that regulate macrophage effector functions and macrophage interplay with other immune cells/tissues/organs which collectively contribute to NAFLD progression.

CURRENT KNOWLEDGE AND DISCUSSION

Immune Responses in NAFLD

Dysregulated immune responsiveness is central to the development and progression of NAFLD (9, 10). In obesity, both liver resident (e.g., Kupffer cells, [KC], hepatic stellate cells, [HSC], hepatocytes) and infiltrating immune cells (e.g., neutrophils, dendritic cells [DC], natural killer [NK] cells, NKT cells, blood monocytes, T cells, B cells, and macrophages) contribute to NAFLD development and progression via systemic and tissue inflammatory mediator production (e.g., interleukin [IL]-17A, IL-6, tumor necrosis factor [TNF], IL-1β) (5, 11). Obesity-associated intestinal permeability and augmented circulating levels of inflammatory ligands (e.g., lipopolysaccharide [LPS]) (12) via activation of pattern recognition receptors (PRRs) on hematopoietic and non-hematopoietic cells activate multiple proinflammatory cascades that in unison promote liver injury (13). The contribution of PRRs to NAFLD progression has been reviewed in detail elsewhere (14, 15). PRR signaling in macrophages, also contributes to activation of adaptive immune responses, with macrophage-T cell interplay having a particularly important role in NAFLD progression (16). In this setting, activated, liver infiltrating T cells produce proinflammatory cytokines and amplify macrophage polarization and activation to in turn propagate overall hepatic inflammation, hepatocellular damage and hepatocyte release of damage associated molecular patterns (DAMPs). Cumulatively these processes fuel and support a chronic inflammatory state in the liver that is a hallmark of NAFLD progression. Due to the extent of various immune processes in NAFLD, here we selectively focus on the role of macrophage-mediated inflammation and their contribution to NAFLD pathology. The contribution of other immune cells (e.g., T cells, neutrophils, DC, NK cells, and NKT cells) and cytokines in NAFLD has been discussed elsewhere (14, 17, 18).

Macrophage Recruitment to the Liver

Liver infiltration by inflammatory monocytes/macrophages is associated with NAFLD progression (19). Increased release of free fatty acid (FFA) by white adipose tissue (WAT) augments triglyceride synthesis and storage in hepatocytes and induces hepatocyte release of inflammatory mediators including proinflammatory cytokines and macrophage recruiting chemokines (e.g., CCL2, CXCL10) (20, 21). In addition to hepatocytes, HSCs, myofibroblast and macrophages themselves can also produce various chemokines (e.g., CCL2, CCL3, CCL4, CCL5, CCL8, and CXCL10) to fuel increased macrophage recruitment (22).

The contribution of hepatic macrophage recruitment to NAFLD pathogenesis is supported in part by increased systemic and hepatic chemokine levels in NAFLD progression in humans (23). One of the most widely explored pathways of recruiting inflammatory and fibrogenic monocytes to the injured liver is the CCR2/CCL2 axis (24). Pharmacological inhibition of CCR2 and genetic deletion of CCL2 reduced liver steatosis in obese mice (25–27). Additionally, use of CCR1-, CCR2-, CCR5-, and CCR8-deficient mice or pharmacological inhibition of these axes reduced hepatic macrophage infiltration, hepatic fibrosis, and hepatocellular damage in experimental models of chronic liver injury (28–32). Recent evidence also suggests that CXCR3-deficient mice are protected from macrophage infiltration and hepatocellular damage in obesity (33, 34). Further, CXCL10-deficient mice exhibit reduced NAFL (34). Despite its promising effects in animal models, targeting of the CCR2 axis in human NALFD using Cenicriviroc (CVC) did not impact hepatic lobular inflammation and only mildly improved fibrosis and decreased circulating levels of sCD14 (a marker of monocyte activation) (35). Characterization of the intrahepatic immune cells will be required to elucidate the effects of CVC on immune cells, monocyte and macrophage recruitment and fibrogenesis. The results of such could give insight to observed difference of effects of CVC on NAFLD progression between murine models and humans.

Like chemokines, inflammatory cytokines can also alter macrophage activation and tissue recruitment in NAFLD. For example, the IL-17A axis impacts macrophage recruitment in the liver (36), while IL-17RA depletion on macrophages ameliorates NAFLD severity (37). However, as IL-17A can also activate liver parenchymal cells to modulate macrophage recruitment. Thus, detailed studies focused on the role of IL-17 family members, their cognate receptors and cell specific expression in NAFLD pathogenesis are needed. The overall role of the IL-17 axis in NAFLD pathogenesis has been reviewed in detail elsewhere (18).

Macrophage Subsets

KCs reside in the anatomical areas that receive venous blood from the gut including hepatic sinusoids, hepatic lymph nodes and portal tract (38). Approximately 80% of the liver blood supply comes from the gut via the portal vein (39). As such, KCs act as key sentinels of the gut-liver interface. Under homeostatic conditions, PRR signaling instructs KCs to govern liver immunity by maintaining tolerance to harmless immunogens and cellular debris while in parallel enabling them to mount a response against pathogenic invaders via phagocytosis, cytokine production and antigen presentation. Collectively, the adaptation to latter events is central to preventing dissemination of microbes into peripheral circulation (5, 39, 40). In obesity, increased intestinal permeability, trafficking of bacteria into the gut lumen and LPS sensing (2, 40, 41) fuel KC activation and alter their function (42). In this context, activated KCs favor inflammatory

responses that contribute to NAFLD pathogenesis (13). *In vitro* treatment of KCs with FFAs (e.g., palmitate) promotes activation and secretion of inflammatory cytokines (e.g., IL-6, TNF, IL-1β) (43) while KC depletion *in vivo* protects from obesity-driven hepatic steatosis (13).

Common bone marrow myeloid progenitors give rise to granulocyte-macrophage progenitors (GMP) from which monocytes are derived. Upon egression from the bone marrow, and following hepatic inflammatory insult, circulating monocytes traffic to the liver. Once in the liver, in response to cytokines and various pathogen associated molecular patterns (PAMPs)/DAMPs, monocytes activate unique transcriptional profiles and differentiate into macrophages. In response to IFNγ or PRR signaling, recruited monocytes differentiate into "classically" activated macrophages that produce proinflammatory cytokines (e.g., IL-6, TNF, IL-1β, IL-12), drive liver recruitment of various immune cells and enhance the overall hepatic inflammation (5, 13, 44). Conversely, in response to either IL-4 or IL-13, tissue recruited monocytes differentiate into "alternatively" activated macrophages that produce anti-inflammatory and wound healing mediators (e.g., IL-8, MCP-1, IL-10) (38, 45, 46). The overall balance of "classically" and "alternatively" activated macrophages in the liver regulates hepatic inflammation, liver scarring and fibrosis. Targeting of inflammatory signaling pathways in macrophages via deletion of JNK, IKKβ, or Toll-like receptor (TLR) 4 is sufficient to reduce hepatic steatosis and inflammation (47–49). A brief summary of the above discussed processes is depicted in **Figure 1**.

Macrophages and Proinflammatory Cytokine Production

Macrophage produced cytokines (e.g., IL-6, TNF, IL-1β) can directly target hepatocytes and promote steatosis, inflammation and hepatocellular damage (5). Systemic increase of these proinflammatory cytokines positively correlates with hepatocellular damage in humans and is recapitulated in NAFLD experimental mouse models (50, 51).

IL-6 is a multifunctional cytokine that regulates immune responses, acute phase reactions, hematopoiesis, and plays key roles in inflammation, host defense and tissue injury (52, 53). IL-6 stimulates hepatic lipogenesis (54), and is associated with obesity (55), impaired insulin signaling (56, 57), and altered insulin sensitivity by activating key steps in the insulin signaling pathway (58). IL-6 is also a biomarker of insulin resistance and cardiovascular diseases risk (50, 59, 60). In humans with NASH, there is a positive correlation between IL-6 expression in hepatocytes and the severity of NAFLD (61). IL-6-deficient mice display a milder NAFLD severity and antibody mediated IL-6 receptor (IL-6R) neutralization improved liver damage in mice fed methionine choline deficient (MCD) diet, despite enhanced steatosis (51, 62).

TNF stimulates hepatic fatty acid synthesis (FAS), increases serum triglyceride (TG) levels (63), stimulates very low density lipoprotein (VLDL) production from liver and contributes to impaired insulin signaling (64, 65). TNF also activates harmful proatherogenic pathways via the reduction of high-density lipoprotein (HDL)-cholesterol, elevated expression of cholesterogenic genes, accompanied by an increase in potentially harmful precholesterol metabolites, and suppression of cholesterol elimination (66). Thus, it is not surprising that TNF sensing by hepatocytes promotes hepatocyte cell death and hepatocyte proliferation (67), and as such directly contributes to NAFLD pathogenesis (68). Further, deletion of TNF in experimental mouse models of NAFLD correlates with decreased steatosis, fibrosis and improved glucose tolerance (69).

IL-1β promotes liver steatosis, inflammation and fibrosis via activation of the IL-1 receptor (IL-1R) signaling (70). IL-1β stimulates TG and cholesterol accumulation in hepatocytes and as such contributes to the development of hepatic steatosis (71). Mechanistically, IL-1β also promotes liver inflammation by inducing IL-6 production, upregulating ICAM-1 and neutrophil infiltration and accrual in the liver (72). IL-1R-deficient mice are protected from liver fibrosis (73). Hepatocyte-specific deletion of IL-1R attenuates liver injury in a model of acute liver disease (74). Whether similar effects are observed in animal models of NAFLD have not been examined. Further, IL-1R activates Myd88 signaling similar to TLRs (75). Thus, the role of IL-1R signaling in NAFLD warrants further investigation. In addition, blockade of IL-1 signaling with anakinra improved glycemic control in patients with T2D (76), suggesting the importance of inflammatory mediators in liver disease pathogenesis.

Macrophages and ROS Production

Reactive oxygen species (ROS) production, a central antimicrobial effector function of macrophages, can be induced in part via macrophage sensing of proinflammatory cytokines. Macrophages generate ROS via numerous mechanisms including ER stress, mitochondrial damage and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs). NOX2, also known as the phagocytic nicotinamide adenine dinucleotide phosphate NADPH oxidase, is constitutively associated with p22phox at the plasma membrane. PRR signaling in KCs and infiltrating macrophages causes complexing of NOX2 with other proteins (p67, P40, Rac GTPases) to generate superoxide, drive proinflammatory cytokine production (e.g., IL-6, TNF, IL-1β, transforming growth factor-β [TGFβ]) and promote hepatic steatosis, hepatocellular damage and fibrosis (43, 77, 78). NOX2-deficient mice display reduced macrophage-associated proinflammatory cytokine production, hepatic steatosis and fibrosis, and overall NAFLD severity in obesity (43, 79, 80). However, the underlying mechanisms regulating macrophage ROS production in NAFLD are not fully understood. Thus, in depth interrogation of the interplay between inflammatory cytokines and NADPH components on macrophage ROS production in NAFLD progression is needed.

Macrophage Cellular Metabolism

Metabolic pathways regulate immune cell function and inflammation (81, 82). Obesity alters cellular metabolism (83). In fact, both obesity-associated and inflammation-driven derangements in cellular metabolism are implicated in NAFLD

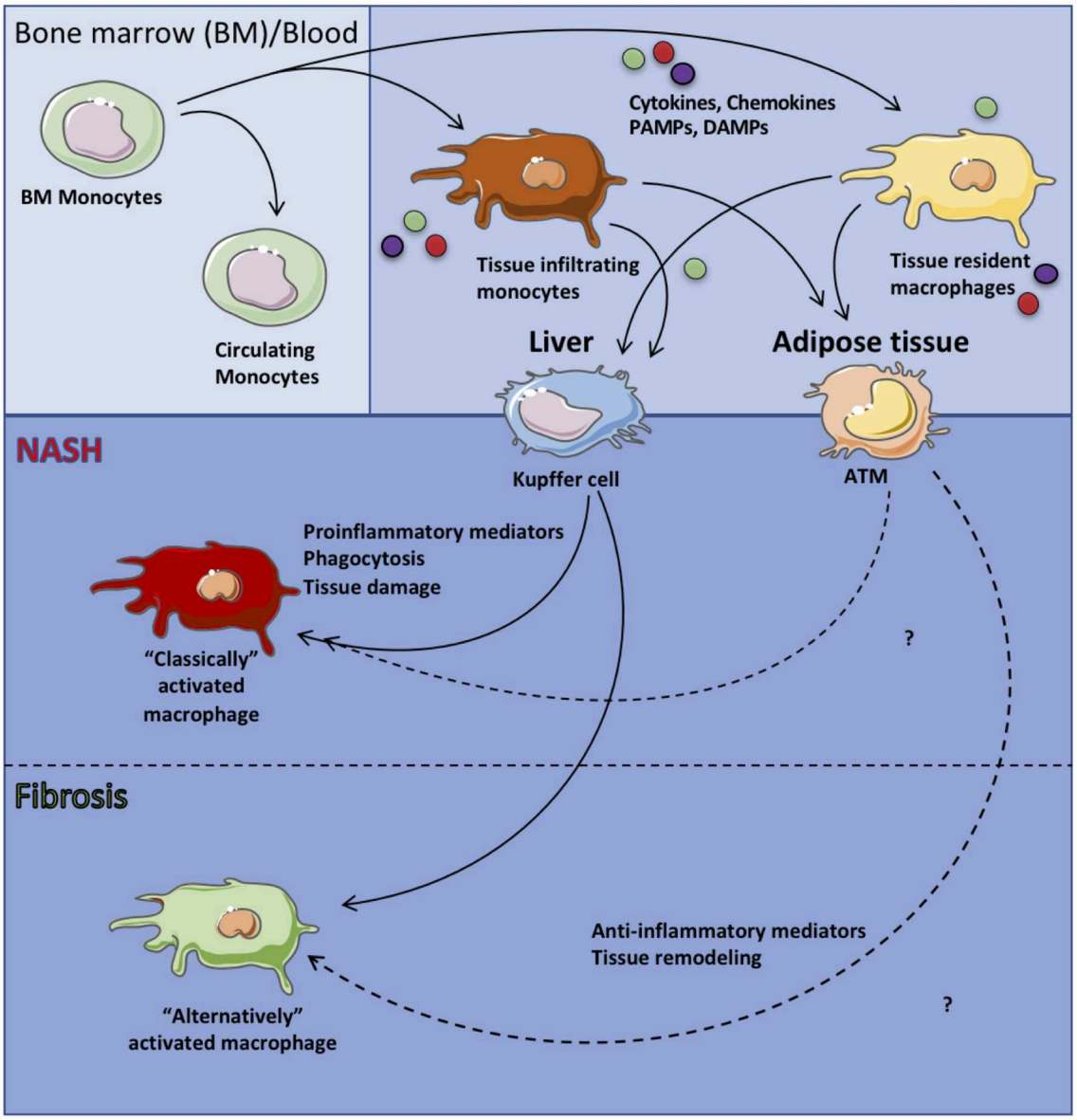

FIGURE 1 | Macrophage subsets in health and disease. Circulating monocytes originating from the bone marrow are recruited to specific tissues and differentiate into tissue resident macrophages. In the context of systemic inflammation, circulating monocytes as well as tissue resident macrophages are activated by sensing of proinflammatory mediators (i.e., IL-6, TNF, IL-1β), chemokines and ROS or anti-inflammatory mediators (i.e., IL-10) leading to "classically" or "alternatively" activated tissue macrophages, respectively which then contribute to tissue pathology.

progression (84–86). The full discovery of specific metabolic pathways and genes detrimental to NAFLD pathogenesis however remains an intense area of investigation. Macrophage-driven inflammation and resolution of inflammation are intricately linked to various metabolic pathways and several clinical phenotypes (i.e., insulin resistance, hyperlipidemia, etc.) (86, 87). Here we review metabolic pathways that contribute to macrophage-intrinsic inflammation and resolution in NAFLD. A brief summary of these processes is depicted in **Figure 2** and **Table 1**.

Glycolysis

Macrophage reliance on glycolysis to meet energetic demands has been demonstrated in several murine models of "classical" macrophage activation (88). Upon stimulation with cytokines or activation of PRR signaling, macrophages acquire a proinflammatory phenotype that correlates with robust upregulation of glycolytic pathways including hypoxia inducing factor alpha (HIF1α) (88). Activation of HIF1α induces transcription of hypoxic genes (e.g., glucose transporters, glycolytic genes) and IL-1β production by macrophages.

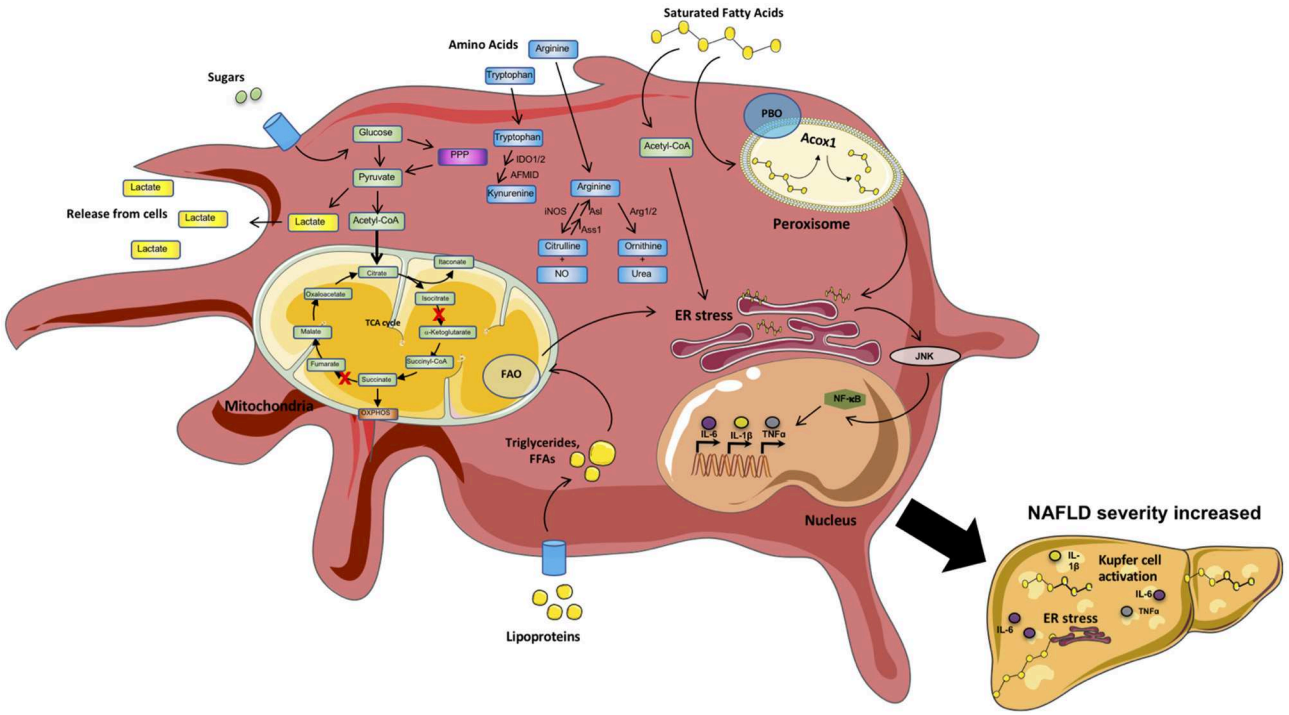

FIGURE 2 | Metabolic processes within inflammatory macrophages. Macrophages are highly metabolically active cells. Their metabolic identity is impacted by inflammatory mediators. In contrast, specific metabolic pathways (Fatty acid synthesis [FAS], Glycolysis, Amino acid [AA] metabolism) regulate the type of mediators produced by these cells. During a proinflammatory state, inflammatory mediators (e.g., sugars, lipoproteins, saturated fatty acids [SFAs], cytokines [IL6, TNF]) trigger "classical" activation of circulating and tissue resident macrophages (e.g., Kupfer cells and adipose tissue macrophages [ATMs]). Circulating sugars are taken up and processed via glycolysis/TCA cycle. In addition, several intermediate metabolites, as well as amino acids L-arginine, L-tryptophan and glutamine, can impact macrophage effector functions. Sensing/uptake of excessive lipoproteins and SFAs activates the mitochondrial fatty acid oxidation (FAO) and peroxisomal fatty acid beta-oxidation (PBO) pathways to breakdown long chain and very long chain fatty acids, respectively. Excessive activation of these pathways triggers ER stress and signaling via JNK and NF-kB, resulting in amplified production of proinflammatory mediators. "Classical" macrophage activation shifts the cells toward preferential utilization of glycolytic pathways with altered enzyme activity within the tricarboxylic acid cycle (TCA) cycle generating more lactate and fast energy production in the form of ATP to generate inflammatory mediators (e.g., IL-6, TNF, IL1). Metabolism of tryptophan (L-TRP) and arginine (L-ARG) by macrophages regulates key immunologic processes. Cumulatively, these inflammatory mechanisms fuel the overall systemic and tissue inflammation, hepatocyte death, and fibrosis in turn amplifying NAFLD pathogenesis.

TABLE 1 | Metabolic function in macrophage subsets.

Pathway	"Classically" activated macrophage	"Alternatively" activated macrophage
Reactive oxygen species	Increased ROS production through mitochondrial ROS and NADPH oxidase	Mitochondrial ROS and NADPH oxidase activity minimal
Glycolysis	High aerobic glycolysis resulting in lactate production	Low glycolytic rate resulting in acetyl-coA production
Pentose phosphate pathway	Increased pentose phosphate pathway	Decreased pentose phosphate pathway
Tricarboxylic Acid Cycle	Fractured TCA cycle, broken at Idh and Sdh	Functional TCA cycle fed by acetyl-coA from beta-oxidation and glycolysis
Electron transport chain	Dysfunctional electron transport chain, resulting in mitochondrial ROS production	Functional electron transport chain resulting in ATP production
Fatty acid	Fatty acid synthesis from fractured TCA	Fatty acid beta-oxidation from lipoproteins
L-Tryptophan catabolism	L-Tryptophan catabolism by IDO results in suppression of aberrant inflammation	L-Tryptophan catabolism not induced
L-Arginine metabolism*	L-Arginine metabolism by iNOS resulting in nitric oxide production	L-Arginine metabolism by arginase resulting in L-ornithine and downstream metabolites

*ATP, adenosine triphosphate; Idh, isocitrate dehydrogenase; IDO, indoleamine-2,3-dioxygenase; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; Sdh, succinate dehydrogenase; TCA, tricarboxylic acid. Unless noted, references are in main text. *Arginase 1 (Arg1) expression occurs along with iNOS in macrophages during intracellular infection, and is not sufficient on its own to define alternative activation.*

Preferential skewing toward glycolysis favors proinflammatory macrophage effector functions as administration of the glucose analog, 2-deoxyglucose (2-DG), decreases macrophage inflammatory polarization, cytokine production and phagocytosis (88). Mice fed MCD diet and patients with NASH display increased hepatic macrophage HIF1α expression and exacerbated hepatic steatosis and inflammation (89). However, given the functional relevance of HIF1α to macrophage-mediated inflammation, additional studies are warranted to determine the impact of macrophage-intrinsic HIF1α in inflammation and NAFLD progression.

Pentose Phosphate Pathway

Pentose phosphate pathway (PPP) branches off glycolysis at glucose-6-phosphate, the second step in glycolysis. Through a series of dehydration and decarboxylation reactions, glucose-6-phosphate is converted to ribulose-5-phosphate. Macrophages upregulate the PPP in response to LPS, which yields two NADPH (used as cofactors for NOXs) and in turn promote inflammation and cellular damage (90–92). Additionally, the PPP is necessary for limiting the dissemination of various pathogens (93). In contrast, macrophages also use the PPP to resolve inflammation and ROS, as PPP results in glutathione reduction and subsequently maintains proper redox balance, limiting the consequences of extraneous ROS activity (94, 95). During hypercholesterolemia, cholesterol inhibits LPS-mediated PPP activity in inflammatory macrophages, leading to a foamy macrophage phenotype (96). Further, adipocytes are known to use NADPH metabolism to regulate lipid metabolism, and dysregulation of enzymes involved in the production of NADPH contributes to obesity and obesity-related pathology (97, 98). Despite the role of PPP in modulating inflammation, the contribution of this metabolic pathway to macrophage-intrinsic inflammation in the context of NAFLD is underdefined and requires in-depth examination.

Tricarboxylic Acid Cycle

Acetyl CoA, generated following glycolysis or beta-oxidation, enters the tricarboxylic acid (TCA) cycle, resulting in H_2O, CO_2, NADH and $FADH_2$ generation. The latter two are utilized in the electron transport chain (ETC) to produce ATP. Despite the high energy yield of the TCA cycle, the process is time consuming and requires oxygen. In the context of a rapid inflammatory response, the TCA is downregulated and fractured in macrophages. Specifically, inflammatory macrophages reduce isocitrate dehydrogenase (Idh) and succinate dehydrogenase (Sdh) activity (90, 91). This results in decreased α-ketoglutarate formation but increased production of itaconate – a key anti-microbial metabolite. Accumulation of succinate in macrophages can inhibit the HIF1α-suppressing molecules prolyl hydroxylases (PHDs), allowing HIF1α to drive IL-1β and other inflammatory processes (90). Sdh is complex II of the ETC, which donates two electrons from $FADH_2$ to produce the electron gradient that drives ATP synthesis. During LPS stimulation, the breakdown of Sdh feeds electrons through complex I, known as reverse electron transport, resulting in mitochondrial ROS production (99). The contribution of Idh and Sdh to NAFLD has not been investigated. However, it can be hypothesized that the hepatic inflammatory environment in NAFLD would drive the breakdown of the TCA cycle, as seen in LPS stimulated macrophages. Hence, additional studies are needed to formally address these postulates.

Fatty Acid Synthesis (FAS)

Excessive hepatocellular uptake of glucose is diverted to FAS pathways where glucose is converted into TGs and secreted to adipose tissue (AT) as VLDL (100). Under pathological conditions, *de novo* FAS by the liver is a primary cause of excessive hepatic steatosis (100). In contrast to glucose, insulin reduces AT lipolysis via suppression of hormone-sensitive lipase (HSL), thus regulating the circulation of FFAs in the periphery (100, 101). However, obesity-and NAFLD-associated insulin resistance limits HSL suppression, leading to increased AT lipolysis and FFA deposition in the liver (102). FAS is essential for immune cell proliferation in response to inflammatory insult. Macrophages upregulate FAS when undergoing "classical" activation. Monocyte treatment with macrophage colony stimulating factor promotes "classical" activation and expression of sterol regulatory element binding transcription protein 1c (SREBP1c), FAS target genes and increases lipid synthesis (103). Inhibition of SREBP1c reduces macrophage inflammatory capacity (104). Increased FAS drives KC inflammasome activation via nucleotide-binding oligomerization (NOD)-like receptor 3 (NLR3) signaling (103, 105). In fact, increased inflammasome activation has been observed in both murine experimental models and human NAFLD (105–107). Inflammasomes, reviewed elsewhere (105), are multiprotein complexes containing nucleotide-binding oligomerization domains NLRs. KCs are a key source of IL-1β and caspase 1, a critical NLR3 component that regulates downstream proinflammatory signaling (e.g., pro-IL-1β, pro-IL-18, ASC), and are elevated in livers from NASH patients (105, 108, 109). However, given the complexity of inflammasome signaling the underlying mechanism unique to macrophage inflammasome-driven inflammation in NAFLD, these processes warrant further examination. Additional studies are also needed to determine if targeted inhibition of FAS pathways in macrophages is sufficient to reverse inflammasome activation and subsequently improve NAFLD pathology.

Beta-Oxidation

Beta-oxidation of fatty acids (FA) is central for ensuring cellular and tissue energetic demands by breakdown and conversion of lipids into ATP. Under homeostatic conditions, fat storage and lipolysis are regulated in part by beta-oxidation. Members of the nuclear hormone receptor superfamily known as peroxisome proliferator activated receptors (PPARα, PPARβ, and PPARγ) are transcriptional modulators of beta-oxidation. PPARα, which is primarily expressed in the liver, has several endogenous ligands (e.g., FA, eicosanoids and other complex lipids) and acts as a master regulator for FA beta-oxidation (110, 111). Given the importance of hepatic steatosis in NAFLD and the relevance of beta-oxidation in immune responses, below we discuss the contribution of both mitochondrial and peroxisomal beta-oxidation to macrophage inflammation.

Mitochondrial beta-oxidation

Mitochondrial-beta oxidation breaks down short ($<C_8$), medium (C_8-C_{14}) and long chain FA (C_{14}-C_{20}) (85). Long chain FA, which are a major component of the standard diet, are shuttled into the mitochondria via carnitine shuttles (carnitine palmitoyltransferase I) by linking with coenzyme A (acyl-CoA) (85, 112). Once acylcarnitine exchanges the carnitine molecule with CoA via exchange with carnitine palmitoyltransferase II the acyl-CoA proceeds into the beta-oxidation cycle. At the inner membrane the enzymatic activity of very long chain acyl-CoA dehydrogenase (VCLAD), shortens long chain acyl-CoAs. Shortened fatty acyl-CoAs are further oxidized by a trifunctional protein complex consisting of: Enoyl-CoA hydratase, 3-hydroxyacyl CoA dehydrogenase, and 3 ketoacyl CoA thiolase. Impaired beta-oxidation in macrophages prevents the degradation of lipids leading to the FA overload and rupture and release of toxic lipid species (85). Although, the traditional view is that FA beta-oxidation is essential for polarization of "alternatively" activated macrophages, recent evidence suggest that inhibition of this pathway may in fact promote a "classical" macrophage phenotype. Etomoxir driven inhibition of beta-oxidation or knockdown of CPT-1 results in reduced fatty acid oxidation (FAO) but increased proinflammatory signaling, cytokine production, ER stress and ROS levels (113–115). Thus, additional studies are needed to formally determine how mitochondrial FAO impacts macrophage inflammation and NAFLD progression.

Peroxisomal beta oxidation

Oxidation of very long chain fatty acids (VLCFAs) ($C>_{21}$) is exclusive to the peroxisome due to the selective presence of very long chain acyl-CoA synthetase (85). Aside from VLCFAs, long chain dicarboxylic acid, eicosanoids, and bile acid precursors are also oxidized within the peroxisomes. Compared to mitochondria, 3 enzymes Acyl-CoA oxidase 1 (Acox1), enoyl CoA hydratase/L-3-hydroxyacyl CoA dehydrogenase bifunctional protein and 3-ketoacyl CoA thiolase regulate peroxisomal beta-oxidation (85). Whole body *Acox1* null mice and $Acox1^{Lampe1}$ mice, which features a point mutation rendering the *Acox1* gene inactive, spontaneously develop steatosis and steatohepatitis (85, 116). $Acox1^{Lampe1}$ mice also exhibit increased systemic inflammation both at baseline and after LPS challenge *in vivo*, increased hepatic expression of macrophage recruiting chemokines and macrophage infiltration into the liver following short term high fat diet (HFD) feeding. HFD feeding combined with secondary LPS insult *in vivo* further exacerbates liver pathologies in $Acox1^{Lampe1}$ mice (86). Together, these data suggest that peroxisomal beta-oxidation regulates macrophage-intrinsic inflammation and NAFLD pathogenesis. However, the underlying processes by which peroxisomal beta-oxidation regulates macrophage function and inflammation remain understudied. Similarly, the contribution of peroxisomal beta-oxidation to inflammatory potential of other immune cells and their contribution to NAFLD progression remains poorly understood.

Amino Acid Metabolism

Amino acids (AA) are critical precursors for several metabolic pathways. For example, glutamine and aspartate are necessary for purine and pyrimidine synthesis as well as feeding the TCA cycle via α-ketoglutarate production. Valine and leucine fuel the synthesis of branched chain FA. Direct metabolism of tryptophan (L-TRP) and arginine (L-ARG) by macrophages and other myeloid cells regulate key immunologic processes. For this reason, below we focus on how metabolism of tryptophan and arginine within macrophages modulates their inflammatory potential.

Tryptophan

In macrophages, L-TRP metabolism is tightly regulated by two isoforms of the enzyme indoleamine 2,3-dioxygenase (IDO1 and IDO2). IDO is the rate limiting enzyme that converts L-TRP into N-formylkynurenine (117). Early reports focused on L-TRP depletion as the central mechanism of immune modulation, yet more recent literature deemphasizes L-TRP depletion and reports kynurenine production as the key regulator of immune responses (117, 118). The downstream products of kynurenine modulate immune responses to infection, cancer, and autoimmune diseases (117, 119, 120). IDO activity is induced in macrophages following IFNγ, LPS, or TNF stimulation and can be further enhanced with IL-1β co-stimulation (121–125). Several studies have begun to address the contribution of IDO-mediated L-TRP metabolism during NAFLD. Kynurenine is increased in the serum of obese subjects, and IDO1 is upregulated in the liver and WAT in obesity (126). IDO-deficient mice displayed elevated liver fibrosis, increased hepatic macrophage infiltration, and higher concentrations of IL-1β, IL-6, and IFNγ in obesity. However, these mice are protected from HFD-driven weight gain, hepatic steatosis, and oxidative stress (127). Interestingly, mice lacking IDO1 expression in macrophages and neutrophils exhibited normal weight gain and insulin sensitivity in obesity (128). Mice with an intact bone-marrow derived immune system, but lacking IDO1 in all other tissues, displayed a similar protection from HFD-induced metabolic disease as the mice with global IDO1 deletion. In sum, these data suggest that IDO contributes to multiple aspects of NAFLD progression and that non-hematopoietic IDO activity may play a key role in regulating NAFLD pathogenesis. Follow-up studies are needed however to dissect the contribution of IDO activity during the full spectrum of NAFLD pathogenesis and to evaluate IDO activity within radiation-resistant tissue macrophages.

L-TRP supplementation has been explored in multiple studies. Mice fed a high fructose diet exhibit reduced liver weight and hepatic lipid accumulation when supplemented with L-TRP (129). Clinical studies examining patients with hepatic steatosis or NASH found supplementation, twice daily, with L-TRP for 14 months result in decreased plasma LDL, TG, and gamma gluthamylo transpeptidase levels with a correlative decrease in plasma IL-1β, IL-6, and TNF (130, 131). Key studies, however, are needed to identify where supplemental L-TRP and its downstream metabolites accumulate, and whether L-TRP is available for IDO activity within the diseased liver. Considering the availability of conditional IDO1 knockout mice (128), it is

now feasible to separate the contribution of IDO activity by macrophages as well as other immune and non-immune cell types during the initiation and progression of NAFLD. These, studies would help determine how IDO modulates immune responses and NAFLD pathogenesis.

Arginine

Historically, macrophage polarization was characterized in part by the ability to metabolize L-ARG. "Classically" activated macrophages upregulate inducible nitric oxide synthase (iNOS) which converts L-ARG to L-citrulline and anti-microbial nitric oxide (NO). In contrast, "alternatively" activated macrophages upregulate arginase 1 (Arg1) to metabolize L-ARG into ornithine and urea (132, 133). It is now appreciated that Arg1 and iNOS expression can occur within similarly stimulated macrophages, adding to the complexity of defining "classical" and "alternative" macrophage activation profiles (134–136). Regardless, macrophage L-ARG metabolism has been documented to restrict intrinsic and extrinsic immune cell function. Blocking arginase activity or eliminating Arg1 within macrophages allows for increased L-arginine availability for NO production and anti-microbial activity but can also be associated with unrestricted lymphocyte activity and increased tissue pathology (132, 136–141). Thus, understanding how L-ARG metabolism is regulated during altered inflammatory and metabolic states, including NAFLD, is of considerable interest.

Limited studies have focused on the contribution of enzymes involved in the breakdown of L-ARG in NAFLD. Mammals possess two arginase isoforms (Arg1, Arg2) that are differentially expressed within tissues. The importance of macrophage Arg1 in NAFLD has not been addressed. The role of Arg2 in NAFLD, despite published studies, remains undefined. Opposing findings employing Arg2-deficient mice have shown that Arg2-deficiency results in development of spontaneous hepatic steatosis and increased liver injury (142) or promotes decreased NAFLD severity in obesity (143). Although NO contributes to NAFLD, additional studies are needed to determine the critical source of NO as NO inhibitors have various specificities and differ in their ability to regulate disease severity (144). L-ARG supplementation has also been shown to reduce NAFLD severity (145). As such, studies determining the contribution not only of L-ARG utilization, but also of L-ARG synthesis during NAFLD are warranted. The necessity of L-ARG synthesis within macrophages has recently been described during infection, suggesting extracellular L-ARG is not available in sufficient concentrations to drive effective macrophage function (135, 146, 147). Accounting for the considerable influx of inflammatory macrophages in NAFLD, future studies aimed at addressing macrophage-specific modulation of L-ARG metabolism with existing molecular tools (e.g., *Arg1*^{flox}, *Asl*^{flox}, Nos2-deficient) (148–150) will be necessary to dissect how various macrophage populations manipulate the liver microenvironment and NAFLD progression.

Macrophages and Trace Metals

Trace metals including iron, zinc and copper are essential for many cellular functions and for optimal adaptive and innate immune responses (151). Among these three metals, iron and copper exert an important influence on the genesis of NAFLD (152–155). Adults, but not children, with NAFLD manifest increased circulating concentrations of ferritin; however, both age groups exhibit increased transferrin saturation (153, 154). Excess iron accrual in the liver, specifically KCs (156) is associated with elevated amounts of hepcidin, which blocks iron egress mediated by ferroportin. Hepcidin binds to ferroportin and enhances its degradation. The accumulation of iron promotes "classically" activated macrophage polarization and production of proinflammatory cytokines that enhance the inflammatory response (157, 158). Excessive iron accumulation in both KCs and hepatocytes is associated with NAFLD (152). Inflammatory mediators, induced by lipid overload, drive increased hepcidin and decreased iron export from KCs and hepatocytes, in turn exacerbating NAFLD severity. Although copper is connected to iron homeostasis, the former metal is diminished in patients with NAFLD. Copper deficiency is associated with a decrease in ceruloplasmin ferroxidase which promotes iron release (152, 159). Aside from the effects of copper on iron regulation, the paucity of copper would reduce generation of copper/zinc superoxide dismutases that scavenge ROS and subsequently impairing the cellular defenses to ROS-mediated damage. Despite the knowledge of low zinc concentrations in chronic liver disease and damage (160) the role of zinc in NAFLD has not been investigated. How zinc deficiency augments liver damage is not well defined. Experiments determining the necessity of superoxide dismutases to combat excess ROS would provide valuable insight but have yet to be performed.

Adipose Tissue/Adipocytes and NAFLD

The traditional perspective of AT was that of a simplistic fat storing/releasing organ playing a role in energy homeostasis (161). It is now well-appreciated that AT is a highly metabolic, active and plastic organ comprised of various types of cells (e.g., adipocytes, progenitor stem cells within the stromal vascular fraction, endothelial cells, fibroblasts, and immune cells) (162, 163). The AT regulates the accommodation of excess energy through storage of circulating dietary lipids and *de novo* lipogenesis. In the case of nutrient shortage, lipolysis controls/regulates the release of hydrolyzed TGs as glycerol and FFAs to provide energy to surrounding tissues (164–166). Obesity-associated changes in AT robustly modify AT function including production of hormones, cytokines and adipokines. Which cells within AT tissue contribute to such changes and how the low-grade systemic and AT inflammatory state in obesity/metabolic disease impacts AT function is poorly understood.

Adipose tissue macrophages (ATMs) are believed to play a major role in regulating AT inflammation. In general, healthy AT, is believed to contain a balance between "alternatively" and "classically" activated ATMs. In contrast, the unhealthy/inflammatory AT, houses an increased number of "classically" activated ATMs. "Classically" activated ATMs produce an array of proinflammatory mediators (e.g., IL-6, TNF, IL-1β, IFNγ) that further amplify AT inflammation and promote

additional macrophage and other immune cell recruitment and activation. Cumulatively, such events, in obesity, fuel a chronic low-grade inflammation within the AT (167, 168). In addition to the enhanced release of soluble mediators, AT inflammation drives expression of integrin α4 on macrophages and vascular cell adhesion molecule 1 (VCAM-1) on adipocytes allowing for AT macrophage accrual (43). The inhibition of integrin α4 reduces ATM retention and AT inflammation. Notably, individuals with NAFLD exhibit high expression of VCAM-1 in AT underlining the importance of this cell-cell interaction pathways (169).

Adipocytes play a pivotal role in metabolic disease by promoting chronic inflammation via release of FFAs in response to increased circulating levels of TNF. These FFAs translocate to the liver and skeletal muscle propagating inflammation and insulin resistance (170, 171). Mechanistically, TNF inhibits PPARγ (172) and CCAAT/enhancer binding protein (C/EBPα) in adipocytes that is needed for the expression of adipocyte-specific GLUT4 and insulin receptor (IR) to maintain insulin sensitivity (173, 174). This suggests that like ATMs, adipocytes themselves play an important role in maintaining AT metabolic processes. Recent studies demonstrate that adipocytes, like immune cells, exhibit immune-like potential (175, 176). Specifically, adipocytes express various innate immune receptors including RIG-I-like receptors (RLR), NLRs, and nucleotide oligomerization domains (NODs) (177, 178). NOD-1 signaling suppresses adipocyte differentiation and contributes to induction of the NF-kB (177, 179). Adipocyte sensing of various PAMPs leads to production of multiple inflammatory mediators (e.g., cytokines, chemokines, adipokines) (180). In obesity, the main mechanisms associated with unlocking adipocyte-intrinsic inflammation are: (a) obesity-associated endotoxemia and (b) AT hypoxic micro-environment which leads to ER stress, inflammatory cytokine production, cell death, release of lipid content and debris and induction of the inflammatory mediators (181). Adipocyte production of inflammatory mediators is potentially sensed by ATMs and leads to their activation (161, 168, 180, 182). Adiponectin, an adipokine, exerts either

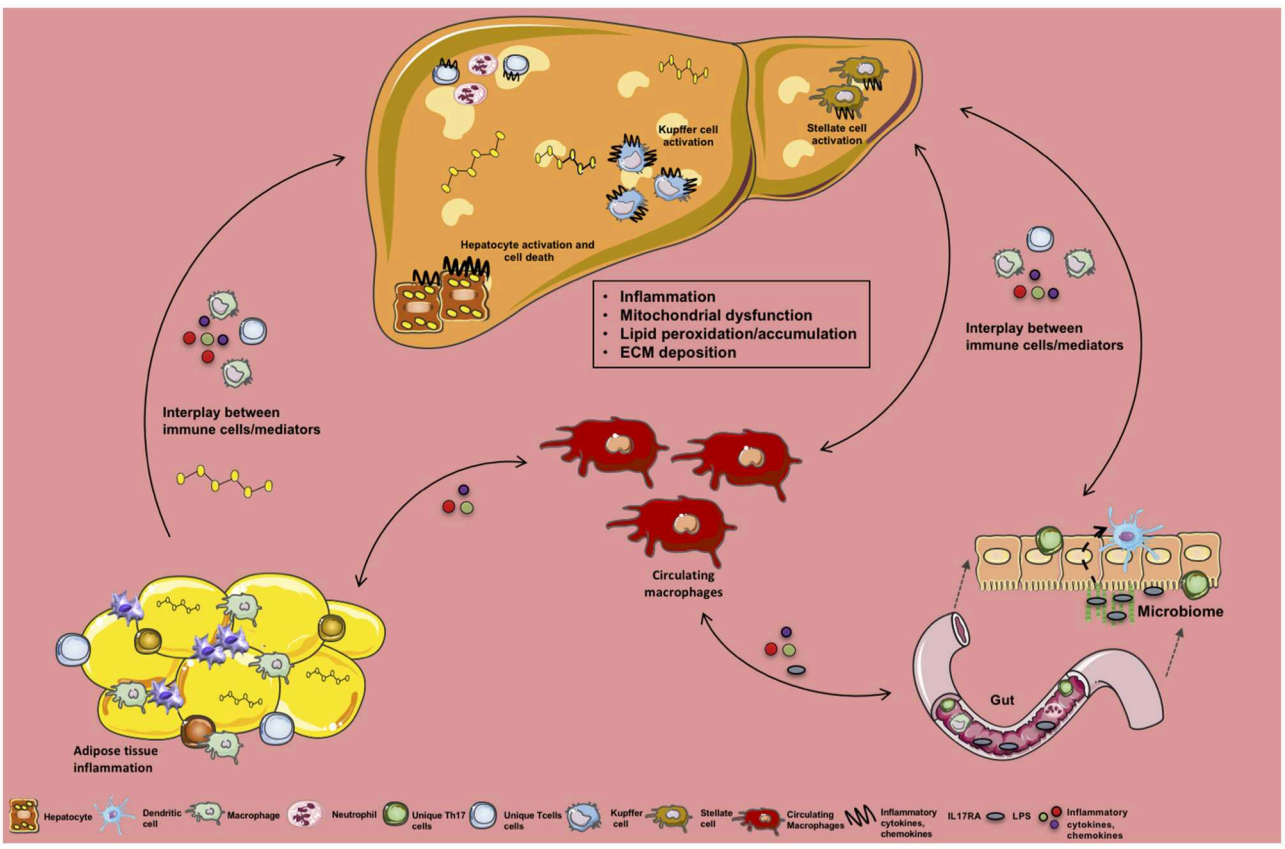

FIGURE 3 | Crosstalk between tissue-specific inflammation and macrophage function in NAFLD. Schematic overview of the crosstalk between various organs, their specific immune cells and inflammatory mediators during NAFLD. Obesity-associated low-grade, chronic inflammation and altered gut microbiome impacts immune cell crosstalk between the gut, circulating monocytes/macrophages, and the liver. In addition, obesity-associated adipocyte expansion promotes hypoxia leading to adipose tissue (AT) inflammation, activation of adipose tissue macropahes (ATMs) and fuels infiltration of various immune cells and inflammatory mediator production (e.g., FFAs, ROS, cytokines, chemokines) to be sensed by circulating macrophages and hepatocytes. Collectively these processes alter hepatocellular lipid metabolism, contributing to steatosis and proinflammatory cytokine (IL-17, TNF, IFNγ, IL-6) and chemokine production (CCL2, CXCL9, CXCL10). Moreover, this inflammatory state activates hepatic stellate cells (HSCs) and Kupffer cells (KCs) in turn contributing to extracellular matrix deposition (collagen fibers) and progression to fibrosis.

anti- or pro-inflammatory effects on macrophages. It inhibits macrophage functions (e.g., phagocytosis, cytokine production) and induces proliferation of "alternatively" activated macrophage in AT (183–185). Conversely, adiponectin also induces pro-inflammatory signaling cascades through NF-kB activation and upregulation of pro-inflammatory cytokines (e.g., TNF-α, IL-6, and IL-8) (186, 187). However, detailed analysis of specific adipocyte mediators and adipokines relevant to altered ATMs polarization and activation is needed. Similarly, whether obesity-activated adipocytes or ATMs directly play a role in NAFLD pathogenesis is not fully understood and should be further investigated. A brief summary of the above discussed processes is depicted in **Figure 3**.

Therapies

Ultimately, the improvement of experimental models to more closely recapitulate human NAFLD would be ideal for the discovery and development of therapies targeting various metabolic pathways in macrophages. Diet and lifestyle changes help in reversing NAFLD progression. Thus, it is not surprising that several pharmacological drugs that target metabolic and inflammatory and molecular mechanisms important in NAFLD progression are currently being examined (188). Hepatic lipid accumulation is an initial driver of NAFLD pathogenesis (9, 84). Intuitively, use of therapeutic drugs that target lipid metabolism is actively pursued. Glitazones, which are a class of insulin-sensitizing drugs, are effective in regulating lipid metabolism. They increase FAS and FA uptake by adipocytes, thus increasing lipid loading in AT instead of ectopic organs (e.g., liver and muscle) (189, 190). However, due to their association with increased risks of heart failure the use of glitazones as a treatment option has not been pursued in the clinic (191, 192). Sodium glucose co-transporter 2 (SGLT2) inhibitors have also proven efficacious in regulating NAFLD-associated dyslipidemia by inhibiting hepatic expression of lipogenic genes (e.g., sterol regulatory-element binding protein 1-c, fatty acid synthase, acetyl-CoA carboxylase 1, and sterol CoA desaturase), hepatic macrophage infiltration and expression of inflammatory cytokine production (190, 193, 194). However, underlying mechanisms that govern this process remains an area of investigation. Upregulation of oxidative stress, inflammation and apoptosis pathways are associated with NAFLD pathogenesis. NASH patients display increased hepatic activation of apoptosis signal-regulating kinase 1 (Ask1). Activation of Ask1 by TNF causes oxidative and ER stress, and induction of p38 and JNK signaling (188, 190, 195). Ask1 inhibition reduced hepatic steatosis, inflammation and fibrosis (196, 197). However, given that phenotypical outcomes of Ask1 inhibition in mice are not often recapitulated in humans, more effective "humanized" mouse models are needed (198, 199). Further, the effects of Ask1 inhibition on macrophage inflammation in NAFLD pathogenesis remains underdefined. Limiting the detrimental effects of obesity-associated microbiome alteration and subsequent systemic endotoxemia which contribute to NAFLD pathogenesis is another active area of investigation for drug development. Excessive PRR activation and inflammation resulting in liver injury is characteristic of NAFLD. JKB-121, a TLR4 antagonist, prevents LPS induced inflammatory liver injury in MCD diet models of NAFLD. However, given that obesity modulates the expression of multiple TLRs, more studies are needed to determine the impact of ablation of other TLRs in NAFLD pathogenesis. In addition, there are several other therapeutic approaches regarding use of ACC inhibitors, fructose inhibitors and obetocholic acid inhibitors (188, 190) for the treatment of NAFLD. In sum, several potential avenues for NAFLD therapies are being pursued. Specifically, there is a need for studies to allow for HFD-driven induction of hepatic fibrosis (41), eliminate gender bias by employing a more "human"-like disease state (e.g., thermoneutrality) (200), CCL4 experimental models of fibrosis (201) use of various murine strains/genotypes to mimic genetic diversity as well as expansion of such findings into non-human primate models of NAFLD (198). Use of such wide ranging experimental models would be beneficial in the development of therapeutic targets that may prove more effective in the clinic. Thus, in sum, given the interplay between metabolism and inflammation, additional therapies targeting macrophage polarization, chemo-attracting, inflammatory and metabolic pathways are needed—something that may be achieved by improving experimental modeling of disease.

CONCLUSION

Overall, this review highlights the inflammatory processes associated with macrophage polarization, tissue recruitment and inflammation and the role of such processes in NAFLD. We also extensively discuss how cellular metabolic pathways may contribute to macrophage-driven inflammation. Given the metabolic changes in obesity and inflammation the potential benefits to be gained from understanding the interplay between various metabolic and inflammatory pathways in macrophages are immense. Further elucidation of the crosstalk between macrophages and other tissues/immune cells similarly remains an exciting area of exploration. However, subsequent to the detailed interrogation of the afore discussed cellular and molecular processes in NAFLD, validation of such processes in multiple experimental models of NAFLD will be required.

AUTHOR CONTRIBUTIONS

JO, MM, MM-F, MD, GD, JQ, and SD wrote the manuscript. All authors have reviewed the manuscript and approve the final version.

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REFERENCES

1. Perumpail BJ, Khan MA, Yoo ER, Cholankeril G, Kim D, Ahmed A. Clinical epidemiology and disease burden of nonalcoholic fatty liver disease. *World J Gastroenterol.* (2017) 23:8263–76. doi: 10.3748/wjg.v23.i47.8263
2. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology.* (2010) 51:679–89. doi: 10.1002/hep.23280
3. Younossi ZM, Blissett D, Blissett R, Henry L, Stepanova M, Younossi Y, et al. The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. *Hepatology.* (2016) 64:1577–86. doi: 10.1002/hep.28785
4. Hardy T, Oakley F, Anstee QM, Day CP. Nonalcoholic Fatty Liver Disease: Pathogenesis and Disease Spectrum. *Annu Rev Pathol.* (2016) 11:451–96. doi: 10.1146/annurev-pathol-012615-044224
5. Arrese M, Cabrera D, Kalergis AM, Feldstein AE. Innate immunity and inflammation in NAFLD/NASH. *Dig Dis Sci.* (2016) 61:1294–303. doi: 10.1007/s10620-016-4049-x
6. Cha JY, Kim DH, Chun KH. The role of hepatic macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Lab Anim Res.* (2018) 34:133–9. doi: 10.5625/lar.2018.34.4.133
7. Kwon H, Pessin JE. Adipokines mediate inflammation and insulin resistance. *Front Endocrinol.* (2013) 4:71. doi: 10.3389/fendo.2013.00071
8. Huang J, Viswakarma N, Yu S, Jia Y, Bai L, Vluggens A, et al. Progressive endoplasmic reticulum stress contributes to hepatocarcinogenesis in fatty acyl-CoA oxidase 1-deficient mice. *Am J Pathol.* (2011) 179:703–13. doi: 10.1016/j.ajpath.2011.04.030
9. Cobbina E, Akhlaghi F. Non-alcoholic fatty liver disease (NAFLD) - pathogenesis, classification, and effect on drug metabolizing enzymes and transporters. *Drug Metab Rev.* (2017) 49:197–211. doi: 10.1080/03602532.2017.1293683
10. Giles DA, Moreno-Fernandez ME, Stankiewicz TE, Cappelletti M, Huppert SS, Iwakura Y, et al. Regulation of Inflammation by IL-17A and IL-17F Modulates Non-Alcoholic Fatty Liver Disease Pathogenesis. *PLoS ONE.* (2016) 11:e0149783. doi: 10.1371/journal.pone.0149783
11. Rosso C, Kazankov K, Younes R, Esmaili S, Marietti M, Sacco M, et al. Crosstalk between adipose tissue insulin resistance and liver macrophages in non-alcoholic fatty liver disease. *J Hepatol.* (2019) 71:1012–21. doi: 10.1016/j.jhep.2019.06.031
12. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes.* (2007) 56:1761–72. doi: 10.2337/db06-1491
13. Grunhut J, Wang W, Aykut B, Gakhal I, Torres-Hernandez A, Miller G. Macrophages in nonalcoholic steatohepatitis: friend or foe? *Eur Med J Hepatol.* (2018) 6:100–9.
14. Wree A, Broderick L, Canbay A, Hoffman HM, Feldstein AE. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. *Nat Rev Gastroenterol Hepatol.* (2013) 10:627–36. doi: 10.1038/nrgastro.2013.149
15. Miura K, Ohnishi H. Role of gut microbiota and Toll-like receptors in nonalcoholic fatty liver disease. *World J Gastroenterol.* (2014) 20:7381–91. doi: 10.3748/wjg.v20.i23.7381
16. Phan AT, Goldrath AW, Glass CK. Metabolic and epigenetic coordination of T cell and macrophage immunity. *Immunity.* (2017) 46:714–29. doi: 10.1016/j.immuni.2017.04.016
17. Braunersreuther V, Viviani GL, Mach F, Montecucco F. Role of cytokines and chemokines in non-alcoholic fatty liver disease. *World J Gastroenterol.* (2012) 18:727–35. doi: 10.3748/wjg.v18.i8.727
18. Giles DA, Moreno-Fernandez ME, Divanovic S. IL-17 Axis driven inflammation in non-alcoholic fatty liver disease progression. *Curr Drug Targets.* (2015) 16:1315–23. doi: 10.2174/1389450116666150531153627
19. Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology.* (2009) 50:261–74. doi: 10.1002/hep.22950
20. Wasmuth HE, Tacke F, Trautwein C. Chemokines in liver inflammation and fibrosis. *Semin Liver Dis.* (2010) 30:215–25. doi: 10.1055/s-0030-1255351
21. Obstfeld AE, Sugaru E, Thearle M, Francisco AM, Gayet C, Ginsberg HN, et al. C-C chemokine receptor 2 (CCR2) regulates the hepatic recruitment of myeloid cells that promote obesity-induced hepatic steatosis. *Diabetes.* (2010) 59:916–25. doi: 10.2337/db09-1403
22. Holt AP, Haughton EL, Lalor PF, Filer A, Buckley CD, Adams DH. Liver myofibroblasts regulate infiltration and positioning of lymphocytes in human liver. *Gastroenterology.* (2009) 136:705–14. doi: 10.1053/j.gastro.2008.10.020
23. Kazankov K, Jorgensen SMD, Thomsen KL, Moller HJ, Vilstrup H, George J, et al. The role of macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Nat Rev Gastroenterol Hepatol.* (2019) 16:145–59. doi: 10.1038/s41575-018-0082-x
24. Mak A, Uetrecht J. Involvement of CCL2/CCR2 macrophage recruitment in amodiaquine-induced liver injury. *J Immunotoxicol.* (2019) 2019:1–6. doi: 10.1080/1547691X.2018.1516014
25. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest.* (2006) 116:1494–505. doi: 10.1172/JCI26498
26. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, et al. CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest.* (2006) 116:115–24. doi: 10.1172/JCI24335
27. Tamura Y, Sugimoto M, Murayama T, Minami M, Nishikaze Y, Ariyasu H, et al. C-C chemokine receptor 2 inhibitor improves diet-induced development of insulin resistance and hepatic steatosis in mice. *J Atheroscler Thromb.* (2010) 17:219–28. doi: 10.5551/jat.3368
28. Seki E, De Minicis S, Gwak GY, Kluwe J, Inokuchi S, Bursill CA, et al. CCR1 and CCR5 promote hepatic fibrosis in mice. *J Clin Invest.* (2009) 119:1858–70. doi: 10.1172/JCI37444
29. Berres ML, Koenen RR, Rueland A, Zaldivar MM, Heinrichs D, Sahin H, et al. Antagonism of the chemokine Ccl5 ameliorates experimental liver fibrosis in mice. *J Clin Invest.* (2010) 120:4129–40. doi: 10.1172/JCI41732
30. Miura K, Yang L, van Rooijen N, Ohnishi H, Seki E. Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2. *Am J Physiol Gastrointest Liver Physiol.* (2012) 302:G1310–21. doi: 10.1152/ajpgi.00365.2011
31. Heymann F, Hammerich L, Storch D, Bartneck M, Huss S, Russeler V, et al. Hepatic macrophage migration and differentiation critical for liver fibrosis is mediated by the chemokine receptor C-C motif chemokine receptor 8 in mice. *Hepatology.* (2012) 55:898–909. doi: 10.1002/hep.24764
32. Baeck C, Wei X, Bartneck M, Fech V, Heymann F, Gassler N, et al. Pharmacological inhibition of the chemokine C-C motif chemokine ligand 2 (monocyte chemoattractant protein 1) accelerates liver fibrosis regression by suppressing Ly-6C(+) macrophage infiltration in mice. *Hepatology.* (2014) 59:1060–72. doi: 10.1002/hep.26783
33. Zhang X, Han J, Man K, Li X, Du J, Chu ES, et al. CXC chemokine receptor 3 promotes steatohepatitis in mice through mediating inflammatory cytokines, macrophages and autophagy. *J Hepatol.* (2016) 64:160–70. doi: 10.1016/j.jhep.2015.09.005

34. Tomita K, Freeman BL, Bronk SF, LeBrasseur NK, White TA, Hirsova P, et al. CXCL10-Mediates Macrophage, but not Other Innate Immune Cells-Associated Inflammation in Murine Nonalcoholic Steatohepatitis. *Sci Rep.* (2016) 6:28786. doi: 10.1038/srep28786
35. Friedman SL, Ratziu V, Harrison SA, Abdelmalek MF, Aithal GP, Caballeria J, et al. A randomized, placebo-controlled trial of cenicriviroc for treatment of nonalcoholic steatohepatitis with fibrosis. *Hepatology.* (2018) 67:1754–67. doi: 10.1002/hep.29477
36. Harley IT, Stankiewicz TE, Giles DA, Softic S, Flick LM, Cappelletti M, et al. IL-17 signaling accelerates the progression of nonalcoholic fatty liver disease in mice. *Hepatology.* (2014) 59:1830–9. doi: 10.1002/hep.26746
37. Gomes AL, Teijeiro A, Buren S, Tummala KS, Yilmaz M, Waisman A, et al. Metabolic inflammation-associated IL-17A causes non-alcoholic steatohepatitis and hepatocellular carcinoma. *Cancer Cell.* (2016) 30:161–75. doi: 10.1016/j.ccell.2016.05.020
38. Baffy G. Kupffer cells in non-alcoholic fatty liver disease: the emerging view. *J Hepatol.* (2009) 51:212–23. doi: 10.1016/j.jhep.2009.03.008
39. Robinson MW, Harmon C, O'Farrelly C. Liver immunology and its role in inflammation and homeostasis. *Cell Mol Immunol.* (2016) 13:267–76. doi: 10.1038/cmi.2016.3
40. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell.* (2010) 140:805–20. doi: 10.1016/j.cell.2010.01.022
41. Kohli R, Kirby M, Xanthakos SA, Softic S, Feldstein AE, Saxena V, et al. High-fructose, medium chain trans fat diet induces liver fibrosis and elevates plasma coenzyme Q9 in a novel murine model of obesity and nonalcoholic steatohepatitis. *Hepatology.* (2010) 52:934–44. doi: 10.1002/hep.23797
42. Mayoral Monibas R, Johnson AM, Osborn O, Traves PG, Mahata SK. Distinct hepatic macrophage populations in lean and obese mice. *Front Endocrinol* (*Lausanne).* (2016) 7:152. doi: 10.3389/fendo.2016.00152
43. Kim SY, Jeong JM, Kim SJ, Seo W, Kim MH, Choi WM, et al. Pro-inflammatory hepatic macrophages generate ROS through NADPH oxidase 2 via endocytosis of monomeric TLR4-MD2 complex. *Nat Commun.* (2017) 8:2247. doi: 10.1038/s41467-017-02325-2
44. Classen A, Lloberas J, Celada A. Macrophage activation: classical versus alternative. *Methods Mol Biol.* (2009) 531:29–43. doi: 10.1007/978-1-59745-396-7_3
45. Kang K, Reilly SM, Karabacak V, Gangl MR, Fitzgerald K, Hatano B, et al. Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. *Cell Metab.* (2008) 7:485–95. doi: 10.1016/j.cmet.2008.04.002
46. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol.* (2003) 3:23–35. doi: 10.1038/nri978
47. Saberi M, Woods NB, de Luca C, Schenk S, Lu JC, Bandyopadhyay G, et al. Hematopoietic cell-specific deletion of toll-like receptor 4 ameliorates hepatic and adipose tissue insulin resistance in high-fat-fed mice. *Cell Metab.* (2009) 10:419–29. doi: 10.1016/j.cmet.2009.09.006
48. Solinas G, Vilcu C, Neels JG, Bandyopadhyay GK, Luo JL, Naugler W, et al. JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. *Cell Metab.* (2007) 6:386–97. doi: 10.1016/j.cmet.2007.09.011
49. Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, et al. IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med.* (2005) 11:191–8. doi: 10.1038/nm1185
50. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med.* (2005) 11:183–90. doi: 10.1038/nm1166
51. Mas E, Danjoux M, Garcia V, Carpentier S, Segui B, Levade T. IL-6 deficiency attenuates murine diet-induced non-alcoholic steatohepatitis. *PLoS ONE.* (2009) 4:e7929. doi: 10.1371/journal.pone.0007929
52. Papanicolaou DA, Wilder RL, Manolagas SC, Chrousos GP. The pathophysiologic roles of interleukin-6 in human disease. *Ann Intern Med.* (1998) 128:127–37. doi: 10.7326/0003-4819-128-2-199801150-00009
53. Ray A, Tatter SB, Santhanam U, Helfgott DC, May LT, Sehgal PB. Regulation of expression of interleukin-6. Molecular and clinical studies. *Ann N Y Acad Sci.* (1989) 557:353–61; discussion 61–2. doi: 10.1111/j.1749-6632.1989.tb24028.x
54. Brass EP, Vetter WH. Interleukin-6, but not tumour necrosis factor-alpha, increases lipogenesis in rat hepatocyte primary cultures. *Biochem J.* (1994) 301 (Pt 1):193–7. doi: 10.1042/bj3010193
55. Fernandez-Real JM, Lopez-Bermejo A, Ricart W. Cross-talk between iron metabolism and diabetes. *Diabetes.* (2002) 51:2348–54. doi: 10.2337/diabetes.51.8.2348
56. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. *J Clin Invest.* (2003) 112:1785–8. doi: 10.1172/JCI20514
57. Senn JJ, Klover PJ, Nowak IA, Mooney RA. Interleukin-6 induces cellular insulin resistance in hepatocytes. *Diabetes.* (2002) 51:3391–9. doi: 10.2337/diabetes.51.12.3391
58. Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur Cytokine Netw.* (2006) 17:4–12.
59. Kugelmas M, Hill DB, Vivian B, Marsano L, McClain CJ. Cytokines and NASH: a pilot study of the effects of lifestyle modification and vitamin E. *Hepatology.* (2003) 38:413–9. doi: 10.1053/jhep.2003.50316
60. Haukeland JW, Damas JK, Konopski Z, Loberg EM, Haaland T, Goverud I, et al. Systemic inflammation in nonalcoholic fatty liver disease is characterized by elevated levels of CCL2. *J Hepatol.* (2006) 44:1167–74. doi: 10.1016/j.jhep.2006.02.011
61. Wieckowska A, Feldstein AE. Diagnosis of nonalcoholic fatty liver disease: invasive versus noninvasive. *Semin Liver Dis.* (2008) 28:386–95. doi: 10.1055/s-0028-1091983
62. Yamaguchi K, Itoh Y, Yokomizo C, Nishimura T, Niimi T, Fujii H, et al. Blockade of interleukin-6 signaling enhances hepatic steatosis but improves liver injury in methionine choline-deficient diet-fed mice. *Lab Invest.* (2010) 90:1169–78. doi: 10.1038/labinvest.2010.75
63. Harris HW, Grunfeld C, Feingold KR, Rapp JH. Human very low density lipoproteins and chylomicrons can protect against endotoxin-induced death in mice. *J Clin Invest.* (1990) 86:696–702. doi: 10.1172/JCI114765
64. Grunfeld C, Pang M, Doerrler W, Shigenaga JK, Jensen P, Feingold KR. Lipids, lipoproteins, triglyceride clearance, and cytokines in human immunodeficiency virus infection and the acquired immunodeficiency syndrome. *J Clin Endocrinol Metab.* (1992) 74:1045–52. doi: 10.1210/jcem.74.5.1373735
65. Borst SE. The role of TNF-alpha in insulin resistance. *Endocrine.* (2004) 23(2–3):177–82. doi: 10.1385/ENDO:23:2-3:177
66. Fon Tacer K, Kuzman D, Seliskar M, Pompon D, Rozman D. TNF-alpha interferes with lipid homeostasis and activates acute and proatherogenic processes. *Physiol Genomics.* (2007) 31:216–27. doi: 10.1152/physiolgenomics.00264.2006
67. Wullaert A, van Loo G, Heyninck K, Beyaert R. Hepatic tumor necrosis factor signaling and nuclear factor-kappaB: effects on liver homeostasis and beyond. *Endocr Rev.* (2007) 28:365–86. doi: 10.1210/er.2006-0031
68. Tomita K, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, et al. Tumour necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. *Gut.* (2006) 55:415–24. doi: 10.1136/gut.2005.071118
69. Kakino S, Ohki T, Nakayama H, Yuan X, Otabe S, Hashinaga T, et al. Pivotal role of TNF-alpha in the development and progression of nonalcoholic fatty liver disease in a murine model. *Horm Metab Res.* (2018) 50:80–7. doi: 10.1055/s-0043-118666
70. Tan Q, Hu J, Yu X, Guan W, Lu H, Yu Y, et al. The role of IL-1 family members and kupffer cells in liver regeneration. *Biomed Res Int.* (2016) 2016:6495793. doi: 10.1155/2016/6495793
71. Negrin KA, Roth Flach RJ, DiStefano MT, Matevossian A, Friedline RH, Jung D, et al. IL-1 signaling in obesity-induced hepatic lipogenesis and steatosis. *PLoS ONE.* (2014) 9:e107265. doi: 10.1371/journal.pone.0107265
72. Garlanda C, Dinarello CA, Mantovani A. The interleukin-1 family: back to the future. *Immunity.* (2013) 39:1003–18. doi: 10.1016/j.immuni.2013.11.010
73. Meier RPH, Meyer J, Montanari E, Lacotte S, Balaphas A, Muller YD, et al. Interleukin-1 receptor antagonist modulates liver inflammation and fibrosis in mice in a model-dependent manner. *Int J Mol Sci.* (2019) 20:E1295. doi: 10.3390/ijms20061295
74. Gehrke N, Hovelmeyer N, Waisman A, Straub BK, Weinmann-Menke J, Worns MA, et al. Hepatocyte-specific deletion of IL1-RI attenuates liver

injury by blocking IL-1 driven autoinflammation. *J Hepatol.* (2018) 68:986–95. doi: 10.1016/j.jhep.2018.01.008
75. Janssens S, Beyaert R. A universal role for MyD88 in TLR/IL-1R-mediated signaling. *Trends Biochem Sci.* (2002) 27:474–82. doi: 10.1016/S0968-0004(02)02145-X
76. Larsen CM, Faulenbach M, Vaag A, Volund A, Ehses JA, Seifert B, et al. Interleukin-1-receptor antagonist in type 2 diabetes mellitus. *N Engl J Med.* (2007) 356:1517–26. doi: 10.1056/NEJMoa065213
77. Gomez R, Villalvilla A, Largo R, Gualillo O, Herrero-Beaumont G. TLR4 signalling in osteoarthritis–finding targets for candidate DMOADs. *Nat Rev Rheumatol.* (2015) 11:159–70. doi: 10.1038/nrrheum.2014.209
78. Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology.* (1998) 114:842–5. doi: 10.1016/S0016-5085(98)70599-2
79. Crosas-Molist E, Fabregat I. Role of NADPH oxidases in the redox biology of liver fibrosis. *Redox Biol.* (2015) 6:106–11. doi: 10.1016/j.redox.2015.07.005
80. Mukherjee R, Moreno-Fernandez ME, Giles DA, Cappelletti M, Stankiewicz TE, Chan CC, et al. Nicotinamide adenine dinucleotide phosphate (reduced) oxidase 2 modulates inflammatory vigor during nonalcoholic fatty liver disease progression in mice. *Hepatol Commun.* (2018) 2:546–60. doi: 10.1002/hep4.1162
81. Chapman NM, Shrestha S, Chi H. Metabolism in immune cell differentiation and function. *Adv Exp Med Biol.* (2017) 1011:1–85. doi: 10.1007/978-94-024-1170-6_1
82. Krzywinska E, Stockmann C. Hypoxia, metabolism and immune cell function. *Biomedicines.* (2018) 6:E56. doi: 10.3390/biomedicines6020056
83. Cirulli ET, Guo L, Leon Swisher C, Shah N, Huang L, Napier LA, et al. Profound perturbation of the metabolome in obesity is associated with health risk. *Cell Metab.* (2019) 29:488–500 e2. doi: 10.1016/j.cmet.2018.09.022
84. Reddy JK, Rao MS. Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol.* (2006) 290:G852–8. doi: 10.1152/ajpgi.00521.2005
85. Reddy JK, Hashimoto T. Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annu Rev Nutr.* (2001) 21:193–230. doi: 10.1146/annurev.nutr.21.1.193
86. Moreno-Fernandez ME, Giles DA, Stankiewicz TE, Sheridan R, Karns R, Cappelletti M, et al. Peroxisomal beta-oxidation regulates whole body metabolism, inflammatory vigor, and pathogenesis of nonalcoholic fatty liver disease. *JCI Insight.* (2018) 3:93626. doi: 10.1172/jci.insight.93626
87. Olefsky JM, Glass CK. Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol.* (2010) 72:219–46. doi: 10.1146/annurev-physiol-021909-135846
88. Diskin C, Palsson-McDermott EM. Metabolic modulation in macrophage effector function. *Front Immunol.* (2018) 9:270. doi: 10.3389/fimmu.2018.00270
89. Wang X, de Carvalho Ribeiro M, Iracheta-Vellve A, Lowe P, Ambade A, Satishchandran A, et al. Macrophage-specific hypoxia-inducible factor-1alpha contributes to impaired autophagic flux in nonalcoholic steatohepatitis. *Hepatology.* (2019) 69:545–63. doi: 10.1002/hep.30215
90. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature.* (2013) 496:238–42. doi: 10.1038/nature11986
91. Jha AK, Huang SC, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, et al. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity.* (2015) 42:419–30. doi: 10.1016/j.immuni.2015.02.005
92. Haschemi A, Kosma P, Gille L, Evans CR, Burant CF, Starkl P, et al. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab.* (2012) 15:813–26. doi: 10.1016/j.cmet.2012.04.023
93. Koo SJ, Szczesny B, Wan X, Putluri N, Garg NJ. Pentose phosphate shunt modulates reactive oxygen species and nitric oxide production controlling trypanosoma cruzi in macrophages. *Front Immunol.* (2018) 9:202. doi: 10.3389/fimmu.2018.00202
94. Pollak N, Dolle C, Ziegler M. The power to reduce: pyridine nucleotides–small molecules with a multitude of functions. *Biochem J.* (2007) 402:205–18. doi: 10.1042/BJ20061638
95. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal.* (2012) 24:981–90. doi: 10.1016/j.cellsig.2012.01.008
96. Baardman J, Verberk SGS, Prange KHM, van Weeghel M, van der Velden S, Ryan DG, et al. A Defective pentose phosphate pathway reduces inflammatory macrophage responses during hypercholesterolemia. *Cell Rep.* (2018) 25:2044–52 e5. doi: 10.1016/j.celrep.2018.10.092
97. Koh HJ, Lee SM, Son BG, Lee SH, Ryoo ZY, Chang KT, et al. Cytosolic NADP+-dependent isocitrate dehydrogenase plays a key role in lipid metabolism. *J Biol Chem.* (2004) 279:39968–74. doi: 10.1074/jbc.M402260200
98. Al-Dwairi A, Pabona JM, Simmen RC, Simmen FA. Cytosolic malic enzyme 1 (ME1) mediates high fat diet-induced adiposity, endocrine profile, and gastrointestinal tract proliferation-associated biomarkers in male mice. *PLoS ONE.* (2012) 7:e46716. doi: 10.1371/journal.pone.0046716
99. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature.* (2014) 515:431–5. doi: 10.1038/nature13909
100. Postic C, Girard J. Contribution of *de novo* fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest.* (2008) 118:829–38. doi: 10.1172/JCI34275
101. Carmen GY, Victor SM. Signalling mechanisms regulating lipolysis. *Cell Signal.* (2006) 18:401–8. doi: 10.1016/j.cellsig.2005.08.009
102. Lewis GF, Carpentier A, Adeli K, Giacca A. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocr Rev.* (2002) 23:201–29. doi: 10.1210/edrv.23.2.0461
103. O'Neill LA, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol.* (2016) 16:553–65. doi: 10.1038/nri.2016.70
104. Ecker J, Liebisch G, Englmaier M, Grandl M, Robenek H, Schmitz G. Induction of fatty acid synthesis is a key requirement for phagocytic differentiation of human monocytes. *Proc Natl Acad Sci USA.* (2010) 107:7817–22. doi: 10.1073/pnas.0912059107
105. Wu X, Dong L, Lin X, Li J. Relevance of the NLRP3 Inflammasome in the pathogenesis of chronic liver disease. *Front Immunol.* (2017) 8:1728. doi: 10.3389/fimmu.2017.01728
106. Csak T, Ganz M, Pespisa J, Kodys K, Dolganiuc A, Szabo G. Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells. *Hepatology.* (2011) 54:133–44. doi: 10.1002/hep.24341
107. Wree A, McGeough MD, Pena CA, Schlattjan M, Li H, Inzaugarat ME, et al. NLRP3 inflammasome activation is required for fibrosis development in NAFLD. *J Mol Med.* (2014) 92:1069–82. doi: 10.1007/s00109-014-1170-1
108. Dixon LJ, Berk M, Thapaliya S, Papouchado BG, Feldstein AE. Caspase-1-mediated regulation of fibrogenesis in diet-induced steatohepatitis. *Lab Invest.* (2012) 92:713–23. doi: 10.1038/labinvest.2012.45
109. Miura K, Kodama Y, Inokuchi S, Schnabl B, Aoyama T, Ohnishi H, et al. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. *Gastroenterology.* (2010) 139:323–34 e7. doi: 10.1053/j.gastro.2010.03.052
110. Wahli W, Michalik L. PPARs at the crossroads of lipid signaling and inflammation. *Trends Endocrinol Metab.* (2012) 23:351–63. doi: 10.1016/j.tem.2012.05.001
111. Yu K, Bayona W, Kallen CB, Harding HP, Ravera CP, McMahon G, et al. Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *J Biol Chem.* (1995) 270:23975–83. doi: 10.1074/jbc.270.41.23975
112. Bjorndal B, Alteras EK, Lindquist C, Svardal A, Skorve J, Berge RK. Associations between fatty acid oxidation, hepatic mitochondrial function, and plasma acylcarnitine levels in mice. *Nutr Metab.* (2018) 15:10. doi: 10.1186/s12986-018-0241-7
113. Namgaladze D, Lips S, Leiker TJ, Murphy RC, Ekroos K, Ferreiros N, et al. Inhibition of macrophage fatty acid beta-oxidation exacerbates palmitate-induced inflammatory and endoplasmic reticulum stress responses. *Diabetologia.* (2014) 57:1067–77. doi: 10.1007/s00125-014-3173-4

114. O'Connor RS, Guo L, Ghassemi S, Snyder NW, Worth AJ, Weng L, et al. The CPT1a inhibitor, etomoxir induces severe oxidative stress at commonly used concentrations. *Sci Rep.* (2018) 8:6289. doi: 10.1038/s41598-018-24676-6
115. Pike LS, Smift AL, Croteau NJ, Ferrick DA, Wu M. Inhibition of fatty acid oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells. *Biochim Biophys Acta.* (2011) 1807:726–34. doi: 10.1016/j.bbabio.2010.10.022
116. Sheridan R, Lampe K, Shanmukhappa SK, Putnam P, Keddache M, Divanovic S, et al. Lampe1: an ENU-germline mutation causing spontaneous hepatosteatosis identified through targeted exon-enrichment and next-generation sequencing. *PLoS ONE.* (2011) 6:e21979. doi: 10.1371/journal.pone.0021979
117. Moffett JR, Namboodiri MA. Tryptophan and the immune response. *Immunol Cell Biol.* (2003) 81:247–65. doi: 10.1046/j.1440-1711.2003.t01-1-01177.x
118. Badawy AA, Namboodiri AM, Moffett JR. The end of the road for the tryptophan depletion concept in pregnancy and infection. *Clin Sci.* (2016) 130:1327–33. doi: 10.1042/CS20160153
119. McGaha TL, Huang L, Lemos H, Metz R, Mautino M, Prendergast GC, et al. Amino acid catabolism: a pivotal regulator of innate and adaptive immunity. *Immunol Rev.* (2012) 249:135–57. doi: 10.1111/j.1600-065X.2012.01149.x
120. Grohmann U, Bronte V. Control of immune response by amino acid metabolism. *Immunol Rev.* (2010) 236:243–64. doi: 10.1111/j.1600-065X.2010.00915.x
121. Hissong BD, Byrne GI, Padilla ML, Carlin JM. Upregulation of interferon-induced indoleamine 2,3-dioxygenase in human macrophage cultures by lipopolysaccharide, muramyl tripeptide, and interleukin-1. *Cell Immunol.* (1995) 160:264–9. doi: 10.1016/0008-8749(95)80037-J
122. Hu B, Hissong BD, Carlin JM. Interleukin-1 enhances indoleamine 2,3-dioxygenase activity by increasing specific mRNA expression in human mononuclear phagocytes. *J Interferon Cytokine Res.* (1995) 15:617–24. doi: 10.1089/jir.1995.15.617
123. Hissong BD, Carlin JM. Potentiation of interferon-induced indoleamine 2,3-dioxygenase mRNA in human mononuclear phagocytes by lipopolysaccharide and interleukin-1. *J Interferon Cytokine Res.* (1997) 17:387–93. doi: 10.1089/jir.1997.17.387
124. Carlin JM, Borden EC, Sondel PM, Byrne GI. Interferon-induced indoleamine 2,3-dioxygenase activity in human mononuclear phagocytes. *J Leukoc Biol.* (1989) 45:29–34. doi: 10.1002/jlb.45.1.29
125. Currier AR, Ziegler MH, Riley MM, Babcock TA, Telbis VP, Carlin JM. Tumor necrosis factor-alpha and lipopolysaccharide enhance interferon-induced antichlamydial indoleamine dioxygenase activity independently. *J Interferon Cytokine Res.* (2000) 20:369–76. doi: 10.1089/107999000312306
126. Wolowczuk I, Hennart B, Leloire A, Bessede A, Soichot M, Taront S, et al. Tryptophan metabolism activation by indoleamine 2,3-dioxygenase in adipose tissue of obese women: an attempt to maintain immune homeostasis and vascular tone. *Am J Physiol Regul Integr Comp Physiol.* (2012) 303:R135–43. doi: 10.1152/ajpregu.00373.2011
127. Nagano J, Shimizu M, Hara T, Shirakami Y, Kochi T, Nakamura N, et al. Effects of indoleamine 2,3-dioxygenase deficiency on high-fat diet-induced hepatic inflammation. *PLoS ONE.* (2013) 8:e73404. doi: 10.1371/journal.pone.0073404
128. Laurans L, Venteclef N, Haddad Y, Chajadine M, Alzaid F, Metghalchi S, et al. Genetic deficiency of indoleamine 2,3-dioxygenase promotes gut microbiota-mediated metabolic health. *Nat Med.* (2018) 24:1113–20. doi: 10.1038/s41591-018-0060-4
129. Ritze Y, Bardos G, Hubert A, Bohle M, Bischoff SC. Effect of tryptophan supplementation on diet-induced non-alcoholic fatty liver disease in mice. *Br J Nutr.* (2014) 112:1–7. doi: 10.1017/S0007114514000440
130. Cichoz-Lach H, Celinski K, Konturek PC, Konturek SJ, Slomka M. The effects of L-tryptophan and melatonin on selected biochemical parameters in patients with steatohepatitis. *J Physiol Pharmacol.* (2010) 61:577–80.
131. Celinski K, Konturek PC, Slomka M, Cichoz-Lach H, Brzozowski T, Konturek SJ, et al. Effects of treatment with melatonin and tryptophan on liver enzymes, parameters of fat metabolism and plasma levels of cytokines in patients with non-alcoholic fatty liver disease−14 months follow up. *J Physiol Pharmacol.* (2014) 65:75–82.
132. Munder M, Eichmann K, Modolell M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. *J Immunol.* (1998) 160:5347–54.
133. Shearer JD, Richards JR, Mills CD, Caldwell MD. Differential regulation of macrophage arginine metabolism: a proposed role in wound healing. *Am J Physiol.* (1997) 272:E181–90. doi: 10.1152/ajpendo.1997.272.2.E181
134. Mattila JT, Ojo OO, Kepka-Lenhart D, Marino S, Kim JH, Eum SY, et al. Microenvironments in tuberculous granulomas are delineated by distinct populations of macrophage subsets and expression of nitric oxide synthase and arginase isoforms. *J Immunol.* (2013) 191:773–84. doi: 10.4049/jimmunol.1300113
135. Qualls JE, Subramanian C, Rafi W, Smith AM, Balouzian L, DeFreitas AA, et al. Sustained generation of nitric oxide and control of mycobacterial infection requires argininosuccinate synthase 1. *Cell Host Microbe.* (2012) 12:313–23. doi: 10.1016/j.chom.2012.07.012
136. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity.* (2014) 41:14–20. doi: 10.1016/j.immuni.2014.06.008
137. Modolell M, Corraliza IM, Link F, Soler G, Eichmann K. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur J Immunol.* (1995) 25:1101–4. doi: 10.1002/eji.1830250436
138. Munder M, Eichmann K, Moran JM, Centeno F, Soler G, Modolell M. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J Immunol.* (1999) 163:3771–7.
139. Rutschman R, Lang R, Hesse M, Ihle JN, Wynn TA, Murray PJ. Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production. *J Immunol.* (2001) 166:2173–7. doi: 10.4049/jimmunol.166.4.2173
140. Barron L, Smith AM, El Kasmi KC, Qualls JE, Huang X, Cheever A, et al. Role of arginase 1 from myeloid cells in th2-dominated lung inflammation. *PLoS ONE.* (2013) 8:e61961. doi: 10.1371/journal.pone.0061961
141. Duque-Correa MA, Kuhl AA, Rodriguez PC, Zedler U, Schommer-Leitner S, Rao M, et al. Macrophage arginase-1 controls bacterial growth and pathology in hypoxic tuberculosis granulomas. *Proc Natl Acad Sci USA.* (2014) 111:E4024–32. doi: 10.1073/pnas.1408839111
142. Navarro LA, Wree A, Povero D, Berk MP, Eguchi A, Ghosh S, et al. Arginase 2 deficiency results in spontaneous steatohepatitis: a novel link between innate immune activation and hepatic *de novo* lipogenesis. *J Hepatol.* (2015) 62:412–20. doi: 10.1016/j.jhep.2014.09.015
143. Liu C, Rajapakse AG, Riedo E, Fellay B, Bernhard MC, Montani JP, et al. Targeting arginase-II protects mice from high-fat-diet-induced hepatic steatosis through suppression of macrophage inflammation. *Sci Rep.* (2016) 6:20405. doi: 10.1038/srep20405
144. Pasarin M, La Mura V, Gracia-Sancho J, Garcia-Caldero H, Rodriguez-Vilarrupla A, Garcia-Pagan JC, et al. Sinusoidal endothelial dysfunction precedes inflammation and fibrosis in a model of NAFLD. *PLoS ONE.* (2012) 7:e32785. doi: 10.1371/journal.pone.0032785
145. Sellmann C, Degen C, Jin CJ, Nier A, Engstler AJ, Hasan Alkhatib D, et al. Oral arginine supplementation protects female mice from the onset of non-alcoholic steatohepatitis. *Amino Acids.* (2017) 49:1215–25. doi: 10.1007/s00726-017-2423-4
146. Rapovy SM, Zhao J, Bricker RL, Schmidt SM, Setchell KD, Qualls JE. Differential Requirements for L-Citrulline and L-Arginine during antimycobacterial macrophage activity. *J Immunol.* (2015) 195:3293–300. doi: 10.4049/jimmunol.1500800
147. Lange SM, McKell MC, Schmidt SM, Zhao J, Crowther RR, Green LC, et al. L-arginine synthesis from l-citrulline in myeloid cells drives host defense against mycobacteria *in vivo*. *J Immunol.* (2019) 202:1747–54. doi: 10.4049/jimmunol.1801569
148. Erez A, Nagamani SC, Shchelochkov OA, Premkumar MH, Campeau PM, Chen Y, et al. Requirement of argininosuccinate lyase for systemic nitric oxide production. *Nat Med.* (2011) 17:1619–26. doi: 10.1038/nm.2544
149. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, et al. Altered responses to bacterial infection and endotoxic shock

in mice lacking inducible nitric oxide synthase. *Cell.* (1995) 81:641–50. doi: 10.1016/0092-8674(95)90085-3
150. El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, Henao-Tamayo M, et al. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol.* (2008) 9:1399–406. doi: 10.1038/ni.1671
151. Failla ML. Trace elements and host defense: recent advances and continuing challenges. *J Nutr.* (2003) 133(5 Suppl 1):1443S–7S. doi: 10.1093/jn/133.5.1443S
152. Aigner E, Theurl I, Haufe H, Seifert M, Hohla F, Scharinger L, et al. Copper availability contributes to iron perturbations in human nonalcoholic fatty liver disease. *Gastroenterology.* (2008) 135:680–8. doi: 10.1053/j.gastro.2008.04.007
153. Aigner E, Weiss G, Datz C. Dysregulation of iron and copper homeostasis in nonalcoholic fatty liver. *World J Hepatol.* (2015) 7:177–88. doi: 10.4254/wjh.v7.i2.177
154. Feldman A, Aigner E, Weghuber D, Paulmichl K. The Potential role of iron and copper in pediatric obesity and nonalcoholic fatty liver disease. *Biomed Res Int.* (2015) 2015:287401. doi: 10.1155/2015/287401
155. Tarantino G, Porcu C, Arciello M, Andreozzi P, Balsano C. Prediction of carotid intima-media thickness in obese patients with low prevalence of comorbidities by serum copper bioavailability. *J Gastroenterol Hepatol.* (2018) 33:1511–7. doi: 10.1111/jgh.14104
156. Turlin B, Mendler MH, Moirand R, Guyader D, Guillygomarc'h A, Deugnier Y. Histologic features of the liver in insulin resistance-associated iron overload. A study of 139 patients. *Am J Clin Pathol.* (2001) 116:263–70. doi: 10.1309/WWNE-KW2C-4KTW-PTJ5
157. Handa P, Thomas S, Morgan-Stevenson V, Maliken BD, Gochanour E, Boukhar S, et al. Iron alters macrophage polarization status and leads to steatohepatitis and fibrogenesis. *J Leukoc Biol.* (2019) 105:1015–26. doi: 10.1002/JLB.3A0318-108R
158. Handa P, Vemulakonda AL, Maliken BD, Morgan-Stevenson V, Nelson JE, Dhillon BK, et al. Differences in hepatic expression of iron, inflammation and stress-related genes in patients with nonalcoholic steatohepatitis. *Ann Hepatol.* (2017) 16:77–85. doi: 10.5604/16652681.1226818
159. Sharp P. The molecular basis of copper and iron interactions. *Proc Nutr Soc.* (2004) 63:563–9. doi: 10.1079/PNS2004386
160. Himoto T, Masaki T. Associations between zinc deficiency and metabolic abnormalities in patients with chronic liver disease. *Nutrients.* (2018) 10:88. doi: 10.3390/nu10010088
161. Trayhurn P. Hypoxia and adipose tissue function and dysfunction in obesity. *Physiol Rev.* (2013) 93:1–21. doi: 10.1152/physrev.00017.2012
162. Ghaben AL, Scherer PE. Adipogenesis and metabolic health. *Nat Rev Mol Cell Biol.* (2019) 20:242–58. doi: 10.1038/s41580-018-0093-z
163. Sarantopoulos CN, Banyard DA, Ziegler ME, Sun B, Shaterian A, Widgerow AD. Elucidating the preadipocyte and its role in adipocyte formation: a comprehensive review. *Stem Cell Rev Rep.* (2018) 14:27–42. doi: 10.1007/s12015-017-9774-9
164. Langin D, Dicker A, Tavernier G, Hoffstedt J, Mairal A, Ryden M, et al. Adipocyte lipases and defect of lipolysis in human obesity. *Diabetes.* (2005) 54:3190–7. doi: 10.2337/diabetes.54.11.3190
165. Ebbert JO, Jensen MD. Fat depots, free fatty acids, and dyslipidemia. *Nutrients.* (2013) 5:498–508. doi: 10.3390/nu5020498
166. Frayn KN. Adipose tissue as a buffer for daily lipid flux. *Diabetologia.* (2002) 45:1201–10. doi: 10.1007/s00125-002-0873-y
167. Kamada Y, Takehara T, Hayashi N. Adipocytokines and liver disease. *J Gastroenterol.* (2008) 43:811–22. doi: 10.1007/s00535-008-2213-6
168. O'Rourke RW, White AE, Metcalf MD, Olivas AS, Mitra P, Larison WG, et al. Hypoxia-induced inflammatory cytokine secretion in human adipose tissue stromovascular cells. *Diabetologia.* (2011) 54:1480–90. doi: 10.1007/s00125-011-2103-y
169. Lefere S, Van de Velde F, Devisscher L, Bekaert M, Raevens S, Verhelst X, et al. Serum vascular cell adhesion molecule-1 predicts significant liver fibrosis in non-alcoholic fatty liver disease. *Int J Obes.* (2017) 41:1207–13. doi: 10.1038/ijo.2017.102
170. van Herpen NA, Schrauwen-Hinderling VB. Lipid accumulation in non-adipose tissue and lipotoxicity. *Physiol Behav.* (2008) 94:231–41. doi: 10.1016/j.physbeh.2007.11.049
171. Ranjit S, Boutet E, Gandhi P, Prot M, Tamori Y, Chawla A, et al. Regulation of fat specific protein 27 by isoproterenol and TNF-alpha to control lipolysis in murine adipocytes. *J Lipid Res.* (2011) 52:221–36. doi: 10.1194/jlr.M008771
172. Sung CK, She H, Xiong S, Tsukamoto H. Tumor necrosis factor-alpha inhibits peroxisome proliferator-activated receptor gamma activity at a posttranslational level in hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol.* (2004) 286:G722–9. doi: 10.1152/ajpgi.00411.2003
173. Stephens JM, Lee J, Pilch PF. Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. *J Biol Chem.* (1997) 272:971–6. doi: 10.1074/jbc.272.2.971
174. Ruan H, Hacohen N, Golub TR, Van Parijs L, Lodish HF. Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. *Diabetes.* (2002) 51:1319–36. doi: 10.2337/diabetes.51.5.1319
175. Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol.* (2006) 6:772–83. doi: 10.1038/nri1937
176. Chan CC, Damen M, Alarcon PC, Sanchez-Gurmaches J, Divanovic S. Inflammation and immunity: from an adipocyte's perspective. *J Interferon Cytokine Res.* (2019) 39:459–71. doi: 10.1089/jir.2019.0014
177. Zhou YJ, Zhou H, Li Y, Song YL. NOD1 activation induces innate immune responses and insulin resistance in human adipocytes. *Diabetes Metab.* (2012) 38:538–43. doi: 10.1016/j.diabet.2012.08.001
178. Ballak DB, van Diepen JA, Moschen AR, Jansen HJ, Hijmans A, Groenhof GJ, et al. IL-37 protects against obesity-induced inflammation and insulin resistance. *Nat Commun.* (2014) 5:4711. doi: 10.1038/ncomms5711
179. Zhou YJ, Liu C, Li CL, Song YL, Tang YS, Zhou H, et al. Increased NOD1, but not NOD2, activity in subcutaneous adipose tissue from patients with metabolic syndrome. *Obesity.* (2015) 23:1394–400. doi: 10.1002/oby.21113
180. Kopp A, Buechler C, Neumeier M, Weigert J, Aslanidis C, Scholmerich J, et al. Innate immunity and adipocyte function: ligand-specific activation of multiple Toll-like receptors modulates cytokine, adipokine, and chemokine secretion in adipocytes. *Obesity.* (2009) 17:648–56. doi: 10.1038/oby.2008.607
181. Marra F, Svegliati-Baroni G. Lipotoxicity and the gut-liver axis in NASH pathogenesis. *J Hepatol.* (2018) 68:280–95. doi: 10.1016/j.jhep.2017.11.014
182. Frasca D, Diaz A, Romero M, Thaller S, Blomberg BB. Secretion of autoimmune antibodies in the human subcutaneous adipose tissue. *PLoS ONE.* (2018) 13:e0197472. doi: 10.1371/journal.pone.0197472
183. Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N, et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood.* (2000) 96:1723–32. doi: 10.1182/blood.V96.5.1723
184. Hui X, Gu P, Zhang J, Nie T, Pan Y, Wu D, et al. Adiponectin enhances cold-induced browning of subcutaneous adipose tissue via promoting M2 macrophage proliferation. *Cell Metab.* (2015) 22:279–90. doi: 10.1016/j.cmet.2015.06.004
185. Luo Y, Liu M. Adiponectin: a versatile player of innate immunity. *J Mol Cell Biol.* (2016) 8:120–8. doi: 10.1093/jmcb/mjw012
186. Lappas M, Yee K, Permezel M, Rice GE. Release and regulation of leptin, resistin and adiponectin from human placenta, fetal membranes, and maternal adipose tissue and skeletal muscle from normal and gestational diabetes mellitus-complicated pregnancies. *J Endocrinol.* (2005) 186:457–65. doi: 10.1677/joe.1.06227
187. Tsatsanis C, Zacharioudaki V, Androulidaki A, Dermitzaki E, Charalampopoulos I, Minas V, et al. Peripheral factors in the metabolic syndrome: the pivotal role of adiponectin. *Ann N Y Acad Sci.* (2006) 1083:185–95. doi: 10.1196/annals.1367.013
188. Sumida Y, Yoneda M. Current and future pharmacological therapies for NAFLD/NASH. *J Gastroenterol.* (2018) 53:362–76. doi: 10.1007/s00535-017-1415-1

189. Munteanu MA, Nagy GA, Mircea PA. Current Management of NAFLD. *Clujul Med.* (2016) 89:19–23. doi: 10.15386/cjmed-539
190. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. *Nat Med.* (2018) 24:908–22. doi: 10.1038/s41591-018-0104-9
191. Wang CH, Weisel RD, Liu PP, Fedak PW, Verma S. Glitazones and heart failure: critical appraisal for the clinician. *Circulation.* (2003) 107:1350–4. doi: 10.1161/01.CIR.0000054675.30348.9A
192. Nesto RW, Bell D, Bonow RO, Fonseca V, Grundy SM, Horton ES, et al. Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. *Diabetes Care.* (2004) 27:256–63. doi: 10.2337/diacare.27.1.256
193. Komiya C, Tsuchiya K, Shiba K, Miyachi Y, Furuke S, Shimazu N, et al. Ipragliflozin improves hepatic steatosis in obese mice and liver dysfunction in type 2 diabetic patients irrespective of body weight reduction. *PLoS ONE.* (2016) 11:e0151511. doi: 10.1371/journal.pone.0151511
194. Takase T, Nakamura A, Miyoshi H, Yamamoto C, Atsumi T. Amelioration of fatty liver index in patients with type 2 diabetes on ipragliflozin: an association with glucose-lowering effects. *Endocr J.* (2017) 64:363–7. doi: 10.1507/endocrj.EJ16-0295
195. Brenner C, Galluzzi L, Kepp O, Kroemer G. Decoding cell death signals in liver inflammation. *J Hepatol.* (2013) 59:583–94. doi: 10.1016/j.jhep.2013.03.033
196. Wang PX, Ji YX, Zhang XJ, Zhao LP, Yan ZZ, Zhang P, et al. Targeting CASP8 and FADD-like apoptosis regulator ameliorates nonalcoholic steatohepatitis in mice and nonhuman primates. *Nat Med.* (2017) 23:439–49. doi: 10.1038/nm.4290
197. Yamamoto E, Dong YF, Kataoka K, Yamashita T, Tokutomi Y, Matsuba S, et al. Olmesartan prevents cardiovascular injury and hepatic steatosis in obesity and diabetes, accompanied by apoptosis signal regulating kinase-1 inhibition. *Hypertension.* (2008) 52:573–80. doi: 10.1161/HYPERTENSIONAHA.108.112292
198. Farrell G, Schattenberg JM, Leclercq I, Yeh MM, Goldin R, Teoh N, et al. Mouse models of nonalcoholic steatohepatitis: toward optimization of their relevance to human nonalcoholic steatohepatitis. *Hepatology.* (2019) 69:2241–57. doi: 10.1002/hep.30333
199. Loomba R, Lawitz E, Mantry PS, Jayakumar S, Caldwell SH, Arnold H, et al. The ASK1 inhibitor selonsertib in patients with nonalcoholic steatohepatitis: a randomized, phase 2 trial. *Hepatology.* (2018) 67:549–59. doi: 10.1002/hep.29514
200. Giles DA, Moreno-Fernandez ME, Stankiewicz TE, Graspeuntner S, Cappelletti M, Wu D, et al. Thermoneutral housing exacerbates nonalcoholic fatty liver disease in mice and allows for sex-independent disease modeling. *Nat Med.* (2017) 23:829–38. doi: 10.1038/nm.4346
201. Yanguas SC, Cogliati B, Willebrords J, Maes M, Colle I, van den Bossche B, et al. Experimental models of liver fibrosis. *Arch Toxicol.* (2016) 90:1025–48. doi: 10.1007/s00204-015-1543-4

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REVIEW
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Interplay Between Macrophages and Angiogenesis: A Double-Edged Sword in Liver Disease

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During chronic liver disease, macrophages support angiogenesis, not only by secreting proangiogenic growth factors and matrix-remodeling proteases, but also by physically interacting with the sprouting vasculature to assist the formation of complex vascular networks. In the liver, macrophages acquire specific characteristics becoming Kupffer cells and working to ensure protection and immunotolerance. Angiogenesis is another double-edged sword in health and disease and it is the biggest ally of macrophages allowing its dissemination. Angiogenesis and fibrosis may occur in parallel in several tissues as macrophages co-localize with newly formed vessels and secrete cytokines, interleukins, and growth factors that will activate other cell types in the liver such as hepatic stellate cells and liver sinusoidal endothelial cells, promoting extracellular matrix accumulation and fibrogenesis. Vascular endothelial growth factor, placental growth factor, and platelet-derived growth factor are the leading secreted factors driving pathological angiogenesis and consequently increasing macrophage infiltration. Tumor development in the liver has been widely linked to macrophage-mediated chronic inflammation in which epidermal growth factors, STAT3 and NF-kβ are some of the most relevant signaling molecules involved. In this article, we review the link between macrophages and angiogenesis at molecular and cellular levels in chronic liver disease.

Keywords: angiogenesis, macrophages, Kupffer cells, hepatic stellate cells, vascular endothelial growth factor

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INTRODUCTION

The liver is both the largest organ of the body and the largest gland, weighing about 1.5 kg. It is situated in the abdominal cavity beneath the diaphragm. It carries out more than 500 essential roles having an impact in both physiology and disease (1). The major functions of the liver may be summarized as follows: Detoxification of metabolic waste products; destruction of spent red cells; and reclamation of their constituents (in conjunction with the spleen); synthesis and secretion of bile into the duodenum via the biliary system; synthesis of the plasma proteins including the clotting factors but excluding the immunoglobulins; synthesis of plasma lipoproteins; and metabolic functions, e.g., glycogen synthesis and gluconeogenesis. Many of these biosynthetic functions directly utilize the products of digestion. With the exception of most lipids (which are transported mainly by lymph vessels), absorbed food products pass directly in the venous blood from the small intestine to the liver via the portal vein before entering the general circulation. Thus, the vascular bed of the liver is perfused by blood rich in amino acids, simple sugars, and

other products of digestion but relatively poor in oxygen. Oxygen required to support the intense metabolic activity of the liver is supplied in the arterial blood via the hepatic artery. The liver, therefore, is unusual in that it has a dual blood supply that is both arterial (20%) and venous (80%). Venous drainage of the liver occurs *via* the hepatic vein and lymph from the liver is drained directly into the thoracic duct. The position of the liver in the circulatory system is therefore optimal for gathering, transforming, and accumulating metabolites and for neutralizing and eliminating toxic substances. This elimination occurs in the bile, an exocrine secretion of the liver that is important in lipid digestion.

The microanatomy of the liver is key for the achievement of the multifaceted hepatic abilities and homeostasis maintenance. The principal and most abundant cells of the liver, the hepatocytes, are arranged into polygonal lobules, the structure of which maximizes contact of hepatocytes with blood flowing through the liver. At the corners of the lobules, there are portal triads, each with a venule (a branch of the portal vein), an arteriole (a branch of the hepatic artery), and a duct (part of the bile duct system). The hepatocytes are radially disposed in the liver lobule. They form a layer of one or two cells thick, arranged like the bricks of a wall. The space between these cellular plates contains the liver sinusoids, composed solely of a discontinuous layer of fenestrated liver sinusoidal endothelial cells (LSECs) (2, 3). The sinusoids arise in the periphery of the lobule, fed by the terminal branches of portal veins and hepatic arterioles at the portal triads, and run in the direction of the hepatic central vein. The endothelial cells are separated from the underlying hepatocytes by a subendothelial space known as space of Disse, which contains microvilli of the hepatocytes. Blood fluids readily percolate through the endothelial wall and make intimate contact with the hepatocyte surface, permitting an easy exchange of macromolecules from the sinusoidal lumen to the liver cell and vice versa. This is physiologically important not only because of the large number of macromolecules (e.g., lipoproteins, albumin, fibrinogen) secreted into the blood by hepatocytes but also because the liver takes up and catabolizes many of these large molecules. In addition to the LSECs, the sinusoids contain phagocytic cells known as Kupffer cells (KCs) (3). The main functions of these hepatic macrophages are to metabolize aged erythrocytes and other particulate debris from the circulation, digest hemoglobin, and secrete proteins related to immunologic processes. The hepatic stellate cells (HSCs), located in the space of Disse, have the capacity to accumulate exogenously administered vitamin A as retinyl esters in lipid droplets (4, 5).

Liver disease comprises different disease stages and is mainly caused by obesity, alcohol consumption, diabetes, or viral infections (6). Non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD) only differ on the etiology; they are the first stages of disease and consist on the accumulation of triglycerides within hepatocytes. This excessive accumulation impairs hepatocyte functionality and promotes tissue inflammation driving toward non-alcoholic steatohepatitis (NASH) development (7). Activation of the immune component and other cellular types such as HSCs and LSECs promotes extracellular fiber deposition (collagen and other matrix constituents) and thus liver fibrosis that will progress toward the next stage of liver disease—cirrhosis—if inflammatory signals remain overexpressed. Hepatocellular carcinoma (HCC) can grow in livers affected by all the etiologies, but it is usually the last stage of disease after cirrhosis (8) (**Figure 1**).

MACROPHAGES IN THE LIVER

Macrophages are myeloid immune cells with the ability to phagocyte pathogens, dead cells, cellular debris, and various components of the extracellular matrix. Ilya Ilyich Mechnikov was the first to describe the process of phagocytosis in 1882, and macrophages were named after this feature as "big eaters" (from ancient Greek, makros "large" + phagein "eat"). However, it is now clear that macrophages are not only big eaters of pathogens and dead cells, but also important components of the stromal architecture of several tissues and organs, where they regulate organ homeostasis and remodeling. For instance, KCs are specialized macrophages that line hepatic sinusoids in the liver, where they scavenge senescent erythrocytes, a process referred to as hemocatheresis (9). During development and tissue healing or regeneration, macrophages stimulate angiogenesis, and facilitate tissue remodeling by secreting a number of proteases and growth factors (9).

The liver bears the biggest proportion of macrophages among all solid organs in the body. The full spectrum of immune cells in the liver is not yet totally clear, but populations of liver-resident macrophages such as KCs have been well-characterized. They present pattern recognition receptors (PRR) for the detection and degradation of microbial-associated molecular patterns (MAMP) and damage-associated molecular patterns (DAMP) (10). KCs in the liver may have different origins: yolk sac, bone marrow or hematopoietic stem cells derived from the ventral wall of the aorta in the aorta–gonad–mesonephros (AGM) region (11) (**Figure 2**). Erythromyeloid progenitors from the yolk sac express macrophage colony-stimulating factor 1 receptor (CSF1R) and allow differentiation into KC in the fetus during development (12). Bone marrow derived macrophages $CCR2^{+}$ (C-C chemokine receptor type 2) $LY6C^{+}$ (lymphocyte antigen 6 complex) can be recruited into the liver and achieve KC-like phenotype. Monocyte recruitment happens mostly after liver injury and under inflammatory conditions as a response to reestablish tissue homeostasis, and when they are excessive they undergo apoptosis (13). Close to week 5 of fetus gestation, hematopoietic stem cells derived from the AGM colonize the liver and give rise to mature erythroid, lymphoid and myeloid cells. Due to the similarities between yolk sac and AGM derived macrophages present in the liver, most studies consider them as the same (13).

Depending on the cell origin and the differentiation process, macrophages acquire different phenotypic (surface marker profile and gene expression) and functional features, which are variable between mice and humans. For instance, $CD14^{++}CD16^{-}$ classical human monocytes or

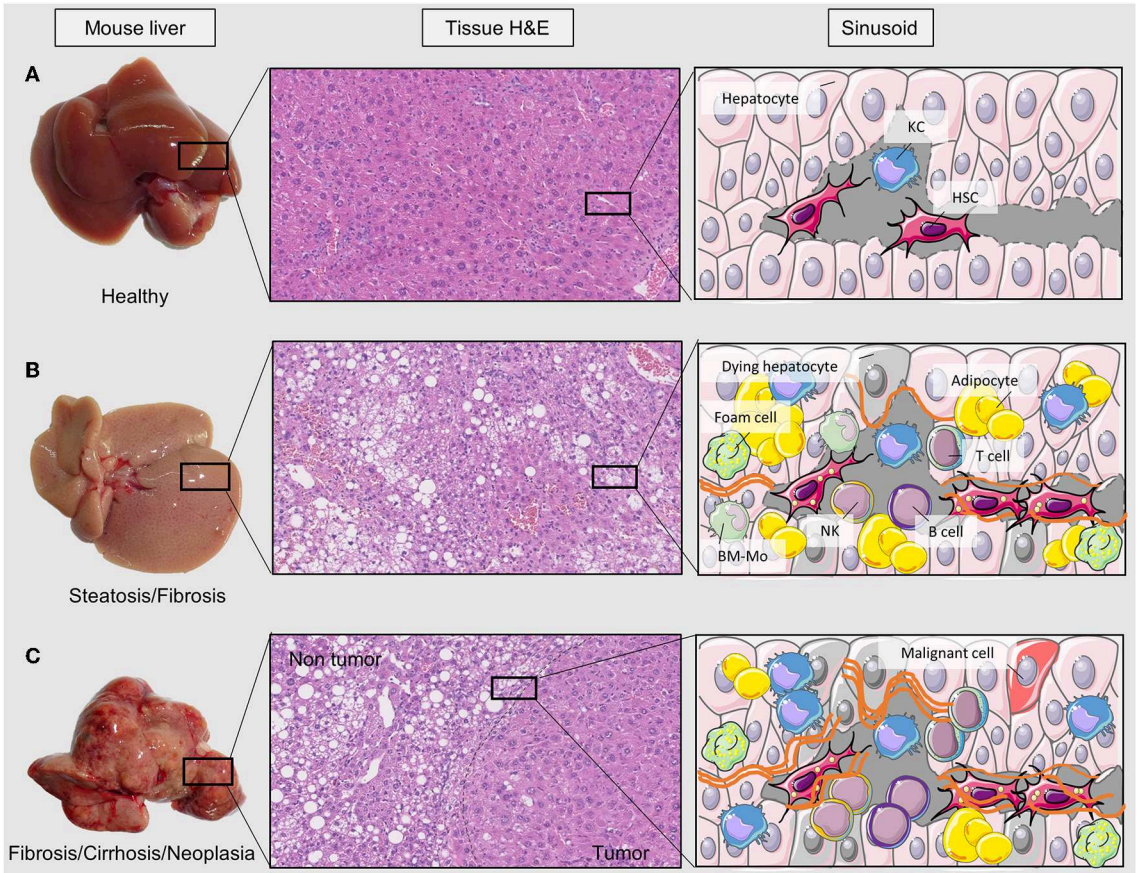

FIGURE 1 | Liver disease stages and tissue alterations. Changes in liver tissue can be detected macroscopically and microscopically in this figure. There are three sets of pictures; **(A)** healthy tissue, **(B)** fibrotic liver, and **(C)** tumorigenic. At the left side of the three sets there are pictures of livers extracted from animal models in our research group. In the next column there are hematoxylin and eosin staining pictures of those tissues where hepatocytes (pink) and adipocytes (round and white) can be seen. At the sinusoid column there is a scheme of tissue cell infiltration and hepatocyte transformation into dying hepatocytes or tumor cells. H&E, hematoxylin eosin staining; KC, Kupffer cell; HSC, hepatic stellate cell; T cell, lymphocyte T; NK, natural killer cell; B cell, lymphocyte B; BM-Mo, bone marrow derived macrophage.

intermediate $CD14^{++}CD16C^{+}$ monocytes correspond to $GR1^{+}/Ly6C^{high}$ inflammatory monocytes in the mouse and are $CCR2^{+}Cx3CR1^{low}$ (14). Cx3CR1, also called fractalkine receptor, is considered a key regulator of macrophage activity (15).

In addition to KCs, other subsets such as monocyte-derived macrophages, myeloid dendritic cells, scar-associated macrophages, inflammatory macrophages, restorative macrophages, tumor-associated macrophages, and monocytic myeloid-derived suppressor cells can be found in the liver. The acquisition of a pro-inflammatory or anti-inflammatory phenotype depends on the local tissue microenvironment (9, 10, 16). Knowing the full spectrum of macrophage activation, the underlying molecular mechanisms, and their implication in either promoting liver disease progression or repairing injured liver tissue is highly relevant from a therapeutic point of view. For instance, scar-associated macrophages positively contribute to fibrosis resolution by producing chemokine (C-X-C motif) ligand 9 and matrix metalloproteinase 13 (17). Other studies have shown the importance of specific receptors involved in macrophage activation such as integrin alphavbeta 3. This is a receptor for vitronectin, and its inhibition *in vivo* decreases angiogenesis and worsens liver fibrosis outstanding the complexity of therapeutic strategies required for patients with liver disease (18). It should also be taken into consideration that animal research, particularly that relating to phagocyte and immune networks, may be poor predictors of human pathophysiology (19).

PATHOPHYSIOLOGY OF LIVER ANGIOGENESIS

Angiogenesis is the process of new vasculature generation from pre-existing blood vessels and is present in health and disease. It is a tightly regulated process as excessive angiogenesis may prelude the establishment of abnormal vasculature and thus disease promotion (20, 21). Little is known about the mechanisms by which the endothelial cells present at the leading edge of vascular sprouts (named endothelial "tip" cells) integrate directional cues from the environment and fuse to

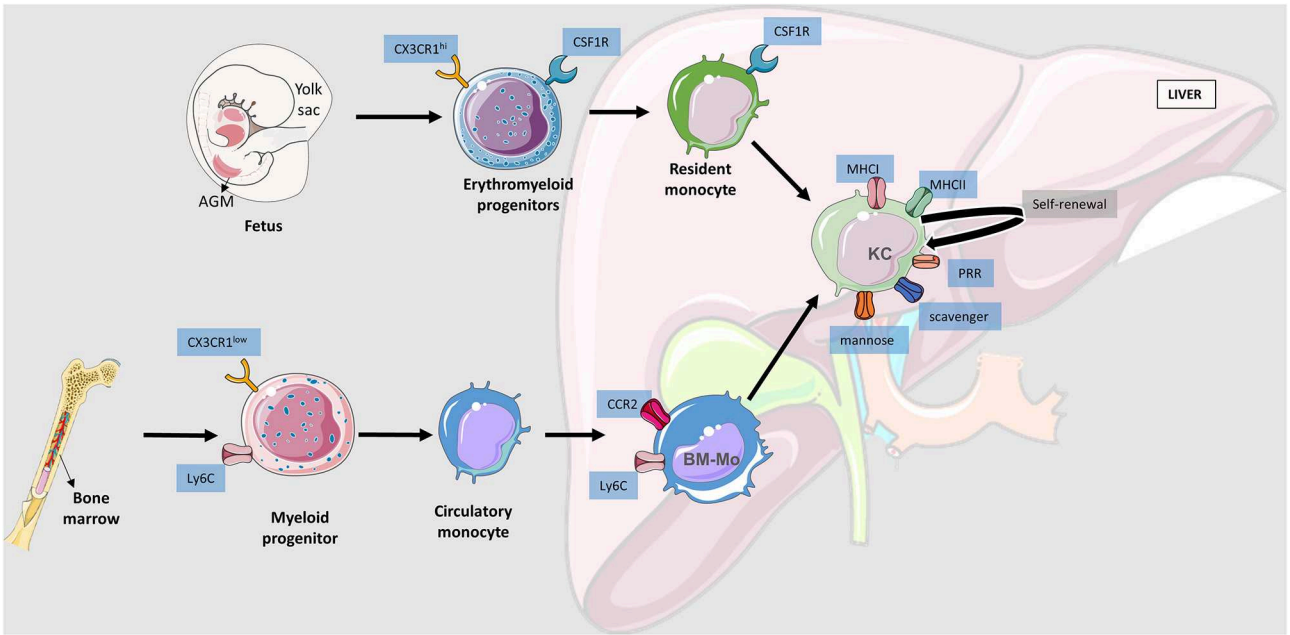

FIGURE 2 | Liver macrophages origin. Schematic representation of macrophages' movement toward the liver. There are three main niches where hematopoietic stem cells evolve and become myeloid progenitors: aorta–gonad–mesonephros (AGM), yolk sac (YS), and bone marrow. YS and AGM derived progenitors are generally considered the same due to their similarities. Erythromyeloid progenitors will go to the fetal liver and become resident monocytes that will evolve into Kupffer Cells (KC) establishing the liver resident macrophage population with self-renewal ability. Bone marrow produced myeloid progenitors that go to the systemic circulatory system and remain there until injury signals activate the cascades for tissue infiltration. Bone marrow derived macrophages (BM-Mo) require CCR2 (C-C chemokine receptor type 2) to be able to infiltrate into the liver and may undergo a conversion into a KC if necessary.

form new functional blood vessels. In the pathological setting of chronic liver disease, angiogenesis has been related to progressive liver inflammation, fibrogenesis, and tumorigenesis (1, 22, 23). Pathological angiogenesis in liver disease also occurs extrahepatically, playing a major role in the formation of porto-systemic collateral vessels and the development and aggravation of splanchnic hemodynamic disturbances and portal hypertension (1, 23). Angiogenesis is also an essential hallmark in liver cancer, allowing not only tumor nourishment and thus growth but also its dissemination toward other organs (23).

Vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and platelet derived growth factor (PDGF) are the leading secreted factors driving pathological angiogenesis in liver disease (1, 23). Increasing the supplying blood vessels of the liver can, in turn, further augment the recruitment of inflammatory cells, which will stimulate inflammation and activate profibrogenic myofibroblasts thereby resulting in fibrogenesis. In addition, the abnormally formed new vessels are very different from the highly specialized intrahepatic sinusoidal vessels. Thus, they are disorganized, chaotic, leaky vessels, which, instead of improving the effective perfusion of hepatocytes, they further compromise the oxygen and nutrient delivery to the liver parenchyma, resulting in further hepatocyte damage and hypoxia. This will exacerbate myofibroblast activation and fibrogenesis and will impair the effectiveness of inflammatory response. Moreover, proangiogenic factors can activate myofibroblasts not only through angiogenesis, but also by a direct activation of these cells, which express receptors for proangiogenic factors. Activated myofibroblasts are also able to produce proangiogenic factors, which further facilitate their own transdifferentiation and also mediate specialized cellular functions such as proliferation, chemotaxis, and production of extracellular matrix. Accordingly, inhibition of angiogenesis, for example, using the multikinase inhibitor sorafenib, causes a marked decrease in the intrahepatic neovascularization, fibrosis, and inflammation observed in animal models of cirrhosis (24).

MECHANISMS LINKING MACROPHAGES AND ANGIOGENESIS

Cellular

Macrophages likely represent the preeminent cells in the body endowed with the ability to migrate within tissues, even in hypoxic conditions, and with the capacity to modify the extracellular matrix and amplify paracrine signals. Macrophages may thus provide temporary scaffolds or paracrine support for the expansion and maturation of vascular networks, both in development and in pathophysiological conditions.

In liver disease, the main producers of pro-angiogenic factors are HSCs, portal fibroblasts, and myofibroblasts (25, 26). Activation of these cells toward angiogenesis promoters is usually stimulated by hypoxia inducible factor 1 (HIF-1α), but the presence of both infiltrated and resident macrophages

also contributes to generate the required vascular growth factor signals. Moreover, endothelial cells also participate in the establishment of a pro-angiogenic microenvironment by leptin secretion easing the stabilization of newly formed vessels in a context of advanced fibrosis where fibrotic septums are more mature (25).

Activated HSCs act, through the expression of VEGF receptor and VEGF family factors, in an autocrine and paracrine manner thus having an impact on LSEC and aggravating fibrosis. VEGFR2 (vascular endothelial growth factor receptor) modulates the pro-fibrogenic activity of HSC and LSECs and, subsequently, angiopoietin-1 activates Tie-2 tyrosine-protein kinase receptors (epidermal growth factor homology-2) on LSEC for vessel stability. The role of LSECs in the development of liver disease is tightly associated with VEGF secreted by hepatocytes and HSC as it determines their fenestrated phenotype that will also have an impact on HSC activation when it becomes abnormal (26).

Differently polarized macrophages have been characterized and associated with several processes involved in liver disease. Macrophages derived from bone marrow are $CCR2^{+}$ and $Ly\text{-}6C^{+}$ and require CCL2 to infiltrate into the liver (27). They play an essential role in angiogenesis regulation as ablation of CCL2 prevented angiogenesis associated with fibrosis, although it does not affect fibrosis development. Myofibroblasts are rather like macrophages as they also present heterogeneous populations in the liver which include HSC, derived cells like periportal fibroblasts, and epithelial-to-mesenchymal transition cells, all of them with different angiogenic capabilities (28).

Kupffer cells are constantly surveilling the liver, being responsible for the removal of damaged red cells from the blood circulation. This process is mediated by polyinosinic acid- and phosphatidylserine-sensitive scavenger receptors, different from scavenger receptor class A type I and II (29, 30). Kupffer cells require the recruitment of additional immune cells to carry out microbial and antigen total clearance. These other immune components are lymphocytes B (B cell) and T (T cell) as well as natural killers (NK) and other types of lymphocytes that are distributed all over the liver parenchyma (31).

Interestingly, the relationship between macrophages and vessels can be carried back to the hematopoietic processes taking place during fetal development and vascular patterning. Liver is one of the most important hematopoietic organs, and this fact becomes of maximum relevance in fetal liver when it becomes a niche for hematopoietic stem cells. Although the signaling pathways switching on maturation or migration programs are yet to be established, an intimate proximity has been set between those hematopoietic stem cells that will give rise to macrophages and pericytes within portal vessels (32).

Molecular

Interestingly, most of the molecules involved in angiogenesis are also involved in inflammation and the other way around. In fact, macrophages derived from the bone marrow are internalized into the liver parenchyma from the vasculature and mature, losing LY6C surface marker. Once there, they achieve the ability to degrade extracellular matrix secreting metalloproteinases (MMP) and become antifibrotic macrophages decreasing HSC activity (33). They can also produce VEGF-A and induce vessel restoration and phagocytosis of dead cells as well as extracellular matrix restoration after acute liver injury (34). Both resident and infiltrating macrophages possess profibrogenic abilities secreting transforming growth factor beta (TGF-β) and PDGF and activating HSCs and myofibroblasts (35). After injury, other cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) and chemokines (CCL-2, CCL5 and CXCL10) are usually secreted by macrophages exacerbating inflammation and boosting angiogenesis (36). Furthermore, KC and bone marrow-derived macrophages can be recruited by HSC secretion of adhesion molecules such as vascular cell adhesion molecular-1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), and E-selectin (37) (**Figure 3**). In addition to expressing classic proangiogenic and tissue-remodeling factors, which may initiate angiogenesis, macrophages appear to support the formation of a functional vascular system by (i) assisting directional vessel growth *via* cell-to-cell contacts and/or their production of guidance factors that act iuxtacrinally on vascular sprouts after the induction of endothelial cell proliferation and angiogenesis; (ii) pruning primitive blood vessels (via secretion of proapoptotic factors) to remodel the vascular network.

The difficulties in targeting angiogenesis reside in the ability of cells to activate compensatory pathways or even the physiological presence of redundant pathways. In this respect, several research groups have shown the relevance of PlGF, a member of the VEGF family involved in endothelial cell and bone marrow-derived cell activation, pathologic angiogenesis, and inflammation (38). PlGF is expressed by macrophages, ECs, and HSCs and specifically binds to VEGFR1. It is upregulated in fibrosis, cirrhosis, and hepatocellular carcinoma (39). Silencing PlGF in mice reduces tumor associated macrophage (TAM)-related chemokines and receptors (CXCL10, ICAM-1, VCAM, and CCR2), pro-inflammatory molecules (TNF-α, IL-1β, CCL2), and even anti-microbial receptors (TLR4 and TLR9), emphasizing the crucial role of this molecule and thus its potential as a therapeutic target (40). Another family of growth factors that may be involved in therapeutic failure is fibroblast growth factors (FGF) which are involved in angiogenesis and inflammation and essential for resolving liver regeneration (41).

IMPACT OF MACROPHAGES AND ANGIOGENESIS ON DISEASE PROGRESSION

Interestingly, liver macrophages derived from circulating monocytes, which invade the tissue after injury, colocalize with newly formed vessels. They present pro-angiogenic genetic profiles with overexpression of VEGF and MMP9 and are mostly found in portal tracts (27). These macrophages require CCL2, linking angiogenesis with inflammation. Indeed, inhibition of CCL2 reduced angiogenesis and monocyte infiltration at the beginning of fiber accumulation in the liver, indicating that there is a tight correlation between the extent of fibrosis and the recruited inflammatory components. These results indicate

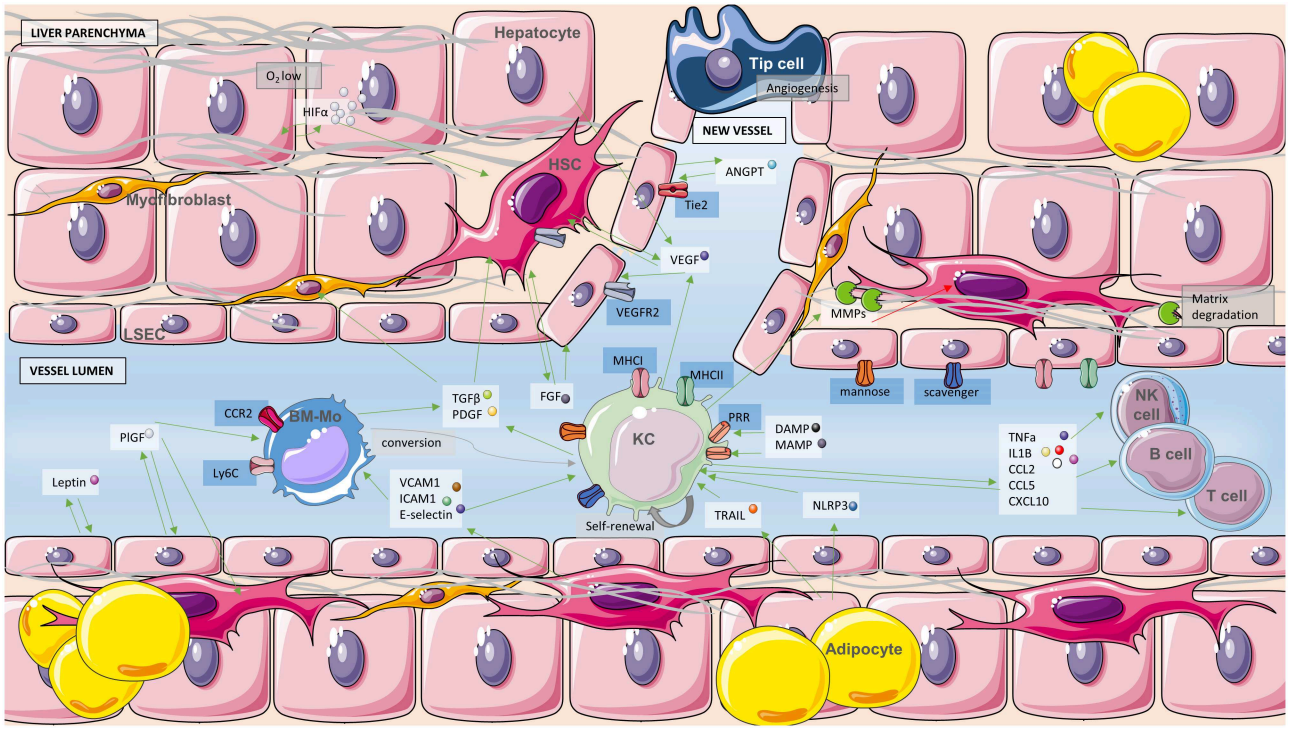

FIGURE 3 | Angiogenesis and macrophages. Schematic representation of the interplay between immune cells and the process of angiogenesis. Green arrows indicate activation of cells or secretion of soluble molecules and red arrows inhibition or inactivation. Soluble molecules are in white boxes, blue boxes refer to receptors and gray boxes to processes. Cell types present in the diagram consist of hepatocytes, myofibroblasts, hepatic stellate cells (HSC), liver sinusoidal cells (LSEC), adipocytes, Kupffer cells (KC), bone marrow-derived macrophages (BM-Mo), lymphocytes NK, T, and B as well as one tip cell leading to angiogenesis. Long gray structures are fibers of collagen. MMPs, metalloproteinases; ANGPT, angiopoietin; CCL2, C-C motif chemokine ligand 2; CCL5, C-C motif chemokine ligand 5; CXCL10, C-X-C motif chemokine ligand 10.

that even though angiogenesis is dependent on macrophage infiltration at the first disease stages, this does not attenuate fibrosis progression. However, when disease progresses toward chronic and even neoplasia development, angiogenesis becomes increasingly a cause for disease progression rather than a consequence (27, 42). The role of macrophages in disease development has been proven to be essential as its depletion causes disease development attenuation in both NAFLD and ALD in animal models (43).

Impact on Steatosis and Fibrosis

Chronic liver disease is characterized by inflammatory and fibrogenic processes that involve pathological angiogenesis, and depending on the origin and etiology of the fibrogenic process development, angiogenesis will have a variable impact on disease progression and reversibility (44). Angiogenesis usually occurs in parallel to fibrosis, and microvessel density has been correlated with the degree of accumulated fiber and vice versa. Even more, liver angiogenesis is different from the same process taking place in other organs or tissues and has intrinsic angiogenic factors such as ANGPTL3 (angiopoietin-like 3) (45). The convergence of increased tissue hypoxia due to fiber deposition and anatomical rearrangement of liver tissue together with wound healing processes that try to reestablish tissue homeostasis generates a characteristic environment full of metalloproteinase, growth factors (PDGF, TGF-1β, FGF, VEGF), cytokines, and adhesion molecules that will promote angiogenesis and fibrosis and settle the perfect conditions for disease chronicity (6).

In the presence of lipid accumulation in the liver and thus in the context of NAFLD, hepatocytes secrete vesicles containing TNF-related apoptosis inducing ligand (TRAIL) among other molecules, and they stimulate macrophages polarizing KC toward inflammation promoters. Conversely, they can switch and trigger apoptosis and autophagy of inflammatory cells ameliorating inflammation and hence fibrosis development (46). Experimental mice models suggest that during non-alcoholic steatohepatitis (NASH) the need for rapid lipid drop through metabolism and translocation gives rise to infiltrated macrophages in the first place instead of KC. Apparently, when injury stimulation remains constant and disease progresses toward chronic liver disease, then resident macrophages play the main role.

In this perspective a tight relationship has been established between macrophages, lipid metabolism, and hepatic steatosis progression. The link has been made through NOD-, LRR-, and pyrin domain-containing 3 (NLRP3) inflammasome (47) which activates pathways that allow an alternative polarization in macrophages. This specific polarization is related to the activation of steatogenic signaling in hepatocytes and

altogether assemble intricate and redundant pathways in which metabolism regulates macrophages and macrophages regulate metabolism (48).

In fibrosis, macrophages play a dual role as their presence increases scarring but at the same time is required for proper tissue repair. Infiltrating macrophages accumulate in the liver and, together with KC, secrete factors, such as TGFβ and PDGF that promote survival and activation of HSC thus acting as profibrogenic cells. This capability is achieved by the influence of NKT cells and other immune components together with factors present in the microenvironment (49).

Impact on Cirrhosis

When liver fibrosis remains constant and other conditions such as obesity, diabetes, malnutrition, and alcoholism contribute to fiber accumulation and chronic inflammation, then liver fibrosis becomes liver cirrhosis which is the next and more severe stage in terms of loss of liver functionality and usually preludes development of hepatocellular carcinoma (50). The presence of macrophages is increased in cirrhosis, similarly observed in liver fibrosis. However, in this advanced disease stage, the need for toxin clearance is dangerously elevated and the state of immunocompetency can move toward an immunodeficiency when cirrhosis is decompensated and gut permeability increases together with PAMP exposure, increasing the risk of mortality (51). The immune system is extremely activated and cytokines are overexpressed in cirrhosis. Distribution and functionality of monocytes are altered; they are mostly pro-inflammatory expressing $CD14^{+}CD16^{+}$, and their phagocytic activity is limited (52).

In cirrhosis, the liver becomes highly hypoxic and angiogenesis is stimulated to compensate the lack of oxygen and nutrients (53). Signals triggering the angiogenesis cascade include HIF-1α, which upregulates the expression of the angiogenic growth factor VEGF. Interestingly, HSCs acquire an angiogenic phenotype stimulated by platelet-derived growth factor (PDGF). This angiogenic phenotype is characterized by enhanced HSC-driven vascular tube formation *in vitro* and enhanced HSC coverage of sinusoids *in vivo* (54). HSC activation becomes excessive because of NO deficiency in cirrhotic livers and consequently, liver perfusion is compromised (55). Increased angiogenesis in portal areas, associated with enhanced inflammatory microenvironment, has been observed in patients with primary biliary cirrhosis (56).

Impact on Tumorigenesis

Hepatocellular carcinoma is the most common type of primary liver cancer and the third leading cause of death related to cancer in the world. Macrophages contribute to growth, angiogenesis, and metastasis in hepatocellular carcinoma (57, 58). Although macrophages resolutely contribute to tumor surveillance they generally become tumor associated macrophages (TAM), which are highly pro-inflammatory, and provide the necessary signals to create a pro-tumorigenic environment and to inhibit immune responses against it (59) (**Figure 4**). This malignant conversion occurs in both KC and infiltrated macrophages. In this context, not only pro-tumorigenic signals are favored by macrophages, but also pro-angiogenic factors such as VEGF, PDGF, TGFβ, and FGF, which allow tumor growth establishment and expansion. TAMs have the ability to be polarized toward pro-inflammatory or anti-inflammatory phenotypes. Essentially, interleukin 6 (IL6) and TGFβ promote tumor growth, IL6 together with TNFα facilitates invasion and metastasis, and TGFβ with IL10 suppresses the immune response against the tumor. But not only this, they even have the ability to activate T helpers type 2 (Th2) and thus recruitment and activation of regulatory T cells (Tregs) which are often involved in self-tolerance (58, 60).

Macrophages associated with hepatocellular carcinoma have a high expression of epidermal growth factor receptor (EGFR), which induces IL6 signaling pathway, and hepatocyte proliferation being thus considered a tumor-promoting factor. IL6 is usually expressed after injury as a response to IL1β derived from dying hepatocytes. However, EGFR-expressing macrophages have also been found in tissues surrounding tumors, indicating a higher level of cirrhosis and poor prognosis (61, 62). The NF-κB signaling pathway plays an important role in linking liver inflammation with cancer (63, 64). In response to pro-inflammatory signals such as TNF or IL1β, IκB kinases (IKK) that have NF-κB kidnapped in the cytoplasm are degraded leaving NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) free (64). It is mainly produced by hepatocytes but Kupffer cells can also induce its activation and it has been related to tumor progression (65). Another transcription factor involved in HCC development and bad prognosis is STAT3 (signal transducer and activator of transcription 3) (63). It is activated by inflammatory signals such as IL6 and epidermal growth factor (EGF) or reactive oxygen species (ROS), and it is expressed by macrophages to prevent chronic inflammation (63). However, once the disease is established, STAT-3 promotes oncogenesis through the Src oncogene (66). Both NF-κB and STAT3 interact to promote tumor growth by inducing activation of pathways related to angiogenesis, hypoxia, chemokines, and immunosuppression.

Angiogenesis is a process whereby new vessels sprout and branch from preexisting blood vessels. Mechanisms for physiological and tumor related angiogenesis are similar but the consequences are far from alike. While physiological angiogenesis allows a homeostatic balance, tumor derived angiogenesis offers tumor cells the ability to survive, propagate, and invade other tissues. In cancer, the new vasculature is structurally and functionally abnormal; blood vessels are immature and leaky (67, 68). Tumor angiogenesis is basically a four-step process: First, the basement membrane in tissues is injured; second, endothelial cells, activated by angiogenic factors, migrate; third, endothelial cells proliferate and stabilize; and four, angiogenic factors continue to influence the angiogenic process. Several studies indicate that the levels of angiogenic factors, mainly VEGF, reflect the aggressiveness of tumor cells and thus have a predictive value in the identification of the high-risk patients with poor prognosis. Angiogenic factors are also attractive therapeutic targets for hepatocellular carcinoma (69, 70). Hypoxia and nutrient

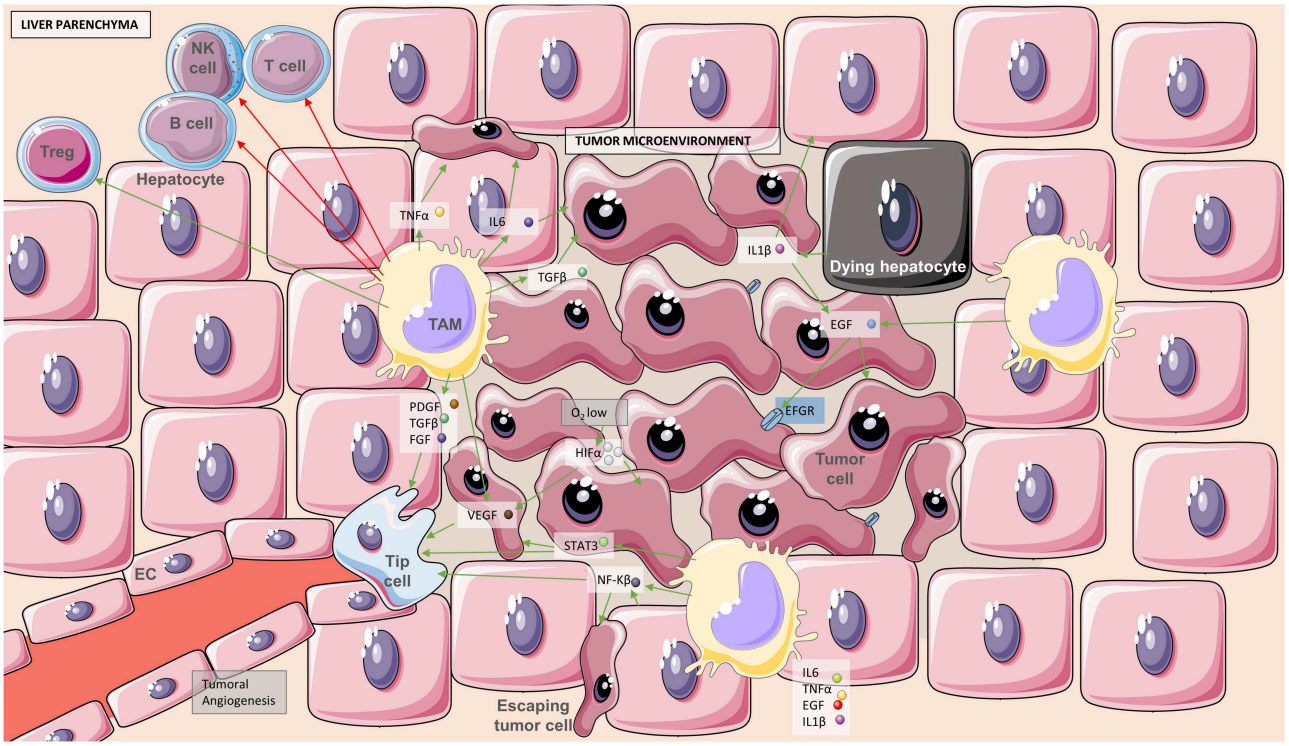

FIGURE 4 | Tumor microenvironment boosts angiogenesis and inflammation. Schematic representation showing the interplay between tumor cells and the processes of immune cell infiltration and angiogenesis. Hypoxic regions in the tumor microenvironment generate the adequate signaling cascades to activate angiogenesis and thus be able to supply tumor cells with the required nutrients. Green arrows indicate activation of cells or secretion of soluble molecules and red arrows inhibition or inactivation. Soluble molecules are in white boxes, blue boxes refer to receptors, and gray boxes to processes. Cellular components consist of hepatocytes, dying hepatocytes, tumor cells, escaping tumor cells, tumor associated macrophages (TAM), endothelial cells (EC), lymphocytes NK, T, and B as well as one tip cell leading to angiogenesis. IL, interleukin.

deprivation trigger the process of neovessel formation by inducing tumor cells to release soluble pro-angiogenic growth factors, chemokines, and cytokines (67, 68). In addition, the tumor microenvironment, including tumor-induced inflammatory responses, recruits multiple cell types and releases the stimulus required to support angiogenesis and allow tumor progression. These immune cells are an important source of matrix metalloproteinase to degrade ECM and promote cell invasion.

Tumor evasion against anti-angiogenic therapy might be propitiated by five mechanisms (71). First, tumor heterogeneity might lead to the co-existence of diverse angiogenic growth factors that can be positively selected in case of a treatment with a single pathway inhibitor. Second, due to therapy, a genetic switch and overexpression of other pro-angiogenic factors can occur. Even more, hypoxic regions can increase and thus upregulate angiogenic growth factors. Another factor to be considered is compensatory programs, which are very coordinated in response to homeostasis perturbation. An example of this is the upregulation of VEGF receptor-2 signaling with the silencing or lack of expression of β3 or β5 integrins; if integrins and VEGF promote maximal signal transduction downstream of the angiogenic pathways, disabling these interactions by impairing both components might therefore be subjected to less compensation or resistance (71).

Impact on Extrahepatic Complications Related to Liver Disease

The crosstalk between angiogenesis and macrophages also plays a role in liver disease-related complications taking place outside the liver such as in splanchnic organs, contributing to progression and aggravation of the pathology. Macrophages and angiogenesis contribute to fibrosis development *via* gut-liver axis activation as both Kupffer cells and HSCs become active through TLR receptors which are dependent on the presence of endotoxins derived from the gut (72). Metabolic disorders contribute to intestinal dysbiosis that leads to gut-vascular barrier leakage and foreign bodies entering the blood stream toward the liver. This process is called bacterial translocation and causes systemic inflammation, macrophages being essential players in both cause and resolution (73). In many cases, liver disease derives from obesity or other metabolic alterations. Fat accumulation in visceral fat depots, including the mesentery, induces a state of chronic inflammation that may reach the liver through the portal venous system, contributing to activate HSCs and increase

fibrogenesis (74). Pathological angiogenesis during chronic liver disease also takes place extrahepatically, playing a major role in the formation of portosystemic collaterals (1, 75–80) and the development and maintenance of splanchnic hyperdynamic circulation and portal hypertension (81–85).

TARGETING ANGIOGENESIS-INFLAMMATION CROSSTALK IN LIVER DISEASE

As mentioned above, inhibition of angiogenesis is at the same time beneficial and damaging, and therapies should ideally affect only pathological angiogenesis leaving the basal level required for wound healing, tissue repair, and other physiological functions of angiogenesis. Due to the relevance of VEGF as a key angiogenic regulator, it is one of the main targets. Approaches used to inhibit angiogenesis in chronic liver disease and portal hypertension include neutralizing monoclonal antibodies (78), tyrosin kinase inhibitors (24, 83–85), or therapeutic small interference RNAs targeting VEGF receptor-2 (76). Attenuation of oxidative stress and inflammation has also been shown to reduce pathological angiogenesis in liver disease and portal hypertension (86–88). Promising results with anti-VEGF therapy have also been demonstrated in hepatocellular carcinoma (69, 89). Another interesting strategy to inhibit pathological angiogenesis is by therapeutically increasing the expression of angioinhibitors that are endogenously present in the body, such as pigment epithelium derived factor (PEDF) or vasohibin (90, 91). A prominent advantage of using these natural inhibitors is that they would not be expected to activate drug resistant genes and thus may offer a promising breakthrough for effective antiangiogenesis therapy. Recent studies also highlight the functional significance of pathologic neovascularization derived from vascular stem/progenitor cells as an important mechanism of formation of new blood vessels in adults, in the setting of chronic liver disease, and identify these stem cells as potential new therapeutic targets (92). In a search for ways to inhibit pathologic production or activities of VEGF without affecting its normal production or functions, our research group has investigated the post-transcriptional regulation of VEGF by the cytoplasmic polyadenylation element-binding proteins CPEB1 and CPEB4 during development of liver disease (93, 94). We have identified a mechanism of VEGF overexpression in the liver and mesentery that promotes pathologic, but not physiologic, angiogenesis, via sequential and non-redundant functions of CPEB1 and CPEB4. Activation of CPEB1 promotes alternative nuclear processing within non-coding 3′-untranslated regions of VEGF and CPEB4 mRNAs, resulting in deletion of translation repressor elements. The subsequent overexpression of CPEB4 promotes cytoplasmic polyadenylation of VEGF mRNA, increasing its translation and generating high levels of VEGF protein, which induces pathologic angiogenesis in chronic liver disease. From a translational point of view, our studies highlight that CPEBs could be promising angiogenesis-disrupting targets in disease. Thus, targeting CPEBs could lead to safer treatment outcomes by specifically reducing excessive pathological VEGF production instead of indiscriminately perturbing both pathological and physiological VEGF synthesis, minimizing potential adverse side-effects. Reduction of pathological angiogenesis in early disease stages could also prevent further disease progression and reduce the risk for developing overt liver cirrhosis. Accordingly, development and evaluation of CPEB inhibitors are currently underway. As better and more specific inhibitors of pathologic angiogenesis are developed, combination strategies continue to evolve, and increased understanding of the complex biology of angiogenesis takes place, antiangiogenic therapy will certainly be evaluated in future clinical trials.

CONCLUSION

The interplay between macrophages and angiogenesis determines the progression of a big number of diseases but, in the liver, this is especially important due to the particularities of the processes of vasculogenesis and inflammation that are taking place. Liver vasculature and microvasculature are essential not only for tissue reoxygenation but also for it to work as the main filter between toxins and the rest of the body, and to accomplish that function the immune system has to maintain its ability to switch from immuno-tolerant to responsive constantly. Essentially, both processes require the presence of the other and when cellular stress remains for a period of time and homeostasis is lost, they boost each other through the detection and secretion of common signaling molecules. For that, it is mandatory to go further in research to decipher more mechanisms that would allow to therapeutically target pathologic levels of both processes without inhibiting immune surveillance and tissue regeneration capabilities. Currently, although there are some drugs approved for all stages of liver disease, there is always the risk of treatment failure due to redundant mechanisms, which claims the need for the detection of specific targets or pathways to avoid the "double-sword" effect.

AUTHOR CONTRIBUTIONS

MR-P: drafting of the manuscript and preparation of figures. MF: review concept, design, and supervision, drafting of the manuscript, and funding.

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REFERENCES

1. Fernandez M. Molecular pathophysiology of portal hypertension. *Hepatology*. (2015) 61:1406–15. doi: 10.1002/hep.27343
2. DeLeve LD. Liver sinusoidal endothelial cells in hepatic fibrosis. *Hepatology*. (2015) 61:1740–6. doi: 10.1002/hep.27376
3. Smedsrød B, De Bleser PJ, Braet F, Lovisetti P, Vanderkerken K, Wisse E, et al. Cell biology of liver endothelial and Kupffer cells. *Gut*. (1994) 35:1509–16. doi: 10.1136/gut.35.11.1509
4. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev*. (2008) 88:125–72. doi: 10.1152/physrev.00013.2007
5. Iwakiri Y, Shah V, Rockey DC. Vascular pathobiology in chronic liver disease and cirrhosis–current status and future directions. *J Hepatol*. (2014) 61:912–24. doi: 10.1016/j.jhep.2014.05.047
6. Friedman SL. Mechanisms of disease: mechanisms of hepatic fibrosis and therapeutic implications. *Nat Clin Pract Gastroenterol Hepatol*. (2004) 1:98–105. doi: 10.1038/ncpgasthep0055
7. Michelotti GA, Machado MV, Diehl AM. NAFLD, NASH and liver cancer. *Nat Rev Gastroenterol Hepatol*. (2013) 10:656–65. doi: 10.1038/nrgastro.2013.183
8. Sun B, Karin M. Obesity, inflammation, and liver cancer. *J Hepatol*. (2012) 56:704–13. doi: 10.1016/j.jhep.2011.09.020
9. Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol*. (2017) 17:306–21. doi: 10.1038/nri.2017.11
10. Bordon Y. (2019) Kupffer cells: finding their niche. *Nat Rev Immunol*. 19:660–1. doi: 10.1038/s41577-019-0231-7
11. De Kleer I, Willems F, Lambrecht B, Goriely S. Ontogeny of myeloid cells. *Front Immunol*. (2014) 5:1–11. doi: 10.3389/fimmu.2014.00423
12. Perdiguero EG, Klapproth K, Schulz C, Busch K, Crozet L, Garner H, et al. Tissue-resident macrophages originate from yolk sac-derived erythro-myeloid progenitors. *Immunity*. (2015) 43:1023–4. doi: 10.1016/j.immuni.2015.11.022
13. Guillot A, Tacke F. Liver macrophages: old dogmas and new insights. *Hepatol Commun*. (2019) 3:730–43. doi: 10.1002/hep4.1356
14. Donnelly DJ, Longbrake EE, Shawler TM, Kigerl KA, Lai W, Amy Tovar C, et al. Deficient CX3CR1 signaling promotes recovery after mouse spinal cord injury by limiting the recruitment and activation of Ly6Clo/iNOS+ macrophages. *J Neurosci*. (2011) 31:9910–22. doi: 10.1523/JNEUROSCI.2114-11.2011
15. Mass E, Ballesteros I, Farlik M, Halbritter F, Günther P, Crozet L, et al. Specification of tissue-resident macrophages during organogenesis. *Science*. (2016) 353:aaf4238. doi: 10.1126/science.aaf4238
16. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*. (2014) 41:14–20. doi: 10.1016/j.immuni.2014.06.008
17. Yang L, Kwon J, Popov Y, Gajdos GB, Ordog T, Brekken RA, et al. Vascular endothelial growth factor promotes fibrosis resolution and repair in mice. *Gastroenterology*. (2014) 146:1339–50. doi: 10.1053/j.gastro.2014.01.061
18. Patsenker E, Popov Y, Stickel F, Schneider V, Ledermann M, Hans S, et al. Pharmacological inhibition of integrin alphavbeta3 aggravates experimental liver fibrosis and suppresses hepatic angiogenesis. *Hepatology*. (2009) 50:1501–11. doi: 10.1002/hep.23144
19. Reynolds G, Haniffa M. Human and mouse mononuclear phagocyte networks: a tale of two species? *Front Immunol*. (2015) 6:1–15. doi: 10.3389/fimmu.2015.00330
20. Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. *Cell*. (2011) 146:873–87. doi: 10.1016/j.cell.2011.08.039
21. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature*. (2011) 473:298–307. doi: 10.1038/nature10144
22. De Palma M, Biziato D, Petrova TV. Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer*. (2017) 17:457–74. doi: 10.1038/nrc.2017.51
23. Fernandez M, Semela D, Bruix J, Colle I, Pinzani M, Bosch J. Angiogenesis in liver disease. *J Hepatol*. (2009) 50:604–20. doi: 10.1016/j.jhep.2008.12.011
24. Mejias M, Garcia-Pras E, Tiani C, Miquel R, Bosch J, Fernandez M. Beneficial effects of sorafenib on splanchnic, intrahepatic, and portocollateral circulations in portal hypertensive and cirrhotic rats. *Hepatology*. (2009) 49:1245–56. doi: 10.1002/hep.22758
25. Aleffi S, Petrai I, Bertolani C, Parola M, Colombatto S, Novo E, et al. Upregulation of proinflammatory and proangiogenic cytokines by leptin in human hepatic stellate cells. *Hepatology*. (2005) 42:1339–48. doi: 10.1002/hep.20965
26. June SL, Semela D, Iredale J, Shah VH. Sinusoidal remodeling and angiogenesis: a new function for the liver-specific pericyte? *Hepatology*. (2007) 45:817–25. doi: 10.1002/hep.21564
27. Ehling J, Gremse F, Möckel D, Kiessling F, Lammers T, Bartneck M, et al. CCl2-dependent infiltrating macrophages promote angiogenesis in progressive liver fibrosis. *Gut*. (2014) 63:1960–71. doi: 10.1136/gutjnl-2013-306294
28. Kisseleva T. The origin of fibrogenic myofibroblasts in fibrotic liver. *Hepatology*. (2017) 65:1039–43. doi: 10.1002/hep.28948
29. Willekens FLA, Werre JM, Kruijt JK, Roerdinkholder-Stoelwinder B, Groenen-Döpp YAM, Van Den Bos AG, et al. Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors. *Blood*. (2005) 105:2141–5. doi: 10.1182/blood-2004-04-1578
30. Terpstra V, van Berkel TJ. Scavenger receptors on liver Kupffer cells mediate the *in vivo* uptake of oxidatively damaged red blood cells in mice. *Blood*. (2000) 95:2157–63. doi: 10.1182/blood.V95.6.2157
31. Albillos A, Lario M, Álvarez-Mon M. Cirrhosis-associated immune dysfunction: distinctive features and clinical relevance. *J Hepatol*. (2014) 61:1385–96. doi: 10.1016/j.jhep.2014.08.010
32. Hoeffel G, Ginhoux F. Fetal monocytes and the origins of tissue-resident macrophages. *Cell Immunol*. (2018) 330:5–15. doi: 10.1016/j.cellimm.2018.01.001
33. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci USA*. (2012) 109:E3186–95. doi: 10.1073/pnas.1119964109
34. Zigmond E, Samia-Grinberg S, Pasmanik-Chor M, Brazowski E, Shibolet O, Halpern Z, et al. Infiltrating monocyte-derived macrophages and resident Kupffer cells display different ontogeny and functions in acute liver injury. *J Immunol*. (2014) 193:344–53. doi: 10.4049/jimmunol.1400574
35. Vannella KM, Wynn TA. Mechanisms of organ injury and repair by macrophages. *Ann Rev Physiol*. (2017) 79:593–617. doi: 10.1146/annurev-physiol-022516-034356
36. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity*. (2016) 44:450–62. doi: 10.1016/j.immuni.2016.02.015
37. Koyama Y, Brenner DA, Koyama Y, Brenner DA. Liver inflammation and fibrosis. *J Clin Invest*. (2017) 127:55–64. doi: 10.1172/JCI88881
38. Van Steenkiste C, Ribera J, Geerts A, Pauta M, Tugues S, Casteleyn C, et al. Inhibition of placental growth factor activity reduces the severity of fibrosis, inflammation, and portal hypertension in cirrhotic mice. *Hepatology*. (2011) 53:1629–40. doi: 10.1002/hep.24238
39. Li X, Yao QY, Liu HC, Jin QW, Xu BL, Zhang SC, et al. Placental growth factor silencing ameliorates liver fibrosis and angiogenesis and inhibits activation of hepatic stellate cells in a murine model of chronic liver disease. *J Cell Mol Med*. (2017) 21:2370–85. doi: 10.1111/jcmm.13158
40. Li X, Jin Q, Yao Q, Zhou Y, Zou Y, Li Z, et al. Placental growth factor contributes to liver inflammation, angiogenesis, fibrosis in mice by promoting hepatic macrophage recruitment and activation. *Front Immunol*. (2017) 8:801. doi: 10.3389/fimmu.2017.00801
41. Schumacher JD, Guo GL. Regulation of hepatic stellate cells and fibrogenesis by fibroblast growth factors. *Biomed Res Int*. (2016) 2016:8323747. doi: 10.1155/2016/8323747
42. Matsubara T, Kanto T, Kuroda S, Yoshio S, Higashitani K, Kakita N, et al. TIE2-expressing monocytes as a diagnostic marker for hepatocellular carcinoma correlates with angiogenesis. *Hepatology*. (2013) 57:1416–25. doi: 10.1002/hep.25965
43. Reid DT, Reyes JL, McDonald BA, Vo T, Reimer RA, Eksteen B. Kupffer cells undergo fundamental changes during the development of experimental NASH and are critical in initiating liver damage and inflammation. *PLoS ONE*. (2016) 11:1–18. doi: 10.1371/journal.pone.0159524
44. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature*. (2000) 470:249–57. doi: 10.1038/35025220
45. Camenisch G, Pisabarro MT, Sherman D, Kowalski J, Nagel M, Hass P, et al. ANGPTL3 stimulates endothelial cell adhesion and migration via integrin

αvβ3 and induces blood vessel formation *in vivo*. *J Biol Chem*. (2002) 277:17281–90. doi: 10.1074/jbc.M109768200
46. Heymann F, Peusquens J, Ludwig-Portugall I, Kohlhepp M, Ergen C, Niemietz P, et al. Liver inflammation abrogates immunological tolerance induced by Kupffer cells. *Hepatology*. (2015) 62:279–91. doi: 10.1002/hep.27793
47. Martinez-Micaelo N, Gonzalez-Abuin N, Pinent M, Ardevol A, Blay M. Dietary fatty acid composition is sensed by the NLRP3 inflammasome: Omega-3 fatty acid (DHA) prevents NLRP3 activation in human macrophages. *Food Funct*. (2016) 7:3480–7. doi: 10.1039/C6FO00477F
48. Huang SCC, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, et al. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat Immunol*. (2014) 15:846–55. doi: 10.1038/ni.2956
49. Pradere JP, Kluwe J, De Minicis S, Jiao JJ, Gwak GY, Dapito DH, et al. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology*. (2013) 58:1461–73. doi: 10.1002/hep.26429
50. Yang JD, Hainaut P, Gores GJ, Amadou A, Plymoth A, Roberts LR. A global view of hepatocellular carcinoma: trends, risk, prevention and management. *Nat Rev Gastroenterol Hepatol*. (2019) 16:589–604. doi: 10.1038/s41575-019-0186-y
51. Riva A, Mehta G. Regulation of monocyte-macrophage responses in cirrhosis—role of innate immune programming and checkpoint receptors. *Front Immunol*. (2019) 10:1–7. doi: 10.3389/fimmu.2019.00167
52. Zimmermann HW, Seidler S, Nattermann J, Gassler N, Hellerbrand C, Zernecke A, et al. Functional contribution of elevated circulating and hepatic non-classical CD14+CD16+ monocytes to inflammation and human liver fibrosis. *PLoS ONE*. (2010) 5:e11049. doi: 10.1371/journal.pone.0011049
53. Lee S, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, et al. Autocrine VEGF signaling is required for vascular homeostasis. *Cell*. (2007) 130:691–703. doi: 10.1016/j.cell.2007.06.054
54. Semela D, Das A, Langer D, Kang N, Leof E, Shah V. PDGF signaling through ephrin-B2 regulates hepatic vascular structure and function. *Gastroenterology*. (2009) 135:671–9. doi: 10.1053/j.gastro.2008.04.010
55. DeLeve LD, Wang X, Guo Y. Sinusoidal endothelial cells prevent rat stellate cell activation and promote reversion to quiescence. *Hepatology*. (2008) 48:920–30. doi: 10.1002/hep.22351
56. Medina J, Arroyo AG, Sanchez-Madrid F, Moreno-Otero R. Angiogenesis in chronic inflammatory liver disease. *Hepatology*. (2004) 39:1185–95. doi: 10.1002/hep.20193
57. Shigeta K, Datta M, Hato T, Kitahara S, Chen IX, Matsui A, et al. Dual programmed death receptor-1 and vascular endothelial growth factor receptor-2 blockade promotes vascular normalization and enhances antitumor immune responses in hepatocellular carcinoma. *Hepatology*. (2019). doi: 10.1002/hep.30889. [Epub ahead of print].
58. Zhang Q, He Y, Luo N, Patel SJ, Han Y, Gao R, et al. Landscape and dynamics of single immune cells in hepatocellular carcinoma. *Cell*. (2019) 179:829–45. doi: 10.1016/j.cell.2019.10.003
59. Meenakshi Sachdeva, Yogesh K Chawla SKA. Immunology of hepatocellular carcinoma. *World J Gastroenterol*. (2015) 7:2080–90. doi: 10.4254/wjh.v7.i17.2080
60. Rudensky AY. Regulatory T cells and Foxp3. *Immunol Rev*. (2011) 241:260–8. doi: 10.1111/j.1600-065X.2011.01018.x
61. Lanaya H, Natarajan A, Komposch K, Li L, Amberg N, Chen L, et al. EGFR has a tumour-promoting role in liver macrophages during hepatocellular carcinoma formation. *Nat Cell Biol*. (2014) 16:972–81. doi: 10.1038/ncb3031
62. Whittaker S, Marais R, Zhu AX. The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene*. (2010) 29:4989–5005. doi: 10.1038/onc.2010.236
63. He G, Karin M. NF-κB and STAT3- key players in liver inflammation and cancer. *Cell Res*. (2011) 21:159–68. doi: 10.1038/cr.2010.183
64. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell*. (2002) 109 (Suppl):S81–96. doi: 10.1016/S0092-8674(02)00703-1
65. Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKβ couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell*. (2005) 121:977–90. doi: 10.1016/j.cell.2005.04.014
66. Gough DJ, Corlett A, Schlessinger K, Wegrzyn J, Larner AC, Levy DE. Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science*. (2009) 324:1713–6. doi: 10.1126/science.1171721
67. Saharinen P, Eklund L, Pulkki K, Bono P, Alitalo K. VEGF and angiopoietin signaling in tumor angiogenesis and metastasis. *Trends Mol Med*. (2011) 17:347–62. doi: 10.1016/j.molmed.2011.01.015
68. Weis SM, Cheresh D. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med*. (2011) 17:1359–70. doi: 10.1038/nm.2537
69. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med*. (2008) 359:378–90. doi: 10.1056/NEJMoa0708857
70. Teufel M, Seidel H, Köchert K, Meinhardt G, Finn RS, Llovet JM, et al. Biomarkers associated with response to regorafenib in patients with hepatocellular carcinoma. *Gastroenterology*. (2019) 156:1731–41. doi: 10.1053/j.gastro.2019.01.261
71. Sennino B, McDonald DM. Controlling escape from angiogenesis inhibitors. *Nat Rev Cancer*. (2012) 12:699–709. doi: 10.1038/nrc3366
72. Dapito DH, Mencin A, Gwak GY, Pradere JP, Jang MK, Mederacke I, et al. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. *Cancer Cell*. (2012) 21:504–16. doi: 10.1016/j.ccr.2012.02.007
73. Grainger JR, Konkel JE, Zangerle-Murray T, Shaw TN. Macrophages in gastrointestinal homeostasis and inflammation. *Pflugers Arch Eur J Physiol*. (2017) 469:527–39. doi: 10.1007/s00424-017-1958-2
74. Marchesini G, Moscatiello S, Di Domizio S, Forlani G. Obesity-associated liver disease. *J Clin Endocrinol Metab*. (2008) 93:74–80. doi: 10.1210/jc.2008-1399
75. Van Steenkiste C, Geerts A, Vanheule E, Van Vlierberghe H, De Vos F, Olievier K, et al. Role of placental growth factor in mesenteric neoangiogenesis in a mouse model of portal hypertension. *Gastroenterology*. (2009) 137:2112–24. doi: 10.1053/j.gastro.2009.08.068
76. Gallego J, Garcia-Pras E, Mejias M, Pell N, Schaeper U, Fernandez M. Therapeutic siRNA targeting endothelial KDR decreases portosystemic collateralization in portal hypertension. *Sci Rep*. (2017) 7:14791–806. doi: 10.1038/s41598-017-14818-7
77. Fernandez M, Abraldes JG. Gastroesophageal varices. In: Muriel P, editors. *Liver Pathophysiology: Therapies & Antioxidants*. Academic Press; Elsevier (2017). p. 914.
78. Fernandez M, Vizzutti F, Garcia-Pagan JC, Rodes J, Bosch J. Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice. *Gastroenterology*. (2004) 126:886–94. doi: 10.1053/j.gastro.2003.12.012
79. Tiani C, Garcia-Pras E, Mejias M, de Gottardi A, Berzigotti A, Bosch J, et al. Apelin signaling modulates splanchnic angiogenesis and portosystemic collateral vessel formation in rats with portal hypertension. *J Hepatol*. (2009) 50:296–305. doi: 10.1016/j.jhep.2008.09.019
80. Schwabl P, Payer BA, Grahovac J, Klein S, Horvatits T, Mitterhauser M, et al. Pioglitazone decreases portosystemic shunting by modulating inflammation and angiogenesis in cirrhotic and non-cirrhotic portal hypertensive rats. *J Hepatol*. (2014) 60:1135–42. doi: 10.1016/j.jhep.2014.01.025
81. Fernandez M, Mejias M, Angermayr B, Garcia-Pagan JC, Rodes J, Bosch J. Inhibition of VEGF receptor-2 decreases the development of hyperdynamic splanchnic circulation and portal-systemic collateral vessels in portal hypertensive rats. *J Hepatol*. (2005) 43:98–103. doi: 10.1016/j.jhep.2005.02.022
82. Fernandez M, Mejias M, Garcia-Pras E, Mendez R, Garcia-Pagan JC, Bosch J. Reversal of portal hypertension and hyperdynamic splanchnic circulation by combined vascular endothelial growth factor and platelet-derived growth factor blockade in rats. *Hepatology*. (2007) 46:1208–17. doi: 10.1002/hep.21785
83. Tugues S, Fernandez-Varo G, Muñoz-Luque J, Ros J, Arroyo V, Rodes J, et al. Antiangiogenic treatment with sunitinib ameliorates inflammatory infiltrate, fibrosis, and portal pressure in cirrhotic rats. *Hepatology*. (2007) 46:1919–26. doi: 10.1002/hep.21921
84. Uschner FE, Schueller F, Nikolova I, Klein S, Schierwagen R, Magdaleno F, et al. The multikinase inhibitor regorafenib decreases angiogenesis and improves portal hypertension. *Oncotarget*. (2018) 9:36220–37. doi: 10.18632/oncotarget.26333
85. Reiberger T, Angermayr B, Schwabl P, Rohr-Udilova N, Mitterhauser M, Gangl A, et al. Sorafenib attenuates the portal hypertensive syndrome in partial portal vein ligated rats. *J Hepatol*. (2009) 51:865–73. doi: 10.1016/j.jhep.2009.06.024
86. Angermayr B, Fernandez M, Mejias M, Gracia-Sancho J, Garcia-Pagan JC, Bosch J. NAD(P)H oxidase modulates angiogenesis and the development of

portosystemic collaterals and splanchnic hyperaemia in portal hypertensive rats. *Gut*. (2007) 56:560–4. doi: 10.1136/gut.2005.088013
87. Angermayr B, Mejias M, Gracia-Sancho J, Garcia-Pagan JC, Bosch J, Fernandez M. Heme oxygenase attenuates oxidative stress and inflammation, and increases VEGF expression in portal hypertensive rats. *J Hepatol*. (2006) 44:1033–9. doi: 10.1016/j.jhep.2005.09.021
88. Lai WK, Adams DH. Angiogenesis and chronic inflammation; the potential for novel therapeutic approaches in chronic liver disease. *J Hepatol*. (2005) 42:7–11. doi: 10.1016/j.jhep.2004.11.008
89. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer*. (2008) 8:579–91. doi: 10.1038/nrc2403
90. Mejias M, Coch L, Berzigotti A, Garcia-Pras E, Gallego J, Bosch J, et al. Antiangiogenic and antifibrogenic activity of pigment epithelium-derived factor (PEDF) in bile duct-ligated portal hypertensive rats. *Gut*. (2015) 64:657–66. doi: 10.1136/gutjnl-2014-307138
91. Coch L, Mejias M, Berzigotti A, Garcia-Pras E, Gallego J, Bosch J, et al. Disruption of negative feedback loop between vasohibin-1 and vascular endothelial growth factor decreases portal pressure, angiogenesis, and fibrosis in cirrhotic rats. *Hepatology*. (2014) 60:633–47. doi: 10.1002/hep.26995
92. Garcia-Pras E, Gallego J, Coch L, Mejias M, Fernandez-Miranda G, Pardal R, et al. Role andtherapeutic potential of vascular stem/progenitor cells in pathological neovascularisation during chronic portal hypertension. *Gut*. (2017) 66:1306–20. doi: 10.1136/gutjnl-2015-311157
93. Calderone V, Gallego J, Fernandez-Miranda G, Garcia-Pras E, Maillo C, Berzigotti A, et al. Sequential functions of CPEB1 and CPEB4 regulate pathologic expression of VEGF and angiogenesis in chronic liver disease. *Gastroenterology*. (2016) 150:982–97. doi: 10.1053/j.gastro.2015.11.038
94. Maillo C, Martin J, Sebastian D, Hernandez-Alvarez M, Garcia-Rocha M, Reina O, et al. Circadian- and UPR-dependent control of CPEB4 mediates a translational response to counteract hepatic steatosis under ER stress. *Nat Cell Biol*. (2017) 19:94–105. doi: 10.1038/ncb3461

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Macrophage Phenotype and Function in Liver Disorder

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Hepatic macrophages are a remarkably heterogeneous population consisting of self-renewing tissue-resident phagocytes, termed Kupffer cells (KCs), and recruited macrophages derived from peritoneal cavity as well as the bone marrow. KCs are located in the liver sinusoid where they scavenge the microbe from the portal vein to maintain liver homeostasis. Liver injury may trigger hepatic recruitment of peritoneal macrophages and monocyte-derived macrophages. Studies describing macrophage accumulation have shown that hepatic macrophages are involved in the initiation and progression of various liver diseases. They act as tolerogenic antigen-presenting cells to inhibit T-cell activation by producing distinct sets of cytokines, chemokines, and mediators to maintain or resolve inflammation. Furthermore, by releasing regenerative growth factors, matrix metalloproteinase arginase, they promote tissue repair. Recent experiments found that KCs and recruited macrophages may play different roles in the development of liver disease. Given that hepatic macrophages are considerably plastic populations, their phenotypes and functions are likely switching along disease progression. In this review, we summarize current knowledge about the role of tissue-resident macrophages and recruited macrophages in pathogenesis of alcoholic liver disease (ALD), non-alcoholic steatohepatitis (NASH), viral hepatitis, and hepatocellular carcinoma (HCC).

Keywords: hepatic macrophages, Kupffer cells, alcoholic liver disease, hepatocellular carcinoma, viral hepatitis, non-alcoholic steatohepatitis

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INTRODUCTION

Hepatic macrophages, consisting of Kupffer cells (KCs) and recruited macrophages, are the largest population of innate immune cells in the liver. In the healthy rodent liver, macrophages comprise around 20–25% of non-parenchymal cells (1, 2); the high occupancy implies that the hepatic macrophages play a vital role in maintaining liver function and homeostasis. KCs, self-renewing tissue-resident phagocytes, are located in the liver sinusoids. During homeostasis, distinct Fc and scavenger receptors are expressed on the KC surface, which allows them to recognize modified self-molecules, resulting in clearing of apoptotic cells, cell debris, and immune complex (3, 4). Additionally, KCs are involved in controlling the iron (5), cholesterol (6), and bilirubin (7) balance of the blood. KCs also express a wide range of pattern recognition receptors (PRRs), including toll-like receptors (TLRs) (8), nucleotide oligomerization (NOD)-like receptors (9), and retinoic acid-inducible gene I (RIG-I)-like receptors (10). These receptors assist KCs to recognize and eliminate invading foreign pathogens.

Hepatic macrophages form highly heterogeneous populations, and several markers have been used to distinguish between KCs and recruited macrophages. In mice, KCs were found to express a unique maker C-Type Lectin Domain Family 4 Member F (CLEC4F) and can be characterized as CD11b+, F4/80+, TIM4+, and CLEC4F+ cell populations (11). The bone-marrow-derived macrophages are CD11b+, F4/80+, CCR2+, and CX3CR1+. MacParland et al. showed that human hepatic macrophages could be classified as CD68+ MACRO+ KCs and CD68+ MACRO– recruited macrophages in the steady state using single-cell analysis (12). According to activation programs, hepatic macrophages can be broadly divided into classically activated pro-inflammatory and alternatively activated anti-inflammatory phenotypes (13, 14). Pro-inflammatory macrophage stimuli lipopolysaccharide (LPS) and interferon (IFN)-γ activate signal transducers and activators of transcription (STAT)1, myeloid differentiation factor 88 (MyD88), Toll-interleukin 1 receptor domain containing adaptor protein (MaL/Tirap), and IFN regulatory factor (IRF)-dependent pathways, resulting in the release of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF), reactive oxygen species (ROS), and nitric oxide synthase (14–16). These macrophages are likely to contribute to hepatic inflammation and damage in distinct liver diseases. Anti-inflammatory macrophages exhibit high phagocytic capacity and produce high levels of arginase 1 as well as IL-10 via activating Janus kinase (JAK)1 and JAK3; they are featured by immunoregulation and tissue remodeling (14, 16).

It has been suggested that hepatic macrophages have two origins (17, 18): recruited macrophages derived from the hematopoietic stem cells and tissue-resident macrophages from the yolk sac. HSC-derived macrophages differentiate from circulating myeloid precursor cells from the bone marrow; this process is mediated by colony-stimulating factor (CSF)-1 (17, 18). The majority of KCs are believed to develop from the yolk sac before the appearance of HSCs (18). However, this theory has been challenged by a recent study that revealed a common progenitor for tissue-resident macrophages, called premacrophages, which were generated early in development and had colonized the whole embryo from embryonic day 9.5. Tissue-specific sets of transcriptional regulators control the differentiation of premacrophages into tissue-resident macrophages, whereby the development of KCs is regulated by Id3, a transcription factor inhibitor of DNA binding 3, and inactivation of Id3 causes KC deficiency in adults (19).

Hepatic recruited macrophages are derived from not only circulating monocytes but also macrophages of different compartments. Circulating monocytes are classified into CD11b+Ly6C^{hi} (20) and CD11b+Ly6C^{low} (21) in mice. CD11b+Ly6C^{hi} subsets can infiltrate into the liver during inflammation (20), whereas the Ly6C^{low} monocytes serve as sentinels to scavenge microparticles and cell debris in the capillaries (21). Monocytes may downregulate Ly6C expression after infiltration and before differentiation (22). Recent findings suggest that self-reviewing peritoneal cavity macrophages, characterized by F4/80^{hi}GATA6+, can rapidly migrate to the liver through the mesothelium in response to a sterile injury (23). This result suggests that the composition of hepatic macrophages may be more complicated than expected. Numerous studies have shown that hepatic macrophages are involved in the progression of inflammation and fibrosis and, therefore, hold the key to controlling the pathogenesis of liver disease (14, 15, 24). In this review, we will summarize current knowledge about hepatic macrophages in pathogenesis of alcoholic liver disease (ALD), non-alcoholic steatohepatitis (NASH), hepatitis B virus/hepatitis C virus (HBV/HCV), and hepatocellular carcinoma (HCC) with a particular focus on KCs and monocyte-derived macrophages.

HEPATIC MACROPHAGES IN ALD

Chronic alcohol consumption, the primary cause of ALD, results in a broad range of disorders, including liver steatosis, alcoholic hepatitis, chronic hepatitis, HCC, liver fibrosis, and/or cirrhosis (25–27). It has been documented that hepatic macrophages accumulate within the portal tracts of ALD patients (28), whereas the depletion of hepatic macrophages via the administration of gadolinium chloride ($GdCl_3$) prevents alcohol-induced liver inflammation in the rat (29). These results suggest that hepatic macrophages play a central role in the pathogenesis of ALD.

One hypothesis for this effect is that ethanol ingestion disrupts the intestinal barrier, which increases the permeability of the gut, thereby enhancing the migration of Gram-negative bacteria into the portal circulation (30, 31) and leading to ALD pathogenesis. The ligation of LPS with the CD14/TLR4 receptor complex on KCs triggers the downstream IL-1 receptor-associated kinase (IRAK) and inhibitor of nuclear factor kappa-B kinase (IKK) pathways, resulting in the release of the inflammatory cytokines IL-6 and TNF-α and chemokines, such as monocyte chemoattractant protein (MCP-1) (32) (**Figure 1A**). These mediators augment inflammation and alcohol-induced liver injury in ALD (32). Compared with wild-type (WT) mice, alcohol-fed mice are more sensitive to LPS and produce more MCP-1 (33) and TNF-α (34) post stimulation. Recent studies showed that a small non-coding RNA, termed microRNA (miRNA), is involved in regulating macrophage infiltration, activation, and ALD progression (**Figure 1A**). Unbiased analysis of miRNA revealed that miR181b-3p released by KCs regulated TLR4 signaling during ethanol consumption (35). In ethanol-fed rats, the overexpression of miR181b-3p inhibited importin α5 expression and suppressed LPS-induced TNF-α expression in KCs (35). In a study in mice, chronic alcohol feeding promoted miR-155 production by KCs via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (36). A later study revealed that macrophage infiltration induced by chronic alcohol consumption was reduced in miR-155-deficient mice (37). In the same study, knockout of miR-155 also alleviated the inflammation and steatosis triggered by chronic alcohol ingestion (37).

During ALD, hepatocytes injured by alcohol consumption can activate KCs. Acute and chronic ethanol exposure stimulates KCs via danger-associated molecular patterns produced by injured hepatocytes (38) (**Figure 1A**). Additionally, Verma et al. found that ethanol exposure stimulated hepatocytes to produce considerably more CD40L-containing extracellular

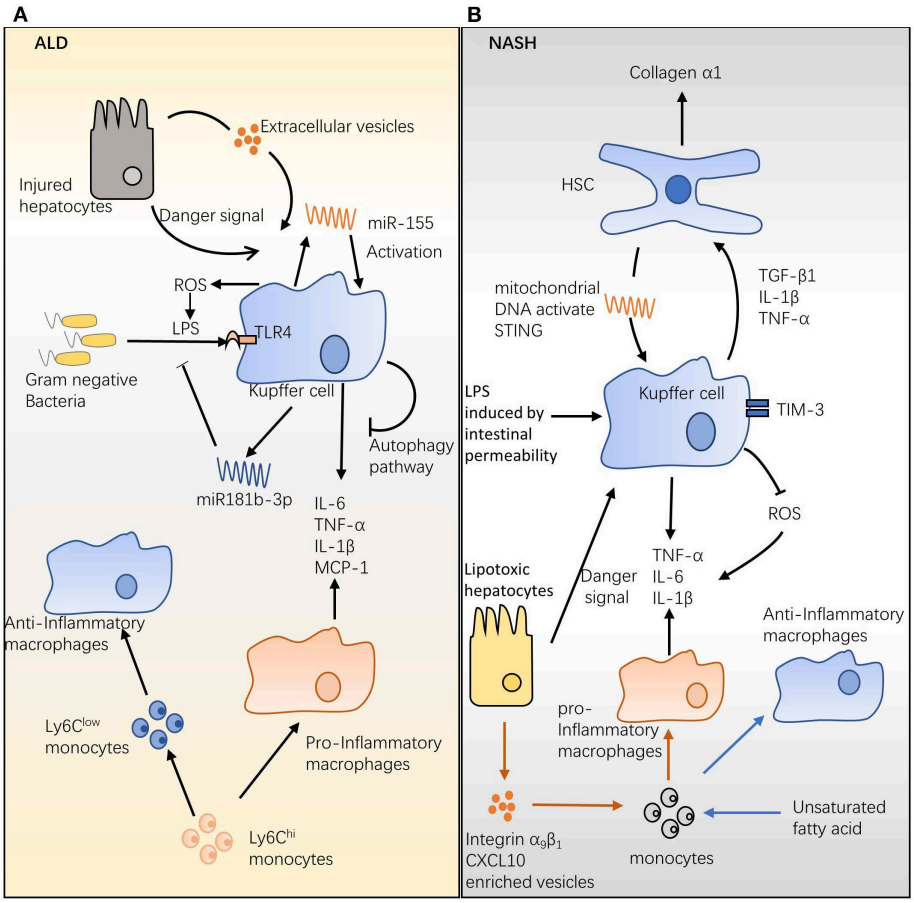

FIGURE 1 | Hepatic macrophages in alcoholic liver disease (ALD) and non-alcoholic steatohepatitis (NASH). **(A)** The role of hepatic macrophages in ALD. Chronic alcohol consumption disrupts the intestinal barrier, which increases the permeability of the gut and allows Gram-negative bacteria to migrate into the portal circulation. Lipopolysaccharide (LPS) expressed on Gram-negative bacteria activates Kupffer cells (KCs) and promotes interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β, and monocyte chemoattractant protein (MCP)-1 release. Hepatocytes injured by alcohol consumption activate KCs via danger signal and CD40-containing extracellular vesicles. Chronic alcohol ingestion induces microRNA (miR)-155 and miR181b-3p expression; the former activates KCs and promotes inflammatory production, while the latter regulates LPS-induced inflammation. The Ly6C^{hi} monocyte can differentiate into pro-inflammatory and anti-inflammatory macrophages during ALD, and the ratio of these two populations may mediate ALD development. **(B)** The role of hepatic macrophages in NASH. High levels of LPS induced by increasing intestinal permeability and/or danger signal from lipotoxic hepatocytes stimulate KCs; activated KCs produce the survival signals, transforming growth factor β, IL-1β, and TNF-α, which stimulate hepatic stellate cells and increase generation of hepatic collagen α1, ultimately triggering fibrosis. Mitochondrial DNA from hepatocytes of high-fat diet (HFD)-fed mice activates KCs and promotes cytokine release, steatosis, and inflammation. Conversely, TIM-3 expressed on hepatic macrophages protects animals from HFD-induced NASH by inhibiting reactive oxygen species production. An HFD augments the infiltration of bone-marrow-derived monocytes into the liver and further differentiates them into protective anti-inflammatory macrophages.

vesicles in a caspase-3-dependent manner, ultimately triggering macrophage activation and production of MCP-1, TNF-α, and ROS (39) (**Figure 1A**). Genetic knockout of CD40 (CD40-/-) or the caspase-activating TNF-related apoptosis-inducing ligand (TRAIL) receptor (TR-/-) protected mice from alcohol-induced injury (39). Notably, during alcohol exposure, KCs are a major source of ROS, which is essential for LPS sensitization (40) and inflammatory cytokine production (41) (**Figure 1A**). In a chronic-plus-binge ethanol-feeding model, KCs show extracellular signal-regulated kinase 1/2 (ERK1/2) signaling attenuation and TNF-α production impairment, when they are pretreated with ROS generation inhibitor NADPH oxidase (40, 41). It has been documented that the cannabinoid receptor 2 (CB2) expressed on KCs protects mice from ALD via an autophagy pathway (**Figure 1A**). This effect is supported by the findings that mice with specifically targeted deletion of the CB2 receptor (CB2$^{Mye-/-}$) or autophagy gene ATG5 (ATG5$^{Mye-/-}$) had exacerbated liver inflammation and alcohol-induced steatosis (42). Upon exposure to LPS, KCs isolated from CB2$^{Mye-/-}$ mice showed a pro-inflammatory phenotype that is characterized by an increased expression of chemokines IL-1β, IL-1α, IL-6, TNF-α, and CCL3 (42). These data suggest that KCs are activated toward a pro-inflammatory phenotype that increases liver inflammation and damage during ALD.

The role of recruited macrophages in ALD is less well studied. Chronic alcohol administration increases the population

of recruited macrophages in the mouse liver (43). In an animal model, ethanol feeding promoted the differentiation of Ly6C^{hi} monocytes into tissue-damaging pro-inflammatory macrophages (43). Moreover, phagocytosis of apoptotic hepatocytes allows Ly6C^{hi} monocytes/macrophages to switch to Ly6C^{low} monocytes/macrophages, which then differentiate into tissue-protective macrophages (43) (**Figure 1A**). It has been suggested that the ratio of these two subsets determines the role of recruited macrophages in the pathogenesis of ALD (43).

HEPATIC MACROPHAGES CONTRIBUTE TO NASH

About 20% of patients who suffer from non-alcoholic fatty liver disease will develop NASH, which is defined by the existence of progressive fibrosis and steatosis with inflammation, ultimately leading to HCC and cirrhosis. To date, the pathogenesis of NASH is still obscure, but several risk factors are known to be involved in the process, ranging from oxidative stress, insulin resistance, cytokines, and epigenetic modification to microbiota alteration and environmental elements (44).

One connection between KCs and NASH is the presence of hepatic stellate cells (HSCs). NASH augments endotoxin influx by increasing intestinal permeability; the high level of endotoxin and/or danger signal from lipotoxic hepatocytes can stimulate KCs (45) (**Figure 1B**). Activated KCs produce transforming growth factor (TGF)-β1, which stimulates HSCs and increases the generation of hepatic collagen-α1(I), eventually triggering fibrosis (46) (**Figure 1B**). In comparison with that in controls, collagen-α1(I) messenger RNA (mRNA) was substantially increased in carbon tetrachloride (CCl_4)-treated mice, and this increase was abolished in TGF-β1-knockout mice (47). In addition, IL-1β and TNF-α production by stimulated KCs was required to maintain HSC survival via the NF-κB pathway (48) (**Figure 1B**). In a low-serum media model, hepatic macrophages protected HSCs from apoptosis, and, in the same model, neutralization of IL-1 and TNF inhibited the protective effects of hepatic macrophages. Additionally, suppression of NF-κB by sulfasalazine induces apoptosis of HSC in humans and rats (49). Furthermore, the depletion of macrophages by clodronate liposome reduced IL-1β and TNF-α mRNA in the fibrotic liver (48). Recent research has shown that mitochondrial DNA from hepatocytes of high-fat diet (HFD)-fed mice activates KCs and induces steatosis and inflammation via the stimulator of IFN genes (STING) pathway (50) (**Figure 1B**). In a mouse model of NASH, fibrosis, inflammation, and steatosis were diminished in the livers of STING-deficient mice (50). The STING agonist, dimethylxanthenone-4-acetic acid, augmented the TNF-α and IL-6 produced by KCs from WT mice, and this increase was attenuated in STING-deficient mice (50). The current literature suggests that activated hepatic macrophages promote the progression of NASH. In contrast, Du et al. found that the expression of TIM-3 on hepatic macrophages is dramatically increased in a methionine- and choline-deficient diet (MCD)-induced NASH model (51). In the same study, TIM-3 deficiency increased the release of ROS by hepatic macrophages and promoted MCD-induced liver fibrosis, as well as steatosis (51) (**Figure 1B**). These results suggest a mechanism by which hepatic macrophages can inhibit NASH development.

RECRUITED MACROPHAGES: FRIEND OR FOE IN NASH PROGRESSION? (51)

Odegaard et al. demonstrated that, in lethally irradiated mice, an HFD promotes the recruitment of bone-marrow-derived monocytes to the liver; these cells then differentiate into anti-inflammatory macrophages, which provide a protective effect against diet-induced insulin resistance via the peroxisome proliferator-activated receptor δ (PPARδ) pathway (52) (**Figure 1B**). The adoptive transfer of PPARδ-/- bone marrow into WT mice failed to activate alternative macrophages or attenuate the induced glucose intolerance caused by the HFD (52). In agreement with these finds, Oliver et al. demonstrated that in an overdose of acetaminophen-induced acute liver damage model, high-fructose, high-fat, and high-cholesterol (FFC)-diet-fed mice shows attenuated liver injury than normal-diet-fed mice (53). In the same model, adopting bone-marrow-derived macrophages (BMDMs) from normal-diet-fed mice into FFC-diet-fed mice increases liver damage (53). Single-cell RNA sequencing reveals that these BMDMs from FFC-diet-fed mice downregulate *S100a8/S100a9*, genes encoding inflammatory marker calprotectin, compared with normal-diet-fed mice (53). Additionally, FFC diet also suppresses the TLR4-dependent inflammatory capacity of BMDMs in the mouse NASH model (53). BMDMs from FFC-diet-fed mice are insensitive to LPS stimulation, reflected by less IL-6 and TNF-α production compared with their normal-diet-fed counterparts (53). In contrast, growing evidence has demonstrated that NASH niche favors pro-inflammatory macrophage/monocyte infiltration, and these infiltrated cells increase liver damage and inflammation (54). The fatty acid palmitate can stimulate death receptor 5 on hepatocytes, resulting in release of extracellular vehicles (EVs) (54). The EVs released from lipotoxic hepatocytes have been shown to promote BMDMs toward the pro-inflammatory phenotype characterized by increasing expression of *Il1b* and *Il6* mRNAs (54). Moreover, hepatocyte-lipotoxicity-induced EVs are enriched with integrin $\alpha_9\beta_1$ (55) and/or CXCL10 (56), which augment pro-inflammatory macrophage infiltration and enhance hepatic fibrosis (**Figure 1B**). Integrin $\alpha_9\beta_1$ is required for monocytes to attach liver sinusoidal endothelial; blockade of this interaction by anti-integrin $\alpha_9\beta_1$ antibody decreases FFC-diet-induced liver fibrosis and injury in NASH mice (55). During hepatic injury, pro-inflammatory macrophages/monocytes are attracted to liver via the CXCL10–CXCR3 axis (57). Compared with those in WT mice, FFC-diet-induced liver injury and inflammation are alleviated in CXCL10–/– mice (56). In a randomized trial, targeting pro-inflammatory monocytes/macrophages by cenicriviroc, a dual antagonist of CCR2 and CCR5, improves hepatic fibrosis in NASH patients (58). One crucial signal that controls the fate of these monocyte-derived macrophages is the type of fatty acids to

which the macrophage is exposed. Exposure by saturated fatty acid causes hepatocyte lipotoxicity that then promotes pro-inflammatory macrophage differentiation, whereas stimulation by unsaturated fatty acids activates PPARδ to enhance anti-inflammatory differentiation in NASH (**Figure 1B**) (52, 59). Taken together, monocytes/macrophages are recruited to the liver during NASH; in response to different compositions of fatty acids, these cells can be differentiated into tissue damage pro-inflammatory macrophages and/or tissue repair anti-inflammatory macrophages; the ratio of two macrophage subsets may determine the role of hepatic macrophage in the pathogenesis of NASH.

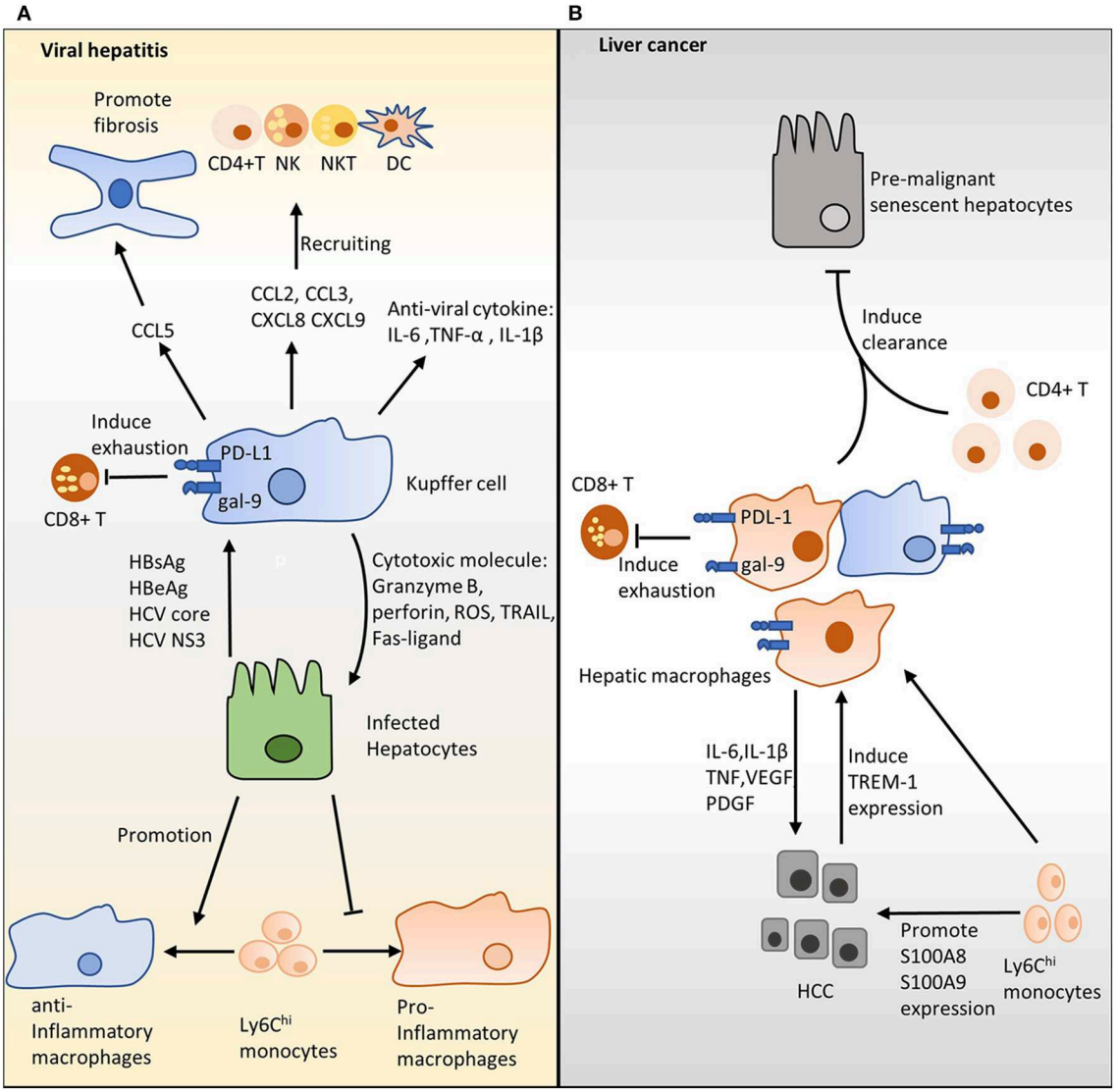

FIGURE 2 | The role of hepatic macrophages in viral hepatitis and hepatocellular carcinoma (HCC). **(A)** Hepatic macrophages and hepatitis B virus (HBV)/hepatitis C virus (HCV). Interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-1β produced by Kupffer cells (KCs) show strong antiviral activities. Additionally, KCs may remove infected hepatocytes by producing cytotoxic molecules, including granzyme B, perforin, reactive oxygen species, TNF-related apoptosis-inducing ligand, and Fas-ligand. KCs produce distinct chemokines, including CC- chemokine ligand (CCL)2, CCL3, CXC-chemokine ligand (CXCL)8, and CXCL9, and, together, these chemokines recruit natural killer cells, natural killer T cells, dendritic cells, and CD4+ T cells to infected sites and enhance infection clearance. HCV stimulation induces hepatic macrophages to generate CCL5, which in turn activates hepatic stellate cells and eventually triggers live inflammation and fibrosis. KCs mediate T-cell dysfunction via PD-1/PD-L1 and TIM-3/galectin-9 pathways. Increased HBV inoculum suppresses polarization of pro-inflammation macrophages. **(B)** Hepatic macrophages contribute to HCC. Hepatic macrophages produce IL-6, IL-1β, TNF, vascular endothelial growth factor, and platelet-derived growth factor to promote tumor growth and angiogenesis during HCC. KCs suppress antitumor activity by inducing T-cell dysfunction through PD-L1/PD-1 and galectin-9/TIM-3 in the HCC setting. In contrast, hepatic macrophages assist CD4+ T cells in removing the premalignant senescent hepatocytes that enhance HCC progression. Ly6C^{hi} monocytes increase the expression of S100A8 and S100A9 on cancer cells and promote tumor migration and invasion.

THE ROLE OF HEPATIC MACROPHAGES IN VIRAL HEPATITIS

The role of hepatic macrophages in the progression of viral hepatitis is still controversial. Activated KCs, characterized by the upregulation of CD33 and CD163, accumulate in the portal tract during chronic HBV/HCV infection, highlighting the importance of these cells in fighting viral hepatitis (60, 61). KCs are the primary source of IL-1β, TNF-α, and IL-6; these inflammatory cytokines exhibit strong antiviral activity during an infection (62) (**Figure 2A**). Additionally, it has been shown that KCs may eliminate infected hepatocytes by releasing cytotoxic molecules, such as granzyme B, perforin, ROS, TRAIL, and Fas ligand (63, 64) (**Figure 2A**). Furthermore, the supernatant from differentiated pro-inflammatory macrophages contains reasonable amounts of IL-1β and IL-6, which inhibit the progression of HBV by decreasing levels of hepatitis B surface antigen (HBsAg) and hepatitis B early antigen (HBeAg) (65).

Several studies have indicated that, in humans, HBV/HCV can directly stimulate hepatic macrophages to trigger inflammatory cytokine secretion, thereby enhancing antiviral activity (15, 66) (**Figure 2A**). *In vitro* stimulation with HBsAg and HBeAg promoted primary human non-parenchymal liver cells to produce IL-6, IL-8, TNF-α, and IL-1β via the NF-κB pathway (67, 68). Similarly, culturing with HCV enhanced the production of IL-1β and IL-18 by KCs and monocyte-derived macrophages (69, 70). It has been documented that HCV core proteins and nonstructural protein 3 trigger monocyte-derived macrophage activation via TLR1, TLR2, and TLR6 signaling (71). In agreement with these findings, immunofluorescence analysis showed that IL-1β and CD68 are co-localized in liver tissues of chronic HCV patients (72). Apart from inflammatory cytokines, activated KCs also produce CCL2 (73), CCL3 (74), CXCL8 (67), and CXCL9 (74, 75). Together, these chemokines recruit natural killer (NK), NKT, dendritic cells (DC), and CD4+ T cells to infected sites to accelerate infection clearance (74, 75). Although uptake of HBV/HCV by KCs *ex vivo* has not been reported, accumulating evidence from *in vitro* experiments suggests that KCs are involved in HBV/HCV clearance via producing inflammatory cytokines and activating other immune cells.

In contrast, it has been shown that hepatic macrophages are involved in the development of HBV/HCV-induced fibrosis. Incubation with HBV significantly enhanced the generation of the pro-fibrotic growth factor TGF-β1 by primary rat KCs (76). Sasaki et al. found that HCV stimulation induced hepatic macrophages to produce CCL5, which in turn activated HSCs and triggered live inflammation as well as fibrosis (77) (**Figure 2A**). In the same study, neutralizing CCL5 with an antibody suppressed HSC activation (77). Furthermore, stimulation with the HCV core protein induces programmed death ligand 1 (PD-L1) expression by KCs (78). Similarly, high galectin-9 expression is seen on the KCs of patients with chronic HBV infections (79). Activation of the programmed cell death protein 1 (PD-1)/PD-L1 and TIM-3/galectin-9 pathways in T cells evokes T-cell dysfunction and, thereby, favors the establishment of a chronic infection (78, 79) (**Figure 2A**).

One hypothesis for these phenomena is that the phenotype of the hepatic macrophages may be shaped by HBV/HCV as the infection progresses. During the early phase of infection, hepatic macrophages are dominated by pro-inflammatory subsets that inhibit virus development by producing cytokines with antiviral activity. In contrast, the chronic hepatitis infection environment suppresses hepatic macrophages polarizing toward the pro-inflammatory phenotype and pushes cells toward the immunoregulation phenotype. Thus, hepatic macrophages show weak antiviral and strong pathological activities in the chronic hepatitis (14). This finding is supported by a recent study showing that an increase in the HBV inoculum attenuated the polarization of monocytes into pro-inflammatory macrophages, evidenced by decreased IL-6 production (65) (**Figure 2A**). In the same study, exposure to the HBV virus enhanced monocyte anti-inflammatory differentiation, evidenced by increased IL-10 production (65) (**Figure 2A**). It is likely that a high virus titer suppresses the antiviral activity of hepatic macrophages and polarizes hepatic macrophages toward a tolerogenic phenotype. In agreement with this hypothesis, Faure-Dupuy et al. demonstrated that exposure to HBV attenuated cytokine release by pro-inflammatory hepatic macrophages and enhanced cytokine production by anti-inflammatory hepatic macrophages (65). This modulation suppresses the antiviral surveillance and favors the establishment of an infection (65). Taken together, a high HBV/HCV titer not only inhibits pro-inflammatory macrophage polarization but also promotes macrophages differentiating toward a tolerogenic phenotype, which favors HBV/HCV development by releasing immunoregulation cytokine IL-10.

HEPATIC MACROPHAGES AND HCC

Hepatic macrophages play a crucial role in the pathogenesis of HCC, as evidenced by the accumulation of hepatic macrophages in resections of HCC patients (80) and the liver tissue of chemically induced HCC mice (81). The majority of studies suggest that hepatic macrophages are pro-inflammatory and pro-tumorigenic cells, which inhibit antitumor immunity and favor the establishment of tumors (82–84). Having a large population of hepatic macrophages is associated with poor survival in HCC patients (80, 85). During HCC, hepatic macrophages produce the pro-angiogenic factors, TGF-β, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), which, together, promote tumor growth (84, 86) (**Figure 2B**). Additionally, it has been documented that hepatic macrophages release different mediators, including IL-6, IL-1β, CCL2, VEGF A (VEGFA), and TNF, to augment tumor cell proliferation in HCC (83, 86) (**Figure 2B**). The evidence for liver macrophage inhibition of HCC growth is limited. The most convincing evidence probably comes from a study of 302 HCC patients, which demonstrated that a high number of CD68+ macrophages is associated with better overall survival (87). Moreover, Kang et al. showed that hepatic macrophages assisted CD4+ T cells

TABLE 1 | Pharmacological agents targeting macrophages in alcoholic liver disease, non-alcoholic steatohepatitis, viral hepatitis, or hepatocellular carcinoma.

Target	Agent	Mechanism of action	Phase	Clinical trial number
ALCOHOLIC LIVER DISEASE				
Gut bacteria	Combined vancomycin and gentamycin and meropenem	Inhibiting macrophage activation by gut bacteria eradication	Ongoing	NCT03157388
NON-ALCOHOLIC STEATOHEPATITIS				
Galectin 3	GR-MD-02	Galectin 3 antagonist on macrophages	Phase 2	NCT02462967
CCR2/CCR5	Cenicriviroc	CCR2/CCR5 antagonist (inhibits monocyte/macrophage infiltration)	Phase 2	NCT02217475
PPARα/δ	Elafibranor	Dual PPARα/δ agonist, PPARδ agonist promotes anti-inflammatory differentiation	Phase 3	NCT02704403
VIRAL HEPATITIS				
GM-CSF	Entecavir plus GM-CSF	GM-CSF promotes macrophage differentiation	Ongoing	NCT03164889
GM-CSF	Y peginterferon alpha-2b plus GM-CSF	GM-CSF promotes macrophage differentiation	Phase 2	NCT02332473
HEPATOCELLULAR CARCINOMA				
CSF1R	Chiauranib	Multi-target inhibitor that suppresses angiogenesis-related kinases, mitosis-related kinase Aurora B, and CSF1R. Blockade of CSF1R decreases the macrophage differentiation.	Phase 1	NCT03245190
CCR2/5	Nivolumab plus CCR2/5 inhibitor	CCR2/CCR5 antagonist (inhibits monocyte/macrophage infiltration)	Phase 2	NCT04123379

CCR2, CC chemokine receptor 2, CSF1R, colony-stimulating factor 1 receptor, GM-CSF, granulocyte-macrophage colony-stimulating factor.

in cleaning the premalignant senescent hepatocytes that promote HCC development in an animal model (88). Therefore, two clinical studies with similar clinical–pathologic characteristics but varied in the number of patients have led to contradictory results (80, 87). It is possible that different therapeutic strategies, in particular, post-recurrence therapies, may have been used in these studies (80, 87). CD68 was used to identify tumor-associated macrophages (TAMs) (80, 87). It is widely accepted that TAMs form heterogeneous populations; therefore, the TAM subset contributions to tumor growth progression or inhibition remain to be investigated. This may help to further evaluate the discrepancy between these two studies.

Studies have found that KCs suppress antitumor activity by inducing T-cell tolerance and dysfunction in an HCC setting. KCs have been demonstrated to function as incomplete antigen-presenting cells (APCs) to induce T-cell tolerance (89). This idea is further supported by a recent study which showed that human KCs might exhibit a tolerogenic phenotype (12); they accumulate at the peritumoral stroma expressing high levels of PD-L1 (90, 91) and galectin-9 (92), thereby inhibiting the antitumor response by activating PD-L1/PD-1 and galectin-9/TIM-3 signaling in T cells (**Figure 2B**). Moreover, the triggering receptor expressed on myeloid cells-1 (TREM-1) is an activating receptor that is widely expressed on monocytes, macrophages, and neutrophils (93). Cancer cell stimulation has been shown to directly increase the expression TREM-1 on KCs, which, in turn, promotes KC activation and HCC progression (93, 94) (**Figure 2B**). In the same study, *Trem1* deficiency diminished IL-1β, IL-6, TNF, CCL2, and CXCL10 release by KCs and suppressed HCC growth (94). Taken together, interaction between T cells and KCs hinders antitumor response by promoting T-cell exhaustion in HCC.

The role of recruited macrophages in HCC development is highlighted by the importance of the CCL2/CCR2 signaling axis, which is crucial for Ly6C^{hi} monocyte recruitment to inflammatory sites (95). It has been suggested that monocyte recruitment during HCC depends on KCs (96), senescent hepatocytes (97), and tumor-associated neutrophils (98). Conditional media from Ly6C^{hi} monocytes increased the expression of S100A8 and S100A9 in cancer cells and promoted tumor migration and invasion in an experimental liver metastasis model (99) (**Figure 2B**). In a preclinical model of HCC, blocking CCL2/CCR2 signaling with a CCR2 antagonist reduced Ly6C^{hi} monocyte numbers in the peripheral blood and suppressed anti-inflammatory macrophage polarization in the liver, ultimately inhibiting tumor growth (100). Indeed, a large number of studies have shown that the CCL2/CCR2 pathway involves the recruitment of myeloid-derived suppressor cells (MDSCs) during inflammation, and Ly6C^{hi} monocytes have been shown to be the precursor of MDSCs (101, 102); therefore, the antitumor effect triggered by blocking the CCL2/CCR2 pathway may be partially due to MDSC depletion. To sum up, during HCC progression, macrophages and MDSCs are recruited to the liver via the CCL2/CCR2 axis; these cells have been shown to promote tumor proliferation and metastasis.

PERSPECTIVE

A tremendous amount of research over the last few decades has revealed that hepatic macrophages play a central role in the pathogenesis of liver disease. Several strategies have been employed to specifically target hepatic macrophages in different liver diseases (**Table 1**). Notably, CD11b, F4/80, and Ly6C in

mice and CD14, HLA-DR, and CD68 in humans have been widely used to identify KCs; however, these markers may be inadequate to distinguish KCs from recruited macrophages. It has been shown that murine KCs express a unique marker, CLEC4F (11). Meanwhile, single-cell RNA-seq analysis showed that KCs are CD68+ Macro+ in healthy humans (12). Therefore, adding these new markers to the conventional hepatic macrophage identification panel should be considered for precise future investigations into the role of liver macrophage subsets in the development of the liver disease. The recently developed mass cytometry Cyto F technique has been used to study hepatic macrophage in liver disease (55); this technique can simultaneously label up to 350 markers on a single cell, therefore providing a powerful platform to investigate in depth the heterogeneity of hepatic macrophages under different liver diseases as well as pharmaceutical intervention conditions.

During inflammation, circulating monocytes infiltrate the liver and are involved in the progression of various liver diseases. The phenotypes and roles of monocyte-derived hepatic macrophages are highly dependent on local stimuli during liver disease (103). For example, during fibrosis, a novel monocyte-derived TREM2+ CD9+ scar-associated macrophage has been discovered; this population is expanded in cirrhotic livers and exhibited a pro-fibrogenic phenotype (104). The current M1–M2 model has limitations; this concept cannot define all cell phenotypes, especially macrophages during chronic inflammation and chronic infection liver disease (16). A recent study suggested an extension to the M1–M2 model by showing that, other than M1 and M2 macrophages, human macrophages can be polarized into distinct phenotypes in response to various stimuli (103). Therefore, it is important to precisely describe macrophage populations based on their origins, stimuli, and identification markers (105).

Self-renewing peritoneal macrophages have been shown to migrate to the liver in response to sterile injury (23). Additionally, the spleen is thought to be a reservoir for inflammatory monocytes, which infiltrate the liver and differentiate into hepatic macrophages during liver injury (106). These studies suggest that recruited macrophages are a highly heterogeneous population, composed of subsets with different origins and functions (23, 107). Currently, monocyte-derived recruited macrophages are extensively studied; however, the contributions of peritoneal cavity and spleen-derived recruited macrophages to the pathogenesis of distinct liver diseases are obscured and remain to be explored in the future.

AUTHOR CONTRIBUTIONS

LD wrote the first draft of the manuscript. YG, XH, and XS contributed to manuscript revision and read and approved the submitted version.

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REFERENCES

1. Lopez BG, Tsai MS, Baratta JL, Longmuir KJ, Robertson RT. Characterization of Kupffer cells in livers of developing mice. *Comp Hepatol.* (2011) 10:2. doi: 10.1186/1476-5926-10-2
2. Dong Z, Wei H, Sun R, Tian Z. The roles of innate immune cells in liver injury and regeneration. *Cell Mol Immunol.* (2007) 4:241–52.
3. Jenne CN, Kubes P. Immune surveillance by the liver. *Nat Immunol.* (2013) 14:996–1006. doi: 10.1038/ni.2691
4. Parker GA, Picut CA. Liver immunobiology. *Toxicol Pathol.* (2005) 33:52–62. doi: 10.1080/01926230590522365
5. Gammella E, Buratti P, Cairo G, Recalcati S. Macrophages: central regulators of iron balance. *Metallomics.* (2014) 6:1336–45. doi: 10.1039/C4MT00104D
6. Wang Y, van der Tuin S, Tjeerdema N, van Dam AD, Rensen SS, Hendrikx T, et al. Plasma cholesteryl ester transfer protein is predominantly derived from Kupffer cells. *Hepatology.* (2015) 62:1710–22. doi: 10.1002/hep.27985
7. Naito M, Hasegawa G, Ebe Y, Yamamoto T. Differentiation and function of Kupffer cells. *Med Electron Microsc.* (2004) 37:16–28. doi: 10.1007/s00795-003-0228-x
8. Nakamoto N, Kanai T. Role of toll-like receptors in immune activation and tolerance in the liver. *Front Immunol.* (2014) 5:221. doi: 10.3389/fimmu.2014.00221
9. Kanneganti T-D, Lamkanfi M, Núñez G. Intracellular NOD-like receptors in host defense and disease. *Immunity.* (2007) 27:549–59. doi: 10.1016/j.immuni.2007.10.002
10. Terpstra V, van Berkel TJC. Scavenger receptors on liver Kupffer cells mediate the *in vivo* uptake of oxidatively damaged red blood cells in mice. *Blood.* (2000) 95:2157. doi: 10.1182/blood.V95.6.2157
11. Scott CL, Zheng F, De Baetselier P, Martens L, Saeys Y, De Prijck S, et al. Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Commun.* (2016) 7:10321. doi: 10.1038/ncomms10321
12. MacParland SA, Liu JC, Ma XZ, Innes BT, Bartczak AM, Gage BK, et al. Single cell RNA sequencing of human liver reveals distinct intrahepatic macrophage populations. *Nat Commun.* (2018) 9:4383. doi: 10.1038/s41467-018-06318-7
13. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol.* (2000) 164:6166–73. doi: 10.4049/jimmunol.164.12.6166
14. Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol.* (2017) 17:306–21. doi: 10.1038/nri.2017.11
15. Tacke F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol.* (2017) 66:1300–12. doi: 10.1016/j.jhep.2017.02.026
16. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime Rep.* (2014) 6:13. doi: 10.12703/P6-13
17. van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. *J Exp Med.* (1968) 128:415–35. doi: 10.1084/jem.128.3.415

18. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science*. (2012) 336:86–90. doi: 10.1126/science.1219179
19. Mass E, Ballesteros I, Farlik M, Halbritter F, Gunther P, Crozet L, et al. Specification of tissue-resident macrophages during organogenesis. *Science*. (2016) 353:aaf4238. doi: 10.1126/science.aaf4238
20. Brempelis KJ, Crispe IN. Infiltrating monocytes in liver injury and repair. *Clin Transl Immunol*. (2016) 5:e113. doi: 10.1038/cti.2016.62
21. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, et al. Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell*. (2013) 153:362–375. doi: 10.1016/j.cell.2013.03.010
22. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci USA*. (2012) 109:E3186–95. doi: 10.1073/pnas.1119964109
23. Wang J, Kubes P. A reservoir of mature cavity macrophages that can rapidly invade visceral organs to affect tissue repair. *Cell*. (2016) 165:668–78. doi: 10.1016/j.cell.2016.03.009
24. Guillot A, Tacke F. Liver macrophages: old dogmas and new insights. *Hepatol Commun*. (2019) 3:730–43. doi: 10.1002/hep4.1356
25. Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology*. (2011) 141:1572–85. doi: 10.1053/j.gastro.2011.09.002
26. Dunn W, Shah VH. Pathogenesis of alcoholic liver disease. *Clin Liver Dis*. (2016) 20:445–56. doi: 10.1016/j.cld.2016.02.004
27. Deng Q-G, She H, Cheng JH, French SW, Koop DR, Xiong S, et al. Steatohepatitis induced by intragastric overfeeding in mice. *Hepatology*. (2005) 42:905–14. doi: 10.1002/hep.20877
28. Karakucuk I, Dilly SA, Maxwell JD. Portal tract macrophages are increased in alcoholic liver disease. *Histopathology*. (1989) 14:245–53. doi: 10.1111/j.1365-2559.1989.tb02143.x
29. Koop DR, Klopfenstein B, Iimuro Y, Thurman RG. Gadolinium chloride blocks alcohol-dependent liver toxicity in rats treated chronically with intragastric alcohol despite the induction of CYP2E1. *Mol Pharmacol*. (1997) 51:944–50. doi: 10.1124/mol.51.6.944
30. Schnabl B, Brenner DA. Interactions between the intestinal microbiome and liver diseases. *Gastroenterology*. (2014) 146:1513–24. doi: 10.1053/j.gastro.2014.01.020
31. Enomoto N, Ikejima K, Yamashina S, Hirose M, Shimizu H, Kitamura T, et al. Kupffer cell sensitization by alcohol involves increased permeability to gut-derived endotoxin. *Alcohol Clin Exp Res*. (2001) 25(6 Suppl):51S−4S. doi: 10.1111/j.1530-0277.2001.tb02418.x
32. Ju C, Mandrekar P. Macrophages and alcohol-related liver inflammation. *Alcohol Res*. (2015) 37:251–62.
33. Mandrekar P, Ambade A, Lim A, Szabo G, Catalano D. An essential role for monocyte chemoattractant protein-1 in alcoholic liver injury: regulation of proinflammatory cytokines and hepatic steatosis in mice. *Hepatology*. (2011) 54:2185–97. doi: 10.1002/hep.24599
34. Nagy LE. Recent insights into the role of the innate immune system in the development of alcoholic liver disease. *Exp Biol Med*. (2003) 228:882–90. doi: 10.1177/153537020322800803
35. Saikia P, Bellos D, McMullen MR, Pollard KA, de la Motte C, Nagy LE. MicroRNA 181b-3p and its target importin α5 regulate toll-like receptor 4 signaling in Kupffer cells and liver injury in mice in response to ethanol. *Hepatology*. (2017) 66:602–15. doi: 10.1002/hep.29144
36. Bala S, Marcos M, Kodys K, Csak T, Catalano D, Mandrekar P, et al. Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor α (TNFα) production via increased mRNA half-life in alcoholic liver disease. *J Biol Chem*. (2011) 286:1436–44. doi: 10.1074/jbc.M110.145870
37. Bala S, Csak T, Saha B, Zatsiorsky J, Kodys K, Catalano D, et al. The pro-inflammatory effects of miR-155 promote liver fibrosis and alcohol-induced steatohepatitis. *J Hepatol*. (2016) 64:1378–87. doi: 10.1016/j.jhep.2016.01.035
38. Petrasek J, Iracheta-Vellve A, Saha B, Satishchandran A, Kodys K, Fitzgerald KA, et al. Metabolic danger signals, uric acid and ATP, mediate inflammatory cross-talk between hepatocytes and immune cells in alcoholic liver disease. *J Leukoc Biol*. (2015) 98:249–56. doi: 10.1189/jlb.3AB1214-590R
39. Verma VK, Li H, Wang R, Hirsova P, Mushref M, Liu Y, et al. Alcohol stimulates macrophage activation through caspase-dependent hepatocyte derived release of CD40L containing extracellular vesicles. *J Hepatol*. (2016) 64:651–60. doi: 10.1016/j.jhep.2015.11.020
40. Thakur V, Pritchard MT, McMullen MR, Wang Q, Nagy LE. Chronic ethanol feeding increases activation of NADPH oxidase by lipopolysaccharide in rat Kupffer cells: role of increased reactive oxygen in LPS-stimulated ERK1/2 activation and TNF-alpha production. *J Leukoc Biol*. (2006) 79:1348–56. doi: 10.1189/jlb.1005613
41. Kono H, Rusyn I, Yin M, Gabele E, Yamashina S, Dikalova A, et al. NADPH oxidase-derived free radicals are key oxidants in alcohol-induced liver disease. *J Clin Invest*. (2000) 106:867–72. doi: 10.1172/JCI9020
42. Denaës T, Lodder J, M.-Chobert N, Ruiz I, J.-Pawlotsky M, Lotersztajn S, et al. The cannabinoid receptor 2 protects against alcoholic liver disease via a macrophage autophagy-dependent pathway. *Sci Rep*. (2016) 6:28806. doi: 10.1038/srep28806
43. Wang M, You Q, Lor K, Chen F, Gao B, Ju C. Chronic alcohol ingestion modulates hepatic macrophage populations and functions in mice. *J Leukoc Biol*. (2014) 96:657–65. doi: 10.1189/jlb.6A0114-004RR
44. Machado MV, Diehl AM. Pathogenesis of nonalcoholic steatohepatitis. *Gastroenterology*. (2016) 150:1769–77. doi: 10.1053/j.gastro.2016.02.066
45. Kazankov K, Jorgensen SMD, Thomsen KL, Moller HJ, Vilstrup H, George J, et al. The role of macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Nat Rev Gastroenterol Hepatol*. (2019) 16:145–59. doi: 10.1038/s41575-018-0082-x
46. Hellerbrand C, Stefanovic B, Giordano F, Burchardt ER, Brenner DA. The role of TGFβ1 in initiating hepatic stellate cell activation *in vivo*. *J Hepatol*. (1999) 30:77–87. doi: 10.1016/S0168-8278(99)80010-5
47. Nakamura T, Sakata R, Ueno T, Sata M, Ueno H. Inhibition of transforming growth factor β prevents progression of liver fibrosis and enhances hepatocyte regeneration in dimethylnitrosamine-treated rats. *Hepatology*. (2000) 32:247–55. doi: 10.1053/jhep.2000.9109
48. Pradere J-P, Kluwe J, De Minicis S, Jiao J-J, G.-Gwak Y, Dapito DH, et al. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology*. (2013) 58:1461–73. doi: 10.1002/hep.26429
49. Oakley F, Meso M, Iredale JP, Green K, Marek CJ, Zhou X, et al. Inhibition of inhibitor of κB kinases stimulates hepatic stellate cell apoptosis and accelerated recovery from rat liver fibrosis. *Gastroenterology*. (2005) 128:108–20. doi: 10.1053/j.gastro.2004.10.003
50. Yu Y, Liu Y, An W, Song J, Zhang Y, Zhao X. STING-mediated inflammation in Kupffer cells contributes to progression of nonalcoholic steatohepatitis. *J Clin Invest*. (2019) 129:546–55. doi: 10.1172/JCI121842
51. Du X, Wu Z, Xu Y, Liu Y, Liu W, Wang T, et al. Increased Tim-3 expression alleviates liver injury by regulating macrophage activation in MCD-induced NASH mice. *Cell Mol Immunol*. (2018) 16:878–86. doi: 10.1038/s41423-018-0032-0
52. Odegaard JI, Ricardo-Gonzalez RR, Red Eagle A, Vats D, Morel CR, Goforth MH, et al. Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. *Cell Metab*. (2008) 7:496–507. doi: 10.1016/j.cmet.2008.04.003
53. Krenkel O, Hundertmark J, Abdallah AT, Kohlhepp M, Puengel T, Roth T, et al. Myeloid cells in liver and bone marrow acquire a functionally distinct inflammatory phenotype during obesity-related steatohepatitis. *Gut*. (2019). doi: 10.1136/gutjnl-2019-318382. [Epub ahead of print].
54. Hirsova P, Ibrahim SH, Krishnan A, Verma VK, Bronk SF, Werneburg NW, et al. Lipid-induced signaling causes release of inflammatory extracellular vesicles from hepatocytes. *Gastroenterology*. (2016) 150:956–67. doi: 10.1053/j.gastro.2015.12.037
55. Guo Q, Furuta K, Lucien F, Gutierrez Sanchez LH, Hirsova P, Krishnan A, et al. Integrin β1-enriched extracellular vesicles mediate monocyte adhesion and promote liver inflammation in murine NASH. *J Hepatol*. (2019) 71:1193–205. doi: 10.1016/j.jhep.2019.07.019
56. Ibrahim SH, Hirsova P, Tomita K, Bronk SF, Werneburg NW, Harrison SA, et al. Mixed lineage kinase 3 mediates release of C-X-C motif ligand

10-bearing chemotactic extracellular vesicles from lipotoxic hepatocytes. *Hepatology.* (2016) 63:731–44. doi: 10.1002/hep.28252
57. Xuan W, Qu Q, Zheng B, Xiong S, Fan G-H. The chemotaxis of M1 and M2 macrophages is regulated by different chemokines. *J Leukoc Biol.* (2015) 97:61–9. doi: 10.1189/jlb.1A0314-170R
58. Friedman SL, Ratziu V, Harrison SA, Abdelmalek MF, Aithal GP, Caballeria J, et al. A randomized, placebo-controlled trial of cenicriviroc for treatment of nonalcoholic steatohepatitis with fibrosis. *Hepatology.* (2018) 67:1754–67. doi: 10.1002/hep.29477
59. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest.* (2006) 116:3015–25. doi: 10.1172/JCI28898
60. McGuinness PH, Painter D, Davies S, McCaughan GW. Increases in intrahepatic CD68 positive cells, MAC387 positive cells, and proinflammatory cytokines (particularly interleukin 18) in chronic hepatitis C infection. *Gut.* (2000) 46:260–9. doi: 10.1136/gut.46.2.260
61. Bility MT, Cheng L, Zhang Z, Luan Y, Li F, Chi L, et al. Hepatitis B virus infection and immunopathogenesis in a humanized mouse model: induction of human-specific liver fibrosis and M2-like macrophages. *PLoS Pathog.* (2014) 10:e1004032. doi: 10.1371/journal.ppat.1004032
62. Isorce N, Testoni B, Locatelli M, Fresquet J, Rivoire M, Luangsay S, et al. Antiviral activity of various interferons and pro-inflammatory cytokines in non-transformed cultured hepatocytes infected with hepatitis B virus. *Antiviral Res.* (2016) 130:36–45. doi: 10.1016/j.antiviral.2016.03.008
63. Tang TJ, Kwekkeboom J, Laman JD, Niesters HG, Zondervan PE, de Man RA, et al. The role of intrahepatic immune effector cells in inflammatory liver injury and viral control during chronic hepatitis B infection. *J Viral Hepat.* (2003) 10:159–67. doi: 10.1046/j.1365-2893.2003.00412.x
64. Kolios G, Valatas V, Kouroumalis E. Role of Kupffer cells in the pathogenesis of liver disease. *World J Gastroenterol.* (2006) 12:7413–20. doi: 10.3748/wjg.v12.i46.7413
65. Faure-Dupuy S, Delphin M, Aillot L, Dimier L, Lebossé F, Fresquet J, et al. Hepatitis B Virus-induced modulation of liver macrophage function promotes hepatocyte infection. *J Hepatol.* (2019) 71:1086–98. doi: 10.1016/j.jhep.2019.06.032
66. Boltjes A, Movita D, Boonstra A, Woltman AM. The role of Kupffer cells in hepatitis B and hepatitis C virus infections. *J Hepatol.* (2014) 61:660–71. doi: 10.1016/j.jhep.2014.04.026
67. Hösel M, Quasdorff M, Wiegmann K, Webb D, Zedler U, Broxtermann M, et al. Not interferon, but interleukin-6 controls early gene expression in hepatitis B virus infection. *Hepatology.* (2009) 50:1773–82. doi: 10.1002/hep.23226
68. Tan AT, Koh S, Goh W, Zhe HY, Gehring AJ, Lim SG, et al. A longitudinal analysis of innate and adaptive immune profile during hepatic flares in chronic hepatitis B. *J Hepatol.* (2010) 52:330–9. doi: 10.1016/j.jhep.2009.12.015
69. Shrivastava S, Mukherjee A, Ray R, Ray RB. Hepatitis C virus induces interleukin-1β (IL-1β)/IL-18 in circulatory and resident liver macrophages. *J Virol.* (2013) 87:12284–90. doi: 10.1128/JVI.01962-13
70. Hosomura N, Kono H, Tsuchiya M, Ishii K, Ogiku M, Matsuda M, et al. HCV-related proteins activate Kupffer cells isolated from human liver tissues. *Dig Dis Sci.* (2011) 56:1057–64. doi: 10.1007/s10620-010-1395-y
71. Chang S, Dolganiuc A, Szabo G. Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins. *J Leukoc Biol.* (2007) 82:479–87. doi: 10.1189/jlb.0207128
72. Negash AA, Ramos HJ, Crochet N, Lau DTY, Doehle B, Papic N, et al. IL-1β production through the NLRP3 inflammasome by hepatic macrophages links hepatitis C virus infection with liver inflammation and disease. *PLoS Pathog.* (2013) 9:e1003330. doi: 10.1371/journal.ppat.1003330
73. Heydtmann M. Macrophages in hepatitis B and hepatitis C virus infections. *J Virol.* (2009) 83:2796–802. doi: 10.1128/JVI.00996-08
74. Salazar-Mather TP, Hokeness KL. Cytokine and chemokine networks: pathways to antiviral defense. *Curr Top Microbiol Immunol.* (2006) 303:29–46. doi: 10.1007/978-3-540-33397-5_2
75. Yoneyama H, Ichida T. Recruitment of dendritic cells to pathological niches in inflamed liver. *Med Mol Morphol.* (2005) 38:136–41. doi: 10.1007/s00795-005-0289-0
76. Li H, Zheng H-W, Chen H, Xing Z-Z, You H, Cong M, et al. Hepatitis B virus particles preferably induce Kupffer cells to produce TGF-β1 over pro-inflammatory cytokines. *Dig Liver Dis.* (2012) 44:328–33. doi: 10.1016/j.dld.2011.11.005
77. Sasaki R, Devhare PB, Steele R, Ray R, Ray RB. Hepatitis C virus–induced CCL5 secretion from macrophages activates hepatic stellate cells. *Hepatology.* (2017) 66:746–57. doi: 10.1002/hep.29170
78. Tu Z, Pierce RH, Kurtis J, Kuroki Y, Crispe IN, Orloff MS. Hepatitis C virus core protein subverts the antiviral activities of human Kupffer cells. *Gastroenterology.* (2010) 138:305–14. doi: 10.1053/j.gastro.2009.09.009
79. Nebbia G, Peppa D, Schurich A, Khanna P, Singh HD, Cheng Y, et al. Upregulation of the Tim-3/galectin-9 pathway of T cell exhaustion in chronic hepatitis B virus infection. *PLoS ONE.* (2012) 7:e47648. doi: 10.1371/journal.pone.0047648
80. Ding T, Xu J, Wang F, Shi M, Zhang Y, Li S-P, et al. High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection. *Hum Pathol.* (2009) 40:381–9. doi: 10.1016/j.humpath.2008.08.011
81. Schneider C, Teufel A, Yevsa T, Staib F, Hohmeyer A, Walenda G, et al. Adaptive immunity suppresses formation and progression of diethylnitrosamine-induced liver cancer. *Gut.* (2012) 61:1733–43. doi: 10.1136/gutjnl-2011-301116
82. Ritz T, Krenkel O, Tacke F. Dynamic plasticity of macrophage functions in diseased liver. *Cell Immunol.* (2018) 330:175–82. doi: 10.1016/j.cellimm.2017.12.007
83. Wan S, Kuo N, Kryczek I, Zou W, Welling TH. Myeloid cells in hepatocellular carcinoma. *Hepatology.* (2015) 62:1304–12. doi: 10.1002/hep.27867
84. Capece D, Fischietti M, Verzella D, Gaggiano A, Cicciarelli G, Tessitore A, et al. The inflammatory microenvironment in hepatocellular carcinoma: a pivotal role for tumor-associated macrophages. *Biomed Res Int.* (2013) 2013:187204. doi: 10.1155/2013/187204
85. Yeung OW, Lo CM, Ling CC, Qi X, Geng W, Li CX, et al. Alternatively activated (M2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. *J Hepatol.* (2015) 62:607–16. doi: 10.1016/j.jhep.2014.10.029
86. Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cell Mol Immunol.* (2016) 13:316–27. doi: 10.1038/cmi.2015.104
87. Li Y-W, Qiu S-J, Fan J, Gao Q, Zhou J, Xiao Y-S, et al. Tumor-infiltrating macrophages can predict favorable prognosis in hepatocellular carcinoma after resection. *J Cancer Res Clin Oncol.* (2008) 135:439. doi: 10.1007/s00432-008-0469-0
88. Kang TW, Yevsa T, Woller N, Hoenicke L, Wuestefeld T, Dauch D, et al. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature.* (2011) 479:547–51. doi: 10.1038/nature10599
89. You Q, Cheng L, Kedl RM, Ju C. Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. *Hepatology.* (2008) 48:978–90. doi: 10.1002/hep.22395
90. Kuang DM, Zhao Q, Peng C, Xu J, Zhang JP, Wu C, et al. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med.* (2009) 206:1327–37. doi: 10.1084/jem.20082173
91. Wu K, Kryczek I, Chen L, Zou W, Welling TH. Kupffer cell suppression of CD8+ T cells in human hepatocellular carcinoma is mediated by B7-H1/programmed death-1 interactions. *Cancer Res.* (2009) 69:8067–75. doi: 10.1158/0008-5472.CAN-09-0901
92. Li H, Wu K, Tao K, Chen L, Zheng Q, Lu X, et al. Tim-3/galectin-9 signaling pathway mediates T-cell dysfunction and predicts poor prognosis in patients with hepatitis B virus-associated hepatocellular carcinoma. *Hepatology.* (2012) 56:1342–51. doi: 10.1002/hep.25777
93. Ho CC, Liao WY, Wang CY, Lu YH, Huang HY, Chen HY, et al. TREM-1 expression in tumor-associated macrophages and clinical outcome in lung cancer. *Am J Respir Crit Care Med.* (2008) 177:763–70. doi: 10.1164/rccm.200704-641OC
94. Wu J, Li J, Salcedo R, Mivechi NF, Trinchieri G, Horuzsko A. The proinflammatory myeloid cell receptor TREM-1 controls Kupffer cell

activation and development of hepatocellular carcinoma. *Cancer Res.* (2012) 72:3977. doi: 10.1158/0008-5472.CAN-12-0938
95. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol.* (2011) 11:762–74. doi: 10.1038/nri3070
96. Elsegood CL, Chan CW, Degli-Esposti MA, Wikstrom ME, Domenichini A, Lazarus K, et al. Kupffer cell–monocyte communication is essential for initiating murine liver progenitor cell–mediated liver regeneration. *Hepatology.* (2015) 62:1272–84. doi: 10.1002/hep.27977
97. Eggert T, Wolter K, Ji J, Ma C, Yevsa T, Klotz S, et al. Distinct functions of senescence-associated immune responses in liver tumor surveillance and tumor progression. *Cancer Cell.* (2016) 30:533–47. doi: 10.1016/j.ccell.2016.09.003
98. Zhou S-L, Zhou Z-J, Hu Z-Q, Huang X-W, Wang Z, Chen E-B, et al. Tumor-associated neutrophils recruit macrophages and T-regulatory cells to promote progression of hepatocellular carcinoma and resistance to Sorafenib. *Gastroenterology.* (2016) 150:1646–58.e17. doi: 10.1053/j.gastro.2016.02.040
99. Lim SY, Yuzhalin AE, Gordon-Weeks AN, Muschel RJ. Tumor-infiltrating monocytes/macrophages promote tumor invasion and migration by upregulating S100A8 and S100A9 expression in cancer cells. *Oncogene.* (2016) 35:5735–45. doi: 10.1038/onc.2016.107
100. Li X, Yao W, Yuan Y, Chen P, Li B, Li J, et al. Targeting of tumour-infiltrating macrophages via CCL2/CCR2 signalling as a therapeutic strategy against hepatocellular carcinoma. *Gut.* (2017) 66:157. doi: 10.1136/gutjnl-2015-310514
101. Lesokhin AM, Hohl TM, Kitano S, Cortez C, Hirschhorn-Cymerman D, Avogadri F, et al. Monocytic CCR2(+) myeloid-derived suppressor cells promote immune escape by limiting activated CD8 T-cell infiltration into the tumor microenvironment. *Cancer Res.* (2012) 72:876–86. doi: 10.1158/0008-5472.CAN-11-1792
102. Zhao Y, Wu T, Shao S, Shi B, Zhao Y. Phenotype, development, and biological function of myeloid-derived suppressor cells. *Oncoimmunology.* (2015) 5:e1004983. doi: 10.1080/2162402X.2015.1004983
103. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity.* (2014) 40:274–88. doi: 10.1016/j.immuni.2014.01.006
104. Ramachandran P, Dobie R, Wilson-Kanamori JR, Dora EF, Henderson EP, Luu NT, et al. Resolving the fibrotic niche of human liver cirrhosis at single-cell level. *Nature.* (2019) 575:512–8. doi: 10.1038/s41586-019-1631-3
105. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity.* (2014) 41:14–20. doi: 10.1016/j.immuni.2014.06.008
106. Mandal M, Gardner CR, Sun R, Choi H, Lad S, Mishin V, et al. The spleen as an extramedullary source of inflammatory cells responding to acetaminophen-induced liver injury. *Toxicol Appl Pharmacol.* (2016) 304:110–20. doi: 10.1016/j.taap.2016.04.019
107. Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N, et al. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science.* (2011) 332:1284–8. doi: 10.1126/science.1204351

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REVIEW
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Innate Immunity and Pathogenesis of Biliary Atresia

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Biliary atresia (BA) is a devastating fibro-inflammatory disease characterized by the obstruction of extrahepatic and intrahepatic bile ducts in infants that can have fatal consequences, when not treated in a timely manner. It is the most common indication of pediatric liver transplantation worldwide and the development of new therapies, to alleviate the need of surgical intervention, has been hindered due to its complexity and lack of understanding of the disease pathogenesis. For that reason, significant efforts have been made toward the development of experimental models and strategies to understand the etiology and disease mechanisms and to identify novel therapeutic targets. The only characterized model of BA, using a Rhesus Rotavirus Type A infection of newborn BALB/c mice, has enabled the identification of key cellular and molecular targets involved in epithelial injury and duct obstruction. However, the establishment of an unleashed chronic inflammation followed by a progressive pathological wound healing process remains poorly understood. Like T cells, macrophages can adopt different functional programs [pro-inflammatory (M1) and resolutive (M2) macrophages] and influence the surrounding cytokine environment and the cell response to injury. In this review, we provide an overview of the immunopathogenesis of BA, discuss the implication of innate immunity in the disease pathogenesis and highlight their suitability as therapeutic targets.

Keywords: biliary atresia, liver fibrosis, rotavirus, innate immunity, macrophages

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INTRODUCTION

Biliary atresia (BA) is a devastating obliterative cholangiopathy that affects exclusively infants and is characterized by a progressive fibro-inflammatory obstruction of the extrahepatic and intrahepatic bile ducts that can lead to cirrhosis and liver failure (1–4). BA occurs in 1 out of 15,000 births in the US (5), affecting all ethnic groups, (6) and with a higher frequency in girls (7). Despite its low incidence, BA is the most common cause of neonatal cholestasis (3), end-stage liver disease in children and the number one indication of pediatric liver transplant worldwide (8, 9). The first disease symptoms include jaundice, alcoholic stools, dark urines (3), and high levels of serum bilirubin (10). A *conclusive* diagnosis of BA is based on an exploratory surgery where obstruction of the extrahepatic biliary tree can be observed and confirmed by a histological analysis of liver or biliary tissue biopsy (3). At the time of diagnosis, about 60 days of life on average (4), the obstructed extrahepatic remnants are removed and hepatoportoenterostomy (HPE, called Kasai) is performed to restore the bile flow (11). However, even if the Kasai procedure is performed during

the first month of life and the cholestasis is resolved, bile duct proliferation, and fibrosis persist (9) resulting in the development of variable degrees of liver fibrosis, cirrhosis, portal hypertension, or other severe hepatic complications (12). Notably, the long-term survival of BA patients has extraordinarily improved in the last decades—from 70% in the 1990s to 80–90% in 2009 (13)—but the treatment still relies on surgery (HPE, transplantation), which is palliative, thereby highlighting the necessity of developing novel targeted therapies to prevent or reverse liver injury.

CLASSIFICATION AND MOLECULAR SIGNATURES

Traditionally, BA patients were divided into "embryonic/developmental" BA (<20%) and "perinatal/acquired" BA (> 80%) depending on their onset (14–16). The former is believed to originate during the first trimester of pregnancy and the accompanying clinical features suggest a developmental origin (4), the latter is thought to appear shortly after birth when the first symptoms become recognizable (10). The presence of splenic malformations—polysplenia but also asplenia—is characteristic of the Biliary Atresia Splenic Malformation (BASM) syndrome, the most representative form of embryonic BA (about 10%). The infants within this group were found to have a worse prognosis than infants with isolated BA (17). The remaining sub-group comprises patients with at least one non-splenic malformation. This group is also often included in the category of non-syndromic BA, since the presence of the underlying defects does not necessarily worsen the disease or implicates different mechanisms of pathogenesis (11, 18). Notably, BASM patients may also have another concomitant defect, such as cardiovascular and laterality defects (17).

In 2012, Davenport proposed the latest reference classification incorporating the cytomegalovirus (CMV)-associated and cystic BA variants to the aforementioned non-syndromic BA and BASM groups (19). CMV-associated BA refers to a subgroup of infants whose liver biopsies stained positive for immunoglobulin M (IgM) antibodies against CMV. The presence of these antibodies has been linked to the poorest HPE outcome and highest mortality, and the tissue biopsies revealed an exacerbated pro-inflammatory response (20): the predominant cellular profile observed in most of the BA patients (16). By contrast, cystic BA, an anatomic variant in which a cyst is formed close to the site of obstruction and a Th2-response is primed, was associated with an improved drainage after HPE and a better long-term outcome (21).

ETIOLOGY

The etiology of BA is heterogeneous and has not been fully elucidated yet. Diverse theories regarding the causes of the disease have been formulated, including embryonic or developmental abnormalities (17, 21), exposure to exogenous triggers such as viruses or toxins (16, 22), immune immaturity (11, 23), immune dysregulation (24, 25), and autoimmunity (26–29). Furthermore, numerous susceptibility factors—such as genetic predisposition (30), maternal diabetes (17), or microchimerism (31)—have also been implicated in the pathogenesis of the disease. This complex cocktail of variables and factors supports the claim that biliary atresia is not a disease with a single etiology but a combination of different phenotypes that share certain clinical features, such as the obliteration of the biliary tree early in life (32).

Animal Models and Etiological Agents

The characteristic lesions of BA such as the obstruction of the extrahepatic biliary tree and cholestasis, have been successfully reproduced and investigated in several animal models—such as lamb, calf, zebrafish, and mouse. The first three forms of experimental BA in lamb, calf and zebrafish are induced through toxins, while the murine models are achieved upon viral infection (5, 33, 34).

One of the first observations of BA-like pathologies in animals was reported in the Australian outbreak in 1964, 1988, and 2007 when lambs were born with cholestasis after pregnant livestock was exposed to unidentified toxic environmental factors in extreme drought conditions (1, 22, 35), which arose the suspicion that the toxic effect could come from the grass. A group of scientists from the university of Pennsylvania imported a plant species characteristic of that area and used zebrafish bioassays to identify the substance responsible: an isoflavonoid that they named biliatresone (22). This toxic compound, capable of inducing biliary atresia phenotype, is the basis of the theory that implicates hepatotoxins as etiological agents.

The other leading theory about the origin of the disease points toward a viral insult (16, 36). The first implication of an hepatotropic virus as causative factor in BA was suggested by Benjamin Landing (37). Despite the initial contradictory findings regarding the presence and role of reovirus in BA (38–41), numerous viruses have been implicated in the pathology of the disease and evidence of preceding viral infection—MxA proteins (Myxovirus resistance protein 1)—could be found even in the absence of viral material (42–44). Whether the virus is the primary causative factor or an accidental secondary event remains unclear (44, 45).

Rhesus Rotavirus-Induced Murine Model

Among all viruses, rhesus rotavirus type A (RRV) is the gold standard to model BA in mice. The use of this murine model has facilitated the study of different aspects of the disease, such as the underlying mechanisms of the pathogenesis (26–28, 46–50) or the identification of novel therapeutic targets (51). This experimental form of BA uses BALB/c newborn mice that, when challenged with RRV within the first hours of life (12–48 h), can recapitulate many aspects of human BA (52) such as time-restricted susceptibility to the viral infection, portal tract infiltration of inflammatory cells and obstruction of both extrahepatic and intrahepatic biliary tree (5, 34). This *in vivo* model allows for the comprehensive study of the early events of the disease that cannot be explored directly in humans, since they happen before the time of diagnosis. However, the RRV model is not yet suitable to study the progression of the disease

after duct obstruction, due to the high mortality rate of the mice before the development of liver fibrosis and related long-term complications (5). Previous studies have examined the fibrogenic response in RRV model and observed insufficient fibrosis (Ishak score 1–2) when determined at 2 weeks' time (**Figure 1A**) (53, 54). These limitations (e.g., high mortality and poor fibrogenic responses), however, could be tackled by optimizing the model induction using reassortant viruses. Recently, a novel RRV-TUCH rotavirus reassortant (TUCH for Tulane University and Cincinnati Children's Hospital) could recapitulate an obstructive jaundice phenotype with lower mortality rates when injected into newborn mice (54). This new model recapitulates the late events of the disease such as liver fibrosis (Ishak score 3–5) and showed a unique resemblance to the human BA, significantly different from CCl_4 and bile duct ligation models (54) (**Figure 1B**). This model, therefore, not only improves our current understanding about BA disease pathogenesis but will also contribute toward the identification of new therapeutic targets.

Other Virus Induced Models

Cytomegalovirus (CMV) has also been used to recapitulate BA in animal models (55). For instance, a regulatory T cell (Treg)-depleted neonatal mouse, when infected with low-dose CMV (LD-CMV) to study BA, induced extensive inflammation, atresia of intrahepatic bile ducts and partial obstruction of the extrahepatic bile ducts. Liver mononuclear cells showed increased percentages of CD3/CD8 T cells and serum autoantibodies (α-enolase) reactive to bile duct epithelial proteins, suggesting the involvement of cellular and humoral autoimmune responses in LD-CMV BA mouse model. There was also an increased hepatic expression of Th1-related genes (tumor necrosis factor α, TNF-α), interferon γ (IFN-γ)-activated genes (signal transducer and activator of transcription 1, STAT-1) and Th1 cytokines/chemokines (lymphotactin, interleukins IL-12p40 and macrophage inflammatory protein 1-alpha, MIP-1α).

Evidence of Viruses as a Causative Agent of BA

As mentioned earlier, viruses have been proposed as etiological agents in BA. These viruses activate pathways that might predispose certain individuals to develop the disease. In the animal model, the RRV Viral Protein 4 (VP4) gene has been demonstrated to be the major determining factor required for the pathogenesis of BA (49). Rotavirus strains with 87% or more homology to RRV's VP4 were capable of infecting murine bile ducts and inducing the disease as well as activating mononuclear cells, independent of viral titers (56). Further research led to the identification of a key amino acid sequence "SRL" in VP4, a sequence specific to those rotavirus strains that cause obstructive cholangiopathy (57). This tripeptide "SRL" on RRV VP4 was found to bind specifically to the cholangiocyte membrane protein heat shock cognate 70 (Hsc70), defining a novel binding site governing VP4 attachment (57). To gain insight into the mechanisms involved upon VP4-mediated infection, a reverse genetics system was developed to create a mutant of RRV with a single amino acid change in the VP4 protein and compared to that of wild-type RRV (where the arginine "R" in "SRL" region was replaced with glycine "G") (58). The mutant virus, when injected to mice, demonstrated reduced symptoms and lower mortality in neonatal mice, resulting in an attenuated form of biliary atresia indicating the importance of "SRL" region (57). This "SRL" peptide was also found either on the capsid or the attachment protein of other viruses including reovirus, cytomegalovirus, human papillomavirus, Epstein-Barr virus, bluetongue virus, polyomavirus, coronavirus, respiratory syncytial virus, adenovirus, rodent paramyxovirus, and herpes

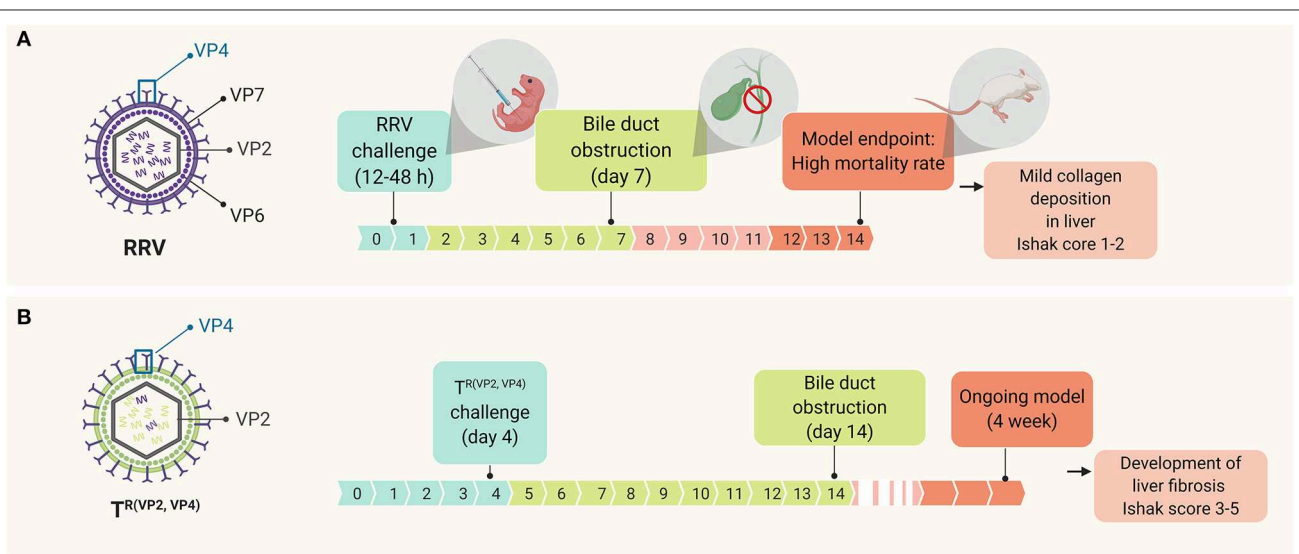

FIGURE 1 | Time line of events in the murine model of BA upon RRV challenging, depicting **(A)** the standard RRV model in comparison with **(B)** the modified model using a novel viral reassortant [$T^{R(VP2, VP4)}$]; this virus reassortant was engineered by replacing the VP2 and VP4 gene of TUCH for the corresponding RRV's VP2 and VP4.

simplex virus 1. Several of these (cytomegalovirus, Epstein-Barr virus, human papillomavirus, and reovirus) have been detected in explanted livers of infants with BA (59–63). Thus, this sequence in the above-mentioned viruses might be involved in cholangiocyte binding in a similar fashion to the RRV "SRL" peptide. Binding of these viruses to Hsc70 might activate the innate immune system through different pathways. The role of Hsc70 binding in human BA induction as a function of these proteins and their influence in oxidative stress and cell metabolism remain largely unexplored.

IMMUNOPATHOGENESIS OF BILIARY ATRESIA

Cholangiocyte Immunobiology

Biliary epithelial cells (cholangiocytes) are not only a physical barrier that drains the bile into the duodenum but they are also immunocompetent cells involved in tissue homeostasis, capable of recognizing microbial conserved motifs known as Pathogen Associated Molecular Patterns (PAMPs) through pattern-recognition receptors (PRRs) and initiating an inflammatory response (64–67). Four main families of PRRs have been described, including toll-like receptors (TLRs), retinoic acid inducible gene 1 (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs) (68).

From the ten types of TLRs that have been identified in mammals, at least 5 of them have been described in mice and human cholangiocytes (64). Among them, TLR-4 is responsible for sensing lipopolysaccharides (LPS) and TLR-3, 7, 8, and 9 are involved in recognition of viral and bacterial RNA or DNA. Activation of these receptors triggers an inflammatory response via Mitogen-activated protein kinases (MAPK), interferon regulatory factor 3 (IRF3) and/or nuclear factor κB (NF-κB) characterized by the production of type I interferons (IFNs) and/or pro-inflammatory cytokines. MAPK signaling is a multifunctional pathway that is pivotal in the innate immune response and viral infection. Among the three central members of the MAPK pathway, extracellular signal-regulated kinase (ERK) 1/2 and p38 activation play the most important roles in RRV infection of cholangiocytes as they seem to be involved in both viral replication and epithelial injury (69). Further studies revealed that ERK phosphorylation and calcium influx appear to be essential to RRV infection, and RRV's viral protein 6 (VP6) drives ERK phosphorylation (70).

TLRs depend on adaptor molecules– myeloid differentiation primary response 88 (MyD88) or toll/interleukin-1 receptor domain-containing adaptor protein (TRIF)—to effectively initiate and transduce the downstream signal to the nuclei, differentiating them into two main TLR signaling pathways (**Figure 2A**) (68). In the MyD88-dependent pathway (associated to TLR 1–5, except for TLR-3), the Interleukin-1 receptor-associated kinase (IRAK)-1, −2 and −4 upregulate the production of Type I IFNs and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) via MAPK, IRF3, and NF-κB pathways (65, 67, 68). It has been demonstrated that the pathogenesis of murine BA is independent of the MyD88 signaling pathway (71). In MyD88/IRAK-M independent pathway, the activation of TLR-3, 7/8 or 9, associated with the TRIF-dependent signaling, results in the activation of NF-κB and IRF3 signaling cascades (65, 68). This different level of regulation could explain why "endotoxin tolerance" to enteric bacteria can be induced in cultured cholangiocytes by treating them with TLR-4 ligands (like LPS) (72) but "viral tolerance" could not be achieved using the same approach (73).

The RLR family (74) is comprised of cytosolic sensors, including RIG-1 and melanoma differentiation-associated protein 5 (MDA-5) that are capable of binding to dsRNA (75–77). This interaction triggers a conformational change that exposes the two caspase activation and recruitment domains (CARDs) at their N-terminus, which are responsible to recruit the complementary protein mitochondrial antiviral-signaling protein (MAVS) and transduce the signal to the nuclei to produce type I interferons and pro-inflammatory cytokines (**Figure 2B**) (75, 78). NLRs (e.g., NLRP3), are also cytosolic innate immune receptors that are activated upon recognition of viral dsRNA. Rather than contributing to the initial events of the acute inflammatory response, they amplify the immune response, release late mediators (IL-1β, IL-18 and high mobility group box 1, HMGB-1) and regulate pyroptosis (pro-inflammatory programmed cell death) through the formation of inflammasomes (**Figure 2C**) (79).

The last group of PRRs described are the large family of CLRs. They are transmembrane receptors, with an immunoreceptor tyrosine-based activation motif (ITAM) or an immunoreceptor tyrosine-based inhibition motif (ITIM), that are able to induce a pro-inflammatory response or modulate it through a crosstalk with other PRRs such as TLRs. CLRs play a crucial role in maintaining immune homeostasis against pathogens and in mounting a pro-inflammatory and/or antiviral response (80–82). Alterations of CLRs have been implicated in different pathological conditions, including gastrointestinal cancers, autoimmune disorders, or allergies (82). It is known that cells from myeloid lineage such as dendritic cells (DCs) and macrophages, as well as some endothelial and epithelial cells, express CLRs; however, it has not been reported in biliary epithelium yet.

Although cholangiocytes play a central role in initiating an immune response upon exposure to the exogenous substances, they are however not capable of mounting an inflammation that is sufficient to induce chemotaxis and recapitulate the obstructing phenotype of BA without the involvement of macrophages and DCs (83–86).

Mechanisms of Epithelial Injury and Duct Obstruction

Upon viral infection, cholangiocytes, macrophages, and DCs (RRV cellular targets) trigger the anti-viral response through type I interferons in an autocrine and paracrine manner in both infected and surrounding cells to prevent the virus from spreading (5). In infected cells, type I IFNs promote biliary apoptosis by upregulation of tumor necrosis factor related

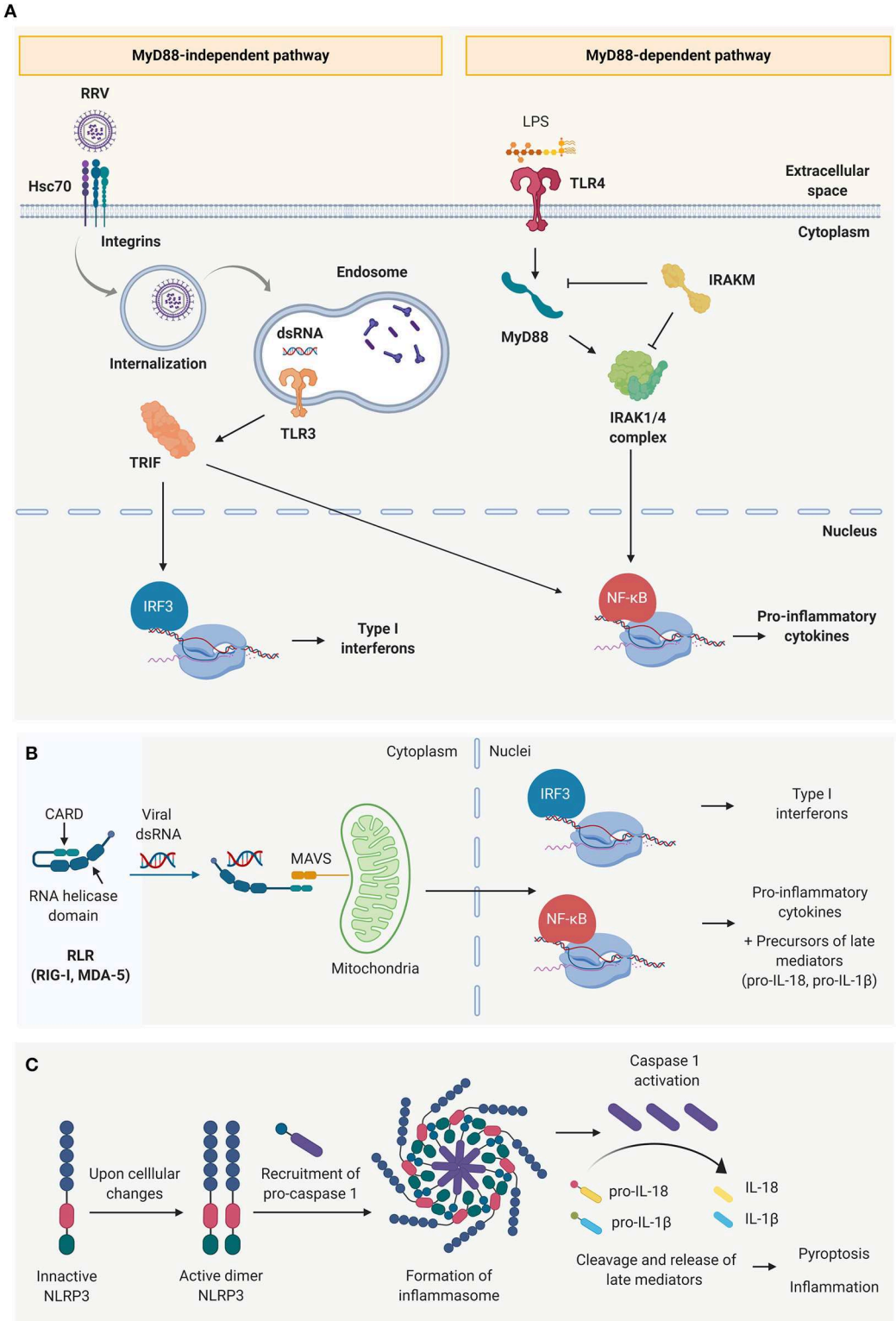

FIGURE 2 | Innate immune receptors present in cholangiocytes. **(A)** Toll-like receptors (TLRs) and schematic representation of the two main signaling pathways: the MyD88 dependent pathway (characteristic of all toll-like receptors except TLR 3) and MYD88 independent pathway (characteristic of TLR3). **(B)** Cytosolic viral sensing of Retinoic-acid-inducible gene I (RIG-I)-like receptors, capable of triggering a pro-inflammatory and antiviral response, and **(C)** nucleotide-binding oligomerization domain (NOD)-like receptors that have the ability to perpetuate the immune response through the formation of inflammasomes, induction of cell death and release of late mediators.

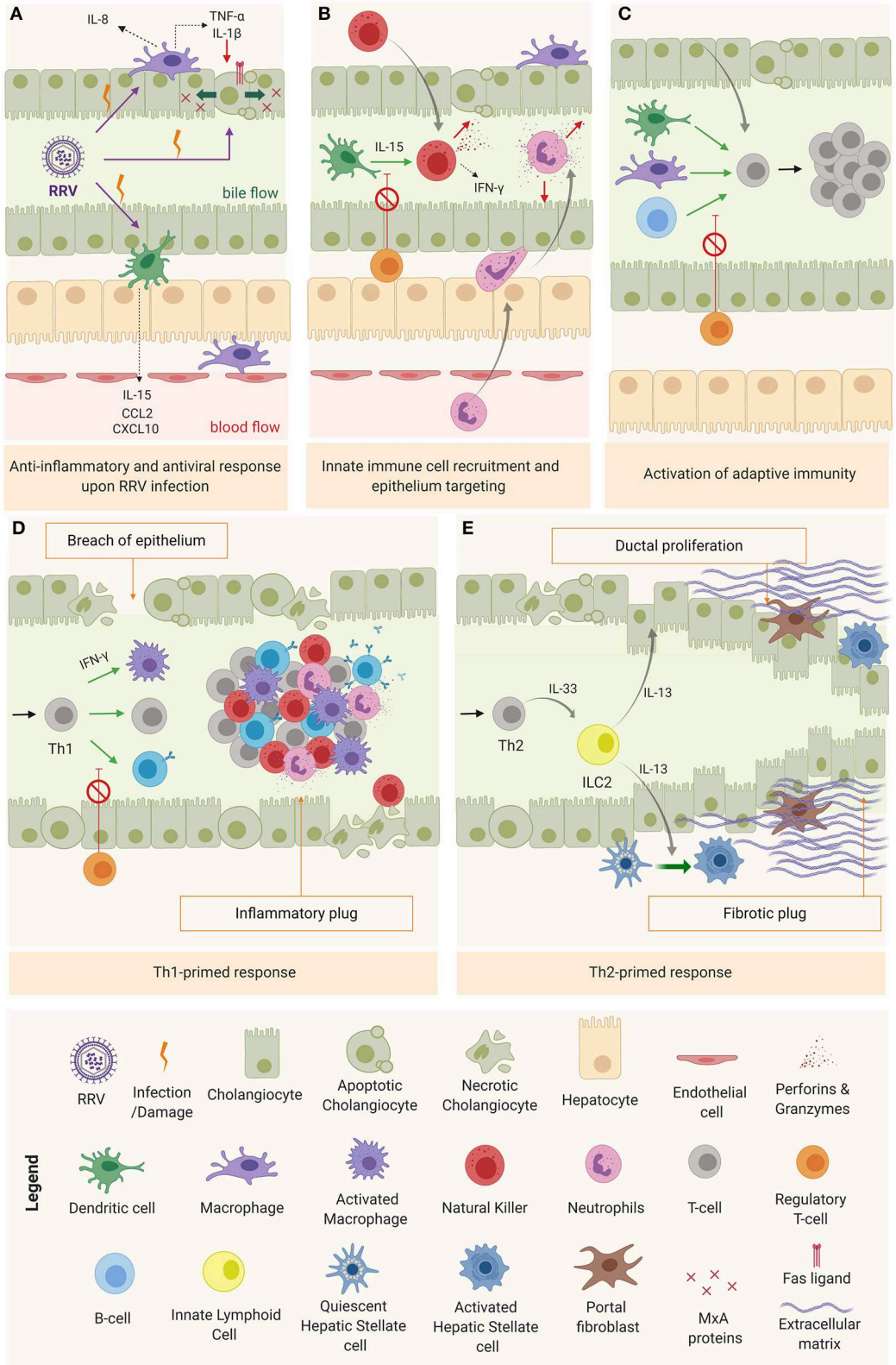

FIGURE 3 | Mechanism of obstruction in biliary atresia. **(A)** RRV infection and activation of the anti-inflammatory and anti-viral response. **(B)** Innate immune cell recruitment & tissue specific attack to epithelia. **(C)** Activation of adaptive immunity **(D)** Th1-primed polarization and alternatively **(E)** Th2 polarization.

apoptosis ligand (TRAIL) (TNF receptor p55) and CD95 (Fas/Apo1 ligand) (87). In surrounding tissue, IFNs trigger the production of antiviral proteins (Mx) that provide protection against viral infection (**Figure 3A**) (88). The production of pro-inflammatory cytokines and chemokines by cholangiocytes, macrophages and DCs creates the favorable microenvironment to recruit and activate inflammatory cells, and to promote an immune effector tissue-specific attack (**Figure 3B**) (84, 85, 89). Among the chemokines produced, the most relevant are IL-8 and IL-15. IL-8, mostly produced by macrophages but also by cholangiocytes (90), recruits and modulates the action of neutrophils (85), basophils, monocytes, and T cells (64, 67, 90); while IL-15, secreted primarily by DCs, attracts and regulates the activity of natural killer (NK), natural killer T (NKT), and gamma-delta cells (89). The recruited inflammatory effector cells are engaged to target specifically the biliary epithelium in a contact dependent manner (91), through IFN-γ-related cytokines (48) and/or cytotoxic agents (perforins, granzymes) (92). Recruited neutrophils produce reactive oxygen species (ROS), leukotrienes, and neutrophil defensins (90). NK cells, activated by DCs via IL-15 (89), induce cholangiocyte death in a contact-dependent manner through Natural killer group 2d (Nkg2d) ligand that interacts with ribonucleic acid export 1 (RAE1) receptors, expressed in infected cells (91) and via the secretion of IFN-γ, perforins, and granzymes (92). In a similar fashion, the cytotoxic power of neonatal $CD8^+$ T cells is exerted through cytotoxic agents (perforin, granzymes, IFN-γ) (92) and in a contact-dependent manner by invading the epithelium (27). Mechanistical studies using the RRV-infected BALB/c murine model showed that depletion of NK cells, blockage of the receptor Nkg2d or depletion of $CD8^+$ T cells (with impairment of IFN-γ mechanisms) reduced cholangiocyte death, evaded rupture of the epithelium and ultimately prevented the obstruction of the extrahepatic biliary tree (27, 91). Likewise, epithelial integrity was preserved by depleting plasmacytoid DCs or blocking the IL-15 signaling, responsible for NK cell activation (86, 89). These results highlight the specific role of DCs, NK, and $CD8^+$T cells in the model.

As the inflammation progresses without being resolved, DCs and macrophages interact chiefly with helper $CD4^+$ T cells (Th0) to promote their activation, oligoclonal expansion (93) and differentiation into a specialized phenotype depending on the predominant cytokine microenvironment at the time (**Figure 3C**). In most of BA patients, this microenvironment is pro-inflammatory (Th1), characterized by IFN-γ production and the activation of effector cells (macrophages, $CD8^+$ T cells and B cells) to perpetuate the tissue damage (**Figure 3D**) (11, 16). In some cases, the infants are not capable of mounting a Th1 response, therefore, the polarization primed is Th2, with IL-13 [produced by type 2 innate lymphoid cells (ILC2)] as a predominant cytokine, responsible for the tissue damage mediated by ductal proliferation and activation of hepatic stellate cell (HSCs) and portal fibroblasts. This is typically the case for the aforementioned cystic variant of BA (94), as depicted in **Figure 3E**.

Humoral Immunity

In contrast to T-cell polarization, very little is known about the implication of humoral immunity in the pathogenesis of BA. In the early stage of the disease, humoral-related genes (i.e., immunoglobulins) are transiently suppressed (95). However, B lymphocytes seem to play a role as antigen presenting cells for effector T cell activation as also shown in **Figure 3C**. An evidence for the role of B lymphocytes has been proposed in a study where the depletion of B-cells in experimental BA was associated with impaired effector T-cell activation and protection against biliary injury (96). Furthermore, humoral duct-specific autoimmunity has been demonstrated in experimental BA (26) but the role of B lymphocytes remains unclear in human BA. Human-based studies regarding humoral activity in BA include the description of immunoglobulins IgM and IgG deposits in the biliary epithelium basement membrane (97) and the detection of autoantibodies (28, 29). Lu et al. (28) detected autoantibodies against α-enolase in the RRV induced mouse model of BA and in serum samples from patients, indicating a role of humoral auto-immunity in disease pathogenesis. The cross-reactivity between an anti-enolase antibody and RRV proteins indicates that molecular mimicry might activate humoral autoimmunity in BA patients. However, further investigation is needed to provide more insight into the implication of humoral immunity in BA.

Immune Dysregulation

A subset of helper $CD4^+$ T cells known as regulatory T cells (Tregs)—that expresses CD25 and forkhead box P3 (FOXP3)—has a pivotal role in immunoregulation and induction of peripheral tolerance. Neonatal Tregs (98, 99) prevent the activation of autoreactive T cells and inhibit the action of several immunocompetent cells (B and T cells, macrophages, dendritic cells, and natural killer cells) (50, 98, 100, 101). In neonatal mice, Tregs populate the spleen from day 3 of life (102) which corresponds the susceptibility time window in the RRV model (100, 103). Moreover, adoptive transfer of Tregs to pups before RRV infection prevented the obstruction of the extrahepatic bile ducts (50, 100, 101). In infants with BA, gene expression of regulatory cytokines (IL-10, transforming growth factor β, TGF-β] and transcription factors (FOXP3) are upregulated in the liver (100), but there is a deficit in number of circulating Tregs in peripheral blood and their regulatory function seems to be impaired (25, 104). Even though the exact underlying mechanisms of Treg malfunctioning and immune dysregulation are not fully understood, epigenetic changes might play a major role. For instance, hypomethylation of FOXP3 promoter was associated with improper functioning of Tregs (25), while hypermethylation of DNA in lymphocytes elicited them to promote an exacerbated inflammatory response (24).

MECHANISMS OF POST-OBSTRUCTION: CHRONIC INFLAMMATION, DUCT PROLIFERATION, AND FIBROSIS

After obstruction, regardless of the restoration of the bile flow, the immune-mediated biliary damage persists (9) and the

initial Th1-predominant milieu shifts toward a Th2 with the simultaneous emergence of the Th17 subset (**Figure 4**).

On one hand, apoptotic and necrotic cells release endogenous molecules known as damage-associated molecular patterns (DAMPs)—recognizable by PRRs—as excessive damage or "danger signals" (68). One of these DAMPs is the interleukin IL-33 that, when released by cholangiocytes and hepatocytes, accumulates in the extracellular matrix (ECM) and promotes inflammation and fibrosis. High levels of IL-33 has been detected in serum and tissue biopsies in both patients and experimental BA (105). In this context, IL-33 in the liver is believed to engage with liver-resident innate helper cells (ILC2) that express IL-33 receptor (ST2 or IL-1R4) to produce pro-fibrotic Th2-related cytokines (IL-4, IL-5, IL-9, and IL-13) (106). Among them, IL-13 upregulates the expression of TGF-β and matrix metalloproteinase 9 (MMP9); activates HSCs via IL-4Ra and STAT6, promoting fibrosis in a TGF-β1/SMAD-independent mechanism (107); and stimulates collagen synthesis by myofibroblasts (activated HSCs and portal fibroblasts). Simultaneously, IL-33 was shown to drive duct proliferation in both intra- and extra-hepatic ducts (105). This IL-33-ILC2-IL13 axis is depicted in **Figure 4A**.

On the other hand, damaged cholangiocytes are shown to produce IL-1β, IL-6, and IL-23 (65). IL-1β, IL-6 are required for Th17 commitment, and IL-23 is needed for the maintenance of this phenotype (108). IL-17A is the representative cytokine of this panel, which induces the production of several pro-inflammatory cytokines and chemokines. Lages et al. identified Th17 cells as the main source of IL-17A after the obstruction of the biliary tree in experimental BA. In this study, a model of biliary injury perpetuation was proposed in which IL-17A stimulated cholangiocytes to produce C-C motif chemokine ligand 2 (CCL2) that recruited inflammatory macrophages expressing IL-17AR to target the epithelium (51), as shown in **Figure 4B**. In this model, depletion of Th17 cells or blockage of CCL2 prevented bile duct paucity and the number of Th17 cells correlated with the concentration of gamma glutamyl transpeptidase (GGT), a biochemical marker of bile duct injury (51). In BA patients, the presence of Th17 in the biliary tree and peripheral blood has been confirmed, as well as Th17-related markers in liver tissue

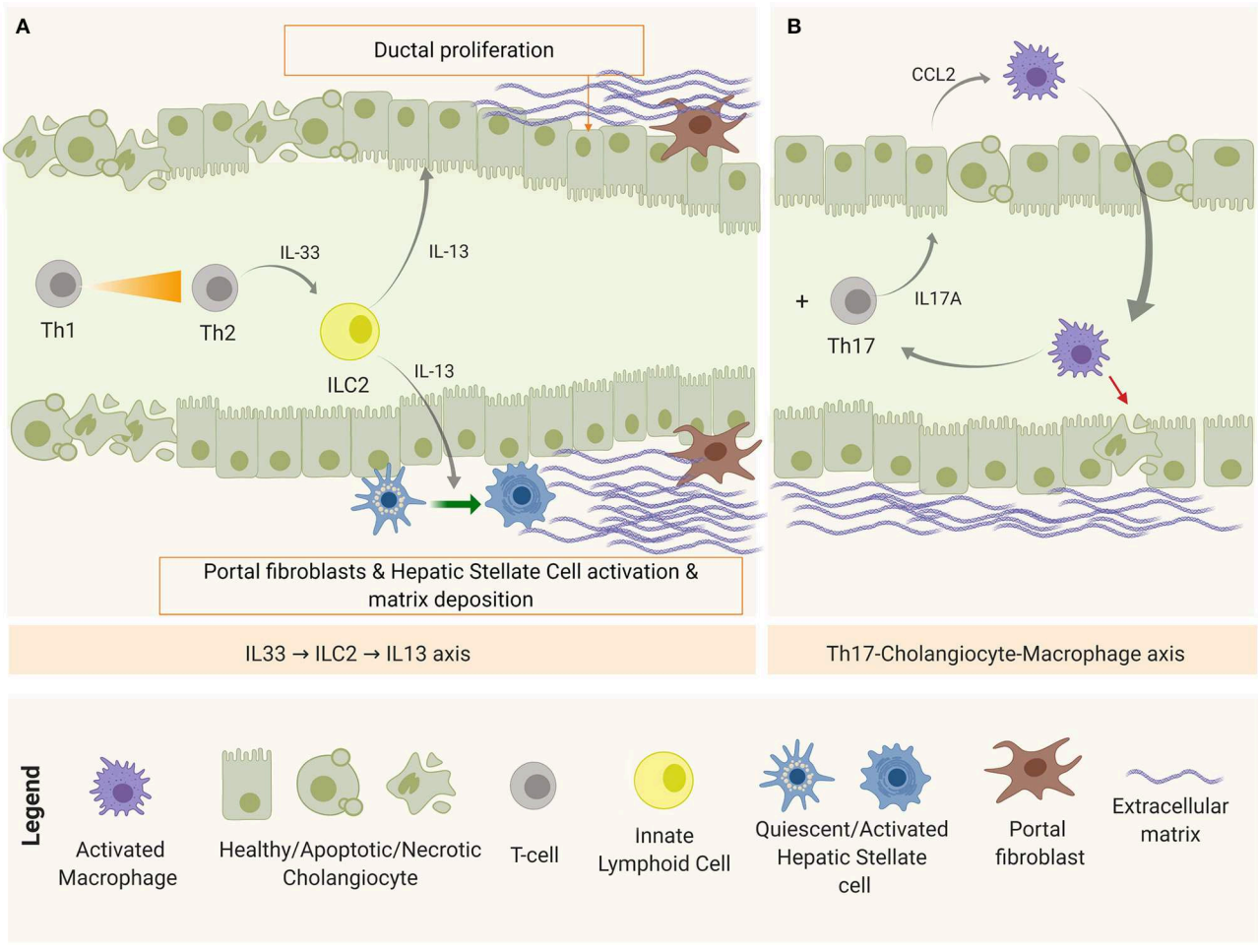

FIGURE 4 | Disease progression mechanisms after bile duct obstruction. **(A)** IL-33-ILC2-IL-13 axis, implicated in fibrosis and duct proliferation and **(B)** Th17-Macrophage axis, as a mechanism of chronic inflammation and damage perpetuation.

[IL-17A and retinoic acid-related orphan receptor (ROR)-γt] and serum IL-23. In addition, a high ratio between Th17 and Tregs has been characterized in peripheral blood (109), a trend that has also been observed in chronic liver diseases such as primary biliary cirrhosis (108).

In addition, damaged or pro-apoptotic as well as inflammatory cells (especially Kupffer cells and macrophages) can express or produce hedgehog (Hh) ligands under pathological conditions (110). Cholangiocytes stimulated with Hh ligands (in an autocrine or paracrine manner) produce a wide assortment of cytokines—including IL-6 and TGF-β (111)—and chemokines that attract different populations of inflammatory cells, including neutrophils, monocytes, and lymphocytes (112). Inflammatory cells stimulated by Hh ligands sustain inflammation, while activated HSCs continue to proliferate in response to this stimulus (113). Abnormal over-activation of the Hedgehog pathway has been observed in the context of chronic inflammation-related fibrosis (114, 115), human cholangiopathies (116), and biliary atresia (117). A characteristic Hh ligand in BA is osteopontin (OPN) that has been correlated with severity of the disease (118).

MACROPHAGES, MICROENVIRONMENT, AND AGE-RAGE

Like T cells, macrophages can adopt different polarization states depending on the surrounding tissue microenvironment (119). Characterization of these functional programs is important since they seem to have vast implications in the outcome of several chronic auto-inflammatory and degenerative diseases (120). Conventionally, they are divided into classically activated M1 (pro-inflammatory) and alternatively activated M2 (restorative) macrophages (119). Polarization into M1 macrophages is driven by activation of TLR signaling through LPS and IFN-γ challenge; while stimulation with regulatory cytokines (IL-4, IL-10) primes a M2 polarization. Several reports have pointed that, in many contexts, the dichotomy M1/M2 may not be sufficient to describe a relevant macrophage population because of its heterogeneity, the complexity of the activation stimuli, and surrounding tissue microenvironment (121–123). However, in the context of fibrosis, two distinct macrophage population have been described for its role in modulating the body response to chronic injury: pro-fibroinflammatory and resolutive macrophages, often associated with M1 and M2 features, respectively. These polarizations have the ability to influence the tissue microenvironment and with it, the net cellular response and outcome of the disease. For instance, pro-inflammatory macrophages, displaying high levels of inflammatory marker lymphocyte antigen 6 complex, locus C (Ly6C), are characterized by a high production of chemokines (such as CCL2) that attract inflammatory cells to the site of injury, pro-inflammatory cytokines (such as TNF-α and IL-1β) that perpetuate hepatic damage and TGF-β that activates HSCs into ECM-producing myofibroblasts. On the contrary, restorative macrophages, displaying low levels of Ly6C, seem to be responsible for inducing HSCs apoptosis (through TRAIL and MMP9), digesting the excess of ECM and promoting clearance of the profibrotic stimuli, thereby facilitating tissue regeneration (122–125). Both tissue-resident and monocyte-derived macrophages can acquire these functional programs. However, the latter is the predominant population during tissue injury (122), highlighting the relevance of infiltration of inflammatory cells in the course of the disease.

Pro-fibroinflammatory macrophages exhibit a wide assortment of mechanisms that allow them to activate and perpetuate inflammation and fibrosis in both TGFβ-dependent and independent circuits. One way to modulate the surrounding cellular response is by influencing the tissue microenvironment. An important component of this microenvironment is the level of oxidative stress, intimately linked to the Advanced Glycation End-Products (AGE)-Receptor of AGEs (RAGE) pathway (120, 122). AGEs refer to a heterogeneous group of toxic by-products that are a result of irreversible non-enzymatic reactions between sugars and proteins as consequence of elevated intra-cellular oxidative species. In normal physiological conditions, AGEs are produced in small amounts, released into the extracellular space, and cleared by specialized phagocytic cells: principally macrophages through scavenger receptors (**Figure 5A**). However, during chronic injury, under continuous oxidative stress, the production of AGEs is higher than their clearance and this leads to their accumulation in the extracellular space, affecting surrounding cells. Interaction of AGEs (or/and other RAGE ligands, such as S100 proteins and HMGB1) with their receptor triggers a signal transduction cascade through different pathways, resulting in numerous cellular responses such as inflammation, fibrosis, or apoptosis (120, 126, 127), as depicted in **Figure 5B**.

In the murine model, RRV has the ability to infect the macrophages, resulting in their activation (85). Activated pro-inflammatory macrophages are one of the main sources of AGEs but damaged cholangiocytes and hepatocytes have also been shown to produce several RAGE ligands in response to injury. In patients with BA, the serum levels of soluble RAGE has been correlated with the severity of the disease (128). A recent network analysis study involving the three main human cholangiopathies (including BA), identified a common connectome in which AGE-RAGE pathways occupy central nodes (129). Remarkably, we have observed an induction of oxidative species and production of AGE-RAGE ligands in RRV-infected cholangiocytes (unpublished work), which suggests an involvement of oxidative stress circuits from the onset of the disease.

THERAPEUTICS AND CLINICAL TRIALS

The routine treatments of BA patients after HPE are ursodeoxycholic acid, antibiotics, and fat-soluble vitamin formulations that have not substantially improved the outcomes of the disease. In a double-blind, placebo-controlled study (START trial) corticosteroid administration within 3 days of the HPE did not change the outcome of the BA cohort while increased the risk of serious adverse effects as compared

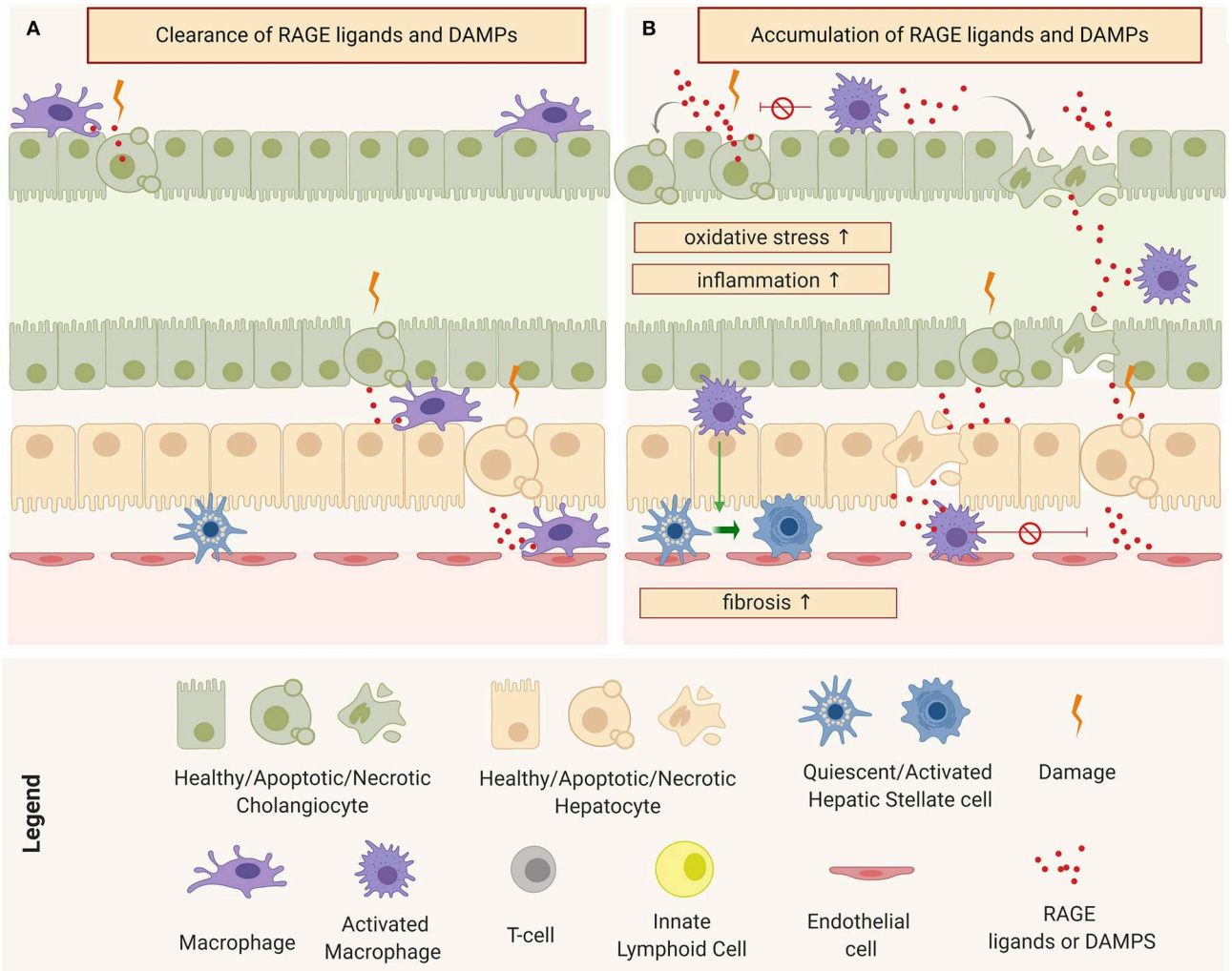

FIGURE 5 | Schematic representation of macrophages and tissue micro-environment: **(A)** Clearance of receptor of advanced glycation end products (RAGE) ligands and Damage-Associated Molecular Patterns (DAMPs) under physiological conditions and **(B)** accumulation of RAGE ligands and DAMPs and consequent perpetuation of damage through the induction of oxidative stress, inflammation, and fibrosis.

to placebo controls (130). Although corticosteroids in BA infants younger than 2 weeks of age did appear to improve biliary drainage, with pending data on native liver survival (131) suggesting a possibility of corticosteroids use on these subsets of infants. In the future, the agents which are currently being tested in cholestatic and fibrotic liver diseases in adults (132) can also be investigated in BA, such as the farnesoid X receptor (FXR) agonist, obeticholic acid, and the modified bile acid norursodeoxycholic acid, which are also currently used in primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) patients (133, 134). Other agent such as apical sodium-dependent bile acid transporter (ASBT) inhibitor may reduce bile acid burden in the liver. The two other agents that are currently used in clinics for pediatric liver diseases—bile acid sequestrants (cholestyramine or colesevelam) and ursodeoxycholic acid—are yet to be thoroughly tested in clinical trials in BA (135).

CONCLUSION AND FUTURE PROSPECTIVE

Due to the establishment of experimental models of BA, especially the RRV murine model, some of the driving mechanisms of epithelial injury and duct obstruction have been elucidated, and the corresponding key cellular and molecular targets have been identified. However, the real applicability of these targets for therapy is hindered due to the lack of early diagnosis and screening tools, and that many questions regarding the etiology of the disease remain unanswered. The molecular and cellular mechanisms in which the disease progresses are still under investigation. Increasing evidence suggests a deeper implication of intricated mechanisms of the innate immunity from the onset of the disease: namely, oxidative stress, altered metabolism, and induction of long-term/abnormal epigenetic changes. Among them, AGE-RAGE

pathway has attracted most of the attention since it encompasses key circuits involved in the pathogenesis of several chronic inflammatory and degenerative diseases, including biliary atresia. Further investigation is needed to determine the extent of implication of the AGE-RAGE pathway and its crosstalk with other fibro-inflammatory circuits. Because macrophages are one of the main drivers of AGE-RAGE and their functional polarizations seem to occupy a central role in the modulation of the tissue response and outcome in chronic conditions, future research should interrogate these cell populations in the context of biliary atresia. Imperatively, there is a need to develop new or improve existing experimental platforms to perform mechanistical studies of later events of the disease and facilitate the identification and implication of cell populations and pathways. In addition, deeper understanding of the model induction through other viruses and/or toxins could shed some light into the etiology of the disease and aid the development of new therapies to manage BA patients without the need of surgery.

AUTHOR CONTRIBUTIONS

AO-P drafted the manuscript. BD, HT, RB, GT, and SM supported the writing of the manuscript, implemented it, and ensured scientific quality. AO-P, RB, and SM designed the figures. AO-P, RB, GT, and SM made the final corrections. All authors corrected and approved the manuscript.

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REFERENCES

1. Davenport M. Biliary atresia: from Australia to the zebrafish. *J Pediatr Surg.* (2016) 51:200–5. doi: 10.1016/j.jpedsurg.2015.10.058
2. Lakshminarayanan B, Davenport M. Biliary atresia: a comprehensive review. *J Autoimmun.* (2016) 73:1–9. doi: 10.1016/j.jaut.2016.06.005
3. Nizery L, Chardot C, Sissaoui S, Capito C, Henrion-Caude A, Debray D, et al. Biliary atresia: clinical advances and perspectives. *Clin Res Hepatol Gastroenterol.* (2016) 40:281–7. doi: 10.1016/j.clinre.2015.11.010
4. Verkade HJ, Bezerra JA, Davenport M, Schreiber RA, Mieli-Vergani G, Hulscher JB, et al. Biliary atresia and other cholestatic childhood diseases: advances and future challenges. *J Hepatol.* (2016) 65:631–42. doi: 10.1016/j.jhep.2016.04.032
5. Asai A, Miethke A, Bezerra JA. Pathogenesis of biliary atresia: defining biology to understand clinical phenotypes. *Nat Rev Gastroenterol Hepatol.* (2015) 12:342–52. doi: 10.1038/nrgastro.2015.74
6. Girard M, Jannot A-S, Besnard M, Jacquemin E, Henrion-Caude A. Biliary atresia: does ethnicity matter? *J Hepatol.* (2012) 57:700–1. doi: 10.1016/j.jhep.2012.03.011
7. Hopkins PC, Yazigi N, Nylund CM. Incidence of biliary atresia and timing of hepatoportoenterostomy in the United States. *J Pediatr.* (2017) 187:253–7. doi: 10.1016/j.jpeds.2017.05.006
8. Suchy FJ, Burdelski M, Tomar BS, Sokol RJ. Cholestatic liver disease: working group report of the first World Congress of pediatric gastroenterology, hepatology, and nutrition. *J Pediatr Gastroenterol Nutr.* (2002) 35:S89–S97. doi: 10.1097/00005176-200208002-00005
9. Lampela H, Kosola S, Heikkilä P, Lohi J, Jalanko H, Pakarinen MP. Native liver histology after successful portoenterostomy in biliary atresia. *J Clin Gastroenterol.* (2014) 48:721–8. doi: 10.1097/MCG.0000000000000013
10. Harpavat S, Finegold MJ, Karpen SJ. Patients with biliary atresia have elevated direct/conjugated bilirubin levels shortly after birth. *Pediatrics.* (2011) 2011:1869. doi: 10.1542/peds.2011-1869
11. Mack CL. What causes biliary atresia? *Unique* aspects of the neonatal immune system provide clues to disease pathogenesis. *Cell Mol Gastroenterol Hepatol.* (2015) 1:267–74. doi: 10.1016/j.jcmgh.2015.04.001
12. Bijl E, Bharwani K, Houwen R, De Man R. The long-term outcome of the Kasai operation in patients with biliary atresia: a systematic review. *Neth J Med.* (2013) 71:170–3.
13. Chardot C, Buet C, Serinet M-O, Golmard J-L, Lachaux A, Roquelaure B, et al. Improving outcomes of biliary atresia: French national series 1986–2009. *J Hepatol.* (2013) 58:1209–17. doi: 10.1016/j.jhep.2013.01.040
14. Zhang DY, Sabla G, Shivakumar P, Tiao G, Sokol RJ, Mack C, et al. Coordinate expression of regulatory genes differentiates embryonic and perinatal forms of biliary atresia. *Hepatology.* (2004) 39:954–62. doi: 10.1002/hep.20135
15. Davenport M. A challenge on the use of the words embryonic and perinatal in the context of biliary atresia. *Hepatology.* (2005) 41:403–4. doi: 10.1002/hep.20549
16. Feldman AG, Mack CL. Biliary atresia: cellular dynamics and immune dysregulation. In: *Seminars in Pediatric Surgery*. *Elsevier*. (2012). p. 192–200. doi: 10.1053/j.sempedsurg.2012.05.003
17. Davenport M, Savage M, Mowat A, Howard E. Biliary atresia splenic malformation syndrome: an etiologic and prognostic subgroup. *Surgery.* (1993) 113:662–8.
18. Schwarz KB, Haber BH, Rosenthal P, Mack CL, Moore J, Bove K, et al. Extrahepatic anomalies in infants with biliary atresia: results of a large prospective North American multicenter study. *Hepatology.* (2013) 58:1724–31. doi: 10.1002/hep.26512
19. Davenport M. Biliary atresia: clinical aspects. In: *Seminars in Pediatric Surgery*. *Elsevier*. (2016). p. 175–84. doi: 10.1053/j.sempedsurg.2012.05.010
20. Zani A, Quaglia A, Hadzić N, Zuckerman M, Davenport M. Cytomegalovirus-associated biliary atresia: an aetiological and prognostic subgroup. *J Pediatr Surg.* (2015) 50:1739–45. doi: 10.1016/j.jpedsurg.2015.03.001
21. Caponcelli E, Knisely AS, Davenport M. Cystic biliary atresia: an etiologic and prognostic subgroup. *J Pediatr Surg.* (2008) 43:1619–24. doi: 10.1016/j.jpedsurg.2007.12.058
22. Lorent K, Gong W, Koo KA, Waisbourd-Zinman O, Karjoo S, Zhao X, et al. Identification of a plant isoflavonoid that causes biliary atresia. *Sci Transl Med.* (2015) 7:286ra267. doi: 10.1126/scitranslmed.aaa1652
23. Mohanty SK, Donnelly B, Bondoc A, Jafri M, Walther A, Coots A, et al. Rotavirus replication in the cholangiocyte mediates the temporal dependence of murine biliary atresia. *PLoS ONE.* (2013) 8:e69069. doi: 10.1371/journal.pone.0069069
24. Dong R, Zhao R, Zheng S. Changes in epigenetic regulation of CD4+ T lymphocytesin biliary atresia. *Pediatr Res.* (2011) 70:555. doi: 10.1203/PDR.0b013e318232a949
25. Li K, Zhang X, Yang L, Wang X-X, Yang D-H, Cao G-Q, et al. Foxp3 promoter methylation impairs suppressive function of regulatory T cells in biliary atresia. *Am J Physiol Gastrointest Liver Physiol.* (2016) 311:G989–97. doi: 10.1152/ajpgi.00032.2016
26. Mack CL, Tucker RM, Lu BR, Sokol RJ, Fontenot AP, Ueno Y, et al. Cellular and humoral autoimmunity directed at bile duct epithelia in murine biliary atresia. *Hepatology.* (2006) 44:1231–9. doi: 10.1002/hep.21366
27. Shivakumar P, Sabla G, Mohanty S, Mcneal M, Ward R, Stringer K, et al. Effector role of neonatal hepatic $CD8^+$ lymphocytes in epithelial injury and autoimmunity in experimental biliary atresia. *Gastroenterology.* (2007) 133:268–77. doi: 10.1053/j.gastro.2007.04.031
28. Lu BR, Brindley SM, Tucker RM, Lambert CL, Mack CL. α-enolase autoantibodies cross-reactive to viral proteins in a mouse

model of biliary atresia. *Gastroenterology.* (2010) 139:1753–61. doi: 10.1053/j.gastro.2010.07.042
29. Pang S-Y, Dai Y-M, Zhang R-Z, Chen Y-H, Peng X-F, Fu J, et al. Autoimmune liver disease-related autoantibodies in patients with biliary atresia. *World J Gastroenterol.* (2018) 24:387. doi: 10.3748/wjg.v24.i3.387
30. Mezina A, Karpen SJ. Genetic contributors and modifiers of biliary atresia. *Digest Dis.* (2015) 33:408–14. doi: 10.1159/000371694
31. Kobayashi H, Tamatani T, Tamura T, Kusafuka J, Yamataka A, Lane GJ, et al. Maternal microchimerism in biliary atresia. *J Pediatr Surg.* (2007) 42:987–91. doi: 10.1016/j.jpedsurg.2007.01.051
32. Petersen C, Davenport M. Aetiology of biliary atresia: what is actually known? *Orphanet J Rare Dis.* (2013) 8:128. doi: 10.1186/1750-1172-8-128
33. Riepenhoff-Talty M, Schaekel K, Clark HF, Mueller W, Uhnoo I, Rossi T, et al. Group A rotaviruses produce extrahepatic biliary obstruction in orally inoculated newborn mice. *Pediatr Res.* (1993) 33:394–9. doi: 10.1203/00006450-199304000-00016
34. Allen SR, Jafri M, Donnelly B, Mcneal M, Witte D, Bezerra J, et al. Effect of rotavirus strain on the murine model of biliary atresia. *J Virol.* (2007) 81:1671–9. doi: 10.1128/JVI.02094-06
35. Harper P, Plant J, Ungers D. Congenital biliary atresia and jaundice in lambs and calves. *Aust Vet J.* (1990) 67:18–22. doi: 10.1111/j.1751-0813.1990.tb07385.x
36. Petersen C, Madadi-Sanjani O. Role of viruses in biliary atresia: news from mice and men. *Innovative Surg Sci.* (2018) 3:101–6. doi: 10.1515/iss-2018-0009
37. Landing B. Considerations of the pathogenesis of neonatal hepatitis, biliary atresia and choledochal cyst-the concept of infantile obstructive cholangiopathy. *Prog Pediatr Surg.* (1974) 6:113–39.
38. Morecki R, Glaser JH, Cho S, Balistreri WF, Horwitz MS. Biliary atresia and reovirus type 3 infection. *N Engl J Med.* (1982) 307:481–4. doi: 10.1056/NEJM198208193070806
39. Brown WR, Sokol RJ, Levin MJ, Silverman A, Tamaru T, Lilly JR, et al. Lack of correlation between infection with reovirus 3 and extrahepatic biliary atresia or neonatal hepatitis. *J Pediatr.* (1988) 113:670–6. doi: 10.1016/S0022-3476(88)80376-7
40. Steele MI, Marshall CM, Lloyd RE, Randolph VE. Reovirus 3 not detected by reverse transcriptase—mediated polymerase chain reaction analysis of preserved tissue from infants with cholestatic liver disease. *Hepatology.* (1995) 21:697–702. doi: 10.1002/hep.1840210315
41. Tyler KL, Sokol RJ, Oberhaus SM, Le M, Karrer FM, Narkewicz MR, et al. Detection of reovirus RNA in hepatobiliary tissues from patients with extrahepatic biliary atresia and choledochal cysts. *Hepatology.* (1998) 27:1475–82. doi: 10.1002/hep.510270603
42. Al-Masri AN, Flemming P, Rodeck B, Melter M, Leonhardt J, Petersen C. Expression of the interferon-induced Mx proteins in biliary atresia. *J Pediatr Surg.* (2006) 41:1139–43. doi: 10.1016/j.jpedsurg.2006.02.022
43. Huang Y-H, Chou M-H, Du Y-Y, Huang C-C, Wu C-L, Chen C-L, et al. Expression of toll-like receptors and type 1 interferon specific protein MxA in biliary atresia. *Lab Invest.* (2007) 87:66. doi: 10.1038/labinvest.3700490
44. Rauschenfels S, Krassmann M, Al-Masri AN, Verhagen W, Leonhardt J, Kuebler JF, et al. Incidence of hepatotropic viruses in biliary atresia. *Eur J Pediatr.* (2009) 168:469–76. doi: 10.1007/s00431-008-0774-2
45. Saito T, Terui K, Mitsunaga T, Nakata M, Ono S, Mise N, et al. Evidence for viral infection as a causative factor of human biliary atresia. *J Pediatr Surg.* (2015) 50:1398–404. doi: 10.1016/j.jpedsurg.2015.04.006
46. Shivakumar P, Campbell KM, Sabla GE, Miethke A, Tiao G, Mcneal MM, et al. Obstruction of extrahepatic bile ducts by lymphocytes is regulated by IFN-γ in experimental biliary atresia. *J Clin Invest.* (2004) 114:322–9. doi: 10.1172/JCI200421153
47. Mack CL, Tucker RM, Sokol RJ, Kotzin BL. Armed CD4+ Th1 effector cells and activated macrophages participate in bile duct injury in murine biliary atresia. *Clin Immunol.* (2005) 115:200–9. doi: 10.1016/j.clim.2005.01.012
48. Erickson N, Mohanty SK, Shivakumar P, Sabla G, Chakraborty R, Bezerra JA. Temporal-spatial activation of apoptosis and epithelial injury in murine experimental biliary atresia. *Hepatology.* (2008) 47:1567–77. doi: 10.1002/hep.22229
49. Wang W, Donnelly B, Bondoc A, Mohanty SK, Mcneal M, Ward R, et al. The rhesus rotavirus gene encoding VP4 is a major determinant in the pathogenesis of biliary atresia in newborn mice. *J Virol.* (2011) 85:9069–77. doi: 10.1128/JVI.02436-10
50. Tucker RM, Feldman AG, Fenner EK, Mack CL. Regulatory T cells inhibit Th1 cell-mediated bile duct injury in murine biliary atresia. *J Hepatol.* (2013) 59:790–6. doi: 10.1016/j.jhep.2013.05.010
51. Lages CS, Simmons J, Maddox A, Jones K, Karns R, Sheridan R, et al. The dendritic cell–T helper 17–macrophage axis controls cholangiocyte injury and disease progression in murine and human biliary atresia. *Hepatology.* (2017) 65:174–88. doi: 10.1002/hep.28851
52. Coots A, Donnelly B, Mohanty SK, Mcneal M, Sestak K, Tiao G. Rotavirus infection of human cholangiocytes parallels the murine model of biliary atresia. *J Surg Res.* (2012) 177:275–81. doi: 10.1016/j.jss.2012.05.082
53. Keyzer-Dekker CM, Lind RC, Kuebler J, Offerhaus G, Ten Kate F, Morsink F, et al. Liver fibrosis during the development of biliary atresia: proof of principle in the murine model. *J Pediatr Surg.* (2015) 50:1304–9. doi: 10.1016/j.jpedsurg.2014.12.027
54. Mohanty SK, Lobeck I, Donnelly B, Dupree P, Walther A, Mowery S, et al. Rotavirus reassortant induced murine model of liver fibrosis parallels human biliary atresia. *Hepatology.* (2019). doi: 10.1002/hep.30907
55. Wen J, Xiao Y, Wang J, Pan W, Zhou Y, Zhang X, et al. Low doses of CMV induce autoimmune-mediated and inflammatory responses in bile duct epithelia of regulatory T cell-depleted neonatal mice. *Lab Invest.* (2015) 95:180–92. doi: 10.1038/labinvest.2014.148
56. Walther A, Mohanty SK, Donnelly B, Coots A, Lages CS, Lobeck I, et al. Rhesus rotavirus VP4 sequence-specific activation of mononuclear cells is associated with cholangiopathy in murine biliary atresia. *Am J Physiol Gastrointest Liver Physiol.* (2015) 309:G466–74. doi: 10.1152/ajpgi.00079.2015
57. Mohanty SK, Donnelly B, Lobeck I, Walther A, Dupree P, Coots A, et al. The SRL peptide of rhesus rotavirus VP4 protein governs cholangiocyte infection and the murine model of biliary atresia. *Hepatology.* (2017) 65:1278–92. doi: 10.1002/hep.28947
58. Mohanty SK, Donnelly B, Dupree P, Lobeck I, Mowery S, Meller J, et al. A point mutation in the rhesus rotavirus VP4 protein generated through a rotavirus reverse genetics system attenuates biliary atresia in the murine model. *J Virol.* (2017) 91:e00510–17. doi: 10.1128/JVI.00510-17
59. Glaser JH, Balistreri WF, Morecki R. Role of reovirus type 3 in persistent infantile cholestasis. *J Pediatr.* (1984) 105:912–5. doi: 10.1016/S0022-3476(84)80076-1
60. Riepenhoff-Talty M, Gouvea V, Evans MJ, Svensson L, Hoffenberg E, Sokol RJ, et al. Detection of group C rotavirus in infants with extrahepatic biliary atresia. *J Infect Dis.* (1996) 174:8–15. doi: 10.1093/infdis/174.1.8
61. Drut R, Drut RM, Gomez MA, Cueto Rua E, Lojo MM. Presence of human papillomavirus in extrahepatic biliary atresia. *J Pediatr Gastroenterol Nutr.* (1998) 27:530–5. doi: 10.1097/00005176-199811000-00007
62. Domiati-Saad R, Dawson DB, Margraf LR, Finegold MJ, Weinberg AG, Rogers BB. Cytomegalovirus and human herpesvirus 6, but not human papillomavirus, are present in neonatal giant cell hepatitis and extrahepatic biliary atresia. *Pediatr Dev Pathol.* (2000) 3:367–73. doi: 10.1007/s100240010045
63. Fjaer RB, Bruu AL, Nordbo SA. Extrahepatic bile duct atresia and viral involvement. *Pediatr Transplant.* (2005) 9:68–73. doi: 10.1111/j.1399-3046.2005.00257.x
64. Harada K, Nakanuma Y. Biliary innate immunity: function and modulation. *Mediat Inflamm.* (2010) 2010:373878. doi: 10.1155/2010/373878
65. Harada K, Nakanuma Y. Biliary innate immunity in the pathogenesis of biliary diseases. *Inflamm Allergy-Drug Targ.* (2010) 9:83–90. doi: 10.2174/187152810791292809
66. Sato K, Meng FY, Giang T, Glaser S, Alpini G. Mechanisms of cholangiocyte responses to injury. *Biochim Biophys Acta-Mol Basis Dis.* (2018) 1864:1262–9. doi: 10.1016/j.bbadis.2017.06.017
67. Zhang HY, Leung PSC, Gershwin ME, Ma X. How the biliary tree maintains immune tolerance? *Biochim Biophys Acta-Mol Basis Dis.* (2018) 1864:1367–73. doi: 10.1016/j.bbadis.2017.08.019
68. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell.* (2010) 140:805–20. doi: 10.1016/j.cell.2010.01.022

69. Jafri M, Donnelly B, Mcneal M, Ward R, Tiao G. MAPK signaling contributes to rotaviral-induced cholangiocyte injury and viral replication. *Surgery.* (2007) 142:192–201. doi: 10.1016/j.surg.2007.03.008
70. Lobeck I, Donnelly B, Dupree P, Mahe MM, Mcneal M, Mohanty SK, et al. Rhesus rotavirus VP6 regulates ERK-dependent calcium influx in cholangiocytes. *Virology.* (2016) 499:185–95. doi: 10.1016/j.virol.2016.09.014
71. Walther AE, Mohanty SK, Donnelly B, Coots A, Mcneal M, Tiao GM. Role of myeloid differentiation factor 88 in Rhesus rotavirus-induced biliary atresia. *J Surg Res.* (2013) 184:322–9. doi: 10.1016/j.jss.2013.05.032
72. Harada K, Isse K, Sato Y, Ozaki S, Nakanuma Y. Endotoxin tolerance in human intrahepatic biliary epithelial cells is induced by upregulation of IRAK-M. *Liver Int.* (2006) 26:935–42. doi: 10.1111/j.1478-3231.2006.01325.x
73. Harada K, Sato Y, Isse K, Ikeda H, Nakanuma Y. Induction of innate immune response and absence of subsequent tolerance to dsRNA in biliary epithelial cells relate to the pathogenesis of biliary atresia. *Liver Int.* (2008) 28:614–21. doi: 10.1111/j.1478-3231.2008.01740.x
74. Harada K, Sato Y, Itatsu K, Isse K, Ikeda H, Yasoshima M, et al. Innate immune response to double-stranded RNA in biliary epithelial cells is associated with the pathogenesis of biliary atresia. *Hepatology.* (2007) 46:1146–54. doi: 10.1002/hep.21797
75. Yoneyama M, Fujita T. Function of RIG-I-like receptors in antiviral innate immunity. *J Biol Chem.* (2007) 282:15315–8. doi: 10.1074/jbc.R700007200
76. Yoneyama M, Fujita T. RNA recognition and signal transduction by RIG-I-like receptors. *Immunol Rev.* (2009) 227:54–65. doi: 10.1111/j.1600-065X.2008.00727.x
77. Loo Y-M, Gale M. Immune signaling by RIG-I-like receptors. *Immunity.* (2011) 34:680–92. doi: 10.1016/j.immuni.2011.05.003
78. Broquet AH, Hirata Y, Mcallister CS, Kagnoff MF. RIG-I/MDA5/MAVS are required to signal a protective IFN response in rotavirus-infected intestinal epithelium. *J Immunol.* (2010) 1002862. doi: 10.4049/jimmunol.1002862
79. Keyel PA. How is inflammation initiated? Individual influences of IL-1, IL-18 and HMGB1. *Cytokine.* (2014) 69:136–45. doi: 10.1016/j.cyto.2014.03.007
80. Bermejo-Jambrina M, Eder J, Helgers LC, Hertoghs N, Nijmeijer BM, Stunnenberg M, et al. C-Type lectin receptors in antiviral immunity and viral escape. *Front Immunol.* (2018) 9:590. doi: 10.3389/fimmu.2018.00590
81. Li TH, Liu L, Hou YY, Shen SN, Wang TT. C-type lectin receptor-mediated immune recognition and response of the microbiota in the gut. *Gastroenterol Rep.* (2019) 7:312–21. doi: 10.1093/gastro/goz028
82. Tang C, Makusheva Y, Sun H, Han W, Iwakura Y. Myeloid C-type lectin receptors in skin/mucoepithelial diseases and tumors. *J Leukoc Biol.* (2019) 106:903–17. doi: 10.1002/JLB.2RI0119-031R
83. Barnes BH, Tucker RM, Wehrmann F, Mack DG, Ueno Y, Mack CL. Cholangiocytes as immune modulators in rotavirus-induced murine biliary atresia. *Liver Int.* (2009) 29:1253–61. doi: 10.1111/j.1478-3231.2008.01921.x
84. Jafri M, Donnelly B, Bondoc A, Allen S, Tiao G. Cholangiocyte secretion of chemokines in experimental biliary atresia. *J Pediatr Surg.* (2009) 44:500–7. doi: 10.1016/j.jpedsurg.2008.07.007
85. Mohanty SK, Ivantes CA, Mourya R, Pacheco C, Bezerra JA. Macrophages are targeted by rotavirus in experimental biliary atresia and induce neutrophil chemotaxis by Mip2/Cxcl2. *Pediatr Res.* (2010) 67:345–51. doi: 10.1203/PDR.0b013e3181d22a73
86. Saxena V, Shivakumar P, Sabla G, Mourya R, Chougnet C, Bezerra JA. Dendritic cells regulate natural killer cell activation and epithelial injury in experimental biliary atresia. *Sci Transl Med.* (2011) 3:102ra194. doi: 10.1126/scitranslmed.3002069
87. Wallach D, Varfolomeev E, Malinin N, Goltsev YV, Kovalenko A, Boldin M. Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol.* (1999) 17:331–67. doi: 10.1146/annurev.immunol.17.1.331
88. Samuel CE. Antiviral actions of interferons. *Clin Microbiol Rev.* (2001) 14:778–809. doi: 10.1128/CMR.14.4.778-809.2001
89. Kuwajima S, Sato T, Ishida K, Tada H, Tezuka H, Ohteki T. Interleukin 15–dependent crosstalk between conventional and plasmacytoid dendritic cells is essential for CpG-induced immune activation. *Nat Immunol.* (2006) 7:740. doi: 10.1038/ni1348
90. Isse K, Harada K, Nakanuma Y. IL-8 expression by biliary epithelial cells is associated with neutrophilic infiltration and reactive bile ductules. *Liver Int.* (2007) 27:672–80. doi: 10.1111/j.1478-3231.2007.01465.x
91. Shivakumar P, Sabla GE, Whitington P, Chougnet CA, Bezerra JA. Neonatal NK cells target the mouse duct epithelium via Nkg2d and drive tissue-specific injury in experimental biliary atresia. *J Clin Invest.* (2009) 119:2281–90. doi: 10.1172/JCI38879
92. Shivakumar P, Mourya R, Bezerra JA. Perforin and granzymes work in synergy to mediate cholangiocyte injury in experimental biliary atresia. *J Hepatol.* (2014) 60:370–6. doi: 10.1016/j.jhep.2013.09.021
93. Mack CL, Falta MT, Sullivan AK, Karrer F, Sokol RJ, Freed BM, et al. Oligoclonal expansions of CD4+ and CD8+ T-cells in the target organ of patients with biliary atresia. *Gastroenterology.* (2007) 133:278–87. doi: 10.1053/j.gastro.2007.04.032
94. Li J, Bessho K, Shivakumar P, Mourya R, Mohanty SK, Dos Santos JL, et al. Th2 signals induce epithelial injury in mice and are compatible with the biliary atresia phenotype. *J Clin Invest.* (2011) 121:4244–56. doi: 10.1172/JCI57728
95. Bezerra JA, Tiao G, Ryckman FC, Alonso M, Sabla GE, Shneider B, et al. Genetic induction of proinflammatory immunity in children with biliary atresia. *Lancet.* (2002) 360:1653–9. doi: 10.1016/S0140-6736(02)11603-5
96. Feldman AG, Tucker RM, Fenner EK, Pelanda R, Mack CL. B cell deficient mice are protected from biliary obstruction in the rotavirus-induced mouse model of biliary atresia. *PLoS ONE.* (2013) 8:e73644. doi: 10.1371/journal.pone.0073644
97. Hadchouel M, Hugon R, Odievre M. Immunoglobulin deposits in the biliary remnants of extrahepatic biliary atresia: a study by immunoperoxidase staining in 128 infants. *Histopathology.* (1981) 5:217–21. doi: 10.1111/j.1365-2559.1981.tb01779.x
98. Bettini M, Vignali DA. Regulatory T cells and inhibitory cytokines in autoimmunity. *Curr Opin Immunol.* (2009) 21:612–8. doi: 10.1016/j.coi.2009.09.011
99. Liberal R, Grant CR, Longhi MS, Mieli-Vergani G, Vergani D. Regulatory T cells: mechanisms of suppression and impairment in autoimmune liver disease. *IUBMB Life.* (2015) 67:88–97. doi: 10.1002/iub.1349
100. Miethke AG, Saxena V, Shivakumar P, Sabla GE, Simmons J, Chougnet CA. Post-natal paucity of regulatory T cells and control of NK cell activation in experimental biliary atresia. *J Hepatol.* (2010) 52:718–26. doi: 10.1016/j.jhep.2009.12.027
101. Lages CS, Simmons J, Chougnet CA, Miethke AG. Regulatory T cells control the CD8 adaptive immune response at the time of ductal obstruction in experimental biliary atresia. *Hepatology.* (2012) 56:219–27. doi: 10.1002/hep.25662
102. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol.* (2004) 22:531–62. doi: 10.1146/annurev.immunol.21.120601.141122
103. Czech-Schmidt G, Verhagen W, Szavay P, Leonhardt J, Petersen C. Immunological gap in the infectious animal model for biliary atresia. *J Surg Res.* (2001) 101:62–7. doi: 10.1006/jsre.2001.6234
104. Brindley SM, Lanham AM, Karrer FM, Tucker RM, Fontenot AP, Mack CL. Cytomegalovirus-specific T-cell reactivity in biliary atresia at the time of diagnosis is associated with deficits in regulatory T cells. *Hepatology.* (2012) 55:1130–8. doi: 10.1002/hep.24807
105. Li J, Razumilava N, Gores GJ, Walters S, Mizuochi T, Mourya R, et al. Biliary repair and carcinogenesis are mediated by IL-33–dependent cholangiocyte proliferation. *J Clin Invest.* (2014) 124:3241–51. doi: 10.1172/JCI73742
106. Mchedlidze T, Waldner M, Zopf S, Walker J, Rankin AL, Schuchmann M, et al. Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity.* (2013) 39:357–71. doi: 10.1016/j.immuni.2013.07.018
107. Liu Y, Meyer C, Müller A, Herweck F, Li Q, Müllenbach R, et al. IL-13 induces connective tissue growth factor in rat hepatic stellate cells via TGF-β-independent Smad signaling. *J Immunol.* (2011) 2011:1003260. doi: 10.4049/jimmunol.1003260
108. Rong G, Zhou Y, Xiong Y, Zhou L, Geng H, Jiang T, et al. Imbalance between T helper type 17 and T regulatory cells in patients with primary biliary cirrhosis: the serum cytokine profile and peripheral cell population. *Clin Exp Immunol.* (2009) 156:217–25. doi: 10.1111/j.1365-2249.2009.03898.x
109. Yang Y, Liu Y-J, Tang S-T, Yang L, Yang J, Cao G-Q, et al. Elevated Th17 cells accompanied by decreased regulatory T cells and cytokine environment in infants with biliary atresia. *Pediatr Surg Int.* (2013) 29:1249–60. doi: 10.1007/s00383-013-3421-6

110. Jung Y, Witek RP, Syn W-K, Choi SS, Omenetti A, Premont R, et al. Signals from dying hepatocytes trigger growth of liver progenitors. *Gut.* (2010) 59:655–65. doi: 10.1136/gut.2009.204354
111. Syal G, Fausther M, Dranoff JA. Advances in cholangiocyte immunobiology. *Am J Physiol Gastrointest Liver Physiol.* (2012) 303:G1077–86. doi: 10.1152/ajpgi.00227.2012
112. Omenetti A, Syn WK, Jung Y, Francis H, Porrello A, Witek RP, et al. Repair-related activation of hedgehog signaling promotes cholangiocyte chemokine production. *Hepatology.* (2009) 50:518–27. doi: 10.1002/hep.23019
113. Yang J-J, Tao H, Li J. Hedgehog signaling pathway as key player in liver fibrosis: new insights and perspectives. *Exp Opin Ther Targets.* (2014) 18:1011–21. doi: 10.1517/14728222.2014.927443
114. Syn WK, Choi SS, Liaskou E, Karaca GF, Agboola KM, Oo YH, et al. Osteopontin is induced by hedgehog pathway activation and promotes fibrosis progression in nonalcoholic steatohepatitis. *Hepatology.* (2011) 53:106–15. doi: 10.1002/hep.23998
115. Shen X, Peng Y, Li H. The injury-related activation of hedgehog signaling pathway modulates the repair-associated inflammation in liver fibrosis. *Front Immunol.* (2017) 8:1450. doi: 10.3389/fimmu.2017.01450
116. Omenetti A, Diehl AM. Hedgehog signaling in cholangiocytes. *Curr Opin Gastroenterol.* (2011) 27:268. doi: 10.1097/MOG.0b013e32834550b4
117. Omenetti A, Bass LM, Anders RA, Clemente MG, Francis HD, et al. Hedgehog activity, epithelial-mesenchymal transitions, and biliary dysmorphogenesis in biliary atresia. *Hepatology.* (2011) 53:1246–58. doi: 10.1002/hep.24156
118. Whitington PF, Malladi P, Melin-Aldana H, Azzam R, Mack CL, Sahai A. Expression of osteopontin correlates with portal biliary proliferation and fibrosis in biliary atresia. *Pediatr Res.* (2005) 57:837. doi: 10.1203/01.PDR.0000161414.99181.61
119. Sica A, Mantovani A. Macrophage plasticity and polarization: *in vivo* veritas. *J Clin Invest.* (2012) 122:787–95. doi: 10.1172/JCI59643
120. Byun K, Yoo Y, Son M, Lee J, Jeong G-B, Park YM, et al. Advanced glycation end-products produced systemically and by macrophages: a common contributor to inflammation and degenerative diseases. *Pharmacol Ther.* (2017) 177:44–55. doi: 10.1016/j.pharmthera.2017.02.030
121. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity.* (2014) 41:14–20. doi: 10.1016/j.immuni.2014.06.008
122. Pellicoro A, Ramachandran P, Iredale JP, Fallowfield JA. Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat Rev Immunol.* (2014) 14:181–94. doi: 10.1038/nri3623
123. Roszer T. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediat Inflamm.* (2015) 2015:816460. doi: 10.1155/2015/816460
124. Seki E, Brenner DA. Recent advancement of molecular mechanisms of liver fibrosis. *J Hepatobiliary Pancreat Sci.* (2015) 22:512–8. doi: 10.1002/jhbp.245
125. Vannella KM, Wynn TA. Mechanisms of organ injury and repair by macrophages. *Annu Rev Physiol.* (2017) 79:593–617. doi: 10.1146/annurev-physiol-022516-034356
126. Yamagishi S-I, Matsui T. Role of receptor for advanced glycation end products (RAGE) in liver disease. *Eur J Med Res.* (2015) 20:15. doi: 10.1186/s40001-015-0090-z
127. Palanissami G, Paul SF. RAGE and its ligands: molecular interplay between glycation, inflammation, and hallmarks of cancer—a review. *Hormones Cancer.* 9:295–325. doi: 10.1007/s12672-018-0342-9
128. Honsawek S, Vejchapipat P, Payungporn S, Theamboonlers A, Chongsrisawat V, Poovorawan Y. Soluble receptor for advanced glycation end products and liver stiffness in postoperative biliary atresia. *Clin Biochem.* (2013) 46:214–8. doi: 10.1016/j.clinbiochem.2012.11.013
129. Luo ZH, Jegga AG, Bezerra JA. Gene-disease associations identify a connectome with shared molecular pathways in human cholangiopathies. *Hepatology.* (2018) 67:676–89. doi: 10.1002/hep.29504
130. Bezerra JA, Spino C, Magee JC, Shneider BL, Rosenthal P, Wang KS, et al. Use of corticosteroids after hepatoportoenterostomy for bile drainage in infants with biliary atresia: the START randomized clinical trial. *JAMA.* (2014) 311:1750–9. doi: 10.1001/jama.2014.2623
131. Tyraskis A, Davenport M. Steroids after the Kasai procedure for biliary atresia: the effect of age at Kasai portoenterostomy. *Pediatr Surg Int.* (2016) 32:193–200. doi: 10.1007/s00383-015-3836-3
132. Arab JP, Karpen SJ, Dawson PA, Arrese M, Trauner M. Bile acids and nonalcoholic fatty liver disease: molecular insights and therapeutic perspectives. *Hepatology.* (2017) 65:350–62. doi: 10.1002/hep.28709
133. Nevens F, Andreone P, Mazzella G, Strasser SI, Bowlus C, Invernizzi P, et al. A placebo-controlled trial of obeticholic acid in primary biliary cholangitis. *N Engl J Med.* (2016) 375:631–43. doi: 10.1056/NEJMoa1509840
134. Fickert P, Hirschfield GM, Denk G, Marschall HU, Altorjay I, Farkkila M, et al. norUrsodeoxycholic acid improves cholestasis in primary sclerosing cholangitis. *J Hepatol.* (2017) 67:549–58. doi: 10.1016/j.jhep.2017.05.009
135. Davenport M. Adjuvant therapy in biliary atresia: hopelessly optimistic or potential for change? *Pediatr Surg Int.* (2017) 33:1263–73. doi: 10.1007/s00383-017-4157-5

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REVIEW
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Targeting of Hepatic Macrophages by Therapeutic Nanoparticles

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Hepatic macrophage populations include different types of cells with plastic properties that can differentiate into diverse phenotypes to modulate their properties in response to different stimuli. They often regulate the activity of other cells and play an important role in many hepatic diseases. In response to those pathological situations, they are activated, releasing cytokines and chemokines; they may attract circulating monocytes and exert functions that can aggravate the symptoms or drive reparation processes. As a result, liver macrophages are potential therapeutic targets that can be oriented toward a variety of aims, with emergent nanotechnology platforms potentially offering new perspectives for macrophage vectorization. Macrophages play an essential role in the final destination of nanoparticles (NPs) in the organism, as they are involved in their uptake and trafficking *in vivo*. Different types of delivery nanosystems for macrophage recognition and targeting, such as liposomes, solid-lipid, polymeric, or metallic nanoparticles, have been developed. Passive targeting promotes the accumulation of the NPs in the liver due to their anatomical and physiological features. This process is modulated by NP characteristics such as size, charge, and surface modifications. Active targeting approaches with specific ligands may also be used to reach liver macrophages. In order to design new systems, the NP recognition mechanism of macrophages must be understood, taking into account that variations in local microenvironment may change the phenotype of macrophages in a way that will affect the uptake and toxicity of NPs. This kind of information may be applied to diseases where macrophages play a pathogenic role, such as metabolic disorders, infections, or cancer. The kinetics of nanoparticles strongly affects their therapeutic efficacy when administered *in vivo*. Release kinetics could predict the behavior of nanosystems targeting macrophages and be applied to improve their characteristics. PBPK models have been developed to characterize nanoparticle biodistribution in organs of the reticuloendothelial system (RES) such as liver or spleen. Another controversial issue is the possible toxicity of non-degradable nanoparticles, which in many cases accumulate in high percentages in macrophage clearance organs such as the liver, spleen, and kidney.

Keywords: hepatic macrophages, nanoparticles, drug delivery, biodistribution, Kupffer cells, toxicity

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INTRODUCTION

The use of particles as carriers of therapeutic agents for liver targeting is not a new idea. Hepatic nanoparticle uptake and distribution was initially studied as a drawback to be avoided because it entails a lack of specific selective distribution to other desired targets as well as toxicity and safety concerns. Nevertheless, from another point of view, strategies of selective delivery to different kinds of hepatic cells have also been explored in the search for specific targets of drugs included in nanoparticulated systems, such as hepatocytes, hepatic stellar cells, endothelial cells, and also the Kupffer cells (KC), the resident liver macrophages.

The liver is a very complex organ with many cells that are different in both morphology and functionality but which are nonetheless strongly inter-related. Although there are some other hepatic cells with phagocytic activity, KCs are undoubtedly the main ones responsible for phagocytosis in the liver. Moreover, it is estimated that they constitute 80–90% of all the macrophages present in the body (1).

Kupffer cells are the liver-resident macrophages, considered professional phagocytes to distinguish them from facultative ones. They are the largest mononuclear phagocyte population in the body and constitute approximately 20% of non-parenchymal liver cells. They present functional heterogeneity, likely due to their different origins and intrinsic plasticity (2).

They have an evident role in monitoring the blood entering the zone in order to endocytose debris, degenerated cells and any potentially harmful materials from the gut and circulation stream. Their strategic location at the luminal side of the hepatic sinusoidal endothelium allows them to act as sentinels that capture and process particles. They are involved in antigen presentation and processing and in the modulation of some hepatocyte functions.

Activation of Kupffer cells can induce a series of events to inhibit pathogen replication, recruit other immune cells into the liver, and activate them. On the other hand, interaction derived from infiltration immune cells leads to KC regulation (2). These complex and multiple inter-relationships with other hepatic cells and their concomitant role in immunological processes provide KCs with a wide variety of receptors that can be harnessed for their specific targeting (3, 4).

Due to the involvement of KCs in the evolution of many liver diseases, modulation of their activity may be used for therapeutic ends and nanosystems constitute promising alternatives for achieve this goal. In the present work, we will focus on the proposed nanosystems for targeting hepatic macrophages in order to improve pathological processes in the liver. The role of macrophages in liver diseases, the types of nanovehicles used, their characteristics, and the influence of the phenotype will be revised. Also, pharmacokinetic models for characterizing the biodistribution of NPs in the liver are addressed.

MONOCYTE–MACROPHAGE SYSTEM (MPS)

Macrophages have been considered to be a part of different body systems throughout history. Previously considered as reticulo-endothelial system (RES) cells, nowadays, macrophages are generally considered to belong to the monocyte mononuclear phagocyte or monocyte macrophage system (MPS), originally defined as a cell lineage of promonocytes that give rise to monocytes that finally becomes macrophages in tissues. This concept was later reformulated to include dendritic cells (5), and recently also the concept of the MPS has been questioned, based on evidence that tissue resident macrophages may be a separate lineage seeded during embryonic development and capable of self-renewal. The newly proposed nomenclature classifies mononuclear phagocytes according to their ontogeny, location, and/or morphology (6). Regarding the phagocytic cells in liver, the main difference of the newly proposed nomenclature is that macrophages and derived monocyte cells are considered to be separate entities (7).

Macrophages

Macrophages are specialized phagocytic cells strategically distributed in the body and specifically adapted to each tissue once they are settled. They are non-migratory cells that monitor their surrounding environment and process the material they engulf. They also recruit other immune cells, playing an important role in immune defense and homeostatic processes (8). To fulfill their functions, macrophages possess a wide range of sensing molecules specialized according to the tissue in which they are nested in each case.

Due to their central role in homeostasis, inflammation, and immunity, macrophages have arisen as interesting targets for therapeutic intervention (9).

Tissue macrophages are a very heterogeneous group of cells due to their different origins and their adaptation to the local environment. The contributions of embryonic origins and adult bone marrow cells vary depending on the tissues, with even tissue-resident macrophages of prenatal origin deriving from different hematopoietic stem cells.

In view of the new evidence that questions the previous MPS model, it is postulated that there are two groups of macrophages in tissues: one coming from prenatally established populations and a second one originating from infiltrated monocytes that are more related to inflammatory conditions. **Figure 1** illustrates the different origins and development processes of tissue-resident macrophages.

It has been demonstrated in mice that there are also several monocyte-derived tissue macrophage populations whose phenotypes reflect different origins. One such population is made up of macrophages originating from Ly-6C high expressing monocytes (classical monocytes), mainly coming from bone marrow, that express inflammatory chemokine receptors (like CCR2), pattern-recognition receptors, and cytokines. Another population is made up of macrophages derived from Ly-6C low expressing monocytes (non-classical monocytes), mainly coming from the spleen, that present a patrolling behavior and express more scavenging receptors. In the steady state, classical ones can leave the bloodstream and patrol extravascular tissues. They can be converted in some proportion into Ly-6C low expressing monocytes and transport antigens to lymph nodes. Non-classical monocytes patrol the intravascular spaces to clear

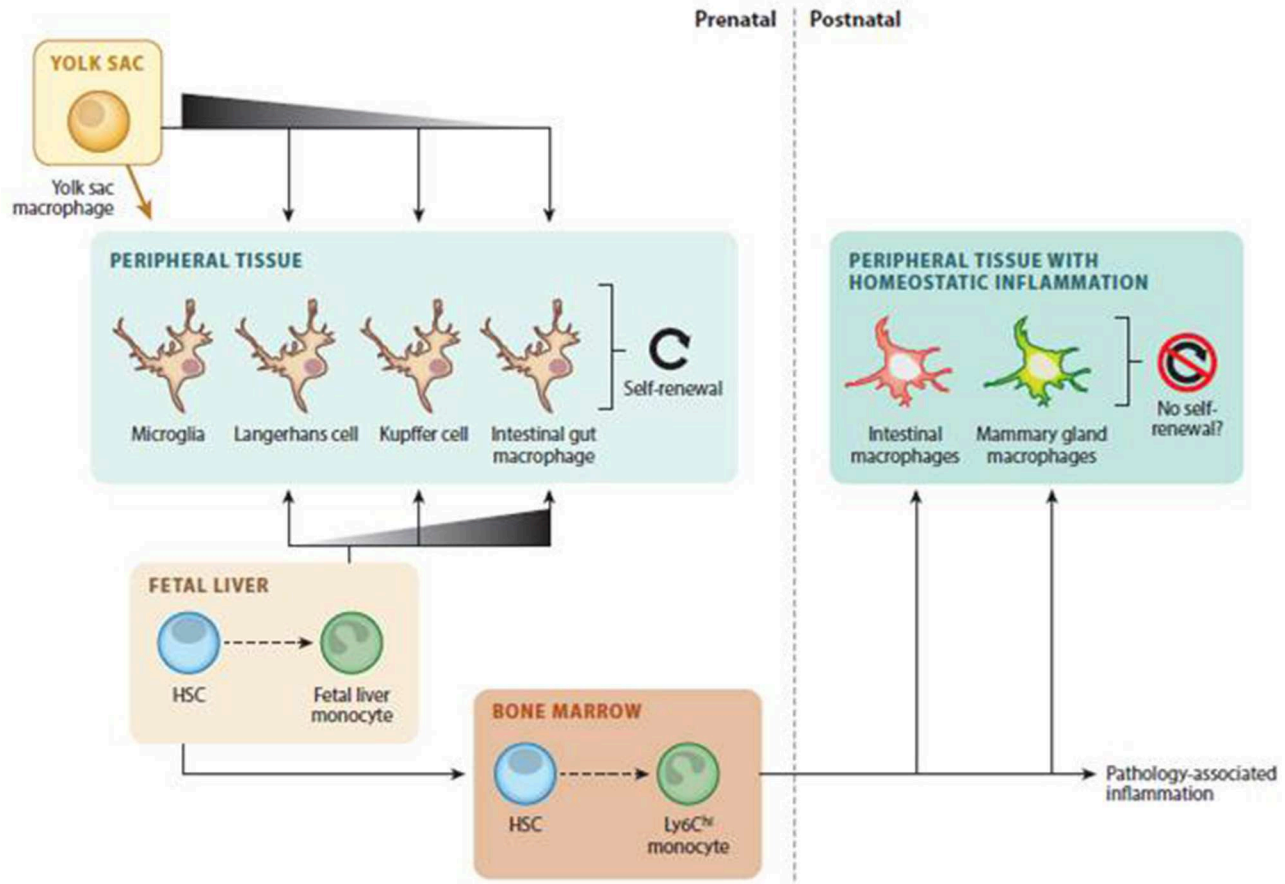

FIGURE 1 | Tissue-resident macrophages development. Adapted with permission (9).

dying endothelial cells. Under inflammation, classical monocytes differentiate to macrophages that are capable of self-renewal (10).

Despite the differences between mice and humans, genetic expression studies have demonstrated that these two subsets of monocytes are also present in humans, together with another intermediate subset between the classical and the non-classical (11, 12).

With respect to the macrophages that are present in the liver, although traditionally the term hepatic macrophages and Kupffer cells (KC) are used almost interchangeably, modifications to the MPS model mentioned above have also affected this assumption. After injury, heterogeneous hepatic macrophages populations can be observed, such as liver-resident macrophages or KCs and two subsets of bone marrow monocyte-derived macrophages (MoMFs), as well as peritoneal macrophages for subcapsular regions of the liver (13).

Classification

It is well-established in mice that macrophages can undergo two different activation states: M1 or classically activated and M2 or alternatively activated. M1 macrophages can produce pro-inflammatory cytokines and chemokines, high levels of reactive nitrogen and oxygen intermediates. They can also facilitate Th1 response and strong antimicrobial and antineoplastic effect. M2 ones are suppressive, involved in cellular repair and characterized by efficient phagocytic activity and high expression of scavenger, galactose, and mannose-type receptors. M1 and M2 even present different iron, glucose, and amino acid metabolism (2, 4). Although not much information is available regarding human beings, there is evidence to suggest a similar behavior in human macrophages (14). This traditional dichotomic classification seems to be too simplistic in view of recent increases in our knowledge of this area due to new sophisticated characterization techniques. Regarding Kupffer cells, their great flexibility and plasticity allow them to adopt a range of multiple intermediate phenotypes depending on the signals, which can lead to a broad spectrum of activation states (13, 15). Nevertheless, this simple classification into two possible extreme activation states is still used as a reference for KC behavior.

Differences not only in activation but also in origin have a great impact on the different subsets that can be defined for liver macrophages. The ontogeny and maintenance of resident hepatic macrophages have been the objects of many studies by important research groups, which have demonstrated that, besides the existence of self-renewal processes from prenatally settled local precursor cells, Kupffer cell populations are maintained by

the infiltration of circulating bone-derived monocytes that differentiate into Kupffer cells in the liver (16, 17). Murine models have provided evidence that MoMF can contribute to regenerating the resident liver macrophage population when KC are massively depleted (18).

On the other hand, even some studies with models of sterile liver injury have shown phagocytes with an expression of the transcription factor GATA-6, suggesting macrophage infiltration from the peritoneal cavity (19).

ROLE OF KUPFFER CELLS IN HEPATIC DISEASES: CYTOKINES AND CHEMOKINES

Due to their physiological functions, Kupffer cells are involved in local cell communication and homeostasis maintenance. The prominent role of KCs in immune processes, particle engulfment, antigen presentation, and the attraction and stimulation of T cells is well-known. They recruit other immune cells in the liver and release mediators to initiate response in other liver cells (2, 13).

Regarding other types of hepatic macrophages, it is known that bone marrow-derived macrophages participate in liver repair and regeneration but functional differences with Kupffer cells have not been clearly established. Following their activation, they produce cytokines that trigger a cascade of responses in other cells. If we accept the classical M1/M2 classification, it can be said that M1 KCs release proinflammatory cytokines, including tumor necrosis factor (TNF)-a, IL-6 and IL-1β, while M2 KCs release IL-4, IL-10, IL-13, and transforming growth factor-β. Consequently, the balance between these two phenotypes can ultimately lead to many different effects such as liver damage and wound repair (20).

The complex roles and expression of different KC phenotypes can lead to both protective and harmful responses (2, 21), and since it is required that their activation be precise, timely and localized, any KC dysregulation can lead to significant pathology (15). However, the results of some studies and hypotheses regarding these opposing effects are disputed. In some cases, KC depletion can be beneficial because of the reduction of inflammation or fibrosis but, on the other hand, the suppression of KC's role against pathogen invasion can clearly be harmful. Further studies must be done in order to conclude if KC depletion could prevent or exacerbate liver damage.

Thus, liver macrophages play an essential role in many pathogenic stages such as acute liver injury, fatty liver disease, fibrosis, cirrhosis, and liver tumors (22), constituting potential therapeutic targets for liver disease treatments. However, the pathogenesis of the disease treated and the phenotype of the macrophages targeted are points to consider when targeting macrophages (23).

In response to liver injuries such as alcoholic liver and non-alcoholic fatty liver disease (NAFLD) diseases, Kupffer cells are activated and a polarization to an M1-like phenotype is promoted in resident as well as monocyte-derived macrophages (24, 25). When injury ends, there is a switch to M2 restorative macrophages that release anti-inflammatory cytokines, regenerative growth factors, and matrix degrading metalloproteinase (MMP) expression, which promote tissue repair (26).

Macrophage polarization also has an important role in the growth and development of tumoral tissues. Macrophages in tumor tissues (TAMs) are mostly M2-like cells that produce tumor-promoting cytokines and growth factors that promote tumor expansion, angiogenesis, metastasis, and immune cell evasion. TAMs also contribute to drug resistance (27, 28).

TARGETING LIVER MACROPHAGES WITH NPs

Delivery Systems for Macrophages Recognition and Targeting

Liver macrophages are specialized in the internalization of foreign nanoparticles, playing an essential role in its destination in the organism, since they are involved in their uptake and trafficking *in vivo*. Therapeutic capacity and clearance mechanisms in clinically relevant nanomedicines have been linked to macrophage activity. However, due to their pathophysiological roles in diseases (29), liver macrophages are also potential therapeutic targets for a variety of aims, from cell activation to monocyte recruitment or macrophage differentiation (30). Emerging nanotechnology systems may offer new perspectives for macrophage vectorization. After intravenous administration, nanoparticles are opsonized in the bloodstream before being phagocytized by macrophages and accumulated in the RES organs. This passive targeting promotes the accumulation of the NPs in the liver, a process that increases within tumors due to the EPR (enhanced permeability and retention) effect (31).

Kupffer cells internalize NPs through multiple scavenger, toll-like, mannose, and Fc receptors (23). The mechanisms involved are macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and additional endocytotic pathways (1, 32, 33). Clathrin-mediated endocytosis has been pointed out as responsible for the internalization of size ranges of approximately 100–350 nm, while caveolin-mediated mechanism is responsible for the endocytosis of 20–100 nm particles (34–36). Macropynocitosis allows for large volume extracellular internalization of 0.5–5 μm nanosystems (23). The internalization process is then modulated by the size of the NPs as well as other characteristics such as charge. Larger nanoparticles generally show more efficient hepatic uptake: a diameter >200 nm is preferred for liver deposition (1, 37–39). Charged NPs, especially those with a positive charge, are taken up to a greater extent than those with a neutral charge (23, 39). Shape also has a great impact on NP uptake and elongated NPs are taken up less by macrophages than spherical ones (40). However, cylindrical silica NPs showed the highest accumulation in the liver compared to other shapes (41). Moreover, surface hydrophobic NPs tend to be opsonized by proteins that make them attractive to the phagocytic cells of the MPS (42–44).

Therefore, the success of therapeutic strategies targeting liver macrophages involves the use of different NP types with

appropriate properties to allow them to be preferentially taken up by the liver. Incorporation of ligands to the surface of NPs increases specificity through active targeting (21, 31).

Liposomes

Liposomes are biodegradable vesicles with an aqueous core and a phospholipidic membrane that can carry hydrophilic as well as hydrophobic compounds. They have the advantage of being both biocompatible and biodegradable, whereas their instability is one of their main drawbacks. Macrophages, especially KCs, readily phagocytose circulating liposomes (45), causing them to accumulate in the liver (46). The liposomes proposed for the vectorization to hepatic macrophages have mainly sizes of ∼100 nm due to restrictions on parenteral formulations. This passive targeting has been exploited for the administration of anti-infective drugs that have an effect on liver macrophages, some examples of which are shown in **Table 1**. For instance, commercial liposomal formulations of amphotericin B (Ambisome) allow the drug to accumulate in spleen and liver macrophages that constitute a reservoir of *Leishmania*, reducing the drug's nephrotoxicity (54, 55). Besides, the encapsulation of vancomycin in liposomes improves its poor penetration into cells, allowing its targeting to Kupffer cells. In a mouse model, such formulations reduced the intracellular Methicillin-resistant *Staphylococcus aureus* (MRSA) reservoir where the bacteria can survive and proliferate, significantly increasing the survival rate of infected mice over those treated with the drug solution (48, 49).

Liposomes are also suitable vehicles for the targeting of anti-inflammatory compounds such as dexamethasone, curcumin, or calcitriol to the liver. They show improved results over the free drug in the treatment of acute and chronic liver disease models in mice. Pharmacokinetic studies show the preferential uptake of the liposomes by the liver although they accumulate not only in Kupffer cells but also in monocytes, infiltrating macrophages and, to a lesser extent, T cells. Liposomes also induce a repolarization of macrophages to a regulatory phenotype (50, 51).

Pathological conditions may influence the liposomes behavior, and recently, changes in spatial distribution and a decrease in the liposomes uptake by macrophages has been described in liver fibrosis (56).

Liposomes can be decorated for active vectorization with surface modifiers, such as mannose that has been proposed for the treatment of liver tumors in order to increase liposome uptake by macrophages via receptor-mediated endocytosis. The increase in mannose ligand concentration leads to a higher accumulation percentage in the livers of mice. Also, active targeting of an immunomodulator to liver by mannose-decorated liposomes resulted in more effective inhibition of metastasis than when delivered by liposomes without mannose (53, 57).

Substances attached to liposome surface may produce other effects. Some arginine-like ligands may switch macrophages to the M1 phenotype in order to achieve an antitumor effect. In a study with a library of this kind of ligands, nitroarginine, and acetylglutamine DOPE:DOPC liposomes were the most effective for the redirection of macrophage phenotypes (52).

In summary, preferentially uptake of liposomes by liver macrophages make them suitable vehicles for the vectorization of anti-inflammatory, anti-infective or other drugs for the treatment of liver diseases. When associated with anti-inflammatory compounds, they are able to promote the macrophages' regulatory state and reduce the dose for the treatment of acute and chronic liver injury. The decoration of the surface of liposomes with arginine-like and mannose ligands may be useful for anti-tumor treatments.

Lipoplexes

Inhibition of regulatory pathways triggered by macrophages via the use of gene therapies is another strategy for the treatment of macrophage-associated diseases. Lipid-based NPs are the most successful non-viral vehicles for targeting RNAi to Kupffer cells and can reach a high efficiency of transfection. Although they are sometimes referred to as liposomes, lipoplexes are usually different in both structure and composition. They are based on cationic lipids that are able to both bind and condense negatively charged iRNA through electrostatic interactions and to deliver the payload into the cytoplasm

TABLE 1 | Characteristics of some liposomes proposed for liver macrophages targeting.

Composition	Size/nm	Active	Effect	References
HSPC, CHOL, DSPG	80	Amphotericin B	Leishmanicide	(47)
DCP, DMPG, CHOL	527.6 ± 58.2	Vancomycin	Improvement of MRSA infection	(48, 49)
DPPC: PEG-(2000)-DSPE: NBD-PE:CHOL	100	Dexamethasone	Switch to M2 phenotype Liver injury and liver fibrosis reduction	(50)
EPC:CHOL	100–150	Curcumin 1,25-dihydroxy-vitamin D3 (calcitriol)	Switch to M2 phenotype Reduction in liver inflammation, fibrosis and fat accumulation	(51)
DOPC: DOPE	83.5–108.8	Arginin-like ligands	Switch to M1 phenotype Antitumor	(52)
DSPC: CHOL: Mannose	∼95	Muramyl dipeptide (MDP)	Increase of Kupffer cells tumoricidal activity	(53)

HSPC, hydrogenated soy phosphatidylcholine; CHOL, cholesterol; DSPG, distearoyl phosphatidylglycerol; DSPC, distearoyl 3 phosphatidylcholine; DCP, dicethylphosphate; DMPG, dimyristoylphoshatidylglycerol; DPPC, dipalmitoyl phosphatidylcholine; PEG-(2000)-DSPE, polyethyleneglycol-(2000)-distearoyl phosphatidylethanolamine; NBD-PE, N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethyl-ammonium salt); EPC, egg phosphatidylcholine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine.

of target cells (58). They also incorporate neutral lipids in order to attenuate the toxicity of cationic lipids. Besides cell selectivity, the success of these kinds of therapies depends on transfection efficacy. Lipoplexes are engulfed by Kupffer cells through macropinocytosis and clathrin-mediated mechanisms after IV administration, but RNAi escape from endosomes is a rate-limiting step for these therapies (59). Proper lipid design allows RNAi to reach the cellular cytoplasm where it exerts its action. In this way, cationic lipid C12-200 eposide would prevent RNAi lysosomal degradation (58) through a micropinocytosis internalization mechanism as macropinosomes do not follow the endosomic degradation pathway (23). It was applied to inhibit the PD-1/PD-L1 pathway that contributes to the persistence of viral liver infections. Lipid NPs of 70–80 nm size were efficiently internalized (66.5%) and expressed by Kupffer cells after *in vivo* IV administration in viral-infected mice. This led to an enhanced antiviral effect. This promising antiviral immunotherapy may be applicable to vaccine development, to treat diverse viral liver infections and other diseases such as hepatocarcinome (60).

However, cationic lipids are used as vehicles for RNAi forming lipoplexes. A high transfection efficacy in Kupffer cells can be achieved with proper lipid selection, showing promising results in immunotherapy.

Inorganic Nanoparticles

Inorganic NPs are a broad group of metallic and non-metallic nanomaterials. Some of them are non-biodegradable, which constitutes a pitfall for their use. However, they possess excellent properties such as small size, high surface area, and easy functionalization and may induce *per se* responses in macrophages with different therapeutic applications as shown in **Table 2**.

Inorganic NPs have been used in the diagnostic and treatment of liver fibrosis and recently this topic has been reviewed in depth (65). For instance, the reduction of inflammatory macrophage activity caused by ceriumoxide NPs has been proposed to prevent hepatic dysfunction in septic rats. The NPs attenuate the expression of a number of different inflammatory macrophage mediators that are associated with sepsis, improving rat survival (61). This downregulation of Kupffer cell activity was also reported for gold nanoparticles (GNPs) in two rat liver-injury models causing antioxidant and antifibrotic effects (62).

Inflammatory diseases may also be treated by the switch of macrophages from an inflammatory ("M1") to an anti-inflammatory ("M2") phenotype. Carbohydrates are able to induce phenotypic changes promoting one or other activation state depending on their physical and chemical characteristics (66). As an example, glucomannan carbohydrate-decorated silicon oxide nanoparticles promote M2 polarization in macrophages by inducing clustering of mannose receptors (MR) on the cell surface. Although this was assayed in a murine inflammatory bowel disease model, it may be applied to other inflammatory diseases (63).

Conversely, induction of an immune response was the aim of calcium phosphate polyetilenimine/SiO_2 nanoparticles used as carriers of a Toll-like-3 ligand. The NPs targeted the liver with 30–40% NP-positive cells when administered intravenously to mice and could be applied to vaccination (67).

Another group of inorganic NPs with applications in liver macrophage vectorization is superparamagnetic iron oxide nanoparticles (SPIONs), which are promising nanomaterials as diagnostic, iron supplement, and drug carrier agents. Surface modifications can render a high biocompatibility (64). They are phagocytized by macrophages and induce a pro-inflammatory response (68–70) through the activation of the Toll-like receptor 4 (71). Recently, they have been proposed for the reeducation of M2 tumor-associated macrophages to an antitumor M1 state in cancer treatment. This effect was studied for carboxymethyldextran-coated iron oxide NPs (Ferumoxytol®), which are approved by the FDA for the treatment of iron deficiency and other clinical uses. In a mouse *in vivo* model, these NPs inhibited tumor growth and prevented metastasis development. This activity was associated with the increase of M1 macrophages that may have been promoted by iron overload (72, 73). The uptake mechanism of carboxy-dextran coated SPIONs by human macrophages is a clathrin-mediated and scavenger receptor endocytosis although macropinocytosis may also contribute to internalization (74). The recognition of SPIONs by macrophages depends on the particle size and surface modifier, and it is better for positively

TABLE 2 | Some examples of inorganic nanoparticles for liver macrophage targeting.

NPs type	Size/nm	Model	Effect	References
CeO_2	53.36 ± 7.04	Lipopolysaccharide induced severe sepsis in rats	Reduced expression of inflammatory macrophage mediators	(61)
Au	7.4 ± 1.6	Rat liver injury with ethanol and methamphetamine	Downregulation of Kupffer cells activity	(62)
Glucomannan-silica	27.6 ± 0.6 −28.89 ± 1.60	Murine inflammatory bowel disease	M2 polarization	(63)
SPIONs				
Dimercaptosuccinic acid	65	Murine and human M2 cells	Modification of M2 activation profile	(64)
3-Aminopropyl-triethoxysilane	54			
Aminodextran	150			

charged particles and for a size of ∼60 nm of size, although such SPIONs show cytotoxicity (71, 74, 75).

The efficient SPION uptake by macrophages allows its use for labeling macrophages in a cellular therapy for the treatment of liver cirrhosis. The labeled cells were tracked *in vivo* by magnetic resonance and no effects on phagocytic activity or cell viability were observed (75). AuNPs have also been used to this end and 50 nm was proposed as the optimal size for labeling without toxicity concerns for both NPs types (76).

In summary, inorganic nanoparticles are able to stimulate and also inhibit the activity of macrophages. Moreover, they can promote a switch to a specific macrophage state. The desired effect depends on the disease to be treated. The interaction of SPIONs with macrophages has been well-characterized and a mathematical model for the prediction of NPs uptake has even been developed (74). However, further insights are needed to clarify the relationship between the characteristics of NPs and their therapeutic and toxic effects.

Polymeric NPs

Polymeric NPs are colloid systems made of natural or synthetic polymers. They consist of a matrix in which the drug is homogeneously distributed or may be structured in a nucleus and a polymeric shell (nanocapsules) (54). They display great versatility. A fundamental feature of these nanosystems is their biodegradability, which is essential for intravenous administration.

Polymers of polylactic–glycolic acids (PLGA) are the most used for drug delivery as they are compatible and biodegradable compounds, and are excipients approved by the FDA. NPs of these polyesthers show excellent properties as drug carriers for liver macrophages vectorization (77) in order to improve efficacy or reduce side effects. PLGA NPs have been proposed as carriers of an inhibitor of the spleen tyrosine kinase SYK. This enzyme is overexpressed in M1 macrophages and shows a positive correlation with the pathogenesis of NASH and alcoholic hepatitis in patients. Although the bare inhibitor was more effective *in vitro*, its incorporation to 160 nm PLGA nanoparticles improved its intrahepatic delivery and therapeutic efficacy *in vivo*. This ameliorated fibrosis, inflammation, and steatosis in mice after IV administration (**Figure 2**) (78).

The objective pursued for rosiglitazone NP incorporation was to reduce their serious side effects such as the increased risk of fatal cardiac arrhythmia. Rosiglitazone vectorization to macrophages using 200 nm PLGA/polyvinylic acid (PVA) nanospheres allows for its selective delivery to circulating

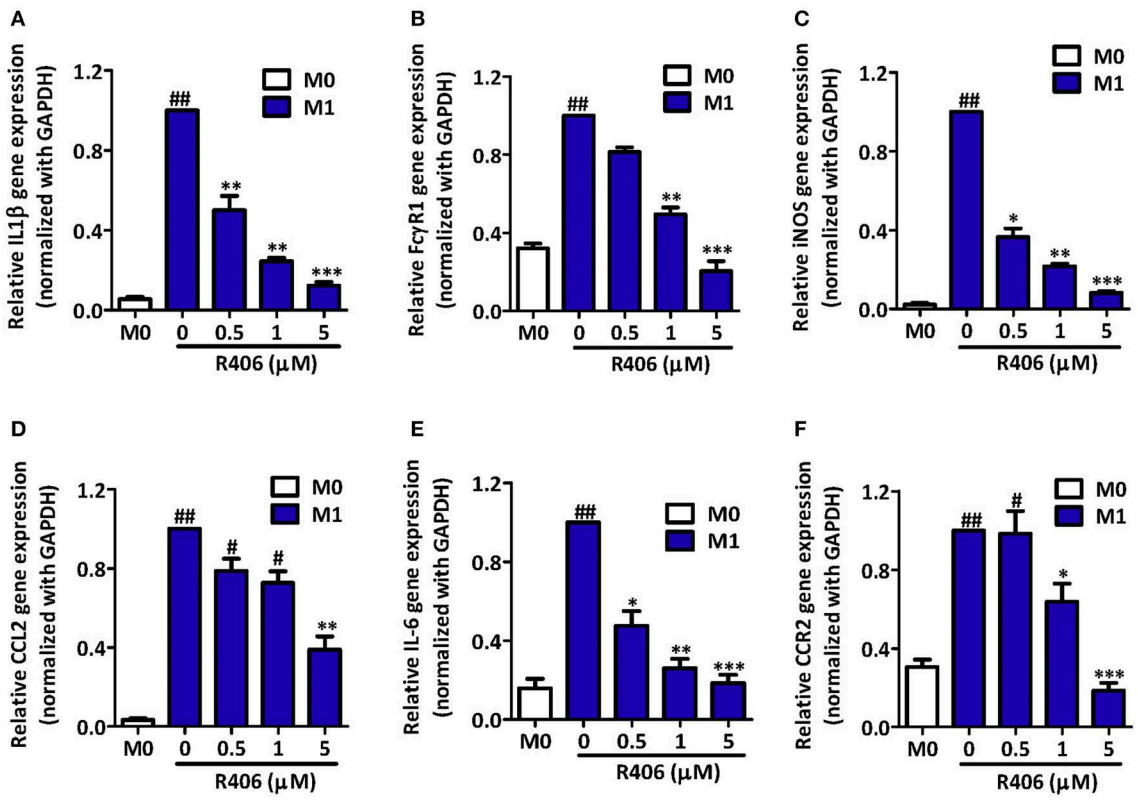

FIGURE 2 | Inhibition of M1-specific differentiation and inflammatory markers by R406 in RAW macrophages. Gene expression of M1 markers IL-1β **(A)**; FcγR1 **(B)**; iNOS **(C)**; CCL2 **(D)**; IL-6 **(E)**; and CCR2 **(F)** in RAW 264.7 cells after incubation with medium alone (M0) or M1 stimulus with R406 (0, 0.5, 1, and 5 μM). Expression values for the respective genes in untreated M1 macrophages were set at 1.0 to calculate the relative gene expression. Data are presented as mean + SEM. #$p < 0.05$, ##$p < 0.01$ denotes significance versus control M0 macrophages. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ denotes significance versus M1-differentiated macrophages. Reproduced with permission (78).

monocytes and Kupffer cells, reducing obesity-related inflammatory reaction in the white adipose tissue and liver and mitigating undesired effects (79).

Cationic polymers have been proposed as carriers of genetic material to reduce the expression of proteins related to liver diseases. This was the objective of RNAi for protein NOGOB encapsulation in [poly(amine-co-ester) (PACE) terpolymers] NPs. NOGOB promotes M1 polarization, stimulating the progression of alcoholic liver disease and liver fibrosis. The *in vivo* spleen administration of the NPs with a size of 240–300 nm allowed up to 60% Nogo-B protein suppression (80). This is a high transfection efficacy although alternative administration routes adequate for clinical use should be assayed. High transfection efficacy was also achieved by PLAcore/PVAshell NPs loaded with PEI-CD98 siRNA. The objective was the downregulation of CD98, a factor that is overexpressed in the livers of non alcoholic fatty liver disease (NAFLD) patients playing as a key inducer in this disease. The IV administration of CD98siRNA NPs with a size of 273.1 ± 19.3 nm effectively targeted liver hepatocytes and Kupffer cells, leading to a significant decrease of major proinflammatory cytokines and markers of NAFLD (81).

Chitosan, a biodegradable, positively charged polymer of natural origin is also widely used as a vehicle of RNAi. Quaternary chitosan NPs were designed for vectorization to macrophages of the RNAi of proinflammatory cytokine tumor necrosis factor TNFα. The NPs with sizes between 210 and 279 nm and zeta potentials from 14 to 22 mV achieved a high cellular uptake efficiency near 100% by RAW-274-7 macrophages. NPs cross-linking with TPP reduced their size and zeta potential, resulting in better transfection abilities (82). Polyethylenimine (PEI) is another cationic polymer with high transfection efficacy due to the proton–sponge phenomenon. In order to increase the chitosan's ability to transfect and reduce the PEI cytotoxicity, both components are mixed. NPs of sizes from 150 to 200 nm with both polymers achieved high macrophages transfection *in vitro* with non-cell toxicity (83). Functionalization of chitosan and other polymers, such as dendrimers with mannose, allows for active vectorization with a better selectivity for macrophage vectorization of RNAi and drugs (84–86).

The applications of polymeric NPs extend to HIV infections, where macrophages play a central role as virus reservoirs. Kutscher et al. designed GLU-decorated chitosan (CS) shell and polylactic-co-glycolic acid (PLGA) core nanoparticles (GLU-CS-PLGA) that recognize special receptors expressed in infected macrophages for the delivery of the antiretroviral drug nevirapine (87). Also, polymeric NPs modified with folic acid may target atazanavir/ritonavir to activated macrophages that overexpress folate receptor at an elevated level (88).

Polyesthers and chitosan are the polymers most often used in order to target drugs and RNAi to liver macrophages. To this end, NPs sizes of 150 to 300 nm have been prepared. Decoration with mannose and other ligands allow NPs to be selectively uptaken.

Other Nanosystems

Exosomes are phospholipidic nanoparticles of endosomal origin that are secreted by cells. They display similar advantages to synthetic nanoparticles, but they usually show higher biocompatibility and physiological activity. Like the majority of nano-sized vesicles, they also accumulate in the liver and may target Kupffer cells. Exosomes derived from mesenchymal stem cells reduce the levels of proinflammatory factors in murine macrophages *in vitro* with a decrease in biochemical and histological damage (89). These effects were also observed in an *in vivo* experimental lethal hepatic injury mouse model. The beneficial effect of these vesicles on reducing mortality implies modulation of the inflammatory response and activation of protective mechanisms to limit cell death (90).

Mesenchymal stem cell exosomes are then new types of nanovehicles with anti-inflammatory and protective effect in liver injury that constitute promising strategies for liver disease treatments.

IMPACT OF MACROPHAGE PHENOTYPE IN NPs UPTAKE AND TOXICITY

Nanosystems that specifically target and deliver therapeutics to polarized macrophages are of interest due to the role that they play in liver diseases. In order to improve the design of those drug delivery systems, the interaction of nanoparticles with macrophages of different phenotypes must be understood, which is why it has been the subject of several studies. Increased NP sequestration by M1 phenotypes has been reported as they are involved in biological processing of foreign materials. Thus, incorporation of phagocytosis promoters in lipid-latex nanoparticles allowed them to target inflammatory M1 macrophages (91). Also, a higher sequestration by inflammatory phenotype was found *in vitro* and *in vivo* for spherical silica nanoparticles. This was attributed to the silanol terminal groups that would attach to the receptors of anionic groups that are overexpressed in M1 macrophages (92).

However, M2 macrophages show increased expression of mannose and galactose receptors (93). This makes it possible to target specifically anti-inflammatory phenotypes with mannose–decorated nanoparticles (94). Pluronic and chitosan-based NPs of 150 to 265 nm, decorated with mannose, with positive and negative zeta potentials, respectively, can selectively target M2 macrophages to treat inflammatory diseases and HIV infections. The charge of the NPs and their degree of internalization are dependent on mannose density (84, 94).

Recently, a comparative study on gold nanoparticle uptake by human monocyte-derived macrophages of different phenotypic polarization showed, in general, higher internalization of gold nanoparticles by M2-polarized human macrophages in comparison with the M1-polarized cells. The extent of the uptake was positively correlated with the expression of M2 markers CD163 and CD206. Further investigation in human Kupffer cells showed comparable internalization of nanoparticles by those unstimulated Kupffer cells with a mixed M1/M2 phenotype and the M2-polarized cells, both of which ingested more nanoparticles than the M1-polarized cells did (28).

In short, various types of nanosystems have been designed for targeting polarized macrophages, although further research

is necessary to define the NP characteristics necessary for promoting preferential M1 or M2 uptake.

HEPATIC BIODISTRIBUTION OF NANOPARTICLES

The kinetics of nanoparticles when administered *in vivo* strongly affects their therapeutic efficacy. Nanoparticular systems are recognized by macrophages of the mononuclear phagocyte system and tend to accumulate mainly in organs such as the liver, the spleen, or the lungs (95). The selective distribution of nanoparticular systems in these types of organs facilitates their use for the diagnosis and treatment of different types of pathologies.

Experimental studies conducted with cell lines and animals have brought about a clarification of the mechanisms of penetration into macrophages and hepatocytes using chitosan nanoparticles (96).

Figure 3 shows the arrangement of chitosan nanoparticles in Kupffer cells, hepatocytes, and in whole animals.

As shown in **Figure 3**, *in vivo* studies in mice demonstrate that, at the level of Kupffer cells, the cellular uptake of chitosan nanoparticles is produced via mechanisms of phagocytosis and clathrin- and caveolin-mediated endocytosis and their release through the lysosomal and multivesicular pathways. In addition, nanoparticles penetrate intracellularly into hepatocytes. The renal and hepatobiliary excretion pathways constitute the main routes of elimination *in vivo*, observing a slow elimination with a nanoparticle half-life of >60 days (96). In addition, *in vitro* studies conducted with murine macrophage cell lines show that chitosan nanoparticle uptake was clathrin-mediated endocytosis as a primary mechanism and also via phagocytosis as a secondary mechanism. After internalization, a large proportion of the nanoparticles may be excreted from the cells by lysosome-mediated and multivesicular body-mediated exocytosis (97).

Short-term biodistribution of silica nanoparticles in mice demonstrates their high accumulation in organs of the reticulo-endothelial system such as liver and spleen. At the same time, the animals in the experiment showed a clear increase in the number of hepatic macrophages over time. Aggregates of macrophages or microgranulomes increased between 6 and 24 h after silica nanoparticle administration. Clearance of silica nanoparticles

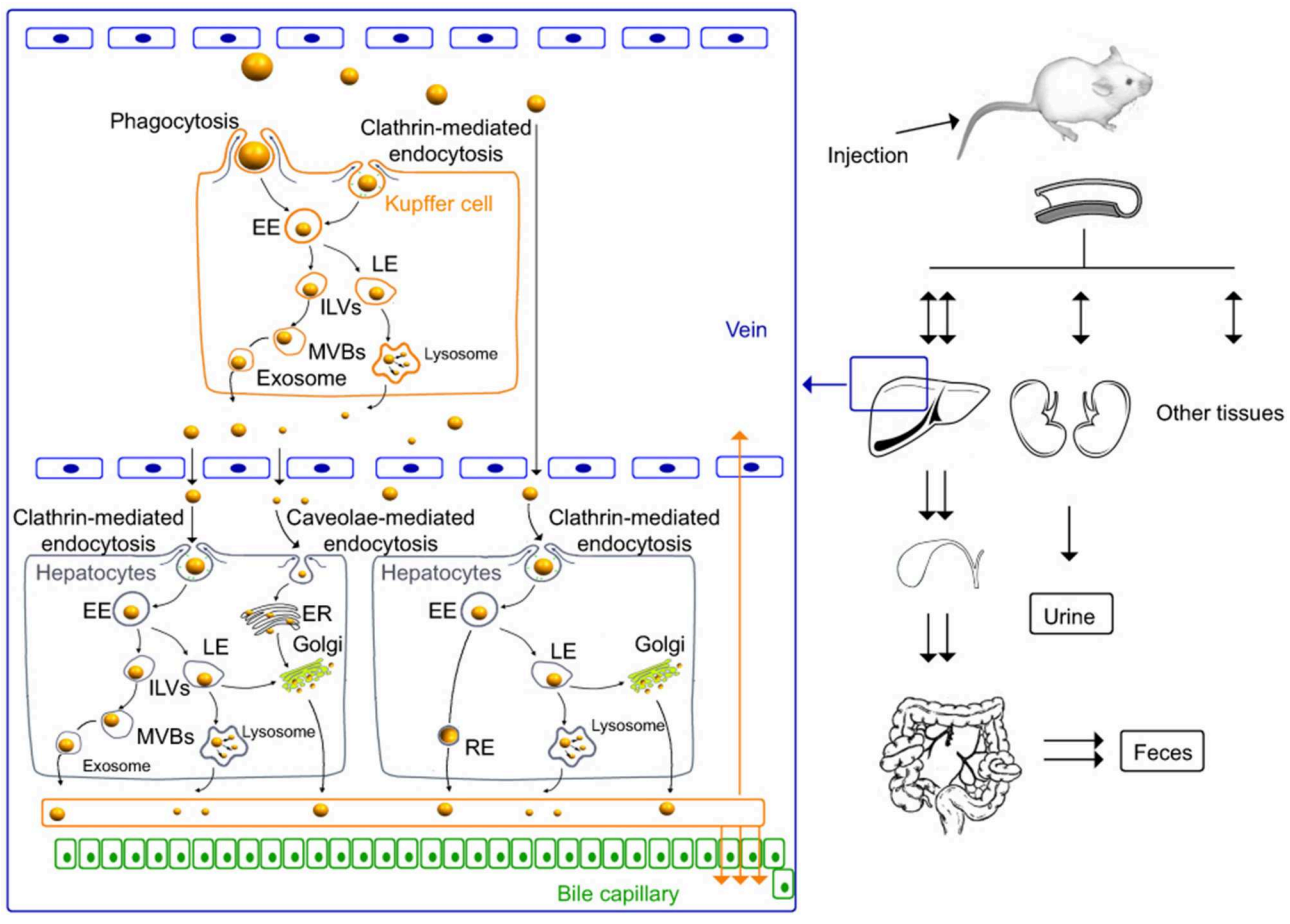

FIGURE 3 | *In vivo* distribution and elimination of chitosan nanoparticles in Kupffer cells and rat hepatocytes (96). International Journal of Nanomedicine. Reproduced with permission from Dove Medical Press Ltd.

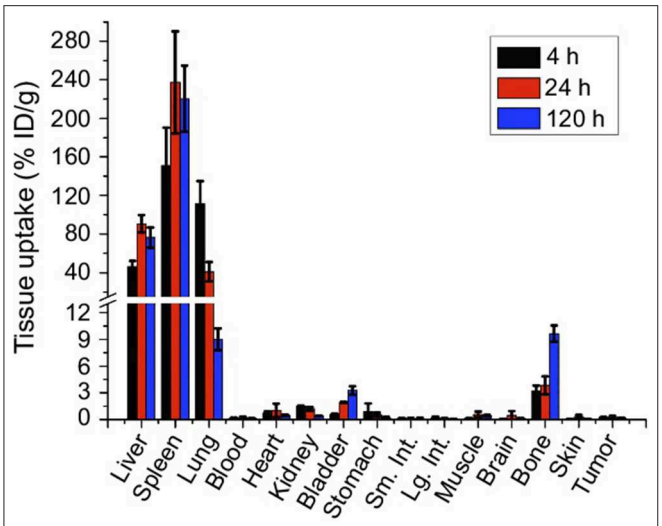

FIGURE 4 | Biodistribution of GNS-labeled macrophage cells at different times after IV administration in mice (102). International Journal of Nanomedicine. Reproduced with permission from Dove Medical Press Ltd.

from the liver appears to be slower than from the spleen, probably due to hepatic processing and biliary excretion (98).

Other authors also describe the retention of silica nanoparticles and the development of fibrosis in rat livers up to 60 days after IV administration (99).

Iron oxide nanoparticles are a type of system increasingly used for magnetic resonance imaging in diagnostic techniques (100). The administration of polyacrylic acid-coated iron oxide nanoparticles is associated with a selective distribution in the liver that produces proinflammatory activation and liver toxicity in mice. A high accumulation of iron was also observed by macrophages phagocytosis in the periportal zone of the hepatic acinus of the liver and in the splenic red pulp of the spleen which demonstrates the specific uptake of this type of nanoparticles by the monocyte–macrophage system (101).

Nanosystems are currently being combined with cell-based platforms with interesting biodistribution properties and with different therapeutic objectives.

As an example, plasmonic gold nanostars (GNS) were incorporated into immune system cells such as dendritic cells or macrophages obtained from bone marrow in order to investigate the biodistribution of these types of cells in a murine lymphoma model (102).

Figure 4 shows biodistribution in organs and tissues at different times in mice after IV administration of GNS-labeled macrophage cells. The quantification of gold in the different tissues was performed using an ICP-MS technique (102).

As shown in **Figure 4**, after IV administration of GNS-labeled macrophage cells, a specific distribution of this cell-based nanosystem preferably in the spleen, liver, and lung was observed, indicating that these organs had high macrophage cell accumulation. This type of cell-based delivery system presents interesting applications for cell-tracking studies (102).

Stabilin (-1 and -2) are specific receptors for the cellular uptake of different substances such as antisense oligonucleotides in the liver through clathrin-mediated endocytosis (103). Recent studies have been conducted to determine the mechanisms involved in the uptake of nanoparticles by hepatic macrophages. For this reason, an embryonic zebrafish model has been used to evaluate the interaction of nanoparticles with macrophages and endothelial cells using liposomes as nanoparticles. This research has demonstrated the role of the stabilin-2 receptor in the uptake of nanoparticles by endothelial cells. The nanoparticle uptake proved to be independent of the type of material and the functional properties of the nanoparticles but was influenced by the surface charge of the nanoparticle. In addition, the interaction between endothelial cells and nanoparticles can be blocked by competitive inhibitors of stabilin-2 such as dextran sulfate (104).

In another work about the mechanisms involved in the anti-inflammatory activity and in the recognition of crystals and nanomaterials by macrophages, cell-surface receptors or membrane cholesterol have been described as mechanisms involved in crystal nanoparticle recognition although other phagocytosis mechanisms are still unknown (105).

Pathological conditions may also influence NPs uptake. Liver fibrosis profoundly changes the myeloid compartment in the liver, with decreasing numbers of Kupffer cells and increasing numbers of MoMF. With the aim of investigating the changes in the targeting properties of different nanosystems in hepatic fibrosis, Ergen et al. studied the biodistribution of three intravenously injected carrier material, i.e., 10 nm poly(N-(2-hydroxypropyl)methacrylamide) polymers, 100-nm PEGylated liposomes, and 2,000 nm poly(butyl cyanoacrylate) microbubbles, in two fibrosis mice models. They found a decreased uptake of polymers and microbubbles by almost all myeloid cells of the fibrotic liver. However, liposomes had an overall higher targeting efficiency for endothelial and myeloid cells, which remained high even in fibrotic livers with around 60% carrier positive cells in healthy livers and after induction of liver fibrosis, although with a low specificity for the various cell populations. In all cases, Kupffer cells and monocyte-derived macrophages were the cells with increased percentage of carrier-positive cells (56).

Pharmacokinetic Models

Different pharmacokinetic models such as compartmental and especially physiologically based pharmacokinetic (PBPK) models have been developed to characterize the disposition of drugs in different organs and tissues, and especially in the liver, when they are administered in different types of nanoparticles (98, 106–112).

Classic pharmacokinetic models, such as the two-compartmental model, have been proposed to characterize the accumulation in the reticulo-endothelial system and in the livers of mice, as well as the elimination of superparamagnetic iron oxide nanoparticles (SPIONs). The model allows binding to Kupffer cells and extrahepatic clearance of nanoparticles to be characterized using dynamic magnetic resonance imaging (MRI) as seen in **Figure 5** (106). The constants K_{in} and K_{out} describe the kinetics of nanoparticles associated with and dissociated from

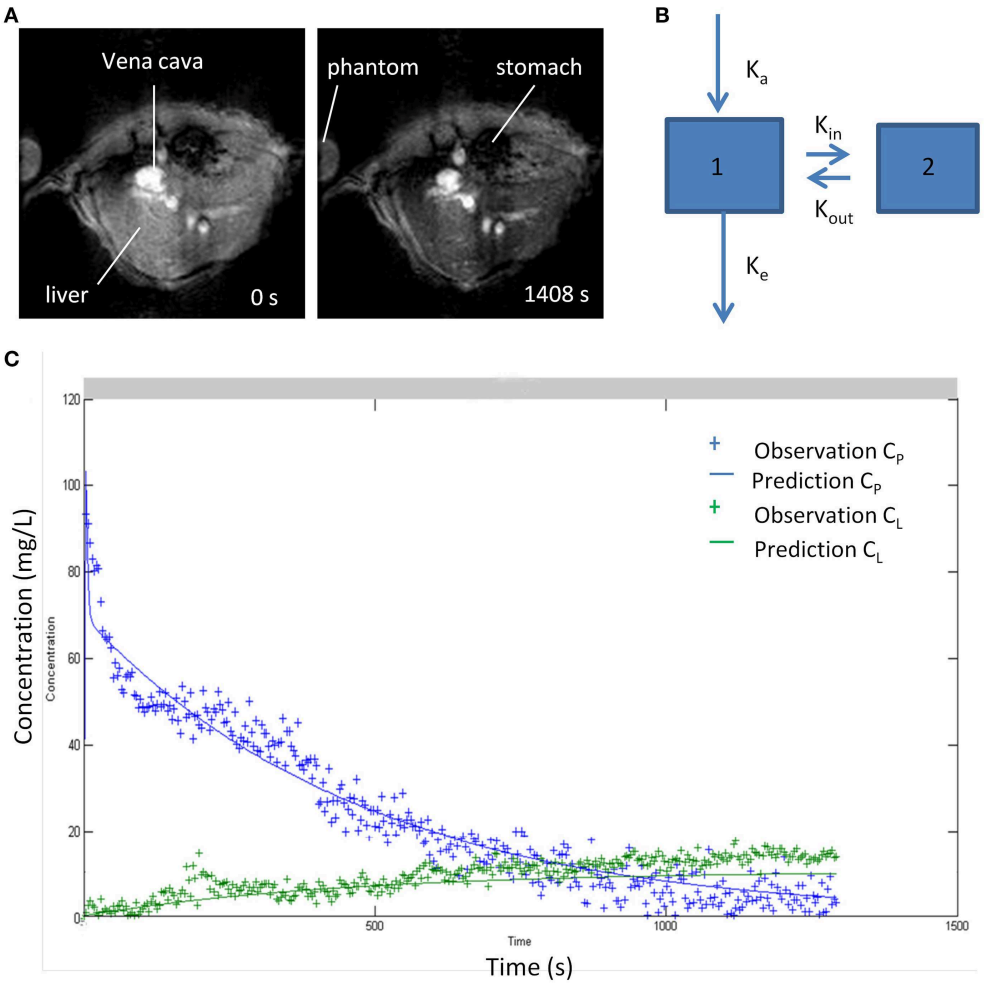

FIGURE 5 | (A) MR images of liver before and after 20 min of intravenous injection of iron oxide nanoparticles **(B)** Two-compartment kinetic model used to characterize blood pharmacokinetics and distribution in liver tissue of superparamagnetic iron oxide nanoparticle (SPIO). Compartments 1 and 2 represent blood and liver. **(C)** Pharmacokinetic profiles of nanoparticles in blood and liver fitted to the two-compartment model (106). Reproduced with permission.

the macrophage, and the constant K_e describes the elimination of nanoparticles from the blood compartment by the extrahepatic RES (97).

The kinetic parameters of distribution in the liver K_{in} and K_{out} are related to Kupffer cell numbers, allowing the function of the reticulo-endothelial system to be evaluated in different situations and presents therapeutic applications in liver disease, allowing the chronic liver injury to be evaluated, taking into account that the macrophages are integrated in different stages of the inflammatory process (106).

Physiologically Based Pharmacokinetic (PBPK) Models

PBPK models have been developed to characterize nanoparticle biodistribution in organs of the reticuloendothelial system (RES) such as the liver or the spleen.

PBPK models constitute an interesting strategy for modeling and simulation that allow the kinetic behavior of drugs in animals and humans to be predicted with a physiological basis. These models are based on grouping the body into different compartments that are assimilated to different organs and tissues. These compartments are defined by the volume of tissue and the blood flow that irrigates it. The mass balance of the drug throughout the body is defined through first-order differential equations. The distribution of the drug in each of the tissues can be perfusion-limited or diffusion-limited (112–114). Perfusion rate-limited kinetics occurs when blood flow limits tissue distribution. In the steady state, the concentration of drug in the tissue is in balance with the concentration of drug in the blood through a specific partition coefficient for each tissue. This type of distribution occurs in lipid molecules that easily penetrate the tissue. Permeability rate-limited kinetics occurs when permeability across the membrane constitutes the limiting process of intratisular distribution and occurs mostly in more polar or hydrophilic molecules (112, 113).

Specific PBPK models that consider the specific disposition properties of nanoparticles have been developed to characterize the biodistribution and elimination of nanoparticulate systems

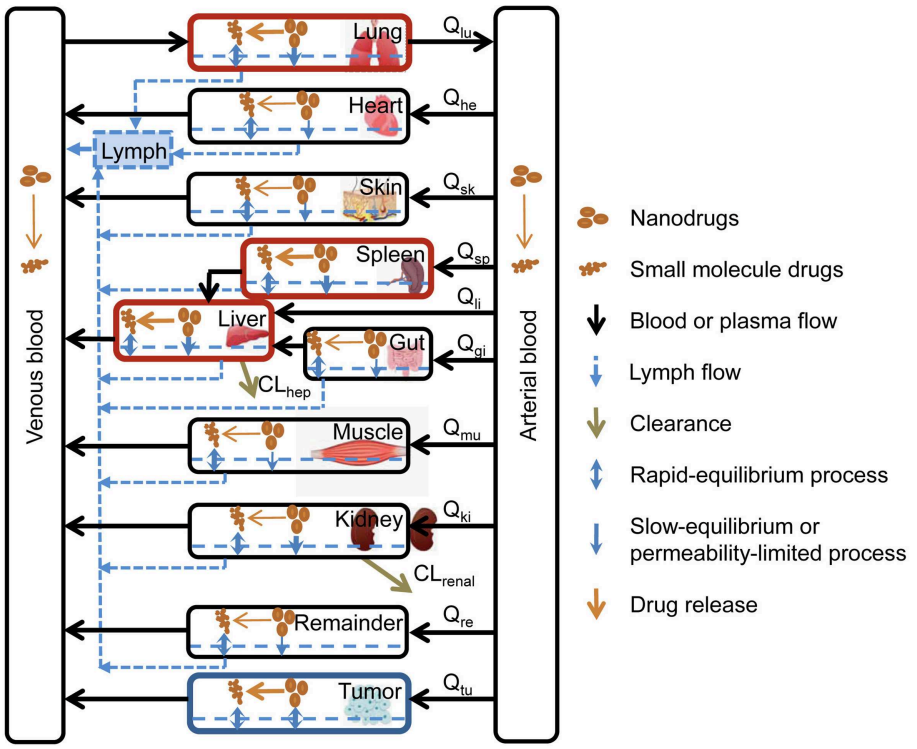

FIGURE 6 | Specific PBPK model to characterize the biodistribution of nanoparticles (112). Reproduced with permission.

in the whole body. This model assumes the existence of two PBPK submodels: one to characterize the disposition of the nanoparticles and another to characterize the disposition of the free drug as shown in **Figure 6**. This model predicts a greater accumulation of nanoparticles in the liver, spleen, and lungs due to the vascular structure of these tissues and the uptake of nanoparticles by the mononuclear phagocytic system. On the other hand, this type of model has some limitations, given that certain processes, such as aggregation or degradation of nanoparticles, among others, can change the properties of nanoparticles and their arrangement in different organs and tissue. In addition, this type of model allows the interaction between the drug, the nanoparticle, and the complex physiology of the organism to be modeled and simulated (112).

Some of these PBPK models have been designed to characterize the role of macrophages in drug tissue concentrations by combining the data from clinical studies with *in vitro* data. The model allows the concentration of the antibiotic moxifloxacin in tissues from biopsies to be predicted, including those from interstitial fluid, intracellular fluid, vascular space, and macrophages. The study showed that macrophages contribute to the accumulation of the drug in tissues from biopsies (109).

This same type of PBPK model has also been used to predict behavior in liver diseases such as liver cirrhosis. This model allows changes in the plasma concentrations of different drugs, such as alfentanil or lidocaine among others associated with Child-Pugh class A, B, and C liver cirrhosis, to be predicted (115).

Previously, general PBPK models that allow the distribution of drugs incorporated in different types of nanoparticles to be simulated have been described (111). The model was tested with different kinds of nanoparticles with differences in drug dose, size, charge, shape, or surface properties. This model also considers saturable phagocytosis of the nanoparticles. This model is based on another previously published model to characterize the biodistribution of PAA-PEG (116).

This model considers 10 anatomical compartments and each compartment is divided into three subcompartments that represent blood, tissue, and phagocytic cells.

Bigger nanoparticles are better recognized by the macrophages than smaller ones. On the other hand, cationic nanoparticles are better recognized by macrophages than anionic or neutral ones (112, 117). Based on this model, when nanoparticles are injected into the blood, they are captured by phagocytic cells of organs of the reticulo-endothelial system (RES) such as the liver and the spleen depending on their electric charge, size, and agglomeration state, among other factors (111).

In the field of toxicokinetics, PBPK models have been applied to characterize the biodistribution of silver nanoparticles compared to ionic silver. The PBPK model predicts a higher accumulation of silver in the liver as silver nanoparticles in comparison with ionic silver (118).

NANOPARTICLE TOXICITY

The toxicity of nanosystems is an important issue that may limit its applicability. Once in the organism, the processing and final fate of nanoparticles is dependent on their composition.

Liposomes are biodegradable phospholipidic-based nanosystems, and once administered, they are degraded by serum proteins in the blood circulation or by intracellular lipases. The degradation products of liposomes are their constituent lipid molecules that can be further metabolized by the body. This also holds for other lipids forming solid lipid nanoparticles. However, positively charged lipids show limited compatibility as they may induce cytokine activation and cellular toxicity with apoptosis (119).

Polymeric nanoparticles are modified in the organism, giving rise to constituent monomeric units or modified polymer chains. For biodegradable polymers, degradation products are smaller than the renal molecular weight cutoff size and can follow renal elimination (120, 121). For non-biodegradable components, the larger molecules may be cleared by hepatobiliary or the mononuclear phagocyte system, but their biotransformation in hepatocytes and macrophages may cause toxicity (122, 123). As their lipidic counterparts, cationic polymers such as PEI show high cellular toxicity (1).

The non-biodegradability of some inorganic NPs is a factor that leads to liver toxicity (124) and limits their applicability in humans. When NPs are not decomposed by the phagocytosis process, they will remain within the cell and be sequestered in the spleen and liver for long periods of time (122, 125–127). Once the nanoparticle-filled phagocyte dies, those nanoparticles are taken up again by other phagocytes of the same organ, resulting in a similar total amount of nanoparticles accumulated (128). NPs accumulate in the liver and especially in Kupffer cells (129) and could cause fibrosis and other histological tissue changes. They produce oxidative stress that in turn modulates the autophagic process in the liver, disrupting liver metabolism and homeostasis (65). This hepatic oxidative damage has been reported in both *in vitro* and *in vivo* models for AuNPs, SiO_2NPs, and AgNPs, with differences among the NP compositions (130–134). However, long-term effects need to be further characterized (65).

Tunable properties of NPs, such as shape, surface charge, and size, may affect their toxicity (135, 136). For instance, cerium oxide (CeO_2) NPs with rod-like shape showed higher and dose-dependently enhanced macrophage cytotoxicity responses with respect to cubic/octahedral NPs (137). The surface charge is also an important parameter that affects NP clearance rate. One study with mesoporous silica NPs (MSNs) showed that positively charged NPs were rapidly excreted from the liver into the gastrointestinal tract while negatively charged NPs remained sequestered in the liver (138). Also, the degradation of silica NPs to silicic acid for their renal excretion depends on the characteristics of the NPs, such as porosity, size, and surface chemistry (139–141).

Toxicity also depends greatly on the dose of the NPs, with cytotoxicity reported over certain doses for inorganic nanoparticles such as cerium oxide and gold NPs (61, 142, 143).

Therefore, tailoring the characteristics of NPs and controlling the doses of inorganic NPs used could overcome the toxicity problems in the liver and allow for their clinical application (65).

Biodegradable SPIONs or SiNPs offer a safer alternative. For instance, SPION administration is well-tolerated, and long-term *in vivo* biodistribution studies have shown that they can be transformed to non-superparamagnetic iron forms and eliminated with no signs of toxicity (64). However, in another study, toxicity of SPIONs and ultrasmall superparamagnetic iron oxide nanoparticles (USPIO) in human macrophages has been described (144).

CONCLUSIONS

Different types of NPs such as liposomes, solid lipid nanoparticles, inorganic NPs, or exosomes have been proposed for targeting liver macrophages in order to treat liver diseases. They are used as delivery systems but may also induce changes in macrophage phenotypes with influence in the progression of the illness. Lipids and polymeric NPs as vectors of RNAi may exert therapeutic effects by inhibiting regulatory pathways triggered by macrophages. Moreover, the interaction of NPs with macrophages depends on their phenotype, although this issue must be studied in more depth. The physiological-based pharmacokinetic models have been mainly used to describe and simulate NPs destination in the organism. Although strategies to vectorize liver macrophages with NPs are promising, compatibility issues, especially long-term toxicity, are drawbacks for certain systems, especially non-biodegradable ones.

AUTHOR CONTRIBUTIONS

All authors have equally contributed to the manuscript conception and preparation. JL and CC elaborated an abstract and an index and then all the three authors organized a well-balanced distribution of the different items. For their part of the work, the responsible person looked for the references, redacted the text and selected the images. CG-M was responsible of references database organization. CG-M and CC adapted the format to the journal template. JL, CG-M, and CC revised the whole content and approved the submitted version.

REFERENCES

1. Zhang YN, Poon W, Tavares AJ, McGilvray ID, Chan WCW. Nanoparticle-liver interactions: cellular uptake and hepatobiliary elimination. *J Control Release*. (2016) 240:332–48. doi: 10.1016/j.jconrel.2016.01.020
2. Ma Y, Yang M, He Z, Wei Q, Li J. The biological function of kupffer cells in liver disease. In: Ghosh A, editor. *Biology of Myelomonocytic Cells*. London: IntechOpen (2017).
3. Meijer DK, Molema G. Targeting of drugs to the liver. *Semin Liver Dis*. (1995) 15:202–56. doi: 10.1055/s-2007-1007278

4. Li P, He K, Li J, Liu Z, Gong J. The role of Kupffer cells in hepatic diseases. *Mol Immunol*. (2017) 85:222–9. doi: 10.1016/j.molimm.2017.02.018
5. Yona S, Gordon S. From the reticuloendothelial to mononuclear phagocyte system—the unaccounted years. *Front Immunol*. (2015) 6:328. doi: 10.3389/fimmu.2015.00328
6. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol*. (2014) 14:571–8. doi: 10.1038/nri3712
7. Hume DA, Irvine KM, Pridans C. The mononuclear phagocyte system: the relationship between monocytes and macrophages. *Trends Immunol*. (2019) 40:98–112. doi: 10.1016/j.it.2018.11.007
8. Mildner A, Yona S, Jung S. A close encounter of the third kind: monocyte-derived cells. *Adv Immunol*. (2013) 120:69–103. doi: 10.1016/B978-0-12-417028-5.00003-X
9. Varol C, Mildner A, Jung S. Macrophages: development and tissue specialization. *Annu Rev Immunol*. (2015) 33:643–75. doi: 10.1146/annurev-immunol-032414-112220
10. Epelman S, Lavine KJ, Randolph GJ. Origin and functions of tissue macrophages. *Immunity*. (2014) 41:21–35. doi: 10.1016/j.immuni.2014.06.013
11. Ingersoll MA, Spanbroek R, Lottaz C, Gautier EL, Frankenberger M, Hoffmann R, et al. Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood*. (2010) 115:e10–9. doi: 10.1182/blood-2009-07-235028
12. Schmidl C, Renner K, Peter K, Eder R, Lassmann T, Balwierz PJ, et al. Transcription and enhancer profiling in human monocyte subsets. *Blood*. (2014) 123:e90–9. doi: 10.1182/blood-2013-02-484188
13. Guillot A, Tacke F. Liver macrophages: old dogmas and new insights. *Hepatol Commun*. (2019) 3:730–43. doi: 10.1002/hep4.1356
14. Martinez FO, Helming L, Milde R, Varin A, Melgert BN, Draijer C, et al. Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. *Blood*. (2013) 121:e57–69. doi: 10.1182/blood-2012-06-436212
15. Dixon LJ, Barnes M, Tang H, Pritchard MT, Nagy LE. Kupffer cells in the liver. *Compr Physiol*. (2013) 3:785–97. doi: 10.1002/cphy.c120026
16. Klein I, Cornejo JC, Polakos NK, John B, Wuensch SA, Topham DJ, et al. Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages. *Blood*. (2007) 110:4077–85. doi: 10.1182/blood-2007-02-073841
17. Zigmond E, Samia-Grinberg S, Pasmanik-Chor M, Brazowski E, Shibolet O, Halpern Z, et al. Infiltrating monocyte-derived macrophages and resident kupffer cells display different ontogeny and functions in acute liver injury. *J Immunol*. (2014) 193:344–53. doi: 10.4049/jimmunol.1400574
18. Scott CL, Zheng F, De Baetselier P, Martens L, Saeys Y, De Prijck S, et al. Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Commun*. (2016) 7:10321. doi: 10.1038/ncomms10321
19. Wang J, Kubes P. A reservoir of mature cavity macrophages that can rapidly invade visceral organs to affect tissue repair. *Cell*. (2016) 165:668–78. doi: 10.1016/j.cell.2016.03.009
20. Sato K, Hall C, Glaser S, Francis H, Meng F, Alpini G. Pathogenesis of Kupffer cells in cholestatic liver injury. *Am J Pathol*. (2016) 186:2238–47. doi: 10.1016/j.ajpath.2016.06.003
21. Peterson KR, Cottam MA, Kennedy AJ, Hasty AH. Macrophage-targeted therapeutics for metabolic disease. *Trends Pharmacol Sci*. (2018) 39:536–46. doi: 10.1016/j.tips.2018.03.001
22. Ritz T, Krenkel O, Tacke F. Dynamic plasticity of macrophage functions in diseased liver. *Cell Immunol*. (2018) 330:175–82. doi: 10.1016/j.cellimm.2017.12.007
23. Gustafson HH, Holt-Casper D, Grainger DW, Ghandehari H. Nanoparticle uptake: the phagocyte problem. *Nano Today*. (2015) 10:487–510. doi: 10.1016/j.nantod.2015.06.006
24. Obstfeld AE, Sugaru E, Thearle M, Francisco AM, Gayet C, Ginsberg HN, et al. C-C chemokine receptor 2 (CCR2) regulates the hepatic recruitment of myeloid cells that promote obesity-induced hepatic steatosis. *Diabetes*. (2010) 59:916–25. doi: 10.2337/db09-1403
25. Krenkel O, Puengel T, Govaere O, Abdallah AT, Mossanen JC, Kohlhepp M, et al. Therapeutic inhibition of inflammatory monocyte recruitment reduces steatohepatitis and liver fibrosis. *Hepatology*. (2018) 67:1270–83. doi: 10.1002/hep.29544
26. Parola M, Pinzani M. Liver fibrosis: pathophysiology, pathogenetic targets and clinical issues. *Mol Aspects Med*. (2019) 65:37–55. doi: 10.1016/j.mam.2018.09.002
27. He H, Ghosh S, Yang H. Nanomedicines for dysfunctional macrophage-associated diseases. *J Control Release*. (2017) 247:106–26. doi: 10.1016/j.jconrel.2016.12.032
28. MacParland SA, Tsoi KM, Ouyang B, Ma XZ, Manuel J, Fawaz A, et al. Phenotype determines nanoparticle uptake by human macrophages from liver and blood. *ACS Nano*. (2017) 11:2428–43. doi: 10.1021/acsnano.6b06245
29. He C, Hu Y, Yin L, Tang C, Yin C. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. *Biomaterials*. (2010) 31:3657–66. doi: 10.1016/j.biomaterials.2010.01.065
30. Tacke F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol*. (2017) 66:1300–12. doi: 10.1016/j.jhep.2017.02.026
31. Li L, Wang H, Ong ZY, Xu K, Ee PLR, Zheng S, et al. Polymer- and lipid-based nanoparticle therapeutics for the treatment of liver diseases. *Nano Today*. (2010) 5:296–312. doi: 10.1016/j.nantod.2010.06.007
32. Dobrovolskaia MA, McNeil SE. Immunological properties of engineered nanomaterials. *Nat Nanotechnol*. (2007) 2:469–78. doi: 10.1038/nnano.2007.223
33. Xiao K, Li Y, Luo J, Lee JS, Xiao W, Gonik AM, et al. The effect of surface charge on *in vivo* biodistribution of PEG-oligocholic acid based micellar nanoparticles. *Biomaterials*. (2011) 32:3435–46. doi: 10.1016/j.biomaterials.2011.01.021
34. Parton RG. Caveolae meet endosomes: a stable relationship? *Dev Cell*. (2004) 7:458–60. doi: 10.1016/j.devcel.2004.09.009
35. Pelkmans L, Burli T, Zerial M, Helenius A. Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. *Cell*. (2004) 118:767–80. doi: 10.1016/j.cell.2004.09.003
36. Gratton SE, Ropp PA, Pohlhaus PD, Luft JC, Madden VJ, Napier ME, et al. The effect of particle design on cellular internalization pathways. *Proc Natl Acad Sci USA*. (2008) 105:11613–8. doi: 10.1073/pnas.0801763105
37. Hoshyar N, Gray S, Han H, Bao G. The effect of nanoparticle size on *in vivo* pharmacokinetics and cellular interaction. *Nanomedicine*. (2016) 11:673–92. doi: 10.2217/nnm.16.5
38. Kulkarni SA, Feng SS. Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery. *Pharm Res*. (2013) 30:2512–22. doi: 10.1007/s11095-012-0958-3
39. Faraji AH, Wipf P. Nanoparticles in cellular drug delivery. *Bioorg Med Chem*. (2009) 17:2950–62. doi: 10.1016/j.bmc.2009.02.043
40. Arnida, Janat-Amsbury MM, Ray A, Peterson CM, Ghandehari H. Geometry and surface characteristics of gold nanoparticles influence their biodistribution and uptake by macrophages. *Eur J Pharm Biopharm*. (2011) 77:417–23. doi: 10.1016/j.ejpb.2010.11.010
41. Decuzzi P, Godin B, Tanaka T, Lee SY, Chiappini C, Liu X, et al. Size and shape effects in the biodistribution of intravascularly injected particles. *J Control Release*. (2010) 141:320–7. doi: 10.1016/j.jconrel.2009.10.014
42. Duan X, Li Y. Physicochemical characteristics of nanoparticles affect circulation, biodistribution, cellular internalization, and trafficking. *Small*. (2013) 9:1521–32. doi: 10.1002/smll.201201390
43. Esmaeili F, Ghahremani MH, Esmaeili B, Khoshayand MR, Atyabi F, Dinarvand R. PLGA nanoparticles of different surface properties: preparation and evaluation of their body distribution. *Int J Pharm*. (2008) 349:249–55. doi: 10.1016/j.ijpharm.2007.07.038
44. Hassan R, Tammam SN, Safy SE, Abdel-Halim M, Asimakopoulou A, Weiskirchen R, et al. Prevention of hepatic stellate cell activation using JQ1- and atorvastatin-loaded chitosan nanoparticles as a promising approach in therapy of liver fibrosis. *Eur J Pharm Biopharm*. (2019) 134:96–106. doi: 10.1016/j.ejpb.2018.11.018
45. Lefere S, Tacke F. Macrophages in obesity and non-alcoholic fatty liver disease: crosstalk with metabolism. *JHEP Reports*. (2019) 1:30–43. doi: 10.1016/j.jhepr.2019.02.004

46. Samuelsson E, Shen H, Blanco E, Ferrari M, Wolfram J. Contribution of Kupffer cells to liposome accumulation in the liver. *Colloids Surf B Biointerfaces*. (2017) 158:356–62. doi: 10.1016/j.colsurfb.2017.07.014
47. Stone NR, Bicanic T, Salim R, Hope W. Liposomal amphotericin B (AmBisome((R))): a review of the pharmacokinetics, pharmacodynamics, clinical experience and future directions. *Drugs*. (2016) 76:485–500. doi: 10.1007/s40265-016-0538-7
48. Sande L, Sanchez M, Montes J, Wolf AJ, Morgan MA, Omri A, et al. Liposomal encapsulation of vancomycin improves killing of methicillin-resistant Staphylococcus aureus in a murine infection model. *J Antimicrob Chemother*. (2012) 67:2191–4. doi: 10.1093/jac/dks212
49. Surewaard BG, Deniset JF, Zemp FJ, Amrein M, Otto M, Conly J, et al. Correction: identification and treatment of the Staphylococcus aureus reservoir *in vivo*. *J Exp Med*. (2016) 213:3087. doi: 10.1084/jem.2016033411032016c
50. Bartneck M, Scheyda KM, Warzecha KT, Rizzo LY, Hittatiya K, Luedde T, et al. Fluorescent cell-traceable dexamethasone-loaded liposomes for the treatment of inflammatory liver diseases. *Biomaterials*. (2015) 37:367–82. doi: 10.1016/j.biomaterials.2014.10.030
51. Maradana MR, Yekollu SK, Zeng B, Ellis J, Clouston A, Miller G, et al. Immunomodulatory liposomes targeting liver macrophages arrest progression of non-alcoholic steatohepatitis. *Metabolism*. (2018) 78:80–94. doi: 10.1016/j.metabol.2017.09.002
52. Bygd HC, Ma L, Bratlie KM. Physicochemical properties of liposomal modifiers that shift macrophage phenotype. *Mater Sci Eng C Mater Biol Appl*. (2017) 79:237–44. doi: 10.1016/j.msec.2017.05.032
53. Opanasopit P, Sakai M, Nishikawa M, Kawakami S, Yamashita F, Hashida M. Inhibition of liver metastasis by targeting of immunomodulators using mannosylated liposome carriers. *J Control Release*. (2002) 80:283–94. doi: 10.1016/S0168-3659(02)00006-8
54. Pei Y, Yeo Y. Drug delivery to macrophages: challenges and opportunities. *J Control Release*. (2016) 240:202–11. doi: 10.1016/j.jconrel.2015.12.014
55. Torrado JJ, Espada R, Ballesteros MP, Torrado-Santiago S. Amphotericin B formulations and drug targeting. *J Pharm Sci*. (2008) 97:2405–25. doi: 10.1002/jps.21179
56. Ergen C, Niemietz PM, Heymann F, Baues M, Gremse F, Pola R, et al. Liver fibrosis affects the targeting properties of drug delivery systems to macrophage subsets *in vivo*. *Biomaterials*. (2019) 206:49–60. doi: 10.1016/j.biomaterials.2019.03.025
57. Hagimori M, Chinda Y, Suga T, Yamanami K, Kato N, Inamine T, et al. Synthesis of high functionality and quality mannose-grafted lipids to produce macrophage-targeted liposomes. *Eur J Pharm Sci*. (2018) 123:153–61. doi: 10.1016/j.ejps.2018.07.036
58. Love KT, Mahon KP, Levins CG, Whitehead KA, Querbes W, Dorkin JR, et al. Lipid-like materials for low-dose, *in vivo* gene silencing. *Proc Natl Acad Sci USA*. (2010) 107:1864–9. doi: 10.1073/pnas.0910603106
59. Gilleron J, Querbes W, Zeigerer A, Borodovsky A, Marsico G, Schubert U, et al. Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nat Biotechnol*. (2013) 31:638–46. doi: 10.1038/nbt.2612
60. Dolina JS, Sung SS, Novobrantseva TI, Nguyen TM, Hahn YS. Lipidoid nanoparticles containing PD-L1 siRNA delivered *in vivo* enter Kupffer cells and enhance NK and CD8(+) T cell-mediated hepatic antiviral immunity. *Mol Ther Nucleic Acids*. (2013) 2:e72. doi: 10.1038/mtna.2012.63
61. Selvaraj V, Nepal N, Rogers S, Manne ND, Arvapalli R, Rice KM, et al. Inhibition of MAP kinase/NF-kB mediated signaling and attenuation of lipopolysaccharide induced severe sepsis by cerium oxide nanoparticles. *Biomaterials*. (2015) 59:160–71. doi: 10.1016/j.biomaterials.2015.04.025
62. de Carvalho TG, Garcia VB, de Araujo AA, da Silva Gasparotto LH, Silva H, Guerra GCB, et al. Spherical neutral gold nanoparticles improve anti-inflammatory response, oxidative stress and fibrosis in alcohol-methamphetamine-induced liver injury in rats. *Int J Pharm*. (2018) 548:1–14. doi: 10.1016/j.ijpharm.2018.06.008
63. Gan J, Dou Y, Li Y, Wang Z, Wang L, Liu S, et al. Producing anti-inflammatory macrophages by nanoparticle-triggered clustering of mannose receptors. *Biomaterials*. (2018) 178:95–108. doi: 10.1016/j.biomaterials.2018.06.015
64. Rojas JM, Sanz-Ortega L, Mulens-Arias V, Gutierrez L, Perez-Yague S, Barber DF. Superparamagnetic iron oxide nanoparticle uptake alters M2 macrophage phenotype, iron metabolism, migration and invasion. *Nanomedicine*. (2016) 12:1127–38. doi: 10.1016/j.nano.2015.11.020
65. Tee JK, Peng F, Ho HK. Effects of inorganic nanoparticles on liver fibrosis: optimizing a double-edged sword for therapeutics. *Biochem Pharmacol*. (2019) 160:24–33. doi: 10.1016/j.bcp.2018.12.003
66. Lundahl MLE, Scanlan EM, Lavelle EC. Therapeutic potential of carbohydrates as regulators of macrophage activation. *Biochem Pharmacol*. (2017) 146:23–41. doi: 10.1016/j.bcp.2017.09.003
67. Sokolova V, Shi Z, Huang S, Du Y, Kopp M, Frede A, et al. Delivery of the TLR ligand poly(I:C) to liver cells *in vitro* and *in vivo* by calcium phosphate nanoparticles leads to a pronounced immunostimulation. *Acta Biomater*. (2017) 64:401–10. doi: 10.1016/j.actbio.2017.09.037
68. Mulens-Arias V, Rojas JM, Perez-Yague S, Morales MP, Barber DF. Polyethylenimine-coated SPIONs trigger macrophage activation through TLR-4 signaling and ROS production and modulate podosome dynamics. *Biomaterials*. (2015) 52:494–506. doi: 10.1016/j.biomaterials.2015.02.068
69. Laskar A, Eilertsen J, Li W, Yuan XM. SPION primes THP1 derived M2 macrophages toward M1-like macrophages. *Biochem Biophys Res Commun*. (2013) 441:737–42. doi: 10.1016/j.bbrc.2013.10.115
70. Kodali V, Littke MH, Tilton SC, Teeguarden JG, Shi L, Frevert CW, et al. Dysregulation of macrophage activation profiles by engineered nanoparticles. *ACS Nano*. (2013) 7:6997–7010. doi: 10.1021/nn402145t
71. Jin R, Liu L, Zhu W, Li D, Yang L, Duan J, et al. Iron oxide nanoparticles promote macrophage autophagy and inflammatory response through activation of toll-like Receptor-4 signaling. *Biomaterials*. (2019) 203:23–30. doi: 10.1016/j.biomaterials.2019.02.026
72. Zanganeh S, Hutter G, Spitler R, Lenkov O, Mahmoudi M, Shaw A, et al. Iron oxide nanoparticles inhibit tumor growth by inducing pro-inflammatory macrophage polarization in tumor tissues. *Nat Nanotechnol*. (2016) 11:986–94. doi: 10.1038/nnano.2016.168
73. Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter H, et al. An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *J Clin Invest*. (2011) 121:985–97. doi: 10.1172/JCI44490
74. Lunov O, Zablotskii V, Syrovets T, Rocker C, Tron K, Nienhaus GU, et al. Modeling receptor-mediated endocytosis of polymer-functionalized iron oxide nanoparticles by human macrophages. *Biomaterials*. (2011) 32:547–55. doi: 10.1016/j.biomaterials.2010.08.111
75. Sharkey J, Starkey Lewis PJ, Barrow M, Alwahsh SM, Noble J, Livingstone E, et al. Functionalized superparamagnetic iron oxide nanoparticles provide highly efficient iron-labeling in macrophages for magnetic resonance-based detection *in vivo*. *Cytotherapy*. (2017) 19:555–69. doi: 10.1016/j.jcyt.2017.01.003
76. Sun X, Gamal M, Nold P, Said A, Chakraborty I, Pelaz B, et al. Tracking stem cells and macrophages with gold and iron oxide nanoparticles – the choice of the best suited particles. *Appl Mater Today*. (2019) 15:267–79. doi: 10.1016/j.apmt.2018.12.006
77. Park JK, Utsumi T, Seo YE, Deng Y, Satoh A, Saltzman WM, et al. Cellular distribution of injected PLGA-nanoparticles in the liver. *Nanomedicine*. (2016) 12:1365–74. doi: 10.1016/j.nano.2016.01.013
78. Kurniawan DW, Jajoriya AK, Dhawan G, Mishra D, Argemi J, Bataller R, et al. Therapeutic inhibition of spleen tyrosine kinase in inflammatory macrophages using PLGA nanoparticles for the treatment of non-alcoholic steatohepatitis. *J Control Release*. (2018) 288:227–38. doi: 10.1016/j.jconrel.2018.09.004
79. Di Mascolo D, C JL, Aryal S, Ramirez MR, Wang J, Candeloro P, et al. Rosiglitazone-loaded nanospheres for modulating macrophage-specific inflammation in obesity. *J Control Release*. (2013) 170:460–8. doi: 10.1016/j.jconrel.2013.06.012
80. Cui J, Piotrowski-Daspit AS, Zhang J, Shao M, Bracaglia LG, Utsumi T, et al. Poly(amine-co-ester) nanoparticles for effective Nogo-B knockdown in the liver. *J Control Release*. (2019) 304:259–67. doi: 10.1016/j.jconrel.2019.04.044
81. Canup BS, Song H, Le Ngo V, Meng X, Denning TL, Garg P, et al. CD98 siRNA-loaded nanoparticles decrease hepatic steatosis in mice. *Dig Liver Dis*. (2017) 49:188–96. doi: 10.1016/j.dld.2016.11.008

82. Xiao B, Ma P, Ma L, Chen Q, Si X, Walter L, et al. Effects of tripolyphosphate on cellular uptake and RNA interference efficiency of chitosan-based nanoparticles in Raw 264.7 macrophages. *J Colloid Interface Sci*. (2017) 490:520–8. doi: 10.1016/j.jcis.2016.11.088
83. Iranpur Mobarakeh V, Modarressi MH, Rahimi P, Bolhassani A, Arefian E, Atyabi F, et al. Optimization of chitosan nanoparticles as an anti-HIV siRNA delivery vehicle. *Int J Biol Macromol*. (2019) 129:305–15. doi: 10.1016/j.ijbiomac.2019.02.036
84. Chu S, Tang C, Yin C. Effects of mannose density on *in vitro* and *in vivo* cellular uptake and RNAi efficiency of polymeric nanoparticles. *Biomaterials*. (2015) 52:229–39. doi: 10.1016/j.biomaterials.2015.02.044
85. Jain K, Verma AK, Mishra PR, Jain NK. Surface-engineered dendrimeric nanoconjugates for macrophage-targeted delivery of amphotericin B: formulation development and *in vitro* and *in vivo* evaluation. *Antimicrob Agents Chemother*. (2015) 59:2479–87. doi: 10.1128/AAC.04213-14
86. Xiao B, Laroui H, Ayyadurai S, Viennois E, Charania MA, Zhang Y, et al. Mannosylated bioreducible nanoparticle-mediated macrophage-specific TNF-alpha RNA interference for IBD therapy. *Biomaterials*. (2013) 34:7471–82. doi: 10.1016/j.biomaterials.2013.06.008
87. Xavier RJ, Podolsky DK. Unraveling the pathogenesis of inflammatory bowel disease. *Nature*. (2007) 448:427–34. doi: 10.1038/nature06005
88. Puligujja P, McMillan J, Kendrick L, Li T, Balkundi S, Smith N, et al. Macrophage folate receptor-targeted antiretroviral therapy facilitates drug entry, retention, antiretroviral activities, and biodistribution for reduction of human immunodeficiency virus infections. *Nanomedicine*. (2013) 9:1263–73. doi: 10.1016/j.nano.2013.05.003
89. Jiang L, Zhang S, Hu H, Yang J, Wang X, Ma Y, et al. Exosomes derived from human umbilical cord mesenchymal stem cells alleviate acute liver failure by reducing the activity of the NLRP3 inflammasome in macrophages. *Biochem Biophys Res Commun*. (2019) 508:735–41. doi: 10.1016/j.bbrc.2018.11.189
90. Haga H, Yan IK, Takahashi K, Matsuda A, Patel T. Extracellular vesicles from bone marrow-derived mesenchymal stem cells improve survival from lethal hepatic failure in mice. *Stem Cells Transl Med*. (2017) 6:1262–72. doi: 10.1002/sctm.16-0226
91. Bagalkot V, Badgeley MA, Kampfrath T, Deiuliis JA, Rajagopalan S, Maiseyeu A. Hybrid nanoparticles improve targeting to inflammatory macrophages through phagocytic signals. *J Control Release*. (2015) 217:243–55. doi: 10.1016/j.jconrel.2015.09.027
92. Herd HL, Bartlett KT, Gustafson JA, McGill LD, Ghandehari H. Macrophage silica nanoparticle response is phenotypically dependent. *Biomaterials*. (2015) 53:574–82. doi: 10.1016/j.biomaterials.2015.02.070
93. Shrivastava R, Shukla N. Attributes of alternatively activated (M2) macrophages. *Life Sci*. (2019) 224:222–31. doi: 10.1016/j.lfs.2019.03.062
94. Hatami E, Mu Y, Shields DN, Chauhan SC, Kumar S, Cory TJ, et al. Mannose-decorated hybrid nanoparticles for enhanced macrophage targeting. *Biochem Biophys Rep*. (2019) 17:197–207. doi: 10.1016/j.bbrep.2019.01.007
95. Walkey CD, Olsen JB, Guo H, Emili A, Chan WC. Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. *J Am Chem Soc*. (2012) 134:2139–47. doi: 10.1021/ja2084338
96. Jiang LQ, Wang TY, Wang Y, Wang ZY, Bai YT. Co-disposition of chitosan nanoparticles by multi types of hepatic cells and their subsequent biological elimination: the mechanism and kinetic studies at the cellular and animal levels. *Int J Nanomedicine*. (2019) 14:6035–60. doi: 10.2147/IJN.S208496
97. Jiang LQ, Wang TY, Webster TJ, Duan HJ, Qiu JY, Zhao ZM, et al. Intracellular disposition of chitosan nanoparticles in macrophages: intracellular uptake, exocytosis, and intercellular transport. *Int J Nanomedicine*. (2017) 12:6383–98. doi: 10.2147/IJN.S142060
98. Waegeneers N, Brasseur A, Van Doren E, Van der Heyden S, Serreyn PJ, Pussemier L, et al. Short-term biodistribution and clearance of intravenously administered silica nanoparticles. *Toxicol Rep*. (2018) 5:632–8. doi: 10.1016/j.toxrep.2018.05.004
99. Zhuravskii S, Yukina G, Kulikova O, Panevin A, Tomson V, Korolev D, et al. Mast cell accumulation precedes tissue fibrosis induced by intravenously administered amorphous silica nanoparticles. *Toxicol Mech Methods*. (2016) 26:260–9. doi: 10.3109/15376516.2016.1169341
100. Farrell BT, Hamilton BE, Dósa E, Rimely E, Nasseri M, Gahramanov S, et al. Using iron oxide nanoparticles to diagnose CNS inflammatory diseases and PCNSL. *Neurology*. (2013) 81:256–63. doi: 10.1212/WNL.0b013e31829bfd8f
101. Couto D, Freitas M, Costa VM, Chisté RC, Almeida A, Lopez-Quintela MA, et al. Biodistribution of polyacrylic acid-coated iron oxide nanoparticles is associated with proinflammatory activation and liver toxicity. *J Appl Toxicol*. (2016) 36:1321–31. doi: 10.1002/jat.3323
102. Liu Y, Huang W, Xiong C, Huang Y, Chen BJ, Racioppi L, et al. Biodistribution and sensitive tracking of immune cells with plasmonic gold nanostars. *Int J Nanomedicine*. (2019) 14:3403–11. doi: 10.2147/IJN.S192189
103. Miller CM, Donner AJ, Blank EE, Egger AW, Kellar BM, Ostergaard ME, et al. Stabilin-1 and stabilin-2 are specific receptors for the cellular internalization of phosphorothioate-modified antisense oligonucleotides (ASOs) in the liver. *Nucleic Acids Res*. (2016) 44:2782–94. doi: 10.1093/nar/gkw112
104. Campbell F, Bos FL, Sieber S, Arias-Alpizar G, Koch BE, Huwyler J, et al. Directing nanoparticle biodistribution through evasion and exploitation of stab2-dependent nanoparticle uptake. *ACS Nano*. (2018) 12:2138–50. doi: 10.1021/acsnano.7b06995
105. Nakayama M. Macrophage recognition of crystals and nanoparticles. *Front Immunol*. (2018) 9:103. doi: 10.3389/fimmu.2018.00103
106. Liu T, Choi H, Zhou R, Chen IW. Quantitative evaluation of the reticuloendothelial system function with dynamic MRI. *PLoS ONE*. (2014) 9:e103576. doi: 10.1371/journal.pone.0103576
107. Sweeney LM, MacCalman L, Haber LT, Kuempel ED, Tran CL. Bayesian evaluation of a physiologically-based pharmacokinetic (PBPK) model of long-term kinetics of metal nanoparticles in rats. *Regul Toxicol Pharmacol*. (2015) 73:151–63. doi: 10.1016/j.yrtph.2015.06.019
108. Cheng YH, Riviere JE, Monteiro-Riviere NA, Lin Z. Probabilistic risk assessment of gold nanoparticles after intravenous administration by integrating *in vitro* and *in vivo* toxicity with physiologically based pharmacokinetic modeling. *Nanotoxicology*. (2018) 12:453–69. doi: 10.1080/17435390.2018.1459922
109. Edginton AN, Ahr G, Willmann S, Stass H. Defining the role of macrophages in local moxifloxacin tissue concentrations using biopsy data and whole-body physiologically based pharmacokinetic modeling. *Clin Pharmacokinet*. (2009) 48:181–7. doi: 10.2165/00003088-200948030-00004
110. Aborig M, Malik PRV, Nambiar S, Chelle P, Darko J, Mutsaers A, et al. Biodistribution and physiologically-based pharmacokinetic modeling of gold nanoparticles in mice with interspecies extrapolation. *Pharmaceutics*. (2019) 11:E179. doi: 10.3390/pharmaceutics11040179
111. Carlander U, Li D, Jolliet O, Emond C, Johanson G. Toward a general physiologically-based pharmacokinetic model for intravenously injected nanoparticles. *Int J Nanomedicine*. (2016) 11:625–40. doi: 10.2147/IJN.S94370
112. Yuan D, He H, Wu Y, Fan J, Cao Y. Physiologically based pharmacokinetic modeling of nanoparticles. *J Pharm Sci*. (2019) 108:58–72. doi: 10.1016/j.xphs.2018.10.037
113. Jones H, Rowland-Yeo K. Basic concepts in physiologically based pharmacokinetic modeling in drug discovery and development. *CPT Pharmacomet Syst Pharmacol*. (2013) 2:e63. doi: 10.1038/psp.2013.41
114. Zhuang X, Lu C. PBPK modeling and simulation in drug research and development. *Acta Pharm Sin B*. (2016) 6:430–40. doi: 10.1016/j.apsb.2016.04.004
115. Edginton AN, Willmann S. Physiology-based simulations of a pathological condition: prediction of pharmacokinetics in patients with liver cirrhosis. *Clin Pharmacokinet*. (2008) 47:743–52. doi: 10.2165/00003088-200847110-00005
116. Li M, Al-Jamal KT, Kostarelos K, Reineke J. Physiologically based pharmacokinetic modeling of nanoparticles. *ACS Nano*. (2010) 4:6303–17. doi: 10.1021/nn1018818
117. Wilhelm S, Tavares AJ, Dai Q, Ohta S, Audet J, Dvorak HF, et al. Analysis of nanoparticle delivery to tumors. *Nat Rev Mater*. (2016) 1:16014. doi: 10.1038/natrevmats.2016.14
118. Bachler G, von Goetz N, Hungerbuhler K. A physiologically based pharmacokinetic model for ionic silver and silver nanoparticles. *Int J Nanomedicine*. (2013) 8:3365–82. doi: 10.2147/IJN.S46624
119. Epstein-Barash H, Gutman D, Markovsky E, Mishan-Eisenberg G, Koroukhov N, Szebeni J, et al. Physicochemical parameters

affecting liposomal bisphosphonates bioactivity for restenosis therapy: internalization, cell inhibition, activation of cytokines and complement, and mechanism of cell death. *J Control Release*. (2010) 146:182–95. doi: 10.1016/j.jconrel.2010.03.011
120. Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Itty Ipe B, et al. Renal clearance of quantum dots. *Nat Biotechnol*. (2007) 25:1165–70. doi: 10.1038/nbt1340
121. Elsabahy M, Wooley KL. Design of polymeric nanoparticles for biomedical delivery applications. *Chem Soc Rev*. (2012) 41:2545–61. doi: 10.1039/c2cs15327k
122. Longmire M, Choyke PL, Kobayashi H. Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats. *Nanomedicine*. (2008) 3:703–17. doi: 10.2217/17435889.3.5.703
123. Zamboni WC, Szebeni J, Kozlov SV, Lucas AT, Piscitelli JA, Dobrovolskaia MA. Animal models for analysis of immunological responses to nanomaterials: challenges and considerations. *Adv Drug Deliv Rev*. (2018) 136–137:82–96. doi: 10.1016/j.addr.2018.09.012
124. Cornu R, Beduneau A, Martin H. Influence of nanoparticles on liver tissue and hepatic functions: a review. *Toxicology*. (2020) 430:152344. doi: 10.1016/j.tox.2019.152344
125. Balasubramanian SK, Jittiwat J, Manikandan J, Ong CN, Yu LE, Ong WY. Biodistribution of gold nanoparticles and gene expression changes in the liver and spleen after intravenous administration in rats. *Biomaterials*. (2010) 31:2034–42. doi: 10.1016/j.biomaterials.2009.11.079
126. Karmali PP, Simberg D. Interactions of nanoparticles with plasma proteins: implication on clearance and toxicity of drug delivery systems. *Expert Opin Drug Deliv*. (2011) 8:343–57. doi: 10.1517/17425247.2011.554818
127. Desai N. Challenges in development of nanoparticle-based therapeutics. *AAPS J*. (2012) 14:282–95. doi: 10.1208/s12248-012-9339-4
128. Sadauskas E, Danscher G, Stoltenberg M, Vogel U, Larsen A, Wallin H. Protracted elimination of gold nanoparticles from mouse liver. *Nanomedicine*. (2009) 5:162–9. doi: 10.1016/j.nano.2008.11.002
129. Fraga S, Brandao A, Soares ME, Morais T, Duarte JA, Pereira L, et al. Short- and long-term distribution and toxicity of gold nanoparticles in the rat after a single-dose intravenous administration. *Nanomedicine*. (2014) 10:1757–66. doi: 10.1016/j.nano.2014.06.005
130. Abdelhalim MAK, Moussa SAA, Qaid HAY. The protective role of quercetin and arginine on gold nanoparticles induced hepatotoxicity in rats. *Int J Nanomedicine*. (2018) 13:2821–5. doi: 10.2147/IJN.S160995
131. Yu Y, Duan J, Li Y, Jing L, Yang M, Wang J, et al. Silica nanoparticles induce liver fibrosis via TGF-beta1/Smad3 pathway in ICR mice. *Int J Nanomedicine*. (2017) 12:6045–57. doi: 10.2147/IJN.S132304
132. Piao MJ, Kang KA, Lee IK, Kim HS, Kim S, Choi JY, et al. Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. *Toxicol Lett*. (2011) 201:92–100. doi: 10.1016/j.toxlet.2010.12.010
133. Ramadi KB, Mohamed YA, Al-Sbiei A, Almarzooqi S, Bashir G, Al Dhanhani A, et al. Acute systemic exposure to silver-based nanoparticles induces hepatotoxicity and NLRP3-dependent inflammation. *Nanotoxicology*. (2016) 10:1061–74. doi: 10.3109/17435390.2016.1163743
134. Gaiser BK, Hirn S, Kermanizadeh A, Kanase N, Fytianos K, Wenk A, et al. Effects of silver nanoparticles on the liver and hepatocytes *in vitro*. *Toxicol Sci*. (2013) 131:537–47. doi: 10.1093/toxsci/kfs306
135. Hu Q, Zhao F, Fan M, He C, Yang X, Huang Z, et al. The influence of titanium dioxide nanoparticles on their cellular response to macrophage cells. *Comp Biochem Physiol C Toxicol Pharmacol*. (2019) 223:42–52. doi: 10.1016/j.cbpc.2019.05.006
136. Mohammadpour R, Yazdimamaghani M, Cheney DL, Jedrzkiewicz J, Ghandehari H. Subchronic toxicity of silica nanoparticles as a function of size and porosity. *J Control Release*. (2019) 304:216–32. doi: 10.1016/j.jconrel.2019.04.041
137. Forest V, Leclerc L, Hochepied JF, Trouve A, Sarry G, Pourchez J. Impact of cerium oxide nanoparticles shape on their *in vitro* cellular toxicity. *Toxicol In Vitro*. (2017) 38:136–41. doi: 10.1016/j.tiv.2016.09.022
138. Souris JS, Lee CH, Cheng SH, Chen CT, Yang CS, Ho JA, et al. Surface charge-mediated rapid hepatobiliary excretion of mesoporous silica nanoparticles. *Biomaterials*. (2010) 31:5564–74. doi: 10.1016/j.biomaterials.2010.03.048
139. King EJ, McGeorge M. The biochemistry of silicic acid: the solution and excretion of silica. *Biochem J*. (1938) 32:426–33. doi: 10.1042/bj0320426
140. Yu T, Hubbard D, Ray A, Ghandehari H. *In vivo* biodistribution and pharmacokinetics of silica nanoparticles as a function of geometry, porosity, and surface characteristics. *J Control Release*. (2012) 163:46–54. doi: 10.1016/j.jconrel.2012.05.046
141. He Q, Zhang Z, Gao Y, Shi J, Li Y. Intracellular localization and cytotoxicity of spherical mesoporous silica nano- and microparticles. *Small*. (2009) 5:2722–9. doi: 10.1002/smll.200900923
142. de Araujo RFJ, de Araujo AA, Pessoa JB, Freire Neto FP, da Silva GR, Leitao Oliveira AL, et al. Anti-inflammatory, analgesic and anti-tumor properties of gold nanoparticles. *Pharmacol Rep*. (2017) 69:119–29. doi: 10.1016/j.pharep.2016.09.017
143. Zhang N, Chen H, Liu AY, Shen JJ, Shah V, Zhang C, et al. Gold conjugate-based liposomes with hybrid cluster bomb structure for liver cancer therapy. *Biomaterials*. (2016) 74:280–91. doi: 10.1016/j.biomaterials.2015.10.004
144. Lunov O, Syrovets T, Buchele B, Jiang X, Rocker C, Tron K, et al. The effect of carboxydextran-coated superparamagnetic iron oxide nanoparticles on c-Jun N-terminal kinase-mediated apoptosis in human macrophages. *Biomaterials*. (2010) 31:5063–71. doi: 10.1016/j.biomaterials.2010.03.023

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Systemic MCP-1 Levels Derive Mainly From Injured Liver and Are Associated With Complications in Cirrhosis

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Background and Aims: Monocyte chemotactic protein-1 (MCP-1) is a potent chemoattractant for monocytes. It is involved in pathogenesis of several inflammatory diseases. Hepatic MCP-1 is a readout of macrophage activation. While inflammation is a major driver of liver disease progression, the origin and role of circulating MCP-1 as a biomarker remains unclear.

Methods: Hepatic CC-chemokine ligand 2 (*CCL2*) expression and F4/80 staining for Kupffer cells were measured and correlated in a mouse model of chronic liver disease (inhalative CCl_4 for 7 weeks). Next, hepatic RNA levels of *CCL2* were measured in explanted livers of 39 patients after transplantation and correlated with severity of disease. Changes in MCP-1 were further evaluated in a rat model of experimental cirrhosis and acute-on-chronic liver failure (ACLF). Finally, we analyzed portal and hepatic vein levels of MCP-1 in patients receiving transjugular intrahepatic portosystemic shunt insertion for complications of portal hypertension.

Results: In this mouse model of fibrotic hepatitis, hepatic expression of *CCL2* ($P = 0.009$) and the amount of F4/80 positive cells in the liver ($P < 0.001$) significantly increased after induction of hepatitis by CCl_4 compared to control animals. Moreover, strong correlation of hepatic *CCL2* expression and F4/80 positive cells were seen ($P = 0.023$). Furthermore, in human liver explants, hepatic transcription levels of *CCL2* correlated with the MELD score of the patients, and thus disease severity ($P = 0.007$). The experimental model of ACLF in rats revealed significantly higher levels of MCP-1 plasma ($P = 0.028$) and correlation of hepatic *CCL2* expression ($R = 0.69$, $P = 0.003$). Particularly, plasma MCP-1 levels did not correlate with peripheral blood monocyte *CCL2* expression. Finally, higher levels of MCP-1 were observed in the hepatic compared to the portal vein ($P = 0.01$) in patients receiving TIPS. Similarly, a positive correlation of MCP-1 with Child-Pugh score was observed ($P = 0.018$). Further, in the presence of ACLF, portal and hepatic vein levels of MCP-1 were significantly higher compared to patients without ACLF (both $P = 0.039$).

Conclusion: Circulating levels of MCP-1 mainly derive from the injured liver and are associated with severity of liver disease. Therefore, liver macrophages contribute significantly to disease progression. Circulating MCP-1 may reflect the extent of hepatic macrophage activation.

Keywords: acute-on-chronic liver failure (ACLF), decompensated liver cirrhosis, inflammation, monocyte chemotactic protein 1 (MCP-1), transjugular intrahepatic portosystemic shunt (TIPS)

INTRODUCTION

Unresolved hepatic inflammation is known to be a major driver of progression in liver disease (1, 2). Hereby, composition of resident and infiltrating monocyte derived macrophages plays a pivotal role in homeostasis of hepatic inflammation and development of fibrosis (3). The phenotype of M2-macrophages (alternative type) is involved in tissue repair and resolution of inflammation in liver disease, whereas M1 phenotype (classic type) leads to pro-inflammatory signaling (4). Thus, monocyte chemotactic protein 1 (MCP-1) recruits peripheral monocytes to the liver and supports takeover of M1 dominant phenotype in hepatic macrophages (5). Systemic inflammation is known to be the key mediator for the development of acute-on-chronic liver failure (ACLF), and an increase in leucocytes and C-reactive protein is strongly associated with the onset of ACLF (6). Nevertheless, activation of Kupffer cells and hepatic monocyte recruitment in ACLF suggest an important role of hepatic inflammation in ACLF development (7, 8). Mechanistically, bacterial translocation takes place in advanced liver disease with portal hypertension, resulting in consecutive inflammation and oxidative stress in the portal venous compartment (9, 10). Early diagnosis and prevention of ACLF is essential, since 28-day mortality rates of 30% and higher are reported (11). Therefore, we hypothesized that levels of MCP-1 in the portal vein and hepatic transcription of *CCL2* may be associated with severity of liver disease and complications of cirrhosis, including ACLF. To address this hypothesis, we measured MCP-1 transcription in explanted cirrhotic livers and MCP-1 levels in portal and hepatic venous blood from patients with decompensated cirrhosis at transjugular intrahepatic portosystemic shunt insertion (TIPS), and further confirmed our findings in animal models of cirrhosis and ACLF.

PATIENTS AND METHODS

Patients, Animal Models, and Methods

Our study was undertaken to investigate the role of MCP-1 as a marker of liver disease progression, associated complications and ACLF. To this end, we performed two animal models of cirrhosis and ACLF, and included two patient cohorts (TIPS and liver transplantation).

Mouse Model of Toxic Liver Fibrosis

As previously described and published (12), male wild-type (WT, C57Bl6/J) mice (12 weeks old) were purchased (Charles River Laboratories Research Model and Services Germany, Sulzfeld, Germany). A total of 11 mice were used for this study. The animals were kept at 22°C with a 12:12-h day-night cycle in individually ventilated cages. Liver injury was induced by inhalative CCl_4 exposure for seven weeks (once/week for the first four weeks, followed by intoxications twice/week for the next three weeks). Briefly, CCl_4 was insufflated with a flow of 2 l/min for 1 min, the cage remaining closed for another minute, and CCl_4 finally removed under the hood for 10 min. Water and chow were provided *ad libitum*. All animals intoxicated with CCl_4 additionally received phenobarbital (0.33 g/l) via drinking water as an inducer of the cytochrome P-450 metabolic activity. Control age-matched untreated mice were used in the experiments. Before being euthanized, mice received ketaminexylazine anesthesia (100 mg ketamine/kg body weight and 10 mg xylazine/kg body weight) via intraperitoneal injection. Liver samples were fixed in formaldehyde (4%) and subsequently embedded in paraffin. All experiments were performed in accordance with the German Animal Welfare Act and the guidelines of the animal care facility of the University Hospital, Bonn (Haus für Experimentelle Therapie, University Hospital Bonn, Germany), and were approved by the North Rhine-Westphalian State Agency for Nature, Environment, and Consumer Protection (LANUV; file reference LANUV NRW, 84–02.04.2015.A491).

F4/80 staining of liver tissue

Stainings were performed on paraffin slides (2–3 μm) for F4/80 immunohistochemistry (IHC). F4/80 IHC was analyzed by counting positive stained cells in high power fields captured at a 200x magnification. A minimum of 12 high power fields were used for analysis.

Patient Cohort 1 (Liver Transplantation)

In patient cohort 1 (liver explants), 39 cirrhotic patients undergoing liver transplantation were included for measurement of hepatic mRNA expression of MCP-1. These patients were enrolled between 1999 and 2005 at the Department of Internal Medicine I, University of Bonn, Germany, as previously described (13). The study protocol was accepted and approved by the local ethics committee of the University of Bonn (029/13).

Quantitative PCR

Total RNA was isolated with ReliaPrep™ RNA Miniprep Systems (Promega, Madison, WI, USA) from shock-frozen liver tissue following the ReliaPrep™ RNA Cell Miniprep System protocol. Hereby, RNA concentration was measured spectrophotometrically at 260 nm. For each sample, 1 μg of total RNA was used. cDNA synthesis was performed by the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA).

Primers and probes for the housekeeping gene (18SrRNA) were provided by Thermo Fisher Scientific, Waltham, MA, USA, as a ready-to-use mix. Every sample underwent two DNase digestion steps to dispose of genomic DNA. Quantitative PCR (qPCR) was carried out using TaqMan gene expression assay for *CCL2* (assay ID Hs00234140_m1, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA. The PCR reaction was performed in a 25-μL volume containing 12.5 μL of 2 × TaqMan-PCR master mix and 2 μL (equivalent to 67 ng of total RNA) of cDNA. The final concentrations were 100 nM for the primers and 200 nM for the probe. Firstly, measurement of cycle threshold values was performed (Ct). Secondly, adjustment with endogenous controls created the ΔCt value was performed. Matching the ΔCt value between study and control group created the ΔΔCt value. The final results of the liver samples were expressed as $2^{-\Delta\Delta Ct}$ and revealed the x-fold change of gene expression compared to the control group. Experiments were carried out in duplicates and results were normalized to 18S rRNA. Duplicate measurements were used as a method to control for loading error. If duplicates were far apart, we either measured once again or we disregarded the sample. Measurement of mRNA levels were performed as previously described (14–16).

Rat Model of Cirrhosis and ACLF

Induction of liver cirrhosis and ACLF

Male wildtype (WT) Sprague Dawley rats were used. The experiments were performed according to the guidelines and regulations approved by LANUV, the responsible committee for animal studies in North Rhine-Westphalia, Germany. All rats were placed in a controlled environment (12 h light/dark; temperature 22–24°C) and received water and standard rat feed (Ssniff, Soest, Germany) *ad libitum*. Bile duct ligation (BDL) was performed in male WT rats with an initial body weight (BW) of 180–200 g to induce cholestatic liver cirrhosis, as previously described (17). ACLF was induced twice via intraperitoneal injection (day 21 and day 25 after BDL) of 6.25 μg/kg bodyweight (BW) lipopolysaccharid (LPS; *E. coli* O55:B5, Sigma-Aldrich, St. Louis, USA). Untreated BDL and sham-operated rats served as controls.

Tissue and blood collection

At the end of the experiment, liver samples were harvested, snap-frozen and stored at −80°C. Peripheral blood was collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) for isolation of peripheral blood mononuclear cells (PBMCs). PBMC were isolated by density gradient centrifugation using Pancoll (PAN-Biotec, Aidenbach, Germany), as previously described (Beyer M, Abdullah, Nat Immunol, 2016). Cells were suspended in RPMI 1640 media with 10% fetal calf serum and 10% dimethyl sulfoxide (Gibco, Carlsbad, USA) and stored at −80°C.

Transcriptome analysis

Transcriptome analysis was performed by PakLabs (Hennigsdorf, Germany) using the Agilent Microarray XS (Agilent Technologies, Santa Clara, USA). Briefly, Low Input QuickAmp Labeling Kit (Agilent Technologies, Santa Clara, USA) was used to create fluorescent complementary RNA (cRNA) followed by hybridization to microarrays using the Gene Expression Hybridization Kit (Agilent Technologies, Santa Clara, USA). Fluorescence signals were detected using SureScan Microarray Scanner (Agilent Technologies, Santa Clara, USA).

Assessment of peripheral blood MCP-1

Peripheral blood circulating MCP-1 in rodents was measured in 25 μl of plasma using a multiplexed bead-based immunoassay (Milliplex MAP Cytokine/Chemokine Magnetic Bead Panel) (Merck Millipore, Darmstadt, Germany) on a Luminex 100 Bioanalyzer (Luminex Corp., Austin, TX), as described previously (6). The readouts were analyzed with Milliplex Analyst software (Merck Millipore) and a five-parameter logistic regression model was used to calculate the concentration of each sample (pg/ml).

Patient Cohort 2 (TIPS)

Patient cohort 2 (TIPS) consisted of 18 patients with diagnosed liver cirrhosis and severe portal hypertension undergoing TIPS-insertion. Patients from this cohort were recruited between May 2000 and April 2003 at the Department of Internal Medicine I, University of Bonn, Germany. Patients older than 18 years, with clinical signs of liver cirrhosis and a multidisciplinary defined indication for TIPS insertion were included in our trial. Exclusion criteria were presence of systemic infection, hepatic encephalopathy (higher than grade I), bilirubinemia (higher than 5mg/dl), or arterial pulmonary hypertension. Once the right branch of the portal vein was cannulated, we harvested blood from the portal and the hepatic vein in EDTA tubes ($N = 18$) for analysis of MCP-1. All TIPS insertions were performed without general anesthesia. After collection of the blood, we centrifuged the samples at 3,000 revolutions per minute for 15 min at 4°C. Afterwards, plasma samples were stored at −80°C, as previously described (18, 19). All patients provided written consent to all procedures, as declared in the study protocol.

Assessment of circulating MCP-1 levels

Plasma concentrations of MCP-1 from the portal and the hepatic vein in humans were also measured in 25 μl of plasma using a multiplexed bead-based immunoassay (Milliplex MAP Cytokine/Chemokine Magnetic Bead Panel) (Merck Millipore, Darmstadt, Germany) on a Luminex 100 Bioanalyzer (Luminex Corp., Austin, TX), as described previously (6, 13, 20). The readouts were analyzed with Milliplex Analyst software (Merck Millipore) and a five-parameter logistic regression model was used to calculate the concentration of each sample (pg/ml).

STATISTICAL ANALYSES

GraphPad Prism 5 for Windows (GraphPad Software, Inc.) or BIAS® for Windows were used for the performance of statistical analyses. Wilcoxon matched-pairs signed rank test was used for paired intra-individual comparisons, namely portal versus hepatic vein MCP-1 concentrations. Group differences of unrelated groups were assessed by the

Mann-Whitney test. Independent associations of variables were assessed in linear regression models. After univariate analyses, multivariate analyses were performed for significant associations using a $P < 0.1$. Variables with a $P > 0.1$ were discharged from the model. Correlations were assessed using Spearmen/Kendall rang correlation. $P < 0.05$ were considered to be statistically significant.

RESULTS

Hepatic Levels of MCP-1 Are Increased in a Toxic Model of Liver Disease in Mice

In order to detect macrophages, we performed F4/80 immunohistochemistry staining in control and CCl_4-intoxicated animals. Representative images show stronger F4/80 positivity in treated mice, which was confirmed via counting F4/80-positive cells (**Figures 1A,B**). Real-time PCR of *CCL2* revealed a statistically higher upregulation in CCl_4-intoxicated animals compared to control mice (**Figure 1C**). Moreover, linear regression analysis showed a strong positive correlation between mRNA levels of *CCL2* and immunohistochemistry quantification of MCP-1 in our animal model (**Figure 1D**).

Baseline Characteristic of Patient Cohort 1 (Liver Transplantation)

Patients undergoing liver transplantation ($N = 39$) were mainly male ($N = 24$; 62%), with a mean age of 47 years. Their mean MELD score was 15 (range 6–27). Most patients had Child Pugh score C ($N = 20$; 51%) and B ($N = 16$; 41%). Viral hepatitis was the main etiology of cirrhosis ($N = 16$; 41%), followed by alcohol ($N = 12$; 30%), and primary sclerosing cholangitis ($N = 7$; 18%). Ascites was present in 21 of the patients (54%). Fifteen patients (38%) had a history of gastrointestinal bleeding. Twenty percent of the patients had hepatorenal syndrome type 1 ($N = 8$), and another 70% ($N = 27$) had hepatorenal syndrome type 2. Fourteen patients (36%) were diagnosed with overt hepatic encephalopathy. All patient information is included in **SI-Table 1**.

Hepatic *CCL2* Transcription Levels Were Associated With Severity of Disease at Liver Transplantation

Transcription levels of *CCL2* in explanted livers showed a correlation to severity of liver disease. Patients with a MELD score above 13 points had significantly higher levels of *CCL2*, compared to patients with a MELD score below 14 points ($P = 0.0066$). Patient stratification by Child-Pugh score also showed increasing levels from Child-Pugh level A to C, even if not statistically significant (**Figure 2**).

MCP-1 Is Increased in Experimental ACLF and Correlates With Hepatic *CCL2* Expression

Bile duct ligation (BDL; 28 days) was performed in rats, and lipopolysaccharid (LPS; from *Escherichia coli*) was injected intraperitoneally (i.p.) on day 21 and day 25 after BDL for induction of ACLF. Circulating MCP-1 levels in peripheral blood were significantly increased in ACLF—($P = 0.028$), compared to BDL—and healthy control animals. Furthermore,

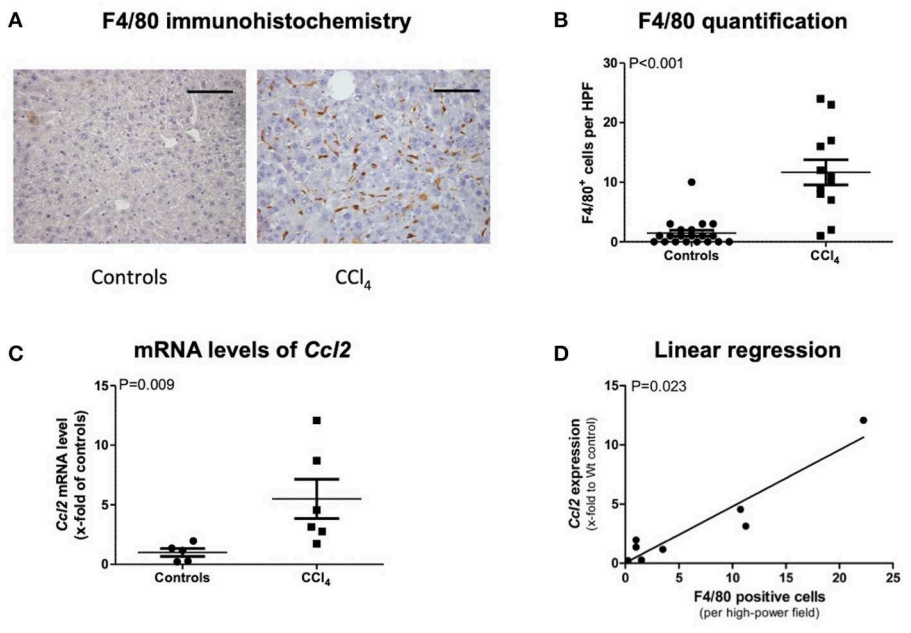

FIGURE 1 | F4/80 immunohistochemistry staining **(A)** and its quantification **(B)**, transcript levels of *CCL2* in livers of CCl_4 intoxicated mice **(C)**, and correlation of both **(D)**. The scale bar is 100μm **(A)**. Results are expressed as mean ± standard error of the mean (SEM); Mann–Whitney test **(B,C)**. Controls: $N = 5$, CCl4 group: $N = 6$. Spearman's rank correlation **(D)**.

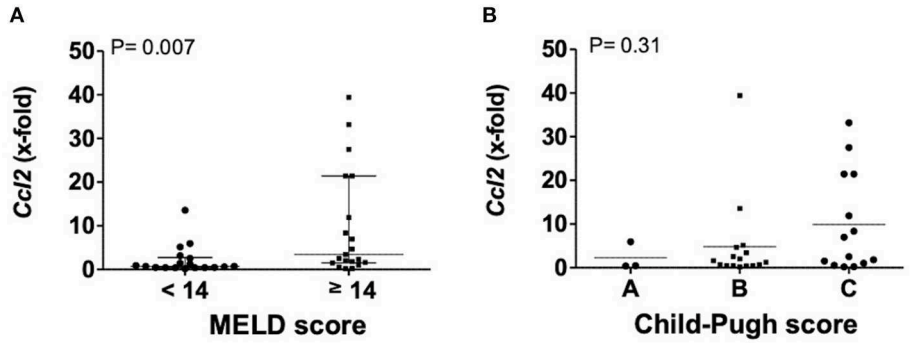

FIGURE 2 | Transcription levels of *CCL2* in explanted livers in dependency of MELD **(A)** or Child-Pugh score **(B)**. Mann–Whitney test with $N = 39$ patients **(A)** and one way ANOVA with $N = 33$ patients **(B)**. Ccl2, (C-C motif) ligand 2; MELD, model for end stage liver disease, $P < 0.05$ were considered statistically significant.

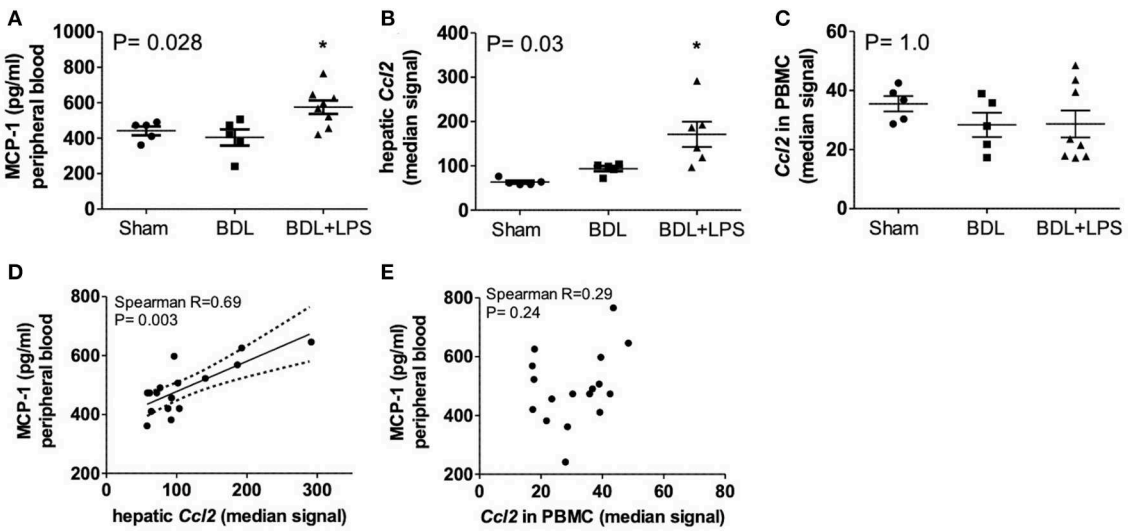

FIGURE 3 | Levels of MCP-1 in peripheral blood **(A)**, expression of CCL2 in liver **(B)**, and PBMC **(C)** and correlation of blood MCP-1 with expression of CCL2 in liver **(D)** and PBMC **(E)** of healthy control, bile-duct ligated and ACLF rats. Mann–Whitney test and Spearman's rank correlation. Rats minimum $N \geq 5$ per group. MCP-1, monocyte chemoattractant protein-1; *CCL2*; ACLF, acute on chronic liver failure; PBMC, peripheral blood mononuclear cells; BDL, bile duct ligation; LPS, lipopolysaccharide; $P < 0.05$ were considered statistically significant.

CCL2 gene expression was highly upregulated in liver tissue ($P = 0.03$) but remained unchanged in isolated PBMCs from ACLF rats. Importantly, *CCL2* expression in liver tissue ($R = 0.69$; $P = 0.003$), but not in PBMC ($R = 0.29$; $P = 0.24$), correlated with the circulating MCP-1 levels in control, BDL and ACLF animals (**Figure 3**).

Baseline Characteristics of Patient Cohort 2 (TIPS)

We included 18 patients undergoing TIPS insertion with a mean age of 59 years in the study. Refractory ascites ($N = 9$; 50%) was the major indication for TIPS insertion, followed by secondary prophylaxis of variceal bleeding ($N = 6$; 33%). In three patients (17%), indication was presence of esophageal variceal bleeding, as well as ascites. Most patients were admitted with alcohol-related liver cirrhosis (61%), followed by viral hepatitis (28%). Patients had a mean MELD score of 14 points (range 7–32), and most patients were classified as Child-Pugh B at the time of study inclusion. Presence of acute-on-chronic liver failure, calculated by the CLIF-C ACLF score, was seen in 5 out of 18 patients at TIPS insertion (28%). All ACLF patients presented with kidney failure−3 out of 8 patients with a CLIF-C organ failure (OF) score of seven points, and 5 out of 8 patients with a CLIF-C OF score of 8 points, respectively (**SI-Table 2**).

Increased Hepatic Vein Levels of MCP-1 in Decompensated Cirrhosis at TIPS Insertion Are Correlated With Disease Severity

Hepatic vein levels of MCP-1 were significantly higher compared to levels in the portal vein at TIPS insertion ($P = 0.01$) (**Figure 4**). Regression analyses revealed hepatic vein levels of MCP-1 associated with systemic levels of leucocytes (univariable

$P = 0.026$, multivariable $P = 0.04$) and INR (univariable $P = 0.01$, multivariable $P = 0.013$). Furthermore, inverse association with systemic levels of albumin (univariable $P = 0.002$, multivariable $P = 0.002$) was observed (**SI-Table 3**). Moreover, portal vein levels of MCP-1 showed association with systemic levels of leucocytes (univariable $P = 0.027$, multivariable $P = 0.036$) and were inversely associated with albumin (univariable $P = 0.004$, multivariable $P = 0.003$) (**SI-Table 4**). Child-Pugh score positively correlated with MCP-1 levels in the hepatic vein ($R = 0.55$; $P = 0.018$) (**Figure 5**). Moreover, patients with ACLF had significantly higher levels of MCP-1 in the hepatic, as well as the portal vein (both $P = 0.039$) (**Figure 6**). Stratification of patients according to presence of gastrointestinal bleeding, ascites or hepatorenal syndrome did not show significant differences in portal or hepatic vein levels of MCP-1 (**SI-Table 5**).

DISCUSSION

The major finding of this study is that circulating MCP-1 is associated with severity of liver cirrhosis, and that it mainly derives from the diseased liver.

We could demonstrate that hepatic transcription of *CCL2* correlates with disease severity and cirrhosis-associated complications. Patients with higher levels of MCP-1 had a reduced survival, predicted by the MELD score and presented with more complications, evaluated by the Child-Pugh score, even in our rather small patient cohort.

To date, several animal studies have provided evidence that MCP-1 reflects monocyte recruitment and inflammation in liver disease (21, 22). Further, pharmacological inhibition of MCP-1 has been shown to result in reduced infiltration of macrophages into the liver and therefore, amelioration of steatohepatitis in rodent models (23).

We could observe that hepatic macrophages are possibly the main source of elevated systemic MCP-1 in decompensated cirrhosis, shown by increased hepatic transcription of *CCL2* in correlation with the number of hepatic macrophages (F4/80 staining) and increased MCP-1 levels in hepatic vein blood. This is of special interest since peripheral blood monocytes are known to be dysfunctional in decompensated cirrhosis with impaired anti-microbial ability (24). Therefore, liver-specific, inflammation-driven monocyte recruitment with tissue dependent MCP-1 activation and release could be a key mechanism of systemic inflammation.

Since the portal vein contains a unique immune composition and is known to be influenced by portal hypertension and bacterial translocation (10, 25), relatively higher MCP-1 levels in the hepatic vein reflect an overwhelming hepatic production with consecutive maintenance in monocyte recruitment and pro-inflammatory signaling. Furthermore, patients with acute-on-chronic liver failure, a syndrome closely associated

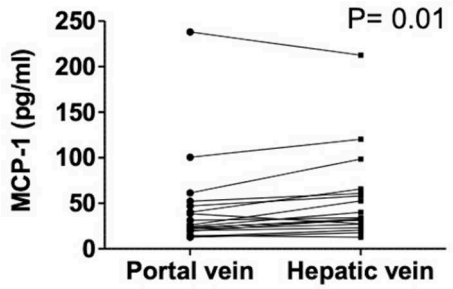

FIGURE 4 | Levels of MCP-1 in portal and hepatic vein in decompensated cirrhosis. Wilcoxon matched-pairs signed rank test. Patients $N = 18$. MCP-1, monocyte chemoattractant protein-1; $P < 0.05$ were considered statistically significant.

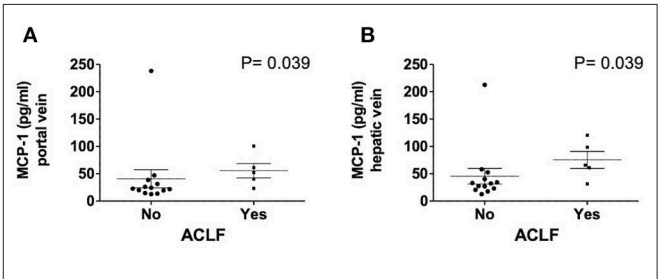

FIGURE 6 | Levels of MCP-1 in portal **(A)** and hepatic vein **(B)** in dependency of ACLF. Mann–Whitney test. Patients $N = 18$. MCP-1, monocyte chemoattractant protein-1; ACLF, acute on chronic liver failure score; $P < 0.05$ were considered statistically significant.

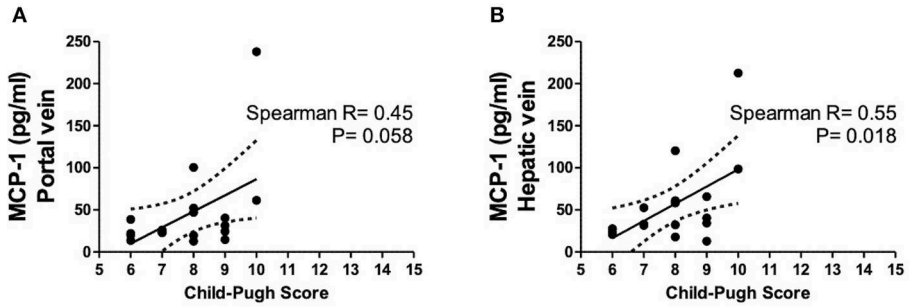

FIGURE 5 | Levels of MCP-1 in portal **(A)** and hepatic vein **(B)** in dependency of Child-Pugh score. Spearman's rank correlation. Patients $N = 8$. MCP-1, monocyte chemoattractant protein-1; $P < 0.05$ were considered statistically significant.

with systemic inflammation and devastating mortality (26), had higher MCP-1 levels compared to patients without ACLF. This could be further confirmed in our rodent model of experimental cirrhosis and ACLF. Thus, ACLF induction led to an upregulation of *CCL2* expression in hepatic tissue, and consequently led to increased MCP-1 plasma levels in ACLF rats. Importantly, our data suggest that changes in MCP-1 plasma level are not associated with PBMC, but rather with diseased liver tissue, since circulating MCP-1 is strongly correlated with hepatic *CCL2* expression, but not with *CCL2* in PBMCs. The positive correlation of MCP-1 to the presence of ACLF further emphasizes the clinical relevance of monocyte recruitment and inflammation in cirrhosis. Interestingly, immune dysfunction in progression of cirrhosis was seen to be disease stage dependent. In a recently published study, patients with advanced, but compensated cirrhosis showed overwhelmingly pro-inflammatory signaling, while patients with ACLF presented signs of immune paralysis with a decrease of several pro-inflammatory cytokines in peripheral blood (27). In our study, presence of sepsis or acute infection are contraindications for TIPS insertion, whereas ACLF correlation to elevated MCP-1 levels in portal and hepatic vein revealed patients with subclinical, but immunological relevant inflammation. This possibly underlines the role of MCP-1 as a biomarker for early prediction of cirrhotic patients with high morbidity and mortality risk. Finally, we found independent inverse correlation of MCP-1 levels in the portal and the hepatic vein and systemic albumin levels. This correlation may also be due to the fact that albumin binds MCP-1 (28, 29).

Our study has several limitations. All our patients are decompensated patients, and TIPS placement was performed in a relatively small cohort of patients. Access to hepatic blood compartments and hepatic tissue in patients with advanced liver disease is rare and difficult to access. Therefore, samples are not paired as this is not possible in clinical practice. Moreover, MCP-1 assessment in our liver samples was only performed on the level of gene expression, but not on the protein level. Furthermore, presence of ACLF was only documented for the TIPS cohort and not for the liver transplantation cohort.

In conclusion, circulating levels of MCP-1 mainly derive from the injured liver and are associated with severity of liver disease. Therefore, liver macrophages contribute significantly to disease progression, and circulating MCP-1 may reflect the extent of hepatic macrophage activation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the University of Bonn. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AQ, HB, JL, and JT contributed to the manuscript by planning and initiating the study. AQ, HB, CG, MS, MB, CJ, MP, RS, FU, SK, CT, HW, M-LB, JL, and JT collected the data. AQ, HB, FU, MB, M-LB, JT, and JL performed the statistics. AQ, HB, FU, M-LB, JT, and JL interpretated data. AQ, HB, FU, RS, M-LB, JL, and JT drafted the manuscript. All authors critically discussed, corrected, and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00354/full#supplementary-material

REFERENCES

1. Gao B, Ahmad MF, Nagy LE, Tsukamoto H. Inflammatory pathways in alcoholic steatohepatitis. *J Hepatol*. (2019) 70:249–59. doi: 10.1016/j.jhep.2018.10.023
2. Bernardi M, Moreau R, Angeli P, Schnabl B, Arroyo V. Mechanisms of decompensation and organ failure in cirrhosis: from peripheral arterial vasodilation to systemic inflammation hypothesis. *J Hepatol*. (2015) 63:1272–84. doi: 10.1016/j.jhep.2015.07.004
3. Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, et al. Hepatic recruitment of the inflammatory Gr1 + monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology*. (2009) 50:261–74. doi: 10.1002/hep.22950
4. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. (2011) 11:723–37. doi: 10.1038/nri3073
5. Li L, Wei W, Li Z, Chen H, Li Y, Jiang W, et al. The spleen promotes the secretion of CCL2 and supports an M1 dominant phenotype in hepatic macrophages during liver fibrosis. *Cell Physiol Biochem*. (2018) 51:557–74. doi: 10.1159/000495276
6. Trebicka J, Amoros A, Pitarch C, Titos E, Alcaraz-Quiles J, Schierwagen R, et al. Addressing profiles of systemic inflammation across the different clinical phenotypes of acutely decompensated cirrhosis. *Front Immunol*. (2019) 10:476. doi: 10.3389/fimmu.2019.00476
7. Triantafyllou E, Woollard KJ, McPhail MJW, Antoniades CG, Possamai LA. The role of monocytes and macrophages in acute and acute-on-chronic liver failure. *Front Immunol*. (2018) 9:2948. doi: 10.3389/fimmu.2018.02948

8. Jansen C, Möller P, Meyer C, Kolbe CC, Bogs C, Pohlmann A, et al. Increase in liver stiffness after transjugular intrahepatic portosystemic shunt is associated with inflammation and predicts mortality. *Hepatol Baltim Md*. (2018) 67:1472–84. doi: 10.1002/hep.29612
9. Queck A, Rueschenbaum S, Kubesch A, Cai C, Zeuzem S, Weigert A, et al. The portal vein as a distinct immunological compartment - A comprehensive immune phenotyping study. *Hum Immunol*. (2018) 79:716–23. doi: 10.1016/j.humimm.2018.07.233
10. Queck A, Carnevale R, Uschner FE, Schierwagen R, Klein S, Jansen C, et al. Role of portal venous platelet activation in patients with decompensated cirrhosis and TIPS. *Gut*. (2019). doi: 10.1136/gutjnl-2019-319044
11. Moreau R, Jalan R, Gines P, Pavesi M, Angeli P, Cordoba J, et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. *Gastroenterology*. (2013) 144:1426–37.e9. doi: 10.1053/j.gastro.2013.02.042
12. Brol MJ, Rösch F, Schierwagen R, Magdaleno F, Uschner FE, Manekeller S, et al. Combination of CCl 4 with alcoholic and metabolic injuries mimics human liver fibrosis. *Am J Physiol Gastrointest Liver Physiol*. (2019) 317:G182–94. doi: 10.1152/ajpgi.00361.2018
13. Lehmann JM, Claus K, Jansen C, Pohlmann A, Schierwagen R, Meyer C, et al. Circulating CXCL10 in cirrhotic portal hypertension might reflect systemic inflammation and predict ACLF and mortality. *Liver Int*. (2018) 38:875–84. doi: 10.1111/liv.13610
14. Klein S, Rick J, Lehmann J, Schierwagen R, Schierwagen IG, Verbeke L, et al. Janus-kinase-2 relates directly to portal hypertension and to complications in rodent and human cirrhosis. *Gut*. (2017) 66:145–55. doi: 10.1136/gutjnl-2015-309600
15. Trebicka J, Hennenberg M, Schulze Pröbsting A, Laleman W, Klein S, Granzow M, et al. Role of β3-adrenoceptors for intrahepatic resistance and portal hypertension in liver cirrhosis. *Hepatology*. (2009) 50:1924–35. doi: 10.1002/hep.23222
16. Trebicka J, Hennenberg M, Laleman W, Shelest N, Biecker E, Schepke M, et al. Atorvastatin lowers portal pressure in cirrhotic rats by inhibition of RhoA/Rho-kinase and activation of endothelial nitric oxide synthase. *Hepatology*. (2007) 46:242–53. doi: 10.1002/hep.21673
17. Klein S, Schierwagen R, Uschner FE, Trebicka J. Mouse rat models of induction of hepatic fibrosis assessment of portal hypertension. In: Rittié L, editor. *Fibrosis*. New York, NY: Springer (2017). p. 91–116. doi: 10.1007/978-1-4939-7113-8_7
18. Trebicka J, Krag A, Gansweid S, Appenrodt B, Schiedermaier P, Sauerbruch T, et al. Endotoxin and tumor necrosis factor-receptor levels in portal and hepatic vein of patients with alcoholic liver cirrhosis receiving elective transjugular intrahepatic portosystemic shunt. *Eur J Gastroenterol Hepatol*. (2011) 23:1218–25. doi: 10.1097/MEG.0b013e32834a75dc
19. Trebicka J, Krag A, Gansweid S, Schiedermaier P, Strunk HM, Fimmers R, et al. Soluble TNF-alpha-receptors I are prognostic markers in TIPS-treated patients with cirrhosis and portal hypertension. *PLoS ONE*. (2013) 8:e83341. doi: 10.1371/journal.pone.0083341
20. Berres M-L, Lehmann J, Jansen C, Görtzen J, Meyer C, Thomas D, et al. Chemokine (C-X-C motif) ligand 11 levels predict survival in cirrhotic patients with transjugular intrahepatic portosystemic shunt. *Liver Int*. (2016) 36:386–94. doi: 10.1111/liv.12922
21. Ehling J, Bartneck M, Wei X, Gremse F, Fech V, Möckel D, et al. CCL2-dependent infiltrating macrophages promote angiogenesis in progressive liver fibrosis. *Gut*. (2014) 63:1960–71. doi: 10.1136/gutjnl-2013-306294
22. Ambade A, Lowe P, Kodys K, Catalano D, Gyongyosi B, Cho Y, et al. Pharmacological inhibition of CCR2/5 signaling prevents and reverses alcohol-induced liver damage, steatosis, and inflammation in mice. *Hepatology*. (2019) 69:1105–21. doi: 10.1002/hep.30249
23. Baeck C, Wei X, Bartneck M, Fech V, Heymann F, Gassler N, et al. Pharmacological inhibition of the chemokine C-C motif chemokine ligand 2 (monocyte chemoattractant protein 1) accelerates liver fibrosis regression by suppressing Ly-6C + macrophage infiltration in mice *Hepatology*. (2014) 59:1060–72. doi: 10.1002/hep.26783
24. Sargenti K, Johansson Å, Bertilsson S, Mattsby-Baltzer I, Klintman D, Kalaitzakis E. Dysfunction of circulating polymorphonuclear leukocytes and monocytes in ambulatory cirrhotics predicts patient outcome. *Dig Dis Sci*. (2016) 61:2294–302. doi: 10.1007/s10620-016-4132-3
25. Schierwagen R, Alvarez-Silva C, Madsen MSA, Kolbe CC, Meyer C, Thomas D, et al. Circulating microbiome in blood of different circulatory compartments. *Gut*. (2019) 68:578–80. doi: 10.1136/gutjnl-2018-316227
26. Clària J, Stauber RE, Coenraad MJ, Moreau R, Jalan R, Pavesi M, et al. Systemic inflammation in decompensated cirrhosis: characterization and role in acute-on-chronic liver failure. *Hepatology*. (2016) 64:1249–64. doi: 10.1002/hep.28740
27. Dirchwolf M, Podhorzer A, Marino M, Shulman C, Cartier M, Zunino M, et al. Immune dysfunction in cirrhosis: distinct cytokines phenotypes according to cirrhosis severity. *Cytokine*. (2016) 77:14–25. doi: 10.1016/j.cyto.2015.10.006
28. Basili S, Carnevale R, Nocella C, Bartimoccia S, Raparelli V, Talerico G, et al. Serum albumin is inversely associated with portal vein thrombosis in cirrhosis. *Hepatol Commun*. (2019) 3:504–12. doi: 10.1002/hep4.1317
29. Huang X, Fan X, Zhang R, Jiang S, Yang K, Chen S. Systemic inflammation and portal vein thrombosis in cirrhotic patients with gastroesophageal varices. *Eur J Gastroenterol Hepatol*. (2020) 32:401–5. doi: 10.1097/MEG.0000000000001526

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REVIEW
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Liver, Tumor and Viral Hepatitis: Key Players in the Complex Balance Between Tolerance and Immune Activation

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Liver cancer is the third most common cause of cancer related death in the World. From an epidemiological point of view the risk factors associated to primary liver cancer are mainly viral hepatitis infection and alcohol consumption. Even though there is a clear correlation between liver inflammation, cirrhosis and cancer, other emerging liver diseases (like fatty liver) could also lead to liver cancer. Moreover, the liver is the major site of metastasis from colon, breast, ovarian and other cancers. In this review we will address the peculiar status of the liver as organ that has to balance between tolerance and immune activation. We will focus on macrophages and other key cellular components of the liver microenvironment that play a central role during tumor progression. We will also discuss how current and future therapies may affect the balance toward immune activation.

Keywords: liver, macrophage, T cell, T cell therapy, cancer

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INTRODUCTION

The liver is a multi-tasking organ responsible for many crucial functions in the body. It is mostly known for its metabolic and detox work, in fact, it participates in the metabolism of fats (making bile), it stores and releases glucose and it clears harmful substances from the blood. The liver receives about 1.5 L of blood every minute coming from the digestive tract and its job is also to prevent and fight infections coming from the bloodstream. With such massive amount of blood, it is easy to imagine the countless potential antigens that the liver encounters at any given time. To prevent unnecessary inflammation and autoimmune disease the liver harbors different cellular components that work together with complex loops of interactions to maintain the organism homeostasis (1). Immune tolerance is therefore needed in the liver as safe measure to prevent tissue damage that would compromise the metabolic functions of this organ (2). The down side of this protective effect is that some infections, like viruses targeting hepatocytes (3) and even cancer have the possibility to escape the immune-response and give rise to potentially lethal liver diseases (4–7). A general view is that tolerance can be induced by both an insufficient/tolerogenic priming of effector T cells (8, 9) and directly through a regulatory T-cell response (10, 11). Kupffer cells, the specific tissue-resident macrophages of the liver, have a key role in promoting tolerance (12). This review will examine the major cellular components of the liver and tumor microenvironment, their role in controlling the balance between tolerance and activation and the potential therapeutic interventions to tilt the balance against liver cancer progression.

ANTIGEN PRESENTATION IN THE LIVER

Role of Kupffer Cells

Kupffer cells (KCs) are liver-resident macrophages, they originate from fetal liver-derived erythromyeloid progenitors and their population is maintained by self-renewal and not by infiltrating monocytes (**Figure 1**; 13, 14). KCs are located in the hepatic sinusoid as represented in **Figure 2**, where pathogens enter the liver via portal or arterial circulation (15). The KC population represent a first line defense against infectious particles and potentially immune reactive particles entering the blood stream from the gut (16, 17). KCs express several scavenger receptors (18) such as Toll-like, complement, antibody receptors which allow them to activate danger-associated molecular patterns and internalize, and kill pathogens (15, 19). They also contribute to the initiation of an innate immune response by secretion of cytokines and chemokines (20). The KCs have a potent phagocytic activity not only for the above-mentioned blood-born pathogens but also for other particles/complexes/debris originating from dead erythrocytes and cells of the hepatic parenchyma. Other functions of KCs are related to the iron (21), bilirubin (22) and cholesterol metabolism (23).

The role of KCs in antigen presentation has been considered to be limited based on experiments where isolated mouse KCs only induced a low T-cell proliferation against soluble antigens *in vitro* (24). Other experimental *in vivo* set-ups (12) showed that KC were able not only to phagocytize particle-bound antigens but also subsequently induce a tolerogenic T-cell response against those antigens. This could be measured by induction of local T-cell proliferation, expansion of Foxp3 + IL-10 + OTII T regs *in vivo*. A high level expression of PDL-1 and secretion of IL-10 by KCs strongly suggests that this cell type can present antigens to CD4+ T cells but then with the goal of inducing tolerance (12, 25).

It is important in this regard to keep in mind that KCs are not a homogenous population. As with other macrophage-like cells, two distinct subsets have been identified, one "M1 immunogenic" type that has phagocytic properties and secretes high levels of IL-12 and low levels of IL-10 (26) and another "M2 alternatively activated" type that secretes high levels of IL-10, TGF-b and low levels of IL-12 (27). When TLRs are engaged the KCs usually act as M2 type (28). When it comes to antigen presentation, several evidences implicate KCs as capable antigen presenting cells (APCs) but due to their up-regulation of inhibitory molecules like PD-L1 (29) and also Fas ligand (30) their major role is likely to be suppression T cell activation and induction of Tregs (10). The classification in M1 and M2 types is not exhaustive and does not reflect the complexity and diversity of functions among this cell population. Further subgrouping of the M2 type in M2a, M2b, and M2c has indeed been proposed. These subgroups express different markers and respond to different stimuli (17, 31). It is important to note that tumor-associated macrophages, which will be discussed later, have a M2 phenotype but a distinct transcriptional profile that promotes tumor angiogenesis (17, 32).

Antigen Presentation by Other Cell Types

A schematic representation of antigen presentation to CD4+ and CD8+ T cells is depicted in **Figure 3**. Kupffer cells are not the only cell type in the liver that function as APC but inducing tolerance instead of immune activation. Human hepatic myeloid dendritic cells (DC) (phenotypically characterized as CD11b+, CD11c+, and CD1c+) secrete IL-10 and induce IL-10 secretion by T cells (33) upon release by hepatic stromal cells of macrophage colony stimulating factor (34). Plasmacytoid DC (phenotypically characterized by expression of BDCA-2 and CD123) are also present in the liver and up-regulate PD-L1 in response to TLR agonists, promoting Treg cell differentiation (35, 36). Even though the liver APCs are skewed toward inducing tolerance there is also a population of myeloid DC (CD141+) that is able to cross-present antigen to CD8+ cells and induce IFN-g secretion (37, 38).

Other liver parenchymal cells also express MHC and costimulatory molecules and are capable of antigen presentation, although most of them still induce tolerance against the antigens presented. Endothelial cells, located at the liver sinusoids and termed liver sinusoidal endothelial cells (LSECs), promote tolerance in CD8+ T cells (39, 40) and lead CD4+ T cells toward Tregs differentiation (11) by secreting IL-10, TGF-b, PGE2 (41) and upregulating PD-L1 (42).

Hepatic stellate cells (HSCs) reside along the sinusoids and regulate the blood flow through these veins. HSCs can present antigens to T cells but again to promote Treg differentiation (43–45).

Lastly hepatocytes seem to be able to present antigens to CD8+ and CD4+ T cells but fail to sustain their proliferation and induce apoptosis instead (46, 47).

Viral Hepatitis Infection: Chronic Inflammation and Liver Cancer

The three major viruses responsible for viral hepatitis are the hepatitis B virus (HBV), the hepatitis C virus (HCV), and the hepatitis D virus (HDV), each estimated to be chronically infect 257 milion, 140 milion, and 15 milion people worldwide, respectively (48). While the viruses are quite different as HBV is a double stranded-DNA virus belonging to the Hepadnaviridae family, HCV is a single-stranded + RNA virus belonging to the Flaviviridae family, and HDV a single stranded circular -RNA viroid, they share common pathological paths. All viruses infect hepatocytes but are not directly cytolytic, the cell damage in fact is due to the anti-viral immune response, such as direct killing by cytotoxic CD8+ T cells or NK cells (49) or the action of inflammatory cytokines (50). During immune activation, liver resident APCs can activate virus-specific T cells which are then responsible for recognition and killing of infected hepatocytes and secretion of inflammatory cytokines (50). Acute hepatitis is associated with viral clearance in 95% of HBV infections in adults but only 5% of vertical infections, and in about (49). 25% of HCV infections For HDV, a satellite virus to HBV, acute infections occur when HBV and HDV coinfect a host (clearance rate is 95%), whereas most become chronically infected by HDV when appearing as a superinfection of HBV carriers. When viral

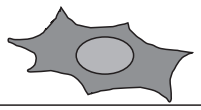
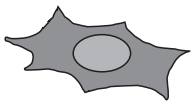
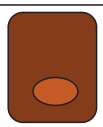
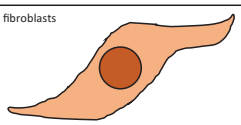
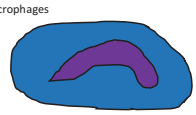
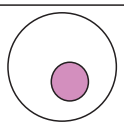
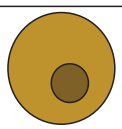
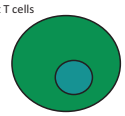
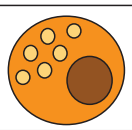
Kupffer cells 	• High level of MHC and costimulatory molecules [7] • High level of PDL-1 and secretion of IL-10 [12, 25] • Not a homogenous population: "M1 immunogenic" type that has phagocytic properties and secretes high levels of IL-12 and low levels of IL-10 [26]; "M2 alternatively-activated" type secretes high levels of IL-10, TGF-b and low levels of IL-12 [27]. Further subgrouping ofthe M2 type in M2a, M2b and M2c has been proposed [17, 31]
Human hepatic myeloid dendritic cells (CD11b+, CD11c+ and CD1c+) 	• Phenotype CD11b+, CD11c+ and CD1c+ • Secretion of IL-10 and induction of IL-10 secretion by T cells [33] upon release by hepatic stromal cells of macrophage colony stimulating factor [34]
Human hepatic myeloid dendritic cells (CD141+) 	• Phenotype CD141+ • Antigen cross-presentation to CD8+ cells and induction of IFN-g secretion [37, 38]
Plasmacytoid dendritic cells (BDCA-2+ and CD123+) 	• Phenotype BDCA-2+ and CD123+ • Up-regulation of PD-L1 in response to TLR agonists, promoting Treg cell differentiation [35, 36]
Liver sinusoidal endothelial cells 	• Promote tolerance in CD8+ T cells [40, 41] • Lead CD4+ T cells toward Tregs differentiation [11] by secreting IL-10, TGF-b, PGE2 [42] and upregulating PD-L1 [43] • In the tumor have an accelerated cell cycle, greater migratory abilities and also secrete factors like IL-6, IL-8, MCP-1, GRO-a that can promote tumor progression and metastasis [51, 66, 67]
Hepatic stellate cells 	• Promote Treg differentiation [44-46] • Secretion of several soluble factors like cytokines (IL-6, IL-1B) [52], chemokines (CCL5, CCL21) [53] and growth factors (TGFa, TGFb, EGF, PDGF, bFGF) [54] • When activated HSCs can infiltrate in the HCC stroma [57] and promote tumor vascularization by secreting PDGF that attracts pericytes which also secrete VEGF [51, 58]
Hepatocytes 	• Able to present antigens to CD8+ and CD4+ T cells butfail to sustain their proliferation and induce apoptosis instead [47, 48]
Cancer-associated fibroblasts 	• Secretion of growth factors, cytokines and chemokines (e.g. EGF, FGF, HGF, TGFb, IL-6) that stimulate also tumor growth and proliferation [59], as well as a more malignant phenotype [60, 61] • Play an important role during HCC growth and metastasis [51, 62-64]
Tumor associated macrophages 	• Promote tumor initiation by secretion ofinflammatory molecules like IFN -g, TNF-a and IL-6 in response to chronic infection or irritation and this chronic inflammation [79] • Promote tumor progression and malignancy by switching to an immunosuppressive phenotype [78] • Supportthe metastatic process by promoting tumor cell migration and angiogenesis [78, 80-82]
Cytotoxic lymphocytes 	• Can kill tumor cells and recruite macrophages • An immune response against common tumor antigens has been identified from both TIL and blood of HCC patients [68]
Tregs 	• Suppression ofimmune response by inhibiting CD8+ T cell effector functions like degranulation, and production of perforin and granzymes [70]
Mucosal-associated invariant T cells 	• Expression of cytokines [71] and other effector molecules [72] • Negatively affected by chronic viral hepatitis infection [73]
Natural Killer Cells 	• Protective role in the liver especially during chronic viral hepatitis infection even though they are still functionally impaired by HBV and HCV [74]

FIGURE 1 | Key cellular components of liver tumor microenvironment. Schematic representation of cell types and summary of functions in tumor microenvironment.

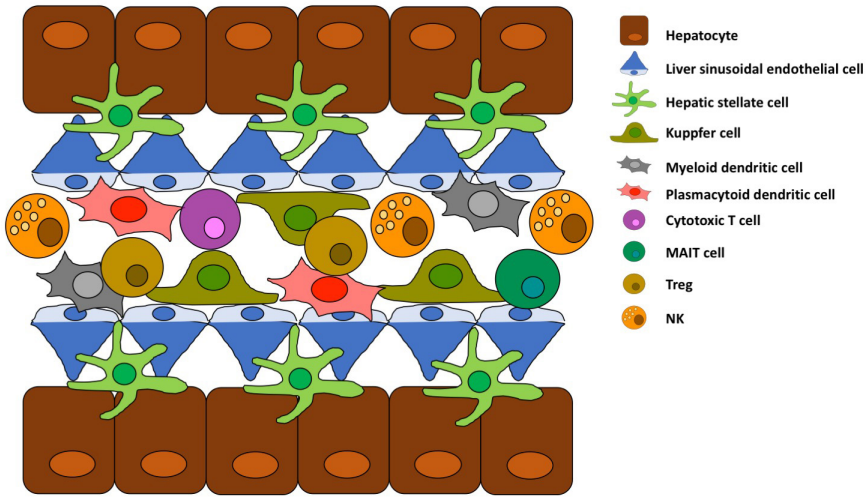


FIGURE 2 | Schematic structure of liver sinusoid. Key cell types and their spatial location are schematically represented.

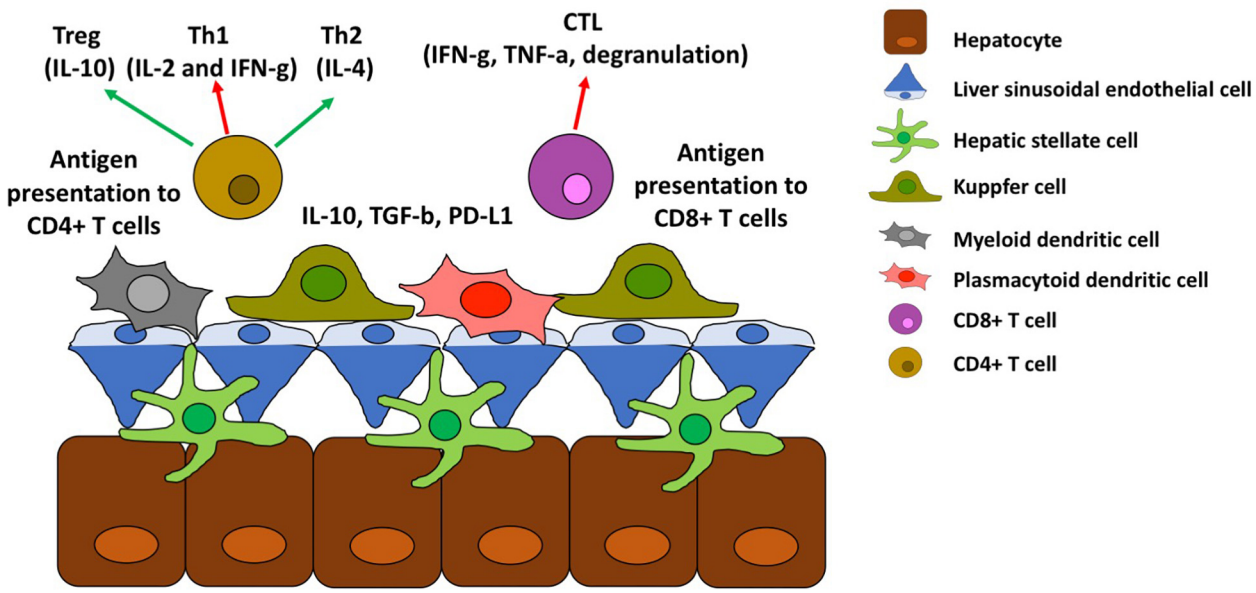


FIGURE 3 | Antigen presentation in the liver. Schematic representation of antigen presentation to CD4+ and CD8+ T cells and its effect toward tolerance (red arrows) or immune activation (green arrow).

clearance is not achieved and the virus is able to persist in the host, this is associated with a chronic inflammation of the liver that can lead to fibrosis, cirrhosis and also cancer. Thus, it is most likely that the persisting hepatic inflammation is the key driver for transformation of hepatocytes to cancer cells. The mechanisms of persistence differs for the different viruses, although a common strategy is to disturb and impair the host immune response. For HBV T cell tolerance, or dysfunction, is a key mechanism of persistence (51). In neonatal vertical infection, the high rate of chronicity is most likely explained by the immature immune response of the host in combination with overproduction of some HBV proteins. In particular one of these proteins is able to pass the placenta and thereby, when presented during schooling of the immunesystem, is perceived as self proteins (52, 53). Hence, these T cell clones are deleted. Other mechanisms of persistence used by HBV, are the constant presence of viral antigens causing immunactivation and then anergy or dysfunction. Finally, the over production and secretion of HBV surface antigen (HBsAg) particles mainly composed of small HBsAg, effectively blocks neutralizing anti-HBs antibodies. Thus, viral particles whose surface is mainly composed of PreS1 and PreS2 can escape these antibodies and infect new cells. Taken together, these factors most likely explain why HBV is able to persist, despite being a genetically stable virus. In contrast,

several observations point to the fact that the higher rate of chronic infections due to HCV as compared to HBV is associated with the better ability of HCV to evade adaptive immunity (54–56), probably due to its extreme mutation rate (57). Thus, HCV persists as a virus constantly changing to evade both B and T cells. For HDV the mechanisms of persistence are less well known, except that HDV accelerates the tissue damage and disease during dual infection. Chronic hepatitis, regardless of the virus causing it, is characterized by a persistent presence of the viral antigens, which cause a continuous T cell stimulation. This chronic stimulation is regulated by immune checkpoint (i.e., expression of PD-1) that limit the effector functions of the virus-specific T cells resulting in a loop of low-grade inflammation and ineffective viral clearance (58, 59). Another important actor in the balance between immune activation and suppression are Tregs. Effective viral clearance is achieved through cytotoxicity that can be very dangerous if uncontrolled, or too massive. An excessive tissue damage needs to be prevented for the sake of host survival, and therefore the role of Tregs can be seen as a safe measure against fatal liver injury. Mouse studies modeling acute HBV infection observed an increased liver damage after Treg depletion (60), also HCV mouse models showed a transient peak of Tregs during acute infection, and a persistent increase in Tregs frequency in chronic infection (61). It is not yet clear wheather Tregs expansion is a cause or a consequence of chronic infection, but certainly this phenomenon is highlighting the complex interplay and immunological balance occurring in the liver during viral infection. The major long-term consequence of chronic infection is the occurrence of fibrosis and cirrhosis that impair liver functionality and also the insurgence of cancer. Treg expansion has been observed in samples of hepatocellular carcinomas (HCCs) and cirrhotic tissue (62). In this study (62), the expression of OX40 (a activation marker) correlates with intratumoral Treg frequency and other markers of proliferation like Ki67; OX40L on the other hand is expressed on myeloid liver infiltrating cells that co-localize with Tregs and directly correlates with HCV viral load. So this is an example of interplay between the host and the virus that is trying to escape the immune system by inducing Tregs via OX40L-OX40 interaction (63, 64).

PRIMARY TUMORS IN THE LIVER (HCC)

Hepatocellular carcinoma is one of the leading causes for cancer-related death in the world. HCC is mostly asymptomatic and the diagnosis is usually made at a late stage, whereby the prognosis generally is poor. Several risk factors have been identified for HCC, such as chronic viral hepatitis, excessive alcohol consumption, obesity, fatty liver, and diabetes (65). Although the presence of these risk factors aid in monitoring specific patient populations, the molecular mechanism(s) behind tumor initiation, progression, and metastasis are poorly understood and this poses a limitation to find efficient therapeutic approaches.

Hepatocellular carcinoma is associated with the dysregulated growth of hepatocytes that form dysplastic nodules resulting in chronic liver damage or cirrhosis. Mouse models have shown that the hepatocytes in this context have a higher expression of vimentin and type I collagen, suggesting that they are acquiring mesenchymal phenotype (66).

Although the specific mechanism has not been clarified, increasing evidence is pointing at the role of tumor microenvironment in HCC pathogenesis (67). In the following sections we will describe the main cellular components of the liver tumor microenvironment considering the primary tumor but also the metastatic deposits originated from other common tumor types like colon and breast cancer.

MAIN CELLULAR COMPONENTS OF THE TUMOR MICROENVIRONMENT

Hepatic Stellate Cells (HSCs)

A summary of cellular components of the tumor microenvironment is represented in **Figure 1**. These cells are part of the liver connective tissue and are located on the perisinusoidal space. HSCs have many homeostatic functions such as vitamin A accumulation, synthesis of collagen, expression of several soluble factors like cytokines (IL-6, IL-1B) (68), chemokines (CCL5, CCL21) (69), and growth factors (TGFa, TGFb, EGF, PDGF, and bFGF) (70). Liver damage can cause proliferation of HSCs and cytoskeleton remodeling (71, 72). When activated HSCs can infiltrate in the HCC stroma (73) and promote tumor vascularization by secreting PDGF that attracts pericytes which also secrete VEGF (67, 74).

Cancer-Associated Fibroblasts (CAFs)

This type of fibroblasts mediates several interactions between the tumor and the liver stroma. They secrete growth factors, cytokines and chemokines (e.g., EGF, FGF, HGF, TGFb, and IL-6) that stimulate also tumor growth and proliferation (75), as well as a more malignant phenotype (76, 77). Several evidences showed that CAFs play an important role during HCC growth and metastasis (67, 78–80).

Endothelial Cells

The main role of these cells is to promote tissue vascularization. The morphology of blood vessels is different in tumors compared to normal tissues, probably due to increased permeability, and this seems to be related to molecular and functional differences of epithelial cells (81). In particular, the endothelial cells present in the tumor have an accelerated cell cycle, greater migratory abilities and also secrete factors like IL-6, IL-8, MCP-1, and GRO-a that can promote tumor progression and metastasis (67, 82, 83).

Immune Cells

Tumor Infiltrating Lymphocytes (TILs)

Tumor Infiltrating Lymphocytes can play an important role for tumor progression, like Tregs, but can also be the source of powerful anti-tumor cells that can be used for therapy. An immune response against common tumor antigens has indeed been identified from both TIL and blood of HCC patients (84). The presence of Tregs in tumor is usually associated with a worse

prognosis (85) since this cell type suppress the immune response by inhibiting CD8+ T cell effector functions like degranulation, and production of perforin and granzymes (86). Recent studies are also highlighting the importance of a mucosal-associated invariant T cell population (MAIT) that is able to activate and express cytokines (87) and other effector molecules (88), MAIT cells seem also to be negatively affected by chronic viral hepatitis infection (89).

Natural killer cells (NK) account for up to 50% of the total number of lymphocytes present in the liver. It has been shown that NK have a protective role in the liver especially during chronic viral hepatitis infection even though they are still functionally impaired by HBV and HCV (90). In HCC, it seems that the NK functionality is also impaired but the use of genetically modified NK cells in combination with kinase inhibitors has provided promising results by *in vivo* models (91, 92).

Tumor Associated Macrophages (TAMs)

Macrophages are frequently found in tumors and according to a high number of clinical and experimental data they seem to promote tumor initiation, progression and even metastasis (93, 94). The mechanism behind the contribution of macrophages to cancer initiation and progression seems to be caused at two major steps: initially macrophages secrete inflammatory molecules like IFN-g, TNF-a and IL-6 in response to chronic infection or irritation and this chronic inflammation seems to be causal to tumor initiation (95). Once the tumor is established, the macrophages switch to an immunosuppressive phenotype promoting progression and malignancy (94). Moreover, TAMs support the metastatic process by promoting tumor cell migration and angiogenesis (94, 96–98). In HCC a role of infiltrating monocytes and KCs seems to be to drive tumor progression and metastasis (99). The presence of TAMs is also associated with increase tumor burden an higher metastasis rate in both HCC patients and mouse models of liver cancer (100). In particular the ability of macrophages to secrete cytokines, chemokines and growth factors seems to be a crucial component of tumor initiation and proliferation (99, 101). KCs have a pro-inflammatory function that seems to be particularly important for HCC initiation (102). At later stages when the tumor is already established their ability to express PD-L1 and secrete immunosuppressive cytokines like IL-10 further contribute to promote tumor progression due to inhibition of effector lymphocytes like CD8+ T cells (14, 103, 104).

Microenvironment of Metastatic Deposits in the Liver

As already mentioned the liver receives a massive amount of blood which may help to explain the transport and hepatic entry of metastatic tumor cells coming from other organs of the body. The liver is indeed the main site of metastasis for several types of cancer, like colon, pancreas, melanoma, breast cancer, and sarcomas (105). The same cellular components that play an important role for tumor initiation and progression of primary liver cancer are also likely to be involved in facilitating the establishment of metastasis (106, 107). Another cell type that has been recently shown to be an important player for development of metastasis are marrow-derived immune cells recruited to the liver. A mouse model of metastatic pancreatic ductal adenocarcinoma revealed an interplay between the different cellular types in the liver that ultimately favored the metastatic deposit formation (108). In this model, tumor cells secrete exosomes that are taken up by KCs that then increase TGFb production affecting HSC that also increase fibronectin production with ultimate recruitment of bone marrow-derived macrophages. Another study found that exosomes from pancreatic cell lines able to metastasize in the liver, contain integrins that can fuse preferentially with KCs. These data also suggest that exosomes may have a role in disease progression and specific organ metastasis depending on the integrins that they contain (109). In general as a metastatic circulating cell survive the first line of defense offered by KCs and HSC (110), the liver milieu, rich of growth factors, proinflammatory molecule such as S100A8, and immune-suppressive cytokines, favors the formation of pre-metastatic niches (109, 111–114). In mouse models, NK cells are shown to play an important role in immune surveillance and prevention or delay of metastatic formation in the liver (115–117). Cancer cells are able to escape direct NK cytotoxicity by forming clusters with other cancer cells (118).

TARGETS FOR THERAPY: CAN WE TILT THE BALANCE?

As described above the level of complexity of the liver and liver-cancer microenvironment is very high. Due to the multiple cellular components, cytokines, chemokines, and physical disposition around the liver sinusoid, it is unlikely that one single approach could be able to break the vicious loop of inflammation, growth stimulation, and immune-tolerance that characterize the interplay between liver and cancer. Despite this, we believe that a combined approach of several therapies targeting different components of the tumor microenvironment may be effective. In the following sessions the available and potential future therapies for liver cancer are reviewed.

Target Microenvironment With Drugs

It is possible to specifically block the signaling pathways used by cancer cells to take advantage of the stroma cells and their growth stimulation (119). For HCC the two most studied pathways are the ones that promotes inflammation and angiogenesis (120). The only approved drug for advanced HCC is Sorafenib, a multi-kines inhibitor that target VEGFR, Raf-kinase and PDGFR, but unfortunately its efficacy is limited (121). Other drugs with similar mechanism of action are currently in experimental evaluation (122, 123).

Target Microenvironment With Physical Agents

One curative option for early stage HCC is ablative surgery. If the tumor is removed completely or the liver is transplanted there is a chance that the tumor will not come back. Most of

patients are unfortunately diagnosed at a very late stage when surgical alternatives are not a possibility anymore (65). Common ablative procedures like radiation, cryoablation, and trans arterial chemoembolization (TACE) are also used at a late stage as palliative treatment. Physically destroying tumor cells may have the advantage to release tumor-antigens that can be taken-up by APCs and be cross-presented to T cells. Increased tumor-specific cytotoxicity has indeed been detected after this type of therapies (124–126). The potential side effect of this treatment is that the presence of PD-L1 on most of APCs in the liver could actually inhibit the tumor-specific T cell response (127) so one obvious measure would be to combine ablation therapy with PD-1 blockade treatment (128, 129). This is currently tested in various clinical trials.

Target Microenvironment With Immunotherapy

Immune suppression and tolerance are key factors in the induction, progression and metastasis of cancer in the liver. It is logical to assume that targeting the immune cells in the liver, reverting what cause their anergic state, may offer a solution for cancer treatment. Several immunotherapy trials have been conducted for HCC with cell therapy (LAK and TIL), cytokines and check-point inhibitors, dendritic cells vaccines and combinations of the above (129). The outcomes of these trials are promising but still not enough to offer a complete remission for HCC. Possibly, the particular microenvironment of the liver presents more challenges compared to other epithelial cancers that were successfully treated by adoptive cell therapy (130–134).

One current promising immunotherapeutic approach is the use of check-point inhibitors that target PD-1 and CTLA-4 promoting T cell activation (135). Recent clinical trials with the anti-PD-1 monoclonal antibodies Nivolumab showed promising anti-tumor activity in some patients with advanced HCC (136).

CONCLUSION

In this review we focused on the interplay within the liver between non-parechymal phagocytig and APCs, viruses, and liver cancer microenvironments. This highlights the complexity and intricate interplay of different factors. Due to the huge number of antigens going through the liver, the physiological need of having a "safe-guard" mechanism to prevent harmful immune-activation and liver damage is very understandable. The drawback of this safe-guard mechanism is that pathogens like viruses or cancer, both primary and metastatic tumor cells, may establish and proliferate. We discussed some of the potential targets in the tumor microenvironment that can promote activation for the immune-system and potentially reverse the tolerance against cancer antigens. The milieu in the liver is highly complex and it is probably unrealistic to think that just a single "magic bullet" therapy could tilt the balance an mediate cure. It is most likely necessary to attack many or all the key players to effectively intervene with tumor progression. A combination of physical ablation, immunotherapy and other molecular drugs may indeed be needed.

One of the key components in the tumor microenvironments are TAMs. The biology of macrophages in general is very complex and to some extent still unknown. There are several evidences that suggest a different role of macrophage subgroups depending on their origin (e.g., fetal liver, bone marrow, etc.) and their location in the organism. What is clear is that they can have multiple, and sometimes opposite functions, that can influence clearance or disease progression. More research is needed to better understand the extent of their influence on tumor formation and progression.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and the writing of the review.

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REFERENCES

1. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol*. (2012) 30:531–64. doi: 10.1146/annurev.immunol.25.022106.141623
2. Medzhitov R, Schneider DS, Soares MP. Disease tolerance as a defense strategy. *Science*. (2012) 335:936–41. doi: 10.1126/science.1214935
3. Rehermann B. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. *Nat Med*. (2013) 19:859–68. doi: 10.1038/nm.3251
4. Racanelli V, Rehermann B. The liver as an immunological organ. *Hepatology*. (2006) 43(2 Suppl. 1):S54–62.
5. Tiegs G, Lohse AW. Immune tolerance: what is unique about the liver. *J Autoimmun*. (2010) 34:1–6. doi: 10.1016/j.jaut.2009.08.008
6. Crispe IN. Immune tolerance in liver disease. *Hepatology*. (2014) 60:2109–17. doi: 10.1002/hep.27254
7. Doherty DG. Immunity, tolerance and autoimmunity in the liver: a comprehensive review. *J Autoimmun*. (2016) 66:60–75. doi: 10.1016/j.jaut.2015.08.020
8. Schurich A, Berg M, Stabenow D, Böttcher J, Kern M, Schild HJ, et al. Dynamic regulation of CD8 T cell tolerance induction by liver sinusoidal endothelial cells. *J Immunol*. (2010) 184:4107–14. doi: 10.4049/jimmunol.0902580
9. Tay SS, Lu B, Sierro F, Benseler V, McGuffog CM, Bishop GA, et al. Differential migration of passenger leukocytes and rapid deletion of naive alloreactive CD8 T cells after mouse liver transplantation. *Liver Transpl*. (2013) 19:1224–35. doi: 10.1002/lt.23720
10. Breous E, Somanathan S, Vandenberghe LH, Wilson JM. Hepatic regulatory T cells and Kupffer cells are crucial mediators of systemic T cell tolerance to antigens targeting murine liver. *Hepatology*. (2009) 50:612–21. doi: 10.1002/hep.23043
11. Kruse N, Neumann K, Schrage A, Derkow K, Schott E, Erben U, et al. Priming of CD4+ T cells by liver sinusoidal endothelial cells induces CD25low forkhead box protein 3- regulatory T cells suppressing autoimmune hepatitis. *Hepatology*. (2009) 50:1904–13. doi: 10.1002/hep.23191
12. Heymann F, Peusquens J, Ludwig-Portugall I, Kohlhepp M, Ergen C, Niemietz P, et al. Liver inflammation abrogates immunological tolerance

induced by Kupffer cells. *Hepatology*. (2015) 62:279–91. doi: 10.1002/hep.27793
13. Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, et al. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature*. (2015) 518:547–51. doi: 10.1038/nature13989
14. Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol*. (2017) 17:306–21.
15. Jenne CN, Kubes P. Immune surveillance by the liver. *Nat Immunol*. (2013) 14:996–1006. doi: 10.1038/ni.2691
16. Thomson AW, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. *Nat Rev Immunol*. (2010) 10:753–66. doi: 10.1038/nri2858
17. Dixon LJ, Barnes M, Tang H, Pritchard MT, Nagy LE. Kupffer cells in the liver. *Compr Physiol*. (2013) 3:785–97.
18. Eckert C, Klein N, Kornek M, Lukacs-Kornek V. The complex myeloid network of the liver with diverse functional capacity at steady state and in inflammation. *Front Immunol*. (2015) 6:179. doi: 10.3389/fimmu.2015.00179
19. Tu Z, Bozorgzadeh A, Pierce RH, Kurtis J, Crispe IN, Orloff MS. TLR-dependent cross talk between human Kupffer cells and NK cells. *J Exp Med*. (2008) 205:233–44. doi: 10.1084/jem.20072195
20. Tacke F, Zimmermann HW. Macrophage heterogeneity in liver injury and fibrosis. *J Hepatol*. (2014) 60:1090–6. doi: 10.1016/j.jhep.2013.12.025
21. Gammella E, Buratti P, Cairo G, Recalcati S. Macrophages: central regulators of iron balance. *Metallomics*. (2014) 6:1336–45. doi: 10.1039/c4mt00104d
22. Naito M, Hasegawa G, Ebe Y, Yamamoto T. Differentiation and function of Kupffer cells. *Med Electron Microsc*. (2004) 37:16–28. doi: 10.1007/s00795-003-0228-x
23. Wang Y, van der Tuin S, Tjeerdema N, van Dam AD, Rensen SS, Hendrikx T, et al. Plasma cholesteryl ester transfer protein is predominantly derived from Kupffer cells. *Hepatology*. (2015) 62:1710–22. doi: 10.1002/hep.27985
24. You Q, Cheng L, Kedl RM, Ju C. Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. *Hepatology*. (2008) 48:978–90. doi: 10.1002/hep.22395
25. Erhardt A, Biburger M, Papadopoulos T, Tiegs G. IL-10, regulatory T cells, and Kupffer cells mediate tolerance in concanavalin a-induced liver injury in mice. *Hepatology*. (2007) 45:475–85.
26. Kinoshita M, Uchida T, Sato A, Nakashima M, Nakashima H, Shono S, et al. Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice. *J Hepatol*. (2010) 53:903–10. doi: 10.1016/j.jhep.2010.04.037
27. Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. *Nat Immunol*. (2013) 14:986–95. doi: 10.1038/ni.2705
28. Zhang M, Xu S, Han Y, Cao X. Apoptotic cells attenuate fulminant hepatitis by priming Kupffer cells to produce interleukin-10 through membrane-bound TGF-beta. *Hepatology*. (2011) 53:306–16. doi: 10.1002/hep.24029
29. Wu K, Kryczek I, Chen L, Zou W, Welling TH. Kupffer cell suppression of CD8+ T cells in human hepatocellular carcinoma is mediated by B7-H1/programmed death-1 interactions. *Cancer Res*. (2009) 69:8067–75. doi: 10.1158/0008-5472.CAN-09-0901
30. Sun Z, Wada T, Maemura K, Uchikura K, Hoshino S, Diehl AM, et al. Hepatic allograft-derived Kupffer cells regulate T cell response in rats. *Liver Transpl*. (2003) 9:489–97.
31. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. (2008) 8:958–69. doi: 10.3389/fpls.2013.00334
32. Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat Rev Immunol*. (2011) 11:750–61. doi: 10.1038/nri3088
33. Bamboat ZM, Stableford JA, Plitas G, Burt BM, Nguyen HM, Welles AP, et al. Human liver dendritic cells promote T cell hyporesponsiveness. *J Immunol*. (2009) 182:1901–11. doi: 10.4049/jimmunol.0803404
34. Xia S, Guo Z, Xu X, Yi H, Wang Q, Cao X. Hepatic microenvironment programs hematopoietic progenitor differentiation into regulatory dendritic cells, maintaining liver tolerance. *Blood*. (2008) 112:3175–85. doi: 10.1182/blood-2008-05-159921
35. Moseman EA, Liang X, Dawson AJ, Panoskaltsis-Mortari A, Krieg AM, Liu YJ, et al. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol*. (2004) 173:4433–42.
36. Castellaneta A, Sumpter TL, Chen L, Tokita D, Thomson AW. NOD2 ligation subverts IFN-alpha production by liver plasmacytoid dendritic cells and inhibits their T cell allostimulatory activity via B7-H1 up-regulation. *J Immunol*. (2009) 183:6922–32. doi: 10.4049/jimmunol.0900582
37. Haniffa M, Shin A, Bigley V, McGovern N, Teo P, See P, et al. Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. *Immunity*. (2012) 37:60–73. doi: 10.1016/j.immuni.2012.04.012
38. Kelly A, Fahey R, Fletcher JM, Keogh C, Carroll AG, Siddachari R, et al. CD141(+) myeloid dendritic cells are enriched in healthy human liver. *J Hepatol*. (2014) 60:135–42. doi: 10.1016/j.jhep.2013.08.007
39. Limmer A, Ohl J, Kurts C, Ljunggren HG, Reiss Y, Groettrup M, et al. Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat Med*. (2000) 6:1348–54.
40. von Oppen N, Schurich A, Hegenbarth S, Stabenow D, Tolba R, Weiskirchen R, et al. Systemic antigen cross-presented by liver sinusoidal endothelial cells induces liver-specific CD8 T-cell retention and tolerization. *Hepatology*. (2009) 49:1664–72. doi: 10.1002/hep.22795
41. Uhrig A, Banafsche R, Kremer M, Hegenbarth S, Hamann A, Neurath M, et al. Development and functional consequences of LPS tolerance in sinusoidal endothelial cells of the liver. *J Leukoc Biol*. (2005) 77:626–33.
42. Diehl L, Schurich A, Grochtmann R, Hegenbarth S, Chen L, Knolle PA. Tolerogenic maturation of liver sinusoidal endothelial cells promotes B7-homolog 1-dependent CD8+ T cell tolerance. *Hepatology*. (2008) 47:296–305. doi: 10.1002/hep.21965
43. Charles R, Chou HS, Wang L, Fung JJ, Lu L, Qian S. Human hepatic stellate cells inhibit T-cell response through B7-H1 pathway. *Transplantation*. (2013) 96:17–24. doi: 10.1097/TP.0b013e318294caae
44. Jiang G, Yang HR, Wang L, Wildey GM, Fung J, Qian S, et al. Hepatic stellate cells preferentially expand allogeneic CD4+ CD25+ FoxP3+ regulatory T cells in an IL-2-dependent manner. *Transplantation*. (2008) 86:1492–502. doi: 10.1097/TP.0b013e31818bfd13
45. Yang HR, Chou HS, Gu X, Wang L, Brown KE, Fung JJ, et al. Mechanistic insights into immunomodulation by hepatic stellate cells in mice: a critical role of interferon-gamma signaling. *Hepatology*. (2009) 50:1981–91. doi: 10.1002/hep.23202
46. Bertolino P, Trescol-Biemont MC, Rabourdin-Combe C. Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival. *Eur J Immunol*. (1998) 28:221–36.
47. Qian S, Wang Z, Lee Y, Chiang Y, Bonham C, Fung J. Hepatocyte-induced apoptosis of activated T cells, a mechanism of liver transplant tolerance, is related to the expression of ICAM-1 and hepatic lectin. *Transplant Proc*. (2001) 33:226. doi: 10.1016/s0041-1345(00)01985-0
48. Lanini S, Pisapia R, Capobianchi MR, Ippolito G. Global epidemiology of viral hepatitis and national needs for complete control. *Expert Rev Anti Infect Ther*. (2018) 16:625–39. doi: 10.1080/14787210.2018.1505503
49. Shin EC, Sung PS, Park SH. Immune responses and immunopathology in acute and chronic viral hepatitis. *Nat Rev Immunol*. (2016) 16:509–23. doi: 10.1038/nri.2016.69
50. Guidotti LG, Inverso D, Sironi L, Di Lucia P, Fioravanti J, Ganzer L, et al. Immunosurveillance of the liver by intravascular effector CD8(+) T cells. *Cell*. (2015) 161:486–500. doi: 10.1016/j.cell.2015.03.005
51. Park JJ, Wong DK, Wahed AS, Lee WM, Feld JJ, Terrault N, et al. Hepatitis B virus–specific and global T-Cell dysfunction in chronic hepatitis B. Gastroenterology. (2016) 150:684–95.e5. doi: 10.1053/j.gastro.2015.11.050
52. Chen MT, Billaud JN, Sällberg M, Guidotti LG, Chisari FV, Jones J, et al. A function of the hepatitis B virus precore protein is to regulate the immune response to the core antigen. *Proc Natl Acad Sci USA*. (2004) 101:14913–8.
53. Chen M, Sällberg M, Hughes J, Jones J, Guidotti LG, Chisari FV, et al. Immune tolerance split between hepatitis B virus precore and core proteins. *J Virol*. (2005) 79:3016–27.
54. Mutz P, Metz P, Lempp FA, Bender S, Qu B, Schöneweis K, et al. HBV bypasses the innate immune response and does not protect HCV from antiviral activity of interferon. *Gastroenterology*. (2018) 154:1791–804.e22. doi: 10.1053/j.gastro.2018.01.044
55. Tan A, Koh S, Bertoletti A. Immune response in hepatitis B virus infection. *Cold Spring Harb Perspect Med*. (2015) 5:a021428. doi: 10.1101/cshperspect.a021428
56. Chen SL, Morgan TR. The natural history of hepatitis C virus (HCV) infection. *Int J Med Sci*. (2006) 3:47–52. doi: 10.7150/ijms.3.47

57. Lazarevic I. Clinical implications of hepatitis B virus mutations: recent advances. *World J Gastroenterol*. (2014) 20:7653–64. doi: 10.3748/wjg.v20.i24.7653
58. Barnaba V. Hepatitis C virus infection: a "liaison a trois" amongst the virus, the host, and chronic low-level inflammation for human survival. *J Hepatol*. (2010) 53:752–61. doi: 10.1016/j.jhep.2010.06.003
59. Guidotti LG, Isogawa M, Chisari FV. Host-virus interactions in hepatitis B virus infection. *Curr Opin Immunol*. (2015) 36:61–6. doi: 10.1016/j.coi.2015.06.016
60. Stross L, Günther J, Gasteiger G, Asen T, Graf S, Aichler M, et al. Foxp3+ regulatory T cells protect the liver from immune damage and compromise virus control during acute experimental hepatitis B virus infection in mice. *Hepatology*. (2012) 56:873–83. doi: 10.1002/hep.25765
61. Billerbeck E, Wolfisberg R, Fahnøe U, Xiao JW, Quirk C, Luna JM, et al. Mouse models of acute and chronic hepacivirus infection. *Science*. (2017) 357:204–8. doi: 10.1126/science.aal1962
62. Piconese S, Timperi E, Pacella I, Schinzari V, Tripodo C, Rossi M, et al. Human OX40 tunes the function of regulatory T cells in tumor and nontumor areas of hepatitis C virus-infected liver tissue. *Hepatology*. (2014) 60:1494–507. doi: 10.1002/hep.27188
63. Zhang JY, Wu XL, Yang B, Wang Y, Feng GH, Jiang TJ, et al. Upregulation of OX40 ligand on monocytes contributes to early virological control in patients with chronic hepatitis C. *Eur J Immunol*. (2013) 43:1953–62. doi: 10.1002/eji.201243097
64. Pollmann J, Götz JJ, Rupp D, Strauss O, Granzin M, Grünvogel O, et al. Hepatitis C virus-induced natural killer cell proliferation involves monocyte-derived cells and the OX40/OX40L axis. *J Hepatol*. (2018) 68:421–30. doi: 10.1016/j.jhep.2017.10.021
65. El-Serag HB. Hepatocellular carcinoma. *N Engl J Med*. (2011) 365:1118–27.
66. Nitta T, Kim JS, Mohuczy D, Behrns KE. Murine cirrhosis induces hepatocyte epithelial mesenchymal transition and alterations in survival signaling pathways. *Hepatology*. (2008) 48:909–19. doi: 10.1002/hep.22397
67. Novikova MV, Khromova NV, Kopnin PB. Components of the hepatocellular carcinoma microenvironment and their role in tumor progression. *Biochemistry (Mosc)*. (2017) 82:861–73.
68. Marra F. Chemokines in liver inflammation and fibrosis. *Front Biosci*. (2002) 7:d1899–914. doi: 10.2741/marra
69. Schwabe RF, Bataller R, Brenner DA. Human hepatic stellate cells express CCR5 and RANTES to induce proliferation and migration. *Am J Physiol Gastrointest Liver Physiol*. (2003) 285:G949–58.
70. Pinzani M, Marra F, Carloni V. Signal transduction in hepatic stellate cells. *Liver*. (1998) 18:2–13. doi: 10.1111/j.1600-0676.1998.tb00120.x
71. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev*. (2008) 88:125–72. doi: 10.1152/physrev.00013.2007
72. Kluwe J, Pradere JP, Gwak GY, Mencin A, De Minicis S, Osterreicher CH, et al. Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition. *Gastroenterology*. (2010) 138:347–59. doi: 10.1053/j.gastro.2009.09.015
73. Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol*. (2008) 214:199–210.
74. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol*. (2005) 7:452–64.
75. Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res*. (2010) 316:1324–31. doi: 10.1016/j.yexcr.2010.02.045
76. Jia CC, Wang TT, Liu W, Fu BS, Hua X, Wang GY, et al. Cancer-associated fibroblasts from hepatocellular carcinoma promote malignant cell proliferation by HGF secretion. *PLoS One*. (2013) 8:e63243. doi: 10.1371/journal.pone.0063243
77. Liu F, Zhang W, Yang F, Feng T, Zhou M, Yu Y, et al. Interleukin-6-stimulated progranulin expression contributes to the malignancy of hepatocellular carcinoma cells by activating mTOR signaling. *Sci Rep*. (2016) 6:21260. doi: 10.1038/srep21260
78. Mazzocca A, Dituri F, Lupo L, Quaranta M, Antonaci S, Giannelli G. Tumor-secreted lysophostatidic acid accelerates hepatocellular carcinoma progression by promoting differentiation of peritumoral fibroblasts in myofibroblasts. *Hepatology*. (2011) 54:920–30. doi: 10.1002/hep.24485
79. Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, et al. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nat Cell Biol*. (2007) 9:1392–400.
80. Fransvea E, Mazzocca A, Antonaci S, Giannelli G. Targeting transforming growth factor (TGF)-betaRI inhibits activation of beta1 integrin and blocks vascular invasion in hepatocellular carcinoma. *Hepatology*. (2009) 49:839–50. doi: 10.1002/hep.22731
81. Baluk P, Morikawa S, Haskell A, Mancuso M, McDonald DM. Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. *Am J Pathol*. (2003) 163:1801–15.
82. Benetti A, Berenzi A, Gambarotti M, Garrafa E, Gelati M, Dessy E, et al. Transforming growth factor-beta1 and CD105 promote the migration of hepatocellular carcinoma-derived endothelium. *Cancer Res*. (2008) 68:8626–34. doi: 10.1158/0008-5472.CAN-08-1218
83. Knipe L, Meli A, Hewlett L, Bierings R, Dempster J, Skehel P, et al. A revised model for the secretion of tPA and cytokines from cultured endothelial cells. *Blood*. (2010) 116:2183–91. doi: 10.1182/blood-2010-03-276170
84. Flecken T, Schmidt N, Hild S, Gostick E, Drognitz O, Zeiser R, et al. Immunodominance and functional alterations of tumor-associated antigen-specific CD8+ T-cell responses in hepatocellular carcinoma. *Hepatology*. (2014) 59:1415–26. doi: 10.1002/hep.26731
85. Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, et al. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol*. (2007) 25:2586–93.
86. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. (2003) 198:1875–86.
87. Sobkowiak MJ, Davanian H, Heymann R, Gibbs A, Emgård J, Dias J, et al. Tissue-resident MAIT cell populations in human oral mucosa exhibit an activated profile and produce IL-17. *Eur J Immunol*. (2019) 49:133–43. doi: 10.1002/eji.201847759
88. Sundström P, Szeponik L, Ahlmanner F, Sundquist M, Wong JSB, Lindskog EB, et al. Tumor-infiltrating mucosal-associated invariant T (MAIT) cells retain expression of cytotoxic effector molecules. *Oncotarget*. (2019) 10:2810–23. doi: 10.18632/oncotarget.26866
89. Dias J, Hengst J, Parrot T, Leeansyah E, Lunemann S, Malone DFG, et al. Chronic hepatitis delta virus infection leads to functional impairment and severe loss of MAIT cells. *J Hepatol*. (2019) 71:301–12. doi: 10.1016/j.jhep.2019.04.009
90. Sun C, Sun HY, Xiao WH, Zhang C, Tian ZG. Natural killer cell dysfunction in hepatocellular carcinoma and NK cell-based immunotherapy. *Acta Pharmacol Sin*. (2015) 36:1191–9. doi: 10.1038/aps.2015.41
91. Kamiya T, Chang YH, Campana D. Expanded and activated natural killer cells for immunotherapy of hepatocellular carcinoma. *Cancer Immunol Res*. (2016) 4:574–81. doi: 10.1158/2326-6066.CIR-15-0229
92. Hong ZF, Zhao WX, Yin ZY, Xie CR, Xu YP, Chi XQ, et al. Natural killer cells inhibit pulmonary metastasis of hepatocellular carcinoma in nude mice. *Oncol Lett*. (2016) 11:2019–26.
93. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest*. (2012) 122:787–95. doi: 10.1172/JCI59643
94. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell*. (2010) 141:39–51. doi: 10.1016/j.cell.2010.03.014
95. Balkwill FR, Mantovani A. Cancer-related inflammation: common themes and therapeutic opportunities. *Semin Cancer Biol*. (2012) 22:33–40. doi: 10.1016/j.semcancer.2011.12.005
96. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev*. (2010) 29:309–16. doi: 10.1007/s10555-010-9223-6
97. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. (2012) 21:309–22. doi: 10.1016/j.ccr.2012.02.022
98. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature*. (2013) 496:445–55. doi: 10.1038/nature12034
99. Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cell Mol Immunol*. (2016) 13:316–27. doi: 10.1038/cmi.2015.104

100. Medina-Echeverz J, Eggert T, Han M, Greten TF. Hepatic myeloid-derived suppressor cells in cancer. *Cancer Immunol Immunother*. (2015) 64:931–40. doi: 10.1007/s00262-015-1736-y
101. Wan S, Kuo N, Kryczek I, Zou W, Welling TH. Myeloid cells in hepatocellular carcinoma. *Hepatology*. (2015) 62:1304–12. doi: 10.1002/hep.27867
102. Wu J, Li J, Salcedo R, Mivechi NF, Trinchieri G, Horuzsko A. The proinflammatory myeloid cell receptor TREM-1 controls Kupffer cell activation and development of hepatocellular carcinoma. *Cancer Res*. (2012) 72:3977–86. doi: 10.1158/0008-5472.CAN-12-0938
103. Eggert T, Wolter K, Ji J, Ma C, Yevsa T, Klotz S, et al. Distinct functions of senescence-associated immune responses in liver tumor surveillance and tumor progression. *Cancer Cell*. (2016) 30:533–47. doi: 10.1016/j.ccell.2016.09.003
104. Makarova-Rusher OV, Medina-Echeverz J, Duffy AG, Greten TF. The yin and yang of evasion and immune activation in HCC. *J Hepatol*. (2015) 62:1420–9. doi: 10.1016/j.jhep.2015.02.038
105. Hess KR, Varadhachary GR, Taylor SH, Wei W, Raber MN, Lenzi R, et al. Metastatic patterns in adenocarcinoma. *Cancer*. (2006) 106:1624–33.
106. Kmiec Z. Cooperation of liver cells in health and disease. *Adv Anat Embryol Cell Biol*. (2001) 161:1–151.
107. Smedsrød B, Le Couteur D, Ikejima K, Jaeschke H, Kawada N, Naito M, et al. Hepatic sinusoidal cells in health and disease: update from the 14th International Symposium. *Liver Int*. (2009) 29:490–501. doi: 10.1111/j.1478-3231.2009.01979.x
108. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol*. (2015) 17:816–26.
109. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. *Nature*. (2015) 527:329–35. doi: 10.1038/nature15756
110. Braet F, Nagatsuma K, Saito M, Soon L, Wisse E, Matsuura T. The hepatic sinusoidal endothelial lining and colorectal liver metastases. *World J Gastroenterol*. (2007) 13:821–5.
111. Kowanetz M, Wu X, Lee J, Tan M, Hagenbeek T, Qu X, et al. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. *Proc Natl Acad Sci USA*. (2010) 107:21248–55. doi: 10.1073/pnas.1015855107
112. Seubert B, Grünwald B, Kobuch J, Cui H, Schelter F, Schaten S, et al. Tissue inhibitor of metalloproteinases (TIMP)-1 creates a premetastatic niche in the liver through SDF-1/CXCR4-dependent neutrophil recruitment in mice. *Hepatology*. (2015) 61:238–48. doi: 10.1002/hep.27378
113. Zhang Y, Davis C, Ryan J, Janney C, Peña MM. Development and characterization of a reliable mouse model of colorectal cancer metastasis to the liver. *Clin Exp Metastasis*. (2013) 30:903–18. doi: 10.1007/s10585-013-9591-8
114. Brodt P. Role of the microenvironment in liver metastasis: from pre- to prometastatic niches. *Clin Cancer Res*. (2016) 22:5971–82.
115. Bertin S, Neves S, Gavelli A, Baqué P, Brossette N, Simões S, et al. Cellular and molecular events associated with the antitumor response induced by the cytosine deaminase/5-fluorocytosine suicide gene therapy system in a rat liver metastasis model. *Cancer Gene Ther*. (2007) 14:858–66.
116. Takehara T, Uemura A, Tatsumi T, Suzuki T, Kimura R, Shiotani A, et al. Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNalpha gene to mice. *Int J Cancer*. (2007) 120:1252–60.
117. Tatsumi T, Takehara T, Yamaguchi S, Sasakawa A, Miyagi T, Jinushi M, et al. Injection of IL-12 gene-transduced dendritic cells into mouse liver tumor lesions activates both innate and acquired immunity. *Gene Ther*. (2007) 14:863–71.
118. Paschos KA, Majeed AW, Bird NC. Natural history of hepatic metastases from colorectal cancer–pathobiological pathways with clinical significance. *World J Gastroenterol*. (2014) 20:3719–37. doi: 10.3748/wjg.v20.i14.3719
119. Coulouarn C, Clement B. Stellate cells and the development of liver cancer: therapeutic potential of targeting the stroma. *J Hepatol*. (2014) 60:1306–9. doi: 10.1016/j.jhep.2014.02.003
120. Taketomi A. Clinical trials of antiangiogenic therapy for hepatocellular carcinoma. *Int J Clin Oncol*. (2016) 21:213–8. doi: 10.1007/s10147-016-0966-0
121. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med*. (2008) 359:378–90. doi: 10.1056/NEJMoa0708857
122. Montella L, Palmieri G, Addeo R, Del Prete S. Hepatocellular carcinoma: will novel targeted drugs really impact the next future? *World J Gastroenterol*. (2016) 22:6114–26. doi: 10.3748/wjg.v22.i27.6114
123. Tahmasebi Birgani M, Carloni V. Tumor microenvironment, a paradigm in hepatocellular carcinoma progression and therapy. *Int J Mol Sci*. (2017) 18:405.
124. Zerbini A, Pilli M, Penna A, Pelosi G, Schianchi C, Molinari A, et al. Radiofrequency thermal ablation of hepatocellular carcinoma liver nodules can activate and enhance tumor-specific T-cell responses. *Cancer Res*. (2006) 66:1139–46.
125. Mizukoshi E, Yamashita T, Arai K, Sunagozaka H, Ueda T, Arihara F, et al. Enhancement of tumor-associated antigen-specific T cell responses by radiofrequency ablation of hepatocellular carcinoma. *Hepatology*. (2013) 57:1448–57. doi: 10.1002/hep.26153
126. Ayaru L, Pereira SP, Alisa A, Pathan AA, Williams R, Davidson B, et al. Unmasking of alpha-fetoprotein-specific CD4(+) T cell responses in hepatocellular carcinoma patients undergoing embolization. *J Immunol*. (2007) 178:1914–22.
127. Zeng Z, Shi F, Zhou L, Zhang MN, Chen Y, Chang XJ, et al. Upregulation of circulating PD-L1/PD-1 is associated with poor post-cryoablation prognosis in patients with HBV-related hepatocellular carcinoma. *PLoS One*. (2011) 6:e23621. doi: 10.1371/journal.pone.0023621
128. Shi L, Chen L, Wu C, Zhu Y, Xu B, Zheng X, et al. PD-1 blockade boosts radiofrequency ablation-elicited adaptive immune responses against tumor. *Clin Cancer Res*. (2016) 22:1173–84. doi: 10.1158/1078-0432.CCR-15-1352
129. Rai V, Abdo J, Alsuwaidan AN, Agrawal S, Sharma P, Agrawal DK. Cellular and molecular targets for the immunotherapy of hepatocellular carcinoma. *Mol Cell Biochem*. (2018) 437:13–36. doi: 10.1007/s11010-017-3092-z
130. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res*. (2011) 17:4550–7. doi: 10.1158/1078-0432.CCR-11-0116
131. Tran E, Turcotte S, Gros A, Robbins PF, Lu YC, Dudley ME, et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science*. (2014) 344:641–5. doi: 10.1126/science.1251102
132. Tran E, Robbins PF, Lu YC, Prickett TD, Gartner JJ, Jia L, et al. T-cell transfer therapy targeting mutant KRAS in cancer. *N Engl J Med*. (2016) 375:2255–62.
133. Stevanoviæ S, Pasetto A, Helman SR, Gartner JJ, Prickett TD, Howie B, et al. Landscape of immunogenic tumor antigens in successful immunotherapy of virally induced epithelial cancer. *Science*. (2017) 356:200–5. doi: 10.1126/science.aak9510
134. Zacharakis N, Chinnasamy H, Black M, Xu H, Lu YC, Zheng Z, et al. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. *Nat Med*. (2018) 24:724–30. doi: 10.1038/s41591-018-0040-8
135. Sangro B, Gomez-Martin C, de la Mata M, Iñarrairaegui M, Garralda E, Barrera P, et al. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. *J Hepatol*. (2013) 59:81–8. doi: 10.1016/j.jhep.2013.02.022
136. Kudo M. Immune checkpoint inhibition in hepatocellular carcinoma: basics and ongoing clinical trials. *Oncology*. (2017) 92(Suppl. 1):50–62. doi: 10.1159/000451016

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LOXL2 Inhibition Paves the Way for Macrophage-Mediated Collagen Degradation in Liver Fibrosis

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Liver fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM) proteins and enzymes, especially fibrillary collagens, and represents a major cause of morbidity and mortality worldwide. Lysyl oxidases (LOXs) drive covalent crosslinking of collagen fibers, thereby promoting stabilization and accumulation of liver fibrosis while limiting its resolution. Here we show in a carbon tetrachloride (CCl_4)-induced liver fibrosis murine model that treatment with a novel anti-lysyl oxidase like 2 (LOXL2) neutralizing antibody, which targets extracellular LOXL2, significantly improves fibrosis resolution. LOXL2 inhibition following the onset of fibrosis accelerated and augmented collagen degradation. This was accompanied by increased localization of reparative monocyte-derived macrophages (MoMFs) in the proximity of fibrotic fibers and their representation in the liver. These cells secreted collagenolytic matrix metalloproteinases (MMPs) and, in particular, the membrane-bound MT1-MMP (MMP-14) collagenase. Inducible and selective ablation of infiltrating MoMFs negated the increased "on-fiber" accumulation of MMP-14-expressing MoMFs and the accelerated collagenolytic activity observed in the anti-LOXL2-treated mice. Many studies of liver fibrosis focus on preventing the progression of the fibrotic process. In contrast, the therapeutic mechanism of LOXL2 inhibition presented herein aims at reversing existing fibrosis and facilitating endogenous liver regeneration by paving the way for collagenolytic macrophages.

Keywords: liver macrophages, lysyl oxidase like 2 (LOXL2), liver fibrosis, matrix metalloproteinases (MMPs), matrix metalloproteinase-14 (MMP-14), monocyte-derived macrophages

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INTRODUCTION

Liver fibrosis is a dynamic process characterized by increased deposition of extracellular matrix (ECM). It emanates from chronic liver injury of any etiology, including chronic viral infection, alcoholic liver disease (ALD), and non-alcoholic steatohepatitis (NASH), a progressive form of fatty liver disease. The liver is primed to respond quickly to injury by activating regenerative feed-forward mechanisms after eliminating the cause of injury. Yet, in the case of liver fibrosis, persistent injury triggers a chronic wound-healing response, leading to the replacement of parenchymal cells by ECM components. Progressive ECM accumulation gradually generates cirrhosis, characterized by disruption of the hepatic architecture and subsequent altered blood flow leading to portal hypertension. Decompensated liver fibrosis may cause further medical complications including ascites, hepatic encephalopathy, variceal hemorrhage, and an increased

individual risk of hepatocellular carcinoma (HCC) (1). While advanced liver fibrosis and cirrhosis have been considered static and irreversible stages, the current paradigm argues that these processes are dynamic and potentially reversible that can be modulated by halting their progression and/or by promoting their resolution (2).

Collagen crosslinking is a hallmark phenotype and an essential process in fibrotic matrix stabilization, contributing to fibrosis progression and limiting its reversibility (3). During fibrosis, activated hepatic stellate cells (HSCs) transdifferentiate into proliferative, contractile, fibrogenic myofibroblasts (4). Together with portal fibroblasts, HSCs secrete large amounts of ECM proteins, predominantly fibrillar type I and III collagens (4, 5), as well as enzymes that stabilize these ECM components via crosslinking (6). Enzymes belonging to the lysyl-oxidase (LOX) family are responsible for collagen as well as elastin crosslinking in pathological conditions like fibrosis (3, 7). Particularly in the liver, crosslinking and overexpression of tissue inhibitors of metalloproteinases (TIMPs) confer resistance to proteolytic degradation, thereby promoting excess ECM accumulation and stability. Among the five variants of the LOX family, lysyl oxidase-like 2 (LOXL2) has been identified as the primary enzyme promoting network formation of collagen and elastin fibers during human and experimental liver fibrosis of various etiologies (3, 6, 8–10) as well as HCC metastasis (11). Indeed, previous studies in rodent models have indicated that LOXL2 inhibition can ameliorate liver fibrosis (9, 12), thus highlighting its therapeutic potential.

Hepatic macrophages are a heterogeneous cell population of resident self-sustaining phagocytes termed Kupffer cells (KCs) and monocyte-derived macrophages (MoMFs) recruited from the circulation to the injured liver (13, 14). Macrophages were shown to play distinct and opposing roles during liver fibrosis, having been critically implicated in both pro-fibrogenic processes and scar-tissue degradation. On the one hand, they promote fibrosis by secreting pro-fibrotic mediators such as transforming growth factor beta (TGF-β) and platelet-derived growth factor (PDGF), and indeed the targeted deletion of liver-infiltrating Ly6C^{hi} monocytes or inhibition of their recruitment ameliorates hepatic inflammation and fibrosis (15–20). On the other hand, it has been shown in mouse models that macrophages can undergo a phenotypic switch during the disease process of liver fibrosis. If chronic injury ceases, local molecular signals trigger the transition of pro-fibrogenic Ly6C^{hi} monocytes into pro-restorative Ly6C^{lo} MoMFs. These cells facilitate the resolution of fibrosis by producing specific matrix metalloproteinases (MMPs) and other proteolytic enzymes like cathepsins and are capable of both degrading and clearing fibrotic ECM (18, 19, 21–24). Indeed, depletion of MoMFs during the resolution phase exacerbates fibrosis (16, 18) while their augmentation accelerates its resolution (22, 25). Accordingly, novel strategies to treat liver disease aimed at targeting macrophages were proposed (26). Yet, with the progression of liver fibrosis, macrophages fail to engage in reparative activities. It has been shown *in vitro* that crosslinking in collagenous scaffolds limits their degradation by macrophages (27). Therefore, LOXL2-driven collagen crosslinking during liver fibrosis may impede the collagenase activity of MoMFs and their reparative behavior.

Here, we used a novel anti-LOXL2 monoclonal antibody, GS341, targeting the catalytic site of extracellular LOXL2 enzymes within the tissue. Its administration following the induction of carbon tetrachloride (CCl_4)-induced liver fibrosis was sufficient to accelerate liver resolution by degrading scar tissue. We show that inhibition of LOXL2-mediated collagen crosslinking facilitates the arrival of MoMFs expressing a unique repertoire of collagenolytic MMPs to the proximity of collagen fibers.

MATERIALS AND METHODS

Animals

The following 8 to 12-week-old mouse strains were used: C57BL/6J wild-type male mice were purchased from Envigo Laboratories (Jerusalem, Israel); *Cx$_3$cr1$^{gfp/+}$* male mice (B6.129P- Cx3cr1tm1Litt/J) (28) were generously provided by Prof. Steffen Jung (Weizmann Institute of Science, Israel). All experiments and procedures were approved by the Weizmann Institute of Science Animal Care and Use Committee (IACUC approval no. 33070117-2).

Liver Injury

Hepatic fibrosis was induced by intraperitoneal (i.p.) injections of CCl_4 (Sigma-Aldrich, Rehovot, Israel) diluted in olive oil (Sigma-Aldrich, Rehovot, Israel) (0.9 CCl_4 μl/g), twice a week for 4 weeks (nine injections in total).

Therapeutic Anti-lysyl Oxidase Like 2 or Immunoglobulin G Control Antibody Treatment

In-house designed and generated antibodies (Abs) anti-LOXL2 (GS341) and anti-glutathione S-transferase [GST, immunoglobulin G (IgG) control antibody] were purified as previously described (29), both in the same conditions. Two weeks after CCl_4-induced fibrosis, mice were injected with GS341 or control Ab every other day at a concentration of 10 mg/kg body weight, so that the last injection was given 24 h after the last injection of CCl_4 (eight injections in total).

MC-21 Administration

Mice received an i.p. injection of 300 μl anti-mouse CCR2 mAb (clone MC-21)-conditioned media (29 μg Ab/ml) for four consecutive days before harvesting the liver tissues.

In situ Zymography

In situ zymography was conducted as previously described (30). Briefly, unfixed 10 μm frozen mouse liver sections were incubated with diluted DQ collagen type I (Invitrogen) (diluted 1/50 in developing buffer: 150 mM NaCl, 5 mM $CaCl_2$, 100 mM Tris-HCl pH 7.6, 20 μM ZnCl, 0.05% Brij 35) for 4 h at 37°C. Next, sections were fixed with 4% paraformaldehyde, then mounting solution (Immu-Mount^{TM} Thermo Scientific) was added, and slides were covered with a coverslip. The slides were imaged under a two-photon microscope (2PM:Zeiss LSM 510

META NLO) or a Nikon Eclipse 8O-I fluorescence microscope equipped with a Nikon digital camera (DXM1200F).

Two-Photon Microscopy, Second Harmonic Generation Imaging

Stained liver sections were imaged using a two-photon microscope in a second harmonic generation (SHG) mode: 1. 2PM:Zeiss LSM 510 META NLO, equipped with a broadband Mai Tai-HP-femtosecond single box tunable Ti-sapphire oscillator with automated broadband wavelength tuning 700–1,020 nm from Spectraphysics, for two-photon excitation. 2. Leica TCS SP8 MP in an upright configuration, equipped with a Chameleon Vision II femtosecond tunable laser (680–1,080 nm) (Coherent Inc., USA) and an Acusto Optical Tunable Filter (Leica Microsystems CMS GmbH, Germany). For second-harmonic imaging of collagen, a wavelength of 800–855 nm was used (detection at 390–450 nm).

Calculating Co-localization of Zymography and Collagen Signals

Images of collagen fibers and zymography signals were obtained using a two-photon 2PM:Zeiss LSM 510 META NLO microscope. Collagen fibers were detected by second-harmonic imaging with a wavelength of 800–855 nm and detection at 390–450 nm. The zymography signal was excited at 488 nm, and its emission was detected at 515 nm. Analysis of the images was done by measuring the intensity of the zymography signal overlapping with the main collagen fiber in the image. Analysis was done with ImageJ software.

Cell Line and Culture

The human dermal fibroblast (HDF) cell line was a gift from the laboratory of Stephen Weiss (University of Michigan, Ann Arbor, MI). HDF cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin, and 100 g/ml streptomycin (Biological Industries). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and the medium was exchanged every 2–3 days and passaged after reaching 80–90% confluence. For ECM synthesis, HDF cells were grown on glass coverslips in 24-well dishes until reaching contact inhibition. Then, the medium was replaced and supplemented with 5 ng/ml epidermal growth factor (EGF), 5 μg/ml insulin, and 100 μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate to induce ECM secretion, in the presence of phosphate buffered saline (PBS) or GS341 in PBS (100 ng/μl) for 14 days.

Immunoprecipitation

Magnetic protein G beads (Genescript) were incubated with GS341 according to the manufacturer's instructions. The GS341-coated beads were incubated with a fibrotic 48 h liver tissue lysate for 1 h at room temperature. Pellet beads were obtained by magnet separation rack and then were washed three times with PBS. The immunoprecipitation complex was eluted by adding 90 μl of elution buffer (Thermo-Scientific) directly to the beads followed by 5-min incubation. pH neutralization was performed by adding 10 μl of 1 M Tris-HCl pH 8.

Histopathological Fibrosis Scoring and Calculation of Collagen Coverage Area

Liver samples were fixed with 4% paraformaldehyde, paraffin embedded, sectioned, and stained with Sirius red. Sirius red-covered areas were analyzed and quantified by ImageJ software: for all images, color de-convolution of FastRed FastBlue 3,3′-diaminobenzidine (DAB) was applied. Then, for each red image, a suitable threshold was applied, and Analyze Particles plugin was used to detect the collagen-covered area. Blind grading of liver fibrosis severity was performed by a trained pathologist based on the Ishak histopathological scoring method (31). In brief, fibrosis was scored as 0 (no fibrosis), 1 (some portal tracts expanded), 2 (most portal tracts expanded), 3 (most portal tracts expanded, ± links), 4 (marked bridging, P-P and P-C links), 5 (marked bridging, occasional nodules, incomplete cirrhosis), and 6 (cirrhosis, probable or definite).

Quantitative Real-Time PCR

Liver tissues were homogenized using a bead beater homogenizer. Total RNA was isolated using the PerfectPure RNA Tissue Kit (5 Prime GmbH) according to the manufacturer's protocol. RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc.). qRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems inc.) on an ABI 7300 instrument (Applied Biosystems). Values were normalized to the *Tbp* or *Rplp0* housekeeping genes. Primer sequences are listed in **Table 1** below. Data are presented as mean fold change using the $2\text{-}\Delta^{CT}$ method (32). The standard error of the mean (SEM) was calculated on the $2^{-\Delta CT}$ data, as was the statistical analysis.

De-cellularization of Liver Tissues

Samples were incubated in a de-cell solution with 3% Triton-100 (6 h, 25°C) and then in a de-cell solution with 0.4% Triton-100 overnight at 4°C [de-cell solution: 1.5 M NaCl, 50 mM Tris pH 8, 50 mM EDTA, protease inhibitor cocktail (Roche)]. Samples were washed three times in ddH_2O and then incubated with 0.5% sodium deoxycholate (60 min, 25°C) to remove lipid remaining.

TABLE 1 | Primer sequences used for qRT-PCR.

Gene	Forward	Reverse
Mouse *tbp*	5′-GAAGCTGCGGTAC AATTCCAG-3′	5′-CCCCTTGTACCCT TCACCAAT-3′
Mouse *rplp0*	5′-TCCAGCAGGTGTT TGACAAC-3′	5′-CCATCTGCAGACA CACACT-3′
Mouse *acta2*	5′-GTCCCAGACATCAG GGAGTAA-3′	5′-TCGGATACTTCAGC GTCAGGA-3′
Mouse *col1a1*	5′- GCTCCTCTTAGG GGCCACT-3′	5′-CCACGTCTCACC ATTGGGG-3′
Mouse *timp1*	5′-CGAGACCACCTTA TACCAGCG-3′	5′-ATGACTGGGTGTA GGCGTA-3′

Samples were washed again three times in ddH_2O and stored at 4°C until use.

Scanning Electron Microscope

De-cellularized mouse liver tissues were fixed using fixative buffer (4% paraformaldehyde, 2% glutaraldehyde in 0.1 M cocodylate buffer with 5 mM $CaCl_2$ pH 7.4) overnight at 4°C. The fixed samples were incubated with 1% uranyl acetate for 30 min in the dark and then dehydrated through increasing concentrations of ethanol ranging from 30 to 100%. Samples were subsequently dried in a critical point dryer and gold sputtered for imaging by a scanning electron microsope (SEM) (Ultra 55 Feg; ZEISS). Fibrotic fiber thickness from high-resolution SEM images was measured. For each image, fibers were randomly chosen and measured using ImageJ software. As this is a continuous model of fibrosis, we assume new fibers are always being formed; therefore, fibers under thickness of 25 μm were omitted from total calculation.

Cell Isolation for Flow Cytometry and Mass Cytometry

Mice were sacrificed, and their livers were harvested after perfusion with PBS via the left ventricle. Livers were weighed, minced into small fragments, and incubated in shaking for 45 min at 37°C with 1 ml PBS (with Mg^{++} and Ca^{++}) containing 0.5 mg/ml collagenase type IV (Sigma-Aldrich, Rehovot, Israel) and 0.1 mg/ml DNase I (Roche). Digested tissue was filtered and mashed with a syringe plunger through a 250-μM nylon sieve in FACS buffer [PBS, 2% fetal calf serum (FCS), 2 mM ethylenediaminetetraacetic acid (EDTA)] to mechanically dissociate the remaining tissue. This was followed by three cycles of washing with FACS buffer at 30 g, each time taking only the supernatant, while omitting the non-leukocyte cell pellet. The supernatant cell pellet was then centrifuged at 390 g, and the pellet cells were lysed for erythrocytes using a red blood cell lysis buffer (Sigma-Aldrich, Rehovot, Israel) (2 min, 25°C).

Flow Cytometry

The following anti-mouse Abs were used: CD45 (clone 30-F11), CD11b (clone M1/70), Ly6C (clone HK1.4), Ly6G (clone 1A8), CX_3CR1 (clone SA011F11), CD64 (clone X54-5/7.1), MHCII (clone M4-114.15.2)—all purchased from BioLegend (San Diego, USA). Anti-mouse F4/80 (clone A3-1) was purchased from BIORAD. Anti MMP-14 was purified in-house from hybridoma cells of LEM-2/15 (33). The cells were incubated with Abs for 30 min in FACS buffer (dark, 4°C) and then washed once with FACS buffer. Cells were analyzed with BD FACSCanto™ II (BD Bioscience). Flow cytometry analysis was performed using FlowJo software (TreeStar, Ashland, OR, USA).

Mass Cytometry (cyTOF)

Subsequent to the cell isolation procedure, cells were stained according to a previously published protocol (34). Individual mice cell suspensions were stained with 0.125 μM Cell-ID Cisplatin for viability and fixed using Maxpar® Fix I Buffer. Samples were then permeabilized using Maxpar® Barcode Perm Buffer and then barcoded using the Cell-ID™ 20-Plex Pd Barcoding Kit, allowing us to join samples for antigen staining. Abs used for staining are listed in **Table 2** below. Before analyzing, the cell suspension was incubated with Cell-ID Intercalator Iridium for 20 min. Cells were analyzed with a cyTOF2® mass cytometer (Fluidigm). Results were normalized and debarcoded using fluidigm cyTOF software (35). Gating and further analysis of the CyTOF results were done with FlowJo software.

TABLE 2 | List of antibodies used in the mass cytometry analysis.

Name	Product	Provider	Isotope conjugation
Anti-human/mouse CD45R/B220	3160012	FLUIDIGM	160Gd–FLUIDIGM
Anti-mouse CD11c	3142003	FLUIDIGM	142Nd–FLUIDIGM
Anti-mouse TER-119	3154005	FLUIDIGM	154Sm–FLUIDIGM
Anti-mouse Ly-6C	3162014	FLUIDIGM	150Nd–FLUIDIGM
Anti-mouse Ly-6G	3141008B	FLUIDIGM	141Pr–FLUIDIGM
Anti-mouse CD64	3151012	FLUIDIGM	151Eu–FLUIDIGM
Anti- mouse F4/80 (BM8)	3159009	FLUIDIGM	159Tb–FLUIDIGM
Anti-mouse CD45	3089005	FLUIDIGM	89Y–FLUIDIGM
Anti-mouse CD3e (maxpar ready)	BLG-100345	Biolegend	153Eu–FLUIDIGM
Anti-mouse CX3CR1 (SA011F11)	3164023	FLUIDIGM	164Dy–FLUIDIGM
Anti-mouse I-A/I-E (M5/114.15.2)	3209006B	FLUIDIGM	209Bi–FLUIDIGM
Anti-mouse CD11b	3143015	FLUIDIGM	143Nd–FLUIDIGM
Anti-MMP-9	ab38898	Abcam	149Sm–Home made
Anti-MMP-14	ab51074	Abcam	156Gd–Home made

Immunofluorescence Staining

Frozen Section

Liver samples embedded in OCT were cross-sectioned (10 μm) on glass microscope slides. Sections were fixed with PBS 4% paraformaldehyde (PFA) for 20 min at 25°C. Samples were blocked in PBS, 20% normal horse serum, and 0.2% Triton x-100 (20 min, 25°C) and then incubated with a primary Ab in PBS, 2% normal horse serum, and 0.2% triton (overnight, 4°C). Samples were then washed three times in PBS and incubated with a secondary antibody (60 min, 25°C). Next, they were mounted in a mounting medium.

Paraffin-Embedded Sections

Liver samples were fixed with PBS 4% PFA, paraffin embedded and sectioned (4 μM). Slides were de-paraffinized using Wcap solution (Bio optica, Milano, Italy) (75°C, 20 min). Antigen retrieval was performed in 0.1 M EDTA, pH 8.0 (Diagnostic BioSystems, CA, USA) using a pressure cooker (125°C, 3 min), followed by washes with worm ddH_2O. Samples were blocked in PBS, 20% normal horse serum, and 0.2% Triton X-100 (20 min, 25°C) and then incubated with primary Ab in PBS, containing 2% normal horse serum and 0.2% Triton X-100 (60 min, 25°C). Next, samples were washed three times in PBS and incubated with a secondary antibody (60 min, 25°C) and mounted in a mounting medium. Primary Abs: anti-GFP antibody (ab6673, Abcam), anti MMP-14 (ab51074, Abcam), TIMP1 (ab86482, Abcam). For

MMP-14 staining, the samples were incubated with a biotin antibody (711-065-152, Jackson ImmunoResearch) following the incubation of the first Ab. Then, a cy3-streptavidin (016-160-084, Jackson ImmunoResearch) was used as a secondary Ab. TIMP1 staining was done following *in situ* zymography after the sections were fixed with 4% PFA.

Tissue Extraction and Western Blotting

Frozen liver tissues were washed in PBS, homogenized in RIPA lysis buffer (EMD Millipore, Burlington, MA, USA) with a protease inhibitor (Roche, Basel, Switzerland) using a hand homogenizer, and centrifuged (14,000 *g*, 15 min, 4°C). Supernatants were then resuspended in a sample buffer [200 mM Tris pH 6.8, 40% glycerol, 8% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), 0.2% bromophenol blue] and boiled for 5 min. Tissue extracts were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes by electro-blotting. Membranes were blocked in Tris-buffered saline with Tween 20 (TBST) buffer (200 mM Tris pH 7.5, 1.5 M NaCl, 0.5% Tween 20) and 2% bovine serum albumin (BSA, 60 min, 25°C) and then incubated with the corresponding primary Ab (60 min, 25°C), washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (60 min, 25°C). Quantification of the band intensities was performed using the ImageJ analysis tool. Abs used in this study: LOXL2 (ab179810, Abcam), LOX (ab ab174316, Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, sc-25778, Santa Cruz). Secondary Abs (both anti-rabbit and mouse) conjugated to HRP were purchased from Jackson ImmunoResearch (cat No.111-001-003 and 115-001-003, respectively). Abs were used at the manufacturer's recommended dilution.

Analysis of Macrophages/MMP-14 Distance From Collagen Fibers

Z-stack images of liver sections were obtained with a Leica TCS SP8 MP microscope. Collagen fibers were detected by second harmonic imaging with a wavelength of 855 nm and detection at 390–450 nm. The macrophage signal [green fluorescent protein (GFP)] was excited at 488 nm, and its emission was detected at 515 nm. The MMP-14 signal (cy3) was excited at 561 nm, and its emission was detected at 570–590 nm. Analysis of the images was done as follows: in Imaris software, surfaces were created for the main fiber in the image (omitting the surrounding short fibrils) and for the macrophages/MMP-14, both with a suitable threshold. A dilate surface of 15 μm and distance transformation map around the main fiber surface were created in order to analyze the distance of the macrophages/MMP-14 from the main fiber. Finally, the total macrophages/MMP-14 volume at a distance of ≤15 μm from fiber was normalized to the volume of the fiber 15 μm dilate surface.

Statistical Analysis

Data were analyzed by unpaired, two-tailed *t*-test to compare between two groups or by one-way ANOVA to compare several groups. After the null hypothesis was rejected ($p < 0.05$), Tukey's Honestly Significant Difference or Dunnett tests were used for follow-up pairwise comparison of groups in the one-way ANOVA. Data are presented as mean ± SEM; values of $p < 0.05$ were considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

RESULTS

Collagenolytic Activity of Matrix metalloproteinases During the Recovery Phase of Carbon Tetrachloride-Induced Liver Fibrosis

Understanding the naturally occurring collagenolytic events in the fibrotic liver necessitates a reproducible model of reversible hepatic fibrosis. Hence, we have utilized a spontaneously reversible murine model of liver fibrosis based on repeated challenges with CCl_4. C57BL/6 mice received nine i.p. injections of CCl_4 over the course of 4 weeks. Liver tissue was then harvested 24, 48, 72, and 120 h after the last injection (**Figure 1A**). Comparison was made with age-matched uninjured livers from mice injected with vehicle (oil) only. Analysis of Sirius red staining, which stains for fibrillary collagen, revealed fibrosis peaks ∼24 h after the last injection, manifested by bridging fibrosis (collagen fibers connecting at least two portal triads) and septa formation (**Figures 1B,C**). Early regeneration took place after 72 h and at 120 h, when a substantial reduction in fibrosis (area covered by collagen) had already occurred (**Figures 1B,C**). Several groups have shown that a myriad of ECM remodeling enzymes are elevated during liver fibrosis, including ECM-degrading enzymes such as MMPs. However, their contribution to the pathogenesis of liver fibrosis or its resolution remains controversial (36, 37). We therefore investigated the localization and kinetics of collagenolytic MMPs following the cessation of CCl_4 administration by testing their *in situ* activity. To this end, non-fixed liver samples were treated with a fluorogenic collagen substrate that emits a fluorescent signal upon its *in situ* digestion by endogenous MMPs. At the peak of fibrosis (24 and 48 h), collagenolytic enzyme activity was demonstrated only at the area surrounding the collagen fibers. However, at 72–120 h, the collagenolytic activity of MMPs was associated with the collagen fibrils as depicted by the co-localization of the zymographic signal with the second harmonic generation (SHG) signal (**Figures 1B,D**). These results highlight that access of natural collagen-degrading enzymes to the fibrotic scar commences at 72–120 h following termination of CCl_4 treatment.

GS341 Relaxes Collagen Packaging *in vitro* and Specifically Targets Lysyl Oxidase Like 2 in the Fibrotic Liver

Targeting specific stages of collagen assembly during liver fibrosis presents a great challenge due to the involvement of various crosslinking enzymes in the multistep, hierarchical process of collagenous matrix buildup. LOXL2 plays a critical role in collagen crosslinking during liver fibrosis development, with its inhibition linked with reduced liver fibrosis (9, 12). Here, we used a novel anti-LOXL2 monoclonal antibody (mAb) developed in our lab, GS341, which targets the catalytic site of the enzyme (29). Grossman et al. (29) have previously shown

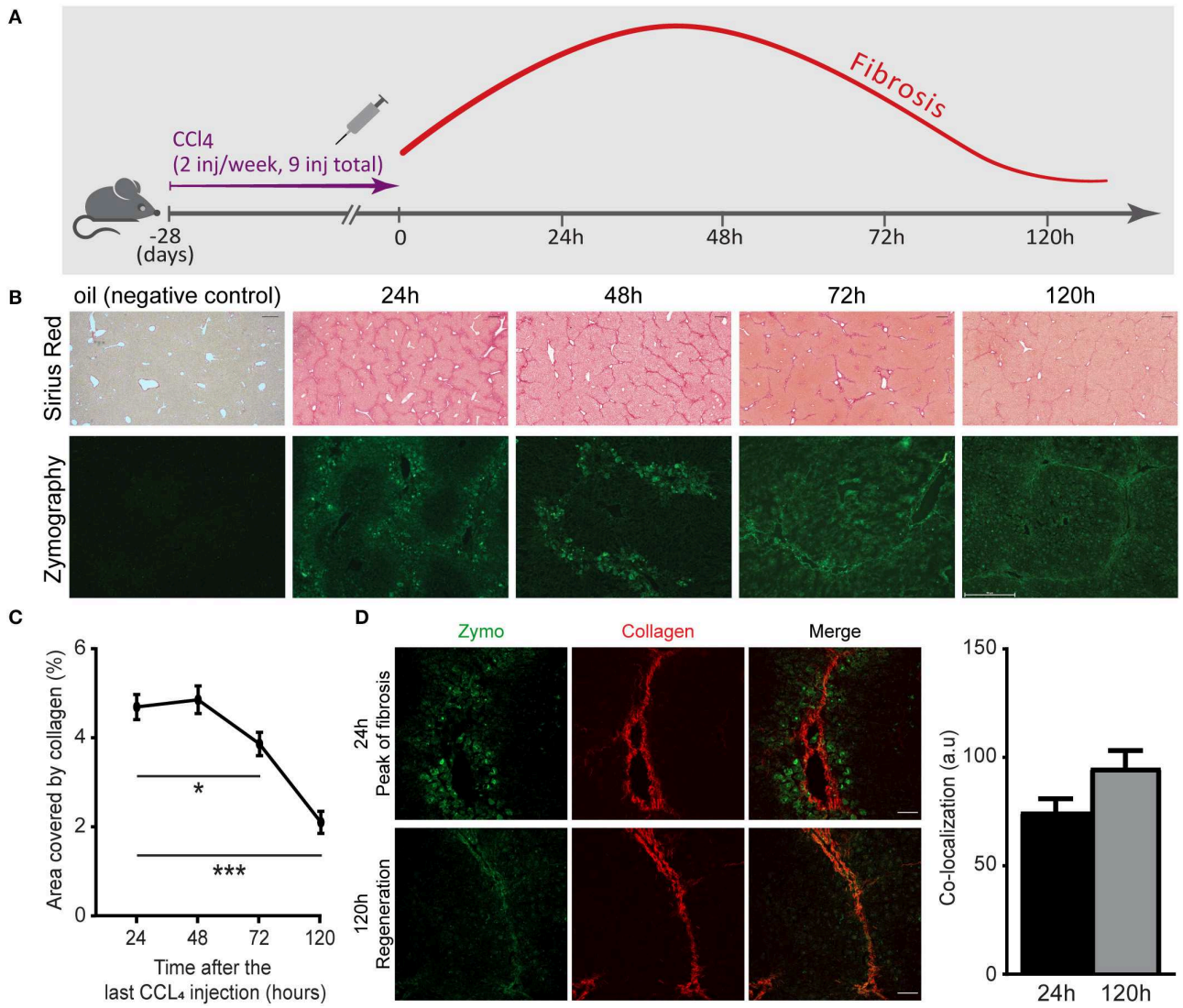

FIGURE 1 | Matrix metalloproteinase (MMP)-governed collagenolytic activity naturally appears at the resolution phase of carbon tetrachloride (CCl_4)-induced fibrosis. **(A)** Schematic representation of the liver fibrosis model. inj, injection. **(B)** Representative images of Sirius red stain (top) and *in situ* zymography (bottom) of samples collected from mice sacrificed at different time points after the last (ninth) injection of CCl_4 or oil control (Sirius red scale−250 μm; zymography scale−50 μm). **(C)** Sirius red quantification by calculating the fraction of collagen-covered area. Analysis was done using ImageJ software ($n = 4$). **(D)** Representative images of livers excised from CCl_4-injected mice at the peak of fibrosis (24 h) and recovery phase (120 h). Zymography signal (green) and collagen signal by second harmonic generation (SHG, red) were obtained using two-photon microscopy (scale−50 μm) ($n \geq 2$). The co-localization of the zymography and collagen signals were quantified by measuring the intensity of the zymography signal that overlapped with the main collagen fiber in the image. Data were analyzed by one-way ANOVA with Dunnett *post hoc* comparison. Results are presented as mean ± SEM with significance: $^{*}p < 0.05$, $^{***}p < 0.001$. Data in **(A–D)** represent a single experiment.

that GS341 changes the natural alignment and diameter of collagen fibers *in vitro* as well as *in vivo* in a breast cancer model. In agreement with these previous results (29), we found that LOXL2 inhibition by GS341 in a native fibroblast-derived three-dimensional (3D) matrix *in vitro* system alters the natural alignment of fibrillary collagen (**Figure 2A**). In addition, ECM scaffolds treated with GS341 were significantly thinner than the control (**Figure 2B**). Next, in order to verify the specificity of the GS341 mAb within the fibrotic liver, immunoprecipitation (IP) was conducted (**Figure 2C**). GS341 specifically precipitated LOXL2 from the lysate and not LOX (**Figure 2C**). Altogether, these results display the specificity of GS341 toward LOXL2 in the fibrotic liver and its ability to interfere with collagen alignment and packaging.

Inhibition of Lysyl Oxidase Like 2-Mediated Collagenous Matrix Remodeling Ameliorates Hepatic Fibrosis

We further investigated the ability of GS341 to ameliorate existing progressive fibrosis. Accordingly, mice on the CCl_4 regimen started after 2 weeks to receive either GS341 or an IgG

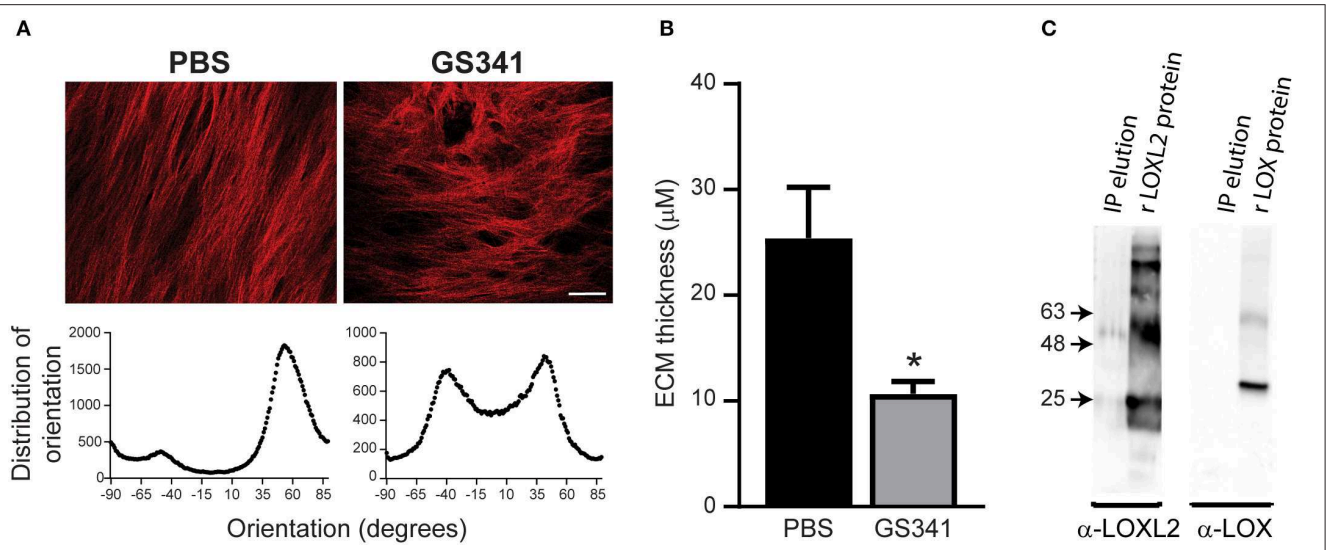

FIGURE 2 | GS341 antibody targets specifically lysyl oxidase like 2 (LOXL2) and affects collagen morphology. **(A)** Upper panel: Representative second harmonic generation (SHG) images of an *in vitro* native fibroblast-derived three-dimensional (3D) matrix treated with GS341 or phosphate buffered saline (PBS) (scale−50 μm). Lower panel: Representative fiber directionality analysis plots depicting the frequency of fibers in a specific orientation. Analysis was done using ImageJ software. **(B)** The thickness of the *in vitro* native fibroblast-derived 3D matrix treated with GS341 or PBS. **(C)** Immunoprecipitation (IP) of fibrotic liver lysate with GS341. Beads conjugated with GS341 were incubated with a fibrotic liver lysate, and the eluted fraction was tested in Western blot against commercial anti-LOX and LOXL2 antibodies (lane 1 in each membrane). LOX and LOXL2 recombinant proteins (lane 2) served as positive controls. IP, immunoprecipitation; r, recombinant. Data were analyzed via an unpaired, two-tailed *t*-test. Results are presented as mean ± SEM with significance: $^*p < 0.05$. Data in **(A–C)** represent one single experiment.

control mAb every other day (eight injections in total), with the last treatment given 24 h after the last CCl_4 injection (**Figure 3A**). Analyses were performed at 48 h after the last CCl_4 injection. This time point is at the peak of the inflammatory/fibrotic phase, while resolution and "on fiber" collagen degradation activity has not yet begun (**Figures 1B–D**). Sirius red staining revealed a significant reduction in the collagen-stained area in mice following GS341 compared to control Ab treatment (**Figures 3B,C**; **Figure S1A**). Blind grading of liver fibrosis severity performed by a trained pathologist based on the Ishak histopathological scoring method (31) showed that mice treated with GS341 exhibited a significantly improved fibrotic score mainly due to reduced bridging scar tissue between portal tracts (**Figure 3D**). We have repeated this experiment, this time looking at 24 h following the last CCL_4 injection, to examine the cumulative effect of GS341 on collagen accumulation. Sirius red staining revealed a significant reduction in collagen-stained area in the GS341 group (**Figures S1B,C**). Interestingly, qRT-PCR of liver tissue 24 h post treatment revealed no difference between the GS341-treated and control groups in the gene expression of key fibrotic elements, including collagen type-I (*Col1a1*), alpha-SMA (*Acta2*), and tissue inhibitor of metalloproteinases 1 (*Timp1*) (**Figure 3E**), suggesting that LOXL2 inhibition in this model and under this treatment regimen does not directly affect the fibrotic activity of HSCs. Following up on the observation that LOXL2 inhibition affected collagen morphology *in vitro* (**Figures 2A,B**), we assessed its effect on collagen arrangement and assembly *in vivo*. High-resolution SEM analysis of decellularized 3D ECM scaffolds extracted from GS341- and control Ab-treated livers 48 h after the last CCl_4 injection uncovered major changes in the morphology and arrangement of collagen fibers (**Figure 3F**). While collagen fibers in the control livers displayed a tightly packed structure of linear fibrils, those in the GS341-treated livers assumed a looser, disoriented structure with gaps between the fibrils (**Figure 3F**). A significant reduction in average collagen fibril diameter was observed in control (37 nm) vs. GS341 (33 nm)-treated groups (**Figure 3G**). Interestingly, reduced levels of both LOXL2 and LOX were observed in the GS341-treated livers, indicating that inhibition of extracellular LOXL2 downregulates both collagen cross-linkers (**Figure 3H**). Overall, these results demonstrate that inhibition of LOXL2 using our novel active-site neutralizing mAb reduces the amount of fibrotic collagenous matrix in liver fibrosis and modifies its arrangement and assembly.

Lysyl Oxidase Like 2 Inhibition Turns Collagen Fibers More Accessible to Macrophages

The ameliorating effects of LOXL2 inhibition on the collagen coverage area, fibrotic score (**Figures 3B–D**), and morphology (**Figures 2A,B**, **3F–G**) prompted us to further explore whether attenuation of LOXL2-governed crosslinking renders collagen fibers more accessible and amenable to degradation. Innate immune cells, especially macrophages, play distinct roles during liver fibrosis but bear the potential to resolve it upon termination of liver injury (16, 18, 19, 21–24). We hypothesized that LOXL2-governed crosslinking makes collagenous matrix less accessible to macrophages and their collagenolytic MMPs. Therefore, we examined the localization of macrophages in livers of $Cx_3cr1^{gfp/+}$ transgenic reporter mice (28) subjected to CCl_4-induced liver

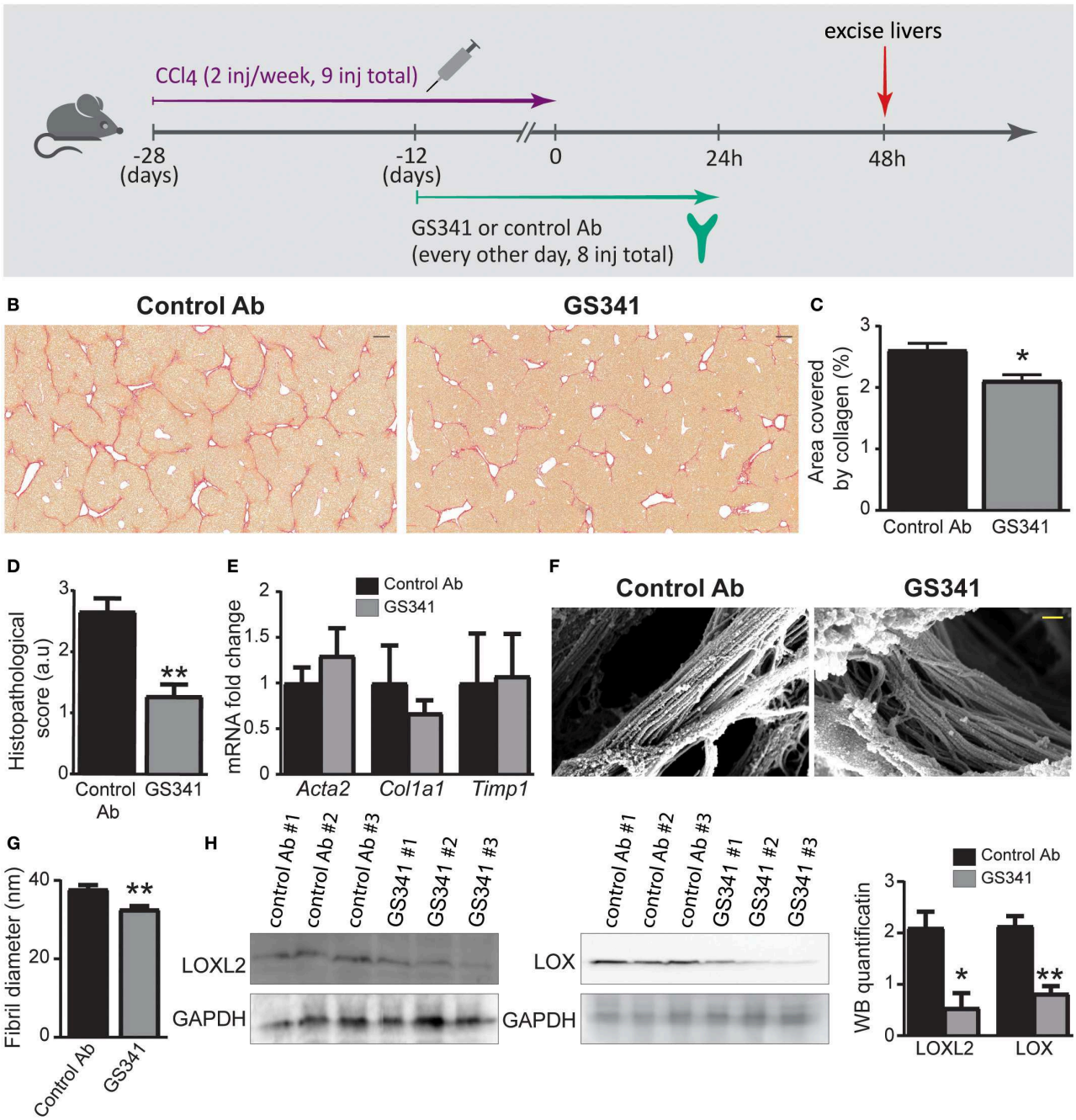

FIGURE 3 | Inhibition by GS341 demonstrates improved recovery in chronic carbon tetrachloride (CCl_4)-induced liver fibrosis and morphological change to fibrotic fibers. **(A)** Schematic representation of the experimental setting. inj, injection. **(B)** Representative images of Sirius red staining performed on paraffin-embedded slides of livers from GS341-treated and control Ab-treated mice. Livers were excised at 48 h after the last (ninth) injection of CCl_4 (scale−200 μm). **(C)** Quantification of the percentage area covered by collagen, performed by ImageJ. **(D)** Graph showing histopathological scoring in GS341-treated mice compared to control Ab-treated mice (**C,D**–$n \geq 6$). **(E)** qRT-PCR analysis of fibrosis marker genes at 24 h after CCl_4-induced liver fibrosis ($n = 6$). **(F)** Representative SEM images of de-cellularized three-dimensional (3D) extracellular matrix (ECM) liver scaffolds excised 48 h after the last CCl_4 injection (scale−200 nm). **(G)** Quantification of fiber thickness in the scanning electron microscope (SEM) images was performed using ImageJ software. Comparison of fibril diameters in control vs. GS341-treated samples shows significant reduction of the fibril diameters of the latter. Statistics are obtained from the four biologic replicates for each case, 12 images per treatment ($n = 4$). **(H)** Western blotting (WB) with commercial anti-lysyl oxidase like 2 (LOXL2) and anti-LOX antibodies (Abs) of liver samples treated with control Ab or GS341, excised 48 h after the last CCl_4 injection. LOXL2/LOX expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Quantification of the WB was done using ImageJ software. Data were analyzed by unpaired, two-tailed *t*-test. Results are presented as mean ± SEM with significance: $^{*}p < 0.05$, $^{**}p < 0.01$. Data in **(A–D)** represent three independent experiments. Data in **(E–G)** represent two independent experiments. Data in **(H)** represent one single experiment.

fibrosis and treated with GS341 or a control Ab. In the liver of these mice, GFP expression can be used to trace infiltrating Ly6C^{hi} monocytes and their MoMF decedents, but not resident KCs (14, 38). Two-photon microscopy imaging was further utilized for the mutual detection of CX_3CR1-GFP expression (mainly MoMFs and some Ly6C^{hi} monocytes

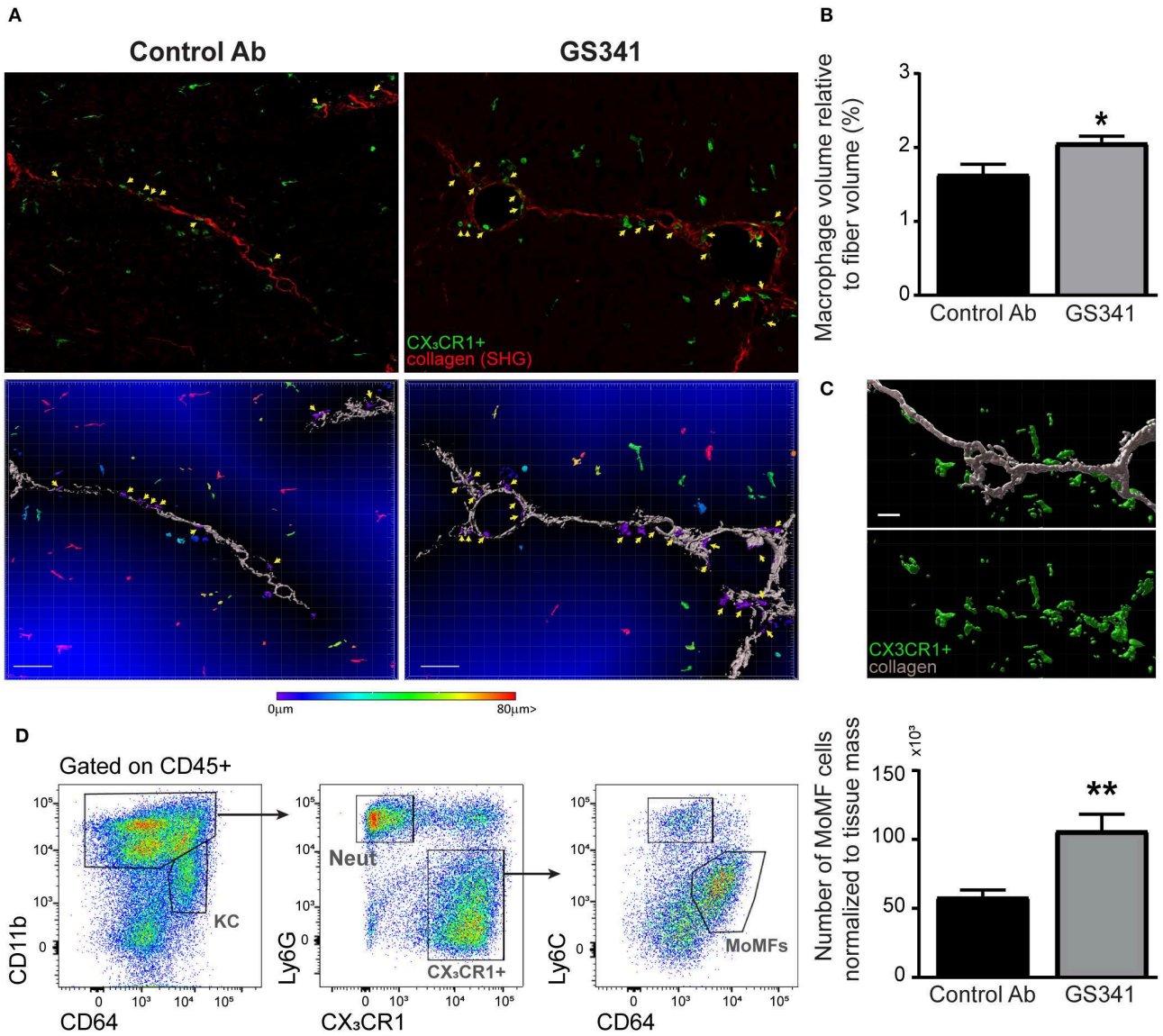

FIGURE 4 | Inhibition of lysyl oxidase like 2 (LOXL2)-governed extracellular matrix (ECM) crosslinking paves the way for macrophages to the fibrotic fibers. *$CX_3CR1^{gfp/+}$* transgenic reporter mice subjected to carbon tetrachloride (CCl_4)-induced liver fibrosis and, after 2 weeks, to treatment with GS341 or a control antibody (Ab). Livers were excised 48 h following the last CCl_4 injection, stained with anti-green fluorescent protein (GFP) antibody, and then two-photon microscopy imaging was used for the mutual detection of CX_3CR1-GFP expression and the second harmonic generation (SHG) signal at the portal tract fibrotic area. **(A)** Upper panel: representative images of the distribution of CX_3CR1-GFP$^+$ monocyte-derived macrophages (MoMFs) (green) near collagen fibers (red) in GS341- and control Ab-treated mice; yellow arrows indicate CX_3CR1-GFP$^+$ MoMFs adjacent to the fibers; scale−50 μm. Lower panel**:** representative images of Imaris analysis for quantification of the distance of infiltrating CX_3CR1-GFP$^+$ MoMFs from the fiber. CX_3CR1-GFP$^+$ MoMFs are color coded according to their distance from the fiber; yellow arrows indicate CX_3CR1-GFP$^+$ macrophages adjacent to the fibers; scale−50 μm. **(B)** Quantification of the CX_3CR1-GFP$^+$ signal's volume within 15 μm from the fiber normalized to the total collagen fiber volume; scale−50 μm ($n \geq 5$). **(C)** Imaris images showing that macrophages adjacent to the fiber are wrapping the fiber and embracing its shape; scale−10 μm. **(D)** Representative flow cytometry images showing the gating strategy used to identify MoMFs (CD45$^+$CD11b$^+$Ly6G$^-$$CX_3CR1^+$Ly6C^{lo}F4/80$^+$CD64$^+$ MHCII$^+$) and a summarizing graph showing their frequency normalized to tissue mass (g) at 48 h following CCl_4-induced liver fibrosis. KC, kupffer cells; Neut, neutrophils; MoMFs, monocyte-derived macrophages. Data were analyzed by an unpaired, two-tailed *t*-test. Results are presented as mean ± SEM with significance: $^*p < 0.05$, $^{**}p < 0.01$. Data in **(A,B)** represent a single experiment, and data in **(D)** represent three independent experiments.

at 48 h) and SHG signaling (collagen fibers) at the portal tract fibrotic area. Forty-eight hours following the last CCl_4 injection, there was a significant increase in the representation of CX_3CR1-GFP$^+$ Ly6C^{hi} monocytes or their MoMF progenies in the vicinity of fibrotic collagen fibers in GS341-treated mice (**Figure 4A**). This was further calculated by the fraction of CX_3CR1-GFP$^+$ MoMF cell volume within a radius of 15 μm from the collagen fiber out of the total collagen fiber volume (**Figure 4B**). Interestingly, the macrophages adjacent to the fiber wrapped the fiber and embraced its shape (**Figure 4C**). Moreover, GS341-treated mice also exhibited increased representation of MoMFs in their livers (**Figure 4D**), suggesting that in the absence of LOXL2 activity, the liver is more attractive or passable for Ly6C^{hi} monocytes.

Ly6C^{hi} Monocytes and Their Monocyte-Derived Macrophage Descendants Express a Unique Repertoire of Collagenolytic Matrix-Degrading Enzymes

Intact fibrillar collagen can only be cleaved by a subset of MMPs (i.e., MMP-1, MMP-8, MMP-13, and MMP-14) (37) and by other proteases, such as cathepsin K (39). Given that inhibition of LOXL2-governed buildup of collagenous matrix during liver fibrosis facilitates the arrival of CX_3CR1^+ Ly6C^{hi} monocytes and their MoMF progenies in the vicinity of fibrous collagen fibers (**Figure 4**), we investigated whether this macrophage subset expresses MMPs that can potentially degrade collagen fibers. To assess the expression of collagenolytic MMPs in these cells, we revisited available gene expression databases. In a model of reversible CCL_4-induced liver fibrosis similar to the one used here, gene expression profiling has been done on liver infiltrating CX_3CR1^+ Ly6C^{hi} monocytes at the necro-inflammatory phase (24 h) and their MoMF progenies at the early resolution phase (72 h) (18). In the original paper, the expression of some collagenolytic MMPs was noted in MoMFs. Here, we revisited this database to further elaborate the full repertoire of collagenolytic matrix enzymes expressed by these cells. We found that Ly6C^{hi} monocytes upregulate the expression of MMP-13 (*Mmp13*) and downregulate that of MMP-8 (*Mmp8*) upon differentiation into pro-restorative MoMFs (**Figure 5A**). Both expressed pronounced levels of the membrane-type 1 (MT1-MMP) (*Mmp14*) (**Figure 5A**). Remarkably, MT1-MMP (MMP-14) is known to efficiently degrade fibrillary collagens *in vivo*. Similar results were obtained in a model of acetaminophen-induced liver injury (AILI) which included, in addition to Ly6C^{hi} monocytes and MoMFs, the gene expression profiling of resident KCs (14). Accordingly, gene expression profiling of liver infiltrating CX_3CR1^+ Ly6C^{hi} monocytes during the necro-inflammatory phase (24 h) and of resident CX_3CR1^- KCs and CX_3CR1^+ MoMFs during the resolution phase (72 h) revealed that Ly6C^{hi} monocytes had higher levels of MMP-8 but lower levels of MMP-13 and similar levels of MMP-14. KCs expressed lower levels of all these MMPs (**Figure 5B**). Cathepsin K gene expression (*Ctsk*) was upregulated upon the differentiation of Ly6C^{hi} monocytes into MoMFs at the resolution phase of both CCl_4-indcued fibrosis (**Figure 5A**) and AILI (**Figure 5B**). Elastin is another ECM protein linked to liver fibrosis progression, and macrophage-derived MMP-12 was shown to mediate its degradation during experimental liver fibrosis (23). We found that Ly6C^{hi} monocytes profoundly upregulate MMP-12 gene expression (*Mmp12*) subsequent to their conversion into the reparative MoMFs at the resolution phase of both CCl_4-induced fibrosis (**Figure 5A**) and AILI (**Figure 5B**). The expression of membrane-bound MMP-14 by Ly6C^{hi} monocytes and MoMFs was especially intriguing, as most studies emphasize the importance of secreted MMPs like MMP-8,−9,−12, and−13 in reducing liver fibrosis (18, 19, 21–23). The enhanced co-localization of CX_3CR1^+ MoMFs with fibrotic collagen fibers as a consequence of GS341 treatment (**Figure 4**) suggests that MMP-14 under these conditions may obtain accessibility to degrade the scar tissue. Hence, we examined the expression pattern of MMP-14 in the fibrotic liver by performing mass cytometry (cyTOF) analysis at the peak of CCl_4-induced fibrosis (48 h). Out of the total MMP-14$^+$ cells in non-parenchymal enriched liver cells, ∼90% were CX_3CR1^+ macrophages. In contrast, gating on MMP-9, another enzyme with collagenolytic properties, revealed that its expression mostly originates from neutrophils (∼70%) but to some degree also from CX_3CR1^+ macrophages (20%) (**Figure 5C**). Flow cytometry analysis further confirmed the expression of MMP-14 mainly in MoMFs (defined as CD11b$^+$Ly6G$^-$$CX_3CR1^+$Ly6C^{lo} CD64$^+$), while other innate immune cell populations present in the portal triad fibrotic area, such as Ly6C^{hi} monocytes and neutrophils, were mostly negative (**Figure 5D**). Interestingly, MoMFs from GS341-treated livers express higher levels of MMP-14 than those isolated from control Ab-treated livers (**Figure 5E**). Immunostaining of *Cx$_3$cr1$^{gfp/+}$* liver sections further demonstrated increased accumulation of CX_3CR1-GFP$^+$ MoMF cells co-expressing MMP-14 in GS341-treated livers (**Figure 5F**). Recently, single-cell transcriptomic analysis of human liver in healthy and cirrhotic states uncovered the existence of scar-associated macrophages (SAMs), which were defined as analogous to MoMFs in mouse liver injury models. These cells, marked by the expression of TREM2 and CD9, expanded in the fibrotic niche and displayed a pro-fibrogenic phenotype (40). Data mining into their available gene expression dataset revealed the expression of MMP-14 among various parenchymal and non-parenchymal lineages mainly by mononuclear macrophages (MPs) and endothelial and epithelial cells. Among the MP populations, the main subpopulation expressing MMP-14 was SAMs (**Figure S2**). Collectively, these results outline that CX_3CR1^+ MoMFs express a myriad of secreted and membrane-bound collagen- and elastin-degrading enzymes.

Ly6C^{hi} Monocytes and Their Monocyte-Derived Macrophage Descendants Drive Collagen Degradation in GS341-Treated Fibrotic Livers

Given that Ly6C^{hi} monocytes and MoMFs express distinct collagenolytic MMPs (**Figure 5**), we conducted a spatially scar-resolving activity-based assay for collagen degradation by performing *in situ* zymography using fresh liver tissue sections. Using this technique, we monitored the localization

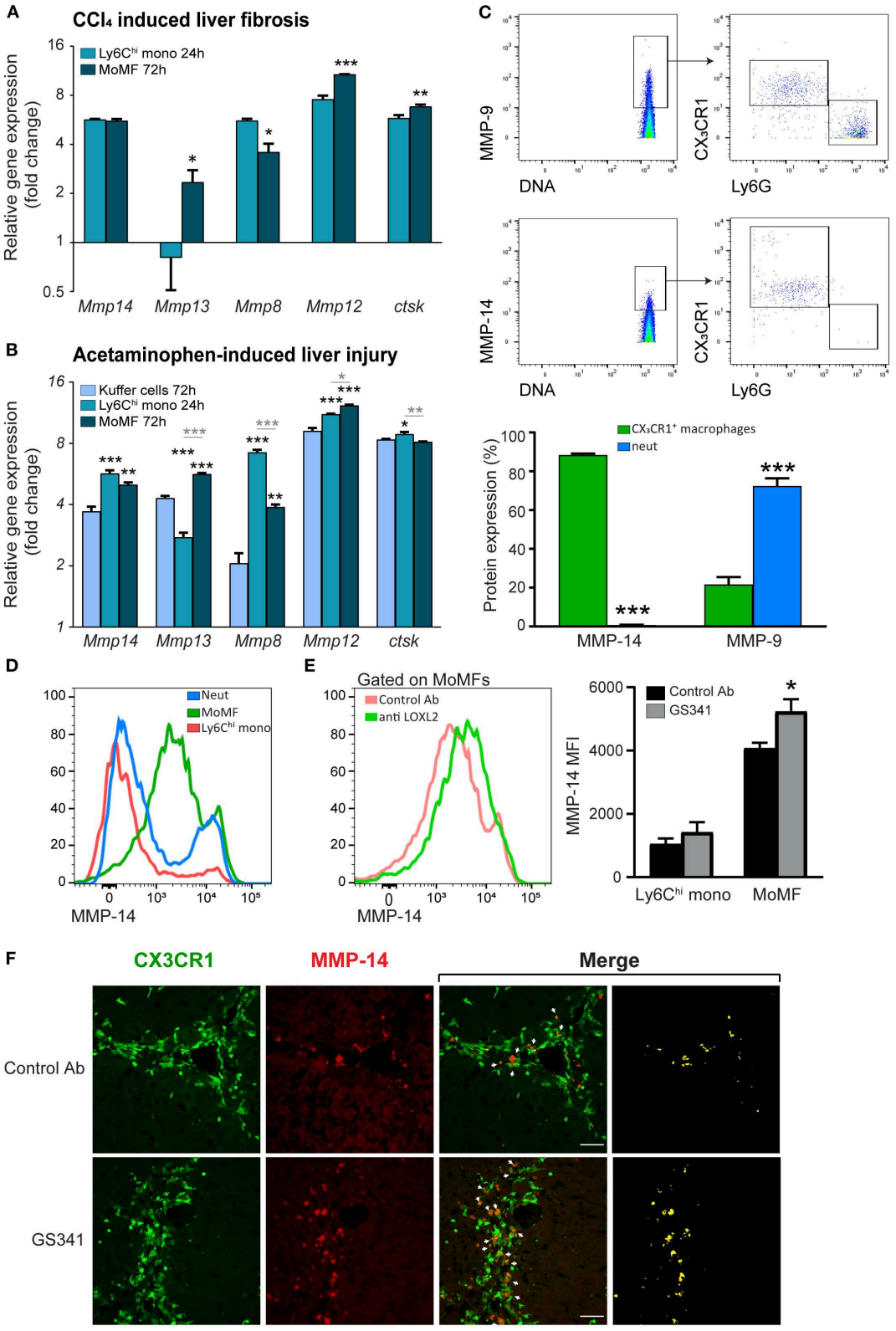

FIGURE 5 | Fibrotic macrophages express different collagenolytic matrix metalloproteinases (MMPs) in injured liver. **(A)** *Mmp8*, *Mmp12*, *Mmp13*, *Mmp14*, and *Cstk* gene expression in sorted Ly6C^{hi} monocytes and monocyte-derived macrophages (MoMFs) 24 and 72 h following carbon tetrachloride (CCl_4)-induced fibrosis, respectively, as extracted from the ArrayExpress database (www.ebi.ac.uk/arrayexpress, accession no. E-MEXP-3177). Their expression was normalized

(Continued)

FIGURE 5 | to the expression of the adipocyte marker *Adipoq*, which served as a background expression level ($n = 3$). **(B)** *Mmp8*, *Mmp12*, *Mmp13*, *Mmp14*, and *Cstk* gene expression in sorted Ly6C^{hi} monocytes 24 h following acetaminophen-induced liver injury (AILI) and in sorted Kupffer cells (KCs) and MoMFs 72 h post AILI. Data were extracted from our existing database (GSE55606). Their expression was normalized to the expression of the adipocyte marker *Adipoq*, which served as a background expression level. Black asterisks indicate a significant difference from KCs at 72 h. Gray asterisks indicate a significant difference between Ly6C^{hi} monocytes at 24 h and MoMFs at 72 h. **(C)** cyTOF analysis of livers following CCl_4-induced liver injury. Mice were injected three times with CCl_4 without any treatment, after which livers were excised 48 h following the last CCl_4 injection. Presented are plots depicting the gating strategy and analysis of neutrophils and macrophages percentage from total MMP-14$^+$ or MMP-9$^+$ cells ($n = 3$). **(D)** Representative flow cytometry images showing the expression of MMP-14 in liver infiltrating neutrophils (CD11b$^+$Ly6G$^+$CX$_3$CR1$^-$CD64$^-$), Ly6C^{hi} monocytes (CD11b$^+$Ly6G$^-$ CX$_3$CR1$^+$Ly6C^{hi}CD64^{lo}), and MoMFs (CD11b$^+$Ly6G$^-$CX$_3$CR1$^+$Ly6C^{lo}CD64$^+$) 48 h following CCl_4-induced liver fibrosis. **(E)** Mean fluorescent intensity (MFI) expression of MMP-14 in MoMFs as depicted by flow cytometry analysis. **(F)** Representative images displaying the localization of CX3CR1-GFP$^+$ MoMFs (green) and MMP-14 (red) in GS341- and control Ab-treated mice. White arrows indicate co-localization of CX3CR1-GFP$^+$ and MMP-14 signals. Mono, monocyte; Neut, neutrophils; MoMFs, monocyte-derived macrophages. Data were analyzed using an unpaired, two-tailed *t*-test **(A,C,E)** or one-way ANOVA with Tukey's Honestly Significant Difference *post hoc* comparison **(B)**. Results are presented as mean ± SEM with significance: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Data in **(A–F)** represent a single experiment.

of fibrillary collagen-degrading enzymes in liver sections of mice treated with the fragment antigen binding (Fab) moiety of GS341 or with control (PBS$^{-/-}$) at the peak of the fibrotic phase (48 h). In the control group, collagenase activity (zymographic signal) exhibited minor co-localization with collagen fibers and was mostly distributed around them (**Figure 6A**). In sharp contrast, in the GS341-Fab-treated livers, collagenase activity co-localized with the collagen fibers (**Figure 6A**), resembling the collagenolytic activity displayed at the resolution phase of CCL_4-induced liver fibrosis (120 h) (**Figure 1D**). Moreover, immunostaining for the endogenous MMP inhibitor TIMP1, a marker of liver fibrosis, together with zymographic analysis of collagenase activity demonstrated that GS341 treatment promotes the disengagement of collagenase activity from TIMP1, while moving toward the fibrotic scar area (**Figure 6B**). We could not detect any difference in the expression of TIMP1, TIMP2, or MMP-2 in whole liver lysates treated with GS341 or control Ab at 48 h (**Figure S3**). To further dissect the contribution of Ly6C^{hi} monocyte-derived MoMFs to this accelerated "on fiber" collagenase activity, we took advantage of the anti-CCR2 MC-21 antibody, which selectively depletes blood-circulating Ly6C$^+$CCR2$^+$ monocytes and thus prevents their infiltration to the injured liver (14, 38). Accordingly, in some of the GS341-treated mice, MC-21 was administered following the last CCl_4 injection. Indeed, the inducible ablation of CX$_3$CR1$^+$ Ly6C^{hi} monocytes and their MoMF descendants eliminated the accelerated and augmented collagenolytic activity adjacent to, or on fibrotic fibers observed following LOXL2 inhibition (**Figure 6C**). Importantly, simultaneous imaging of both MMP-14 and collagenous matrix revealed higher expression of MMP-14 around the fibrillary collagen in the GS341-treated liver compared to the control, and these MMP-14-expressing cells were cleared by MC-21, confirming their Ly6C^{hi} monocyte ontogeny (**Figure 6D**). Further calculation of the vicinity of MMP-14 and the collagen fiber (up to a radius of 15 μm) revealed its higher proximity in the GS341-treated group, which was eliminated following MC-21-induced ablation of MoMFs (**Figure 6E**). Altogether, these results suggest that CX$_3$CR1$^+$ MoMFs gain greater accessibility to fibrotic fibers by virtue of LOXL2 inhibition, where they can use their unique repertoire of secreted and membrane-bound MMPs to degrade the fibrotic fibers.

DISCUSSION

Liver fibrosis-associated morbidity and mortality are progressively increasing worldwide, with no successful anti-fibrotic treatment available to date. Several reports implicate LOXL2 in ECM buildup and stabilization during liver fibrosis through crosslinking of structural proteins such as type I and III collagens and elastin (3, 6, 8–10). The development and accumulation of crosslinked fibers stabilize the fibrotic scar produced during the process of liver injury, rendering it more stable and therefore more resistant to degradation by matrix-degrading enzymes. Liver macrophages are very plastic and display opposing functions during liver fibrosis. A great deal of research has been invested in trying to prevent their pro-fibrotic behavior, while other therapeutic approaches have focused on elucidating switches that can promote and augment their reparative activity (26). Yet, with the progression of liver fibrosis, macrophages fail to resolve the formed scar tissue. The results presented here generate a direct link between LOXL2-governed ECM crosslinking and the impaired accessibility and scar-degrading activity of Ly6C^{hi} monocyte-derived MoMFs in the context of liver fibrosis. In this respect, therapeutic inhibition of extracellular LOXL2 activity in livers with preestablished fibrosis interferes with collagen packaging, rendering it more accessible to infiltrating Ly6C^{hi} monocyte-derived MoMFs, which uniquely bring to the fibrotic scar a set of membrane-bound and secreted collagen- and elastin-degrading enzymes. We especially outline here the provision by these cells of MMP-14 collagenase, which as being membrane tethered, depends upon the proximity and accessibility to collagen fibers.

Studies trying to understand the biochemical changes affecting fibrosis irreversibility have pinpointed LOXL2 as a key fibrogenic enzyme in liver fibrosis of various etiologies (3, 6, 8–10). In one study, the benefit of concurrent treatment with an allosteric anti-LOXL2 mAb (AB0023) in a reversible model of CCL_4-induced liver fibrosis was assessed (9). AB0023 improved mouse survival and significantly reduced portoportal and portocentral bridging fibrosis, as well as the representation of α-SMA$^+$ myofibroblasts in the portoportal septa (9). Yet, it remained unclear whether delayed LOXL2 neutralization would be as effective in the settings of preestablished biliary and non-biliary fibrosis. In this respect, in a follow-up study (12), delayed AB0023 treatment during progression (7–12 weeks) of thioacetamide

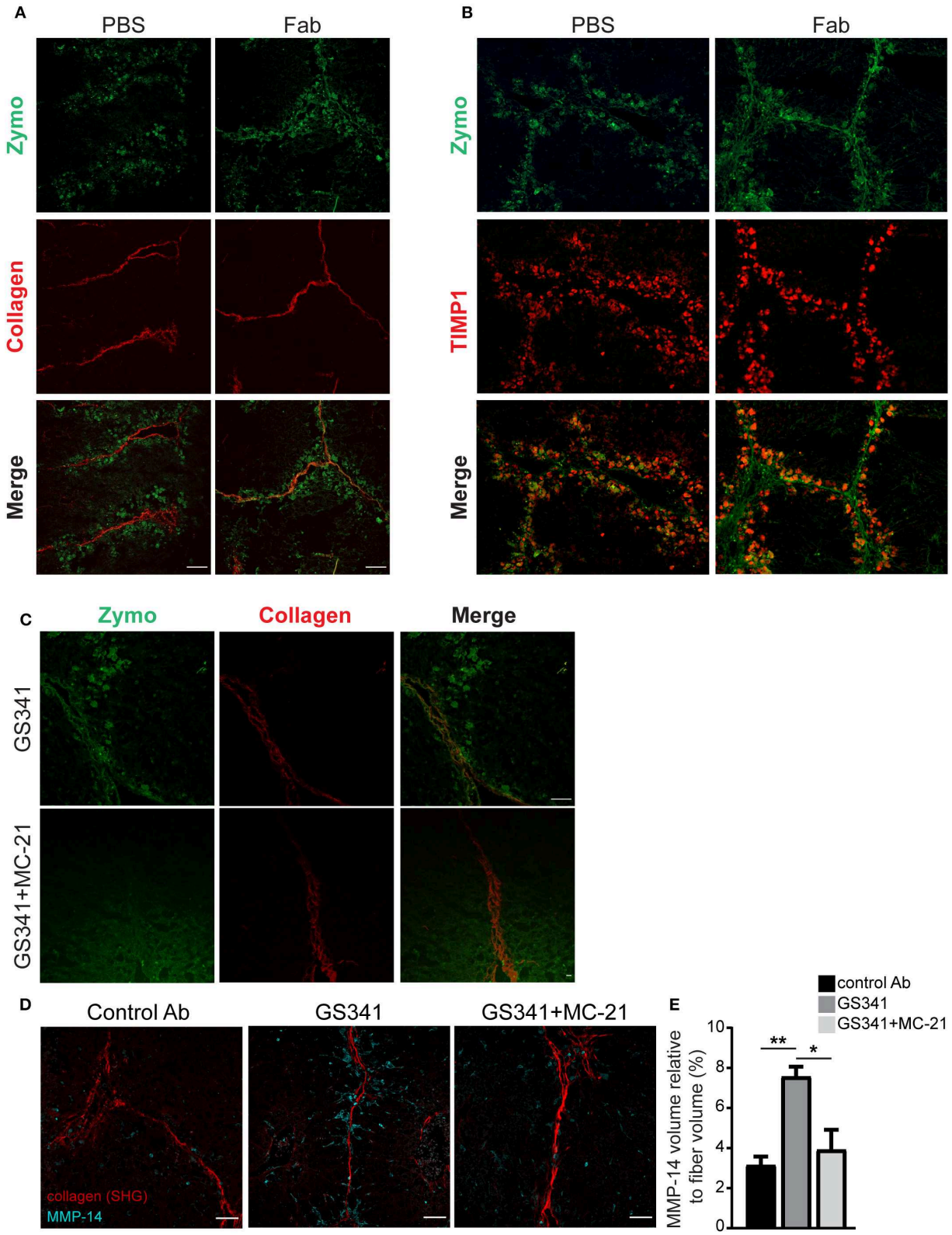

FIGURE 6 | Ly6C^{hi} monocytes and monocyte-derived macrophages (MoMFs) facilitate collagen degradation following lysyl oxidase like 2 (LOXL2) inhibition. **(A)** Representative images of *in situ* zymography of fragment antigen binding (Fab)-treated and control livers 48 h after the last carbon tetrachloride (CCl_4) injection.

(Continued)

FIGURE 6 | Spatial localization differences are demonstrated in collagen type I-degrading enzymes in Fab-treated livers compared to control [$n = 5$, green–zymography; red–second harmonic generation (SHG); scale−50 μm]. **(B)** Representative images of *in situ* zymography and tissue inhibitor of metalloproteinase (TIMP)1 staining of Fab-treated and control livers 48 h after the last CCl_4 injection. **(C)** Representative images of *in situ* zymography of GS341– and GS341+ MC-21-treated mice 48 h after the last CCl_4 injection (green–zymography; red–SHG; scale−50 μm). **(D)** Representative SHG images of 48 h liver samples treated with control antibody (Ab)/GS341/GS341+MC-21 Abs stained with MMP-14 antibody (scale−50 μm). **(E)** Quantification of the MMP-14 signal volume within 15 μm from the fiber, normalized to the total collagen fiber volume ($n = 3$). Data were analyzed by one-way ANOVA with Dunnett *post hoc* comparison. Results are presented as mean ± SEM with significance: $^{*}p < 0.05$, $^{**}p < 0.01$. Data in **(A–E)** represent a single experiment.

(TAA)-induced fibrosis significantly reduced histological signs of bridging fibrosis. AB0023 treatment also promoted fibrosis reversal, with enhanced splitting and thinning of fibrotic septa, and a 45% decrease in collagen area 4 weeks after recovery from established TAA fibrosis (12). Furthermore, in two mouse models of biliary fibrosis, AB0023 similarly achieved significant anti-fibrotic efficacy and suppressed the ductular reaction, while hepatocyte replication increased (12).

Although these studies clearly associate between LOXL2 inhibition and reduction in hepatic collagen levels, mechanistic comprehension of this anti-fibrotic effect remains largely elusive. On one hand, LOXL2 may perpetuate fibrotic responses via HSC activation. Supporting this is the finding that exposure of both HSCs and portal fibroblasts in culture to increased mechanical tension on artificial polyacrylamide promotes their transition to myofibroblasts (41). Moreover, increased liver stiffness as a result of LOX-governed collagen crosslinking precedes fibrosis and potentially drives the HSC-myofibroblast transition (42). An additional mechanism was offered in an elegant study by the Popov group showing that blockage of LOXL2 by AB0023 can directly promote the differentiation of $EpCAM^{+}$ hepatic progenitor cells (HPCs) toward regenerative hepatocytes, rather than fibrogenic ductal cell lineage commitment (12). LOXL2 expression by HPCs further suggests possible intracellular effects for this enzyme in determining the differentiation fates of these cells. Moreover, conditioned medium from HSC cultures promoted the growth of $K19^{+}$ cholangiocytes from HPC cells and was likewise inhibited by AB0023. Therefore, the authors suggested that in the setting of fibrosis, autocrine/paracrine LOXL2 may favor HPC differentiation toward fibrogenic cholangiocytes. We present here an additional disease-modifying scheme. We show that LOXL2-governed collagen crosslinking limits scar degradation by liver-infiltrating, scar-destroying MoMFs. Previously, our lab demonstrated a role for LOXL2 in mediating morphological changes to collagen fibers *in vitro* and *in vivo* in a breast cancer model (29). Fibers grown in the presence of our novel active site anti-LOXL2 mAb (GS341) grow thinner and lose their directionality. Here we show for the first time, using high-resolution imaging, that inhibition of LOXL2 under conditions of progressive liver fibrosis changes the spatial organization of the fibrotic fibers. The resultant loosening of collagen assembly paves the way for the arrival of CX_3CR1^{+} $Ly6C^{hi}$ monocyte-derived MoMFs, which can now degrade the unraveled collagen and facilitate liver regeneration.

A previous study performed in a model of TAA-induced experimental liver fibrosis has explored the specific contribution of LOX and LOXL2 to fibrotic matrix stabilization utilizing specific blocking mAbs (M64 and AB0023, respectively) (12). Of these, only LOXL2 inhibition reduced the levels of insoluble collagen, a marker for collagen crosslinking. Here, we have used advanced high-resolution SEM imaging to uncover the effects of LOXL2 inhibition on collagen assembly *in situ* in a different model of liver fibrosis. Our results indicate reduced collagen packaging following treatment with GS341, suggesting indeed that LOXL2 is a key mediator of collagen remodeling during experimental liver fibrosis.

The increased matrix rigidity, which presumably accompanies LOXL2-mediated collagen crosslinking, may have direct implications on the migration and behavior of macrophages. Macrophages express mechanosensors such as integrins that can sense the ECM in their vicinity and transmit force from the extracellular environment via their interaction with numerous cytoskeletal and signaling proteins (43). Moreover, collagen and its digestion products can act as chemotactic stimuli for monocytes and macrophages (44, 45). Macrophages also adapt their migration mode to the matrix architecture and the biophysical parameters of the collagenous matrix (46). For example, fibrillar type I collagen favors an amoeboid migration mode, while denser 3D collagen I matrices promote a mesenchymal migration mode of human monocyte-derived macrophages (47). Nevertheless, the effects of the fibrotic matrix in general, and LOXL2-mediated modification of collagenous matrix in particular, on liver $Ly6C^{hi}$ monocytes and MoMF migration and intra-hepatic motility have so far been overlooked. We show here that inhibition of LOXL2 activity during progressive liver fibrosis leads to increased accumulation of MoMFs within the liver parenchyma, particularly in the vicinity (up to 15 μ) of fibrotic collagen fibrils. Some of these macrophages actually adopt the shape of the collagen fiber while wrapping it. MoMFs may be actively attracted to the liver due to cues emanating from the exposure of buried epitopes as a result of the morphological changes in collagen fibers inflicted by LOXL2 inhibition, which favors collagen degradation by utilizing a membrane-bound effective protease. Alternatively, the reduced stiffness of the tissue may allow their easier entry into the liver. These options warrant further in-depth elucidation. In addition, a dense collagenous matrix may also dictate the behavior of MoMFs. Indeed, monocytes and macrophages grown on a collagen-rich matrix exhibit increased proliferation and acquire an alternatively activated M2 polarization state (48–50). Our results demonstrate accelerated collagen-degrading activity by MoMFs following LOXL2 inhibition and specifically increased expression of MMP-14. Further studies are required to determine whether MoMFs can directly sense collagenous matrix and to characterize the molecular reprogramming they are subjected to in the absence vs. presence of LOXL2-governed collagen

remodeling. Overall, these results suggest that extracellular LOXL2-governed collagen remodeling impedes the arrival of these reparative macrophages to the sites of fibrosis, providing a possible mechanistic explanation for the failure of these cells to resolve scar tissue in settings of advanced fibrosis.

Gene expression analyses supported by mass and flow cytometry data and immunostaining revealed here that Ly6C^{hi} monocytes acquire a unique repertoire of scar-degrading enzymes upon differentiating into reparative MoMFs. These include secreted MMPs, such as MMP-12 and−13. These results are in agreement with previous studies (18, 19, 21–24). Yet, we also show that CX_3CR1^+ MoMFs uniquely express the membrane-bound MMP-14 gene and protein and that its expression by MoMFs is increased by LOXL2 inhibition. This inhibition leads to increased proximity of MoMFs to fibrous collagen fibers, which may provide access for membrane-based MMP-14 to facilitate focal collagen proteolysis. In human cirrhotic livers, MMP-14 expression is profoundly elevated in comparison with normal liver tissue. It is also increased during experimental CCL_4-induced fibrosis, and its expression persists at the resolution phase (51). Yet, the involvement of MMP-14 in the pathogenesis and resolution of liver fibrosis remains so far ambiguous. Studies in a model of liver ischemia–reperfusion injury have indicated a role for MMP-14 in facilitating macrophage infiltration into the injured liver via interactions with fibronectin (52). Therefore, there may be a connection between the increased representation of MoMFs in GS341-treated livers and their higher expression of MMP-14. MMP-14 may also participate in the activation of MMP-13 (53), which is expressed by MoMFs as well.

Emerging evidence indicates that various cells composing the fibrotic niche can produce matrix cross-linkers belonging to the LOX family. Originally, HSCs and portal fibroblasts were noted as the major sources of LOXL1, LOXL2, and LOXL3. LOXL4 is widely expressed, while LOX in the normal liver appeared to be expressed primarily by hepatocytes and portal fibroblasts (6). As mentioned before, HPC cells can also produce LOXL2 and react to it in an autocrine and paracrine manner (12). Using immunoprecipitation assays, we show that GS341 specifically binds LOXL2. Yet, GS341 treatment reduces the protein expression levels of both LOXL2 and LOX collagen cross-linkers. Further studies are required to explain how inhibition of LOXL2 activity downregulates the production of LOX. In a recent study, single cell RNASeq transcriptomic profiling in human cirrhotic patients has actually detected the expression of both LOX and LOXL2 in the $CD34^+PLVAP^+VWA1^+$ endothelial cell (EC) population, which expands in cirrhotic liver tissue and was found to be restricted to the fibrotic niche (40). The fact that these cells also express the fractalkine chemokine CX_3CL1 (40) argues for their possible interaction with CX_3CR1^+ MoMFs. Using the gene expression dataset in this study, we show here that the expression of MMP-14 in human cirrhotic livers is associated with SAMs among MPs but is also expressed by ECs and epithelial cells, all constituents of the fibrotic niche. These results highlight that better comprehension of the LOXL2 and MMP-14 producing cells within the fibrotic niche is important for elucidating their pathophysiological crosstalk in the cirrhotic liver. For example, one may argue that in the context of progressive fibrosis, LOXL2 production by ECs and/or myo-fibroblasts within the fibrotic niche turns the collagen scar less accessible for degradation by MMP-14 expressed on SAMs.

It is worth noting that the proteolytic activity of MMPs can be regulated at multiple levels, including transcription, conversion from zymogen to active enzyme, compartmentalization and restraining by endogenous inhibitors such as TIMPs. Therefore, when judging the pathophysiological relevance of MMP expression in MoMFs, their collagenase activity has to be verified. In this respect, by using a spatially resolving activity-based assay for collagen degradation, we identified that collagen type I-degrading enzymes, such as MMPs, are already produced and active during the course of fibrosis progression and at the fibrosis peak (24–48 h). Nevertheless, their activity was not localized to the collagen fibers but rather to cells around the fibrotic areas and portal triads. Only during the regeneration phase (72–120 h), the zymographic activity of collagen type I degradation co-localized with the fibrotic collagen fibers, i.e., the bridging fibrotic fibers. Importantly, we show that delayed LOXL2 inhibition during the course of CCL_4-induced fibrosis is sufficient to accelerate the appearance of this regeneration-like phenotype of co-localization already at the peak of fibrosis (48 h), concomitantly with uncoupling from the endogenous MMP inhibitor TIMP1. This "on fiber" transition of collagenase activity is diminished by the inducible ablation of Ly6C^{hi} monocytes and their MoMF descendants. Therefore, these results highlight that MoMFs are the primary source for collagen-degrading enzymes following LOXL2 inhibition.

As mentioned above, preclinical studies with the allosteric anti-LOXL2 AB0023 mAb have proved its efficacy in ameliorating biliary and non-biliary fibrosis (9, 12). Unfortunately, a humanized IgG4 monoclonal antibody against LOXL2, Simtuzumab® (Gilead Sciences SA), has failed so far to achieve a significant clinical benefit in patients with idiopathic pulmonary fibrosis (54), NASH (55, 56), or primary sclerosing cholangitis (57). Various factors may explain this failure to translate the overall positive preclinical results to the clinic: first, rodent models exhibit higher reversibility of liver fibrosis than do humans, especially with respect to patients suffering from cirrhosis and portal hypertension. Second, other compensatory pathways can drive collagen crosslinking, including other LOX isoforms and tissue transglutaminases. Third, there may be genetic and epigenetic changes in human patients that affect fibrosis progression and regression. We demonstrate here the use of a novel anti-LOXL2 mAb that directly targets the catalytic site of this enzyme, but its translational potency remains elusive. Moreover, we provide a new mechanistic view of how LOXL2 inhibition encourages the arrival of reparative MoMFs. Therefore, it will be extremely important in the future to delineate the molecular programs that favor macrophage restorative vs. pathological activity in liver fibrosis. In addition, immunotherapies that aim to enhance MoMF levels and/or reparative activity are already being clinically pursued (58, 59) and could have a synergistic effect with the anti-LOXL2 mAb presented here on the recovery process from liver fibrosis.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by The Weizmann Institute of Science animal care and use committee protocol# 33070117-2.

AUTHOR CONTRIBUTIONS

MK, TG, NA, CV, and ISa designed, performed, and analyzed all experiments and wrote the manuscript. MV substantially assisted MK and TG with some of the major *in vivo* experiments and gene expression analyses. EB is a liver pathologist, who has performed histopathological assessment. SH-L and ISo helped with image and data analyses.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00480/full#supplementary-material

REFERENCES

1. Trautwein C, Friedman SL, Schuppan D, Pinzani M. Hepatic fibrosis: concept to treatment. *J Hepatol.* (2015) 62:S15–24. doi: 10.1016/j.jhep.2015.02.039
2. Weiskirchen R, Tacke F. Liver fibrosis: from pathogenesis to novel therapies. *Dig Dis.* (2016) 34:410–22. doi: 10.1159/000444556
3. Liu SB, Ikenaga N, Peng ZW, Sverdlov DY, Greenstein A, Smith V, et al. Lysyl oxidase activity contributes to collagen stabilization during liver fibrosis progression and limits spontaneous fibrosis reversal in mice. *FASEB J.* (2016) 30:1599–609. doi: 10.1096/fj.14-268425
4. Tsuchida T, Friedman SL. Mechanisms of hepatic stellate cell activation. *Nat Rev Gastroenterol Hepatol.* (2017) 14:397–411. doi: 10.1038/nrgastro.2017.38
5. Schuppan D, Ruehl M, Somasundaram R, Hahn EG. Matrix as a modulator of hepatic fibrogenesis. *Semin Liver Dis.* (2001) 21:351–72. doi: 10.1055/s-2001-17556
6. Perepelyuk M, Terajima M, Wang AY, Georges PC, Janmey PA, Yamauchi M, et al. Hepatic stellate cells and portal fibroblasts are the major cellular sources of collagens and lysyl oxidases in normal liver and early after injury. *Am J Physiol Gastrointest Liver Physiol.* (2013) 304:G605–14. doi: 10.1152/ajpgi.00222.2012
7. Kagan HM, Li W. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem.* (2003) 88:660–72. doi: 10.1002/jcb.10413
8. Dongiovanni P, Meroni M, Baselli GA, Bassani GA, Rametta R, Pietrelli A, et al. Insulin resistance promotes lysyl oxidase like 2 induction and fibrosis accumulation in non-alcoholic fatty liver disease. *Clin Sci.* (2017) 131:1301–15. doi: 10.1042/CS20170175
9. Barry-Hamilton V, Spangler R, Marshall D, McCauley S, Rodriguez HM, Oyasu M, et al. Allosteric inhibition of lysyl oxidase-like-2 impedes the development of a pathologic microenvironment. *Nat Med.* (2010) 16:1009–17. doi: 10.1038/nm.2208
10. Vadasz Z, Kessler O, Akiri G, Gengrinovitch S, Kagan HM, Baruch Y, et al. Abnormal deposition of collagen around hepatocytes in Wilson's disease is associated with hepatocyte specific expression of lysyl oxidase and lysyl oxidase like protein-2. *J Hepatol.* (2005) 43:499–507. doi: 10.1016/j.jhep.2005.02.052
11. Wong CC, Tse AP, Huang YP, Zhu YT, Chiu DK, Lai RK, et al. Lysyl oxidase-like 2 is critical to tumor microenvironment and metastatic niche formation in hepatocellular carcinoma. *Hepatology.* (2014) 60:1645–58. doi: 10.1002/hep.27320
12. Ikenaga N, Z.-Peng W, Vaid KA, Liu SB, Yoshida S, Sverdlov DY, et al. Selective targeting of lysyl oxidase-like 2 (LOXL2) suppresses hepatic fibrosis progression and accelerates its reversal. *Gut.* (2017) 66:1697–708. doi: 10.1136/gutjnl-2016-312473
13. Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol.* (2017) 17:306–21. doi: 10.1038/nri.2017.11
14. Zigmond E, Samia-Grinberg S, Pasmanik-Chor M, Brazowski E, Shibolet O, Halpern Z, et al. Infiltrating monocyte-derived macrophages and resident kupffer cells display different ontogeny and functions in acute liver injury. *J Immunol.* (2014) 193:344–53. doi: 10.4049/jimmunol.1400574
15. Krenkel O, Puengel T, Govaere O, Abdallah AT, Mossanen JC, Kohlhepp M, et al. Therapeutic inhibition of inflammatory monocyte recruitment reduces steatohepatitis and liver fibrosis. *Hepatology.* (2017) 67:1270–83. doi: 10.1002/hep.29544
16. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Investig.* (2005) 56:115. doi: 10.1172/JCI200522675

17. Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, et al. Hepatic recruitment of the inflammatory Gr1^{+} monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology.* (2009) 50:261–74. doi: 10.1002/hep.22950
18. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci USA.* (2012) 109:E3186–95. doi: 10.1073/pnas.1119964109
19. Mitchell C, Couton D, Couty JP, Anson M, Crain AM, Bizet V, et al. Dual role of CCR2 in the constitution and the resolution of liver fibrosis in mice. *Am J Pathol.* (2009) 174:1766–75. doi: 10.2353/ajpath.2009.080632
20. Pradere JP, Kluwe J, De Minicis S, Jiao JJ, Gwak GY, Dapito DH, et al. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology.* (2013) 58:1461–73. doi: 10.1002/hep.26429
21. Fallowfield JA, Mizuno M, Kendall TJ, Constandinou CM, Benyon RC, Duffield JS, et al. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol.* (2007) 178:5288–95. doi: 10.4049/jimmunol.178.8.5288
22. Popov Y, Sverdlov DY, Bhaskar KR, Sharma AK, Millonig G, Patsenker E, et al. Macrophage-mediated phagocytosis of apoptotic cholangiocytes contributes to reversal of experimental biliary fibrosis. *J Physiol Gastrointest Liver Physiol.* (2010) 298:323–34. doi: 10.1152/ajpgi.00394.2009
23. Pellicoro A, Aucott RL, Ramachandran P, Robson AJ, Fallowfield JA, Snowdon VK, et al. Elastin accumulation is regulated at the level of degradation by macrophage metalloelastase (MMP-12) during experimental liver fibrosis. *Hepatology.* (2012) 55:1965–75. doi: 10.1002/hep.25567
24. Thomas JA, Pope C, Wojtacha D, Robson AJ, Gordon-Walker TT, Hartland S, et al. Macrophage therapy for murine liver fibrosis recruits host effector cells improving fibrosis, regeneration, and function. *Hepatology.* (2011) 53:2003–15. doi: 10.1002/hep.24315
25. Ma PF, Gao CC, Yi J, Zhao JL, Liang SQ, Zhao Y, et al. Cytotherapy with M1-polarized macrophages ameliorates liver fibrosis by modulating immune microenvironment in mice. *J Hepatol.* (2017) 67:770–9. doi: 10.1016/j.jhep.2017.05.022
26. Tacke F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol.* (2017) 66:1300–12. doi: 10.1016/j.jhep.2017.02.026
27. Yahyouche A, Zhidao X, Czernuszka JT, Clover AJ. Macrophage-mediated degradation of crosslinked collagen scaffolds. *Acta Biomater.* (2011) 7:278–86. doi: 10.1016/j.actbio.2010.08.005
28. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol.* (2000) 20:4106–14. doi: 10.1128/MCB.20.11.4106-4114.2000
29. Grossman M, Ben-Chetrit N, Zhuravlev A, Afik R, Bassat E, Solomonov I, et al. Tumor cell invasion can be blocked by modulators of collagen fibril alignment that control assembly of the extracellular matrix. *Cancer Res.* (2016) 76:4249–58. doi: 10.1158/0008-5472.CAN-15-2813
30. George SJ, Johnson JL. *In situ* zymography. *Methods Mol Biol.* (2010) 622:271–7. doi: 10.1007/978-1-60327-299-5_17
31. Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, et al. Histological grading and staging of chronic hepatitis. *J Hepatol.* (1995) 22:696–9. doi: 10.1016/0168-8278(95)80226-6
32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ Method. *Methods.* (2001) 25:402–8. doi: 10.1006/meth.2001.1262
33. Udi Y, Grossman M, Solomonov I, Dym O, Rozenberg H, Moreno V, et al. Inhibition mechanism of membrane metalloprotease by an exosite-swiveling conformational antibody. *Structure.* (2015) 23:104–15. doi: 10.1016/j.str.2014.10.012
34. Behbehani GK, Thom C, Zunder ER, Finck R, Gaudilliere B, Fragiadakis GK, et al. Transient partial permeabilization with saponin enables cellular barcoding prior to surface marker staining. *Cytometry A.* (2014) 85:1011–9. doi: 10.1002/cyto.a.22573
35. Zunder ER, Finck R, Behbehani GK, Amir el AD, Krishnaswamy S, Gonzalez VD, et al. Palladium-based mass tag cell barcoding with a doublet-filtering scheme and single-cell deconvolution algorithm. *Nat Protoc.* (2015) 10:316–33. doi: 10.1038/nprot.2015.020
36. Afratis NA, Selman M, Pardo A, Sagi I. Emerging insights into the role of matrix metalloproteases as therapeutic targets in fibrosis. *Matrix Biol.* (2018) 68–9:167–79. doi: 10.1016/j.matbio.2018.02.007
37. Roeb E. Matrix metalloproteinases and liver fibrosis (translational aspects). *Matrix Biol.* (2018) 68–9:463–73. doi: 10.1016/j.matbio.2017.12.012
38. Graubardt N, Vugman M, Mouhadeb O, Caliari G, Pasmanik-Chor M, Reuveni D, et al. Ly6C(hi) monocytes and their macrophage descendants regulate neutrophil function and clearance in acetaminophen-induced liver injury. *Front Immunol.* (2017) 8:626. doi: 10.3389/fimmu.2017.00626
39. Fonovic M, Turk B. Cysteine cathepsins and extracellular matrix degradation. *Biochim Biophys Acta.* (2014) 1840:2560–70. doi: 10.1016/j.bbagen.2014.03.017
40. Ramachandran P, Dobie R, Wilson-Kanamori JR, Dora EF, B.Henderson EP, Luu NT, et al. Resolving the fibrotic niche of human liver cirrhosis at single-cell level. *Nature.* (2019) 575:512–8. doi: 10.1101/766113
41. Li Z, Dranoff JA, Chan EP, Uemura M, Sévigny J, Wells RG. Transforming growth factor-β and substrate stiffness regulate portal fibroblast activation in culture. *Hepatology.* (2007) 46:1246–56. doi: 10.1002/hep.21792
42. Georges PC, Hui J-J, Gombos Z, McCormick ME, Wang AY, Uemura M, et al. Increased stiffness of the rat liver precedes matrix deposition: implications for fibrosis. *Am J Physiol.* (2007) 293:G1147–54. doi: 10.1152/ajpgi.00032.2007
43. Doyle AD, Yamada KM. Mechanosensing via cell-matrix adhesions in 3D microenvironments. *Exp Cell Res.* (2016) 343:60–6. doi: 10.1016/j.yexcr.2015.10.033
44. Franco C, Britto K, Wong E, Hou G, Zhu SN, Chen M, et al. Discoidin domain receptor 1 on bone marrow-derived cells promotes macrophage accumulation during atherogenesis. *Circ Res.* (2009) 105:1141–8. doi: 10.1161/CIRCRESAHA.109.207357
45. Postlethwaite AE, Kang AH. Collagen-and collagen peptide-induced chemotaxis of human blood monocytes. *J Exp Med.* (1976) 143:1299–307. doi: 10.1084/jem.143.6.1299
46. Wiesner C, Le-Cabec V, El Azzouzi K, Maridonneau-Parini I, Linder S. Podosomes in space: macrophage migration and matrix degradation in 2D and 3D settings. *Cell Adhes Migr.* (2014) 8:179–91. doi: 10.4161/cam.28116
47. Van Goethem E, Poincloux R, Gauffre F, Maridonneau-Parini I, Le Cabec V. Matrix architecture dictates three-dimensional migration modes of human macrophages: differential involvement of proteases and podosome-like structures. *J Immunol.* (2010) 184:1049–61. doi: 10.4049/jimmunol.0902223
48. Kaplan G. *In vitro* differentiation of human monocytes. Monocytes cultured on glass are cytotoxic to tumor cells but monocytes cultured on collagen are not. *J Exp Med.* (1983) 157:2061–72. doi: 10.1084/jem.157.6.2061
49. Wesley RB, 2nd, Meng X, Godin D, Galis ZS. Extracellular matrix modulates macrophage functions characteristic to atheroma: collagen type I enhances acquisition of resident macrophage traits by human peripheral blood monocytes in vitro. *Arterioscler Thromb Vasc Biol.* (1998) 18:432–40. doi: 10.1161/01.ATV.18.3.432
50. Patel NR, Bole M, Chen C, Hardin CC, Kho AT, Mih J, et al. Cell elasticity determines macrophage function. *PLoS ONE.* (2012) 7:e41024. doi: 10.1371/journal.pone.0041024
51. Zhou X, Hovell CJ, Pawley S, Hutchings MI, Arthur MJ, Iredale JP, et al. Expression of matrix metalloproteinase-2 and−14 persists during early resolution of experimental liver fibrosis and might contribute to fibrolysis. *Liver Int.* (2004) 24:492–501. doi: 10.1111/j.1478-3231.2004.0946.x
52. Duarte S, Shen XD, Fondevila C, Busuttil RW, Coito AJ. Fibronectin-alpha4beta1 interactions in hepatic cold ischemia and reperfusion injury: regulation of MMP-9 and MT1-MMP via the p38 MAPK pathway. *Am J Transplant.* (2012) 12:2689–99. doi: 10.1111/j.1600-6143.2012.04161.x
53. Knauper V, Will H, Lopez-Otin C, Smith B, Atkinson SJ, Stanton H, et al. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. *J Biol Chem.* (1996) 271:17124–31. doi: 10.1074/jbc.271.29.17124
54. Raghu G, Brown KK, Collard HR, Cottin V, Gibson KF, Kaner RJ, et al. Efficacy of simtuzumab versus placebo in patients with idiopathic pulmonary fibrosis: a randomised, double-blind, controlled, phase 2 trial. *Lancet Respir Med.* (2017) 5:22–32. doi: 10.1016/S2213-2600(16)30421-0
55. Harrison SA, Abdelmalek MF, Caldwell S, Shiffman ML, Diehl AM, Ghalib R, et al. Simtuzumab is ineffective for patients with bridging

fibrosis or compensated cirrhosis caused by nonalcoholic steatohepatitis. *Gastroenterology*. (2018) 155:1140–53. doi: 10.1053/j.gastro.2018.07.006
56. Loomba R, Lawitz E, Mantry PS, Jayakumar S, Caldwell SH, Arnold H, et al. The ASK1 inhibitor selonsertib in patients with nonalcoholic steatohepatitis: A randomized, phase 2 trial. *Hepatology*. (2018) 67:549–59. doi: 10.1002/hep.29514
57. Muir AJ, Levy C, Janssen HLA, Montano-Loza AJ, Shiffman ML, Caldwell S, et al. Simtuzumab for primary sclerosing cholangitis: phase 2 study results with insights on the natural history of the disease. *Hepatology*. (2019) 69:684–98. doi: 10.1002/hep.30237
58. Fraser AR, Pass C, Burgoyne P, Atkinson A, Bailey L, Laurie A, et al. Development, functional characterization and validation of methodology for GMP-compliant manufacture of phagocytic macrophages: a novel cellular therapeutic for liver cirrhosis. *Cytotherapy*. (2017) 19:1113–24. doi: 10.1016/j.jcyt.2017.05.009
59. Moroni F, Dwyer BJ, Graham C, Pass C, Bailey L, Ritchie L, et al. Safety profile of autologous macrophage therapy for liver cirrhosis. *Nat Med.* (2019) 25:1560–5. doi: 10.1038/s41591-019-0599-8

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REVIEW
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Hepatic Macrophages in Liver Injury

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Ample evidence suggests that hepatic macrophages play key roles in the injury and repair mechanisms during liver disease progression. There are two major populations of hepatic macrophages: the liver resident Kupffer cells and the monocyte-derived macrophages, which rapidly infiltrate the liver during injury. Under different disease conditions, the tissue microenvironmental cues of the liver critically influence the phenotypes and functions of hepatic macrophages. Furthermore, hepatic macrophages interact with multiple cells types in the liver, such as hepatocytes, neutrophils, endothelial cells, and platelets. These crosstalk interactions are of paramount importance in regulating the extents of liver injury, repair, and ultimately liver disease progression. In this review, we summarize the novel findings highlighting the impact of injury-induced microenvironmental signals that determine the phenotype and function of hepatic macrophages. Moreover, we discuss the role of hepatic macrophages in homeostasis and pathological conditions through crosstalk interactions with other cells of the liver.

Keywords: Kupffer cells, monocyte-derived macrophages, liver injury, microenvironmental cues, cellular crosstalk

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INTRODUCTION

Hepatic macrophages, consisting of liver resident Kupffer cells (KCs) and monocyte-derived macrophages (MoMϕs), play a central role in maintaining homeostasis of the liver as well as contributing to the progression of acute or chronic liver injury (1). Kupffer cells are the most abundant tissue macrophages in mammalian bodies, accounting for 80–90% of total tissue macrophages (2). In the liver, KCs are distributed along the hepatic sinusoids, preferentially near the periportal areas (3). In the healthy liver, a small number of MoMϕs are located mainly in the portal triad areas (4, 5). In mouse, KCs and MoMϕs can be distinguished by their differential expression of certain cell surface markers. Kupffer cells are defined as $CD11b^{low}$, $F4/80^{hi}$, C-type lectin domain family 4 member F, $CD68^{+}$, and CX3C chemokine receptor 1 $(CX_3CR1)^{-}$ (6, 7). MoMϕs are $CD11b^{+}$, $F4/80^{+}$, $Ly6C^{+}$, macrophage colony-stimulating factor 1 receptor $(CSF1R)^{+}$. Human KCs are not as well-characterized and normally identified as $CD68^{+}$ cells (8). Human MoMϕs are normally identified as $CD14^{+}$, CC-chemokine receptor 2 $(CCR2)^{+}$ cells (**Table 1**) (8, 9).

A salient function of macrophages is to remove pathogens and dead cells (3). In a healthy liver, KCs constantly phagocytose aged erythrocytes, neutrophils, and effector $CD8^{+}$ T cells, thereby maintaining homeostasis within the liver (10–12). Monocyte-derived macrophages are also able to phagocytose dead endothelial cells and maintain integrity of vasculature under normal condition (13). The phagocytosis function of macrophages and its contribution to liver homeostasis and disease have been recently highlighted in an excellent review (14). Aside from phagocytosis, macrophages act as antigen-presenting cells and regulate adaptive immune responses. It is known that KCs play a central role in inducing immune tolerance to innocuous antigens that reached the liver from the intestine (15). In contrast, MoMϕs that are recruited into the liver lose the tolerogenic phenotype and instead contribute to antigen-specific proinflammatory immune

TABLE 1 | Difference between KCs and MoMϕs.

	KCs	MoMϕs
Origins	Fetal liver-derived erythromyeloid progenitors	Circulatory monocytes from bone marrow-derived haematopoietic stem cells
Location	Along the hepatic sinusoids, preferentially near the periportal area	Mainly in the portal triad of the healthy liver
Cell surface markers in mice	$CD11b^{low}$, $F4/80^{hi}$, $Clec4F^+$, $CD68^+$, $CX3CR1^-$	$CD11b^+$, $F4/80^+$, $Ly6C^+$, $CSF1R^+$
Cell surface markers in humans	$CD68^+$	$CD14^+$, $CCR2^+$
Functional differences	Induce immune tolerance to innocuous antigens; defend against pathogens; sense alterations in tissue integrity and maintain tissue homeostasis; orchestrate tissue repair after injury; facilitate leukocyte recruitment	Contribute to anti-bacterial responses; replenish KCs after injury; co-existence of subsets with opposing functions of pro-inflammatory and anti-inflammatory; pro-fibrogenic and anti-fibrogenetic

KCs, Kuppfer cells; MoMϕs, Monocytes-derived macrophages; CD11b, cluster of differentiation molecule 11B; Clec4f, C-type lectin domain family 4 member f; CX3CR1, CX3C chemokine receptor 1; CSF1R, Colony-stimulating factor 1 receptor; CCR2, Chemokine (C-C motif) receptor 2.

responses (**Table 1**) (16). During liver injury, a large number of circulating monocytes infiltrate into the liver and further differentiate into MoMϕs (17). Injury-induced modulations of the liver tissue microenvironment, such as soluble mediators released by activated/stress cells and accumulation of dead cells, influence the phenotypes of both KCs and MoMϕs and determine their involvement in aggravation of liver injury or restoration of liver functions (6, 16, 17). Under healthy and disease conditions, we call any signals received from local microenvironment and prompted functional differentiation of hepatic macrophages as microenvironmental signals. Understanding how microenvironmental signals affect the heterogeneity of hepatic macrophages and how hepatic macrophages interact with other cells during liver injury is instrumental not only in gaining basic knowledge but also in designing therapeutic strategies to treat liver diseases. In this review, we summarize the latest findings about specific environmental signals that impact the phenotype and function of hepatic macrophages and discuss the functions of hepatic macrophages in healthy and diseased liver through interacting with other cells.

ORIGIN OF HEPATIC MACROPHAGES

The recent development and improvement of research tools have allowed us to gain novel insights into the origin of hepatic macrophages. Contrary to the notion that KCs are originated solely from bone marrow–derived circulating monocytes (18), recent evidence suggests that KCs are mainly derived from yolk sac erythromyeloid progenitors (EMPs). Erythromyeloid progenitors colonize the fetal liver at E8.5 and give rise to macrophage precursors (pMacs) at E9.5 in a CX3CR1-dependent manner. A distinct transcriptional programming involving the upregulation of DNA binding 3 (Id3) is important in the further differentiation of pMacs into KCs (19). With regard to the maintenance of tissue-resident macrophages including KCs, an interesting "niche competition" model has been put forth. This model proposes that bone marrow–derived circulating monocytes and EMPs have an almost identical potential to develop into KCs and that they compete for a restricted number of niches. However, during liver development, the majority of liver niches are taken by EMPs and few by monocytes. At steady state, the niches in liver are self-maintained and tightly controlled by specific Maf transcription repressors and enhancers (20). However, when a significant number of KCs die or depleted experimentally, circulating monocytes have been shown to contribute to the KCs pool, suggesting that circulating monocytes have the potential as their embryonic counterparts to develop into KCs (21–23).

Monocyte-derived macrophages are derived from circulatory monocytes, which are developed from bone marrow–resident hematopoietic stem cells (23). Hematopoietic stem cells first differentiate into common lymphoid progenitors and then into granulocyte–monocyte progenitors (24, 25). Granulocyte–monocyte progenitors further give rise to common monocyte progenitors (cMoPs) under the regulation of macrophage CSF-1 (26). Eventually, cMoPs differentiate into circulatory monocytes. These distinct steps of hepatic stellate cell (HSC) differentiation into monocytes are tightly regulated by a number of transcription factors and epigenetic DNA methylation mechanisms (23). In mice, there are two subsets of monocytes in the liver: $Ly6C^{hi}$ and $Ly6C^{low}$ monocytes. The $Ly6C^{hi}$ monocytes can differentiate into $Ly6C^{low}$ monocytes, which can further differentiate into MoMϕs (27, 28). In humans, the $CD14^{hi}CD16^-$ and $CD14^-CD16^{hi}$ monocytes correlate with $Ly6C^{hi}$ and $Ly6C^{low}$ monocytes in mice, respectively (29).

IMPACT OF TISSUE MICROENVIRONMENTAL FACTORS ON HEPATIC MACROPHAGES

Irrespective of the cellular origins of hepatic macrophages, they differentiate into different phenotypes depending on the stimulatory signals in the tissue environment (30). In disease conditions, there are two major types of stimulatory signals (**Table 2**), including danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs).

Danger-associated molecular patterns are a diverse group of molecules including nucleic acid in various conformations (e.g., single-/double-stranded RNA or DNA), nuclear proteins

TABLE 2 | Microenvironment in the liver.

	Examples	Reported in liver diseases	References
DAMPs	HMGB-1	AILI, I/R injury, NASH	(31–36)
	HSP-70	AILI	(37, 38)
	Bile acids	Cholestatic liver disease	(39–43)
	Histones	I/R injury	(44)
	FFAs	NASH	(45)
	mtDNA	NASH	(45)
PAMPs	Bacteria-derived products, e.g. LPS	ALD, NASH	(46–49)
	Hepatitis virus, e.g. HBV, HCV, HEV	Hepatitis virus infection	(50–52)
Phagocytosis of cellular debris	Apoptotic cells	LPS/D-gal-induced hepatitis, CCL4-induced liver fibrosis, ALD	(53–55)

DAMPs, danger-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; HMGB-1, high mobility group box 1; HSP-70, heat shock protein 70; FFAs, free fatty acids; mtDNA, mitochondrial DNA; LPS, Lipopolysaccharides; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; AILI, Acetaminophen-induced liver injury; I/R, Ischemia/Reperfusion; NASH, Non-alcoholic steatohepatitis; ALD, Alcoholic liver disease.

[e.g., high mobility group box 1 (HMGB1)], cytosolic proteins [e.g., heat shock protein 70 (HSP-70)], purine nucleotides [e.g., adenosine triphosphate (ATP)], or mitochondrial compounds (e.g., mtDNA, N-formyl peptides). Danger-associated molecular patterns are recognized by pathogen recognition receptors (PRRs), which are expressed on hepatic macrophages and can activate the cells (56, 57). These PRR receptors include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain–like receptors (NLRs), and retinoic acid-inducible gene 1–like receptors (56). Danger-associated molecular patterns play a critical role in activating macrophages during sterile liver injury, which can be caused by a variety of stimuli, such as overdose of acetaminophen (APAP), cholestatic obstruction, excess accumulation of free fatty acids (FFAs), or hepatic ischemia, and reperfusion (I/R) (31, 58). The release of DAMPs is common in these situations, and it may be an active process from viable or dying cells, or a passive process leaking out from necrotic cells (56).

In APAP-induced liver injury (AILI), hepatocyte damage leads to the release of HMGB1, which activates KCs to produce proinflammatory cytokines and chemokines (31). High mobility group box 1 is a nuclear-binding protein that can function as a DNA chaperone in nucleus, sustaining autophagy in cytosol and DAMP molecule outside the cell (32). High mobility group box 1–deficient mice show less inflammation and neutrophil recruitment post-APAP treatment when compared to their wild-type counterparts (31, 33). Mice lacking the receptor for advanced glycation end products, a receptor for HMGB1, on bone marrow–derived cells display a similar phenotype as the HMGB1-deficient mice (31, 33). Together, these data indicate that HMGB1 activates KCs and contributes to AILI (31, 33). Aside from HMGB1, HSP-70 has also been reported to be released by necrotic hepatocytes during AILI (37). Heat shock protein 70 is an important component of cellular machinery, facilitating protein folding, but also an inducible stress-response protein that can undergo translocation to the cell surface or is released into the extracellular milieu during cellular stress or necrotic death (38). Heat shock protein 70 can stimulate macrophages to promote immune response and inflammation (37).

Similar to the necroinflammatory injury pattern in AILI, cholestatic liver disease is also characterized by hepatocytes necrosis (59). Lesions in bile duct canaliculi cause an increase of bile acids, which result in liver injury, but appear to drive KCs into anti-inflammatory state (39, 59). Bile acids can bind to G-protein–coupled bile acid receptor (TGR5), which is expressed on hepatic macrophages (40). Activation of TGR5 in macrophages reduces proinflammatory cytokines while maintaining anti-inflammatory cytokines (41). The anti-inflammatory effect of TGR5 on macrophages is mediated by inhibiting nuclear factor κB and JNK signaling pathways and NLRP3 inflammasome (41, 43).

Hepatic I/R injury is another example of sterile tissue injury in which DAMPs are released. It is reported that after I/R injury hepatocytes actively secrete HMGB1, which binds to TLR4 and activates KCs (34). In addition to HMGB1, other DAMPs, such as histones, DNA fragments, ATP, and mitochondrial reactive oxygen species (ROS), also activate KCs via different PRRs, such as TLR9 (recognizing histones) (44), TLR3 (recognizing RNA) (60, 61), TLR4 (recognizing HSP-70) (62), and nucleotide-binding domain leucine-rich repeat containing family pyrin domain containing 3 (NLRP3) (recognizing ATP) (63, 64). As a result, KCs secrete ample proinflammatory cytokines to exacerbate I/R injury (65).

Non-alcoholic steatohepatitis (NASH) is characterized by steatosis with tissue injury and inflammation (35). Increased accumulation of fat in hepatocytes leads to lipotoxicity, which induces apoptotic and necrotic death of hepatocytes with concomitant release of DAMPs, such as HMGB1 and FFAs (35). Released HMGB1 activates KCs via TLR4 as in other liver injury cases (35, 36). Free fatty acids decrease the mitochondrial membrane potential and subsequently induce mtDNA release from the mitochondria to the cytoplasm (45). Released mtDNA causes NLRP3 inflammasome activation in KCs, which further promotes the production of proinflammatory interleukin 1β (IL-1β) (45).

Pathogen-associated molecular patterns include pathogen-specific polysaccharides, lipoproteins, and nucleic acids. They bind to PPRs expressed on hepatic macrophages and trigger inflammatory activation of these cells (66). In alcoholic liver disease (ALD), excess chronic ingestion of alcohol increases gut permeability and causes translocation of bacteria-derived products, such as lipopolysaccharides (LPSs), from the gut to the liver (67). Lipopolysaccharide binds to CD14 in association with TLR4 on KCs to initiate proinflammatory signaling (46). Numerous studies have demonstrated that KCs play a central role in alcohol-induced inflammation and injury of the liver (27, 28). Kupffer cells produce cytokines such as tumor necrosis factor α (TNF-α) and free radicals to promote ALD (68, 69). In addition to KCs, MoMϕs can be differentiated from the circulating monocytes that are recruited into the liver as a result of the inflammatory response during liver injury (28). Two types of MoMϕs ($Ly6C^{low}$ MoMϕs and $Ly6C^{hi}$ MoMϕs) have been reported to coexist in the liver of mice after chronic alcohol feeding. The $Ly6C^{low}$ MoMϕs exhibit restorative phenotype, whereas $Ly6C^{hi}$ MoMϕs exhibit proinflammatory phenotype. A large dose of alcohol binge after chronic alcohol feeding enhances liver inflammation and injury with an increased ratio of $Ly6C^{hi}$/$Ly6C^{low}$ MoMϕ. Moreover, $Ly6C^{hi}$ MoMϕ switches to $Ly6C^{low}$ MoMϕ upon phagocytosis of apoptotic hepatocytes. Taken together, KCs and MoMϕ are involved in the pathogenesis of ALD (27, 28, 70).

Whereas, translocation of LPS from the gut lumen to portal circulation in ALD may result from direct alcohol toxicity to intestinal epithelium (67), increased exposure to intestinal LPS has also been considered in the pathogenesis of NASH (71). Increased levels of LPS in the portal circulation have been observed during NASH development (72). The role of LPS-TLR4 signaling in non-alcoholic fatty liver disease (NAFLD) has been demonstrated in a number of studies (47). Toll-like receptor 4 expression on KCs is elevated both in patients with NASH and mice fed with methionine–choline–deficient diet (47, 48). Depletion of KCs by clodronate liposomes significantly reduced TLR4 expression and steatohepatitis (47). Together, endotoxin appears to play an important role in NAFLD through TLRs on KCs, particularly TLR4. However, the function of MoMϕ in response to endotoxin during NAFLD remains unclear.

Hepatitis virus infection is a leading cause of chronic liver diseases (49). Hepatitis B virus (HBV) modulates liver macrophage functions to favor viral replication and survival. These modulations include reduced production of antiviral cytokine IL-1β and enhanced levels of immunosuppressive IL-10 (73). Hepatitis C virus (HCV) can enter KCs through phagocytic uptake. Subsequently, viral RNA triggers myeloid differentiation primary response gene 88 (MyD88)–mediated TLR7 signaling to induce IL-1β expression. Hepatitis C virus uptake concomitantly induces a potassium efflux that activates the NLRP3 inflammasome and promotes the secretion of activated IL-1β. Interleukin 1β produced by KCs contributes to HCV disease severity (74). It has also been reported that the function of monocytes/macrophages is impaired in pregnant patients with hepatitis E virus (HEV)–induced acute hepatitis (50). Toll-like receptor 3 and TLR7 expressions are reduced, and MyD88 downstream molecules, IRF3 and IRF7, are decreased in macrophages from these patients (50). The macrophage phagocytic activity and *Escherichia coli*–induced ROS production are significantly impaired in macrophages from HEV patients as well (50). These data suggest that the impact of viruses on hepatic macrophages could result in immune suppression and/or inflammation and tissue damage.

Phagocytosis of Dead Cells

A salient function of macrophages is to remove dead cells and cellular debris, which is a key initial step in resolving tissue inflammation and promoting tissue repair from injury. It has been widely reported that phagocytosis of dead cells triggers transcriptional reprogramming of macrophages to switch from a proinflammatory to an anti-inflammatory and tissue restorative phenotype. For example, it is shown that the membrane-bound transforming growth factor β (TGF-β) on apoptotic cells can trigger KCs to produce IL-10, which suppresses the proinflammatory immune response in a mouse model of LPS/D-galactosamine–induced hepatitis (51, 52). In a mouse model of ALD, it is reported that phagocytosis of cellular debris triggers hepatic macrophages to switch from proinflammatory to an anti-inflammatory phenotype (28). Similarly, in a mouse model of carbon tetrachloride–induced liver fibrosis, phagocytosis of cellular debris induces $Ly6C^{hi}$ proinflammatory, profibrotic MoMϕs to differentiate into antifibrotic macrophages (52). The restorative macrophages are the most abundant subset in the liver during maximal fibrosis resolution and represent the predominant source of matrix metalloproteinases. Moreover, it has been demonstrated that phagocytosis of necrotic hepatocytes by macrophages activates Wnt ligand secretion, thereby promoting liver regeneration (53).

In summary, the particular function of KCs and MoMϕ is tightly regulated by microenvironmental signals, such as DAMPs and PAMPs. The same signals may play a similar role in different liver diseases; for example, HMGB1 released by hepatocytes triggers KC activation in AILI, I/R, and NASH, indicating similar functions of macrophages in various liver diseases (32–34, 37, 54, 56, 58, 75, 76). In certain liver diseases, such as NAFLD and ALD, DAMPs and PAMPs coexist to regulate macrophage functions (35, 56, 77, 78) However, more studies are warranted to better understand the differential functions of KCs and MoMϕs in a given disease situation.

COMMUNICATION BETWEEN HEPATIC MACROPHAGES AND OTHER CELLS IN THE LIVER

There are five major cell types in the liver including hepatocytes, biliary epithelial cells (cholangiocytes), liver sinusoidal endothelial cells (LSECs), KCs, and HSCs (66, 79). Hepatocytes account for roughly 80% of the liver's mass (79), and they perform a number of vital functions, including protein synthesis, detoxification, and metabolism of lipids and carbohydrates (79). Cholangiocytes are another type of epithelial cells in the liver, lining the lumen of the

TABLE 3 | The crosstalk of hepatic macrophages with other cells in the liver.

	Interacted cell types	Roles of hepatic macrophages in interaction	Reported in liver diseases	References
KCs	Hepatocytes	KCs activate Notch signaling in hepatocytes	ICC	(81)
	Hepatocytes	KCs are essential for proliferation of liver progenitor cells	Choline-deficient, ethionine-supplemented diet-mediated liver injury	(82)
	Hepatocytes	KCs trigger hepatocytes senescence	ALD	(83)
	Hepatocytes	KC are activated by hepatocytes-derived extracellular vesicles	ALD	(84, 85)
	HSCs	KCs activate HSCs and modulate fibrogenic responses in HSCs	Liver fibrosis	(86, 87)
	HSCs	KCs secrete IFN-γ to induce apoptosis of HSCs	Liver fibrosis	(88)
	HSCs	HSCs induce KCs differentiation and decrease its cytokine secretion	Liver fibrosis	(89)
	LSECs	KCs are essential for LSECs' uptake of hyaluronic acid	*In vitro* culture	(90)
	MoMϕs	KCs produce CCL2 to recruit MoMϕs	Amodiaquine-induced liver injury	(91)
	Neutrophils	The production of TNF-α and TGF-β by KCs is promoted by neutrophil-secreted IL-17; Express adhesion molecules to recruit neutrophils	Cholestatic liver injury, LPS-induced liver injury	(92, 93)
	NKT cells	KCs produce IL-1β to recruit and activate NKT cells	Alcoholic steatosis	(94)
	$CD4^+$ T cells	KCs produce ROS, IL-6 and TNF-α to recruit $CD4^+$ T cells.	Hepatic I/R injury	(95)
	T cells	KCs produce IL-10, TGF-β, ROS, IDO, PGE2/J2 to induce and maintain T cell tolerance or apoptosis	Liver transplantation, HBV infection, *in vitro* culture	(96–101)
	$CD8^+$ T cells	KCs prime $CD8^+$ T cells to differentiate into effector cells to kill viruses	HBV infection	
	Platelets	KCs promote adhesion of platelets on the KCs to encase the bacteria and facilitate anti-bacterial responses	Bacteria infection in the liver	
MoMϕs	cholangiocytes	MoMϕs release IL-6 to promote the proliferation of cholangiocytes	Cholestatic liver disease	(102, 103)
	cholangiocytes	MoMϕs are recruited by cholangiocytes-derived osteopontin and MCP-1	Partial Hepatectomy	(104)
	LSECs	LSECs are activated by MoMϕs	Partial Hepatectomy	(105)
	NKT cells	MoMϕs promote NKT cells over-activation and cell death	NAFLD	(106)
	NKT cells	MoMϕs produce IL-12 to activate NKT cells, which inhibits liver regeneration	Partial hepatectomy	(107)

KCs, Kuppfer cells; MoMϕs, Monocytes-derived macrophages; LSECs, liver sinusoidal endothelial cells; HSCs, hepatic stellate cells; NKT cells, natural killer T cell; IFN-γ, Interferon-gamma; CCL2, chemokine (C-C motif) ligand 2; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; IL-17, interleukin 17; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-10, interleukin 10; ROS, reactive oxygen species; IDO, indoleamine 2,3-dioxygenase; PGE2/J2, prostaglandin E2/J2; MCP-1, monocyte chemoattractant protein-1; ICC, intrahepatic cholangiocarcinoma; LPS, lipopolysaccharides; HBV, hepatitis B virus; I/R, ischemia/reperfusion.

bile ducts (79). Liver sinusoidal endothelial cells form the lining of the smallest blood vessels in the liver, part of the reticuloendothelial system (79). Liver sinusoidal endothelial cells regulate the passage of molecules from the blood vessels into the liver (79). Hepatic stellate cells are found in the space of Disse, between hepatocytes and LSECs. Hepatic stellate cells can transdifferentiate from a quiescent state to an activated, highly proliferative, and wound-healing myofibroblast (80). In addition, there are a variety of other immune cells such as monocytes, neutrophils, and platelets that contribute to liver homeostasis as well as disease progression (66). In different types and stages of liver diseases, hepatic macrophages are critically involved in the progression and regression via close communication with other cells in the liver (**Table 3**) (108).

Crosstalk Between Hepatic Macrophages and Hepatocytes

The crosstalk between hepatic macrophages and hepatocytes has been indicated in a number of studies. In a mouse model of thioacetamide (TAA)–induced intrahepatic cholangiocarcinoma, KCs accumulate around the central vein area and express Notch ligand Jagged-1 rapidly after the initiation of the TAA treatment, coinciding with the activation of Notch signaling in pericentral hepatocytes. Depletion of KCs prevents the Notch-mediated hepatocytes transformation to cholangiocytes, suggesting that KCs contribute to the cell fate change in hepatocytes (109). Another study has also demonstrated that KCs are required for the proliferation of liver progenitor cells that can differentiate into hepatocytes in a model of choline-deficient, ethionine-supplemented diet–mediated liver injury and regeneration (110). In ALD, it is shown that KC-produced IL-6 triggers hepatocyte senescence, which becomes resistant to apoptosis (81). Interestingly, recent studies also support that hepatocytes communicate with macrophages through the release of extracellular vesicles (EVs) that contain proteins and microRNAs (82, 83). *In vitro* studies using HepG2 cells have demonstrated that alcohol treatment induces an elevated release of EVs, which activate THP-1 cells, a human leukemia monocytic cell line into a proinflammatory phenotype through CD40 ligand (83). Another study also showed that exosomes derived from alcohol-treated hepatocytes mediated the transfer of liver-specific miRNA-122 to monocytes and sensitized monocytes to LPS stimulation (82). These studies suggest that hepatocytes release EVs that contain altered proteins and miRNAs to regulate the activation of monocytes/macrophages.

Interactions of Hepatic Macrophages With Cholangiocytes, HSCs, and LSECs

Macrophages secrete IL-6 during infection, and IL-6 can induce cholangiocyte proliferation leading to ductular reaction (84, 85). On the other hand, cholangiocytes are the major source of osteopontin and macrophage chemoattractant protein 1, which acts as chemotaxis to recruit MoMϕs during partial hepatectomy (102). Hepatic stellate cells and KCs are located in close proximity to each other (86, 103). In a mouse model of CCl_4-induced liver fibrosis, it is shown that depletion of hypoxia-inducible factor 1α in HSCs inhibits KC activation and reduces the release of proinflammatory cytokines, suggesting a function of HSCs in regulating KCs during liver fibrosis (87). On the other hand, KCs have also been reported to modulate HSC functions. Chemokine (C-X-C motif) ligand 6 stimulates the phosphorylation of epidermal growth factor receptor and the expression of TGF-β in KCs, which further activates HSCs and results in liver fibrosis (103). It is reported that ROS and IL-6 activate KCs, which in turn modulate fibrogenic responses of HSCs (104). Activated KCs secrete interferon γ, which subsequently induces HSC apoptosis in a STAT1-dependent manner and reduces liver fibrosis (111). Liver sinusoidal endothelial cells are the major source of intercellular adhesion molecule 1 (ICAM-1). In partial hepatectomy, ICAM-1 expressed on KCs and LSECs recruits leukocytes, which leads to TNF-α and IL-6 production, thereby promoting hepatocyte proliferation (89). Moreover, MoMϕs also play an important role in activating LSECs and contributing to vascular growth and liver regeneration (88). Kupffer cell depletion inhibits hyaluronic acid uptake by LSECs and impairs sinusoidal integrity, suggesting there is a crosstalk between KCs and LSECs (37, 112).

Interactions of Macrophages With Other Hepatic Immune Cells

Kupffer cell activation by pathogens results in CCL2 secretion, which promotes the recruitment of monocytes into the injured liver (105). It has been reported that alcohol treatment of THP-1 cells or human primary monocytes triggers the secretion of EVs, which induce the differentiation of naive monocytes into anti-inflammatory macrophages by delivering cargos, such as miR-27a (90).

Neutrophils are the most abundant white blood cells in the circulation, and they are recruited to the liver in various injury conditions (91). During cholestatic liver injury, neutrophils secrete IL-17, which promotes the production of TNF-α and TGF-β by KCs. On the other hand, KCs express adhesion molecules that induce neutrophil attachment and facilitate bacterial clearance in the liver (113). Another study reported that KCs coordinate with LSECs for neutrophil adhesion, recruitment, and activation through TLR4 during LPS-induced liver injury (78).

Natural killer T (NKT) cells are a group of "unconventional" T cells that express both natural killer (NK) cell receptors and T cell receptors. In the NASH liver, lipid accumulation causes an increase of the number of MoMϕs and activates the cells to a proinflammatory phenotype. Activated MoMϕs promote NKT cell activation and induce NKT cell deficiency, contributing to the pathogenesis of NAFLD (93). The importance of KC/NKT interaction in liver regeneration has also been described. After partial hepatectomy, MoMϕs produce IL-12 to activate hepatic NKT cells, which prohibit liver regeneration (114). In ALD, NLRP3 inflammasome activation results in IL-1β release by KCs. Released IL-1β recruits and activates hepatic invariant NKT cells, which promote liver inflammation, neutrophil infiltration, and liver injury (106). Furthermore, ample evidence supports the importance of macrophages in interacting with conventional T cells. It has been shown that KCs trigger the recruitment of $CD4^+$ T cells by ROS, IL-6, and TNF-α (107). Kupffer cells express high levels of MHC class II and act as antigen-presenting cells. In fact, a number of studies have shown that KCs play an important role in inducing and maintaining T cell tolerance, through producing tolerogenic mediators such as IL-10, TGF-β, ROS, indoleamine 2,3-dioxygenase, prostaglandin E_2/J_2 (94, 97). Moreover, it is reported that during liver transplantation KCs induce T cell apoptosis and thus promote immune tolerance (98). After HBV infection, KCs produce IL-10 and support liver tolerance by inducing anti-HBV $CD8^+$ T cell exhaustion (99). A recent study demonstrated that the induction of endoplasmic reticulum stress in HCC cell lines induces the secretion of EVs. The HCC-derived EVs induce elevated expression of programmed death ligand 1 on macrophages *in vivo* and *in vitro*, which causes T cell dysfunction and impaired proliferation (100). Recent studies showed that priming $CD8^+$ T cells by KCs leads to the differentiation of $CD8^+$ T cells into effector cells that have powerful killing activities against hepatitis viruses, such as HBV (101).

Moreover, interesting interactions between macrophages and platelets have been unveiled (115). Under basal conditions, platelets form transient "touch-and-go" interactions with von Willebrand factor constitutively expressed on KCs (115). During bacterial infection, platelets switch from "tough-and-go" to sustained GPIIb-mediated adhesion on the KCs to encase the bacteria and facilitate anti-bacterial responses (115).

In summary, hepatic macrophages interact with almost all cell types of the liver. In naive state, KCs, through these interactions, play a critical role in maintaining tissue homeostasis. Under injury or disease conditions, the crosstalk of macrophages with hepatocytes, cholangiocytes, LSECs, HSCs, and other immune cells contributes to exacerbation of tissue damage or promotion of liver repair and regeneration, depending on the cell type and signaling pathways involved.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. ZS wrote the draft. CJ revised the manuscript.

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REFERENCES

1. Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cell Mol Immunol.* (2016) 13:316–27. doi: 10.1038/cmi.2015.104
2. Bilzer M, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. *Liver Int.* (2006) 26:1175–86. doi: 10.1111/j.1478-3231.2006.01342.x
3. Abdullah Z, Knolle PA. Liver macrophages in healthy and diseased liver. *Pflugers Arch.* (2017) 469:553–60. doi: 10.1007/s00424-017-1954-6
4. Theurl I, Hilgendorf I, Nairz M, Tymoszuk P, Haschka D, Asshoff M, et al. On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. *Nat Med.* (2016) 22:945–51. doi: 10.1038/nm.4146
5. Gammella E, Buratti P, Cairo G, Recalcati S. Macrophages: central regulators of iron balance. *Metallomics.* (2014) 6:1336–45. doi: 10.1039/C4MT00104D
6. Scott CL, Zheng F, De Baetselier P, Martens L, Saeys Y, De Prijck S, et al. Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Commun.* (2016) 7:10321. doi: 10.1038/ncomms10321
7. Lavin Y, Winter D, Blecher-Gonen R, David E, Keren-Shaul H, Merad M, et al. Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. *Cell.* (2014) 159:1312–26. doi: 10.1016/j.cell.2014.11.018
8. Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol.* (2017) 17:306–21. doi: 10.1038/nri.2017.11
9. Dal-Secco D, Wang J, Zeng Z, Kolaczkowska E, Wong CH, Petri B, et al. A dynamic spectrum of monocytes arising from the in situ reprogramming of CCR2+ monocytes at a site of sterile injury. *J Exp Med.* (2015) 212:447–56. doi: 10.1084/jem.20141539
10. Terpstra V, van Berkel TJ. Scavenger receptors on liver Kupffer cells mediate the *in vivo* uptake of oxidatively damaged red blood cells in mice. *Blood.* (2000) 95:2157–63. doi: 10.1182/blood.V95.6.2157
11. Shi JL, Fujieda H, Kokubo Y, Wake K. Apoptosis of neutrophils and their elimination by Kupffer cells in rat liver. *Hepatology.* (1996) 24:1256–63. doi: 10.1002/hep.510240545
12. Crispe IN, Dao T, Klugewitz K, Mehal WZ, Metz DP. The liver as a site of T-cell apoptosis: graveyard, or krilling field? *Immunol. Rev*. (2000) 174:47–62. doi: 10.1034/j.1600-0528.2002.017412.x
13. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, et al. Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell.* (2013) 153:362–75. doi: 10.1016/j.cell.2013.03.010
14. Horst AK, Tiegs G, Diehl L. Contribution of macrophage efferocytosis to liver homeostasis and disease. *Front Immunol.* (2019) 10. doi: 10.3389/fimmu.2019.02670
15. Crispe IN. Immune tolerance in liver disease. *Hepatology.* (2014) 60:2109–17. doi: 10.1002/hep.27254
16. Heymann F, Peusquens J, Ludwig-Portugall I, Kohlhepp M, Ergen C, Niemietz P, et al. Liver inflammation abrogates immunological tolerance induced by Kupffer cells. *Hepatology.* (2015) 62:279–91. doi: 10.1002/hep.27793
17. Zigmond E, Samia-Grinberg S, Pasmanik-Chor M, Brazowski E, Shibolet O, Halpern Z, et al. Infiltrating monocyte-derived macrophages and resident kupffer cells display different ontogeny and functions in acute liver injury. *J Immunol.* (2014) 193:344–53. doi: 10.4049/jimmunol.1400574
18. van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. *J Exp Med.* (1968) 128:415–35. doi: 10.1084/jem.128.3.415
19. Mass E, Ballesteros I, Farlik M, Halbritter F, Gunther P, Crozet L, et al. Contribution of macrophage efferocytosis to liver homeostasis and disease. *Science.* (2016) 353:aaf4238. doi: 10.1126/science.aaf4238
20. Soucie EL, Weng Z, Geirsdottir L, Molawi K, Maurizio J, Fenouil R, et al. Lineage-specific enhancers activate self-renewal genes in macrophages and embryonic stem cells. *Science.* (2016) 351:aad5510. doi: 10.1126/science.aad5510
21. Sawai CM, Babovic S, Upadhaya S, Knapp D, Lavin Y, Lau CM, et al. Hematopoietic stem cells are the major source of multilineage hematopoiesis in adult animals. *Immunity.* (2016) 45:597–609. doi: 10.1016/j.immuni.2016.08.007
22. Guilliams M, Scott CL. Does niche competition determine the origin of tissue-resident macrophages? *Nat Rev Immunol.* (2017) 17:451–60. doi: 10.1038/nri.2017.42
23. Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new reality. *Blood.* (2015) 125:2605–13. doi: 10.1182/blood-2014-12-570200
24. Auffray C, Fogg DK, Narni-Mancinelli E, Senechal B, Trouillet C, Saederup N, et al. CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation. *J Exp Med.* (2009) 206:595–606. doi: 10.1084/jem.20081385
25. Liu K, Victora GD, Schwickert TA, Guermonprez P, Meredith MM, Yao K, et al. *In vivo* analysis of dendritic cell development and homeostasis. *Science.* (2009) 324:392–7. doi: 10.1126/science.1170540
26. Dai XM, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, et al. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood.* (2002) 99:111–20. doi: 10.1182/blood.V99.1.111
27. Wang M, Frasch SC, Li G, Feng D, Gao B, Xu L, et al. Role of gp91(phox) in hepatic macrophage programming and alcoholic liver disease. *Hepatol Commun.* (2017) 1:765–779. doi: 10.1002/hep4.1078
28. Wang M, You Q, Lor K, Chen F, Gao B, Ju C. Chronic alcohol ingestion modulates hepatic macrophage populations and functions in mice. *J Leukoc Biol.* (2014) 96:657–65. doi: 10.1189/jlb.6A0114-004RR
29. Ingersoll MA, Spanbroek R, Lottaz C, Gautier EL, Frankenberger M, Hoffmann R, et al. Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood.* (2010) 115:e10–9. doi: 10.1182/blood-2009-07-235028
30. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity.* (2014) 40:274–88. doi: 10.1016/j.immuni.2014.01.006
31. Huebener P, Hernandez C, Schwabe RF. HMGB1 and injury amplification. *Oncotarget.* (2015) 6:23048–9. doi: 10.18632/oncotarget.5243
32. Chen RC, Hou W, Zhang QH, Kang R, Fan XG, Tang DL. Emerging role of high-mobility group box 1 (HMGB1) in liver diseases. *Mol. Med.* (2013) 19:357–66. doi: 10.2119/molmed.2013.00099
33. Huebener P, Pradere JP, Hernandez C, Gwak GY, Caviglia JM, Mu X, et al. The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis. *J Clin Invest.* (2015) 125:539–50. doi: 10.1172/JCI76887
34. Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J Exp Med*. (2005) 201:1135–43. doi: 10.1084/jem.20042614
35. Arrese M, Cabrera D, Kalergis AM, Feldstein AE. Innate immunity and inflammation in NAFLD/NASH. *Dig Dis Sci.* (2016) 61:1294–303. doi: 10.1007/s10620-016-4049-x
36. Li L, Chen L, Hu L, Liu Y, Sun HY, Tang J, et al. Nuclear factor high-mobility group box1 mediating the activation of Toll-like receptor 4 signaling in hepatocytes in the early stage of nonalcoholic fatty liver disease in mice. *Hepatology.* (2011) 54:1620–30. doi: 10.1002/hep.24552
37. Martin-Murphy BV, Holt MP, Ju C. The role of damage associated molecular pattern molecules in acetaminophen-induced liver injury in mice. *Toxicol Lett.* (2010) 192:387–94. doi: 10.1016/j.toxlet.2009.11.016
38. Wallin RP, Lundqvist A, More SH, von Bonin A, Kiessling R, Ljunggren HG. Heat-shock proteins as activators of the innate immune system. *Trends Immunol.* (2002) 23:130–5. doi: 10.1016/S1471-4906(01)02168-8
39. Fiorucci S, Biagioli M, Zampella A, Distrutti E. Bile acids activated receptors regulate innate immunity. *Front Immunol.* (2018) 9:1853. doi: 10.3389/fimmu.2018.01853
40. Perino A, Schoonjans K. TGR5 and Immunometabolism: Insights from Physiology and Pharmacology. *Trends Pharmacol Sci.* (2015) 36:847–57. doi: 10.1016/j.tips.2015.08.002
41. Reich M, Klindt C, Deutschmann K, Spomer L, Haussinger D, Keitel V. Role of the G protein-coupled bile acid receptor TGR5 in liver damage. *Dig Dis.* (2017) 35:235–40. doi: 10.1159/000450917
42. Lou G, Ma X, Fu X, Meng Z, Zhang W, Wang YD, et al. GPBAR1/TGR5 mediates bile acid-induced cytokine expression in murine Kupffer cells. *PLoS ONE.* (2014) 9:e93567. doi: 10.1371/journal.pone.0093567

43. Wang YD, Chen WD, Yu D, Forman BM, Huang W. The G-protein-coupled bile acid receptor, Gpbar1 (TGR5), negatively regulates hepatic inflammatory response through antagonizing nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) in mice. *Hepatology*. (2011) 54:1421–32. doi: 10.1002/hep.24525
44. Huang H, Evankovich J, Yan W, Nace G, Zhang L, Ross M, et al. Endogenous histones function as alarmins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. *Hepatology*. (2011) 54:999–1008. doi: 10.1002/hep.24501
45. Pan J, Ou Z, Cai C, Li P, Gong J, Ruan XZ, et al. Fatty acid activates NLRP3 inflammasomes in mouse Kupffer cells through mitochondrial DNA release. *Cell Immunol*. (2018) 332:111–20. doi: 10.1016/j.cellimm.2018.08.006
46. Rao R. Endotoxemia and gut barrier dysfunction in alcoholic liver disease. *Hepatology*. (2009) 50:638–44. doi: 10.1002/hep.23009
47. Rivera CA, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. *J Hepatol*. (2007) 47:571–9. doi: 10.1016/j.jhep.2007.04.019
48. Emontzpohl C, Stoppe C, Theissen A, Beckers C, Neumann UP, Lurje G, et al. The role of macrophage migration inhibitory factor in remote ischemic conditioning induced hepatoprotection in a rodent model of liver transplantation. *Shock*. (2019) 52:e124–e34. doi: 10.1097/SHK.0000000000001307
49. Bernal W, Auzinger G, Dhawan A, Wendon J. Acute liver failure. *Lancet*. (2010) 376:190–201. doi: 10.1016/S0140-6736(10)60274-7
50. Sehgal R, Patra S, David P, Vyas A, Khanam A, Hissar S, et al. Impaired monocyte-macrophage functions and defective Toll-like receptor signaling in hepatitis E virus-infected pregnant women with acute liver failure. *Hepatology*. (2015) 62:1683–96. doi: 10.1002/hep.28143
51. Zhang M, Xu S, Han Y, Cao X. Apoptotic cells attenuate fulminant hepatitis by priming Kupffer cells to produce interleukin-10 through membrane-bound TGF-beta. *Hepatology*. (2011) 53:306–16. doi: 10.1002/hep.24029
52. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci USA*. (2012) 109:E3186–95. doi: 10.1073/pnas.1119964109
53. Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, et al. Macrophage-derived Wnt opposes notch signaling to specify hepatic progenitor cell fate in chronic liver disease. *Nat Med*. (2012) 18:572–9. doi: 10.1038/nm.2667
54. Alisi A, Carsetti R, Nobili V. Pathogen- or damage-associated molecular patterns during nonalcoholic fatty liver disease development. *Hepatology*. (2011) 54:1500–2. doi: 10.1002/hep.24611
55. Evankovich J, Cho SW, Zhang R, Cardinal J, Dhupar R, Zhang L, et al. High mobility group box 1 release from hepatocytes during ischemia and reperfusion injury is mediated by decreased histone deacetylase activity. *J Biol Chem*. (2010) 285:39888–97. doi: 10.1074/jbc.M110.128348
56. Mihm S. Danger-Associated Molecular Patterns (DAMPs): Molecular Triggers for Sterile Inflammation in the Liver. *Int J Mol Sci*. (2018) 19:E3104. doi: 10.3390/ijms19103104
57. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature*. (2003) 425:516–21. doi: 10.1038/nature01991
58. Antoine DJ, Williams DP, Kipar A, Jenkins RE, Regan SL, Sathish JG, et al. High-mobility group box-1 protein and keratin-18, circulating serum proteins informative of acetaminophen-induced necrosis and apoptosis *in vivo*. *Toxicol Sci*. (2009) 112:521–31. doi: 10.1093/toxsci/kfp235
59. Li M, Cai SY, Boyer JL. Mechanisms of bile acid mediated inflammation in the liver. *Mol Aspects Med*. (2017) 56l:45–53. doi: 10.1016/j.mam.2017.06.001
60. Kariko K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem*. (2004) 279:12542–50. doi: 10.1074/jbc.M310175200
61. Cavassani KA, Ishii M, Wen H, Schaller MA, Lincoln PM, Lukacs NW, et al. TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. *J Exp Med*. (2008) 205:2609–21. doi: 10.1084/jem.20081370
62. Calderwood SK, Gong J, Murshid A. Extracellular HSPs: the complicated roles of extracellular HSPs in immunity. *Front Immunol*. (2016) 7:159. doi: 10.3389/fimmu.2016.00159
63. Nomura J, So A, Tamura M, Busso N. Intracellular ATP decrease mediates NLRP3 inflammasome activation upon nigericin and crystal stimulation. *J Immunol*. (2015) 195:5718–24. doi: 10.4049/jimmunol.1402512
64. Baron L, Gombault A, Fanny M, Villeret B, Savigny F, Guillou N, et al. The NLRP3 inflammasome is activated by nanoparticles through ATP, ADP and adenosine. *Cell Death Dis*. (2015) 6:e1629. doi: 10.1038/cddis.2014.576
65. Chang WJ, Toledo-Pereyra LH. Toll-like receptor signaling in liver ischemia and reperfusion. *J Invest Surg*. (2012) 25:271–7. doi: 10.3109/08941939.2012.687802
66. Gao B, Jeong WI, Tian Z. Liver: an organ with predominant innate immunity. *Hepatology*. (2008) 47:729–36. doi: 10.1002/hep.22034
67. Parlesak A, Schafer C, Schutz T, Bode JC, Bode C. Increased intestinal permeability to macromolecules and endotoxemia in patients with chronic alcohol abuse in different stages of alcohol-induced liver disease. *J Hepatol*. (2000) 32:742–7. doi: 10.1016/S0168-8278(00)80242-1
68. Iimuro Y, Gallucci RM, Luster MI, Kono H, Thurman RG. Antibodies to tumor necrosis factor alfa attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat. *Hepatology*. (1997) 26:1530–37. doi: 10.1002/hep.510260621
69. Zhong Z, Connor H, Stachlewitz RF, VonFrankenberg M, Mason RP, Lemasters JJ, et al. Role of free radicals in primary nonfunction of marginal fatty grafts from rats treated acutely with ethanol. *Mol Pharmacol*. (1997) 52:912–19. doi: 10.1124/mol.52.5.912
70. Ju C, Liangpunsakul S. Role of hepatic macrophages in alcoholic liver disease. *J Investig Med*. (2016) 64:1075–7. doi: 10.1136/jim-2016-000210
71. Thuy S, Ladurner R, Volynets V, Wagner S, Strahl S, Konigsrainer A, et al. Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake. *J Nutr*. (2008) 138:1452–5. doi: 10.1093/jn/138.8.1452
72. Baffy G. Kupffer cells in non-alcoholic fatty liver disease: the emerging view. *J Hepatol*. (2009) 51:212–23. doi: 10.1016/j.jhep.2009.03.008
73. Faure-Dupuy S, Delphin M, Aillot L, Dimier L, Lebosse F, Fresquet J, et al. Hepatitis B virus-induced modulation of liver macrophage function promotes hepatocyte infection. *J Hepatol*. (2019) 71:1086–98. doi: 10.1016/j.jhep.2019.06.032
74. Negash AA, Ramos HJ, Crochet N, Lau DT, Doehle B, Papic N, et al. IL-1beta production through the NLRP3 inflammasome by hepatic macrophages links hepatitis C virus infection with liver inflammation and disease. *PLoS Pathog*. (2013) 9:e1003330. doi: 10.1371/journal.ppat.1003330
75. Dragomir AC, Laskin JD, Laskin DL. Macrophage activation by factors released from acetaminophen-injured hepatocytes: potential role of HMGB1. *Toxicol Appl Pharmacol*. (2011) 253:170–7. doi: 10.1016/j.taap.2011.04.003
76. McDonald KA, Huang H, Tohme S, Loughran P, Ferrero K, Billiar T, et al. Toll-like receptor 4 (TLR4) antagonist eritoran tetrasodium attenuates liver ischemia and reperfusion injury through inhibition of high-mobility group box protein B1 (HMGB1) signaling. *Mol Med*. (2015) 20:639–48. doi: 10.2119/molmed.2014.00076
77. Ganz M, Szabo G. Immune and inflammatory pathways in NASH. *Hepatol Int*. (2013) 7(Suppl 2):771–81. doi: 10.1007/s12072-013-9468-6
78. McDonald B, Jenne CN, Zhuo L, Kimata K, Kubes P. Kupffer cells and activation of endothelial TLR4 coordinate neutrophil adhesion within liver sinusoids during endotoxemia. *Am J Physiol Gastrointest Liver Physiol*. (2013) 305:G797–806. doi: 10.1152/ajpgi.00058.2013
79. Trefts E, Gannon M, Wasserman DH. The liver. *Curr Biol*. (2017) 27:R1147–51. doi: 10.1016/j.cub.2017.09.019
80. Tsuchida T, Friedman SL. Mechanisms of hepatic stellate cell activation. *Nat Rev Gastro Hepat*. (2017) 14:397–411. doi: 10.1038/nrgastro.2017.38
81. Wan J, Benkdane M, Alons E, Lotersztajn S, Pavoine C. M2 kupffer cells promote hepatocyte senescence: an IL-6-dependent protective mechanism against alcoholic liver disease. *Am J Pathol*. (2014) 184:1763–72. doi: 10.1016/j.ajpath.2014.02.014
82. Momen-Heravi F, Bala S, Kodys K, Szabo G. Exosomes derived from alcohol-treated hepatocytes horizontally transfer liver specific miRNA-122 and sensitize monocytes to LPS. *Sci Rep*. (2015) 5:9991. doi: 10.1038/srep09991
83. Verma VK, Li H, Wang R, Hirsova P, Mushref M, Liu Y, et al. Alcohol stimulates macrophage activation through caspase-dependent hepatocyte

derived release of CD40L containing extracellular vesicles. *J Hepatol*. (2016) 64:651–60. doi: 10.1016/j.jhep.2015.11.020
84. Park J, Tadlock L, Gores GJ, Patel T. Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line. *Hepatology*. (1999) 30:1128–33. doi: 10.1002/hep.510300522
85. Xiao Y, Wang J, Yan W, Zhou Y, Chen Y, Zhou K, et al. Dysregulated miR-124 and miR-200 expression contribute to cholangiocyte proliferation in the cholestatic liver by targeting IL-6/STAT3 signalling. *J Hepatol*. (2015) 62:889–96. doi: 10.1016/j.jhep.2014.10.033
86. Weiskirchen R, Tacke F. Cellular and molecular functions of hepatic stellate cells in inflammatory responses and liver immunology. *Hepatobiliary Surg Nutr*. (2014) 3:344–63.
87. Mochizuki A, Pace A, Rockwell CE, Roth KJ, Chow A, O'Brien KM, et al. Hepatic stellate cells orchestrate clearance of necrotic cells in a hypoxia-inducible factor-1alpha-dependent manner by modulating macrophage phenotype in mice. *J Immunol*. (2014) 192:3847–57. doi: 10.4049/jimmunol.1303195
88. Melgar-Lesmes P, Edelman ER. Monocyte-endothelial cell interactions in the regulation of vascular sprouting and liver regeneration in mouse. *J Hepatol*. (2015) 63:917–25. doi: 10.1016/j.jhep.2015.05.011
89. Selzner N, Selzner M, Odermatt B, Tian Y, Van Rooijen N, Clavien PA. ICAM-1 triggers liver regeneration through leukocyte recruitment and Kupffer cell-dependent release of TNF-alpha/IL-6 in mice. *Gastroenterology*. (2003) 124:692–700. doi: 10.1053/gast.2003.50098
90. Saha B, Momen-Heravi F, Kodys K, Szabo G. MicroRNA cargo of extracellular vesicles from alcohol-exposed monocytes signals naive monocytes to differentiate into M2 macrophages. *J Biol Chem*. (2016) 291:149–59. doi: 10.1074/jbc.M115.694133
91. Prame Kumar K, Nicholls AJ, Wong CHY. Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease. *Cell Tissue Res*. (2018) 371:551–65. doi: 10.1007/s00441-017-2753-2
92. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol*. (2007) 25:297–336. doi: 10.1146/annurev.immunol.25.022106.141711
93. Tang T, Sui Y, Lian M, Li Z, Hua J. Pro-inflammatory activated Kupffer cells by lipids induce hepatic NKT cells deficiency through activation-induced cell death. *PLoS ONE*. (2013) 8:e81949. doi: 10.1371/journal.pone.0081949
94. You Q, Cheng L, Kedl RM, Ju C. Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. *Hepatology*. (2008) 48:978–90. doi: 10.1002/hep.22395
95. Grohmann U, Fallarino F, Bianchi R, Belladonna ML, Vacca C, Orabona C, et al. IL-6 inhibits the tolerogenic function of CD8 alpha+ dendritic cells expressing indoleamine 2,3-dioxygenase. *J Immunol*. (2001) 167:708–14. doi: 10.4049/jimmunol.167.2.708
96. Bissell DM, Wang SS, Jarnagin WR, Roll FJ. Cell-specific expression of transforming growth factor-beta in rat liver. Evidence for autocrine regulation of hepatocyte proliferation. *J Clin Invest*. (1995) 96:447–55. doi: 10.1172/JCI118055
97. Knolle PA, Uhrig A, Protzer U, Trippler M, Duchmann R, zum Buschenfelde KHM, et al. Interleukin-10 expression is autoregulated at the transcriptional level in human and murine Kupffer cells. *Hepatology*. (1998) 27:93–9. doi: 10.1002/hep.510270116
98. Chen GS, Qi HZ. Effect of Kupffer cells on immune tolerance in liver transplantation. *Asian Pac J Trop Med*. (2012) 5:970–972. doi: 10.1016/S1995-7645(12)60184-9
99. Li M, Sun R, Xu L, Yin WW, Chen YY, Zheng XD, et al. Kupffer cells support hepatitis B virus-mediated CD8(+) T cell exhaustion via hepatitis B core antigen-TLR2 interactions in mice. *J Immunol*. (2015) 195:3100–9. doi: 10.4049/jimmunol.1500839
100. Liu J, Fan L, Yu H, Zhang J, He Y, Feng D, et al. Endoplasmic reticulum stress causes liver cancer cells to release exosomal miR-23a-3p and up-regulate programmed death ligand 1 expression in macrophages. *Hepatology*. (2019) 70:241–58. doi: 10.1002/hep.30607
101. Benechet AP, De Simone G, Di Lucia P, Cilenti F, Barbiera G, Le Bert N, Fumagalli V, et al. Dynamics and genomic landscape of CD8(+) T cells undergoing hepatic priming. *Nature*. (2019) 574:200–5. doi: 10.1038/s41586-019-1620-6
102. Wen Y, Feng D, Wu H, Liu W, Li H, Wang F, et al. Defective initiation of liver regeneration in osteopontin-deficient mice after partial hepatectomy due to insufficient activation of IL-6/Stat3 pathway. *Int J Biol Sci*. (2015) 11:1236–47. doi: 10.7150/ijbs.12118
103. Cai X, Li Z, Zhang Q, Qu Y, Xu M, Wan X, et al. CXCL6-EGFR-induced Kupffer cells secrete TGF-beta1 promoting hepatic stellate cell activation via the SMAD2/BRD4/C-MYC/EZH2 pathway in liver fibrosis. *J Cell Mol Med*. (2018) 22:5050–61. doi: 10.1111/jcmm.13787
104. Nieto N. Oxidative-stress and IL-6 mediate the fibrogenic effects of [corrected] Kupffer cells on stellate cells. *Hepatology*. (2006) 44:1487–501. doi: 10.1002/hep.21427
105. Mak A, Uetrecht J. Involvement of CCL2/CCR2 macrophage recruitment in amodiaquine-induced liver injury. *J Immunotoxicol*. (2019) 16:28–33. doi: 10.1080/1547691X.2018.1516014
106. Cui KL, Yan GX, Xu CF, Chen YY, Wang J, Zhou RB, et al. Invariant NKT cells promote alcohol-induced steatohepatitis through interleukin-1 beta in mice. *J Hepatology*. (2015) 62:1311–8. doi: 10.1016/j.jhep.2014.12.027
107. Hanschen M, Zahler S, Krombach F, Khandoga A. Reciprocal activation between CD4(+) T cells and Kupffer cells during hepatic ischemia-reperfusion. *Transplantation*. (2008) 86:710–8. doi: 10.1097/TP.0b013e3181821aa7
108. Tacke F, Weiskirchen R. Liver immunology: new perspectives. *Hepatobiliary Surg Nutr*. (2014) 3:330. doi: 10.3978/j.issn.2304-3881.2014.11.07
109. Terada M, Horisawa K, Miura S, Takashima Y, Ohkawa Y, Sekiya S, et al. Kupffer cells induce Notch-mediated hepatocyte conversion in a common mouse model of intrahepatic cholangiocarcinoma. *Sci Rep*. (2016) 6:34691. doi: 10.1038/srep34691
110. Elsegood CL, Chan CW, Degli-Esposti MA, Wikstrom ME, Domenichini A, Lazarus K, et al. Kupffer cell-monocyte communication is essential for initiating murine liver progenitor cell-mediated liver regeneration. *Hepatology*. (2015) 62:1272–84. doi: 10.1002/hep.27977
111. Jeong WI, Park O, Radaeva S, Gao B. STAT1 inhibits liver fibrosis in mice by inhibiting stellate cell proliferation and stimulating NK cell cytotoxicity. *Hepatology*. (2006) 44:1441–51. doi: 10.1002/hep.21419
112. Deaciuc IV, Bagby GJ, Lang CH, Spitzer JJ. Hyaluronic acid uptake by the isolated, perfused rat liver: an index of hepatic sinusoidal endothelial cell function. *Hepatology*. (1993) 17:266–72. doi: 10.1002/hep.1840170217
113. Gregory SH, Cousens LP, van Rooijen N, Dopp EA, Carlos TM, Wing EJ. Complementary adhesion molecules promote neutrophil-Kupffer cell interaction and the elimination of bacteria taken up by the liver. *J Immunol*. (2002) 168:308–15. doi: 10.4049/jimmunol.168.1.308
114. Wu X, Sun R, Chen Y, Zheng X, Bai L, Lian Z, et al. Oral ampicillin inhibits liver regeneration by breaking hepatic innate immune tolerance normally maintained by gut commensal bacteria. *Hepatology*. (2015) 62:253–64. doi: 10.1002/hep.27791
115. Wong CH, Jenne CN, Petri B, Chrobok NL, Kubes P, Nucleation of platelets with blood-borne pathogens on Kupffer cells precedes other innate immunity and contributes to bacterial clearance. *Nat Immunol*. (2013) 14:785–92. doi: 10.1038/ni.2631

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frontiers in Immunology

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The Role of Macrophage-Inducible C-Type Lectin in Different Stages of Chronic Liver Disease

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The macrophage-inducible C-type lectin (mincle) is part of the innate immune system and acts as a pattern recognition receptor for pathogen-associated molecular patterns (PAMPS) and damage-associated molecular patterns (DAMPs). Ligand binding induces mincle activation which consequently interacts with the signaling adapter Fc receptor, SYK, and NF-kappa-B. There is also evidence that mincle expressed on macrophages promotes intestinal barrier integrity. However, little is known about the role of mincle in hepatic fibrosis, especially in more advanced disease stages. Mincle expression was measured in human liver samples from cirrhotic patients and donors collected at liver transplantation and in patients undergoing bariatric surgery. Human results were confirmed in rodent models of cirrhosis and acute-on-chronic liver failure (ACLF). In these models, the role of mincle was investigated in liver samples as well as in peripheral blood monocytes (PBMC), tissues from the kidney, spleen, small intestine, and heart. Additionally, mincle activation was stimulated in experimental non-alcoholic steatohepatitis (NASH) by treatment with mincle agonist trehalose-6,6-dibehenate (TDB). In human NASH, mincle is upregulated with increased collagen production. In ApoE deficient mice fed high-fat western diet (NASH model), mincle activation significantly increases hepatic collagen production. In human cirrhosis, mincle expression is also significantly upregulated. Furthermore, mincle expression is associated with the stage of chronic liver disease. This could be confirmed in rat models of cirrhosis and ACLF. ACLF was induced by LPS injection in cirrhotic rats. While mincle expression and downstream signaling via FC receptor gamma, SYK, and NF-kappa-B are upregulated in the liver, they are downregulated in PBMCs of these rats. Although mincle expressed on macrophages might be beneficial for intestinal barrier integrity, it seems to contribute to inflammation and fibrosis once the intestinal barrier becomes leaky in advanced stages of chronic liver disease.

Keywords: ACLF, bacterial translocation, fibrosis, inflammation, NASH

INTRODUCTION

The macrophage-inducible Ca^{2+}-dependent lectin receptor (mincle) is a primary component of the innate immune response and acts as a sensor for pathogen-associated molecular patterns (PAMPS) and damage-associated molecular patterns (DAMPS) and is expressed mainly on cell types of the myeloid lineage (e.g., macrophages) (1). Trehalose-6,6-dimycolate (TDM) stands out among the PAMPS that interact with mincle (2). The mycobacterial cell wall glycolipid has strong immunomodulatory functions. Binding of the ligand to mincle leads to interaction with the signaling adapter FC receptor gamma chain (FCER1G) (3). The formed receptor complex enables intracellular signaling molecules to dock on the immunoreceptor tyrosine-based activation motif (ITAM) and thereby transduce signaling in immune cells. The downstream signaling of the complex proceeds inter alia via spleen associated tyrosine kinase (SYK) and the nuclear factor kappa-light-chain-enhancer (NF-κB) to induce the gene expression of pro-inflammatory cytokines, chemokines, and enzymes (4, 5). On the cellular level, mincle stimulation promotes the inflammatory phenotype of mainly M1 macrophages (6, 7).

Mincle is known for its pro-inflammatory properties, especially in non-alcoholic (NASH) and alcoholic steatohepatitis (ASH) (8, 9), and might be involved in the activation of myofibroblasts (8). In NASH, mincle is predominantly reported to be involved in the formation of crown-like structures. In these structures, dying cells are surrounded by infiltrating macrophages, which promote adipose tissue inflammation and fibrosis (10, 11). Similar structures have been described in NASH livers, but not in other etiologies of chronic liver disease (12). To date, only one study has investigated the role of mincle in ASH in a murine model with mild fibrosis. This study identified Kupffer cells, the macrophages of the liver, as the main source for mincle expression. The inhibition of downstream signaling by SYK inhibitors led to decreased production of inflammatory markers (9).

In more advanced stages of chronic liver disease, an impaired intestinal barrier followed by bacterial translocation into the blood circulation are important events in the development from stable liver cirrhosis toward acute-on-chronic liver failure (ACLF) (13). Infiltrating bacteria or bacterial products lead to chronic systemic inflammation (14). Mincle possibly plays an important role in this process in two respects, namely integrity of the intestinal barrier and systemic inflammation. It recognizes gut bacteria that penetrate the Peyer's patches and induces the activation of immune cells to reinforce the intestinal barrier and thus prevents systemic inflammation. Furthermore, mincle deficiency leads to increased bacterial translocation and hepatic inflammation (15). Since ACLF is accompanied by a high short-term mortality of 30% within 28 days (16), targeting mincle in advanced chronic liver disease potentially restores intestinal barrier integrity and reduces bacterial translocation, thereby possibly preventing ACLF development.

Since to date, there are only a few reports published about the role of mincle in fibrosis and none about its role in more advanced stages of chronic liver injury, this study aims to assess the role of mincle in cirrhosis and ACLF in the liver and in other organs that are highly affected by systemic inflammation.

MATERIALS AND METHODS

Human Liver Samples

Liver samples from cirrhotic patients were taken during liver transplantation at the University of Bonn between 1999 and 2005. Liver samples of non-cirrhotic donors were used as controls. This study was approved by the local ethics committee of the University of Bonn (029/13).

Liver samples of NASH patients were taken during bariatric surgery at the St. Franziskus-Hospital (Cologne, Germany) between 2018 and 2019. NASH was diagnosed in liver biopsies independently by two experienced pathologists using the non-alcoholic fatty liver disease activity score after Kleiner (17). Non-alcoholic fatty liver diseases were ruled out for control individuals with <5% of parenchymal steatosis. NASH patients had a Kleiner score of 6 or 7, with at least 33% of parenchymal steatosis, hepatocyte ballooning, lobular inflammation, and grade 2 or 3 fibrosis. This study was approved by the ethics committees of the North Rhine-Westphalian Chamber of Medicine (2017110) and of the University of Bonn (194/17).

Liver samples were immediately stored at −80°C and cut on dry ice to avoid thawing. The studies were performed in accordance with the declaration of Helsinki and patients gave their written informed consent.

Animals

Twelve-week-old apolipoprotein E knockout mice ($ApoE^{-/-}$, C57BL/6J background; Charles River, Wilmington, USA) were used for induction of experimental NASH.

For induction of chronic liver disease and ACLF, male wild type Sprague Dawley rats with an initial body weight of 180–200 g were used. Rats were fed standard rat chow (Ssniff, Soest, Germany).

All animals received water and chow *ad libitum*. The animals were kept in individually ventilated cages at 22°C with a 12 h day/night cycle. All experiments were performed in accordance with the German Animal Protection Law and the guidelines of the animal care unit at the University of Bonn (Haus für experimentelle Therapie, Bonn, Germany) and approved by the relevant North Rhine-Westphalian state agency for Nature, Environment, and Consumer Protection (LANUV, Germany) (LANUV84-02.04.2014.A137).

Induction of Experimental NASH and Administration of Mincle Agonist TDB

Mice were fed a high-fat, cholesterol-rich diet (western diet; WD) containing 21% fat (with coconut oil), 19.5% casein, and 1.25% cholesterol (Ssniff, Soest, Germany) for seven weeks to induce NASH as described previously (18–21).

Trehalose-6,6-dibehenate (TDB) is a synthetic analog of the mycobacterial cell wall glycolipid of trehalose-6,6-dimycolate (TDM). TDB (dose 50 μg, Invitrogen, Karlsbad, CA, USA) was injected s.c. once per week for 7 weeks. Control mice received solvent (sodium chloride).

Induction of Experimental Cirrhosis and Acute-on-Chronic Liver Failure

To induce cirrhosis, bile duct ligation (BDL) was performed as a model for cholestatic liver disease as described previously (22, 23). To induce ACLF, rats received a single intraperitoneal dose of lipopolysaccharide (LPS from *E. coli* O111:B4, Sigma–Aldrich, St. Louis, USA, 6.25 mg/kg body weight) 25 days after BDL and were sacrificed 72 h after injection. BDL and sham-operated rats without LPS injection served as controls. Sodium chloride was used as solvent.

Tissue and Blood Collection

At the end of the experiment, animals were anesthetized and laparotomy was performed for tissue collection. Liver, kidney, spleen, small intestine, and heart were stored at −80°C until further use as described previously (18, 24). Blood samples were collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) for isolation of peripheral blood mononuclear cells (PBMC). PBMCs were isolated by density gradient centrifugation using Pancoll (PAN-Biotec, Aidenbach, Germany) as described previously (25). Cells were suspended in RPMI 1640 media with 10% fetal calf serum and 10% dimethyl sulfoxide (Gibco, Carlsbad, USA) and stored at −80°C until further use.

Human liver samples were snap-frozen and stored at −80°C following excision as described previously (26).

Hepatic Hydroxyproline Content

Hydroxyproline content measurement was performed as described previously (24, 26). Briefly, analog segments of 200 mg snap-frozen liver samples were dissolved and homogenized in 12 N hydrochloric acid at 110°C. Homogenized samples were later dissolved in methanol, oxidized with chloramine T, and finally reacted with Ehrlich's reagent. Samples were measured photometrically at 558 nm.

Quantitative Polymerase Chain Reaction

RNA isolation and quantitative polymerase chain reaction (qPCR) were performed as described previously (24). Briefly, total RNA was isolated with ReliaPrep RNA Miniprep Systems and cDNA synthesis was performed by the ImProm-II Reverse Transcription System (both Promega, Madison, WI, USA). DNase digestion was performed to dispose of genomic DNA. TaqMan gene expression assays (Thermo Fisher Scientific, Waltham, MA, USA) were used (**Supplemental Table 1**) for qPCR according to the manufacturer's protocol on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Experiments were carried out in duplicates. Gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method and results were standardized against 18S rRNA expression. Gene expression levels were shown as x-fold expression compared with the respective control group.

Transcriptome Analysis

Transcriptome analysis was performed by OakLabs (Hennigsdorf, Germany) using the Agilent Microarray XS (Agilent Technologies, Santa Clara, USA). Briefly, Low Input QuickAmp Labeling Kit (Agilent Technologies, Santa Clara, USA) was used to create fluorescent complementary RNA (cRNA) followed by hybridization to microarrays using the Gene Expression Hybridization Kit (Agilent Technologies, Santa Clara, USA). Fluorescence signals were detected using SureScan Microarray Scanner (Agilent Technologies, Santa Clara, USA).

Western Blotting

Protein levels were analyzed by Western blot as described previously (24, 26). Briefly, snap-frozen livers were homogenized and diluted. The protein content of homogenates was determined with the DC assay kit (Bio-Rad, Munich, Germany). Forty micrograms of protein samples was subjected to SDS-PAGE under reducing conditions (10% gels), and proteins were blotted on nitrocellulose membranes. The membranes were blocked and incubated with primary antibody against mincle (NBP1-49311, Novus Biologicals, Littleton, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an endogenous control (sc-166545 for human samples and sc-47724 for rodent samples; both Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with the corresponding secondary antibody, and blots were developed using enhanced chemiluminescence. Protein quantification was performed by ImageJ (version 1.51q, NIH, USA) and results were corrected for GAPDH levels.

Statistics

Statistical analyses were performed using Prism V.5.0 (GraphPad, San Diego, CA). Data were expressed as mean ± standard error of the mean (SEM). Groups were tested by Shapiro–Wilk-test for normal distribution. Comparisons between two groups were done by unpaired *t*-test or by non-parametric Mann–Whitney *U t*-tests. Correlations were performed using SPSS V25 (IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY, USA). Statistical analysis of patient characteristics and gene expressions were done by calculation of Spearman's correlation coefficient with *p*-value. $P < 0.05$ were considered statistically significant.

For transcriptome analysis, statistical parameters were computed between groups, and results are shown as log2-fold change and visualized by heatmaps. *P*-values were calculated using paired *t*-test and corrected according to the adaptive Benjamini–Hochberg procedure. A FDR-adjusted *p*-value below 0.05 was considered statistically significant.

RESULTS

Mincle in NASH

The expression of mincle (*gene CLEC4E*) was assessed in human liver samples from patients with NASH who underwent bariatric surgery and in liver samples from donors for liver transplantation. Mincle shows a tendency toward upregulation in patients with NASH without reaching statistical significance (**Figure 1A**). NASH patients show significantly higher fibrogenesis (**Figure 1B**), as demonstrated by collagen 1 (gene *COL1A1*), and inflammation (**Figure 1C**) than control patients, as demonstrated by macrophage and Kupffer cell marker EMR1 (gene *ADGRE1*). Furthermore, there is a trend toward upregulation of αSMA (gene *ACTA2*), a surrogate marker of hepatic stellate cell activation (**Figure 1D**). Interestingly, mincle expression is upregulated with increased collagen 1 expression

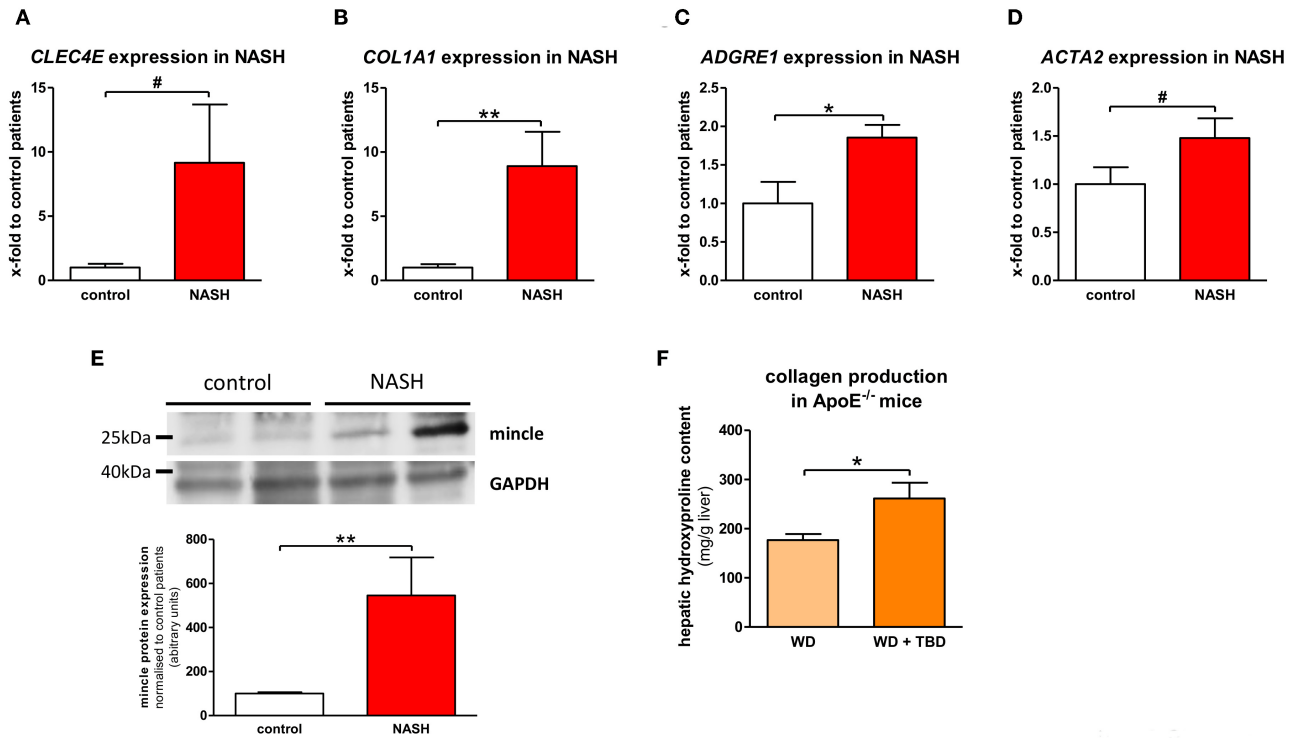

FIGURE 1 | Mincle in NASH. **(A–D)** Gene expression of mincle [gene *CLEC4E*; **(A)**], collagen [gene *COL1A1*; **(B)**], EMR1 [gene *ADGRE1*; **(C)**], and αSMA [gene *ACTA2*; **(D)**] in liver samples of non-alcoholic steatohepatitis (NASH) patients and non-NASH controls (control). **(E)** Representative western blot showing protein levels of mincle in NASH patients and non-NASH controls and quantification of the Western blots. **(F)** Hepatic collagen production, shown by measurement of hepatic hydroxyproline content, in ApoE$^{-/-}$ fed WD with or without TDB treatment. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#}p < 0.1$ using unpaired *t*-test **(A,D)** or non-parametric Mann–Whitney *U t*-test **(B,C,E,F)** after testing for normal distribution.

($r = 0.550$, $p \leq 0.001$, **Supplemental Figure 1A**) and with EMR1 expression ($r = 0.398$, $p = 0.082$, **Supplemental Figure 1B**).

In line with gene expression results, mincle protein was significantly upregulated in liver samples of NASH patients in comparison to liver samples of control patients (**Figure 1E**). To further substantiate a close interaction of mincle and fibrosis in NASH, we injected the mincle ligand Trehalose-6,6-dibehenate (TDB) in a murine model of NASH that was established in our lab (18) to stimulate mincle signaling. TDB is a synthetic analog of the mycobacterial cell wall glycolipid TDM. TDB significantly increases hepatic collagen accumulation in comparison to untreated littermates as shown by measurement of the hepatic hydroxyproline, a major component of collagen (**Figure 1F**).

Hepatic Mincle in Cirrhosis and ACLF

Since the upregulation of mincle and its activity enhances hepatic fibrogenesis in NASH, the more severe stages of chronic liver disease were investigated. Mincle expression was highly upregulated in human liver samples of cirrhotic patients compared to donor livers and is associated with the severity of chronic liver disease assessed by Child–Pugh score, reflecting the prognosis of cirrhosis (**Figure 2A**). Furthermore, hepatic mincle expression in cirrhotic patients is inversely associated with platelet count ($r = -0.372$, $p = 0.023$), as a surrogate for the severity of portal hypertension.

ACLF develops from liver cirrhosis and is associated with systemic inflammation, multi-organ failure, and high short-term mortality. To assess hepatic levels of mincle in this condition, ACLF was triggered by lipopolysaccharide (LPS) administration in a well-established rat model of cholestatic cirrhosis. Protein levels of mincle were increased in liver samples of cirrhotic and ACLF rats in comparison to sham-operated control animals (**Figure 2B**).

The rodent models of cirrhosis and ACLF were also used to examine downstream signaling of mincle via FC receptor gamma chain (gene *Fcer1g*), spleen associated tyrosine kinase (gene *Syk*), caspase recruitment domain family member 9 (gene *Card9*) and nuclear factor kappa B (genes *Nfkb1* and *NFkb2*) in liver and in circulating immune cells via transcriptome analysis (**Figure 2C**). In liver samples, the components of the mincle downstream signaling were significantly upregulated in both cirrhosis and ACLF compared to sham-operated control animals (**Figure 2D**). Upregulation of the downstream signaling in experimental cirrhosis and ACLF were confirmed by qPCR for *Fcer1g* and *Nfkb1* (**Figure 2E**). In PBMC, the expression

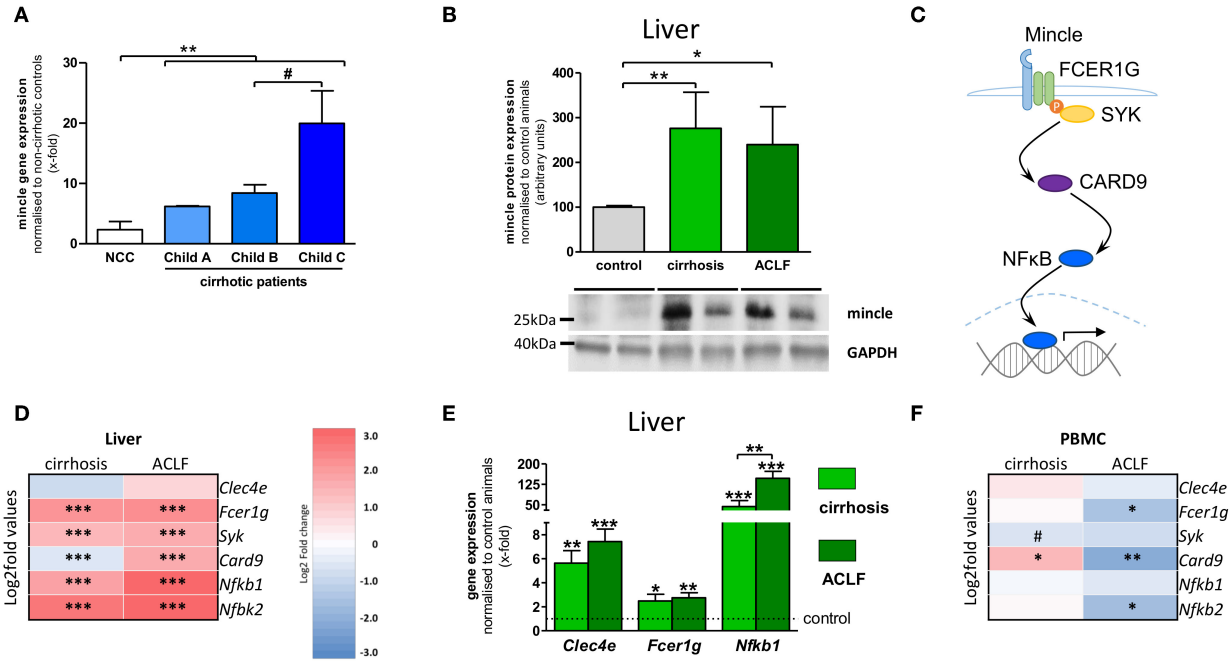

FIGURE 2 | Hepatic mincle in cirrhosis and ACLF. **(A)** Gene expression of mincle (gene *CLEC4E*) in cirrhotic patients stratified by severity of chronic liver disease compared to non-cirrhotic controls (NCC). **(B)** Representative Western blot showing protein levels of mincle in control, cirrhotic induced by bile-duct ligation (BDL), and rats with acute-on-chronic liver failure (ACLF) induced by lipopolysaccharide (LPS) within cirrhosis and quantification of the Western blots. **(C)** Diagram of mincle downstream signaling via FC receptor gamma chain (FCER1G), spleen associated tyrosine kinase (SYK), caspase recruitment domain family member 9 (CARD9) and nuclear factor kappa B (NFκB) leading to expression of pro-inflammatory genes. **(D)** Heatmap of mincle and downstream signaling maker expression in cirrhotic and ACLF livers. **(E)** Gene expression analysis of *Clec4e*, *Fcer1g*, and *Nfkb1* by qPCR in cirrhotic and ACLF liver compared to sham-operated control. **(F)** Heatmap of mincle and downstream signaling maker expression in cirrhotic and ACLF peripheral blood mononuclear cells (PBMC). Arrows indicate the respective band used for quantification in cases when more than one band is shown in the representative Western blots. In heatmaps, statistical comparison was performed between cirrhosis or ACLF and sham-operated control rats. Upregulation is marked by red colors and downregulation by blue colors. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.1$ using non-parametric Mann–Whitney *U t*-test or FDR-adjusted paired *t*-test for data from microarray assay.

profile of mincle signaling was almost unchanged in cirrhosis. By contrast, a clear downregulation of the gene was observed in ACLF (**Figure 2F**).

There is a clear dysregulation of mincle between liver and PBMCs in cirrhosis and ACLF. Since cirrhosis and ACLF are systemic diseases we also investigated mincle and its downstream signaling in extrahepatic tissues.

Mincle in Extrahepatic Tissues in Cirrhosis and ACLF

Inflammation in cirrhosis and, especially, in ACLF affects extrahepatic organs (kidney, spleen) but is also maintained and triggered by them (e.g., bacterial translocation from the intestine). Therefore, mincle expression and signaling profile were investigated in extrahepatic tissues. In spleen, protein levels of mincle significantly increased in cirrhosis and showed a stronger enhancement in ACLF (**Figure 3A**). The downstream signaling profile was similar to the one observed in PBMC, with minor changes of gene expression in cirrhosis but a significant downregulation of the signaling components in ACLF (**Figure 3B**). This could be mainly confirmed in qPCR experiments. However, it seems that there was also a mincle-independent splenic upregulation of *Nfkb1 in ACLF* (**Figure 3C**). In kidney, protein levels of mincle increased in cirrhosis and in ACLF (**Figure 3D**). As with higher renal mincle levels in cirrhosis and ACLF, downstream signaling profile showed an upregulation of the components in cirrhosis and in ACLF (**Figure 3E**) and was confirmed by pPCR (**Figure 3F**). In tissue samples of the small intestine, protein levels of mincle seemed to bring about a relative increase comparable to the one observed in spleen and kidney tissue samples (**Figure 3G**). However, in transcriptome analysis of the small intestine, the mincle upregulation failed to translate into an upregulation of the downstream signaling (**Figure 3H**). Results from qPCR confirmed the transcriptome analysis, except for a mincle-independent upregulation of *Nfkb1* (**Figure 3I**). In heart tissue, protein levels of mincle showed a similar increase in the disease stages from healthy control via cirrhosis to ACLF (**Figure 3J**). The downstream signaling profile showed a mild increase, at least in some of the components in cirrhosis, but a marked significant increase in ACLF (**Figure 3K**). These results could be confirmed by qPCR experiments (**Figure 3L**).

Taken together, protein levels of mincle are increased in cirrhosis and ACLF in all observed organs, with the highest expression in ACLF. However, this upregulation does not lead to enhanced downstream signaling in all organs. It rather seems that there are two different expression profiles, one

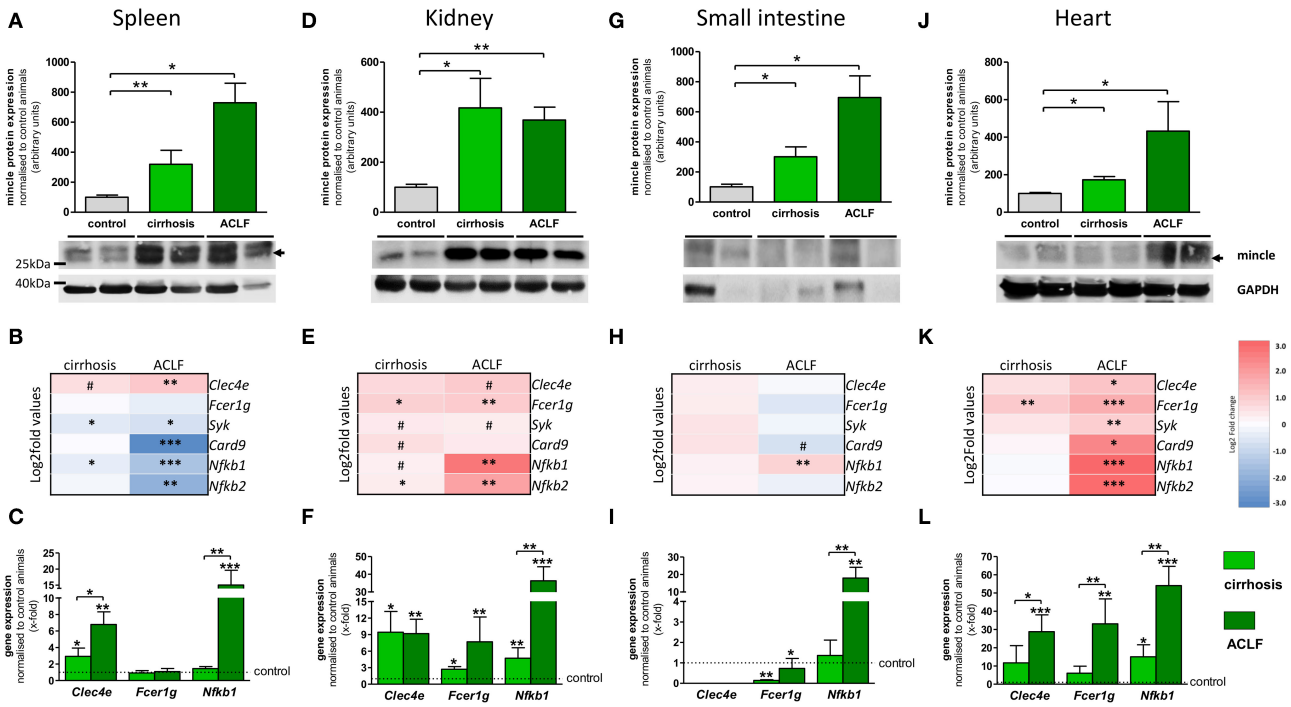

FIGURE 3 | Extrahepatic mincle in cirrhosis and ACLF. **(A–C)** Mincle in the spleen. **(A)** Representative Western blot showing protein levels of mincle in control, cirrhotic, and ACLF rats and quantification of the Western blots. **(B)** Heatmap of mincle and downstream signaling maker expression in cirrhosis and ACLF. **(C)** Gene expression of Clec4e, Fcer1g and Nfkb1 in cirrhosis and ACLF. **(D–F)** Mincle in kidney. **(D)** Representative Western blot showing protein levels of mincle in control, cirrhotic, and ACLF rats and quantification of the Western blots. **(E)** Heatmap of mincle and downstream signaling maker expression in cirrhotic and ACLF. **(F)** Gene expression of Clec4e, Fcer1g, and Nfkb1 in cirrhosis and ACLF. **(G–I)** Mincle in small intestine. **(G)** Representative Western blot showing protein levels of mincle in control, cirrhotic and ACLF rats and quantification of the Western blots. **(H)** Heatmap of mincle and downstream signaling maker expression in cirrhotic and ACLF. **(I)** Gene expression of Clec4e, Fcer1g, and Nfkb1 in cirrhosis and ACLF. **(J–L)** Mincle in heart. **(J)** Representative Western blot showing protein levels of mincle in control, cirrhotic, and ACLF rats and quantification of the Western blots. **(K)** Heatmap of mincle and downstream signaling maker expression in cirrhotic and ACLF. **(L)** Gene expression of Clec4e, Fcer1g, and Nfkb1 in cirrhosis and ACLF. Arrows indicate the respective band used for quantification in cases when more than one band is shown in the representative Western blots. In heatmaps, statistical comparison was performed between cirrhosis, respectively, ACLF and sham-operated control rats. Upregulation is marked by red colors and downregulation by blue colors. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.1$ using using non-parametric Mann–Whitney *U t*-test or FDR-adjusted paired *t*-test for data from microarray assay.

that shows a marked upregulation of the mincle downstream signaling, as in liver, kidney, and heart, and one that shows a dysregulation of the downstream signaling, as in PBMC, spleen, and the small intestine. Gene expression experiments revealed a mincle-independent increase of *Nfkb1* in organs with dysregulated downstream signaling, which, however, was not as prominent as *Nfkb1* upregulation in organs with mincle-dependent upregulation of the downstream signaling, such as liver, kidney, and heart.

DISCUSSION

This study describes for the first time the mincle expression in different stages of chronic liver disease especially in more advanced stages of chronic liver disease and ACLF. Interestingly, the downstream signaling of mincle under these conditions seems not systemic. While mincle signaling is downregulated in compartments of the immune system and the intestinal barrier, it is upregulated in the other observed tissues including the liver.

To date, very little is known about the role of mincle in chronic liver disease, particularly its hepatic function. In NASH, mincle is involved in the formation of crown-like structures, in which infiltrating macrophages attracted from dying adipocytes surround these cells and thereby promote adipose tissue inflammation and fibrosis (10, 11). Similar structures have also been identified in liver tissue in experimental and human NASH when macrophages aggregate around hepatocytes that contain large lipid droplets, which significantly correlated with fibrosis. Of note, these structures were primarily described for NASH—but rarely for viral hepatitis—as a general mechanism for chronic liver diseases. However, the impact of mincle on the progression of liver disease was not investigated (12). This study complements previous findings by providing additional data, especially in human samples and provides evidence that mincle is upregulated in NASH and its pro-fibrotic properties.

Moreover, mincle seems to play a role in chronic liver diseases in general. Our study confirms previous data from the murine model (9) and demonstrates that mincle is also upregulated in more advanced stages of human chronic liver

disease and that it is associated with disease progression regardless of etiology. The correlation of mincle expression with Child-Pugh score and platelet count as readouts for portal hypertension, suggest that mincle is associated with the major complication in cirrhosis promoting the development of systemic inflammation and ACLF. Thereby, the degree of activation of systemic inflammation can be used as a predictor for disease progression and mortality. While in decompensated cirrhosis, a partial activation of systemic inflammation with low short-term mortality can be observed, ACLF is characterized by complete activation of systemic inflammation and high short-term mortality (27–29). The upregulation of inflammatory markers is mediated primarily by myeloid cells and is associated with activation of the immune response due to bacterial translocation (30–32). Therefore, the assessment of mincle in ACLF was of special interest for this study, since systemic inflammation concurs with local inflammation in the liver. Interestingly, Martínez-López et al. (15) reported that mincle deficient mice showed increased bacterial translocation into the circulation and increased hepatic inflammation. In fact, the current study demonstrates a dysregulated mincle downstream signaling in compartments of the immune system and intestinal barrier in ACLF, namely PBMC, spleen and the small intestine. In previous studies, we could demonstrate a specific blood microbiome in decompensated cirrhosis (31). Therefore, the dysregulated mincle signaling might contribute to bacterial translocation and the development toward ACLF. Thus, there exists a clear relationship between portal hypertension and development of ACLF mediated by systemic inflammation (13, 33). This relationship is supported by the fact that systemic inflammation can be reduced if the underlying portal hypertension is ameliorated by insertion of a transjugular intrahepatic portosystemic shunt (34–36).

Although advanced chronic liver disease features systemic inflammation, "sepsis-like" immune paralysis develops in more severe stages facilitating secondary infections and organ failure (37, 38). Mincle possibly plays an important role in these apparently opposing observations, since the dysregulation of mincle signaling in spleen and PBMC, as important compartments of the immune system, could be responsible for the failure to clear invading gut bacteria and consequent systemic inflammation. This might also contribute to the compartmentalization of the immune response in decompensated cirrhosis and ACLF (32). This study proves that upregulation of mincle in ACLF is not restricted to the liver but is also found in other important compartments. However, mincle upregulation does not necessarily translate into an upregulation of the downstream signaling via FCER1G/SYK/CARD9/NFκB. Downstream signaling of mincle is differently regulated dependent on the compartment of the immune system and the intestinal barrier. This finding indicates that mincle possibly plays an important role in immune paralysis observed in ACLF. The role of mincle in spleen and small intestine upon progression of chronic liver disease remains uncertain and requires further clarification, since some of the data from the microarray could not be confirmed by qPCR. However, we can not exclude that these discrepancies in the results are due to the two different used methods as lack of convertibility between results generated by microarray or qPCR has been reported (39, 40).

The co-occurrence of complications of advanced chronic liver disease and systemic inflammation leads to extrahepatic organ dysfunction promoting the development of ACLF. Renal dysfunction in cirrhosis leads to ascites formation and implicates inflammation by bacterial infections promoting progression toward ACLF (41). Moreover, kidney failure is the most common additional organ failure in ACLF originating probably from inflammation and systemic hemodynamic instability (42). The combination of vasoconstrictor terlipressin and albumin provides the best treatment, probably by combining hemodynamic as well as immunomodulatory properties (43). Interestingly, in subarachnoid hemorrhage, a subtype of stroke, albumin treatment attenuated activation of the immune response in parenchymal macrophages of the central nervous system (44). Therefore, the interaction of albumin and mincle in tissue-resident macrophages may also be one of the beneficial effects of albumin treatment in patients with advanced chronic liver disease (38).

In addition to renal dysfunction, circulatory dysfunction also plays an important role in the development of ACLF. Reduced heart rate variability is a common feature of cirrhosis due to systemic inflammation. Heart rate variability is further reduced in patients developing ACLF and can be used to predict disease progression and short-term mortality (45). Interestingly, mincle is also expressed in cardiac tissue (46). We also observed an upregulation of mincle in cardiac tissue in the model of ACLF. This may be explained by cell dysfunction, whereby mincle induces necroptosis (46).

The major limitation of this study is that mincle expression was only measured in whole organ lysates but not in the respective resident macrophage populations of each organ.

Mincle could be another integral component in the cross-talk between macrophages and hepatic stellate cells that translates macrophage-induced fibrosis. This study opens avenues for the investigation of the processes behind dysregulated mincle signaling in different compartments of the immune system.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee at the University of Bonn and ethics committees of the North Rhine-Westphalian Chamber of Medicine. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by North Rhine-Westphalian State Agency for Nature, Environment, and Consumer Protection (LANUV, Germany).

AUTHOR CONTRIBUTIONS

RS, JT, and SK: conceptualization. RS, FU, CO, ST, MB, OT, WG, CG, SZe, AP, PP, SZi, CW, LS, KR, JT, and SK: methodology. RS, FU, CO, ST, JT, and SK: formal analysis. RS, FU, CO, JT, and SK: investigation. RS, JT, and SK: writing-original draft. RS, FU, CO, ST, MB, OT, WG, CG, SZe, AP, PP, SZi, CW, LS, KR, JT, and SK: approved final version of the manuscript. RS, JT, and SK: visualization. RS, JT, and SK: supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01352/full#supplementary-material

REFERENCES

1. Braganza CD, Teunissen T, Timmer MSM, Stocker BL. Identification and biological activity of synthetic macrophage inducible C-type lectin ligands. *Front Immunol.* (2018) 8:1940. doi: 10.3389/fimmu.2017.01940
2. Ishikawa E, Ishikawa T, Morita YS, Toyonaga K, Yamada H, Takeuchi O, et al. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *J Exp Med.* (2009) 206:2879–88. doi: 10.1084/jem.20091750
3. Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat Immunol.* (2008) 9:1179–88. doi: 10.1038/ni.1651
4. Hsu Y-MS, Zhang Y, You Y, Wang D, Li H, Duramad O, et al. The adaptor protein CARD9 is required for innate immune responses to intracellular pathogens. *Nat Immunol.* (2007) 8:198–205. doi: 10.1038/ni1426
5. Hara H, Ishihara C, Takeuchi A, Imanishi T, Xue L, Morris SW, et al. The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors. *Nat Immunol.* (2007) 8:619–29. doi: 10.1038/ni1466
6. Lv LL, Tang PM-K, Li CJ, You YK, Li J, Huang X-R, et al. The pattern recognition receptor, Mincle, is essential for maintaining the M1 macrophage phenotype in acute renal inflammation. *Kidney Int.* (2017) 91:587–602. doi: 10.1016/j.kint.2016.10.020
7. Kodar K, Harper JL, McConnell MJ, Timmer MSM, Stocker BL. The Mincle ligand trehalose dibehenate differentially modulates M1-like and M2-like macrophage phenotype and function via Syk signaling. *Immun Inflamm Dis.* (2017) 5:503–14. doi: 10.1002/iid3.186
8. Tanaka M, Ikeda K, Suganami T, Komiya C, Ochi K, Shirakawa I, et al. Macrophage-inducible C-type lectin underlies obesity-induced adipose tissue fibrosis. *Nat Commun.* (2014) 5:4982. doi: 10.1038/ncomms5982
9. Kim J-W, Roh Y-S, Jeong H, Yi H-K, Lee M-H, Lim C-W, et al. Spliceosome-associated protein 130 exacerbates alcohol-induced liver injury by inducing NLRP3 inflammasome-mediated IL-1β in mice. *Am J Pathol.* (2018) 188:967–980. doi: 10.1016/j.ajpath.2017.12.010
10. Watanabe Y, Nagai Y, Honda H, Okamoto N, Yamamoto S, Hamashima T, et al. Isoliquiritigenin attenuates adipose tissue inflammation *in vitro* and adipose tissue fibrosis through inhibition of innate immune responses in mice. *Sci Rep.* (2016) 6:23097. doi: 10.1038/srep23097
11. Ogawa Y, Suganami T, Itoh M, Tanaka M. Pathogenesis of non-alcoholic steatohepatitis its potential therapeutic strategies. In Nakao K, Minato N, Uemoto S, editors. *Innovative Medicine: Basic Research Development*. Tokyo: Springer. Available online at: http://www.ncbi.nlm.nih.gov/books/NBK500329/ (accessed October 11, 2019).
12. Itoh M, Kato H, Suganami T, Konuma K, Marumoto Y, Terai S, et al. Hepatic crown-like structure: a unique histological feature in non-alcoholic steatohepatitis in mice and humans. *PLoS One.* (2013) 8:e82163. doi: 10.1371/journal.pone.0082163
13. Trebicka J, Reiberger T, Laleman W. Gut-liver axis links portal hypertension to acute-on-chronic liver failure. *Visc Med.* (2018) 34:270–75. doi: 10.1159/000490262
14. Wiest R, Garcia-Tsao G. Bacterial translocation (BT) in cirrhosis. *Hepatology.* (2005) 41:422–33. doi: 10.1002/hep.20632
15. Martínez-López M, Iborra S, Conde-Garrosa R, Mastrangelo A, Danne C, Mann ER, et al. Microbiota sensing by Mincle-Syk axis in dendritic cells regulates interleukin-17 and−22 production and promotes intestinal barrier integrity. *Immunity.* (2019) 50:446–61.e9. doi: 10.1016/j.immuni.2018.12.020
16. Arroyo V, Moreau R, Kamath PS, Jalan R, Ginès P, Nevens F, et al. Acute-on-chronic liver failure in cirrhosis. *Nat Rev Dis Primers.* (2016) 2:16041. doi: 10.1038/nrdp.2016.41
17. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology.* (2005) 41:1313–321. doi: 10.1002/hep.20701
18. Schierwagen R, Maybüchen L, Zimmer S, Hittatiya K, Bäck C, Klein S, et al. Seven weeks of Western diet in apolipoprotein-E-deficient mice induce metabolic syndrome and non-alcoholic steatohepatitis with liver fibrosis. *Sci Rep.* (2015) 5:12931. doi: 10.1038/srep12931
19. Tiyerili V, Zimmer S, Jung S, Wassmann K, Naehle CP, Lütjohann D, et al. CB1 receptor inhibition leads to decreased vascular AT1 receptor expression, inhibition of oxidative stress and improved endothelial function. *Basic Res Cardiol.* (2010) 105:465–77. doi: 10.1007/s00395-010-0090-7
20. Hoyer FF, Steinmetz M, Zimmer S, Becker A, Lütjohann D, Buchalla R, et al. Atheroprotection via cannabinoid receptor-2 is mediated by circulating and vascular cells in vivo. *J Mol Cell Cardiol.* (2011) 51:1007–14. doi: 10.1016/j.yjmcc.2011.08.008
21. Schierwagen R, Maybüchen L, Hittatiya K, Klein S, Uschner FE, Braga TT, et al. Statins improve NASH via inhibition of RhoA and Ras. *Am J Physiol Gastrointest Liver Physiol.* (2016) 311:G724–G33. doi: 10.1152/ajpgi.00063.2016
22. Trebicka J, Hennenberg M, Odenthal M, Shir K, Klein S, Granzow M, et al. Atorvastatin attenuates hepatic fibrosis in rats after bile duct ligation

via decreased turnover of hepatic stellate cells. *J Hepatol.* (2010) 53:702–12. doi: 10.1016/j.jhep.2010.04.025
23. Trebicka J, Hennenberg M, Laleman W, Shelest N, Biecker E, Schepke M, et al. Atorvastatin lowers portal pressure in cirrhotic rats by inhibition of RhoA/Rho-kinase and activation of endothelial nitric oxide synthase. *Hepatology.* (2007) 46:242–53. doi: 10.1002/hep.21673
24. Brol MJ, Rösch F, Schierwagen R, Magdaleno F, Uschner FE, Manekeller S, et al. Combination of CCl4with alcoholic and metabolic injuries mimics human liver fibrosis. *Am J Physiol Gastrointest Liver Physiol.* (2019) 317:G182–G94. doi: 10.1152/ajpgi.00361.2018
25. Beyer M, Abdullah Z, Chemnitz JM, Maisel D, Sander J, Lehmann C, et al. Tumor-necrosis factor impairs $CD4^+$ T cell-mediated immunological control in chronic viral infection. *Nat Immunol.* (2016) 17:593–603. doi: 10.1038/ni.3399
26. Granzow M, Schierwagen R, Klein S, Kowallick B, Huss S, Linhart M, et al. Angiotensin-II type 1 receptor-mediated Janus kinase 2 activation induces liver fibrosis. *Hepatology.* (2014) 60:334–48. doi: 10.1002/hep.27117
27. Trebicka J, Amoros A, Pitarch C, Titos E, Alcaraz-Quiles J, Schierwagen R, et al. Addressing profiles of systemic inflammation across the different clinical phenotypes of acutely decompensated cirrhosis. *Front Immunol.* (2019) 10:476. doi: 10.3389/fimmu.2019.00476
28. Clària J, Stauber RE, Coenraad MJ, Moreau R, Jalan R, Pavesi M, et al. Systemic inflammation in decompensated cirrhosis: characterization and role in acute-on-chronic liver failure. *Hepatology.* (2016) 64:1249–64. doi: 10.1002/hep.28740
29. Jansen C, Möller P, Meyer C, Kolbe CC, Bogs C, Pohlmann A, et al. Increase in liver stiffness after transjugular intrahepatic portosystemic shunt is associated with inflammation and predicts mortality. *Hepatology.* (2018) 67:1472–84. doi: 10.1002/hep.29612
30. Hackstein C-P, Assmus LM, Welz M, Klein S, Schwandt T, Schultze J, et al. Gut microbial translocation corrupts myeloid cell function to control bacterial infection during liver cirrhosis. *Gut.* (2017) 66:507–18. doi: 10.1136/gutjnl-2015-311224
31. Schierwagen R, Alvarez-Silva C, Madsen MSA, Kolbe CC, Meyer C, Thomas D, et al. Circulating microbiome in blood of different circulatory compartments. *Gut.* (2018) 68:578–80. doi: 10.1136/gutjnl-2018-316227
32. Alvarez-Silva C, Schierwagen R, Pohlmann A, Magdaleno F, Uschner FE, Ryan P, et al. Compartmentalization of immune response and microbial translocation in decompensated cirrhosis. *Front Immunol.* (2019) 10:69. doi: 10.3389/fimmu.2019.00069
33. Trebicka J. Predisposing factors in acute-on-chronic liver failure. *Semin Liver Dis.* (2016) 36:167–73. doi: 10.1055/s-0036-1583195
34. Berres M-L, Asmacher S, Lehmann J, Jansen C, Görtzen J, Klein S, et al. CXCL9 is a prognostic marker in patients with liver cirrhosis receiving transjugular intrahepatic portosystemic shunt. *J Hepatol.* (2015) 62:332–9. doi: 10.1016/j.jhep.2014.09.032
35. Lehmann JM, Claus K, Jansen C, Pohlmann A, Schierwagen R, Meyer C, et al. Circulating CXCL10 in cirrhotic portal hypertension might reflect systemic inflammation and predict ACLF and mortality. *Liver Int.* (2018) 38:875–84. doi: 10.1111/liv.13610
36. Berres M-L, Lehmann J, Jansen C, Görtzen J, Meyer C, Thomas D, et al. Chemokine (C-X-C motif) ligand 11 levels predict survival in cirrhotic patients with transjugular intrahepatic portosystemic shunt. *Liver Int.* (2016) 36:386–94. doi: 10.1111/liv.12922
37. Wasmuth HE, Kunz D, Yagmur E, Timmer-Stranghöner A, Vidacek D, Siewert E, et al. Patients with acute on chronic liver failure display "sepsis-like" immune paralysis. *J Hepatol.* (2005) 42:195–201. doi: 10.1016/j.jhep.2004.10.019
38. Laleman W, Claria J, Van der Merwe S, Moreau R, Trebicka J. Systemic inflammation and acute-on-chronic liver failure: too much, not enough. *Can J Gastroenterol Hepatol.* (2018) 2018:1027152. doi: 10.1155/2018/1027152
39. Allanach K, Mengel M, Einecke G, Sis B, Hidalgo LG, Mueller T, et al. Comparing microarray versus RT-PCR assessment of renal allograft biopsies: similar performance despite different dynamic ranges. *Am J Transpl.* (2008) 8:1006–15. doi: 10.1111/j.1600-6143.2008.02199.x
40. Etienne W, Meyer MH, Peppers J, Meyer RA. Comparison of mRNA gene expression by RT-PCR and DNA microarray. *BioTechniques.* (2004) 36:618–26. doi: 10.2144/04364ST02
41. Cárdenas A, Ginès P. Acute-on-chronic liver failure: the kidneys. *Curr Opin Crit Care.* (2011) 17:184. doi: 10.1097/MCC.0b013e328344b3da
42. Bajaj JS, Moreau R, Kamath PS, Vargas HE, Arroyo V, Reddy KR, et al. Acute-on-chronic liver failure: getting ready for prime time? *Hepatology.* (2018) 68:1621–32. doi: 10.1002/hep.30056
43. Bernardi M, Ricci CS, Zaccherini G. Role of human albumin in the management of complications of liver cirrhosis. *J Clin Exp Hepatol.* (2014) 4:302–11. doi: 10.1016/j.jceh.2014.08.007
44. Xie Y, Guo H, Wang L, Xu L, Zhang X, Yu L, et al. Human albumin attenuates excessive innate immunity via inhibition of microglial Mincle/Syk signaling in subarachnoid hemorrhage. *Brain Behav Immun.* (2017) 60:346–60. doi: 10.1016/j.bbi.2016.11.004
45. Jansen C, Chatterjee DA, Thomsen KL, Al-Kassou B, Sawhney R, Jones H, et al. Significant reduction in heart rate variability is a feature of acute decompensation of cirrhosis and predicts 90-day mortality. *Aliment Pharmacol Ther.* (2019) 50:568–79. doi: 10.1111/apt.15365
46. Soppert J, Kraemer S, Beckers C, Averdunk L, Möllmann J, Denecke B, et al. Soluble CD74 reroutes MIF/CXCR4/AKT-mediated survival of cardiac myofibroblasts to necroptosis. *J Am Heart Assoc.* (2018) 7:e009384. doi: 10.1161/JAHA.118.009384

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