THE ROLE OF MYELOID-DERIVED CELLS IN THE PROGRESSION OF LIVER DISEASE

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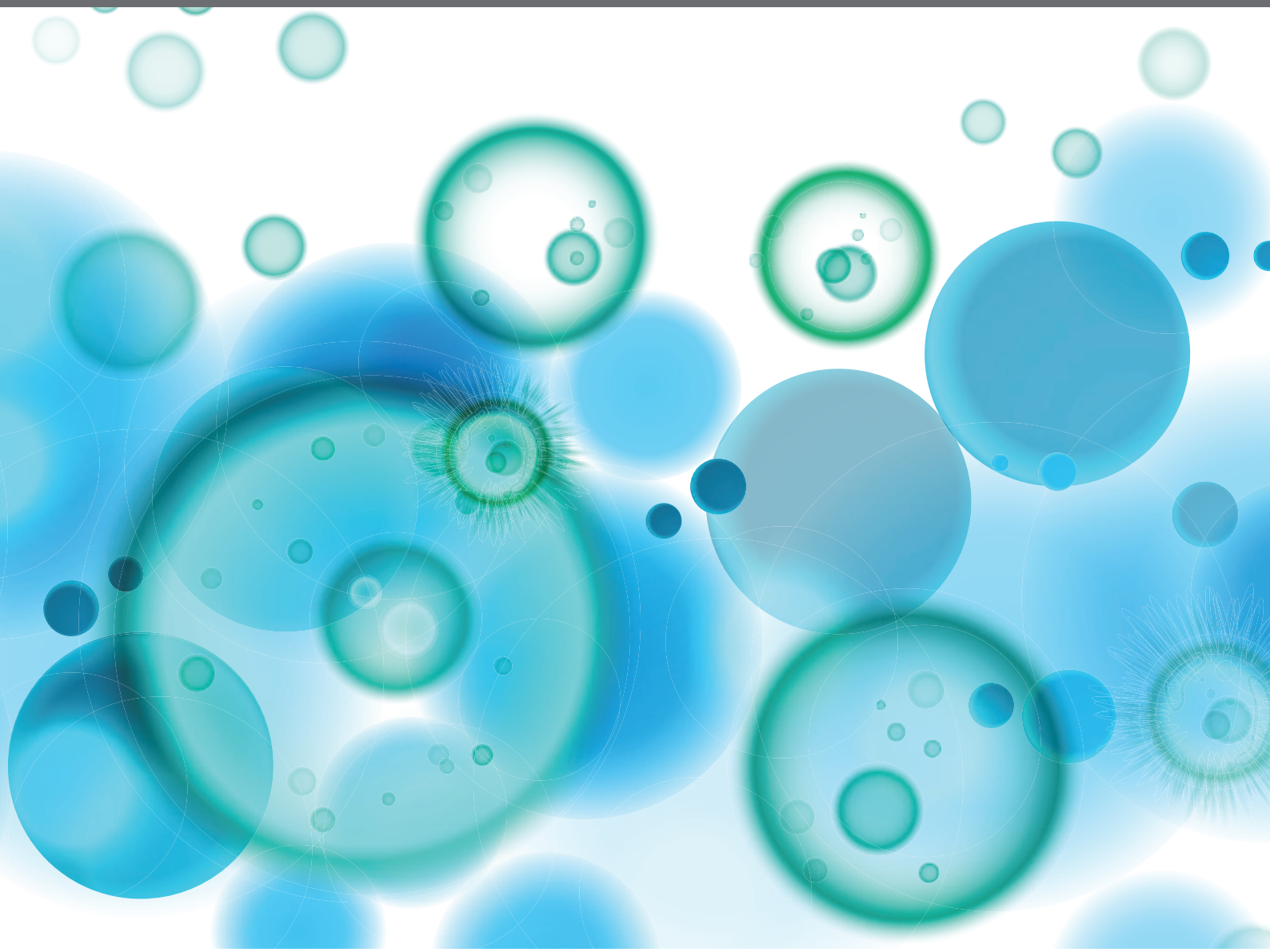

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THE ROLE OF MYELOID-DERIVED CELLS IN THE PROGRESSION OF LIVER DISEASE

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Table of Contents

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EDITORIAL
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Editorial: The Role of Myeloid-Derived Cells in the Progression of Liver Disease

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Editorial on the Research Topic

The Role of Myeloid-Derived Cells in the Progression of Liver Disease

The liver is strategically positioned to drain the intra-abdominal organs through the portal vein. As such, the liver is constantly challenged with foreign food antigens, bacterial products that require a high degree of tolerance and restraint. In chronic liver disease, when hepatocyte damage occurs, this tolerant state is often lost resulting in local and systemic inflammation and fibrosis development, which may be detrimental to the host. It is well-established that macrophages, as highly diverse immune cells, play a central role in both the initiation as well as restoration of inflammation and fibrogenesis. Macrophages can be either embryonically seeded in organs where they are maintained through self-renewal (1) or derived from infiltrating bone marrow monocyte precursors (2, 3). Regardless of their origin, they adapt to microenvironmental cues within the niche they reside in and become imprinted with a unique transcriptional signature (4, 5). Technologies such as single-cell RNA sequencing accelerated discoveries in the field by uncovering the diversity of monocyte/macrophage functions even during steady-state conditions (6). Nevertheless, our understanding of the molecular mechanisms that goes astray during disease states, resulting in the loss of macrophages' ability to maintain homeostatic functions, remains incomplete. In this special Research Topic, experts in the field dissect the landscape of myeloid derived cells as well as neutrophils during chronic liver diseases. Understanding how myeloid cells contribute to injury and repair will enable the design of new therapies.

INNATE IMMUNE CELL CROSSTALK TO GOVERN INFLAMMATION AND REPAIR DURING ACUTE AND CHRONIC LIVER INJURY

In this Research Topic, Weston et al. provide a comprehensive overview regarding the role for myeloid-derived cells during acute and chronic hepatic injury. More specifically, they describe how tissue-resident Kupffer cells, infiltrating monocytes/macrophages, dendritic cells, and neutrophils act in concert to initiate an inflammatory response but also to regenerate tissue following injury.

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Finally, they touch upon the idea that strategies targeting liver macrophages would require precision medicine to specifically target pathogenic subsets only.

OUTLINING THE EXTENT OF INNATE IMMUNE DEFECTS DURING CIRRHOSIS DEVELOPMENT AND ACUTE-ON-CHRONIC LIVER FAILURE

Patients with acute decompensated cirrhosis and acute-on-chronic liver failure (ACLF), display evidence of hepatic and systemic inflammation but paradoxically also features of immunosuppression, rendering them highly susceptible to infections. Within this Research Topic, a clinical investigation by Trebicka et al. demonstrated that the extent of systemic immune inflammation in acute decompensated cirrhotics correlates with a higher risk of disease progression toward ACLF or death of the patient. Another study by Alvarez-Silva et al. demonstrated that the levels of inflammatory cytokines and microbial richness are significantly higher in ascites fluid compared to plasma samples from patients with decompensated cirrhosis. However, the authors could not find any correlation between the bacterial DNA abundance and/or richness and the extent of systemic inflammation in these patients.

Providing some insight into the molecular mechanisms, Irvine et al. and Martin-Mateos et al. review the respective innate immune dysfuntions observed in cirrhosis and ACLF, within this Research Topic. Furthermore, Irvine et al. propose mechanisms that improve susceptibility to infections, while Martin-Mateos et al. highlights mechanisms targeting the gut, -high-grade systemic inflammation and reverting immune paralysis as therapeutic opportunities to improve clinical outcome of these patients. Also within this Research Topic, Triantafyllou et al. provide an overview of the role of monocytes/macrophages in driving systemic immunosuppression and hepatic inflammation thereby contributing to the pathophysiology of acute decompensated cirrhosis as well as ACLF. Additionally the authors describe the opportunities and challenges of therapeutic strategies aiming at reverting Kupffer cell activation, hampering monocyte recruitment to the liver or manipulating macrophage polarization to interfere in disease progression. Along the same line of investigation, Riva and Mehta provide evidence for epigenetic mechanisms, and the role of checkpoint receptors in regulating monocyte function. In combination with recent advances in the field whereby metabolic rewiring can influence immunological functions (7), these studies open up new opportunities of targetable pathways that may be exploited to improve monocyte function in ACLF.

Finally, Moreau et al. placed the spotlight on dysfunctional neutrophils during cirrhosis within the Research Topic. More particularly, they provide an overview of defects in intracellular signaling pathways, impaired activation of the NADPH oxidase complex, myeloperoxidase (MPO) release and defective bactericidal activity within neutrophils during cirrhosis. Importantly, they report some studies that suggest that defective neutrophil functions, at least *in vitro*, could be rescued by TLR7/8 agonists.

TAKING THE BREAK OFF MONOCYTES, DRIVES INFLAMMATION, AND INTESTINAL BARRIER BREACH

Patients with advanced stages of cirrhosis often exhibit a dysfunctional intestinal barrier, whereby luminal bacteria and their products translocate into the circulation and reach the liver via the portal vein (gut-liver axis). Very exciting recent research elucidate for the first time the mechanisms of intestinal barrier breach during cirrhosis (8). Nevertheless, how exactly monocytes/macrophages contribute to intestinal barrier dysfunction remains incompletely understood. Although not directly linked to an experimental liver disease model, an interesting study by Mouhadeb et al., may shed some light on this aspect by demonstrating that disruption of COMMD10, a protein with yet unknown function, unleashes the inflammatory capacity of circulating Ly6C^{hi} monocytes, resulting in intestinal barrier dysfunction and elevated bacterial translocation to the mouse liver. It would be intriguing to verify whether similar mechanisms set the stage for pathogenic bacterial translocation during cirrhosis development.

TARGETING MACROPHAGES DURING NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) is hallmarked by chronic low-grade inflammation and lipid accumulation in the liver as well as in extra-hepatic sites such as adipose tissue. Furthermore, a complex inter-organ crosstalk fuels the onset and progression of hepatic injury and fibrosis development. Within this disease setting, it is well-established that myeloid-derived cells play a prominent role in regulating inflammation and metabolism. In this Research Topic, Hundertmark et al. provide a comprehensive overview of the functional and phenotypic versatility of myeloid cells as well as the microenvironmental signals that trigger their activation during NAFLD progression. Additionally, they point out the existence of an orchestrated interplay between myeloid cells in different compartments, such as circulation, gut, adipose tissue, and the liver. Extending along the same line of investigation, Vonghia et al. review current data on experimental NAFLD treatment strategies whereby myeloid-derived cells constitute the targeted population.

In another manuscript within this Research Topic, Liao et al. demonstrated that palmitate-induced hepatocyte stress resulted in the release of extracellular vesicles enriched in the lipotoxic molecule, sphingosine 1-phosphate (S1P). These extracellular vesicles promoted macrophage chemotaxis through interaction with the S1P1 receptor on the surface of the macrophages. Although awaiting further *in vivo* confirmation, the authors suggest this to be a novel signaling axis for macrophage recruitment during NAFLD, where hepatic lipotoxicity prevails.

MACROPHAGES GOING OFF TRACK IN THE AGING LIVER

The process of aging is closely associated with a number of degenerative modifications in the liver, where hepatic structure and cell function tend to decline. In this Research Topic, Stahl et al. review macrophage deficits in mitochondrial function,—decline in autophagy and altered proinflammatory function, are discussed as possible mechanisms that may be relevant during age-related liver diseases.

NEUTROPHILS FUELING LIVER ISCHEMIA AND REPERFUSION INJURY

Hepatic ischemia/reperfusion (I/R) is an important cause of liver damage occurring during hepatic resection and liver transplantation. Neutrophils have been shown to be one of the cellular players contributing towards tissue injury, however the molecular mechanisms involved have not been completely defined. Within this Research Topic, Sun et al. implicate that MAPK-activated protein kinase 2 (MK2) contribute to hepatic I/R since its ablation protects against hepatic I/R injury in a murine model. This result implicates MK2 as a potential novel therapeutic target for I/R injury.

CONCLUSIONS

Emerging evidence demonstrated that myeloid-derived cells play a key role in the initiation, and progression of liver disease. This Research Topic provides multiple examples of how different myeloid cell subsets but also neutrophils can contribute to the inflammatory processes that underlie the clinical manifestations of various liver diseases. Such a compilation of relevant information may uncover new therapeutic targets and ultimately lead to improved outcomes in patients with advanced liver disease.

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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REVIEW
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Adapted Immune Responses of Myeloid-Derived Cells in Fatty Liver Disease

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Non-alcoholic fatty liver disease (NAFLD) is considered to be one of the most frequent chronic liver diseases worldwide and is associated with an increased risk of developing liver cirrhosis and hepatocellular carcinoma. Hepatic macrophages, mainly comprising monocyte derived macrophages and tissue resident Kupffer cells, are characterized by a high diversity and plasticity and act as key regulators during NAFLD progression, in conjunction with other infiltrating myeloid cells like neutrophils or dendritic cells. The activation and polarization of myeloid immune cells is influenced by dietary components, inflammatory signals like danger-associated molecular patterns (DAMPs) or cytokines as well as gut-derived inflammatory factors such as pathogen-associated molecular patterns (PAMPs). The functionality of myeloid leukocytes in the liver is directly linked to their inflammatory polarization, which is shaped by local and systemic inflammatory mediators such as cytokines, chemokines, PAMPs, and DAMPs. These environmental signals provoke intracellular adaptations in myeloid cells, including inflammasome and transcription factor activation, inflammatory signaling pathways, or switches in cellular metabolism. Dietary changes and obesity also promote a dysbalance in intestinal microbiota, which can facilitate intestinal permeability and bacterial translocation. The aim of this review is to highlight recent findings on the activating pathways of innate immune cells during the progression of NAFLD, dissecting local hepatic and systemic signals, dietary and metabolic factors as well as pathways of the gut-liver axis. Understanding the mechanism by which plasticity of myeloid-derived leukocytes is related to metabolic changes and NAFLD progression may provide options for new therapeutic approaches.

Keywords: NASH, innate immunity, macrophages, metabolism, steatosis, fibrosis, PAMPs, lipotoxicity

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a multifactorial disease affecting around 25% of the population in industrialized countries. It is commonly associated with conditions like insulin resistance, impaired glucose tolerance, dyslipidemia, hypertension, or atherosclerosis (1). The spectrum of the disease reaches from simple steatosis to non-alcoholic steatohepatitis (NASH) up to liver cirrhosis or hepatocellular carcinoma. It has been suggested that during NASH a state of chronic low-grade inflammation is induced as the consequence of multiple exogenous as well as endogenous hits originating from systemic changes as well as from liver, gut, and adipose tissue (2). One of the key components in the development of NAFLD is excess energy

intake that results in an altered composition of circulating fatty acids with significantly higher levels of saturated fatty acids. Superfluous peripheral fat is stored in the form of triglycerides in adipose tissue promoting the release of adipokines such as leptin and inflammatory cytokines that regulate local as well as systemic inflammatory processes (3). Likewise, the liver as the primary metabolic organ is strongly affected by increased availability of carbohydrates and fatty acids, resulting in the accumulation and storage of toxic lipids as well as hepatic stress responses, ultimately resulting in activation of immune pathways and eventually cell death (4). Immune signals are mostly recognized through pattern recognition receptors (PRRs) by myeloid immune cells that sense pattern-associated molecular patterns (PAMPs), such as gut-derived pathogens, or danger-associated-molecular-patterns (DAMPs) released from damaged or dying cells (5). Besides cytokines, external signals including toxic lipid species or elevated levels of carbohydrates can drive activation of myeloid immune cells that may result in a broad range of immune responses. Inflammatory responses often involve chemokine-mediated recruitment of innate immune cells such as neutrophils, monocytes, or dendritic cells (DCs) to sites of inflammation, thereby affecting myeloid cell composition in metabolically important organs such as liver, gut, and adipose tissue. In response to environmental stimuli, myeloid cells are able to adapt their phenotype thus exhibiting functionally versatile roles that may contribute to initiation and progression of NAFLD. In this review, we aim to delineate the various and diverse roles myeloid cells play in the progression of fatty liver diseases and present recent insights into the crosstalk between local and systemic immune signals derived from liver, gut, and adipose tissue. The mechanisms discussed in this review have been primarily unraveled in mouse models of NAFLD and NASH, while fewer data exist from human patients supporting the relevance of these mechanisms. In addition, not all principal findings on mechanisms of macrophage activation and functional responses have been validated for macrophages in the liver, which are characterized by a remarkable heterogeneity (6). Hence, macrophages derived from bone marrow monocytes (or other tissues) and liver macrophages might not share all activation pathways. Nonetheless, the tremendous progress in recent research supports a crucial and well-orchestrated role of myeloid cells in fatty liver disease, providing exciting insights into the immune pathogenesis of NAFLD and indicating promising therapeutic targets.

Abbreviations: DAMP, danger-associated-molecular pattern; DCs, dendritic cells; FXR, farnesoid X receptor; GPR, G-protein-coupled-receptor; IL, interleukin; JNK, c-jun N-terminal kinase; KC, Kupffer cell; LPS, lipopolysaccharide; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; NLRP3, NOD-like receptor protein 3; NOD, nucleotide oligomerization domain; PAMP, pathogen-associated-molecular pattern; PPAR, peroxisome proliferator-activated receptor; PRR, pattern recognition receptor; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; SCFA, short chain fatty acids; STAT, signal transducer and activator of transcription; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAIL, TNF related apoptosis inducing ligand.

ACTIVATING SIGNALS OF MYELOID IMMUNE CELLS IN FATTY LIVER DISEASE

Danger Signals

During NAFLD development, endogenous DAMPs that are released from damaged or stressed cells initiate sterile inflammation and contribute to the activation of myeloid cells (7, 8). The pathology of NASH is linked to a chronic state of sterile inflammation induced by high levels of DAMPs that lead to the activation of PRRs (**Figure 1A**). Molecules that have been recognized as danger signals in fatty liver disease comprise nuclear factors e.g., high mobility group box protein (HMGB1), nucleic acids, lipid mediators, histones, ATP, and uric acid (9). Additionally, gut-derived PAMPs such as lipopolysaccharide (LPS), bacterial DNA, or peptidoglycans can enter the enterohepatic circulation due to increased intestinal permeability and hence stimulate immune cells in the liver (5). Inflammasome-dependent mechanisms further control diversity and composition of the gut microbiota, and NAFLD-associated dysbiosis facilitates DAMPs and PAMPs entering portal circulation, thereby promoting inflammatory responses in the liver and aggravating steatohepatitis (10).

DAMPs are typically recognized by toll-like receptors (TLRs) or P2X purinoceptor 7 (P2X7) that in turn result in activation of the inflammasome (9). In mouse models of NASH, an increase in accumulation of HMGB1, uric acid, ATP, and exogenous LPS has been discovered along with increasing populations of pro-inflammatory macrophages (11). Consistent with this, increased extracellular ATP released by damaged cells was shown to stimulate the inflammasome via P2X7 receptor signaling in NASH models. Interestingly, ATP released from hepatic cells upon liver injury further resulted in the recruitment of peritoneal cavity macrophages exhibiting an anti-inflammatory like phenotype (12). HMGB1 and IL-33 are released from dying hepatocytes and showed significant upregulation in mice during NASH progression (13), in which HMGB1 activates TLR4 and the adaptor protein myeloid differentiation primary response 88 (MyD88) (14). HMGB1 was further shown to be necessary for neutrophil recruitment in acute liver injury (15). Similarly, elevated levels of HMGB1 were found in obese adipose tissue (16) and may aggravate inflammatory activation of adipose tissue macrophages. Moreover, in response to cytokine stimulation through LPS, TNF or TGF-β, HMGB1 was shown to be released by activated macrophages or DCs that contributed to the activation of pro-inflammatory pathways (17). Further, the extracellular alarmins S100A8 and S100A9 were shown to promote inflammation in monocytes and macrophages *in vivo* as well as *in vitro* via the receptor advanced glycation end-products (RAGE) and TLR4 dependent pathways in mice and men (18, 19). Most receptors targeted by DAMPs can also be activated by PAMPs, which is of special interest in the context of alterations in the gut microbiome and bacterial defense mechanisms during NAFLD. Intestinal permeability in NASH further favors translocation of microbial products such as bacterial DNA, LPS from gram-negative bacteria or β-Glucan from fungi that may act as microbial-associated molecular patterns (MAMPs) or PAMPs. Indeed, several studies

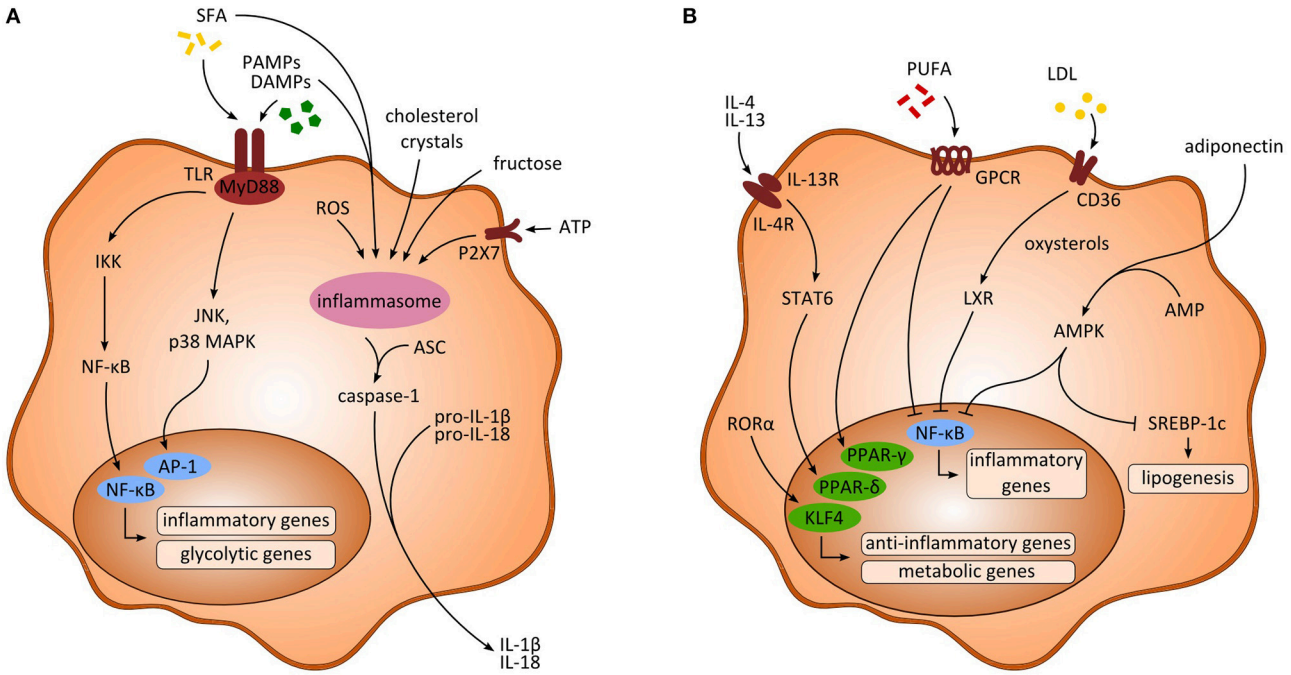

FIGURE 1 | Activating signals and intracellular pathways of myeloid-derived cells. **(A)** Signaling pathways upon inflammatory activation of macrophages. Saturated fatty acids (SFA) and pathogen-associated-molecular-patterns (PAMPs) as well as danger-associated-molecular-patterns (DAMPs) activate the transcription factors NF-κB and activator protein 1 (AP-1), ultimately leading to the transcription of inflammatory and glycolytic genes. Further, activation of the inflammasome through multiple activating signals results in enhanced production of inflammatory cytokines (e.g., IL-1β, IL-18) via caspase-1. **(B)** Anti-inflammatory signaling pathways in macrophages. Anti-inflammatory cytokines (e.g., IL-4, IL-13) as well as polyunsaturated fatty acids (PUFA) and RORα lead to the transcription of PPARs and KLF4, respectively, enhancing the transcription of anti-inflammatory and metabolic genes. Moreover, inhibition of NF-κB is induced through PUFA, low density lipoprotein (LDL) and adiponectin, thereby impeding the transcription of inflammatory associated genes. AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ASC, apoptosis-associated speck like protein containing a caspase recruitment domain; ATP, adenosine triphosphate; IKK, inhibitory κB kinase; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species.

indicate a high relevance of elevated gut-derived LPS and TLR signaling in mouse models of NASH (20–22). Additionally, altered bile acid homeostasis might promote the progression of NAFLD by shaping macrophage function (23). Bile acids are important mediators of lipid and glucose metabolism in the liver acting through nuclear receptors, farnesoid X receptor (FXR), and G-protein-coupled-receptor TGR5. In Kupffer cells (KCs), sensing of bile acids through TGR5 resulted in secretion of anti-inflammatory cytokines. Moreover, activation of TGR5 was also associated with inhibition of the inflammasome (24).

Lipotoxicity

Bioactive lipids have substantial functions in energy homeostasis, cell communication, regulation of inflammation, and maintaining structural integrity. In NAFLD an increased availability of dietary lipids, increased hepatic *de novo* lipogenesis and increased lipolysis in adipose tissue jointly lead to accumulation of triglycerides in hepatocytes. A dysbalance of extra- and intracellular lipid composition caused by excess free fatty acids may result in the accumulation of toxic lipids with ensuing cellular injury involving organelle dysfunction or cell death (25). Toxic lipids are known to affect the endoplasmic reticulum (ER) by causing ER stress and activation of the unfolded protein response, whereas in mitochondria, excess lipids contribute to the production of reactive oxygen species (ROS) (25). Circulating fatty acids in the blood stream mainly comprise saturated fatty acids such as palmitic or stearic acid that are thought to induce inflammation via TLR dependent pathways, mainly involving TLR4. *In vitro*, palmitate induced TLR2 and TLR4 dependent pro-inflammatory signaling in both monocytes and macrophages (26). However, recent findings indicated that palmitate does not act as a TLR4 agonist directly, but that TLR4-dependent priming is necessary for immune signaling through palmitate (27). A recent study further investigated the effect of palmitate induced death receptor 5 signaling on the secretion of extracellular vesicles from hepatocytes and showed that these vesicles promoted a pro-inflammatory phenotype in macrophages (28). Further, pro-inflammatory hepatic macrophages release ROS upon stimulation with palmitate through internalization of TLR4/MD2 complexes (29). However, short chain fatty acids (SCFA) protect against diet-induced obesity by inducing a PPAR-γ mediated switch to fat oxidation (30). Additionally, mouse models showed elevated levels of ceramides enhanced by the cytokines IL-6 and IL-10. Consistently, inhibition of ceramide synthesis reduced steatosis in hepatocytes (31). In the liver, free fatty acids have been shown to activate KC through

TLR2 and TLR4 signaling (32). Further, intracellular storage of lipids has been observed in KCs during experimental NASH that was associated with an inflammatory phenotype *in vivo* as well as *in vitro* (33, 34).

In contrast, polyunsaturated fatty acids (PUFA) such as omega-3 and omega-6 fatty acids appear to play a protective role regarding inflammation (**Figure 1B**). In macrophages, docosahexaenoic acid has been shown to activate PPAR-γ and AMP-activated protein kinase (AMPK) thus inhibiting expression of the transcription factor nuclear factor kappa beta (NF-κB) (35, 36). Docosahexaenoic acid and eicosapentaenoic acid bind to G-protein-coupled-receptor 120 (GPR120) on macrophages and in turn inhibit NF-κB and improve insulin resistance (37). Interestingly, macrophages and other myeloid cells (monocytes, neutrophils) also express receptors for medium chain fatty acids, especially GPR84, which is induced by LPS, high glucose concentrations and oxidized LDL (38). The functional consequences of this pathway are not fully understood. Potentially, medium chain fatty acids can amplify inflammatory and/or fibrogenic responses via GPR84 in macrophages (38, 39).

Moreover, lipotoxicity essentially involves mitochondrial dysfunction due to elevated levels of free fatty acids leading to increased production of ROS as well as lipid peroxidation. In the mitochondria, redox nodes (e.g., NADPH/NADP+) contribute to oxidative phosphorylation and also balance the redox state of the cell. In this context, it was shown that mice deficient in NADPH were protected from dysfunction in oxidative phosphorylation and NASH induced by a high-fat diet (40). Increased levels of ROS in NAFLD result in depletion and inhibition of antioxidative molecules and enzymes such as thioredoxin and glutathione. Accordingly, experimental evidence suggests that treatment with antioxidants, e.g., glutathione, may prevent the progression toward NASH by reducing hepatic oxidative stress (41).

Glucotoxicity

Aside lipids, also an increased uptake of sugars and other carbohydrates is associated with sustained NAFLD progression (1). Most interestingly, a few studies have established a link between sugars and activation of myeloid immune cells. Fructose has been shown to induce NOD-like receptor protein 3 (NLRP3) inflammasome formation in human macrophage cell lines via thioredoxin-interacting protein (TXNIP) to mitochondria shuttling, resulting in an increased production of ROS as well as IL-1β and IL-18 (42). Moreover, upregulation of various glucose transporters (GLUT) has been described during monocyte to macrophage differentiation (43). In line, increased GLUT1 expression leads to an upregulated pentose-phosphate pathway and an inflammatory macrophage phenotype, characterized by a high expression of CCL2, CXCL2, TNF-α, and IL-6 (44). GLUT3 further mediates glucose uptake following hypoxia in human macrophages, leading to *de novo* lipogenesis and subsequent lipid droplet accumulation (45).

Inflammatory Cytokines

One key mechanism of relaying inflammatory processes between fat, liver, and gut during NAFLD is the release of cytokines such as IL-1β, IL-6, IFN-γ, and TNF-α, which aggravate local inflammation, thereby worsening insulin resistance and triglyceride accumulation in fat and liver tissue. Inflammatory cytokines and chemokines, particularly TNF-α, IL-8 and CCL3, were also shown to be correlated with disease severity in human NAFLD patients (46), and myeloid immune cells, such as monocytes, neutrophils, and DC are well-known sources of both cytokines and chemokines (47).

IL-1β is released by myeloid immune cells following inflammatory polarization and inflammasome activation (48). In the liver, KCs are well-known sources of IL-1β and IL-18 during NAFLD (49, 50), and it has been shown that cholesterol treatment induces IL-1β and IL-18 production in both KCs and monocyte derived macrophages in a dose dependent manner (51). In line, also adipose tissue macrophages and neutrophils secrete IL-1β, thereby promoting inflammation during NAFLD progression (52). IL-6 is known to be secreted by macrophages and DC during NAFLD (53, 54), and has been associated with increased risk for insulin resistance in men (55). TNF-α is another often named inflammatory cytokine in NAFLD (56), and can be secreted by neutrophils (57), monocytes, adipose tissue macrophages (58), KCs (59), and DCs (53). Although TNF-α secretion enhances local inflammation thereby worsening insulin resistance and hepatic steatosis (60), early studies in humans exploring TNF-α blockade as a therapeutic target in metabolic diseases did not show beneficial effects (61). Another important group of cytokines are interferons, which have been shown to be relevant during NAFLD progression (62). While interferon secretion is typically associated with T, NK, and NKT cells, it induces an inflammatory phenotype in macrophages (13). Moreover, cytosine-phosphate-guanosine (CpG) microbial motif stimulation mediates autocrine type I IFN signaling, and subsequently induces fatty acid oxidation in plasmacytoid DCs (63). Furthermore, Galectin-3, a lectin secreted by macrophages is increased in obesity and stimulates pro-inflammatory pathways in adipose tissue (64).

INTRACELLULAR RESPONSES OF MYELOID IMMUNE CELLS UPON ACTIVATION

Intracellular Signaling

Intracellular pathways following activation by PAMPs and DAMPs mainly involve the activation of PRRs, e.g., TLRs, that immediately lead to an inflammatory response and, accordingly, several studies demonstrated TLR upregulations associated with metabolic diseases. Upon binding to its corresponding ligand TLRs associate with MyD88, which results in an activation of c-Jun N-terminal kinase (JNK), inhibitor of nuclear factor kappa-B kinase 2 (IKK2) and mitogen-activated protein kinase (MAPK) p38 (**Figure 1A**). As a consequence, expression of NF-κB and activator protein 1 (AP-1) is upregulated and ultimately results in transcription of pro-inflammatory cytokines such as IL-6 or TNF-α (5, 65) (**Figure 1A**). Consequently, interfering with NF-κB signaling by pharmacological inhibition of IKK2 significantly reduced hepatic steatosis and inflammatory

responses in NASH mice (66). Diet-induced activation of JNK was further shown to be enhanced by mixed-lineage kinase 3 (MLK3) thus increasing hepatic steatosis as well as insulin resistance (67). In line, expression of JNK in adipose tissue macrophages was found to be necessary for the induction of insulin resistance and inflammation in obese mice (68). Danger signal HMGB1 further enhances LPS induced pro-inflammatory activation of macrophages through phosphorylation of MAPK p38 and activation of NF-κB (69).

In addition, hematopoietic knockout of TLR4 in obese mice demonstrated a crucial role of TLR4 in inducing insulin resistance in liver and adipose tissue, especially in KCs and macrophages (70). In murine KCs, TLR4 further promotes ROS dependent activation of X-box binding protein thus regulating diet-induced NASH (71). Moreover, translocated bacterial DNA binds to TLR9 expressed by KCs thereby enhancing secretion of IL-1β, which subsequently induces steatosis in mice and promotes fibrogenesis (50).

Activation of the Inflammasome

Recent studies suggest an important role of the inflammasome in the progression of NAFLD, as it is expressed in innate immune cells including monocytes, macrophages, DCs, and neutrophils. Inflammasomes are intracellular (cytosolic) multiprotein oligomers that recognize danger signals via the nucleotide oligomerization domain (NOD)-like receptor (NLR). The sensing of PAMPs and DAMPs results in complex formation of the NLR sensor, ASC and pro-caspase-1. After activation, caspase-1 leads to maturation and subsequent secretion of inflammatory IL-1β and IL-18 (72) (**Figure 1A**). In experimental NASH, mice fed a high-fat diet showed accumulation of cholesterol crystals in KCs leading to activation of the inflammasome (73). Similarly, *in vitro* exposure of phagocytes to cholesterol crystals induced assembly of NLRP3 inflammasomes (74). In contrast, lack of the NLRP3 inflammasome results in decreased levels of IL-18 and other cytokines in the intestine and is related to an altered gut microbiota and derangement of the gut-liver-axis (10, 75). Consistently, NLRP3 blockade improved NAFLD in obese mice presumably by inhibiting inflammasome activation mediated by cholesterol crystals in myeloid cells (51). Of note, a derivate of cholesterol, 27-hydroxycholesterol, was shown to reduce cholesterol accumulation in KCs and thereby attenuated steatosis in mice (76). Interestingly, the NLRP3 inflammasome also seems to be critical in Western diet mediated reprogramming of myeloid immune cells, which suggests a possible role in inducing trained immunity in these cells (77). Wen et al. further demonstrated that the saturated fatty acid palmitate induced NLRP3-ASC inflammasome assembly in macrophages involving AMPK and ROS signaling (78). Aside palmitate, also stearate could induce assembly of the inflammasome in human macrophages, whereas unsaturated fatty acids as oleate and linoleate prevented activation of NLRP3 (79). Inflammasome complexes are also expressed in human KCs and respond to common liver pathogens as *S. thyphimurium* and *F. novicida*, whereas hepatitis B virus (HBV) infection inhibited inflammasome formation (80). Deficiency of caspase-1 and caspase-11 in macrophages reduces hepatic inflammation through reduced formation of cholesterol crystal and enhanced cholesterol efflux in obese mice (81).

Cellular Metabolism

Adaptations in the cellular metabolism of myeloid immune cells essentially shape their plasticity and functionality through the interaction of several transcription factors, signaling molecules, posttranscriptional, and epigenetic regulation. Varying availability of nutrients requires metabolic reprogramming in myeloid cells to ensure appropriate activation and function of metabolic processes (e.g., glycolysis, citric acid cycle).

Inflammatory activation of macrophages (e.g., through LPS or IFN-γ) is associated with enhanced aerobic glycolysis leading to increased lactate production as well as activation of the pentose phosphate pathway (82). The transcription factors hypoxia-inducible factor-1α (HIF-1α) and HIF-2α control the transcription of inflammatory associated NOS2 and anti-inflammatory arginase-1 (ARG1), respectively. Increased expression of inducible nitric oxide synthase (iNOS) in inflammatory macrophages and DCs is linked to enhanced production of nitrogen species that may inhibit mitochondrial respiration. The increased influx of glucose in pro-inflammatory macrophages is regulated by HIF-1α, whereas loss of HIF-1α is associated with reduced secretion of inflammatory cytokines (83). This suggests a possible role of HIF-1α in the pathology of NAFLD, as chronic hypoxia was shown to directly trigger NAFLD by increasing macrophage accumulation in adipose tissue (84). In DCs, LPS stimulation drives glucose oxidation leading to enhanced synthesis of phospholipids and production of pro-inflammatory cytokines (85). On the other side, during homeostasis resting cells show increased consumption of glucose as well as fatty acids that trigger activation of citric acid cycle and oxidative phosphorylation (82). Furthermore, the carbohydrate kinase like (CARKL) protein controls metabolic reprogramming via the pentose phosphate pathways and was found to be downregulated in anti-inflammatory macrophages in both mice and humans (86). Increased glucose metabolism in anti-inflammatory macrophages is promoted through mTORC2 and IRF4 signaling that is essential for metabolic remodeling (87).

Moreover, regulation of lipid metabolism as well as transcription of inflammatory and anti-inflammatory genes significantly shapes the functional phenotype of myeloid cells. Inflammatory signaling in NAFLD is associated with activation of NF-κB by saturated fatty acids that activate sterol regulatory element-binding protein-1 (SREBP-1), which then leads to increased lipogenesis via acetyl-CoA carboxylase (88, 89). Polarization toward an anti-inflammatory phenotype can be regulated by different ways. The scavenger receptor CD36 takes up oxidized low density lipoprotein (LDL), which is then converted to oxysterol by cytochrome P450 oxidase. Next, the nuclear liver X receptor (LXR) is activated by oxysterols and in turn leads to inhibition of NF-κB signaling and hence an attenuation of inflammation (90) (**Figure 1B**). Additionally, levels of adenosine monophosphate (AMP) can be elevated due to metabolic factors (e.g., adiponectin) and cause activation of AMP kinase (AMPK) that inhibits Acetyl-CoA carboxylase. This results in decreased levels of Malonyl-CoA and consequently

increased fatty acid oxidation and inhibition of SREBP-1C (**Figure 1B**). Activation of AMPK was shown to induce an anti-inflammatory phenotype in macrophages through inhibiting inflammatory polarization and crucially contributes to immune function in macrophages (91).

Nuclear factors peroxisome proliferator-activator receptors (PPARs) are activated by fatty acids or their intermediates and regulate transcription of metabolic processes. In mice, PPAR-γ specific knockout in macrophages protected against diet-induced hepatic steatosis (92). In addition, retinoic acid receptor-related orphan receptor-α (RORα) regulates hepatic lipid homeostasis by reducing PPAR-γ activity. In the liver, it was recently shown that RORα increases anti-inflammatory polarization of liver macrophages through Kruppel-like factor 4 (KLF4) and accordingly ablation of RORα in myeloid cells predisposed mice to high-fat diet-induced NASH (93). Anti-inflammatory cytokines (e.g., IL-4, IL-13) released by adipocytes or hepatocytes activate PPAR-δ expression via STAT6 signaling and thereby induce an anti-inflammatory phenotype in adipose tissue as well as hepatic macrophages (94) (**Figure 1B**). In line, myeloid specific deletion of PPAR-δ resulted in increased insulin resistance and development of steatohepatitis (95).

However, chronic low grade inflammation in NAFLD is perpetuated by several factors (e.g., dietary fats, chronic changes to the gut microbiome, persisting changes in metabolism, adipose tissue mediators), which likely explain why anti-inflammatory pathways fail to achieve adequate resolution of NAFLD (unlike after an acute injury to the liver). In this context, the dual inflammatory and pro-resolving role of Kupffer cells and infiltrating macrophages is of great importance (96). Reprogramming of inflammatory macrophages toward an anti-inflammatory phenotype might represent an interesting therapeutic target to strengthen anti-inflammatory signaling and improve NAFLD progression, yet it is unclear if this would be able to overcome the inflammatory signals, if the injurious trigger (e.g., overnutrition) persists.

Cellular metabolism has further been shown to be controlled by epigenetic factors that are involved in the development and progression of NAFLD (97). In obese subjects, progression toward NASH was linked to modified methylation of genes associated with insulin metabolism (98). Moreover, epigenetic alteration of hepatic DNA were shown to significantly affect energy metabolism in mitochondria and influence fibrogenic signaling with increasing age (99).

MYELOID CELL COMPOSITION, DIFFERENTIATION, AND POLARIZATION IN DIFFERENT COMPARTMENTS

Liver

The liver harbors multiple distinct subsets of macrophages and DCs. Two major types of macrophages are located in the liver: monocyte-derived macrophages and yolk sac-derived tissue-resident KCs. Although myeloid cells in general and macrophages in particular exhibit a remarkable heterogeneity in the liver (6), many studies oftentimes simplistically denote these cells as KCs. The unique vascular supply of the liver and the sinusoidal localization of many hepatic myeloid cells imply that these cells are among the first to encounter gut-derived PAMPs via the hepatic port vein, but are also exposed to local DAMPs (**Figure 2**). During the progression of NAFLD, myeloid cell composition changes fundamentally. In the early phase of NAFLD associated inflammation, the most important immune cell population in the liver are the tissue resident KCs (100), which survey their surrounding for stress or danger signals like e.g., DAMPs released by steatotic hepatocytes. Depletion of KCs was consequently shown to ameliorate NASH in mice, underlining their importance in the progression of the disease (60). Interestingly, during NASH, a preponderance of anti-inflammatory polarized KCs was found that initiated apoptosis of inflammatory KCs by the secretion of IL-10 (101) (**Figure 2**). Moreover, mice supplied with probiotics, e.g., *L. paracasei*, exhibited enhanced anti-inflammatory macrophages in the liver that were involved in attenuation of hepatic steatosis in NASH (21). These results highlight that the state of polarization of KCs and other macrophage populations is crucial in the progression of NAFLD. Following DAMP stimulation release of TNF-α by hepatic macrophages is necessary to activate NF-kB signaling in hepatocytes and CXCL1 production for the recruitment of neutrophils (102). In mice with steatohepatitis, diet-derived lipids activated pro-inflammatory KCs that in turn lead to reduced hepatic NKT cells due to cell death (103).

Upon inflammation monocytes are primarily recruited via CCR2-CCL2 as well as CXCR3 and CXCL10 dependent signaling (6, 104). Accordingly, the infiltration of Ly-6C+ monocytes has been identified as a crucial factor in the progression toward NASH and fibrosis in mice (100, 105). The most widely explored pathway of recruiting inflammatory and fibrogenic monocytes to injured liver is via the chemokine CCL2 (106), which can also be targeted therapeutically in experimental models of steatohepatitis (107). Besides CCL2 secreted by KCs and steatotic hepatocytes, TNF related apoptosis inducing ligand (TRAIL) might be involved in the recruitment of circulating monocytes (108). Additionally, damaged hepatocytes may release extracellular vesicles containing CXCL10 or ceramides, thereby initiating recruitment of myeloid cells to the liver (109, 110). Ceramides were shown to affect macrophage chemotaxis via sphingosine-1-phosphate; consequently, inhibition of sphingosine-1-phosphate attenuated steatosis in experimental NASH (111). Moreover, dysregulation of lipid synthesis due to steatosis can also lead to accumulation of toxic lipids in KCs that in turn exhibit a pro-inflammatory phenotype, as characterized by the secretion of inflammatory cytokines and chemokines as well as enhanced recruitment of lymphocytes (33). Interestingly, arginase 2 deficient mice, lacking anti-inflammatory macrophages, develop spontaneous steatosis and show characteristics of early steatohepatitis without high-fat diet feeding (112). Furthermore, CD44 appears to be involved in the induction of a pro-inflammatory phenotype in hepatic macrophages thereby aggravating progression toward NASH in mice and men (113). Additionally, palmitate-induced release of extracellular vesicles (containing TRAIL) from hepatocytes was shown to promote a pro-inflammatory phenotype in macrophages, thus exposing an interesting therapeutic target for NASH in patients (28).

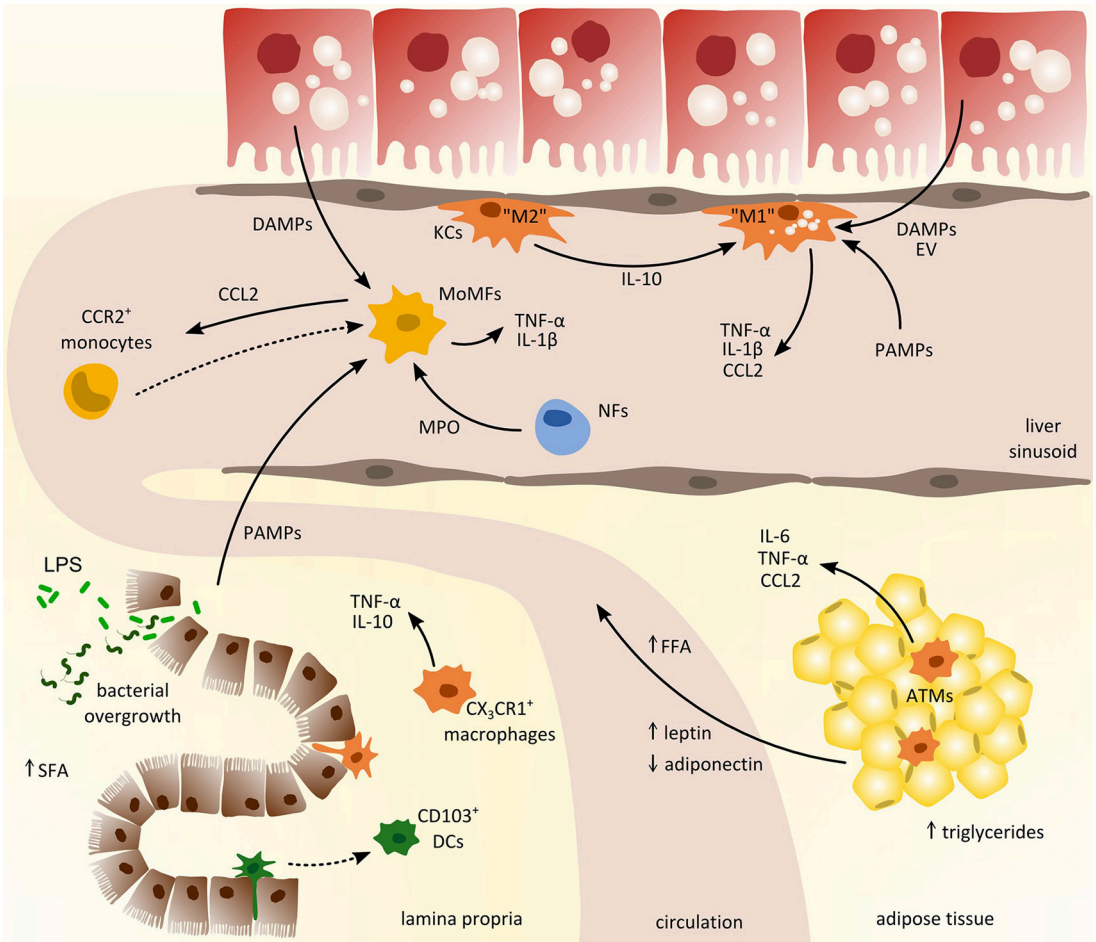

FIGURE 2 | Cross-talk of myeloid-derived cells from different compartments in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Inflammatory activation promoting the progression of NAFLD involves the interaction between liver, gut, and adipose tissue. From the gastrointestinal tract (left lower corner), pathogen-associated-molecular-patterns (PAMPs) enter the circulation due to increased intestinal permeability and promote hepatic inflammation together with local danger-associated-molecular-patterns (DAMPs). In adipose tissue (right lower corner), chronic inflammation induces the release of pro-inflammatory cytokines (e.g., IL-6, TNF-α) and free fatty acids (FFA) that trigger hepatic injury and activation of hepatic macrophages such as monocyte-derived macrophages (MoMFs) and tissue-resident Kupffer cells (KCs). Activated macrophages in the liver produce CCL2 (and other pro-inflammatory cytokines) to recruit circulating monocytes from the blood that contribute to sustained inflammation. ATMs, adipose tissue macrophages; DCs, dendritic cells; EV, extracellular vesicles; LPS, lipopolysaccharide; MPO, myeloperoxidases; NFs, neutrophils; SFA, saturated fatty acids.

Neutrophils are mainly recruited toward areas of inflammation via the CXCR1/2 receptors and CXCL1/2 axis (in mice) or IL-8 (in humans), respectively (47). The induction of CXCL2 secretion by hepatic cells has been shown to depend on both TLR2 and S100A9 (114). Furthermore, myeloperoxidases secreted by neutrophils are increased in NASH patients (115). Myeloperoxidases were shown to be toxic to macrophages, thereby contributing to the progression of inflammation and insulin resistance (57, 116). Consequently, in NAFLD models mice lacking myeloperoxidases showed reduced hepatic inflammation as well as improved insulin resistance (115). In addition, neutrophils may contribute to suppression of bacterial infections via extracellular traps activated by the release of DAMPs in liver injury (117, 118).

Recently, an influx of inflammatory DCs has been demonstrated in experimental NASH that was associated with a regulatory phenotype contributing to clearance of apoptotic cells and thus limiting inflammation (53). Furthermore, inflammatory DCs expressing CX_3CR1 were shown to contribute to sustained inflammation in mice with diet-induced NASH and were linked to increased levels of TNF-α (119). Moreover, capability of hepatic DCs to respond to immunogenic stimuli seems to be dependent of their lipid content and concentration in mice and men (120).

Adipose Tissue

During fatty liver disease progression, the adipose tissue is not simply an organ for excess lipid storage. In fact, adipose tissue actively contributes to systemic metabolic disturbances and to

inflammatory changes in NAFLD, not only in the adipose tissue itself but also in the liver. Compared to homeostatic conditions the composition as well as the number of myeloid immune cells in adipose tissue is altered in obese patients. Increased expression of CCL2 in adipocytes leads to monocyte recruitment and accumulation of macrophages in adipose tissue (3). Macrophage infiltration then results in enhanced accumulation of triglycerides in adipocytes further enhancing adipose tissue inflammation. The infiltration of monocytes is typically accompanied by an increase in adipose DCs and an early influx of neutrophils. In addition to an increased infiltration of macrophages in adipose tissue under obese conditions, an altered inflammatory phenotype can be observed in these cells characterized by enhanced TNF production (**Figure 2**). In this context Chung et al. showed that induction of inflammation is dependent on the interaction of adipocytes and macrophages through α_4-integrin (58). It was further demonstrated that gut-derived LPS induced inflammatory pathways in adipose tissue through TLR signaling and contributed to the recruitment of macrophages by increasing CCL2 expression in adipocytes (22). In line with this, ablation of TLR4 in adipose tissue reduced the infiltration and macrophages that exhibited an M2 like phenotype (121).

Adipose tissue inflammation is further driven by an infiltration of neutrophils. Neutrophils are characterized by high numbers of cytoplasmic granules containing neutrophil elastase, myeloperoxidase, lysozymes ,and other antibacterial defensins. Following tissue infiltration, neutrophils also secrete cytokines as IL-1β, IL-8, and TNF-α, inducing local inflammatory processes, especially during early stages of adipose tissue inflammation following high-fat diet feeding (52, 57).

In experimental NASH, adipose tissue derived leptin has been shown to promote KC activation in the liver via iNOS and NADPH oxidase that cause oxidative stress and was further linked to increased levels of TGF-β (122, 123). High levels of leptin also enhanced expression of the LPS receptor CD14 via STAT3 signaling in KCs, thus increasing their reactivity to low doses of gut derived bacterial LPS and ultimately leading to increased hepatic inflammation (124). On the other hand, adiponectin which is reduced in NAFLD, had anti-inflammatory effects on macrophages and induced reduction of TNF-α (125). Furthermore, insulin like growth factor 1 (IGF1) is produced by IL-4 mediated alternatively activated macrophages, and IGF1 receptor (IGF1R) positive macrophages attenuate adipose tissue inflammation (126). In obese mice, exosomes containing miRNA (e.g., mi-R155) secreted by adipose tissue macrophages were shown to promote glucose tolerance and insulin resistance suggesting a possible role in in the progression of NAFLD (127), especially regarding crosstalk between organs (128). Moreover during obesity, adipose tissue derived S100A8 and S100A9 stimulated IL-1β production in adipose tissue macrophages through TLR4 and NLRP3 dependent pathways then promoting an increase of myeloid precursors in the bone marrow via IL-1R in mice (129).

Gut

Evidence from mice and men demonstrate the great importance of the cross-talk between liver and the intestine in the development of metabolic diseases (130, 131). NAFLD is often accompanied by an altered gut microbiota and bacterial overgrowth that is strongly associated with increased intestinal permeability as well as enhanced pathological bacterial translocation. In the gut, mononuclear phagocytes, comprising macrophages, DCs, and neutrophils, are tightly controlled to ensure intestinal homeostasis (132). During homeostasis and inflammation, intestinal macrophages, characterized by their expression of F4/80 and CX_3CR1, secrete IL-10 and low levels of TNF-α stimulating regulatory T-cells as well as newly infiltrating monocytes (133) (**Figure 2**). Myeloid cells were further shown to contribute to the progression of bacterial infections during liver fibrosis by production of IFN-γ and increased secretion of IL-10 (20). Studies in CX_3CR1-deficient mice further depict a contribution to intestinal homeostasis through intestinal macrophages thus ameliorating diet-induced steatohepatitis (134). Moreover, intestinal macrophages exert a high phagocytic activity and may be also involved in limiting bacterial translocation (135). Intestinal $CD103^+$ DCs are mainly responsible to transport bacteria to mesenteric lymph nodes. In line, in CCl_4 induced liver cirrhosis in rats enhanced bacterial translocation was linked to increased levels of $CD103^+$ DCs (136). DCs in the gut are further known to express tight junction proteins (e.g., occludin) and thereby influence bacterial translocation in response to oxidative stress and inflammatory stimulation (137).

Several studies indicate a high relevance of gut microbiota dysbiosis and imbalanced microbiota-produced metabolites (e.g., SCFA or ethanol) through increasing the susceptibility for inflammatory liver diseases (138). This is underlined by a recent study in which germ free mice inoculated with feces from patients suffering from NAFLD developed more severe NASH after a high-fat diet compared to control mice (139). Further, gut-derived bacterial metabolites (e.g., tryptamine and indole-3-acetate), that are depleted upon high-fat feeding can mediate inflammation in macrophages and hepatocytes by lowering levels of pro-inflammatory cytokines as TNF-α, IL-1β, and CCL2 (140). Studies by Caesar et al. could also demonstrate that gut microbiota derived LPS promotes macrophage recruitment into adipose tissue and pro-inflammatory TNF-α expression (141). Early evidence by Turnbaugh et al. demonstrated that the gut microbiota of obese mice further possesses an increased ability to harvest energy from their diet underlining its major role in metabolism and hence metabolic diseases (142). Moreover, in recent metagenomic studies, hepatic steatosis was associated with a reduction in microbial gene richness with an increase in *Proteobacteria* and elevated levels of the microbial metabolite phenylacetic acid that promotes hepatic lipid accumulation (143). Even though several microbial metabolites have been shown to strongly influence the progression of NAFLD, the diverse interactions between host and microbiome most likely comprise more complex factors and require further analyses.

Moreover, SCFA are significantly involved in the pathology of NAFLD, as they are the major fermentation products of the gut microbiome including acetate, propionate, and butyrate. Compared to long chain fatty acids that lead to the initiation of inflammatory pathways, short chain fatty acids seem to have a countervailing effect on the progression of NAFLD. Diet-derived SCFA inhibit the expression of PPAR-γ and

hence lead to the activation of AMPK reducing glycolysis and lipogenesis (30). These effects are primarily mediated via the GPR43 receptor. However, the precise role of the potential anti-inflammatory effects SCFA in fatty liver disease and their potential therapeutic value need to be investigated more detailed.

MODULATING MYELOID-DERIVED CELLS IN FATTY LIVER DISEASE

Therapeutic Modulation of Activating Signals

The thorough understanding of activating pathways and functional roles of myeloid cells in fatty liver diseases offers the opportunity to develop novel treatment strategies. Therapeutic targeting of activating signals of myeloid cells, especially macrophages, has been a promising approach to treat liver diseases. Recent trials target bile acid receptors to influence the immune phenotype of macrophages. In mice, a dual TGR5/FXR agonist, INT-767, was shown to protect against steatohepatitis and increase numbers of anti-inflammatory monocytes and macrophages (144). Further, the semi-synthetic bile acid analog obeticholic acid is a strong FXR agonist with promising results from an early phase clinical trial in patients with NASH (145). While FXR antagonism has many beneficial effects on metabolism, it was also shown to reduce activation of murine KCs *in vitro* (146). The multi-ligand PRR RAGE (receptor for S100 proteins and HMGB1) is also considered as potential therapeutic target, as it is implicated in several chronic inflammatory diseases. However, blocking RAGE in rats could not inhibit LPS induced hepatic oxidative damage (147). Additionally, dietary lipid composition can be modulated to counteract inflammatory processes. Although NASH patients show decreased levels of n-3 PUFA, clinical trials supplementing PUFAs were not able to prove improvement of steatosis or fibrosis (148). Further there have been investigations interfering with TNF-α related signaling to treat alcoholic hepatitis or NASH by using monoclonal antibodies or inhibiting TNF-α (e.g., pentoxifylline or etanercept), however such drastic interventions are unlikely to become clinical routine for long-term treatment due to safety concerns (149, 150).

Interfering with gut-derived signals, particularly PAMPs, might also reduce activation of myeloid cells in fatty liver disease in the liver as well as in the gut. Microbiome-based strategies to treat NAFLD predominantly include the use of antibiotics, prebiotics or probiotics. Studies by Jiang et al. demonstrate that combined treatment with bacitracin, neomycin, and streptomycin interferes with the gut microbiota and regulates NAFLD progression through decreased FXR signaling as well as reduced ceramide levels (151). Another novel approach involves fecal microbial transplantation to modulate the gut microbiota. In a recent study, fecal microbial transplantation was shown to limit high-fat diet-induced NAFLD (152). Similarly, intestinal microbiota transfer determined the susceptibility to NASH induced by high-fat diet feeding in mice (153). In addition, orlistat, a gut lipase inhibitor, can attenuate diet-induced obesity by limiting absorption of dietary fatty acids from the gut (154).

Therapeutic Modulation of Intracellular Responses in Myeloid Cells

Key intracellular signaling pathways in NASH are intensively investigated at the moment and, amongst others, include PPARs as they essentially regulate glucose and lipid metabolism. Agonists for PPAR-γ and PPAR-δ (e.g., elafibranor) reduced inflammation and fibrosis in mouse models of NAFLD (155) and also demonstrated promising results in an early clinical trial (156). These findings were associated with reduced release of pro-inflammatory cytokines such as TNF-α (94, 156). Further, a pan-PPAR agonist, IVA337, was shown to markedly improve NASH features in mice (157). PPAR-γ agonists further ameliorated lipid-induced polarization of inflammatory KCs in mice resulting in an improvement of steatohepatitis (158) and also reduced components of NASH in a long term clinical study (159). Many other drugs affecting intracellular inflammatory or metabolic pathways are currently being evaluated as potential treatments for NAFLD (4). Among them, the apoptosis signal-regulating kinase 1 (ASK1) inhibitor selonsertib blocks inflammatory signaling (JNK and p38 MAPK) in KCs (160), which might explain antifibrotic signals observed in an early clinical trial (161).

Therapeutic Modulation of Myeloid Cell Composition, Differentiation, and Polarization

The interplay between recruited and tissue-resident myeloid-derived cells allows the interference with recruiting signals (e.g., chemokines) to reduce myeloid cell accumulation. Inhibition of hepatic macrophage infiltration can be realized by specifically blocking distinct chemokine receptors, e.g., through cenicriviroc, a dual antagonist against the chemokine receptors CCR2 and CCR5 (162–164). Due to the primarily inflammatory and fibrogenic phenotype of infiltrating monocyte-derived macrophages in steatohepatitis (165), this principle was found effective in reducing fibrosis in animal models (166) as well as in NASH patients treated for 1 year with cenicriviroc (167). However, such a strategy would not directly affect KCs or non-CCR2 dependent myeloid cells. KCs can be targeted by many drug carrier systems (including nanoparticles) due to their characteristic scavenging capacity (168). This concept was validated in studies targeting macrophages and KCs with anti-inflammatory dexamethasone and thus reducing hepatic fibrosis in rodent models (169, 170). Furthermore, cholesterol lowering drugs such as ezetimibe or atorvastatin were shown to reduce the accumulation of cholesterol in murine KCs resulting in the resolution of NASH (171). In a more specific approach, inhibiting the lectin galectin-3 has yielded promising results in animal models and is currently being evaluated in clinical trials (172).

CONCLUSIONS

Accumulating research from recent years has revealed an immense heterogeneity of myeloid subsets with diverse roles in homeostasis and disease. Myeloid-derived cells hold key

functions in metabolic diseases, particularly in the establishment and progression of fatty liver disease. Activation, differentiation as well as polarization of immune cells are significantly influenced by distinct local and systemic signals, and the progression of NAFLD is driven by inflammatory immune cells. Importantly, there is a orchestrated interplay between myeloid cells in different compartments, such as circulation/bone marrow, gut, adipose tissue, and liver. In particular, the functional and phenotypic versatility of myeloid cells plays a central role in the pathology of NAFLD and related diseases. Targeting myeloid cells to overcome metabolic disorders has therefore emerged as a promising approach in treating fatty liver disease.

AUTHOR CONTRIBUTIONS

JH wrote the manuscript and designed the figures. OK contributed to the manuscript text, reviewed, and edited the article. FT reviewed, edited, and finalized the review. All authors approved the manuscript and agreed to be accountable for the content of the work.

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A Role for MK2 in Enhancing Neutrophil-Derived ROS Production and Aggravating Liver Ischemia/Reperfusion Injury

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Increased inflammatory responses and enhanced reactive oxygen species contribute to hepatic ischemia/reperfusion (I/R) injury, however the modulatory mechanisms haven't been completely unveiled. Here, we report that genetic deficiency of MAPK-activated protein kinase 2 (MK2) protected against hepatic I/R injury and decreased hepatic neutrophil accumulation in $MK2^{-/-}$ mice. Depletion of neutrophil attenuated hepatic I/R injury in wide type mice. In response to C5a stimulation, $MK2^{-/-}$ neutrophils generated less superoxide in which both NADPH oxidase activation and $p47^{phox}$ phosphorylation were decreased. Furthermore, Ser329 of $p47^{phox}$ was identified for enhancement of superoxide production. The Ser329 phosphorylation was reduced in $MK2^{-/-}$ neutrophils. To determine whether MK2 modulates hepatic I/R injury via activating neutrophils, we generated myeloid-specific MK2 deletion mice ($MK2^{Lyz2-KO}$) and liver I/R injury was reduced in $MK2^{Lyz2-KO}$ mice. Our results indicate that MK2 augments hepatic I/R injury and induces ROS production with increased $p47^{phox}$ phosphorylation and MK2 is a potential drug target for treating hepatic I/R injury.

Keywords: MK2, ROS, $p47^{phox}$, neutrophils, hepatic ischemia/reperfusion injury

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INTRODUCTION

Hepatic ischemia/reperfusion (I/R) is a pathophysiologic process that can be triggered by liver transplantation, elective liver surgery, toxic liver injury, and hepatic sinusoidal obstruction syndrome (1). Hepatic I/R injury is induced by reperfusion of blood flow and hypoxia accentuation in ischemic tissues, which leading to traumatic hemorrhagic shock, live damage, and graft dysfunction (2). Despite the recent improvements in liver preservation and surgical techniques, hepatic I/R injury remains an important clinical problem during liver surgery. Therefore, it is of importance to comprehensively understand the mechanisms of hepatic I/R and develop novel therapeutic approaches.

Hepatic I/R injury is a complex pathophysiological process. Initial phase of hepatic I/R injury is associated with oxidative stress, anaerobic metabolism, and calcium overload. At late phase, an increase in the cellular damage and, immune response contribute to the progression of

parenchymal injury (3). Overall, reactive oxygen species (ROS), pro-inflammatory cytokines and chemokines as well as Kupffer cells (KCs), neutrophils and lymphocytes are involved in the process and are closely interlocked (4–6).

Neutrophils have a pivotal role in the pathogenesis of hepatic I/R injury (7). During hepatic I/R injury, neutrophils are first activated by chemokines, and migrate across the endothelium to the hepatocytes (7, 8). Then neutrophils further damage endothelial cells and destroy the integrity of the microvasculature through release of ROS, proteinases (cathepsin G, granulocytes elastase) and cationic peptides. Inhibition of neutrophil infiltration can protect against hepatocellular injury following I/R (7). However, the molecular mechanisms and regulation of neutrophils-derived ROS production have not been defined.

MAPK-activating protein kinase 2 (MK2) is a major effector serine/threonine-protein kinase downstream of p38-alpha (p38α) MAPK (9, 10). Once activated, MK2 phosphorylates many substrates and is implicated in many cellular processes including stress and inflammatory responses, cytoskeleton modulation, nuclear export, gene expression and cell proliferation (10, 11). MK2 plays a vital role in several diseases, such as cancer (12, 13), neurodegenerative disease (14), and inflammatory diseases (10, 15). Recently, Ashraf et al. reported that p38 MAPK activity is increased upon reperfusion and p38 MAPK inhibition prevents severe functional impairment caused by I/R (16). However, It has not been demonstrated how p38 MAPK modulates hepatic I/R and whether MK2 is a critical gene in modulation of hepatic I/R injury.

Using a mouse model of hepatic I/R injury, we found that complete MK2 deficiency markedly alleviated liver damage, serum alanine aminotransferase levels, intrahepatic macrophage/neutrophil trafficking, and pro-infiammatory cytokine production. Depletion of neutrophils *in vivo* reduced hepatic I/R injury and MK2 was required for NADPH oxidase activation and superoxide production. We also identify $p47^{phox}$ as a substrate of MK2, in which MK2 phosphorylated serine 329 residue of $p47^{phox}$ and this modification was required for NADPH oxidase activation. Furthermore, we proved that conditional depletion of MK2 in neutrophils also protected against hepatic I/R injury. Collectively, our findings reveal a critical role of MK2 in promoting ROS production and accentuating hepatic I/R injury.

RESULTS

Genetic MK2 Deficiency Alleviates Liver Injury Caused by Hepatic I/R in Mice

MK2 plays a critical role in inflammation and cell proliferation (10), however its role in hepatic I/R injury remains unknown. To determine the effect of MK2 on hepatic I/R, we performed a hepatic I/R mouse model in genetic MK2 deficiency mice (referred to as $MK2^{-/-}$ mice) with 60 min of partial liver warm ischemia followed by reperfusion for 6 and 18 h. Compared to that in $MK2^{+/+}$ mice, the serum ALT levels in $MK2^{-/-}$ mice were significantly reduced at 6 and 18 h (**Figure 1A**). Based on H&E staining, the liver damage was concordant with the change of serum ALT so that liver necrosis area was attenuated in $MK2^{-/-}$ mice (**Figure 1B**). Image-Pro Plus software analysis revealed statistically significant decreased necrosis area in livers from $MK2^{-/-}$ mice, compared to that from $MK2^{+/+}$ mice (**Figure 1C**). Collectively, these results indicated that MK2 deficiency dramatically ameliorates liver damage during hepatic I/R injury.

MK2 Deficiency Reduces Inflammatory Cytokine Production

Given that inflammatory cytokines contribute to the occurrence and development of hepatic I/R injury (6), we further determined whether MK2 modulates cytokine production in hepatic I/R injury. Six hours after hepatic I/R injury, significant increases of IL-6, TNF-α and KC mRNA of liver were observed in $MK2^{+/+}$ mice (**Figures 1D,E**). However, deletion of MK2 resulted in dramatic decrease of these cytokine at 6 h after I/R injury (**Figure 1D**). Similarly, compared with that in $MK2^{+/+}$ mice, the levels of IL-6, TNF-α and KC in serum were remarkably attenuated in $MK2^{-/-}$ mice at 6 h after hepatic I/R (**Figure 1E**). These results indicate that MK2 contributes to the hepatic I/R-induced increase of inflammatory cytokines in mice.

MK2 Is Required for Neutrophil Infiltration During Hepatic I/R Injury

Because neutrophil infiltration is associated with hepatic I/R injury, we firstly determined whether neutrophils contribute to hepatic I/R injury by depleting neutrophils with anti-Gr-1 antibody (1A8). As showed in **Figure 2A**, administration of anti-Gr-1 intraperitoneally (i.p.) effectively reduced neutrophil count in peripheral blood to 0.3%, compared to control mice receiving isotype-IgG which showed 39.8% of neutrophils in peripheral blood (**Figure 2A**). Then, mice received anti-Gr-1 antibody or an IgG control were subjected to hepatic I/R. As shown in **Figure 2B**, mice receiving anti-Gr-1 antibody displayed significantly reduced ALT in the serum. In the I/R groups, less necrotic areas were observed after neutrophil depletion based on histologic analysis and statistical analysis (**Figures 2C,D**). Additionally, to evaluate the infiltration and activation of neutrophils, we assessed the myeloperoxidase (MPO) activity. As shown in **Figure 2E**, mice receiving anti-Gr-1 antibody displayed a lower MPO activity. Furthermore, we investigated the role for MK2 in the accumulation of neutrophils during hepatic I/R injury. As showed in **Figure 2F**, increased MPO activity was significantly decreased after MK2 deletion during hepatic I/R injury (**Figure 2F**). Taken together, these results indicate that neutrophils are required for hepatic I/R injury and MK2 contributes to local accumulation of neutrophils during hepatic I/R injury.

Abbreviations: I/R injury, Hepatic ischemia/reperfusion injury; ROS, reactive oxygen species; MK2, MAPK-activating protein kinase 2; ALT, alanine amino transferase; AST, aspartate amino transferase; PMA, phorbol ester phorbol 12-myristate 13-acetate; fMLF, N-formyl-Met-Leu-Phe; MPO, Myeloperoxidase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; PMN, polymorphonuclear neutrophils; KCs, Kupffer cells.

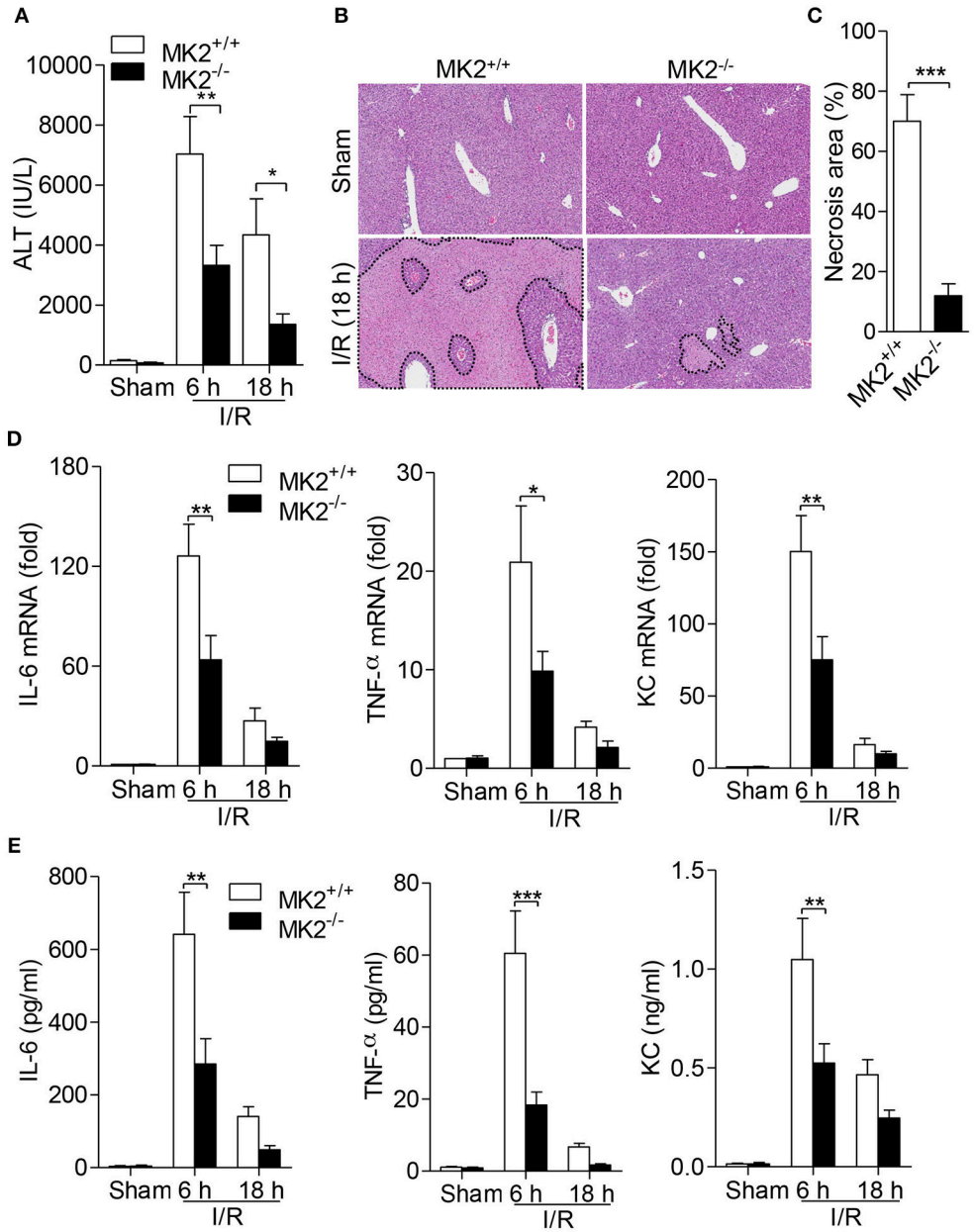

FIGURE 1 | Genetic MK2 deficiency alleviated hepatic I/R injury and inflammatory cytokine production. $MK2^{+/+}$ and $MK2^{-/-}$ mice were subjected to 60 min of partial liver warm ischemia, followed by reperfusion for 6 and 18 h. **(A)** Mouse blood was collected and serum ALT was detected. **(B)** Hepatic I/R injury was evaluated by hematoxylin-and-eosin (H&E) staining of injury liver tissues. Original magnification ×100. **(C)** The necrosis area was quantified by using ImageJ software. **(D)** The mRNA levels of IL-6, TNF-α, and KC in injury livers were detected by qPCR. **(E)** The protein levels of IL-6, TNF-α, and KC in serum were detected by ELISA. The results are shown as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, based on 5 mice in each group.

MK2 Is Required for Neutrophil Superoxide Production

It has been demonstrated that oxidant stress, especially the excessive reactive oxygen species (ROS), acts as one of the main modulators of hepatic I/R. Neutrophils are one of main sources of oxygen radicals in the post-ischemic liver, in which nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated upon adhesion or by pro-inflammatory cytokines and the complement system (17). To determine the role of MK2 in oxygen radical production by neutrophils, we isolated polymorphonuclear neutrophils (PMN) from bone marrow of $MK2^{-/-}$ and $MK2^{+/+}$ mice. As shown in **Figures 3A,C,E**, the baseline of superoxide generation in neutrophils from $MK2^{-/-}$ were not changed compared with $MK2^{+/+}$ mice. Upon

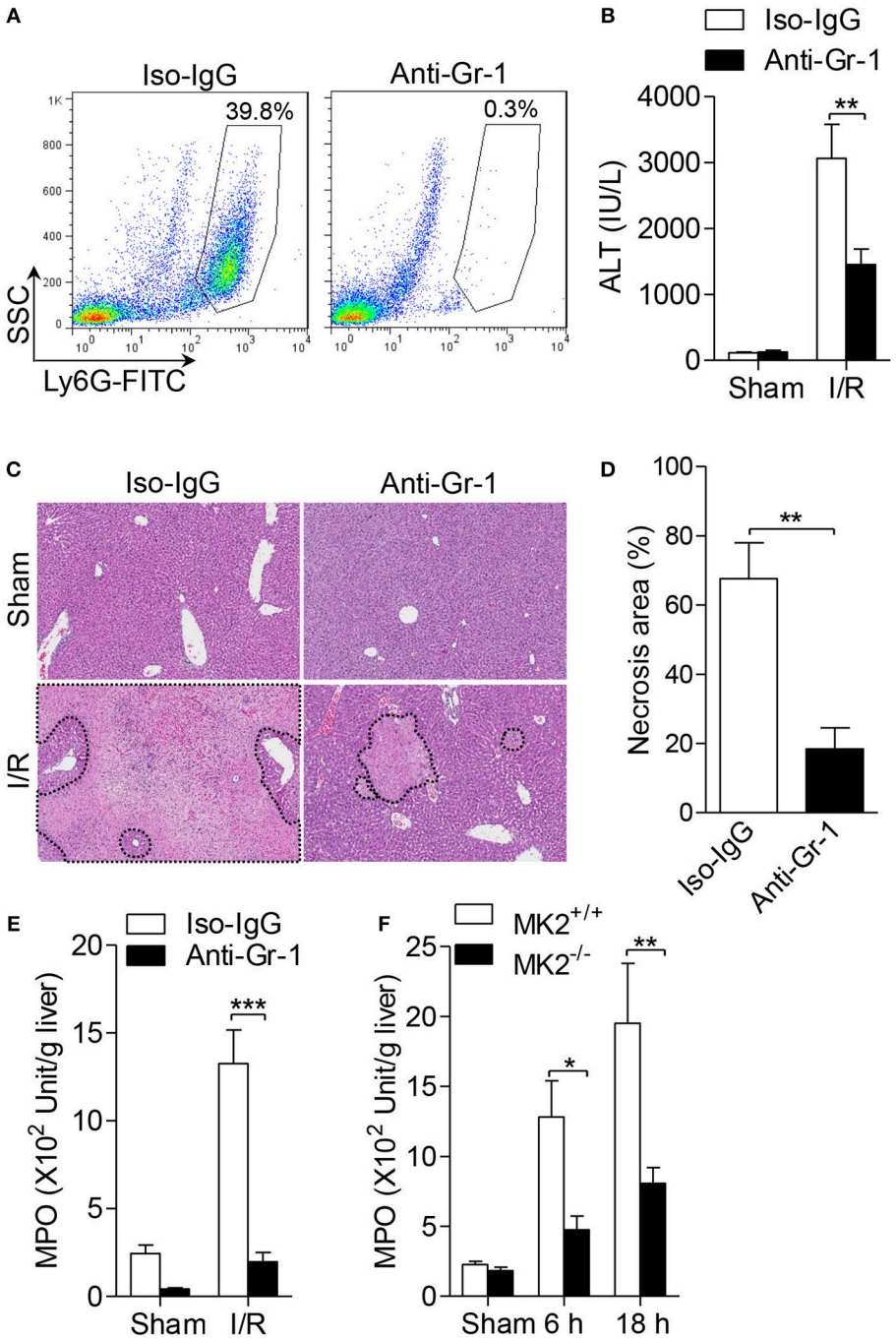

FIGURE 2 | Genetic MK2 deficiency reduced neutrophil trafficking in hepatic I/R injury. **(A)** The wild type mice ($MK2^{+/+}$) were intraperitoneally injected with anti-Gr-1 antibody (1A8), or isotype-matched IgG as control for 24 h, and then the percentage of neutrophils in peripheral blood were shown, as determined by anti-Ly6G-FITC FACS staining. **(B)** After injected with anti-Gr-1 antibody as in **(A)**, mice were subjected to hepatic ischemia for 60 min and reperfusion for 18 h. Serum ALT was detected for evaluation of liver injury. **(C)** Representative histological staining (H&E) of ischemic liver tissue was displayed. Original magnification ×100. **(D)** The necrosis area was quantified by ImageJ software. **(E)** The activities of MPO activity of injury liver tissues were detected. **(F)** $MK2^{+/+}$ and $MK2^{-/-}$ mice were subjected to hepatic I/R injury as in **Figure 1**, the activities of MPO in liver tissue extract were evaluated. The results are shown as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, based on 5 mice in each group.

treatment with C5a, neutrophils from $MK2^{-/-}$ mice generated significantly less superoxide compared to that from $MK2^{+/+}$ mice (**Figures 3A,B**). Besides activated complement component, bacterial derived formylated peptide formyl-methionyl-leucyl-phenylalanine (fMLF) also tightly regulates the activation of the NAPDH oxidase in a receptor specific manner (18). Here,

$MK2^{-/-}$ neutrophils treated with fMLF showed significantly impaired superoxide production compared to that from $MK2^{+/+}$ mice (**Figures 3C,D**). PMA is another NADPH oxidase agonist, which bypasses receptors and directly activates PKC. It is interesting that PMA-stimulated superoxide production showed no significant difference between neutrophils from $MK2^{+/+}$ and $MK2^{-/-}$ mice (**Figures 3E,F**). Collectively, these findings indicated that MK2 is involved in the regulation of chemoattractant receptor-dependent neutrophil NADPH oxidase activation and superoxide production.

MK2 Regulates AKT and P38 MAPK Phosphorylation in Neutrophils

Since several signaling pathways contribute to the activity of NADPH oxidase, we therefore reasoned whether MK2 has crosstalk with these signals. As shown in **Figure 4A**, neutrophils purified from the $MK2^{+/+}$ and $MK2^{-/-}$ mice were stimulated with 100 nM C5a for 5 min. Phosphorylation of AKT and p38 MAPK was determined by western blotting using respective antibodies. In response to C5a stimulation, the $MK2^{-/-}$ neutrophils exhibited attenuated phosphorylation of AKT (Ser473) and p38 MAPK with unaltered kinetics, compared to $MK2^{+/+}$ neutrophils (**Figures 4A–C**). In addition, the total level of p38 MAPK was also greatly decreased in $MK2^{-/-}$ neutrophils (**Figures 4A,D**). Altogether, these findings suggest that MK2 is required for C5a-induced activation of AKT and p38 MAPK, two critical kinases for modulation of NADPH oxidase activation.

MK2 Directly Phosphorylates P47 ^{phox} and Regulates Its Membrane Translocation

Phosphorylation of $p47^{phox}$ leads to its membrane translocation that is critical for the formation of the active NADPH oxidase (19). Therefore, we further determined whether MK2 regulated $p47^{phox}$ activation and membrane translocation. As shown in **Figures 5A,B**, C5a significantly induced $p47^{phox}$ phosphorylation which was abolished after the neutrophils were pre-treated with the p38 inhibitor SB203580 for 15 min. In response to C5a stimulation, phospho-$p47^{phox}$ was evaluated in neutrophils in 1 min, whereas the phospho-$p47^{phox}$ was greatly decreased in neutrophils from $MK2^{-/-}$ mice, compared to that from $MK2^{+/+}$ mice (**Figures 5C,D**). Furthermore, to test whether MK2 directly phosphorylated $p47^{phox}$, we generated and purified human GST-$p47^{phox}$ ($hp47^{phox}$) and mouse GST-$p47^{phox}$ ($mp47^{phox}$) protein (20), which were then subjected MK2. Phosphorylation of $p47^{phox}$ was evaluated by *in vitro* kinase assays. As showed in **Figure 6A**, both human $p47^{phox}$ and mouse $p47^{phox}$ protein were phosphorylated by MK2. The results suggested that MK2 could directly phosphorylate $p47^{phox}$.

Because $p47^{phox}$ is responsible for transporting the whole cytosolic complex ($p47^{phox}$-$p67^{phox}$-$p40^{phox}$) to the docking site during NADPH oxidase activation (21), it is possible that MK2 modulates $p47^{phox}$ membrane translocation via phosphorylation of $p47^{phox}$. In naïve neutrophils, the majority of $p47^{phox}$ proteins were located in the cytoplasmic compartment (green) in image a (**Figure 6B**). Upon treatment with C5a for 2 min, $p47^{phox}$ was translocated to the plasma membrane as shown in image b, which was greatly impaired in $MK2^{-/-}$ neutrophils in image d (**Figure 6B**). Thus, these data indicated that MK2 directly phosphorylates $p47^{phox}$ and induces membrane translocation of $p47^{phox}$.

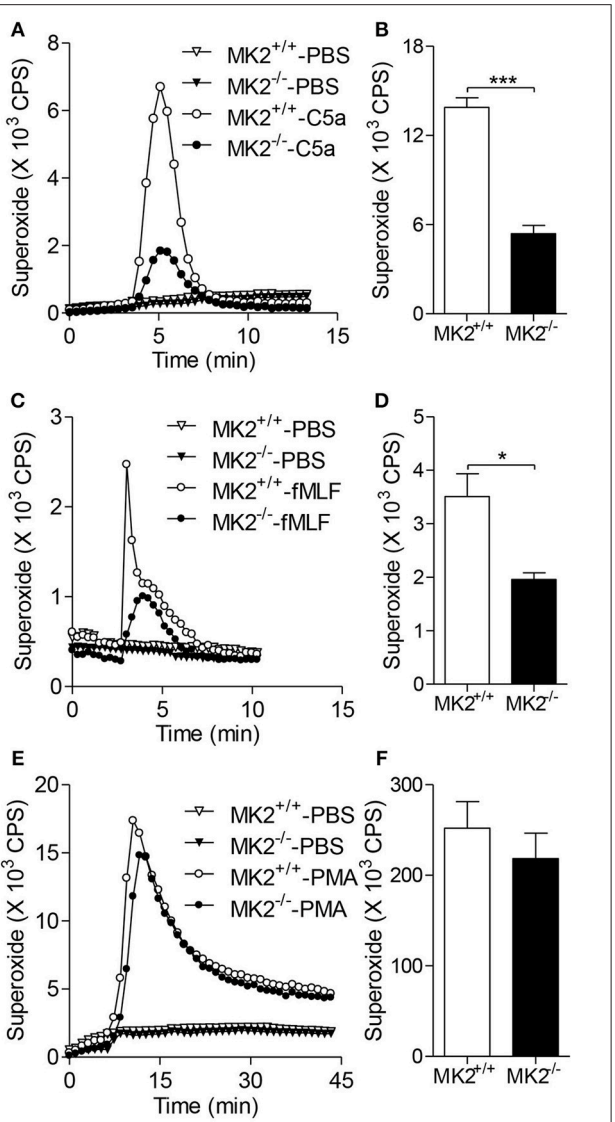

FIGURE 3 | MK2 modulated neutrophil superoxide production. Neutrophils were isolated from mice and isoluminol-ECL was used to measure superoxide production. Representative tracing showing the production of superoxide (left) and cumulative superoxide production was quantified based the area under tracing line (right). The $MK2^{+/+}$ and $MK2^{-/-}$ neutrophils were stimulated with 100 nM of C5a or PBS **(A,B)**, 10 μM of fMLF or PBS **(C,D)**, and 200 ng/ml of PMA or PBS **(E,F)**. The results were presented as means ± SEM, $^{*}P < 0.05$; $^{***}P < 0.001$, compared with similarly treated cells from $MK2^{+/+}$ mice. The experiments were repeated at least 3 times.

MK2 Phosphorylates P47^{Phox} on Ser329

The C terminus (between Ser303 and Ser379) of $p47^{phox}$ is a regulatory domain for $p47^{phox}$ activation and membrane

translocation (19, 22, 23). To determine the specific phosphorylated site of $p47^{phox}$ by MK2, we compared mouse $p47^{phox}$ with human $p47^{phox}$ and found that Ser329 on mouse $p47^{phox}$ was conservative to human $p47^{phox}$ serine 328 (**Figure 7A**). To further identify whether Ser329 of p47phox is required for NAPDH oxidase activation, we generated lentivirus with full-length $p47^{phox}$ ($p47^{phox}$-WT) or Ala substitution Ser329 of $p47^{phox}$ ($p47^{phox}$-S329A) mutant and infected them into $p47^{phox}$ deficient neutrophils that were defective for superoxide generation. In response to C5a stimulation, $p47^{phox}$ deficient neutrophils with wide type $p47^{phox}$ ($p47^{phox}$-WT) produced more superoxide than that with $p47^{phox}$-S329A, although neutrophils with $p47^{phox}$-S329A still had capability to generate superoxide (**Figures 7B,C**). To evaluate whether MK2 modulated $p47^{phox}$ Ser329 phosphorylation, we used antibody specific to phosphorylated $p47^{phox}$ Ser329. Upon treatment with C5a, the phosphorylation of $p47^{phox}$ Ser329 were induced within 0.5 min and reached peak at 1 min (**Figures 7D,E**). $MK2^{-/-}$ neutrophils displayed an attenuated phosphorylation at Ser329 of $p47^{phox}$ compared to $MK2^{+/+}$ neutrophils. Thus, these data indicate that Ser329 in $p47^{phox}$ sequence is an important regulatory site for NADPH oxidase activation and can be modulated by MK2.

Myeloid-Specific Deletion of MK2 Alleviated Hepatic I/R Injury

To further prove that the loss of MK2 in neutrophils regulates hepatic I/R injury, we generated myeloid-specific deficiency of MK2 mice (referred to as $MK2^{Lyz2-KO}$) by mating $MK2^{loxP/loxP}$ mice with lysozyme 2-Cre mice (**Figure 8A**). The expression of MK2 was abolished in neutrophils from $MK2^{Lyz2-KO}$ mice compared with that from $MK2^{Lyz2-WT}$ mice (**Figure 8B**), which was no significant difference between the liver tissues from $MK2^{Lyz2-KO}$ or $MK2^{Lyz2-WT}$ mice (**Figure 8C**), suggesting MK2 was deficient in neutrophils. Then, the $MK2^{Lyz2-KO}$ and $MK2^{Lyz2-WT}$ mice were subjected to hepatic I/R. Compared to $MK2^{Lyz2-WT}$ mice, $MK2^{Lyz2-KO}$ mice displayed reduced ALT activities (**Figure 8D**) and decreased liver necrosis injury (**Figures 8E,F**) 6 h after hepatic I/R injury. Taken together, these data confirm that MK2 contributes to hepatic I/R injury and modulates neutrophil activation and ROS production.

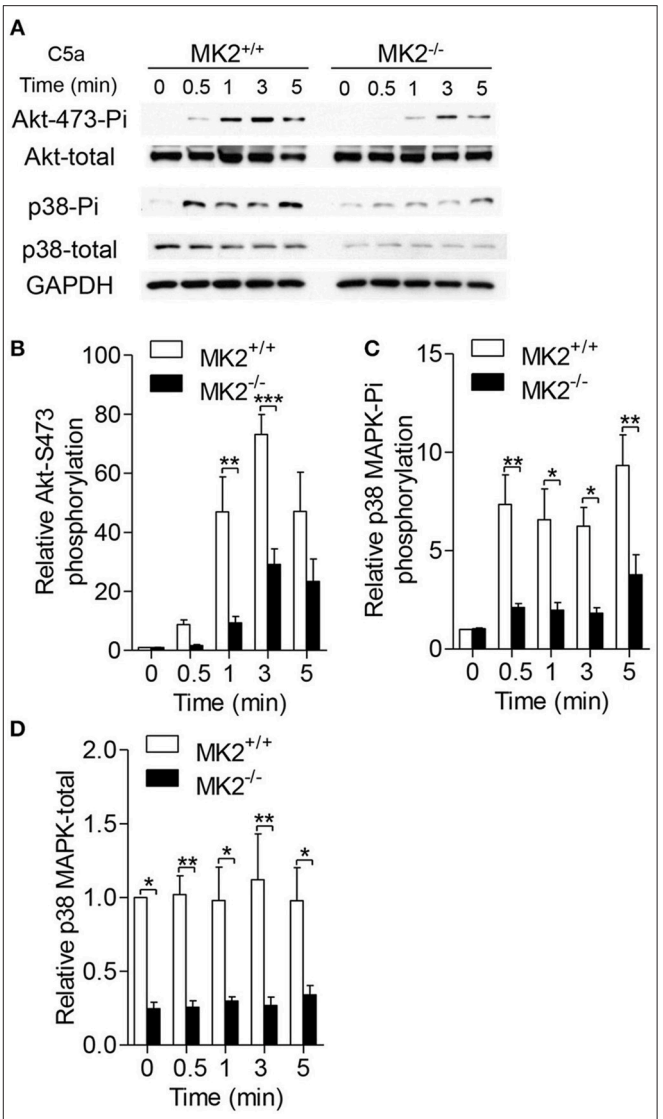

FIGURE 4 | MK2 was required for AKT and p38 MAPK phosphorylation in neutrophils. **(A)** Neutrophils from $MK2^{+/+}$ and $MK2^{-/-}$ mice were challenged with C5a (100 nM) for indicated time. Phosphorylation of AKT (Ser473) and p38 MAPK was determined by western blotting using anti-phospho-antibodies against the phospho-AKT (Ser473), total AKT, phospho-p38 MAPK, and total p38 MAPK. **(B–D)** Densitometry analysis was conducted to determine the relative level of induced AKT phosphorylation, p38 MAPK phosphorylation, and total p38 MAPK. Data shown are means ± SEM from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

DISCUSSION

Hepatic ischemia/reperfusion (I/R) injury is a common pathological process in liver surgery and transplantation, influencing the patient's outcome post-surgery (8). Although p38 MAPK has been implicated in the pathogenesis of hepatic I/R injury (16), the modulatory mechanisms remain elusive. Here, we found that MK2, one of the downstream kinases of p38 MAPK, contributed to hepatic I/R. Genetic MK2 deficiency dramatically limited pathological damage, reduced serum ALT activities, decreased inflammatory cytokine production, and impaired neutrophil infiltration in mice. In *in vitro* study, MK2 deficiency abrogated superoxide production and activation of AKT and p38 MAPK in neutrophils. Furthermore, we identified that NADPH oxidase activation was regulated by MK2 that directly phosphorylated Ser329 of $p47^{phox}$. The myeloid-specific deletion of MK2 mice ($MK2^{Lyz2-KO}$) also displayed a reduced liver injury after I/R injury. Therefore, our findings demonstrated for the first time that down-regulation of MK2 protects against hepatic I/R injury, which could be a novel therapeutic target for I/R injury.

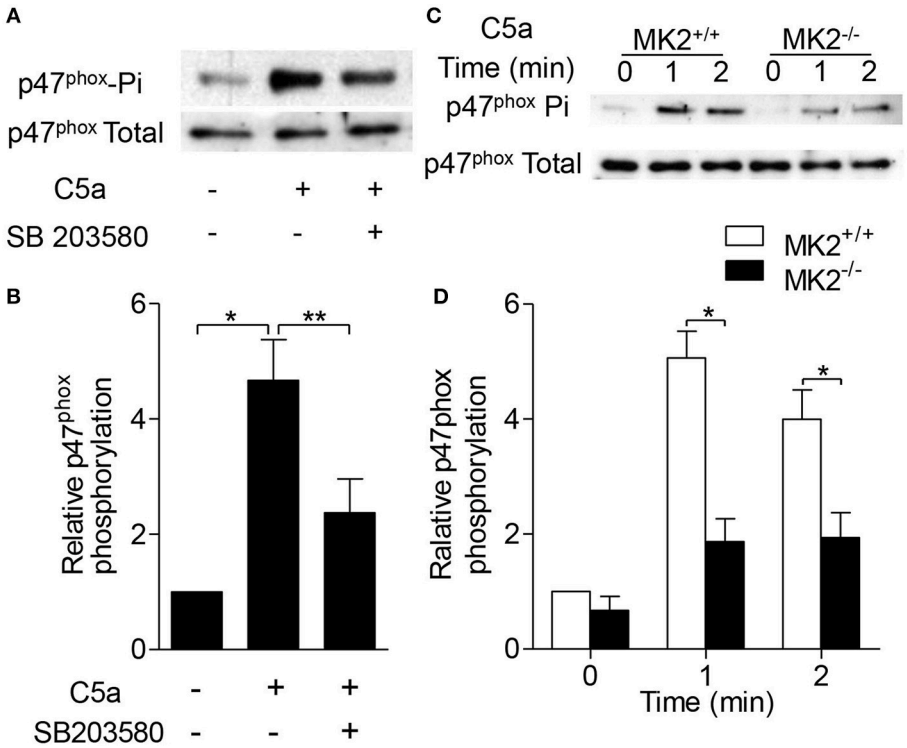

FIGURE 5 | MK2 was essential for $p47^{phox}$ phosphorylation in response to C5a stimulation. **(A)** Neutrophils from WT mice were pre-treated with SB203580 (3 μM) for 15 min, and then stimulated with C5a (100 nM) for 1 min. The phosphorylation of $p47^{phox}$ was determined. **(B)** Densitometry analysis was conducted to determine the 47^{phox} phosphorylation. **(C)** Neutrophils from $MK2^{+/+}$ and $MK2^{-/-}$ mice were stimulated with C5a (100 nM) for 1 and 2 min. The phosphorylation (Phospho) of $p47^{phox}$ was determined. **(D)** Densitometry analysis was conducted to determine 47^{phox} phosphorylation in $MK2^{+/+}$ and $MK2^{-/-}$ neutrophils. The results are shown as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$, based on three independent experiments.

Hepatic I/R injury is closely related to innate immune cell activation and inflammatory processes. Although dendritic cells, natural killer T (NKT) cells, Kupffer cells are all involved in modulation of hepatic I/R injury (4), neutrophils play an nonredundant role in hepatic I/R injury because depletion of neutrophils or neutrophil specific deficiency of MK2 alleviating I/R injury (**Figures 2, 8**). Coincidentally, recent studies also reveal that neutrophils participate in the hepatic I/R injury. Honda M et al found that formyl-peptide receptor 1 (FPR1)-mediated neutrophil recruitment modulate hepatic I/R injury (24). Infiltrated neutrophils induce hepatocyte death and enhance Kupffer cells to produce inflammatory cytokines by releasing neutrophil extracellular trap (NET) (25). In addition to generation of NET, neutrophils can exacerbate tissue injury though releasing ROS, proteinases and cationic peptides (20). Although we couldn't exclude that MK2 may regulate hepatic I/R injury via modulating Kupffer cells or dendritic cells, our results suggest that MK2-mediated neutrophil activation is involved in hepatic I/R injury.

MK2 is one of the downstream kinases of p38 MAPK that is activated during hepatic I/R injury and regulates liver injury (16). Coxon et al. proved that MK2 regulates human neutrophil activation in p38-dependent and ERK-dependent signal pathways (26). Furthermore, MK2 is involved in neutrophils polarization and chemotaxis and regulates infectious diseases (27, 28). However, a particular role of MK2 in sterile inflammatory responses was not well investigated. In this study, we uncovered the alleviation of liver damage and reduction of liver neutrophil accumulation in genetic MK2 deficiency mice ($MK2^{-/-}$) and myeloid-specific deletion of MK2 mice ($MK2^{Lyz2-KO}$) during haptic I/R injury. These results indicate that MK2 regulates hepatic I/R injury through modulating neutrophil functions. However, it should be noted that, in our *in vivo* data, we only examined that several inflammatory cytokines and chemokines such as IL-6, TNF-α and KC within the liver tissue were decreased in $MK2^{-/-}$ mice. More adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), integrin need to be further studied to explore the molecular mechanism for the regulatory effect of MK2 in neutrophils chemotaxis, recruitment and transendothelial migration.

Oxidative stress has been considered to be a major part of liver damage induced by I/R injury, and oxygen free radicals are the principal components that lead to hepatocellular necrosis and apoptosis in I/R injury (8). Therefore, antioxidants including superoxide dismutase, catalase, glutathione, vitamin E, and beta-carotene protect against hepatic I/R injury (29). During reperfusion after liver ischemia, neutrophils are a potential source of oxygen free radicals by activation of

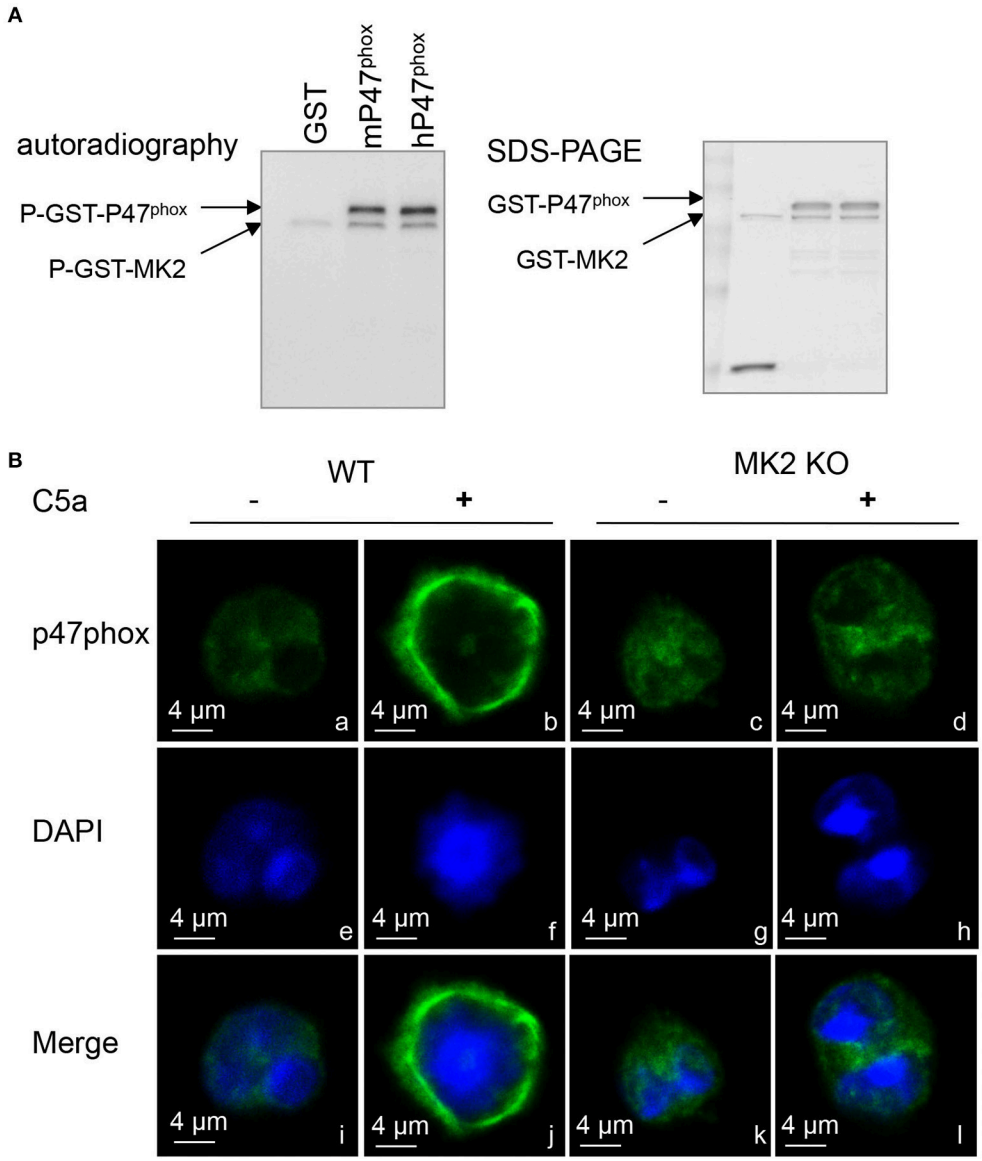

FIGURE 6 | MK2 directly regulated p47^{phox} phosphorylation and membrane translocation. **(A)** Autoradiograph of *in vitro* kinase assay using full-length human p47^{phox} (hp47^{phox}) or mouse p47^{phox} (mp47^{phox}) fused to GST as substrates for GST-MK2. GST without mp47^{phox} was used as a negative control. Two phosphorylated bands were identified in the autoradiograph: a phosphorylated GST–p47^{phox}, and an autophosphorylated GST-MK2. The loading levels of each protein were indicated in coomassie blue staining (right). **(B)** Neutrophils from MK2$^{+/+}$ and MK2$^{-/-}$ mice were treated with C5a (100 nM) for 2 min and immunofluorescence imaging was performed to detect the localization of p47^{phox} (green). Nuclei were stained with DAPI (blue). The results are representatives of three independent experiments, scale bar is 4 μm.

NADPH oxidase. Here, our data showed that MK2 deficient neutrophils produced less superoxide production in response to C5a or fMLF stimulation but not to PMA. Given that PMA bypasses receptors and directly activates protein kinase C (PKC), MK2 mediates NADPH oxidase activation through specific cell-surface receptors and related molecules, such as phosphoinositide 3-kinase (PI3K), G protein-coupled receptor kinases (GRKs), small GTP-binding proteins, and mitogen-activated protein kinases (MAPK). Consistently, we also found that phosphorylation of AKT and p38 MAPK is reduced in MK2-deficient neutrophils in response to C5a stimulation (**Figure 4**). Thus, our data suggests that MK2 accentuate superoxide generation and hepatic I/R injury via modulating AKT and p38 MAPK signals.

MK2 is a stress-activated serine/threonine-protein kinase involved in different cellular functions through its kinase activity (9). NADPH oxidase is an inducible electron transport system assembled by several subunits (gp91^{phox}, gp22^{phox},

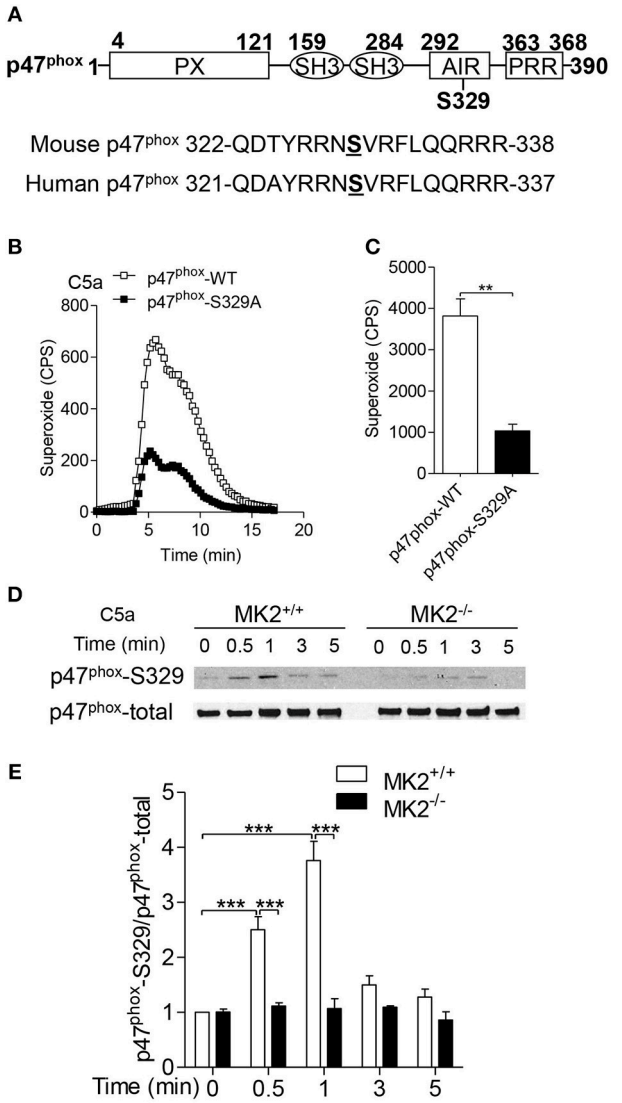

FIGURE 7 | MK2 regulated mouse p47^{phox} at Ser329 residue. **(A)** Alignment of sequence of human and mouse p47^{phox} proteins surrounding the potential MK2 phosphorylation site. **(B)** Neutrophils from *p47$^{phox-/-}$* mice were transiently transfected with full-length p47phox or its S329 mutant for 6 h, and followed challenge with 100 nM of C5a. The production of superoxide production was measured as described. **(C)** The quantitative analysis was performed based on results of B superoxide. **(D)** Neutrophils from MK2$^{+/+}$ and MK2$^{-/-}$ mice were challenged with 100 nM of C5a for 0.5, 1, 3, and 5 min, the phosphorylation of p47^{phox} (Ser329) was determined by western blotting with anti- p47phox-S329 antibody and p47^{phox}-total antibody. **(E)** Densitometry analysis was conducted to determine the relative level of p47^{phox}-S329. Data shown are means ± SEM from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

p47^{phox}, p67^{phox}, small GTPase Rac, and p40^{phox}) (17). The phosphorylation and membrane translocation of the subunit p47^{phox} is necessary for NADPH oxidase activation and regulation (21, 22). Although several kinases are implicated in regulating the phosphorylation of p47^{phox}, the mechanisms and dynamics of p47^{phox}-orchestrated NADPH activation haven't been completely revealed. In human neutrophils, phorbol myristate acetate (PMA), and fMLF induce MK2 phosphorylation at Ser334 and further increase NADPH oxidase activation (30). TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and C5a can induce p47^{phox} phosphorylation through p38 MAPK (31). Thus, p38 MAPK and MK2 are involved in the modulation of NADPH oxidase activation. However, the regulatory mechanisms have not yet been defined. Our *in vitro* kinase assay showed that MK2 can directly regulated the phosphorylation of p47^{phox}, thereby reducing neutrophil superoxide production. Moreover, membrane translocation of p47^{phox} was impaired in MK2$^{-/-}$ neutrophils. Our data first demonstrate that MK2 regulates the ROS production in neutrophils through the direct regulatory effect on the phosphorylation of p47^{phox}.

Several phosphorylated sites of p47^{phox} are involved in NADPH oxidase activation. The p47^{phox} phosphorylation at Ser345 serves as a point of convergence for different MAPKs to induce priming of ROS production (23). We previously proved that Thr356 of p47^{phox} is a phosphorylation site for p38 MAPK (20). Here, we identified that Ser329 was a new phosphorylation site and could be modulated by MK2. The phosphorylation level of p47^{phox} on Ser329 was abrogated in MK2$^{-/-}$ neutrophils. The p47^{phox} has multiple serine residues ranging from Ser303 to Ser379 (23). Phosphorylation of some serine sites, such as Ser359 or Ser370, is an important initial step to activate p47^{phox} (32, 33). Other residue such as Ser379 can cause inhibition of NADPH oxidase activation (34). Although we identified Ser329 as a new residue that can be regulated by MK2, the synergistic and dynamic effects of these residues on p47^{phox} activation and ROS production should be further explored.

In conclusion, we demonstrated substantial and significant protection against the hepatic I/R injury in genetic MK2 knockout mice and MK2 myeloid-specific deletion mice. In addition, we found MK2 played a vital role in NADPH oxidase activation and ROS production through AKT and p38 signal pathways. Moreover, MK2 regulated NADPH oxidase activation by phosphorylation of p47^{phox} on Ser329 under complement component stimulation. Our findings not only reveal a novel regulatory mechanism for hepatic I/R injury, but also identify a novel therapeutic target for ameliorating hepatic I/R injury in liver transplantation.

METHODS

Mice

MK2 deficient mice, MK2$^{loxP/loxP}$ mice and lysozyme 2-Cre mice were purchased from The Jackson Laboratory. *MK2*$^{Lyz2-KO}$ Mice were generated by mating MK2$^{loxP/loxP}$ mice with lysozyme 2-Cre mice (35). The p47$^{phox-/-}$ mice was provided by Dr. Steven M Holland (NIH, Bethesda, MD, USA). All mice used in the study were on C57BL/6 background and 8–12 weeks of age. Mice were housed in a climate-controlled room (25°C, 55% humidity and 12 h light/darkness cycles) and all procedures were conducted with the use of protocols approved by the Institutional Animal Care and Use Committee at Shanghai Jiao Tong University.

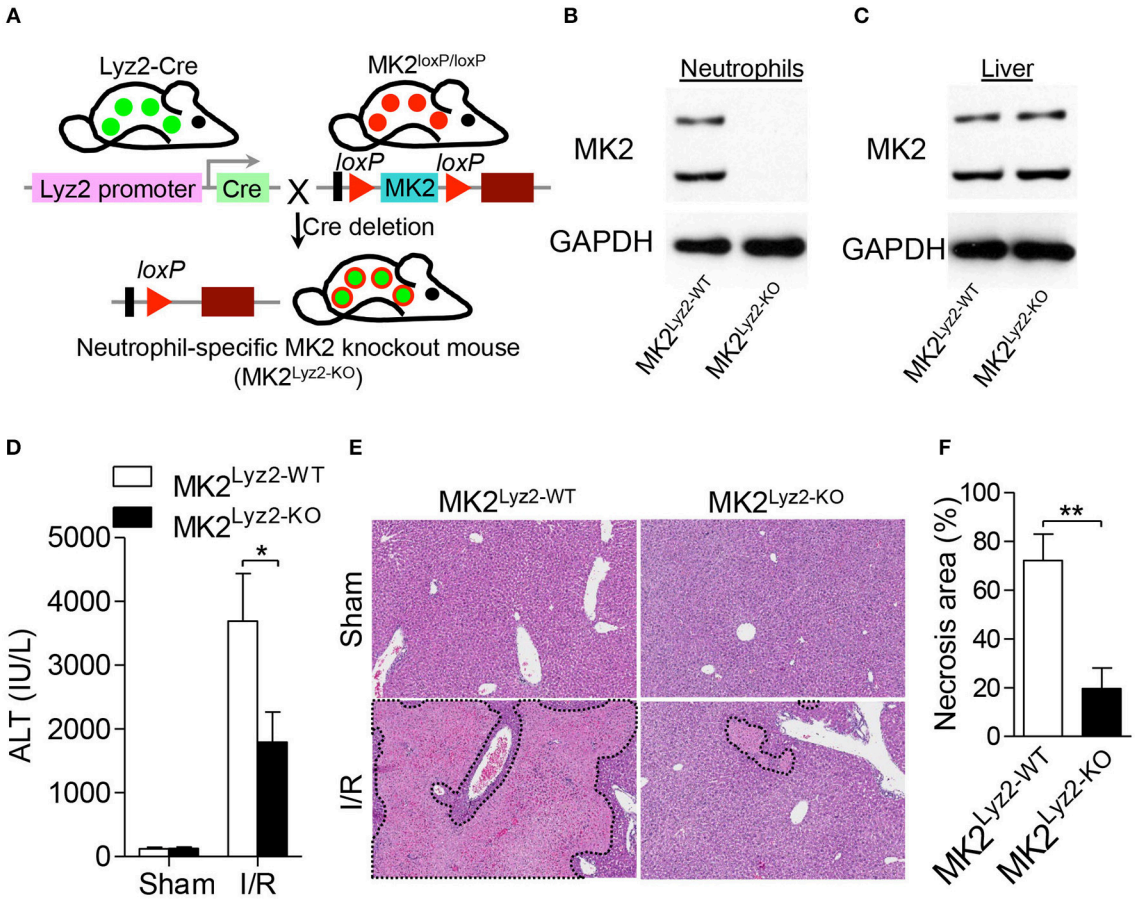

FIGURE 8 | Myeloid-specific deletion of MK2 decreased hepatocellular damage in hepatic I/R injury. **(A)** Schematic representation of the generation of $MK2^{Lyz2-KO}$ and $MK2^{Lyz2-WT}$ mice that $MK2^{loxP/loxP}$ mice were bred with Lysozyme (Lyz2)-Cre transgenic mice. The protein level of MK2 in neutrophils **(B)** and livers **(C)** from $MK2^{Lyz2-KO}$ and $MK2^{Lyz2-WT}$ mice was detected by western blot. $MK2^{Lyz2-KO}$ and $MK2^{Lyz2-WT}$ mice were subjected to hepatic I/R injury with 60 min of ischemia and 16 h of reperfusion. Serum and injury livers were collected for analysis. **(D)** Serum ALT levels were measured. **(E)** Representative H&E stained sections of injury livers were shown. Original magnification ×100. **(F)** The necrosis area was measured by ImageJ software. The results are shown as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$, based on 5 mice in each group.

Reagents

Phorbol ester phorbol 12-myristate 13-acetate (PMA), *N*-formyl-Met-Leu-Phe (fMLF), C5a and isoluminol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The p38 MAPK inhibitor SB203580 was ordered from Selleck (Houston, TX, USA). Antibodies including Phosphor-Akt, Akt, Phosphor-p38 MAPK, p38 MAPK, MK2, and GAPDH were ordered from Cell Signaling Technology (Danvers, MA, USA). Anti-$p47^{phox}$ antibody was purchased from Santa Cruz Biotechnology and anti-phospho-$p47^{phox}$ (Ser329) antibody was generated by N.J. Compass Biotechnology (Nanjing, China). Other reagents were ordered from Sigma-Aldrich (St. Louis, MO, USA).

Partial Liver Ischemia/Reperfusion Injury (IRI) Mouse Model

All mice were anesthetized with pentobarbital (5 mg/kg) by intraperitoneal injection. The mouse abdominal cavities were opened with operating scissors. All structures in the portal triad (hepatic artery, portal vein, bile duct) to the left and median liver lobes were occluded with a microvascular clamp (Fine Science Tools) for 60 min; reperfusion was initiated by removal of the clamp. After 6 or 18 h of reperfusion, anesthetized animals were sacrificed, and liver tissue and serum were collected for analysis. Sham-operated groups underwent the same surgical procedure, except that the blood supply to the liver lobes was not interrupted.

Histopathological Analysis

Following euthanasia, representative pieces of ischemic lobes were quickly removed and fixed in ice cold 10% phosphate-buffered formalin for 24 h at 4°C, and then embedded in paraffin. Tissue blocks were sectioned at 4 μm thickness and slices were baked at 60°C for 4 h. After removal of the paraffin by using xylene and a graded ethanol series, the sections were cut to 4-μm-thick sections, stained with hematoxylin and eosin (Beyotime Institute of Biotechnology, China). Following staining,

the observer was blinded to treatment group. The slides were viewed under a microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

Activity Assay of ALT

Blood samples were centrifuged for 10 min at 3,000 rpm and serum were collected. The activities of serum alanine aminotransferase (ALT) were measured using the ALT Assay Reagent kit (NJJCBIO, Nanjing, China) according to the manufacturer's instructions with colorimetric evaluation on the microplate reader (FlexStation 3, Molecular Devices, CA, USA).

Myeloperoxidase Activity Assay

Frozen mouse liver was homogenized with a Teflon homogenizer in 50 mM phosphate buffer. After centrifugation at 13,000 *g* for 30 min, the cell pellet was resuspended in 1 ml 0.5% hexadecyl trimethylammonium bromide (Sigma-Aldrich, St. Louis, MO, USA), and treated with three cycles of freeze, thaw, and sonication. After centrifugation at 13,000 *g* for 20 min at 4°C, the supernatant was incubated with 16 mM 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO, USA) and 15 mM H_2O_2, and absorbance at 655 nm was determined. One unit of MPO activity was defined as the change of absorbance of 1.0 per min.

Superoxide Production Assays

Mouse polymorphonuclear neutrophils (PMN) were purified from bone marrow cell suspensions as described previously (20). Isolated neutrophils were incubated with 10 μM isoluminol at 37°C for 30 min, and then challenged with PMA (200 ng/mL) for 45 min, fMLF (10 μM) or C5a (100 nM) for 15–60 min. The generation of superoxide was detected by using a Wallac 1420 Multilabel Counter (PerkinElmer, Houston, TX, USA).

Neutrophil Depletion

To deplete neutrophils, mice were intraperitoneally injected with 5 mg/kg (100 μg/mouse) anti-Gr-1 antibody (1A8) that specifically recognized neutrophil surface mark Ly6G (Bio X Cell, West Lebanon, NH, USA) 24 h before ischemia/reperfusion challenge. Isotype-matched IgG of the same amount was used as a negative control.

Flow Cytometric Analysis

After treating with anti-Gr-1 antibody for neutrophils deletion, the mice were performed liver ischemia/reperfusion injury (IRI), the percentages of neutrophils were measured using Ly6G-FITC (BD Biosciences, West Lebanon, NH, USA) for flow cytometry. Data was analyzed using FlowJo 7.5 (Tree Star Software).

Immunofluorescence Microscopy

Neutrophils on glass coverslips coated with fibrinogen were stimulated with or without C5a, washed once with ice-cold phosphate-buffered saline (PBS), and then fixed with 3% paraformaldehyde in PBS. The anti-$p47^{phox}$ antibody were used at 1:250 dilution overnight, cells were incubated with a rhodamine green-X-conjugated goat anti-mouse IgG (secondary antibody) at room temperature for 1 h. After washing, the coverslips were mounted on glass slides with the use of ProLong Gold anti-fade reagent with DAPI. Fluorescence images were captured with a confocal microscope with custom software (Leica Micro-systems, CMS GMBH, and LAS AF).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from liver tissues, and first-strand cDNA was synthesized with Moloney murine leukaemia virus reverse transcriptase (Qiagen, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with SYBR Green (Invitrogen) in a Bio-Rad real-time PCR detection system with primer sets for IL-6, forward 5′-TAGTCCTTCCTACCC CAATTTCC-3′, reverse 5′- TTGGTCCTTAGCCACTCCTTC-3′; TNF-α, forward 5′-CCCTCACACTCAGATC ATCTTCT-3′, reverse 5′-GCTACGACGTGGGCTACAG-3′; KC, forward 5′-TCCA GAGCTTGAAGGTGTTGCC-3′, reverse 5′-AACCAA GGGAGCTTCAGGGTCA-3′. The expression of each gene was normalized to GAPDH mRNA, and calculated with respect to the baseline control using $\Delta\Delta Ct$ method *via* StepOne 2.0.

Cytokine Production Detection

The concentration of TNF-α, IL-6, and KC in supernatants from liver tissues of liver ischemia/reperfusion injury (IRI) mice were evaluated by ELISA, according to manufacturer's instruction (R&D Systems, Minneapolis, MN, USA).

Kinase Assay

In vitro kinase assays were performed as previously described by using activate and a recombinant GST-mouse $p47^{phox}$ or GST-human $p47^{phox}$ fusion protein as substrates (20). The samples were analyzed by SDS-PAG autoradiography.

Western Blot Analysis

The neutrophils after treatment were washed with DPBS and lysed with RIPA lysis buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, United States) and phosphatase inhibitor cocktail (Roche Applied Science, Indianapolis, IN, United States). The samples were resolved on SDS-PAGE, and underwent immunoblotting analysis using the indicated antibodies. The protein band intensities were normalized to those of GAPDH. The intensity was quantified by Image J software.

Lentiviral Production and Transduction

Full-length $p47^{phox}$ ($p47^{phox}$-WT) and Ala substitution Ser329 of $p47^{phox}$ ($p47^{phox}$-S329A) mutant were cloned into the pLVX-IRES-mCherry vector (Takara CA, USA). HEK293T cells were planted into 90 mm dish at 80% confluence and co-transfected with 10 μg of $p47^{phox}$ plasmid, 7.5 μg of the psPAX2 plasmid and 2.5 μg of pMD2.G plasmid (Addgene, Cambridge, MA) by using polyethyleneimine (Polyscience,USA). Culture supernatants of the HEK293T cells were collected after 72 h and concentrated to 300 μl with a concentration tube. For infection,, $p47^{phox}$ deificient neutrophils were incubated with lentiviral supernatants

of different concentrations and Polybrene (Sigma, St. Louis, MO), followed by centrifugation at 2,000 x g for 90 min at 30°C and incubation at 37°C for 4 h. The supernatant was exchanged with fresh complete medium. The cells were stimulated with C5a (100 nM) and harvested for experiment after 6 h.

Statistical Analysis

Each experiment was performed independently for at least 3 times. The results are presented as the mean ± SEM. Statistical significance of differences between groups was analyzed with unpaired Student's *t*-test or one-way ANOVA when more than two groups were compared. Statistical significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Analysis and graphing were performed using the Prism software (ver. 5.0; GraphPad, San Diego, CA).

AUTHOR CONTRIBUTIONS

LS, QW, YN, NC, RW, GW, DZ, and HH performed the experiments. LS, NC, YN, and FQ analyzed the data. LS, RY, and FQ prepared the manuscript.

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Impaired COMMD10-Mediated Regulation of Ly6C^{hi} Monocyte-Driven Inflammation Disrupts Gut Barrier Function

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Ly6C^{hi} monocyte tissue infiltrates play important roles in mediating local inflammation, bacterial elimination and resolution during sepsis and inflammatory bowel disease (IBD). Yet, the immunoregulatory pathways dictating their activity remain poorly understood. COMMD family proteins are emerging as key regulators of signaling and protein trafficking events during inflammation, but the specific role of COMMD10 in governing Ly6C^{hi} monocyte-driven inflammation is unknown. Here we report that COMMD10 curbs canonical and non-canonical inflammasome activity in Ly6C^{hi} monocytes in a model of LPS-induced systemic inflammation. Accordingly, its deficiency in myeloid cells, but not in tissue resident macrophages, resulted in increased Ly6C^{hi} monocyte liver and colonic infiltrates, elevated systemic cytokine storm, increased activation of caspase-1 and-11 in the liver and colon, and augmented IL-1β production systemically and specifically in LPS-challenged circulating Ly6C^{hi} monocytes. These inflammatory manifestations were accompanied by impaired intestinal barrier function with ensuing bacterial dissemination to the mesenteric lymph nodes and liver leading to increased mortality. The increased inflammasome activity and intestinal barrier leakage were ameliorated by the inducible ablation of COMMD10-deficient Ly6C^{hi} monocytes. In consistence with these results, COMMD10-deficiency in Ly6C^{hi} monocytes, but not in intestinal-resident lamina propria macrophages, led to increased IL-1β production and aggravated colonic inflammation in a model of DSS-induced colitis. Finally, COMMD10 expression was reduced in Ly6C^{hi} monocytes and their corresponding human CD14^{hi} monocytes sorted from mice subjected to DSS-induced colitis or from IBD patients, respectively. Collectively, these results highlight COMMD10 as a negative regulator of Ly6C^{hi} monocyte inflammasome activity during systemic inflammation and IBD.

Keywords: COMMD10, Ly6C^{hi} monocytes, inflammasome, systemic inflammation, colitis

INTRODUCTION

Macrophages are myeloid immune cells that are strategically positioned throughout the body tissues, where they play key innate immune effector functions during development and adulthood, while orchestrating processes related to tissue maintenance and repair, surveillance of hazardous foreign material and tissue specific functions (1). A long lasting dogma held that tissue-resident macrophages rely on replenishment by bone marrow (BM)-derived monocytes (2). However, it is now well-established that most tissue-resident macrophages are established prenatally and maintained through adulthood by longevity and limited self-renewal (1). Ly6C^{hi} monocytes have instead emerged as an additional highly plastic and dynamic emergency squad that can be rapidly recruited to sites of inflammation and complement classical tissue-resident macrophages by pro-inflammatory or resolving activities (1, 3). In the particular examples of drug-induced liver injury (4, 5) and steatohepatitis (6), recruited inflammatory Ly6C^{hi} monocytes differentiate into an ephemeral macrophage subset that is functionally distinct from the resident Kupffer cell (KC) population. Matching the dynamic gut landscape with its constant tissue renewal, intestinal lamina propria macrophages (lpMF) form a unique paradigm of tissue resident macrophages, as their majority are replenished under homeostatic conditions by circulating Ly6C^{hi} monocytes (7–12). Yet, under inflammatory settings, the same monocytes give rise to distinct effector cells that actively promote gut inflammation (10, 13).

Inflammasomes are key signaling platforms composed of multimeric protein complexes that orchestrate host defense mechanisms in response to pathogenic microorganisms or death-associated molecular patterns (DAMPs). Upon assembly of different inflammasomes, caspase-1 is self-cleaved and subsequently proteolytically activates the pro-inflammatory cytokines IL-1β and IL-18 (14). In general, inflammasome activation employs a two-step mechanism; recognition of a first signal e.g., via Toll-like receptor (TLR) signaling provides transcriptional upregulation of pro-IL-1β, whereas a second signal is recognized for proteolytic activation (15). Recently, a "non-canonical" inflammasome pathway has become evident in which lipopolysaccharide (LPS) from Gram-negative bacteria is directly sensed by cytosolic caspase-11 or its human orthologs caspase-4 and caspase-5 to trigger caspase-1-independent pyroptosis, a form of cell death, and caspase-1-dependent secretion of IL-1β and IL-18 (16, 17). Blood monocytes and macrophages are considered as primary sources of IL-1β. Yet, while macrophages seem to rely on the two-step model, blood monocytes are mechanistically competent of releasing mature IL-1β even following a single stimulus of TLR-2 or−4 (18, 19). Interestingly also, classical human CD14^{hi} monocytes, which are equivalent to the murine Ly6C^{hi} monocytes, produce higher levels of IL-1β in response to LPS in comparison with the non-classical monocyte subset as a result of increased *Il1b* mRNA decay in the latter (20). Therefore, Ly6C^{hi} monocyte dwelling in the circulation and their rapid tissue recruitment in various inflammatory disorders necessitates tight regulation of their inflammasome activation to support appropriate immunity and avoid immune-pathology.

The COMMD (copper metabolism MURR1 domain) protein family includes 10 evolutionarily conserved proteins present in eukaryotic multicellular organisms. All share the structurally conserved COMM domain, which serves as an interface for the regulation of protein-protein interactions. The specific functions of COMMD proteins are poorly defined, but they seem to play distinct and non-redundant roles involved with transcriptional regulation and protein trafficking in various physiological processes (21, 22). COMMD1 is the best characterized member of the family and has been implicated in many different cellular functions, such as copper and cholesterol homeostasis, ionic transport, transcription regulation, and oxidative stress (23). With respect to immune cells, COMMD1-targeted deficiency in myeloid cells results in increased NF-κB activation, thereby exacerbating the course of LPS-induced systemic inflammation and colitis (24). Aside from their effects on transcription factor function, COMMD proteins have been identified as essential components of the COMMD/CCDC22/CCDC93 (CCC) protein complex, which modulates endolysosome architecture and is required for the correct trafficking of different transmembrane proteins that traverse through this compartment (22, 25). Collectively, these features of COMMD proteins mark them as candidate mediators of monocyte and macrophage immune responses. Studies on the specific immunoregulatory role of COMMD10 however, have so far been hindered by the embryonic lethality of COMMD10-knockout mice.

Here we utilized conditional COMMD10-knokcout mice allowing the targeting of COMMD10-deficiency to distinct myeloid immune cells. We show that COMMD10-deficiency dramatically augments canonical and non-canonical inflammasome activation in Ly6C^{hi} monocytes, but not tissue-resident macrophages, thus fueling inflammation in mice with LPS-induced systemic inflammation or colitis.

MATERIALS AND METHODS

Mice

Animal experiments were performed with male adult C57BL/6J mice (8–12-weeks old). Animals were maintained in specific pathogen-free animal facility. *LysM*$^{\Delta Commd10}$ and *Cx3cr1*$^{\Delta Commd10}$ mice were generated by crossing *Lyz2*^{cre} and *Cx3cr1*^{cre} (26) mice with Commd10$^{fl/fl}$ mice, respectively, which were purchased from the EUCOMM consortium (strain EM: 05951) (C57BL/6J background). Experiments with *LysM*$^{\Delta Commd10}$ and *Cx3cr1*$^{\Delta Commd10}$ mice were performed on mice heterozygous for these genes.

Human Biopsies and Blood Monocyte Isolation

Intestinal biopsies were obtained from 16 terminal ileums and colons of IBD patients with active disease according to pathological assessment of nearby biopsies and from 14 age and sex-matched healthy controls. Biopsies were immediately frozen in liquid nitrogen for further analyses. Peripheral blood (20 ml) was obtained from 15 IBD patients or healthy

controls. PBMCs were enriched from whole blood by Ficoll density gradient media (Ficoll-PaqueTM PLUS, GE Healthcare). $CD14^+CD16^-HLA\text{-}DR^+$ cells were sorted using FACS-Aria machine (BD Biosciences) and used immediately for RNA isolation.

Ethics Statement

Studies with human cells and tissues were carried out in accordance with the recommendations of Tel-Aviv Sourasky Medical Center Helsinki committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki. All procedures involving human subjects were reviewed and approved by the Institutional Review Boards (Tel-Aviv Sourasky Medical Center IRB approval # TLV-0579-10). All mouse studies were carried out in accordance with the recommendations of Tel-Aviv Sourasky Medical Center ethical committee for animal studies. The protocols were approved by the local committee (# 16-6-13).

BMDM and BM Neutrophil Preparation and Stimulation

BMDM were prepared by flushing BM from the femur and tibia and culturing in RPMI medium containing FBS (10%), penicillin (100 IU/ml), streptomycin (100 μg/ml) and macrophage-colony stimulating factor (M-CSF, 20 ng/ml), at 37°C in 5% CO_2. Media was supplemented every 2–3 days. On day 6, 500,000 BMDM were seeded and subjected to LPS stimulation (Sigma-Aldrich, L2880 Lot #025M4040V) (100 ng/ml) for 3 h, or to *Escherichia coli* (ATCC 25922) at multiplicity of infection (MOI) = 1, with early log phase bacteria. 30 min after infection cells were washed and supplemented with gentamicin (100 ng/ml) to eliminate extracellular bacteria. At indicated times macrophages were washed twice with $PBS^{-/-}$ (without Ca^{++} and Mg^{++}), and collected for western blot analyses. Neutrophils were isolated from BM by magnetic separation using the neutrophil isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany cat# 130-092-332). Neutrophils were enriched to high purity (above 99%) and identified using flow cytometry as $CD11b^+Ly6G^+$ cells. About 2,000,000 neutrophils were seeded in RPMI containing 10% FBS, 1% L-Glutamine and 0.1% penicillin streptomycin, and incubated for 30 min in a 5% CO_2 at 37°C. Non-adherent cells were removed and neutrophils were subjected to LPS (100 ng/ml) for 3 h. ATP (InvivoGen, cat# Tlrl-atp1) was added in the last 30 min of the experiment.

Sorting of Splenic Ly6C^{hi} Monocytes

Spleens were subjected to mechanical meshing and filtered through 200 μM wire mesh. The cell sediments were washed and pellet was lysed for erythrocytes by 2 min incubation with ACK buffer composed of 0.15 M NH_4Cl, and 0.01 M $KHCO_3$, and then washed again with $PBS^{-/-}$ (without Ca^{2+} and Mg^{2+}). Ly6C^{hi} monocytes were defined as $CD45^+CD11b^+CD115^+$ Ly6C^{hi} and sorted to high purity (above 90%) using the SH800 sorter machine (Sony Biotechnology Inc). Cells were subjected to LPS as detailed above for BMDM.

Lymphocyte Isolation From Spleen and Thymus

Spleen and thymus were subjected to mechanical meshing into a sterile RPMI containing 10% FBS, 1% L-Glutamine and 0.1% penicillin streptomycin. Erythrocytes were lysed by incubation with ACK buffer for 2 min. Cells were plated and selective adherence to plastic was used to enrich non-adherent lymphocytes.

LPS-Induced Systemic Inflammation

Mice at the same range of body mass were i.p. injected with LPS, 0.2 mg/mouse, in 100 μl saline. Mice were sacrificed at indicated time points.

DSS-Induced Colitis

Dextran sulfate sodium (DSS, MP Biomedicals, cat# 160110) was administered in drinking water (1.5%) for 4 and 7 days. Colitis severity was assessed using the murine colonoscopy system (IMAGE1 SPIES^{TM} series, Karl Storz, Germany). Quantification of colitis severity was graded as previously described (27). Digitally recorded video files were processed with Windows Movie Maker software (Microsoft).

Inducible Ablation of Ly6C^{hi} Monocytes

In the LPS-induced systemic inflammation experiments, some of the mice received an i.p. injection of 400 μl anti-mouse CCR2 mAb (clone MC-21)-conditioned media (30 μg Ab/ml), as previously described. Injections were started at 12 h prior to LPS challenge, and repeated every 24 h until sacrifice. In the DSS-induced colitis experiment, MC-21 was injected every 24 h starting at day 1 of DSS, as previously described (10).

Isolation of Hepatic Non-parenchymal Cells

Hepatic non-parenchymal cells were isolated from liver as previously described (4). In brief, mice were perfused with 10 ml of cold $PBS^{-/-}$ and the liver was excised. Livers were cut into small fragments, incubated with shaking (37°C, 250 rpm for 45 min) with 5 ml digestion buffer [5% FCS, 0.5 mg/ml collagenase VIII (Sigma-Aldrich, Rehovot, Israel, C5138-500MG) in $PBS^{+/+}$ (with Ca^{2+} and Mg^{2+})]. Subsequently, livers were subjected to mechanical meshing and filtered through 200 μM wire mesh. This was followed by three cycles of washings with $PBS^{-/-}$ at 40 g from which the supernatant was taken, omitting the parenchymal cell pellet. The supernatant was then centrifuge (400 g) and cell pellet was lysed for erythrocytes by 2 min incubation with ACK buffer (0.15 M NH_4C and 0.01 M $KHCO_3$).

Isolation of Colonic Lamina Propria Cells

Colonic lamina propria phagocytes were isolated as previously described (10). In brief, extra-intestinal fat tissue was carefully removed and colons were then flushed of their luminal content with cold $PBS^{-/-}$, opened longitudinally, and cut into 3–5 mm pieces. Epithelial cells and mucus were removed by 40 min incubation with 5 ml $HBSS^{-/-}$ (without Ca^{2+} and Mg^{2+}) containing 5% FBS, 2 mM EDTA, and 1 mM DTT (Sigma) at

37°C shaking at 250 rpm. Colon pieces were then incubated with shaking (37°C, 250 rpm for 45 min) with 5 ml digestion buffer [5% FCS, 1 mg/ml collagenase VIII (Sigma-Aldrich, Rehovot, Israel, C5138-500MG) in PBS$^{+/+}$]. The cell suspension was then filtered through 200 μM wire mesh and washed with PBS$^{-/-}$.

Protein Immunoblotting

Total protein from BMDM, neutrophils, lymphocytes and mentioned tissues was extracted in ice cold RIPA buffer (C-9806S, Cell Signaling Tech. Beverly, Massachusetts) containing protease inhibitors (P8340, Sigma Aldrich St. Louis, Missouri). Proteins were detected by immunoblotting using standard techniques. Antibodies that were used: caspase-11 (sc-374615), GAPDH (sc-47724), β-ACTIN (sc-47778), Pol II (sc-9001), GCN5 (sc-20698) from Santa Cruz; caspase-1(AG-20B-0042) from adipogen; anti-COMMD10 antibody (GTX121488) from Genetex, pP65 (3033) and P65 (6956) from Cell Signaling. Blots were incubated with HRP-conjugated secondary antibodies, and subjected to chemiluminescent detection using the MicroChemi imaging system (DNR Bio-Imaging Systems, Israel). P65 subcellular fractionation was performed using NE-PER nuclear/cytoplasmic extraction kit (78835, Thermo scientific, Paisley, UK) per manufacturer's instructions. Equivalent protein amounts were loaded for both nuclear and cytoplasmic fractions.

Commd10 Gene Silencing in Human Monocyte Derived Macrophages

Human blood CD14^{+} monocytes were isolated using the magnetic monocyte isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany cat# 130-091-153). Cells were cultured in RPMI medium containing FBS (10%), penicillin (100 IU/ml), streptomycin (100 μg/ml) and macrophage-colony stimulating factor (M-CSF, 20 ng/ml), at 37°C in 5% CO_2. Media was supplemented every 2–3 days. On day 6 cells were plated in 6-well plates (∼5 × 10^5 cells/well) for 18–24 h before transfection. Cells were transfected with 200 nM ON-TARGETplus SMARTpool siRNA targeting *Commd10* or with non-targeting scrambled siRNA [Thermo Scientific, Dharmacon (Illkirch, France)] using HiPerFect transfectant reagent (Qiagen, Courtaboeuf, France) for 48 h, per manufacturer's instructions. Cells were then stimulated with 100 ng/ml LPS for 1 h.

Quantitative Real-Time PCR (RT-PCR)

Total RNA was extracted from tissues with TRIzol® reagent (Invitrogen, United States), and from BMDM or sorted Ly6C^{hi} monocytes using the RNeasy Mini Kit (QIAGEN, Germany). RNA was reverse transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, California). All PCR reactions were performed with SYBR Green PCR Master Mix kit (Applied Biosystems) and Applied Biosystems 7300 Real-Time PCR machine. Quantification of PCR signals of each sample was performed by the ΔCt method normalized to *Gapdh* housekeeping gene. For the list of gene specific primers see **Table S1**.

Flow Cytometry Analysis

The following anti-mouse antibodies were used (dilutions are indicated): CD45 (clone 30-F11, 1:100), CD11b (clone M1/70, 1:300), Ly6C (clone HK1.4, 1:300), CD115 (clone AFS98, 1:100), CD64 (clone X54-5/7.1, 1:50), Ly6G (clone 1A8, 1:100), and Tim4 (clone RMT4-54, 1:50), MHCII (clone M4-114.15.2, 1:200), CX_3CR1 (clone SA011F11, 1:100), CD14 (clone M5E2, 1:100), CD16 (clone 3G8, 1:100) all purchased from BioLegend, San Diego, CA, USA. Anti-mouse F4/80 (clone A3-1, 1:50) was purchased from BIORAD. Cells were analyzed with BD FACSCanto™ II (BD Bioscience). Flow cytometry analysis was performed using FlowJo software (TreeStar, Ashland, OR, United States).

ELISA

Mouse plasma and supernatants from BMDM, BM neutrophils or sorted Ly6C^{hi} monocytes were collected and kept in −80°C until assessed. The levels of the cytokines TNFα, IL-1β, and IFNγ were assessed with the DuoSet ELISA kit (R&D Systems). Plasma from mice at 4 h following LPS challenge was subjected to multiplex cytokine array using the mouse cytokine array Q1 (R&D Systems), and analyzed by Q-analyzer software for QAM-CYT-1.

Fitc Dextran Assay for Intestinal Barrier Function

Mice received FITC-conjugated dextran by gavage (Sigma-Aldrich, Cat# FD4, 160 mg/100 g of body mass) 20 h after LPS stimuli. Four hours later, plasma was collected and FITC fluorescence signal was analyzed using the Synergy 2 Multi-Mode Reader (BioTek Instruments Inc).

Colony Formation Unit (CFU) Assay

Tissues were weighed and briefly homogenized (Polytron PT-MR 2100, Kinematica AG) in 1 ml PBS$^{-/-}$. Appropriate dilutions were seeded on LB agar plates and incubated at 37°C for 24 h. The number of colonies was counted and CFU per gram-tissue mass was calculated.

Data Analysis Using the Immgen Database

Raw microarray data were downloaded from the Immunological Genome Project data Phase 2 (GSE37448 microarray datasets) using Partek GS 6.6 (http://www.partek.com/pgs). Blood Ly6C^{hi} monocytes ($n = 3$) and neutrophils ($n = 4$) were selected. Raw gene expression data were obtained for *Commd10*. The gene expressions of the adipocyte marker adiponectin (*Adipoq*) and of the hepatocyte and cholangiocyte marker Cytokeratin18 (*Krt18*) were also obtained to set background expression levels.

Statistical Analysis

In most experiments, statistical differences between two groups were determined using the unpaired two tailed *t*-test with GraphPad. Survival data (**Figure 2C**) were analyzed by Logrank (Mental-cox method) and Gehan-Breslow-Wilcoxon tests using GraphPad, comparing the *Commd10$^{fl/fl}$* and *LysM$^{\Delta Commd10}$* groups. Significance was defined if *p*-value was <0.05 as following: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS

COMMD10-Deficient Monocyte-Derived Macrophages Exhibit Increased Pro-Inflammatory Response to LPS

The role of COMMD proteins in the regulation of myeloid cell function has been so far overlooked, in particular with respect to COMMD10, given the embryonic lethality of COMMD10-knockout mice. Therefore, we generated conditional COMMD10 knockout mice (*Commd10$^{fl/fl}$*) that allow specific targeting of COMMD10-deficiency to myeloid cells by crossing them with *Lyz2^{cre}* mice. The resulting *LysM$^{\Delta Commd10}$* mice exhibited normal birth rate and life expectancy (*data not shown*). Efficient COMMD10 protein-deficiency was evident in macrophages generated from the spleen and BM, but not in lymphoid cells extracted from the spleen and thymus (**Figure 1A**). Both splenic and bone marrow-derived macrophages (BMDM) exhibited specific reduction in *Commd10* expression, while the expression of all other *Commd* genes was intact (**Figure 1B**). Therefore, the *LysM$^{\Delta Commd10}$* mice provided us with a model to study the immunoregulatory role of COMMD10 in myeloid cells. We assessed the effect of COMMD10-deficiency on LPS-induced innate immune responses in BMDM. LPS-stimulated BMDM from *LysM$^{\Delta Commd10}$* mice showed increased transcription of pro-inflammatory cytokines including TNFα (*Tnf),* IL-1β (*Il1b*) and the MX Dynamin Like GTPase 1 (*Mx1*) (**Figure 1C**). Supernatants from overnight cultures of *LysM$^{\Delta Commd10}$* BMDM contained higher levels of the secreted cytokines TNFα, IFNγ and IL-1β (**Figure 1D**). Therefore, COMMD10-deficiecny in Ly6C^{hi} monocyte-derived BMDM exhibit increased inflammatory cytokine production in response to LPS.

COMMD1, the prototype member of the COMMD family, was initially reported to inhibit NF-κB-mediated transcription (28). Further studies in human embryonic kidney cells genetically manipulated to overexpress distinct COMMD proteins revealed that COMMD10 is also capable of inhibiting TNF-mediated NF-κB activation (21). Yet, the physiological relevance of this finding to immune cells, specifically monocytes, remains elusive. In this respect, LPS-challenged COMMD10-deficient BMDM exhibited intensified and more persistent phosphorylation of P65 (**Figure 1E**) as well as increased translocation of P65 to the nucleus (**Figure 1F**). Increased levels of phosphorylated P65 (pP65) and IL-1β secretion were also evident in COMMD10-deficient BMDM infected with the Gram^{neg} bacteria *Escherichia coli* (*E. coli*) (**Figures 1G,H,I**). To further validate these findings in human CD14^{hi} monocytes, which are the equivalent of mouse Ly6C^{hi} monocytes, we utilized siRNA to silence *Commd10* gene expression in M-CSF-differentiated CD14^{hi} monocyte-derived macrophages. Even partial reduction in COMMD10 expression was sufficient to induce greater activation of NF-κB in response to LPS, as manifested by increased nuclear translocation of P65 (**Figure 1J**). Therefore, these results establish a role for COMMD10 in suppressing NF-κB activation.

COMMD10-Deficiency in Myeloid Cells Exacerbates LPS-Induced Systemic Inflammation

We next examined the response of *LysM$^{\Delta Commd10}$* mice to systemic LPS-induced systemic inflammation. Analysis of an array of plasma cytokines at 4 h following LPS challenge revealed higher levels of pro-inflammatory cytokines and chemokines including IFNγ, IL-6, IL-9, CCL5 (RANTES) and IL-1β in the *LysM$^{\Delta Commd10}$* vs. *Commd10$^{fl/fl}$* mice (**Figure 2A**). More direct analysis of TNFα levels further revealed its elevated expression in the plasma of *LysM$^{\Delta Commd10}$* mice (**Figure 2B**). This augmented cytokine storm witnessed in the *LysM$^{\Delta Commd10}$* mice may induce tissue damage and impair survival. Indeed, mortality rates of *LysM$^{\Delta Commd10}$* mice were significantly higher over a period of 72 h in comparison with *Commd10$^{fl/fl}$* control mice ($p = 0.02$; **Figure 2C**). These results suggest that COMMD10-deficiency induces a hyper-inflammatory state that impairs overall survival following acute endotoxic shock.

COMMD10 Negatively Regulates Inflammasome Activity in Ly6C^{hi} Monocytes During LPS-Induced Systemic Inflammation

Inflammasome activation in macrophages is essential to ensure adequate host defense against invading microbes, yet it must be finely controlled to avoid overt tissue damage (14). While both blood monocytes and macrophages are prime producers of IL-1β, monocytes naturally express higher levels of activated caspase-1 allowing its maturation and release even following a single stimulus of TLR-2 or−4 (18). To determine whether Ly6C^{hi} monocytes were responsible for the excessive IL-1β production in the serum of LPS-challenged *LysM$^{\Delta Commd10}$* mice (**Figure 2A**), we induced their ablation by preemptive treatment with the anti-CCR2 MC-21 antibody (10, 29). Indeed, MC-21 treatment specifically depleted Ly6C^{hi} monocytes tissue infiltrates in the liver (**Figure S1A**) and colon (**Figure S1B**) of LPS-challenged mice, but was inert to tissue-resident macrophages or neutrophils in these compartments. In addition, we crossed *Commd10$^{fl/fl}$* with *Cx3cr1^{cre}* mice giving rise to *Cx3cr1$^{\Delta Commd10}$* mice. In these mice, cre-driven recombination (and the ensuing COMMD10-deficiency) is efficient in tissue-resident macrophages, significantly less in circulating CX_3CR1^{lo}Ly6C^{hi} monocytes and hardly affects CX_3CR1^{neg} neutrophils (26). Plasma IL-1β levels were profoundly elevated in *LysM$^{\Delta Commd10}$* vs. *Commd10$^{fl/fl}$* mice at 4 h following LPS challenge (**Figure 3A**). Strikingly, this elevation was ameliorated following Ly6C^{hi} monocyte ablation (**Figure 3A**). Moreover, plasma IL-1β levels were similar in both *Commd10$^{fl/fl}$* and *Cx3cr1$^{\Delta Commd10}$* mice (**Figure 3A**). These phenomena were mirrored in the transcription of *Il1b* in the liver (**Figure 3B**). The activation of caspases-1 and-11 can both drive IL-1β release (15, 16). In this respect, in comparison with *Commd10$^{fl/fl}$* mice, there was significantly higher expression of pro-caspase-11 as well as activation of both caspase-1 and-11 in the liver of *LysM$^{\Delta Commd10}$*, but not *Cx3cr1$^{\Delta Commd10}$* mice, at 4 h following LPS challenge

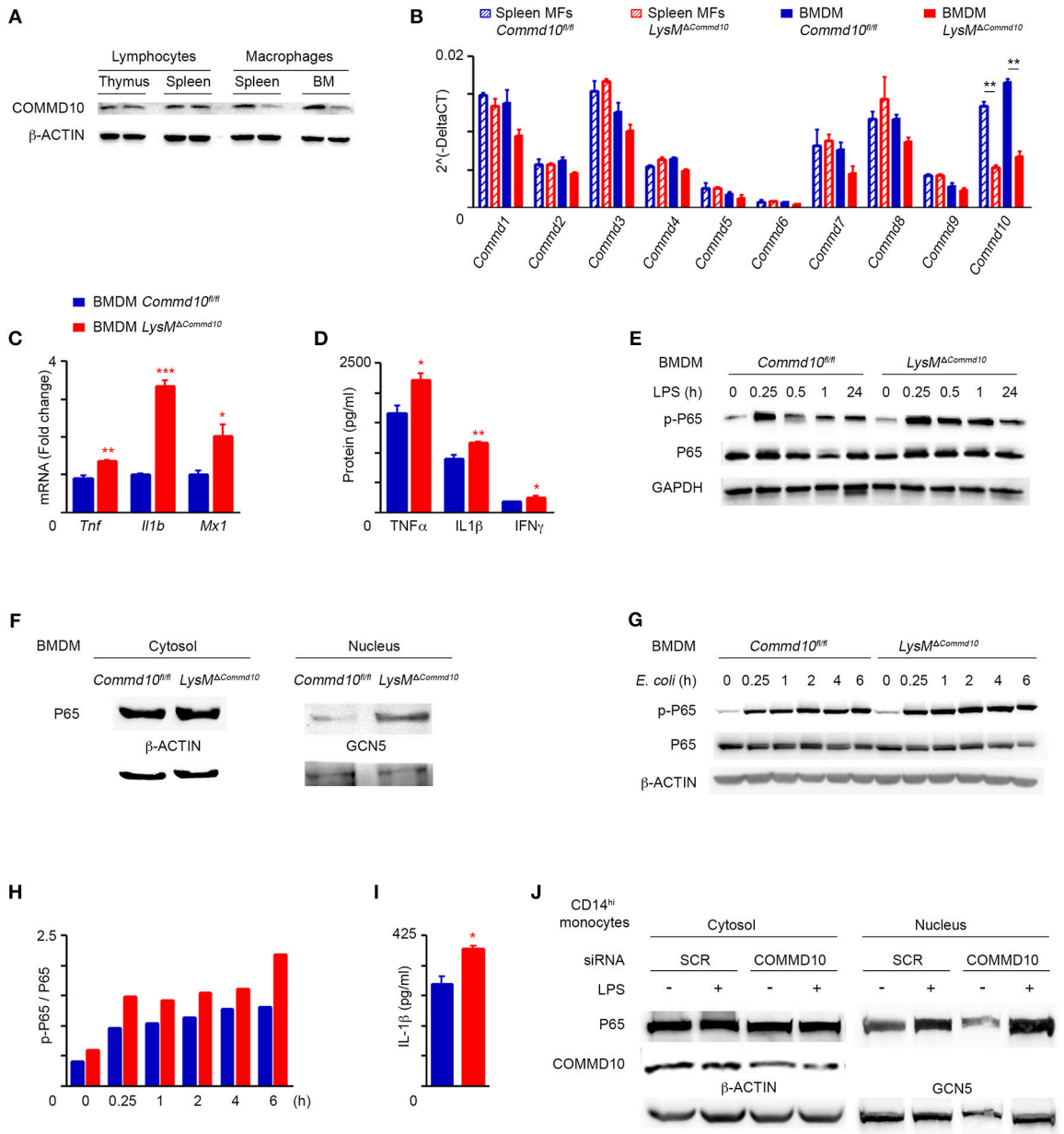

FIGURE 1 | *LysM*$^{\Delta Commd10}$ BMDM respond to LPS by higher production of pro-inflammatory cytokines. **(A,B)** Cells were isolated from spleen and BM of *Commd10*$^{fl/fl}$ (blue) or *LysM*$^{\Delta Commd10}$ (red) mice. **(A)** Immunoblots showing the expression of COMMD10 in cell lysates of thymic or splenic lymphocytes vs. splenic or BM-derived macrophages. β-actin was utilized as control. **(B)** qRT-PCR gene expression of all COMMD family members in splenic macrophages and BMDM ($n = 3$). **(C,D)** BMDM were stimulated with LPS (100 ng/ml) for 3 h. **(C)** qRT-PCR gene expression of pro-inflammatory cytokine in cell lysate ($n = 3$). **(D)** ELISA of pro-inflammatory cytokine levels in cell free supernatants ($n = 3$). **(E)** Representative immunoblot of BMDM cell lysates following LPS challenge (100 ng/ml) showing the protein expression of phospho-P65 (p-P65) and P65 over time course. GAPDH was used as control. **(F)** Immunoblots showing P65 expression in cytosolic and nuclear fraction lysates of LPS-treated BMDM. β-ACTIN and Polymerase II antibodies, respectively, were used as controls ($n = 3$). **(G–I)** BMDM from *Commd10*$^{fl/fl}$ or *LysM*$^{\Delta Commd10}$ mice were infected with *E. Coli* at multiplicity of infection (MOI) = 1. **(G)** Immunoblots showing the protein expression of p-P65 and P65 over time course. β-ACTIN was used as control. **(H)** Respective densitometry-based quantification of p-P65/P65 ratio in *Commd10*$^{fl/fl}$ vs. *LysM*$^{\Delta Commd10}$ BMDM over time course following *E. coli* infection. **(I)** ELISA analysis of IL-1β from supernatants at 6 h post-infection with *E. coli* ($n = 4$). **(J)** Immunoblots showing cytosolic COMMD10 protein expression following siRNA-based gene silencing vs. scrambled siRNA control, β-ACTIN was used as control, and immunoblots showing P65 expression in cytosolic and nuclear fraction lysates of LPS-treated human CD14^{hi} monocyte-derived macrophages. GCN5 was used as control in the nuclear fraction ($n = 3$). Data were analyzed by unpaired, two-tailed *t*-test, comparing each time between *Commd10*$^{fl/fl}$ and *LysM*$^{\Delta Commd10}$ (red stars), and are presented as mean ± SEM with significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data in **(A,B,E)** represents two-independent experiments. Data in **(C,D,F–J)** are from a single experiment.

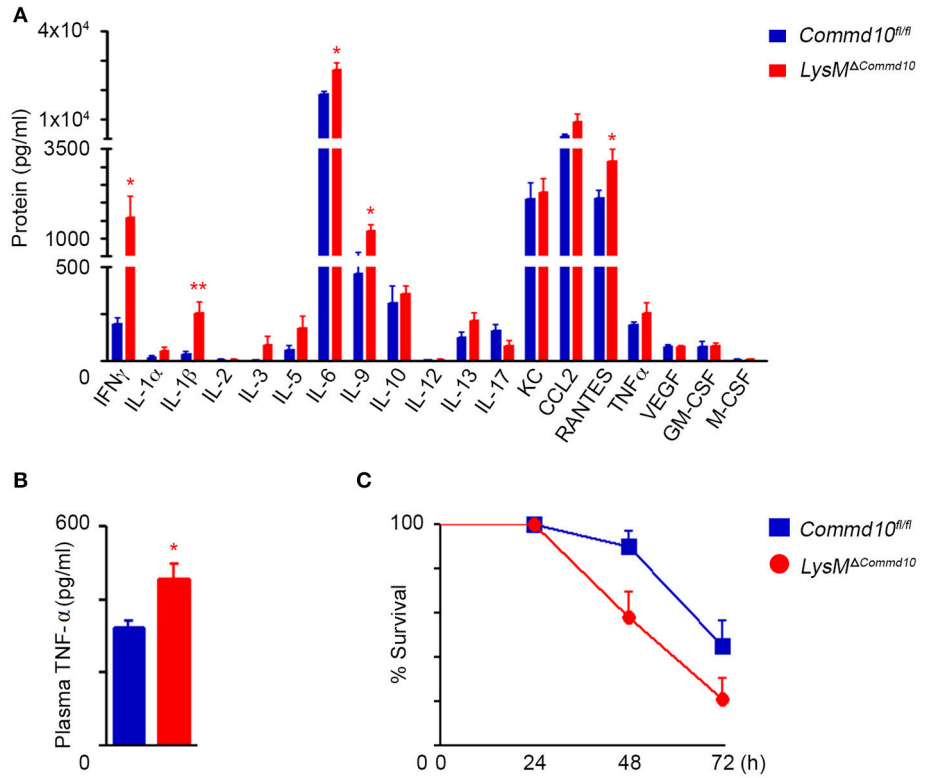

FIGURE 2 | *LysM*$^{\Delta Commd10}$ mice exhibit aggravated cytokine storm and mortality in response to systemic LPS challenge. **(A,B)** *Commd10*$^{fl/fl}$ (blue) and *LysM*$^{\Delta Commd10}$ (red) mice were i.p. injected with LPS (0.2 mg per mouse matched for body mass) and sacrificed 4 h later. **(A)** Multiplex ELISA array of plasma pro-inflammatory cytokines ($n = 4$). **(B)** ELISA analysis of plasma TNF-α ($n \geq 3$). **(C)** Survival curve over 72 h ($n = 10$). Data in **(A,B)** were analyzed by unpaired, two-tailed *t*-test, comparing each time between *Commd10*$^{fl/fl}$ and *LysM*$^{\Delta Commd10}$ (red stars), and are presented as mean ± SEM with significance: $^*p < 0.05$, $^{**}p < 0.01$. Data in **(C)** were analyzed by Logrank (Mental-cox method) ($P = 0.03$) and Gehan-Breslow-Wilcoxon ($P = 0.02$) tests comparing between the *Commd10*$^{fl/fl}$ and *LysM*$^{\Delta Commd10}$ groups. Data in **(A,B)** represent a single experiment. Data in **(C)** represent three independent experiments.

(**Figures 3C,D**). Strikingly, MC-21-mediated Ly6C^{hi} monocyte ablation completely abolished the activation of these caspases in the liver (**Figures 3C,D**). In alignment, flow cytometry analysis showed increased infiltration of Ly6C^{hi} monocytes, defined as CD11b$^+$Ly6C^{hi}Ly6G$^-$, to the livers of *LysM*$^{\Delta Commd10}$ vs. *Commd10*$^{fl/fl}$ mice. In contrast, COMMD10-deficiency had no effect on the abundancy of resident CD11b^{int}F4/80$^+$Tim4$^+$ KCs or infiltrating CD11b$^+$Ly6G$^+$Ly6C^{lo} neutrophils (**Figure 3E**). Similarly, increased activation of caspases-1 and-11 was observed in *LysM*$^{\Delta Commd10}$ spleen (**Figure 3F**) and colon (**Figure 3G**), and in both cases, it was ameliorated by MC-21-driven ablation of Ly6C^{hi} monocytes. Moreover, there was a trend for increased infiltration of Ly6C^{hi} monocytes to the *LysM*$^{\Delta Commd10}$ colon with no clear difference in the abundance of CD11b$^+$F4/80$^+$CD64$^+$ resident lpMF and Ly6G$^+$ neutrophils (**Figure 3H**). To directly delineate the effect of COMMD10-deficiency on inflammasome activation in mature circulating Ly6C^{hi} monocytes, these cells were sorted to high purity from their splenic reservoir according to their simultaneous expression of CD45, CD11b, CD115 (Csf-1R) and Ly6C (*data not shown*). In the absence of COMMD10, Ly6C^{hi} monocytes exhibited a strong trend toward increased transcription of *Il1b* and significantly increased secretion of IL-1β in response to LPS (**Figure 3I**). Of note, in contrast to Ly6C^{hi} monocytes, there were reduced levels of IL-1β production in the supernatants of LPS and adenosine triphosphate (ATP)-stimulated COMMD10-deficient BM neutrophils (**Figures S2A,B**). Using published data sets available from the ImmGen Consortium database we could further show that *Commd10* gene expression was near background levels in blood neutrophils and significantly higher in blood Ly6C^{hi} monocytes (**Figure S3**). Taken together, these findings implicate COMMD10 as a negative regulator of inflammasome activity in Ly6C^{hi} monocytes, but not in tissue-resident macrophages and neutrophils, during LPS-induced systemic inflammation.

COMMD10-Deficiency in Ly6C^{hi} Monocytes Leads to Increased Intestinal Barrier Dysfunction in LPS-Induced Systemic Inflammation

Defective intestinal epithelial tight junction (TJ) barrier has been implicated as an important pathogenic factor in sepsis (30). It may lead to bacterial translocation from the intestinal lumen to the mesenteric nodes, liver and systemic

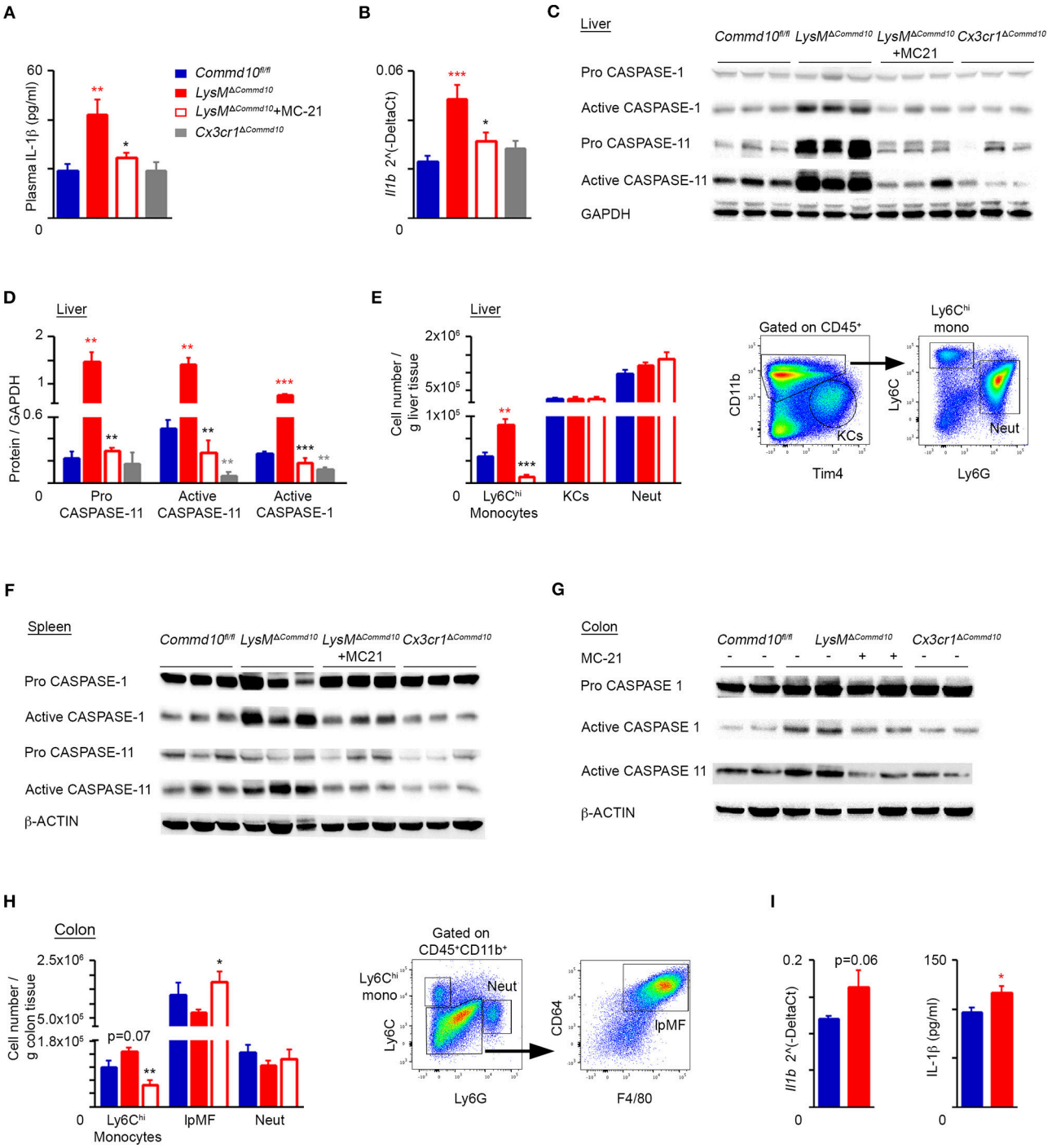

FIGURE 3 | Increased canonical and non-canonical inflammasome activity in COMMD10-deficient Ly6C^{hi} monocytes during LPS-induced systemic inflammation. **(A–H)** *Commd10$^{fl/fl}$* (blue), *LysM$^{\Delta Commd10}$* (red) or *Cx3cr1$^{\Delta Commd10}$* (gray) mice were i.p. injected with LPS (0.2 mg per mouse of similar body mass) and sacrificed 4 h later. Where indicated, MC-21 was injected 12 h prior to LPS stimulation (red border). **(A)** ELISA analysis of plasma IL-1β ($n \geq 5$). **(B)** qRT-PCR gene expression of *Il1b* in liver extracts ($n \geq 5$). **(C)** Immunoblots of liver lysates demonstrating the expression of pro- and active- caspase-1 and−11. GAPDH was utilized as control ($n = 3$). **(D)** Respective densitometry-based quantification of pro-caspase-11, active caspase-1 and active caspase-11, normalized to GAPDH. **(E)** Left panel: flow cytometry-based assessment of liver Ly6C^{hi} monocyte, KC and neutrophil abundance normalized to tissue mass (g) ($n = 5$). Right panel: representative flow cytometry images showing gating strategy of liver Ly6C^{hi} monocytes, KCs and neutrophils ($n = 5$). **(F,G)** Immunoblots of **(F)** spleen and **(G)** colon lysates demonstrating the expression of pro- and/or active- caspase-1 and−11. β-actin was utilized as control ($n = 3$ for **E**, $n = 2$ for **F**). **(H)** Left panel: flow cytometry-based assessment of colonic Ly6C^{hi} monocyte, resident lpMF and neutrophil abundance normalized to tissue mass (g) ($n = 5$). Right panel: representative flow cytometry

(Continued)

FIGURE 3 | images showing gating strategy of colonic Ly6C^{hi} monocytes, lpMFs and neutrophils out of CD45^{+}CD11b^{+} cells ($n = 5$). **(I)** Circulating Ly6C^{hi} monocytes were sorted from their splenic reservoir and stimulated with LPS (100 ng/ml) for 10 h. Left panel: qRT-PCR gene expression of *Il1b*. Right panel: ELISA analysis of IL-1β from cell free supernatants (biological repeats: $n = 3$ for *Commd10*$^{fl/fl}$ and $n = 4$ for *LysM*$^{\Delta Commd10}$, each repeat is composed from a pool of three mice). Data were analyzed by unpaired, two-tailed *t-test*, comparing each time between one of the mouse groups with *Commd10*$^{fl/fl}$ mice (colored stars), or between *LysM*$^{\Delta Commd10}$ mice with and without MC-21 treatment (black stars). Results are presented as mean ± SEM with significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data in **(A–C,E,F,H)** represent two independent experiments. Data in **(D,G)** represent a single experiment.

circulation, and induce severe complications resulting in high mortality rates. Excess production of inflammatory cytokines, and specifically IL-1β, can damage TJ protein expression and function (31, 32). Therefore, we hypothesized that the increased mortality rates of LPS-challenged *LysM*$^{\Delta Commd10}$ mice (**Figure 2C**) may be the result of impaired intestinal barrier function. Indeed, a significant breach in barrier function was evident in *LysM*$^{\Delta Commd10}$ mice at 24 h following LPS challenge, as confirmed by markedly increased intestinal translocation of the orally administered fluorescent probe FITC-dextran (**Figure 4A**). Ly6C^{hi} monocyte depletion partially mitigated barrier leakage (**Figure 4A**). In agreement with the impaired barrier function, expression of colonic tight-junction claudin genes *Cldn* 1, 2 and 15 was decreased in the colons of *LysM*$^{\Delta Commd10}$ mice at 48 h following LPS challenge (**Figure 4B**). Ly6C^{hi} monocyte depletion restored their expression to the level present in *Commd10*$^{fl/fl}$ colons (**Figure 4B**). In alignment with their increased mortality (**Figure 2A**), bacterial counts were elevated in mesenteric lymph nodes (MLN), liver and spleen of *LysM*$^{\Delta Commd10}$ mice (**Figure 4C**). Collectively, these findings mark an important role for COMMD10 in the protection of intestinal barrier function from Ly6C^{hi} monocyte-driven inflammation during sepsis.

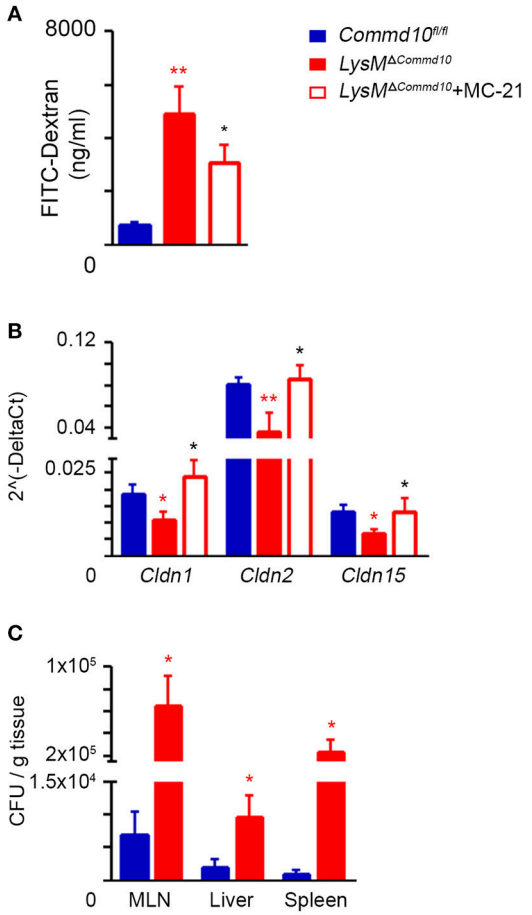

FIGURE 4 | Increased intestinal barrier dysfunction in LPS-challenged *LysM*$^{\Delta Commd10}$ mice driven by Ly6C^{hi} monocytes. *Commd10*$^{fl/fl}$ (blue) and *LysM*$^{\Delta Commd10}$ (red) mice were i.p. injected with LPS (0.2 mg per mouse of similar weight) and sacrificed 24 or 48 h later. Where indicated, MC-21 was injected 12 h prior to LPS stimulation and every 24 h (red border). **(A)** Fluorometric assessment of FITC dextran plasma signal at 4 h following its oral administration to mice after 24 h of LPS challenge ($n \geq 6$). **(B)** Graphical summary of qRT-PCR assessment of *Claudin 1, 2,* and *15* gene expressions at 48 h following LPS challenge ($n \geq 7$). **(C)** Indicated tissues were extracted at 48 h following LPS injection. Colony forming units (CFU) were determined and normalized to tissue mass ($n = 4$). Data were analyzed by unpaired, two-tailed *t*-test, comparing each time between *Commd10*$^{fl/fl}$ and *LysM*$^{\Delta Commd10}$ (red stars) or between *LysM*$^{\Delta Commd10}$ mice with and without MC-21 treatment (black stars). Data are presented as mean ± SEM with significance: $^{*}p < 0.05$, $^{**}p < 0.01$. Data in **(A–C)** represent 2–3 independent experiments.

COMMD10 Restrains Inflammasome Activity in Ly6C^{hi} Monocytes During Colitis

The majority of colonic-resident lpMFs rely on constant replenishment by blood Ly6C^{hi} monocytes attracted by local tonic low-grade inflammatory stimuli (8, 9, 11, 12). Newly arriving Ly6C^{hi} blood monocytes are normally conditioned by IL-10 to acquire a non-inflammatory gene-expression profile, while differentiating into CX$_3$CR1^{hi} lpMFs (33, 34). However, this conditioning process fails when they enter the inflamed gut, and hence differentiate instead into pro-inflammatory CX$_3$CR1^{lo} effector cells that actively promote gut inflammation (10, 13). Interestingly, IL-1β was recently shown to be the key mediator driving intestinal inflammation in mice and patients with IL-10 receptor deficiency (35). Therefore, we next studied the effect of COMMD10-deficiency in myeloid cells on intestinal inflammation in an acute model of dextran sodium sulfate (DSS)-induced colitis. Remarkably, live colonoscopy assessment revealed significantly aggravated colitis in *LysM*$^{\Delta Commd10}$ vs. *Commd10*$^{fl/fl}$ control mice (**Figure 5A**; **Movie S1**). In the colon of *Cx3cr1*^{cre} mice, cre-mediated recombination is mainly targeted to resident lpMFs (34). Notably, the colitis score of *Cx3cr1*$^{\Delta Commd10}$ mice was similar to that of *Commd10*$^{fl/fl}$ control mice (**Figure 5A**; **Movie S1**), suggesting that COMMD10 does not play a critical anti-inflammatory role in CX$_3$CR1^{hi} lpMFs. The aggravated colitis in *LysM*$^{\Delta Commd10}$ mice was also reflected in the reduced length of their colon

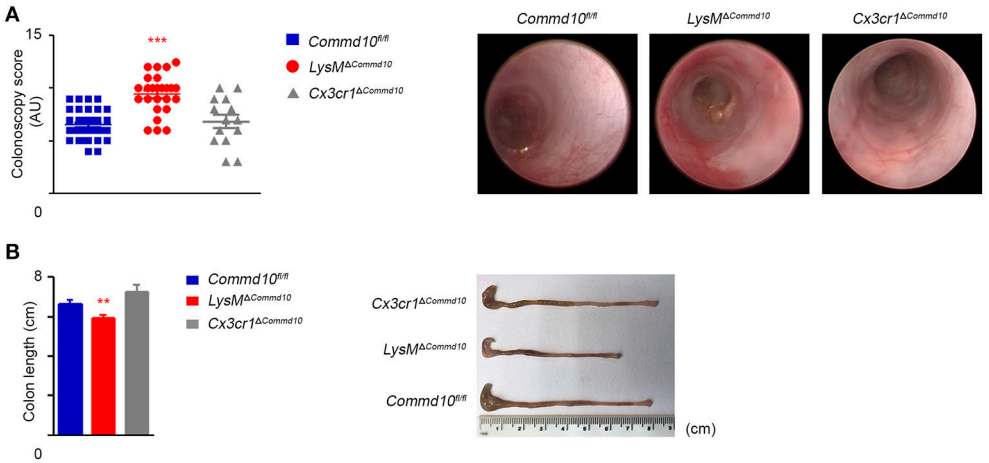

FIGURE 5 | *LysM*$^{\Delta Commd10}$, but not *Cx3cr1*$^{\Delta Commd10}$ mice exhibit augmented DSS-induced colitis. *Commd10*$^{fl/fl}$ (blue), *LysM*$^{\Delta Commd10}$ (red) or *Cx3cr1*$^{\Delta Commd10}$ (gray) mice were treated with DSS (1.5% in drinking water) for 7 days. **(A)** Left panel: Scatter plot graph demonstrating colonoscopy scores as determined by live colonoscopy ($n \geq 14$). Right panel: Representative colonoscopy images. **(B)** Left panel: graph demonstrating colon length. Right panel: Representative colon images ($n \geq 17$ for *Commd10*$^{fl/fl}$ and *LysM*$^{\Delta Commd10}$ groups, $n = 5$ for *Cx3cr1*$^{\Delta Commd10}$ group)**.** Data were analyzed by unpaired, two-tailed *t*-test, comparing each time between one of the mouse groups with *Commd10*$^{fl/fl}$ mice (colored stars). Results are presented as mean ± SEM with significance: $^{**}p < 0.01$, $^{***}p < 0.001$. Data in **(A,B)** represent 2–3 independent experiments.

in comparison with that of *Commd10*$^{fl/fl}$ or *Cx3cr1*$^{\Delta Commd10}$ (**Figure 5B**).

Colonic infiltrating Ly6C^{hi} monocytes become the dominant pro-inflammatory myeloid effector cells at day 4 of DSS-induced colitis (10). Therefore, we next investigated the effect of COMMD10-deficiency on Ly6C^{hi} monocyte inflammasome activity at this time point. Similarly to their response to LPS-induced systemic inflammation (**Figure 3A**), *LysM*$^{\Delta Commd10}$ mice exhibited a prominent increase in plasma levels of IL-1β, which was completely ameliorated upon MC-21-governed Ly6C^{hi} monocyte ablation. Further emphasizing that Ly6C^{hi} monocytes are the main source for the augmented plasma IL-1β production, *Cx3cr1*$^{\Delta Commd10}$ mice exhibited a significant reduction in plasma IL-1β, even in comparison to control *Commd10*$^{fl/fl}$ mice. Thus, these results exclude a contribution of tissue-resident macrophages to the overall increase of plasma IL-1β observed in the *LysM*$^{\Delta Commd10}$ mice (**Figure 6A**). At day 7, the augmented plasma IL-1β production in *LysM*$^{\Delta Commd10}$ mice was still apparent (**Figure 6B**). Plasma TNFα levels were also elevated in the *LysM*$^{\Delta Commd10}$ mice at day 4 and 7 (**Figure 6C**). We next examined whether generation of Ly6C^{hi} monocytes and neutrophils in the BM or their egress to the circulation were affected by the specific COMMD10-deficiencies in *LysM*$^{\Delta Commd10}$ and *Cx3cr1*$^{\Delta Commd10}$ mice. However, flow cytometry analysis of BM and blood of these transgenic mice at day 4 of DSS-induced colitis revealed no clear differences in their levels (**Figures 6D,E**). Hence, these results suggest that Ly6C^{hi} monocyte hyper-inflammasome activity, rather than availability in the circulation, is responsible for the increased plasma levels of IL-1β in *LysM*$^{\Delta Commd10}$ mice. Surprisingly in the colon, there were significantly reduced fractions of Ly6C^{hi} monocytes and neutrophils in both *LysM*$^{\Delta Commd10}$ and *Cx3cr1*$^{\Delta Commd10}$ mice, with no clear difference in the abundance of colonic lpMFs (**Figure 6F**). This was further corroborated by showing reduced number per tissue mass of colonic Ly6C^{hi} monocytes, but not of lpMFs, in both *LysM*$^{\Delta Commd10}$ and *Cx3cr1*$^{\Delta Commd10}$ mice (**Figure 6G**). In agreement with this, the colonic expression of the genes encoding for the Ly6C^{hi} monocyte and neutrophil recruitment chemokines *Ccl2*, *Cxcl1*, and *Cxcl2*, respectively, was markedly reduced in both mice (**Figure 6H**). Finally, *LysM*$^{\Delta Commd10}$ colons exhibited reduced local transcription of *Il1b* gene, which is in alignment with the declined accumulation of Ly6C^{hi} monocytes (**Figure 6H**). Therefore, these results suggest that circulating Ly6C^{hi} monocytes are responsible for the augmented systemic production of IL-1β.

COMMD10 Is Downregulated in Mouse Ly6C^{hi} Monocytes and Their Equivalent Human CD14^{hi} Monocytes During Intestinal Inflammation

The results above highlight an important negative immunoregulatory role for COMMD10 in tuning Ly6C^{hi} monocyte-driven inflammation during colitis (**Figure 6**). Therefore, downregulation of COMMD10 levels may be essential to license their inflammatory behavior. Indeed, in comparison to Ly6C^{hi} monocytes sorted from steady state splenic reservoirs, there was a profound decrease in the transcription of *Commd10* in their Ly6C^{hi} monocyte counterparts sorted from splenic reservoirs and inflammatory colons at day 4 of DSS-induced colitis (**Figure 7A**). Furthermore, *Commd10* gene expression was also profoundly reduced in the transition of Ly6C^{hi} monocytes from circulation (splenic reservoir) to colonic tissue in the context of colitis (**Figure 7A**). A functionally similar pro-inflammatory CD14^{hi} monocyte population has been identified within inflamed tissues of IBD patients (36, 37). Notably, there

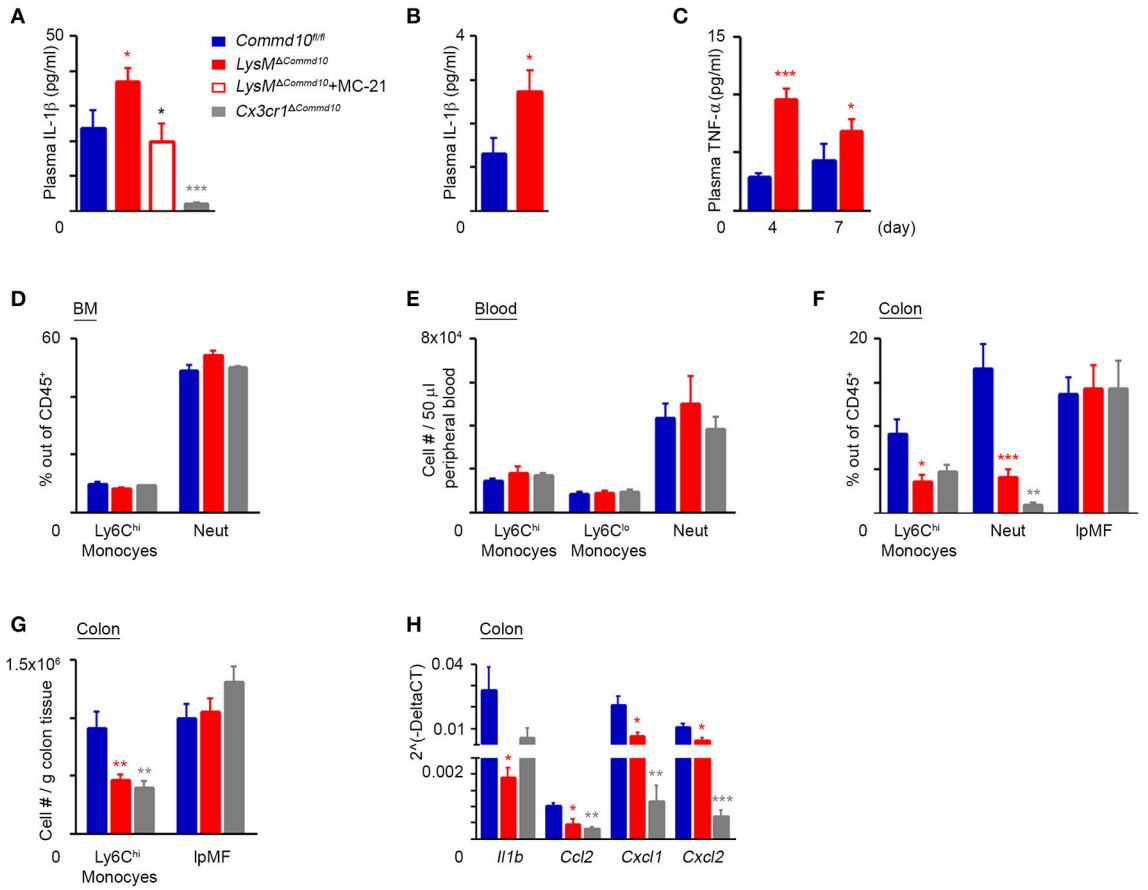

FIGURE 6 | COMMD10 negatively regulates inflammasome activity in Ly6C^{hi} monocytes during colitis. *Commd10$^{fl/fl}$* (blue), *LysM$^{\Delta Commd10}$* (red) or *Cx3cr1$^{\Delta Commd10}$* (gray) mice were treated with DSS (1.5% in drinking water) for 4 or 7 days. Where indicated, MC-21 was injected every day, starting at 24 h after DSS administration (red border). **(A)** ELISA analysis of plasma IL-1β at day 4 and **(B)** day 7 of DSS ($n \geq 5$). **(C)** ELISA analysis of plasma TNF-α (n≥4). **(D)** Flow cytometry-based assessment of BM Ly6C^{hi} monocyte and neutrophil fractions out of total CD45^{+} immune cells at day 4 of DSS ($n \geq 5$). **(E)** Flow cytometry-based assessment of Ly6C^{hi} and Ly6C^{lo} monocytes and neutrophils in 50 µl peripheral blood at day 4 of DSS ($n = 6$). **(F)** Flow cytometry-based assessment of colonic Ly6C^{hi} monocyte, neutrophil and resident lpMF fractions out of total CD45^{+} immune cells at day 4 of DSS ($n = 6$). **(G)** Flow cytometry-based assessment of colonic Ly6C^{hi} monocyte and resident lpMF abundance normalized to tissue mass at day 4 of DSS ($n \geq 10$). **(H)** qRT-PCR gene expression of *Il1b*, *Ccl2, Cxcl1*, and *Cxcl2* in colon tissue at day 4 of DSS ($n \geq 4$). Data were analyzed by unpaired, two-tailed *t*-test, comparing each time between one of the mouse groups with *Commd10$^{fl/fl}$* mice (colored stars), or between *LysM$^{\Delta Commd10}$* mice with and without MC-21 treatment (black stars). Results are presented as mean ± SEM with significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data in **(A,C,F–H)** represent two independent experiments. Data in **(D** and **E)** represent a single experiment.

was also significant decline in the expression of COMMD10 gene (**Figure 7B**) and protein (**Figure 7C**) in circulating CD14^{hi} monocytes sorted from patients with active IBD vs. healthy subjects. This reduction in COMMD10 expression was further evident in colonic or ileal tissue biopsies extracted from IBD patients vs. healthy subjects at both gene (**Figure 7D**) and protein (**Figure 7E**) levels.

DISCUSSION

We have identified a novel role for COMMD10, a protein with unknown function, in the regulation of Ly6C^{hi} monocyte-driven inflammation during systemic inflammation. In particular, lack of COMMD10 in circulating and tissue-infiltrating Ly6C^{hi} monocytes leads to augmented inflammasome-induced caspase-1 and 11 activity during LPS-induced systemic inflammation. The hyper-inflammatory activity of COMMD10-deficient monocytes compromises intestinal barrier function culminating in increased bacterial translocation to internal organs including the liver. In the setting of colitis, we demonstrate that disrupted COMMD10 activity unleashes the inflammatory behavior of circulating Ly6C^{hi} monocytes. Our results also indicate for the first time the involvement of COMMD10 in the inhibition of NF-κB activation in monocytes and their macrophage descendants.

Inflammasomes are key signaling platforms that detect pathogenic microorganisms and sterile stressors, and activate in response the highly pro-inflammatory cytokine IL-1β (14, 15). This cytokine is controlled by two checkpoints: NF-κB-driven transcription of pro-IL-1β as well as inflammasome-dependent caspase-1-mediated maturation and release. An additional non-canonical inflammasome activation pathway has been described in macrophages responding to Gram^{neg} bacteria, by which

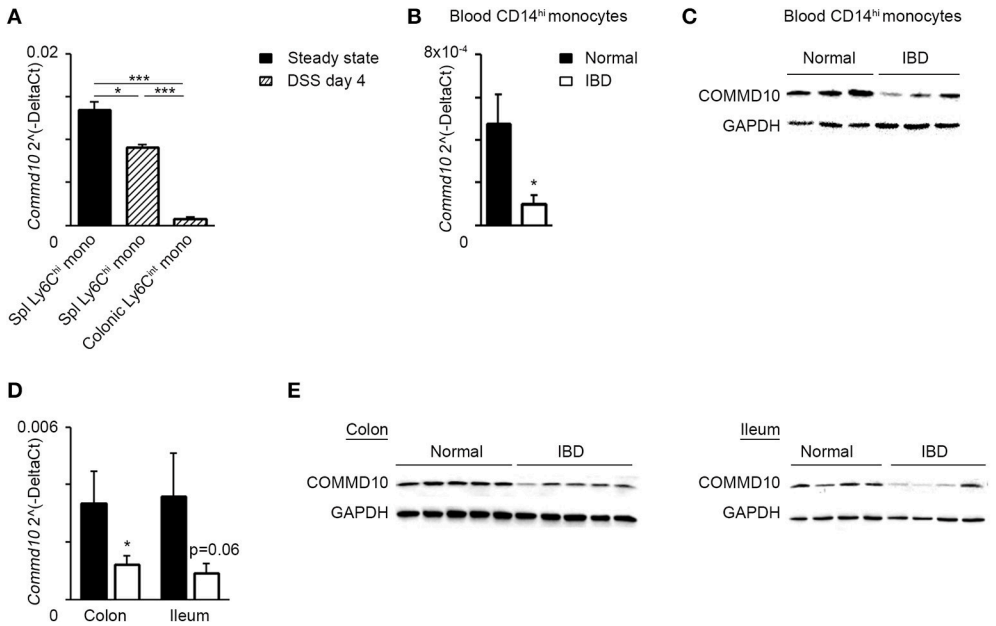

FIGURE 7 | COMMD10 is downregulated in mouse Ly6C^{hi} monocytes and their equivalent human CD14^{hi} monocytes during intestinal inflammation. **(A)** Splenic and colonic Ly6C^{hi} monocytes were sorted from steady state mice (solid) or at day 4 of DSS (diagonal). **(B,C)** Circulating CD14^{hi} monocytes were sorted from patients with active IBD (black border) or healthy controls (solid). **(B)** qRT-PCR gene expression of *Commd10* and **(C)** immunoblot demonstrating COMMD10 protein expression. GAPDH was utilized as control ($n \geq 3$). **(D,E)** Biopsies were obtained from colons and terminal ileums of IBD patients with active disease and healthy controls. **(D)** qRT-PCR analysis of *Commd10* gene expression. **(E)** Immunoblots demonstrating COMMD10 protein expression in colon (left panel) and ileum (right panel) biopsies obtained from IBD patients vs. healthy controls. GAPDH was utilized as controls ($n \geq 14$). Data were analyzed by unpaired, two-tailed *t*-test, comparing each time between IBD with healthy controls. Results are presented as mean ± SEM with significance: $^{*}p < 0.05$, $^{***}p < 0.001$. Data in **(A–C)** represent a single experiment.

caspase-11 directly senses cytosolic LPS to induce IL-1β secretion (16). Utilizing an unbiased multiplex cytokine and chemokine array we show that COMMD10-deficiency in myeloid cells leads to a profound elevation in plasma levels of various pro-inflammatory cytokines, including IL-1β, during endotoxemia. This was further consolidated by a specific ELISA analysis for IL-1β in the plasma and activation of caspase-1 and 11 in the liver, spleen and colon. Strikingly, this phenotype was abolished upon the specific ablation of Ly6C^{hi} monocytes. Moreover, *Cx3cr1*$^{\Delta Commd10}$ mice, in which COMMD10-deficiency is mostly targeted to tissue-resident macrophages (26), showed similar inflammasome activity to that of control *Commd10*$^{fl/fl}$ mice. COMMD10-deficiency had no effect also on inflammasome activity in isolated BM neutrophils responding to LPS/ATP stimulation. In alignment, we demonstrate that *Commd10* gene expression is significantly higher in circulating Ly6C^{hi} monocytes vs. neutrophils. Therefore, these results point to COMMD10 as a negative regulator of both canonical and non-canonical inflammasome pathways uniquely in Ly6C^{hi} monocytes among the various phagocytic cells targeted by *Lyz2*^{cre}. Synthesis and release of IL-1β differ between monocytes and macrophages as the former have constitutively activated caspase-1, and sole transcriptional stimulation by bacterial ligands, such as LPS leads to the release of active IL-1β (18). In this respect, we show that LPS stimulation is solely sufficient to induce augmented IL-1β transcription and secretion in both COMMD10-deficient Ly6C^{hi} monocyte-derived BMDM and COMMD10-deficient sorted circulating Ly6C^{hi} monocytes.

Several studies have linked members of the COMMD protein family to transcription factor regulation. The first transcription factor to be associated with COMMD proteins was NF-κB (21, 28), a master regulator of inflammation and cell survival. COMMD1 is a negative regulator of this pathway, acting to promote the ubiquitination and proteasomal degradation of transcriptionally active NF-κB subunits (38). Consistent with this, COMMD1 deficiency in myeloid cells results in increased NF-κB activation, thereby exacerbating the course of LPS-induced systemic inflammation (24). Studies in non-immune cells genetically manipulated to overexpress COMMD10 have indicated its ability to inhibit NF-κB-mediated transcription as well (21), though the physiological relevance of this finding in general, and in immune cells in particular, remains elusive. Here we show that COMMD10-deficiency in Ly6C^{hi} monocytes and their macrophage descendants, as well as in their human CD14^{hi} monocyte equivalents, leads to increased and persistent activation of NF-κB in response to LPS or *E. coli* infection. This may explain the augmented transcription of NF-κB-target genes in these cells. Moreover, the unleashed NF-κB activity in COMMD10-deficient Ly6C^{hi} monocytes and their macrophage progenies may also be responsible for the increased inflammasome activation in

these cells. Of particular interest is the increased expression of pro-caspase-11 in the *LysM*$^{\Delta Commd10}$ livers following LPS treatment, which was abolished by Ly6C^{hi} monocyte ablation. Pro-caspase-11 transcription via LPS-TLR4 signaling can be induced in parallel by either MyD88-NF-κB or Trif-IRF3 signaling (39). While we provide evidence for increased NF-kB involvement, the potential contribution of the Trif-IRF3 pathway requires further studies. COMMD1 suppresses NF-κB activity by promoting the ubiquitination and degradation of its subunits (38). Yet, the mechanism coupling COMMD10 to NF-κB inhibition remains unknown. An additional transcriptional program shown to be influenced by COMMD1 is hypoxia inducible factor (HIF). Accordingly, constitutional deficiency of COMMD1 leads to broad activation of HIF-1α and embryonic lethality (40), while its deficiency in cancer cells promotes HIF-1α-dependent gene expression and tumor cell invasion (41). Sepsis is characterized by a dysregulated inflammatory response to infection (42). Interestingly, monocytes from septic patients acquire an immunosuppressive phenotype by upregulating HIF-1α activity (43). With respect to COMMD10, there is no evidence that it is involved with HIF-1α regulation. Overall, further studies are needed to delineate the task division between distinct COMMD proteins in shaping the transcriptional programing of Ly6C^{hi} monocytes in response to endotoxin stimulation. Importantly also, given the role of different COMMD proteins in mediating intracellular protein trafficking events (22, 25), COMMD10 may potentially regulate the inflammasome complex activity by affecting its assembly or stability.

LysM$^{\Delta Commd10}$ mice exhibit increased mortality in response to systemic LPS challenge. Intestinal barrier function may be disrupted by various pro-inflammatory cytokines, such as IL-1β (31, 32), leading to systemic bacterial dissemination. Therefore, the augmented pro-inflammatory cytokine response in the *LysM*$^{\Delta Commd10}$ mice at 4 h after LPS challenge, and in particular the systemic elevated inflammasome activation and IL-1β production by Ly6C^{hi} monocytes, may compromise intestinal epithelial cell layer integrity. Indeed, we show severe intestinal barrier leakage in the *LysM*$^{\Delta Commd10}$ mice and reduced transcription of TJ genes in the colon. The ablation of Ly6C^{hi} monocytes partially restores intestinal barrier function and the expression of selected TJ genes, further confirming a role for COMMD10-deficient monocytes in impairing gut barrier function. IFNγ and TNFα are additional pro-inflammatory cytokines known to increase intestinal TJ permeability (44). The expression of both was also significantly higher in the plasma of LPS-challenged *LysM*$^{\Delta Commd10}$ mice and supernatants of *LysM*$^{\Delta Commd10}$ BMDM, therefore suggesting their possible contribution to the observed intestinal barrier disruption in these mice. An alternative explanation for the increased bacterial dissemination and mortality in LPS-challenged *LysM*$^{\Delta Commd10}$ mice may be that over-activation of caspases-1 and −11 in these mice facilitates pyroptosis. This lytic form of cell death releases DAMPs that can further incite multiple organ dysfunction and mortality, and also lead to deficiency in intestinal epithelial cells and various immunocytes (45).

We and others have indicated a pivotal role for Ly6C^{hi} monocytes and their CD14^{hi} human counterparts in the initiation and propagation of intestinal inflammation (10, 13, 36, 37). Our results mark COMMD10 as a negative regulator of inflammasome activity in these cells in the context of colitis as well. We also demonstrate that COMMD10 expression is reduced in circulating Ly6C^{hi} monocytes and their equivalent human CD14^{hi} monocyte subset in the context of gut inflammation. Hence, regulation of COMMD10 expression or activity may be an essential mechanism to tune the physiological inflammasome activity in these cells. The increased colitis score observed in the *LysM*$^{\Delta Commd10}$, but not in *Cx3cr1*$^{\Delta Commd10}$ mice, further substantiates that COMMD10-governed immunoregulation is important already at the level of these circulating monocytes. Surprising was the finding of reduced numbers of effector Ly6C^{hi} monocytes and neutrophils in the inflamed colons in both *LysM*$^{\Delta Commd10}$ and *Cx3cr1*$^{\Delta Commd10}$ mice vs. the control *Commd10*$^{fl/fl}$ mice. A possible explanation may be that COMMD10-deficiency disturbs the migratory capacity of Ly6C^{hi} monocytes and neutrophils. However, our data showing similar representation of Ly6C^{hi} monocytes in the BM and blood compartments of these mice argue against any affect for COMMD10-deficiency on their generation or migration to the circulation. The low expression of *Commd10* gene in neutrophils decreases the probability of an intrinsic effect for COMMD10-deficiency on the migration of these cells. Furthermore, the paucity of Ly6C^{hi} monocytes and neutrophils in the *Cx3cr1*$^{\Delta Commd10}$ colons also excludes cell-intrinsic migratory effects of COMMD10-deficiency, as in these mice cre-mediated recombination (and ensuing COMMD10-deficiency) is mainly targeted to resident macrophages (26). Instead, their paucity in both *LysM*$^{\Delta Commd10}$ and *Cx3cr1*$^{\Delta Commd10}$ mice is in alignment with reduced colonic expression of their recruiting chemokines in these mice. We have previously shown that colonic lpMFs express these chemokines at day 4 of DSS (10). Therefore, COMMD10-deficiency may have an anti-inflammatory effect in colonic-resident lpMFs. This argument is also supported by the profound reduction in plasma IL-1β in the *Cx3cr1*$^{\Delta Commd10}$ vs. *Commd10*$^{fl/fl}$ mice. Further studies are required to delineate the immunoregulatory circuits governed by COMMD10 in resident lpMF.

Robust, and yet balanced inflammatory responses are involved in detection of tissue injury or infection, subsequent inflammatory reaction, and ultimate resolution. Monocytes seem perfectly suited for this task since they are equipped with a large repertoire of scavenger and pattern recognition receptors enabling them to rapidly react to local danger signals (46). Their high plasticity empowers their prompt molecular adaptation in response to altering cues and production of effector molecules capable of driving both inflammation and resolution (1, 3). Although the recent past has seen great advancement in our comprehension of Ly6C^{hi} monocyte biology, the molecular mechanisms that allow monocytes to act in restorative rather than pathological manner remain largely elusive. Here we report on the important role of COMMD10 in restraining Ly6C^{hi} monocyte-driven inflammation.

AUTHOR CONTRIBUTIONS

The corresponding authors (CV and NG) confirm that all authors agree to be accountable for the content of the work. OM, SB, NG, and CV designed, performed, and analyzed all experiments and wrote the manuscript. KC, IF, and SG substantially assisted OM and SB with some of the major *in vivo* experiments and gene expression analyses. NM and ZH greatly assisted with the recruitment and collection of IBD patient samples. EB contributed to the design of the original idea in this 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.2018.02623/full#supplementary-material

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REVIEW
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Macrophages in the Aging Liver and Age-Related Liver Disease

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The number of individuals aged 65 or older is projected to increase globally from 524 million in 2010 to nearly 1. 5 billion in 2050. Aged individuals are particularly at risk for developing chronic illness, while being less able to regenerate healthy tissue and tolerate whole organ transplantation procedures. In the liver, these age-related diseases include non-alcoholic fatty liver disease, alcoholic liver disease, hepatitis, fibrosis, and cirrhosis. Hepatic macrophages, a population comprised of both Kupffer cells and infiltrating monocyte derived macrophages, are implicated in several chronic liver diseases and also play important roles in the homeostatic functions of the liver. The effects of aging on hepatic macrophage population dynamics, polarization, and function are not well understood. Studies performed on macrophages derived from other aged sources, such as the bone marrow, peritoneal cavity, lungs, and brain, demonstrate general reductions in autophagy and phagocytosis, dysfunction in cytokine signaling, and altered morphology and distribution, likely mediated by epigenetic changes and mitochondrial defects, that may be applicable to hepatic macrophages. This review highlights recent findings in macrophage developmental biology and function, particularly in the liver, and discusses the role of macrophages in various age-related liver diseases. A better understanding of the biology of aging that influences hepatic macrophages and thus the progression of chronic liver disease will be crucial in order to develop new interventions and treatments for liver disease in aging populations.

Keywords: Kupffer cell, monocyte-derived macrophage, chronic liver disease, inflammation, fibrosis, senescence, mitochondria

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INTRODUCTION TO MACROPHAGES IN THE LIVER

The liver is an important immunological organ, serving as a surveillance system for gut-derived pathogens and producing several key immune components—complement, acute phase, and coagulation proteins (1, 2). While hepatocytes and certain non-parenchymal cells possess some inherent immunological properties [see reviews (3) and (4)], multiple populations of $CD45^+$ immune cells are transiently or permanently located in the liver.

The healthy liver is home to several populations of lymphocytes, including natural killer (NK) cells, NK T-cells, B-cells, mucosal associated T-cells and γδ-T-cells (5–8). In humans, 40% of the resident lymphocyte population are composed of NK cells, while in mice 40% of the resident lymphocytes are NK T-cells.

Innate immune cells also have a large presence in the liver. The majority of immune cells in the liver are myeloid derived cells (9). Dendritic cell (DC) populations, including both myeloid and plasmacytoid DCs, are present in the liver and can activate T-cells under appropriate

conditions (10). Myeloid-derived suppressor cells (MDSC) are also present and suppress T-cell activation (11). Macrophages derived from circulating monocytes have been identified as a motile population of myeloid cells infiltrating the liver during inflammation and at varying levels during homeostasis (12–14). The resident macrophage, known as the Kupffer cell (KC), is the most highly represented immune cell, comprising nearly one-third of non-parenchymal cells in the liver (15).

Macrophages play a key role in the homeostatic functions of the liver as well as in disease states and are among the most widely studied immune cells in the liver (15). The prevalence and severity of many chronic liver diseases increases with age, including alcoholic hepatitis, non-alcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma (16). However, mechanisms underlying changes in the liver structure and cellular function, including hepatocytes and resident immune cell populations, are not well understood. This review will cover the developmental origin and function of macrophages in the liver, as well as their implications in several age-related liver diseases, in order to better understand and expose gaps in the knowledge of the biology of liver aging and disease. As most of the experimental data has been collected from murine animal models, this review focuses largely on preclinical studies in mice and extends these findings to chronic liver disease in humans.

Development and Polarization of Macrophages

Macrophage development begins in the extraembryonic yolk sac from erythro-myeloid progenitors, a process known as primitive hematopoiesis, prior to the appearance of hematopoietic stem cells or monocytic precursors (17, 18). After this transient wave of primitive hematopoiesis, hematopoietic stem cells appear in the aorto-gonado-mesonephric region and migrate to the fetal liver, marking the start of definitive hematopoiesis and development of naïve macrophages through monocytic precursors (17). Definitive hematopoiesis remains in the fetal liver until approximately E18 in mice or 12 weeks post-conception in humans, after which hematopoietic stem cells migrate from the fetal liver to the bone marrow niche, where they will remain throughout adulthood (19).

Macrophages can be subdivided into tissue resident or monocyte derived populations. The number of resident macrophages varies considerably based on the tissue, and approximately 80% of total tissue resident macrophages are located in the liver (20). Resident macrophages can be colonized from a single developmental source, such as microglia and Kupffer cells, which are derived from yolk sac primitive macrophages. Alternatively, resident macrophages may be repopulated several times during development, such as Langerhans cells in the skin, which are originally derived from yolk-sac macrophages and later replaced by fetal liver monocytes (13, 21). Macrophage populations in the gut, spleen, and lungs are continuously replenished by monocyte input throughout adulthood (12, 13).

During inflammation, both resident and monocyte-derived macrophages participate in migration, expansion, and signaling depending on the tissue type and stimulus. Resident tissue macrophages, including Kupffer cells, peritoneal macrophages, and pleural macrophages, have been shown to undergo rapid proliferation in response to Th2 cytokines, such as interleukin-4 (22). Infiltrating macrophages can be derived from either classical ($CD14^+CD16^-$ human, $Ly6C^{hi}$ mouse) or non-classical ($CD14^+CD16^+$ human, $Ly6C^{lo}$ mouse) monocytes, which extravasate into tissue sites under chemokine concentration gradient guidance (17, 23). Following extravasation, monocytes differentiate into macrophages whose functionality are largely based on the integration of the various signaling molecules present in the local microenvironment (24, 25).

In addition to developmental heterogeneity, macrophages are extraordinarily dynamic cells that exhibit various phenotypes ranging from a pro-inflammatory, classically activated "M1" polarization state to an anti-inflammatory, alternatively activated "M2" polarization state, as shown in **Figure 1** (26, 27). M1/M2 polarization refers to extreme phenotypes that can be simulated *in vitro* with the addition of various stimuli (lipopolysaccharide and/or interferon-gamma for M1 activation; interleukin-4 for M2 activation), but the scenario *in vivo* is often more complex and thus polarization is a spectrum of phenotypes, including but not limited to M2a, M2b, and M2c sub-groups (27, 28).

In general, M1 polarization leads to production of canonical pro-inflammatory cytokines (IL-1, IL-6, IL-12, IL-18, TNF, IL-23, IL-27), reactive oxygen species such as nitric oxide, and chemokines (CCL2, CCL5, CXCL9, CXCL10, CXCL11, IL-8) (27). Macrophage pro-inflammatory cytokine maturation can occur through inflammasome-independent or inflammasome-dependent mechanisms (29, 30). Inflammasome formation is induced by damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) binding to nod-like receptors, including NLRP3 and NLRP1, or to Pyrin receptor family members such as Pyrin and AIM2 on the surface of macrophages (31). Docking proteins, including apoptosis-associated speck-like protein, assemble and recruit pro-caspase 1, where proximity-based autoproteolytic cleavage catalyzes the activation of caspase-1 (29, 32) Caspase-1 activation subsequently catalyzes the cleavage of the pro-forms of IL-1β and IL-18 into activated forms. Caspase-1 may also induce pyroptosis, a programmed cell death pathway characterized by cellular lysis and endogenous DAMP release into the environment, to induce additional inflammatory cell recruitment (33).

M2-polarized macrophages typically appear following M1-polarized macrophages in an injury setting and serve as a counterbalance to resolve inflammation and promote tissue repair (34). The M2 polarization state leads to secretion of cytokines commonly associated with anti-inflammatory properties, such as IL-10, as well as various matrix-modulatory factors including transforming growth factor-β (TGF-β) and matrix metalloproteases (MMPs) (35). The overproduction of remodeling growth factors can lead to excessive deposition of matrix proteins (i.e., fibrosis), or excessive angiogenesis and immunosuppression in the case of tumor-associated macrophages (TAMs) (36). Therefore, the temporal regulation of M1 and M2 macrophage responses are extremely important for appropriate outcomes following

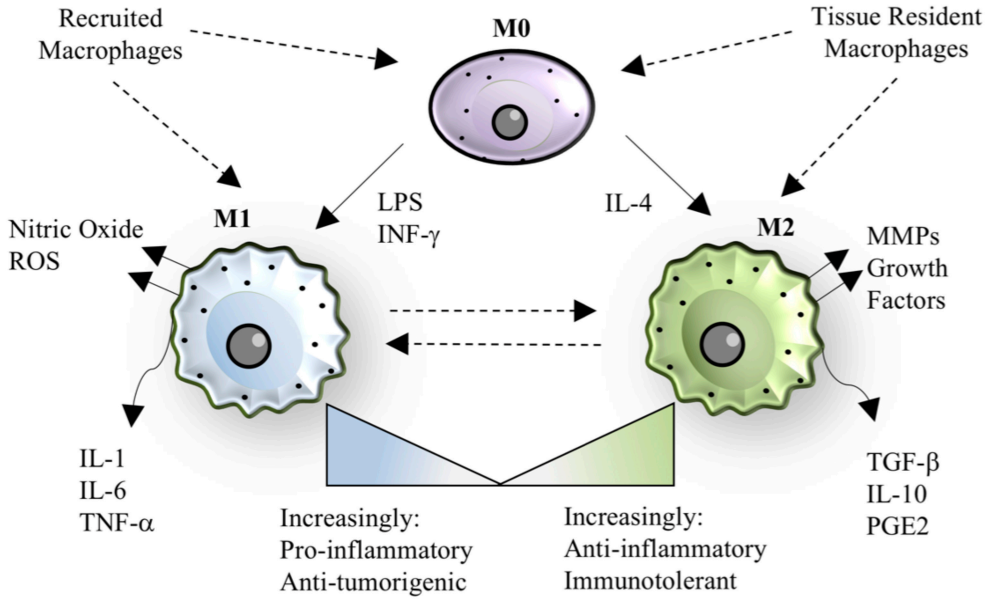

FIGURE 1 | Macrophage polarization. The paradigm of macrophage polarization begins with a quiescent or patrolling M0 cell, that may be derived from either circulating monocytes or tissue-resident populations. The transition from M0 to M1 can be stimulated by lipopolysaccharide (LPS) or interferon-gamma, resulting in a cell that has pro-inflammatory and anti-tumorigenic properties. M1 macrophages also produce nitric oxide and other reactive oxygen species (ROS). The transition from M0 to M2 can be stimulated by interleukin-4, resulting in a cell that has anti-inflammatory or immunotolerant properties. M2 macrophages also produce matrix metalloproteinases (MMPs) and growth factors that aid in tissue regeneration. M1 and M2 phenotypes are dynamic and exist on a spectrum, where recruited macrophages tend to be more "M1-like" in the case of bacterial infection, and tissue-resident macrophages tend to be more "M2-like" during homeostasis. In specific instances, such as helminth infection, recruited macrophages take on an M2 phenotype, demonstrating the versatility and adaptability of the macrophage polarization spectrum in response to the signals in a given context.

injury and infection and are often dysregulated in chronic disease.

The Origin and Function of the Kupffer Cell

The Kupffer cell is a primitive cell, appearing early during embryogenesis (E9.5-E12.5 in mice), derived primarily from the yolk sac (14, 37, 38). Importantly, the KC population is maintained through self-proliferation, with minimal input from circulating monocytes during homeostasis (12–14, 22).

The tissue-resident Kupffer cell is the key detector of commensal or pathogenic microbial signals, danger signals, and tumor cells moving through the hepatic circulation (15, 39, 40). KCs are located along the hepatic sinusoids allowing for the low-pressure blood supply come into contact with both KCs and hepatocytes through the fenestrated endothelium (2). KCs express the complement receptor CR1g, which binds complement fragments C3b and iC3b, allowing phagocytosis of complement C3-opsonized particles even under low-pressure blood flow (41, 42). Bacterial clearance by KCs is crucial for host defense as 80% of blood-borne bacteria accumulate in the liver and are destroyed there (15, 39, 40).

PAMPs and DAMPs are present in relatively high concentrations in blood entering the liver from the gut, via the portal vein, and engage with pattern recognition receptors (PRRs), including Toll-like receptors, on the surface of macrophages and hepatocytes (43–45). With low levels of bacterial endotoxins, KCs promote immune tolerance by secreting anti-inflammatory factors including IL-10, TGF-β, and prostaglandin-E2 (PGE2), thereby inducing regulatory T-cells (46, 47). In the presence of higher concentrations of damage or pathogen-associated signaling molecules, KCs can become polarized toward an M1 phenotype and produce a variety of inflammatory cytokines including IL-1, IL-6, IL-12, TNF-α (15, 39, 40). Several liver diseases are influenced by KC activation and expansion, but their individual role has been difficult to dissect from more recently identified macrophage populations in the liver.

The Origin and Function of Other Hepatic Macrophage Populations

Within the past decade, the heterogeneity of hepatic macrophages, i.e., Kupffer cells and monocyte-derived macrophages (mMØs), has become an emerging topic in hepatology. Fate tracing experiments in the brain first determined that resident microglia are established prenatally and maintained independently from monocyte input, which was later translated to the liver (48). Holt et al. were among the first to identify two populations of macrophages in the liver (49). Through bone marrow chimera experiments, cells expressing $F4/80^{hi}CD11b^{lo}$ were identified as KCs, while cells expressing $F4/80^{lo}CD11b^{hi}$ were found to be derived from circulating monocytic progenitors (49). Shortly after, Klein et al. classified

two macrophage populations in the liver: KCs as immobile, "sessile" macrophages and mMØs as motile cells (50).

In most cases, murine mMØs in the liver are derived from an influx of bone marrow derived, Ly6C^{hi} monocytes, primarily driven by monocyte chemoattractant protein, which can be produced by KCs, stellate cells, or hepatocytes (also known as CCR2-CCL2 interactions) (51, 52). Secondary pathways of monocyte recruitment to the liver are CXCR3-CXCL10, CCR1-CCL5, and CCR8-CCL1 dependent (53–55). Murine mMØs may also be derived from Ly6C^{lo} monocytes trafficking from the spleen, which express CD11b, but are thought to take on a more patrolling or regulatory macrophage phenotype (56). Phagocytes from the peritoneal cavity, which express F4/80, CD11b, and GATA6, have been shown to cross into the liver after subcapsular liver lesion (57), but it remains unclear if this infiltration occurs in other liver injury settings.

Kinoshita et al. determined that murine mMØs and KCs could be distinguished by CD11b and CD68 expression, respectively (40). The CD11b^{hi} mMØs were radiosensitive and particularly efficient at producing IL-12, protecting the host against tumor xenografts, while the CD68^{hi} KCs were radioresistant and highly efficient at phagocytosis, protecting the host against bacterial challenge (40). The use of these differentiating markers has been expanded to several disease models, identifying a damaging role of TNF/FasL production by CD11b^{+} mMØs in carbon-tetrachloride acute liver injury and hepatitis in hypercholesterolemic mice (58, 59). The CD11b^{+} mMØs were also found to accumulate in the liver following repeated lipopolysaccharide injections, but suppressed TNF efflux into the systemic circulation, thereby reducing lethal septicemia (60). In addition, the CD11b^{+} mMØs were recruited during diet-induced steatohepatitis in FGF5 deficient mice and following partial hepatectomy, where they were crucial for liver regeneration (61, 62). Others have observed that monocyte derived cells protect the liver from iron toxicity in hemolytic anemia by ingesting senescent and dying erythrocytes (63).

More recently, the C-type lectin, Clec4f, has been identified as a selective murine KC marker (64). Interesting, ablating KCs via Clec4f-driven diphtheria toxin causes an influx of mMØs, which can differentiate into nearly identical KCs when this "niche" is made available. Only 12 genes remained differentially expressed between monocyte derived KCs and embryonically derived KCs, including *CD209f, CD163, C2, CCR3, Timd4,* and *Snrpn* at the time points examined (64).

Taken together, KCs and mMØs play heterogenous roles in various liver disease states and cannot be considered wholly harmful or beneficial without deciphering the given context. Importantly, infiltrating monocytes may also differentiate into dendritic cells, which play distinct immunostimulatory and antigen presentation roles in the liver (65). The heterogeneity of hepatic macrophages is less well defined in humans as compared to murine animal models. The role of these diverse hepatic macrophage populations in a given disease context is just beginning to be understood and has not yet been examined in the natural aging process, a major risk factor for several chronic liver diseases.

CELLULAR MANIFESTATIONS OF AGING ON HEPATOCYTES AND MACROPHAGES

The process of aging is closely associated with a number of degenerative modifications in the liver, where hepatic structure and cell function are observed to decline. Both hepatocytes and macrophages exhibit deficits in mitochondrial function, linked to a decline in autophagy and production of pro-inflammatory molecules (66, 67). While the effects of aging on hepatic macrophages have not been well characterized on a cellular and molecular level, several studies have examined macrophages from alternative tissue sources and may offer some insight about hepatic macrophages.

Changes in Hepatic Structure and Hepatocyte Function

Several studies have shown that the volume of blood in the liver decreases in elderly individuals, leading to a total volume loss of 20–40% (68). In addition, the thickness of liver sinusoidal endothelial cells fenestrations increases, limiting the exchange of molecules to and from the liver (69). At the serum level, aging is associated with reductions in albumin and bilirubin, increases in alkaline phosphatase, and minimal changes in aminotransferase levels (70). The metabolism of cholesterol in the liver also decreases, leading to increased blood cholesterol and neutral fat levels over time (70). However, recent investigations suggest the most essential change in liver aging is loss of the functional liver cell mass (71).

Changes in the morphology of hepatocytes may be related to increased polyploidy, accumulation of lipofuscin in the cytoplasm, and declining surface area of endoplasmic reticulum and number of mitochondria, ultimately negatively affecting the function of hepatocytes (66, 72, 73). The decline in hepatocyte mitochondrial function has been suggested to enhance the vulnerability of aged livers to acute injury and to cause delays in liver regeneration (74). The oxidative capacity of the liver also declines with aging, and therefore medications that require oxidation, such as benzodiazepines, may accumulate in toxic levels and are not prescribed to elderly individuals (75). In addition, aging livers are known to accumulate a multiprotein C/EBPalpha-Brm-HDAC1 complex that silences elongation factor 2 (E2F)-dependent promoters, thereby reducing proliferation and regenerative capacity of hepatocytes (76).

Senescent cells accumulate in the liver during aging and in chronic liver disease; and may include hepatocytes, cholangiocytes, stellate cells, and immune cells [reviewed here: (77)]. Cells become senescent as a result of replicative exhaustion (telomere shortening), DNA damage, or oxidative stress, among other mechanisms (78). Senescent cells are characterized by the expression of cell cycle inhibitors p21, p16, and p53, which prevent replication and apoptosis, as well as secretion of pro-inflammatory cytokines that signal for their removal but can lead to tissue damage when chronically expressed (79, 80). Senescent hepatocytes accumulate with age and appear as an almost "universal phenomenon" in chronic liver diseases

including hepatitis B and C infection, alcoholic and non-alcoholic liver disease, and genetic haemochromatosis (77). Senescent hepatocytes undergo metabolic changes, such as increased transport of conjugated bilirubin into the hepatic sinusoids and insulin resistance through dysregulated glucose transporter expression and Akt signaling among others, which dysregulates normal function (81). Interestingly, the length of telomeres was found to be conserved in aged hepatocytes and bile duct cholangiocytes but decreased in aged Kupffer cells and stellate cells, suggesting cell-specific mechanisms of senescent phenotype acquisition in the liver (82).

Overall, the effects of aging on non-parenchymal cell subsets, particularly hepatic macrophages, is an understudied area. Hilmer et al. determined that the number and basal activity of Kupffer cells was increased with old age in a rat model (83), however this work was performed before the heterogeneity of liver macrophages was fully contemplated. Similarly, Singh et al. identified a change in liver macrophage distribution with aging, where old mice had more F4/80^{+} macrophages located in large lymphoid structures (also containing T-cells and B-cells), and a reduction in the number of spindle shaped macrophages throughout the parenchyma (84).

Recent work by Maeso-Diaz et al. characterized molecular changes in LSECs, stellate cells, and hepatic macrophages in the livers of aged rats (85). The authors found a significant deregulation of LSEC phenotype with aging as demonstrated by downregulation of vasodilatory pathways (nitric oxide, heme oxygenase), increase in oxidative stress, and decrease in angiocrine markers (stabilin-2, CD32b, and VEGFR2), in addition to increased portal pressure and vascular resistance (85). Aged stellate cells had increased markers of activation (alpha smooth muscle actin and collagen I), increased intracellular lipid stores, and alterations in retinoid metabolism (85). Finally, the authors noted an increase in recruitment of proinflammatory cells in aged livers, including increased IL-6 expression in isolated hepatic macrophages, but no change in the traditional macrophage polarization markers (85). Human livers showed similar trends in downregulation of endothelial markers, but substantial work remains to extend and decode these findings in both aged murine and human systems.

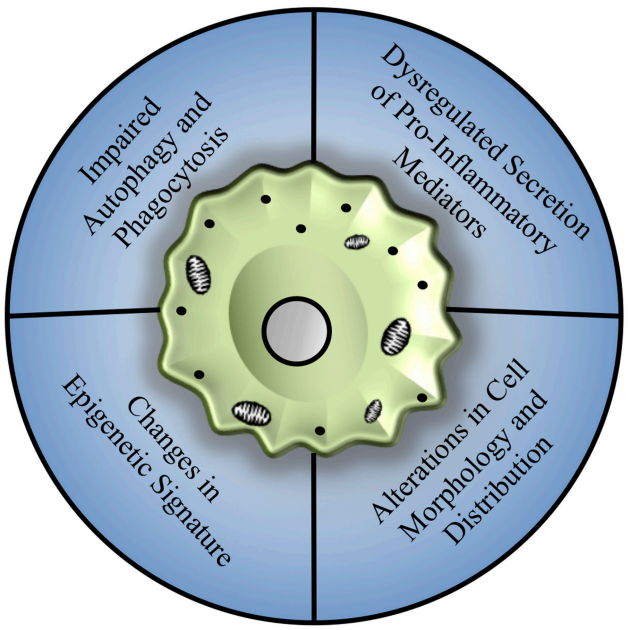

FIGURE 2 | Mechanisms of age-associated decline in macrophage function. Macrophages from various tissue sources, including the bone marrow, peritoneal cavity, lungs, and brain, show an array of dysfunction including a general reduction in autophagy and phagocytosis, dysregulation of pro-inflammatory cytokine production associated with changes in toll-like receptor expression, and alterations in cell morphology and distribution that may be related to repopulation of tissues by infiltrating monocyte populations, largely mediated by changes in epigenetic signature and mitochondrial dysfunction.

Declines in Cell Intrinsic Macrophage Function With Aging

The process of aging has been shown to affect macrophage polarization and function in multiple tissues and disease models, with compelling underlying mechanisms that may be broadly applicable to hepatic macrophages, as summarized in **Figure 2**.

Autophagy, or the intracellular degradation of damaged proteins and organelles in autophagosomes, is one such process which has been shown to become dysregulated with increasing age in macrophage populations (86). Deletion of autophagy genes *Atg5* and *Atg7* in bone marrow-derived macrophages results in decreased antigen presentation capacity, impaired maturation, altered mitochondrial metabolism, downregulation of surface receptors such as toll-like receptor 4 (TLR4), and increased secretion of pro-inflammatory cytokines (86, 87). The phenotype of autophagy deficient bone marrow macrophages closely mimics the phenotype of macrophages isolated from aged individuals (86), which may be driven by the hypermethylation of autophagy associated genes (88). It remains to be seen if restoring autophagic flux to aged macrophages can improve their function and polarization status, however caloric restriction has been shown to improve both longevity and autophagic capacity in animal models (89).

Phagocytic clearance of extracellular pathogens and antigen presentation tends to be attenuated in aged macrophage populations as well (90). While no difference in phagocytic capacity was found between young and aged bone marrow derived macrophages, peritoneal macrophages exhibit an age-associated decline in phagocytic capacity and antigen presentation (90). This reduction in phagocytic ability was driven by age-related alterations in the local microenvironment, as young peritoneal macrophages transplanted into aged peritoneal space exhibited reduced phagocytic and antigen presentation capabilities (90). Alveolar macrophages isolated from aged individuals have been shown to exhibit reduced phagocytic capacity as well as attenuated expression of genes associated with macrophage proliferation (91). This reduction both in cell number and phagocytic ability contributes to the age-related mortality risk following pathogen infection, such as

in the case of an influenza lung infection model (91). In the liver, there have been mixed reports on the maintenance of Kupffer cell phagocytosis, where one study reported a deficit (92) and another reported an increase in phagocytosis with aging (83).

Furthermore, the reduction in expression levels of autophagosome components prevents the endocytosis of both inflammasome components and damaged mitochondria and can thereby promote chronic activation of pro-inflammatory signals in aged cells (87). This phenomenon has been termed "inflamm-aging," one of the hallmarks of aging, and has been strongly correlated to morbidity (93). In addition to dysregulated cytokine production, aging can promote alterations in the secretion of oxidative species (94–96). Bone marrow-derived macrophages isolated from aged murine and human donors have been shown to produce greater concentrations of nitric oxide and reactive oxidative species than macrophages isolated from young donors (96). However, this upregulation in the secretion of reactive oxidative species was not observed following pro-inflammatory stimulation of peritoneal macrophages (94, 95). Further studies will be necessary to fully understand tissue-specific changes in respiratory burst characteristics.

The morphology of macrophages can also be influenced by the aging process. Microglia have been shown to increase cell soma volume while reducing the length of cellular processes in aged brains (97). These morphological changes reduce the capability of the microglia to interact with neural cells and perform routine surveillance of the local microenvironment (97). While microglial population sizes increase to account for this reduction in cell process size (17), the population expansion tends to occur in a non-homogenous manner, disrupting the uniform microglial distributions commonly observed in young animals (97). In addition, aged microglia exhibit enhanced secretion of reactive oxidative species following central nervous system injury through upregulation of NADPH oxidase 2 (NOX2) (98), and secrete elevated concentrations of IL-1β mediated by hypomethylation of CpG sires in the IL-1β proximal promoter (99).

Infiltration of monocyte-derived macrophages to multiple tissue sites has been observed in age-related diseases, including Alzheimer's disease (100), which leads to increased inflammation and phagocytosis. In the cardiovascular system, the tissue resident macrophage population (yolk-sac derived, M2-like) is replaced by bone marrow-derived macrophages over time (101, 102). This shift in macrophage populations contributes to deleterious outcomes following cardiac injury, such as chronic inflammation, fibrotic scar deposition, and reduced re-vascularization of ischemic tissue following injury (101, 103). It remains unclear whether age-related changes in macrophage responses are a primary result of shifting cell origins and polarization states driven by the microenvironment or by cell intrinsic functions, although both are likely in play.

The Role of the Aged Microenvironment on Macrophage Function

While cell intrinsic properties, such as mitochondrial capacity and autophagy, may drive changes in age-associated macrophage polarization, this only represents a partial view of the complex innate and adaptive immune dysfunction which occurs with age. For example, age-related alterations in the T cell compartment, which are numerous [reviewed here (104, 105)], can shift the relative Th1 and Th2 cytokine concentrations and directly impact macrophage polarization (106, 107).

Non-immune cell types can also contribute to the age-associated immune dysfunction. With age, increasingly large numbers of pre-adipocytes differentiate into mature adipocytes, with enhanced secretion of leptin, TNF-α, and IL-6, and reduced secretion of adiponectin—a factor commonly associated with M2 macrophage polarization, thereby promoting M1 phenotypes (108–110). Numerous DAMPs, which have been shown to promote inflammasome formation and activation, also have age-related increases in concentration, including cholesterol (111), amyloid-β (112, 113), hydroxyapatite crystals (114), purine catabolic end products such as uric acid (115), and ATP (116). Radical oxidative species, which tend to exhibit an increase in production and secretion with increasing age, have also been shown to play an important role in inflammasome activation through oxidation of proteins, lipids, and DNA, leading to inflammatory cytokine secretion into the aged microenvironment (29, 117, 118). More recently, the effect of the aging gut microbiome on macrophage function has been considered (119). Raising mice under germ-free conditions preserves the bactericidal capacity of alveolar macrophages and reduces secretion of IL-6 in old age, mediated by reduced dysbiosis and improved gut permeability (119).

While this is only a brief consideration of additional factors that have been observed to influence macrophage function, it serves to highlight the complexity and tissue specificity that defines age-related immunomodulation. The mechanisms governing the acquisition of age-related dysfunction in infiltrating and tissue resident macrophage populations continues to be an active area of research, and it will be essential for investigators to utilize an array of cell markers and descriptive methods to appropriately place cells along the spectrum of polarization states.

THE ROLE OF MACROPHAGES IN AGE-RELATED LIVER DISEASE

Aging is a major risk factor for the development and prognosis of several chronic liver diseases and conditions, including nonalcoholic fatty liver disease, alcoholic liver disease, hepatitis C, and an increased susceptibility to develop fibrosis and cirrhosis (16). The reduced capacity of aged livers to regenerate and to tolerate transplantation leads to increased risk of mortality from chronic liver diseases, as demonstrated in **Figure 3**.

Both Kupffer cells and infiltrating monocytes are implicated to various levels in the etiology of chronic liver disease, however the distinction between these two cell populations is less clear in human clinical liver samples compared to animal studies. Importantly, most animal models of chronic liver disease are induced in young, healthy animals through genetic manipulation or by administering specific diets or chemical

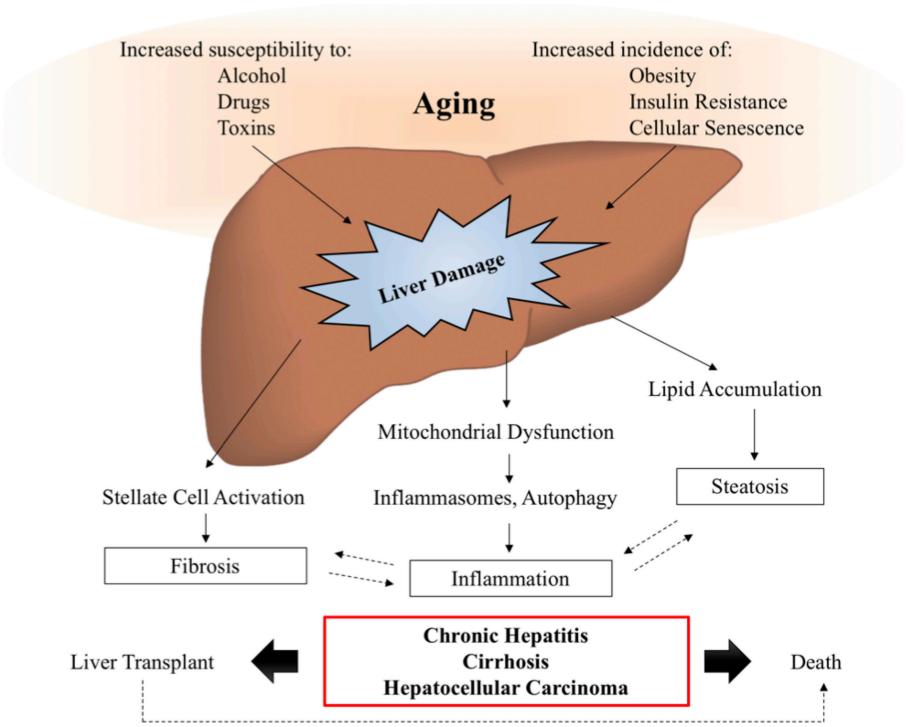

FIGURE 3 | Aging and liver disease**.** With increasing age, the liver becomes more susceptible to damage from alcohol, drugs, and toxins, while the prevalence of metabolic disease, obesity, and cellular senescence are known to increase. These insults lead to the activation of signaling pathways driving liver pathology, such as inflammation, steatosis, and fibrosis, which are often involved in positive feedback loops, further exacerbating the symptoms of liver disease. In a subset of patients, liver disease will progress to chronic hepatitis, cirrhosis, and hepatocellular carcinoma, also known as end stage liver disease, for which transplant is the only viable therapy. Studies suggest that elderly patients have decreased survival post-transplantation, demonstrating the need for additional therapies to intervene and prevent the onset of age-related liver disease symptoms.

toxins. Findings from these animal studies are important to identify novel mechanisms regulating liver disease but may not prove relevant or all-encompassing when translating the findings to older, human populations, as evidenced by the moderate success of several anti-fibrotic therapies in clinical trials (120). Distinguishing between the biology of aging and age-associated pathologies can be a difficult task but is an important effort in the field of hepatology. Additional studies are needed to assess the effect of aging on the liver prior to the onset of age-associated pathologies in order to dissect mechanisms of aging from the manifestation of the pathology itself.

Liver Fibrosis

Aging is a major risk factor for the progression of liver fibrosis, particularly in hepatitis, which can advance to portal hypertension and cirrhosis (121). Fibrosis occurs when excessive connective tissue is deposited following acute or chronic liver damage, and is often the starting point of architectural distortion and dysfunction that prevents normal functioning of the liver (122). Age-related dysfunction in hepatic macrophages and stellate cells are heavily implicated in development of fibrosis and may be related to oxidative stress and macrophage polarization, but these mechanisms are not well understood (123).

In general, an increase in M1-polarized macrophages appears at the early stages of liver disease and promotes or exacerbates fibrosis, cirrhosis, and eventual liver failure. In clinical studies, the progression from hepatitis to fibrosis and eventually cirrhosis was associated with enrichment of $CD14^{+}CD16^{+}$ non-classical monocytes in the liver, which have been shown to activate stellate cells *in vitro* based on TGF-beta release (124). These cells may be derived from classical $CD14^{+}CD16^{-}$ monocytes or infiltrate directly (125). Activation of the CCL2-CCR2 axis is associated with monocyte infiltration in both rodent models and patients with fibrosis and liver disease (126). A recent study found that the acquisition of M1 phenotypes in liver fibrosis may be regulated by interferon regulatory factor 5 (IRF5), which is significantly induced in liver macrophages in both mouse and human subjects developing fibrosis, and may represent a novel target for therapeutic intervention (127).

Alcoholic Liver Disease

Elderly individuals are more likely to feel socially isolated and depressed, resulting in a rising alcohol consumption rate and prevalence of alcoholic liver disease in this population (128). Alcohol and its breakdown products are toxic to the liver, which can cause a disorder in lipid metabolism by increasing

the synthesis of fatty acids and suppressing mitochondrial β-oxidation (129). The risk of alcohol toxicity is further increased in elderly populations due to alterations in metabolism or by the intake of certain medications (130).

Alcoholic liver disease can be classified into three stages: accumulation of extra fat in the liver (steatosis), alcoholic hepatitis, and cirrhosis. Macrophages were found to increase in both the early and late stages of alcoholic liver disease. Increased gut permeability and high levels of endotoxin in the portal blood results in activation of Kupffer cells (131). In addition, Ly6C$^+$ macrophages were found to accumulate in murine models of alcoholic liver injury, regulated by CCL2 chemokine signaling (132). Clinical symptoms of alcoholic liver disease among the elderly are similar to younger counterparts, yet the prevalence of advancing to irreversible stages of the disease is greater in older individuals (133). Oxidative stress and production of TNF by hepatic macrophages, as a result of steatosis or leaky gut, causes progression to later stages (130). Furthermore, the expression of M1 pro-inflammatory genes was found to be higher in peritoneal macrophages from patients with alcohol-related cirrhosis and ascites compared to hepatitis-C related cirrhosis, suggesting systemic differences in macrophage polarization (134). Reducing leaky gut and inflammation have been identified as potential therapeutic targets for treating alcoholic liver disease (135).

Non-alcoholic Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are leading causes of chronic liver disease globally and are projected to become the leading indication for liver transplantation in the United States (136–138). NAFLD often presents with metabolic syndrome, such as obesity and excessive visceral fat, hyperlipidemia, hypertension, insulin resistance and an increased secretion of pro-inflammatory cytokines (139). All of these components of metabolic syndrome are generally observed in elderly populations and many studies find increased rates of NAFLD among elderly people compared with their younger counterparts (140–142).

The mechanism for developing age-related steatosis is not fully understood but has been attributed to hepatocyte senescence driving a reduction in mitochondrial metabolism (143), decreased transport of insulin across the sinusoidal endothelium (144), reduction in autophagic flux (145), or chronic low-level inflammation (146, 147), leading to the build-up of toxic free fatty acids in the liver. Lifestyle choices, such as high fat diet (HFD), may also lead to the development of NAFLD in aged individuals. Work by Fontana et al. found that mice were equally susceptible to steatosis following HFD regardless of age. However, the older mice exhibited more severe hepatocellular injury and inflammation following administration of HFD that was attributed to increased M1 macrophage polarization in both the liver and white adipose tissues (148).

As in other chronic liver diseases, macrophages accumulate in the livers of NAFLD patients (149). Experimental mouse models have shown an accumulation of Ly6C$^+$ monocytes is a critical step in the development of progressive fibrosis from steatohepatitis in a CCL2-dependent mechanism (131). In addition, lipids and free fatty acids contribute to DAMP-induced Kupffer cell activation (150). Patients with low-grade steatosis were found to have higher mRNA expression of M2 markers, CD206 and CD163, compared to patients with advanced steatosis. The M2 macrophages were hypothesized to promote apoptosis of M1 polarized hepatic macrophages and protect against disease progression of NAFLD or alcoholic hepatitis (149). Interestingly, soluble CD163 (sCD163), a marker of M1 macrophage polarization, increases systemically with severity of NAFLD in human patients (151), again suggesting that M1 polarization is associated with more advanced stages of the disease.

Traditional treatments for NAFLD are more challenging for elderly populations as they require lifestyle changes in exercise, diet, and medication or liver transplantation (152, 153). Findings from a recent clinical trial using a CCR2/CCR5 antagonist to block inflammation resulted in twice as many subjects achieving improvement in fibrosis and no worsening of steatohepatitis compared with placebo (154, 155). Subgroup analysis showed the therapy to be similarly effective in patients below and above 56 years of age, demonstrating promise in the context of age-associated disease.

CONCLUSION

The decline in mitochondrial capacity with aging has emerged as a key mechanism underlying several of the observed changes in the function of both hepatocytes and macrophages, likely contributing to the increased prevalence and severity of chronic liver diseases in elderly populations. In general, the accumulation of infiltrating or M1-polarized monocytes/macrophages tends to exacerbate liver disease and contribute to fibrosis, cirrhosis, and liver failure. However, very few, if any, studies have systematically identified changes in the hepatic macrophage populations of aging livers in either animal models or human patients, representing a hugely understudied area of research.

Liver transplantation is the standard treatment for patients with end stage liver disease. The number of elderly people with liver cirrhosis requiring transplantation has grown over the past 20 years and is expected to increase further (66). Overall survival rates after 1 year are approximately 90% and 10-year survival rates may be more than 70% for recipients (156). However, some studies have found a reduced survival rate in transplant recipients over 60 years of age (from 90 to 64% 1 year survival rate), primarily due to kidney dysfunction and cardiopulmonary disease complications (157). Survival rates improve for elderly patients with few comorbidities (158), thus age alone is not an exclusion criterion from liver transplantation, but all disease risk factors must be considered to devise effective treatment strategies for these age-related chronic liver diseases.

Therapies that suppress M1-polarization or infiltration of macrophages might reduce the progression of liver disease from manageable early stages to chronic end-stages requiring liver transplantation. In the context of aging specifically, promoting M2-gene expression might delay the advancement of liver disease to later stages. However, M2 macrophages have

been associated with tumor progression, including clinical hepatocellular carcinoma (HCC) specimens (159). Thus, modulating macrophage polarization has been considered a double-edged sword, and the appearance of liver fibrosis or HCC would need to be closely monitored. Treating the primary drivers of age-associated liver diseases such as alcohol consumption, visceral and ectopic fat accumulation, or deficits in mitochondrial capacity and other age-related mechanisms, will be just as important as targeting the inflammatory symptoms to combat these age-related liver diseases.

Elderly populations continue to increase globally and are particularly at risk for succumbing to liver failure due to the declines in regenerative capacity, reduced survival post-liver transplantation, and tendencies to develop inflammation and fibrosis. Between 1999 and 2016, deaths in the United States from cirrhosis increased by 65% and deaths from HCC doubled (160). A better understanding of the biology of liver aging that influences the onset and progression of chronic liver disease will be crucial in order to develop new interventions and treatments for our aging populations.

AUTHOR CONTRIBUTIONS

ES, MH, BP, and BB contributed to the writing and editing of the manuscript and figure preparation.

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REVIEW
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The Role of Monocytes and Macrophages in Acute and Acute-on-Chronic Liver Failure

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Acute and acute-on-chronic liver failure (ALF and ACLF), though distinct clinical entities, are considered syndromes of innate immune dysfunction. Patients with ALF and ACLF display evidence of a pro-inflammatory state with local liver inflammation, features of systemic inflammatory response syndrome (SIRS) and vascular endothelial dysfunction that drive progression to multi-organ failure. In an apparent paradox, these patients are concurrently immunosuppressed, exhibiting acquired immune defects that render them highly susceptible to infections. This paradigm of tissue injury succeeded by immunosuppression is seen in other inflammatory conditions such as sepsis, which share poor outcomes and infective complications that account for high morbidity and mortality. Monocyte and macrophage dysfunction are central to disease progression of ALF and ACLF. Activation of liver-resident macrophages (Kupffer cells) by pathogen and damage associated molecular patterns leads to the recruitment of innate effector cells to the injured liver. Early monocyte infiltration may contribute to local tissue destruction during the propagation phase and results in secretion of pro-inflammatory cytokines that drive SIRS. In the hepatic microenvironment, recruited monocytes mature into macrophages following local reprogramming so as to promote resolution responses in a drive to maintain tissue integrity. Intra-hepatic events may affect circulating monocytes through spill over of soluble mediators and exposure to apoptotic cell debris during passage through the liver. Hence, peripheral monocytes show numerous acquired defects in acute liver failure syndromes that impair their anti-microbial programmes and contribute to enhanced susceptibility to sepsis. This review will highlight the cellular and molecular mechanisms by which monocytes and macrophages contribute to the pathophysiology of ALF and ACLF, considering both hepatic inflammation and systemic immunosuppression. We identify areas for further research and potential targets for immune-based therapies to treat these devastating conditions.

Keywords: acute liver failure, acute-on-chronic liver failure, monocytes, macrophages, immunosuppression, liver inflammation, damage-associated molecular patterns, pathogen-associated molecular patterns

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INTRODUCTION

The liver is a unique innate immune environment and exerts crucial immune surveillance functions during homeostasis (1, 2). It has a dual blood supply, thus it is constantly exposed to circulating antigens, pathogens, pathogen-associated toxins, and danger signals which reach the liver from the gastrointestinal tract, via the portal vein, or from the systemic circulation via arterial blood (3). Hence, it is an important line of defense and plays a central role in regulating tolerogenic and inflammatory responses (1, 2). For such purposes, the liver shows a high degree of vascularization, slow blood flow through the sinusoidal system and highly permeable fenestrated endothelia allowing direct access to liver immune cells from the blood stream (1, 2). The liver houses an abundant population of tissue-resident macrophages, as intrasinusoidal Kupffer cells (KC), which function as the dominant phagocytes in the liver and compose over 80% of the body's macrophages in states of health. It also contains other myeloid [neutrophils and dendritic cells (DCs)] and lymphoid [T cells, natural killer (NK) cells, and NK T cells] cells that shape innate and adaptive immune responses (2, 4). They are organized in a manner designed to maximize screening for both systemic and gut-derived pathogens, thereby avoiding their systemic spread (5).

While macrophages contribute during the maintenance of homeostasis, they are equally relevant in responses to liver injury, playing key roles in the initiation and progression of liver diseases (1, 2). During injury, the liver macrophage pool is augmented by recruitment of bone-marrow derived monocytes which mature *in situ* into macrophages and contribute to the development and resolution of hepatic inflammation. Macrophage-mediated inflammation may also give rise to systemic consequences. This is perhaps best typified by the conditions of acute liver failure (ALF) and acute-on-chronic liver failure (ACLF), which are characterized by local hepatic inflammation complicated by a systemic inflammation and subsequent systemic immunosuppression. Patients therefore experience symptoms of liver decompensation, accompanied by a systemic inflammatory response that involves endothelial dysfunction and may progress to multi-organ failure along with susceptibility to secondary infections. In this review, we will consider how monocytes and macrophages contribute to the initiation and propagation of local liver inflammation in ALF and ACLF; how they drive systemic immune dysfunction and what immunotherapeutic strategies could be used to target their role.

THE CLINICAL SYNDROMES OF ALF AND ACLF

Acute liver failure (ALF) is a rare condition in which coagulopathy, jaundice, and hepatic encephalopathy arise in the context of an acute hepatic injury and the absence of chronic liver disease (CLD) (6). Various sub-categorizations of ALF have been proposed which use the interval between the development of jaundice and emergence of hepatic encephalopathy to differentiate patients with rapidly progressive "hyperacute" disease from those with a more indolent ("subacute") clinical course in whom the outcome is generally poorer. In the UK and USA, acetaminophen (paracetamol, APAP) overdose is the commonest cause of ALF and is characterized by the rapid progression of symptoms over a few days (6). In many other parts of the world acute viral hepatitis is the dominant cause of ALF (7). Idiosyncratic drug reactions, hepatic ischemic insults, autoimmune hepatitis, and seronegative disease account for a significant minority of cases and tend to run a slower clinical course (**Table 1**). A common feature which is shared by all these conditions is hepatocellular loss of a magnitude and at a rate which exceeds the liver's regenerative capacity. Hepatocyte death results in synthetic "loss of function" features such as jaundice and coagulopathy. Significantly, this overwhelming cell death also provokes a robust innate immune response which drives many of the other clinical features of ALF, as will be discussed below.

Acute on chronic liver failure (ACLF), in contrast, occurs in patients with pre-existing liver disease. The exact definition of ACLF has been debated in the community, with the Asian Pacific Association for the Study of the Liver (APASL),

TABLE 1 | Comparison of features of human acute and acute-on-chronic liver failure syndromes.

	ALF	ACLF
Background liver	Normal	Chronic liver disease ± cirrhosis
Demographics*	Mean age: 36 Female preponderance	Mean age: 56 Male preponderance
Causes	Paracetamol/Acetaminophen Other drug-induced liver injury (DILI) Acute viral hepatitis Ischaemia Pregnancy related Autoimmune hepatitis	CLD: Any: Alcohol, chronic viral hepatitis, NASH, other Precipitant: Bacterial infection, alcohol consumption, GI bleed, viral reactivation, *de novo* viral hepatitis, ischaemia, DILI
Clinical features	Coagulopathy, jaundice, hepatic encephalopathy High incidence of SIRS, extrahepatic organ failure and susceptibility to infection	Coagulopathy, jaundice, and extrahepatic organ failure. High incidence of hepatic encephalopathy, SIRS, and susceptibility to infection
Infection susceptibility	Bacterial infection 35–40% Fungal infection 11.2%	37% bacterial infection at diagnosis, increasing to 66% by 4 weeks 2–3.5% fungal infections
Infection onset	Late (>5 days)	Early (< 5 days) and late
Mortality	40% hospital mortality	40–80% hospital mortality
DAMPs/Alarmins	IL-1α, IL-33, ATP, formyl peptides, mitochondrial DNA, cyclophilin A, histones, HMGB1	IL-33, histones, HMGB1

**Patient demographics from large European cohorts, reflecting disease trends in this region. ACLF, acute-on-chronic liver failure; ALF, acute liver failure; ATP, adenosine triphosphate; CLD, chronic liver disease; DAMPs, damage-associated molecular patterns, DILI, drug-induced liver injury; GI, gastrointestinal bleeding; HMGB-1, high-mobility group box-1; IL, interleukin; NASH, non-alcoholic steatohepatitis; SIRS, systemic inflammatory response syndrome*

American Association for the Study of Liver Diseases (AASLD), The European Association for the Study of the Liver-chronic liver failure (EASL-CLIF) consortium and the World Gastroenterology Organization (WGO), all having formalized definitions within the last decade. The WGO working party definition, published in 2014, states: "*ACLF is a syndrome in patients with chronic liver disease with or without previously diagnosed cirrhosis which is characterized by acute hepatic decompensation resulting in liver failure (jaundice + prolonged international normalized ratio) and one or more extrahepatic organ failure that is associated with increased mortality within a period of 28 days and up to 3 months from onset*" (8). Key features of this definition are the presence of chronic liver disease and occurrence of acute hepatic dysfunction with synthetic failure that progresses to cause extrahepatic organ failure. The EASL-CLIF definition is important in its distinction that the CLD must be cirrhosis. This more selective population, of patients developing ACLF in the context of cirrhosis, is the best characterized, with much of the evidence base coming from studies that use the EASL-CLIF definition.

Typical events that precipitate ACLF are infections, gastrointestinal bleeds, viral reactivation, and superimposed drug, viral, or ischaemic insults (9, 10) (**Table 1**). Patients with ACLF experience a high mortality compared to those with uncomplicated decompensation of chronic liver disease and, as would be expected, mortality increases with the severity of extra hepatic organ failure. The 28 day and 90 day mortality from an acute decompensation of cirrhosis are 5 and 14% respectively, whereas for ACLF they range from 22–78 to 41–79% depending on the grade (11).

In both ALF and ACLF, infections are key drivers or life-threatening complicating factors in these syndromes. Discounting the etiological hepatotropic viruses, bacterial infections are the primary microbial clinical issue in liver failure. In ALF bacterial infection occurs in up to a third of patients (12) and is late-onset (>5 days since hospital admission) and predominantly related to gram positive organisms (13). This is postulated to be a consequence of ALF-associated immunosuppression and the invasive nature of critical care support predisposing to nosocomial infections. By contrast, patients with ACLF can suffer from mainly gram negative bacterial infection (14) as a cause for their deterioration, potentiated by high levels of bacterial translocation from the gut, but are also at further risk of secondary nosocomial sepsis during hospital episodes for similar reasons as ALF patients.

MONOCYTES AND MACROPHAGES IN THE INITIATION OF HEPATIC INFLAMMATION: SENSING DAMPS AND PAMPS

The innate immune system is primed to respond to invading pathogens, through recognition of unique microbial molecular motifs, known as pathogen-associated molecular patterns (PAMPs) (15). PAMPs are recognized via pattern-recognition receptors (PRRs), in a process called structural feature recognition. However, innate immune-mediated inflammation also occurs in the absence of infection. Termed sterile inflammation, this state is induced by release of host-derived products, called damage-associated molecular patterns (DAMPs) during tissue damage (2, 15). DAMPs, which are normally sequestered inside cells, interact with PRRs on immune cells and initiate an inflammatory response (4). In the liver, well described DAMPs include the high-mobility group box-1 (HMGB-1) protein, IL-1α, IL-33, ATP, S100-calcium-binding-protein-A8/A9 (S100A8/9), mitochondrial DNA, histone-associated DNA, purines, heat-shock proteins, and bile acids (2, 3, 15–24). Their activity is mediated through PRRs expressed on liver immune cells such as the toll-like receptors (TLRs), purinergic receptors and the receptor for advanced glycation end-products (RAGE) (2, 15). Ligation of DAMPs to their receptors results in activation of immune cells, which shifts them toward a pro-inflammatory phenotype, thus initiating an inflammatory signal through cytokine and chemokine release, which in turn amplify and sustain the inflammatory response (25).

Liver inflammation can be initiated by various DAMPs and PAMPs and is a major component of the immunopathology of a variety of liver diseases including ALF, ACLF, liver cirrhosis, alcoholic liver disease (ALD), liver fibrosis and cancer (2, 15). As described above, ALF is characterized by massive, rapid hepatocyte death which can occur by either necrotic or apoptotic pathways (26, 27). The result of hepatocyte necrosis is the release of DAMPs (25). Liver-resident KCs highly express various DAMP receptors (e.g., P2X7, TLR4, TLR9 and RAGE) thus mediate the initial response to injury (25). After acetaminophen overdose, oxidative stress and direct mitochondrial damage are induced in hepatocytes, which consequently release DAMPs that can be recognized by KCs (**Figure 1**). In turn, activated KCs secrete pro-inflammatory cytokines (e.g., TNF-α), reactive oxygen species (ROS), and chemokines (e.g., CCL2) that amplify the pro-inflammatory signal and increase the recruitment of bone-marrow derived cells into the liver, mainly neutrophils and monocytes, thereby enhancing the inflammatory process (2, 15). The sustained KC-released cytokines can also recruit other inflammatory cell subsets, such as eosinophils, DCs, and T cells. While the specific roles of these cell are not fully investigated in ALF, they have been implicated in drug-induced liver injury (2, 15). In ACLF, with a reduced baseline hepatic reserve and longstanding circulatory dysfunction, the initiating event may be lower volume hepatocyte death, which nonetheless causes release of DAMPs and incitement of inflammation **(Figure 1)**. Alternatively, systemic infection may lead to development of ACLF in which inflammation is triggered by an enhanced systemic exposure to PAMPs (10, 28).

In experimental models of ALF and ACLF and in patients with both conditions, a number of DAMPs have been implicated in disease pathogenesis. HMGB-1, perhaps one of the best characterized DAMPs, is a highly conserved chromatin binding protein that is usually located in the cell nucleus (29). During necrotic cell death HMGB-1, along with other nuclear contents, is passively released as cell membrane integrity fails. Released into the local tissue environment and the circulation, HMGB-1 signals through TLR-4 and RAGE receptor on KCs to upregulate NF-κB dependent pro-inflammatory cytokine secretion (20). HMGB-1 in a hyper-acetylated form may also be released by

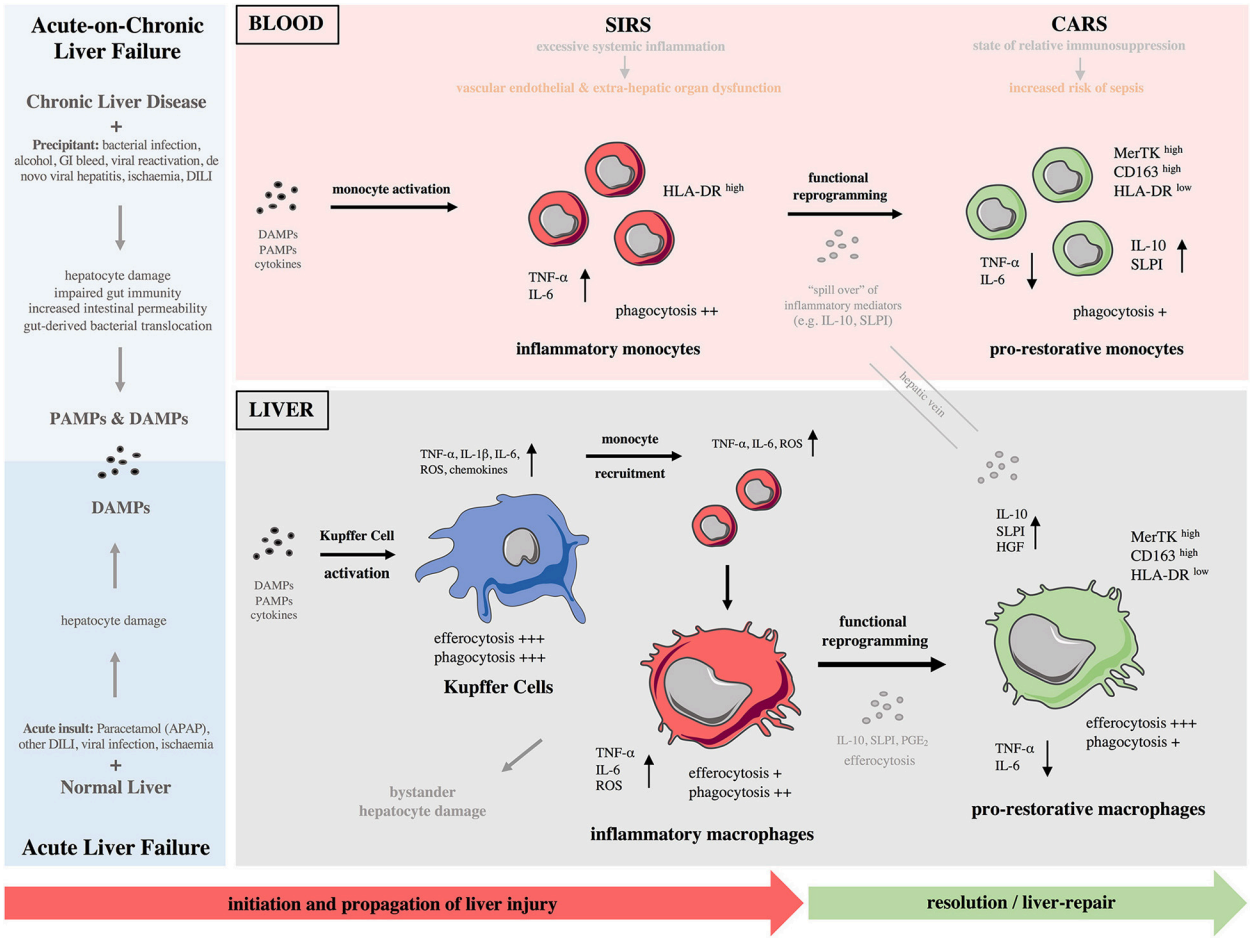

FIGURE 1 | Monocytes and macrophages in the immunopathology of acute and acute-on-chronic liver failure**. (Left)** Different causes lead to development of acute (bottom) and acute-on-chronic (top) liver failure. A major component of the immunopathology of both syndromes is liver inflammation initiated by release of various DAMPs and DAMPs/PAMPs, respectively. **(Right)** During these syndromes, there is a reciprocal interaction of the immune responses between the liver and systemic circulation throughout the different phases. Initiation phase: Kupffer cells become activated after recognition of PAMPs/DAMPs and initiate a pro-inflammatory response. Propagation phase: Bone-marrow derived monocytes are recruited to the liver and differentiate into inflammatory macrophages, expanding the macrophage pool and promoting tissue destruction. During the propagation phase, innate immune activation is self-perpetuating with recruitment of effector cells driving further cytokine and chemokine production; their release to systemic circulation provokes SIRS. These macrophage-derived mediators contribute to vascular endothelial dysfunction and microcirculatory disturbances, resulting in extra-hepatic organ dysfunction. In parallel to SIRS, a CARS develops that is due to release of anti-inflammatory mediators from the liver. Resolution/tissue-repair phase: In response to anti-inflammatory cytokines/mediators and efferocytosis of apoptotic cells, macrophages undergo functional reprogramming toward a pro-restorative phenotype, favoring resolution, and tissue recovery. "Spill over" of anti-inflammatory mediators from the liver to systemic circulation enhances CARS and causes monocyte functional reprogramming toward a pro-restorative phenotype, eventually leading to relative immunosuppression that predisposes susceptibility to infectious complications. CARS, compensatory anti-inflammatory response syndrome; CD, cluster of differentiation; DAMP, damage-associated molecular pattern; DILI, drug-induced liver injury; GI, gastrointestinal bleeding; HGF, hepatocyte growth factor; HLA-DR, human leukocyte antigen-DR; IL, interleukin; MerTK, Mer Tyrosine Kinase receptor; NASH, non-alcoholic steatohepatitis; PAMPs, pathogens-associated molecular patterns; PGE2, prostaglandin E2; ROS, reactive oxygen species; SIRS, systemic inflammatory response syndrome; SLPI, secretory leukocyte protease inhibitor; TNF-α, tumor necrosis factor-alpha.

activated monocytes and macrophages. In human paracetamol-induced ALF, HMGB-1 has been shown to be an early biomarker, predicting which patients at presentation will go on to develop acute liver injury (30). High levels of total and acetylated HMGB-1 are also correlated with worse prognosis in patients with ALF (31). Evidence from experimental models suggest HMGB-1 is of mechanistic importance in the pathogenesis of ALF, not merely an epiphenomenon. HMGB-1 neutralizing antibodies are shown to ameliorate injury and reduce bacterial translocation in murine models of paracetamol-induced ALF (21, 32). In patients with hepatitis B related ACLF, HMGB-1 has been shown to be significantly elevated compared with CLD patients, however does not have prognostic value in these patients (33, 34).

Histones are other nuclear structural proteins that when released in an uncontrolled manner during necrotic cell death can act as DAMPs to initiate inflammation. In a study of a cohort of ALF patients predominantly with acute viral hepatitis, extracellular histones were shown to be elevated and correlate with disease severity and outcome (35). In hepatitis B related

ACLF levels of extracellular histones were significantly elevated compared with patients with CLD and correlated with clinical evidence of systemic inflammation, severity, and patient outcome (36). Histones act to initiate inflammation through the TLR2 and TLR4 receptors and are shown to activate the nod-like receptor family pyrin-domain containing-3 (NLRP3) inflammasome in immune cells, a signaling pathway leading to IL-1β production. Extracellular histones are directly toxic to endothelial cells and have been linked to the propagation of tissue injury and systemic endothelial dysfunction in sepsis (37). Animal studies and *ex vivo* human work has shown that targeted anti-histone treatments can reduce monocyte pro-inflammatory cytokine production and reduce the severity of ALF (24). Other DAMPs associated with initiation of inflammation in ALF/ACLF include the extracellular ATP signaling via the purinergic P2X7 receptor, extracellular DNA signaling through TLR9, and cyclophilin A (10, 15, 23, 25, 38, 39), as summarized in **(Table 1)**.

PAMPs and DAMPs can collectively contribute to local and systemic inflammation in a variety of liver diseases (25, 40). Several pathologies, including ALF and ACLF, straddle the border between sterile and pathogen-induced inflammation which although are conceptually distinct, largely overlap at a functional level (4). For example, in ALD ethanol-induced liver damage results in released of various DAMPs; however, ethanol exposure also increases intestinal permeability and results in lipopolysaccharide (LPS) leakage, a bacterial-derived PAMP, from commensal intestinal flora to the blood supply to the liver (4, 41). LPS binds to and activates KCs that in turn produce inflammatory cytokines and promote hepatocyte damage (4, 41). In addition, the innate immune system has evolved to use shared PRRs to detect both sterile and infectious insults. Bacterial constituents, such as lipopolysaccharide (LPS), or free bacterial DNA may stimulate the innate immune system via the same PRRs that are used by DAMPs. For instance, TLR4 can be activated by both LPS and HMGB-1 (37, 39). Bacterial infection is one of the commonest precipitating factors in ACLF, and even in the absence of overt sepsis, ACLF may be initiated by events that increase bacterial translocation from the gut into the portal circulation. Taken together, although some forms of sterile liver injury may in fact respond solely to DAMP release, many of these can be complicated by a response to PAMPs.

MONOCYTES AND MACROPHAGES IN THE DEVELOPMENT OF LOCAL INFLAMMATION IN LIVER FAILURE SYNDROMES: RECRUITMENT AND FUNCTIONAL DIVERSITY

Liver Macrophage Plasticity

Macrophages are characterized by their broad diversity and plasticity; in response to injury or infection, they secrete pro-inflammatory cytokines and reactive oxygen/nitrogen species that aid their antimicrobial responses (42, 43). During homeostatic conditions, local micro-environmental cues induce macrophages to adopt phenotypes linked with tissue repair and remodeling (42, 43). Based on their differentiation status, macrophages were traditionally categorized into pro-inflammatory (M1) or anti-inflammatory/wound-healing (M2) (42, 44). However, extensive transcriptomic analyses of human MoMFs, cultured with different stimuli (e.g., cytokines, fatty acids, or lipopolysaccharides), revealed a spectrum of macrophage activation states that are not adequately described by the M1/M2 dichotomy (45). Firstly, the signals received by macrophages in their local microenvironment are diverse and temporally and spatially dynamic (42, 43). Secondly, macrophages not only respond with diverse phenotypes but can also reversibly switch from one type to another (46, 47). A newly proposed way of looking at macrophage polarization is to consider a multidimensional model including the source of macrophages, their specific microenvironments with local signals and a collection of macrophage markers (42, 43).

The liver contains the largest proportion of macrophages among all solid organs in the body (48, 49). Macrophages are a key cellular component of the liver; studies in mouse livers estimate that every 100 hepatocytes are accompanied by 20–40 macrophages (1). Due to their inherent plasticity, liver macrophages flexibly respond to differential environments and adopt to the educative signals arising from parenchymal and other immune cells within the liver (50, 51). Hence, they execute diverse functions during liver inflammation, ranging from tissue-destructive to resolution and pro-restorative roles (47). Liver macrophages secrete high levels of reactive oxygen/nitrogen species and inflammatory cytokines and chemokines, and therefore can regulate both innate and adaptive immune responses. They influence the different phases following liver injury by promoting the clearance of cell debris, extracellular matrix remodeling, tissue regeneration, and inflammatory resolution (2). In line with the multidimensional macrophage model (42, 43), macrophages isolated from injured murine livers express inflammatory (M1-like) and pro-restorative (M2-like) markers simultaneously (52, 53) while can rapidly change their phenotype depending on the local hepatic micro-environmental milieu (54, 55).

Monocyte Recruitment Into the Liver Following Acute Injury

A prominent feature of acute liver injury is the increased numbers of hepatic macrophages (56–59). Following injury, the macrophage pool of the liver is expanded due to the infiltration of bone marrow derived CCR2+ Ly6C^{high} monocytes which develop into MoMFs (56–59). The CCR2/CCL2 axis is crucial for their recruitment to the liver. Dambach et al. first showed that CCL2 is highly secreted in the mouse liver after APAP overdose while latter work, with several liver injury models applied in CCR2$^{-/-}$ mice, confirmed the importance of CCR2 for monocyte recruitment to the liver (58–61). CCR5 has also been described as crucial for monocyte recruitment in experimental APAP toxicity (62). Human data show that serum and liver tissue CCL2 levels are increased in acetaminophen-induced ALF (AALF) patients who also have increased numbers of S100A8/9+ newly infiltrating monocytes

or MoMFs, in necrosis areas within their livers (63). Of note, these patients show increased CCR2 expression in their intermediate, but not classical/non-classical, monocytes (63). The anti-inflammatory liver micro-environment (e.g., CCL2, IL-10, and TGF-β) in human AALF implicates these cells in pro-restorative responses (63). However, the severity of AALF patients correlates inversely with blood monocyte numbers and directly with serum CCL2 levels, suggesting that patients with poorer outcomes recruited more monocytes to the liver (63). In accordance, Mossanen et al. show that CCR2+ cells are increased in the liver of AALF patients and express inflammatory markers, such as S100A9, thus implicating them in propagation of injury (53). Taken together, liver-recruited monocytes play dual roles during liver injury; depending on the disease stage, they can perpetuate inflammation but also promote resolution of inflammation (64).

In situ Development of Liver Monocyte-Derived Macrophages

Liver MoMFs form a developmentally, phenotypically and functionally distinct subset of macrophages compared to liver-resident KCs (65). Numerous human and mouse studies suggest a potential immune-regulatory role for MoMFs during liver injury. Liver MoMFs are CX3CR1+, derive from blood Ly6C^{high} monocytes while following their infiltration they undergo a maturation process toward Ly6C$_{low}$ MoMFs (52, 65) **(Figure 2A).** These initially inflammatory CCR2^{high} CX3CR1^{low} monocytes form ring-like structures around injured areas where they differentiate *in situ* into CCR2^{low} CX3CR1^{high} cells, that in turn guide resolution and tissue-repair (8, 29, 66). This maturation process into pro-restorative macrophages is suggested to be driven by the ingestion of cellular debris and augmented by secreted growth factors and cytokines [e.g., macrophage colony-stimulating-factor-1 (CSF1), IL-4, and IL-10] (52, 58, 66, 67). Both resident and infiltrating macrophage populations proliferate in the liver and this expands their numbers during inflammatory conditions (67). Following APAP hepatotoxicity, there is a massive expansion of liver macrophages at 12 h post APAP in mice, that is mainly due to recruitment of MoMFs rather than proliferation of KCs (57, 59, 65). Of note, mouse liver MoMFs are characterized by a gene expression profile that is distinct to circulating Ly6C^{high} monocytes and liver-resident KCs (52, 65). In support of this, increased numbers of newly-infiltrating (S100A8/9+CD68+) macrophages are detected in the liver of AALF patients (53, 63).

In addition to resident KCs, MoMFs apparently contribute to the resolution and tissue-repair processes, as their depletion at different stages of liver injury highlights their critical immune-regulatory roles (68). In both mouse and human ALF, liver-infiltrating MoMFs exhibit a largely anti-inflammatory phenotype and transcriptional signature indicative of a pro-restorative, wound-healing, and regenerative functions (63–66). For instance, mature (CX3CR1^{high} Ly6C^{low}) liver MoMFs highly secrete mediators involved in extracellular matrix remodeling, angiogenesis and hepatocyte regeneration (52, 53, 58). This pro-restorative phenotype may be induced by local micro-environmental cues, including CSF1 and secretory leukocyte protease inhibitor (SLPI), that are secreted in the liver of APAP-mice and patients with AALF (64, 66, 67). In keeping with their role in resolving inflammation, two recent studies described that MoMFs regulate neutrophil survival and clearance in APAP liver injury, that is apparently mediated through Mer-Tyrosine-Kinase (MerTK) (66, 69). Recently, Kubes et al. described the infiltration of macrophages from the peritoneal cavity to the liver. Using the thermal and carbon-tetrachloride (CCl_4)-induced acute liver injury models, they identified a subset of GATA6-expressing peritoneal macrophages as a third population responding to injury (70). These GATA6+ peritoneal macrophages migrate directly through the liver visceral endothelium, and not the vasculature, and aid tissue healing and regeneration processes (70). It remains unclear if this mechanism is restricted to sub-capsular liver lesions in close proximity to the peritoneal cavity. These findings may extend to other models of liver injury, and this new paradigm of avascular macrophage recruitment has opened new avenues for research and potential therapeutic applications (70).

Kupffer Cell Depletion Following Acute Injury

Mouse liver-resident KCs have an F4/80^{high} CD11b^{+} expression profile in contrast to MoMFs which are F4/80^{+} CD11b^{high} cells (57, 65, 67, 71) **(Figure 2A)**. Owing to their non-monocytic origin, KCs are CX3CR1^{neg} while their expression of other surface markers overlaps with other phagocytes (72, 73). Recent lineage-tracing and transcriptional analyses revealed that KCs express the prototypical markers C-type-lectin-domain-family-4-member-F (Clec4F^{+}) and T-cell-immunoglobulin-and-mucin-domain-containing-4 (Tim4^{+}) (51, 65, 73, 74) **(Figure 2A).** The heterogeneity of human liver macrophages is less characterized. There are currently no lineage-specific markers to clearly distinguish human KCs from MoMFs (1, 75). CD68 and CD14 are used as macrophage markers but are commonly expressed by both subsets (1, 53, 76). Most human studies have stained liver tissue of AALF and ACLF patients for CD68 and the pro-inflammatory marker S100A8/9 to discriminate the circulation-derived S100A8/9^{+} MoMFs from resident KCs (53, 63, 64, 66, 77).

Following liver damage in acute liver failure, the absolute number of KCs decreases whereas the liver-recruited monocytes and MoMFs significantly increase as described above (52, 53, 57, 59, 60, 65, 66). In murine models KC depletion occurs at 24–48 h post APAP but full recovery takes place by 120 h, through self-renewal (52, 60, 65). These findings concur with human data showing increased proliferative activity (Ki67+CD68+) of resident macrophages in AALF patients (63). Of note, recent studies revealed that infiltrating bone marrow derived monocytes can replace the KC population, if KCs are completely ablated (54, 74, 78, 79). Following KC depletion in mice, using clodronate-loaded liposomes (CLL), monocytes are able to repopulate the KC and DC populations, giving rise to the full liver myeloid cell heterogeneity within weeks. However,

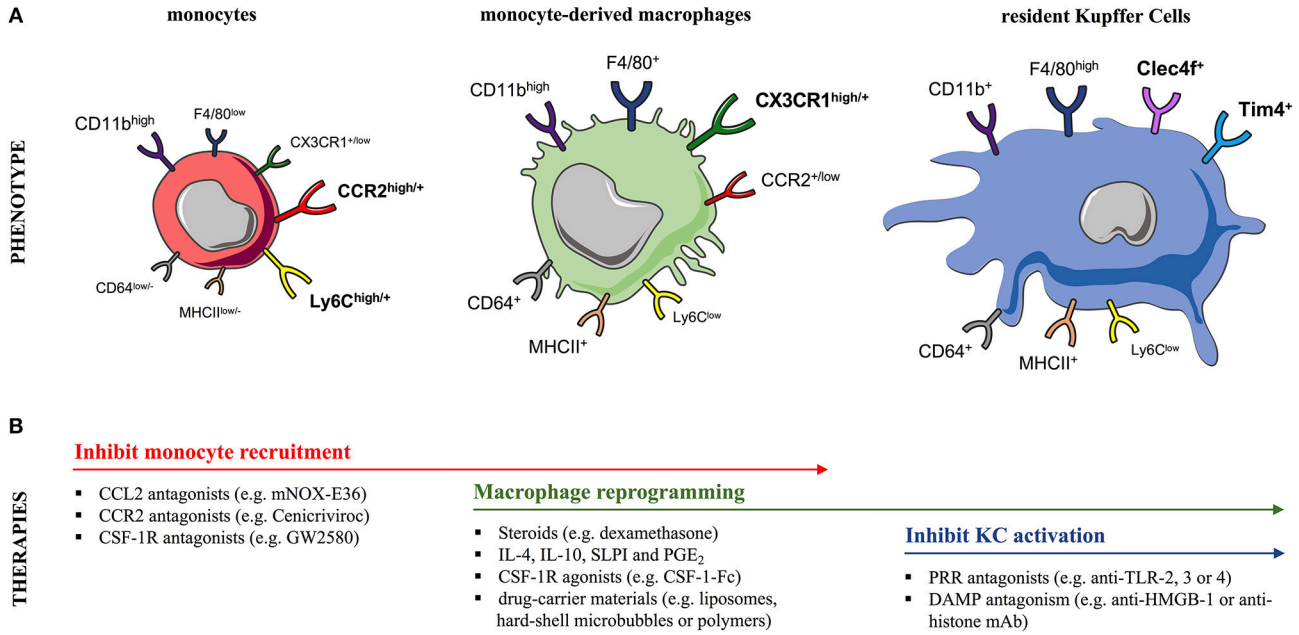

FIGURE 2 | Murine monocyte and liver macrophage subsets and targeted therapeutic strategies**. (A)** In mice, (left) blood and liver-infiltrating monocytes differentially express the markers Ly6C, CCR2, and CX3CR1. During steady-state, the liver macrophage pool can be expanded due to recruitment of circulating ($CCR2^{+}$) $Ly6C^{high}$ monocytes, a process markedly increased after injury. Following their infiltration, monocytes undergo a maturation process into (middle) ($CX3CR1^{+}$) $Ly6C_{low}$ monocyte-derived macrophages (MoMFs) that exhibit a $CD11b^{high}$ $F4/80^{+}$ profile. In contrast, the (right) embryonically derived ($CX3CR1^{-}$) liver-resident Kupffer cells (KCs) are $CD11b^{+}$ $F4/80^{high}$ cells expressing the prototypical markers Clec4F and Tim4. Markers designated in bold are currently used to distinguish these two subsets. **(B)** The table summarizes therapeutic interventions targeting monocyte recruitment, macrophage polarization/differentiation or KC activation in experimental models of acute liver injury. CCL2, CC-chemokine ligand 2; CCR2, CC-chemokine receptor 2; CD, cluster of differentiation; Clec4F, C-type-lectin-domain-family-4-member-F; CSF1R, macrophage colony-stimulating-factor-1 receptor; CX3CR1, CX3C-chemokine receptor 1; DAMP, damage-associated molecular pattern; HMGB-1, high-mobility group box-1; IL, interleukin; MHCII, major histocompatibility complex class II; PRR, pattern-recognition receptor; PGE2, prostaglandin E2; SLPI, secretory leukocyte protease inhibitor; Tim-4, T-cell-immunoglobulin-and-mucin-domain-containing-4; TLR, Toll-like receptor.

these observations were made in models involving extensive experimental KC depletion (79). In contrast, after KC depletion in APAP injury, KC repopulation relies on self-renewal rather than contribution from monocytes (65). Additional studies have shown that the replacement of KCs by monocytes can result in self-renewing macrophages with similar functional and transcriptional profiles to yolk-sac derived KCs (54, 74). Thus, MoMFs are plastic and are influenced by local signals received within the hepatic microenvironment as they repopulate the KC niche (54, 74).

Kupffer Cells Are Pivotal for Antimicrobial Defense

The liver is essential for antimicrobial defense and macrophages play a fundamental role in this (3). KCs are highly effective phagocytes that not only recognize, ingest, and degrade cellular debris but also clear foreign material and pathogens (1, 75, 80, 81). Regardless their origin, steady-state KCs are a homogeneous macrophage population with a specific transcriptional program defined by their unique liver niche (54). KCs are exclusively located intravascularly, seeded along the hepatic sinusoidal endothelial cells (HSECs), whereas monocytes and MoMFs can be located extravascularly (58, 70). This optimal location underscores their crucial role in ensuring liver homeostasis by constantly clearing blood-borne pathogens, associated toxins (e.g., LPS) and cellular debris (3). Accordingly, KCs are fully equipped with high expression of Fc (e.g., CD64), scavenger (e.g., CD163), complement, or PRR (e.g., TLR4, TLR9) receptors so they exert antimicrobial responses (1, 75, 82).

Following intravenous injection of *E. coli* bacteria in mice more than 60% of them become trapped in the liver after 10 min, and this proportion can rise to more than 80% in 6 h. Also, intravascular administration of fluorescent-labeled latex microbeads leads to rapid uptake by liver phagocytes with minimal uptake by circulating immune cells or other tissue compartments (e.g., spleen, lung) (67, 79). Mouse experimental studies utilizing flow cytometry and intravital confocal microscopy show that steady-state KCs are the dominant phagocyte in the liver, characterized by enhanced uptake of pH-sensitive *E. coli* bioparticles or GFP-expressing *E. coli*, compared to liver MoMFs and neutrophils (67, 79). However, phagocyte depletion by using CLL in mice revealed that newly-arrived, immature KCs have a reduced phagocytic uptake of *E. coli* for at least 1 month after CCL administration, suggesting a window of hepatic phagocytic dysfunction during the KC maturation process in the liver (79).

KCs can also cooperate with platelets and other non-parenchymal cells (e.g., HSECs or neutrophils) in order to promote pathogen clearance. For example, aggregation of

patrolling platelets with KCs facilitates bacterial recognition and clearance during *B. cereus* and *S. aureus* systemic infections in mice, thus conferring protection against sepsis (83). However, recognition and scavenging of bacteria by KCs does not necessarily translate into bacterial clearance. Another recent study has highlighted that KCs can serve as a potential reservoir for *S. aureus,* showing that a small proportion of *S. aureus* overcomes the antimicrobial defense of KCs and replicates within their phagolysosomes, thereby efficiently evading from recruited neutrophils (84). Over time, KCs lyse and release bacteria into the circulation, enabling dissemination to other organs (84). Similarly, KCs were not able to eliminate intracellular bacteria *L. monocytogenes* infection in mice (16). Instead, *L. monocytogenes* induced KC death by necroptosis which triggered the release of hepatocyte-derived danger signals, such as IL-33, and led to monocyte recruitment to the liver; MoMFs ultimately eliminate bacteria and restore homeostasis (16). Work by Sierro et al. recently described an additional liver-resident macrophage population, occupying the hepatic capsule, that is pivotal for the liver antimicrobial defense (73). These liver capsular macrophages (LCMs) are replenished from circulating monocytes at the steady state unlike the embryonically derived KCs. LCMs play a key role in immune surveillance, by sensing peritoneal pathogens and promoting the recruitment of neutrophils toward the capsule to control intrahepatic bacterial dissemination (73).

The depletion of KCs during acute liver injury is likely to have functional and clinical significance in ALF and ACLF. Given that KCs, as the dominant intravascular phagocyte, are responsible for removing live bacterial and microbial products from the circulation and exerting a tolerogenic effect in steady state, their depletion may permit enhanced systemic exposure to DAMPs and persisting bacteraemia. This is an area where further research is required, to define the mechanism of KC depletion and functional significance of the resulting phagocytic defect.

Liver Macrophages: Drivers of Injury or Promoters of Repair?

The role of liver macrophages in acute liver injury has been controversial. The experimental model of APAP injury in mice is the best explored example, with detailed characterization of macrophage subsets. Initially, KC activation was thought to exacerbate APAP hepatotoxicity in mice (85). However, it is now well-established that KCs are largely depleted after APAP overdose and MoMFs represent the largest macrophage population in the liver (52, 53, 57, 59, 65, 66). The first experiments using gadolinium chloride, a potent KC inactivator, noted protective effects against APAP toxicity, however latter work applying KC depletion prior to APAP demonstrated a beneficial role of KCs (85, 86). Others, by using CLL to deplete most tissue macrophages, showed that macrophage inhibition had a detrimental effect on liver injury (59, 87). Also, mice deficient for NADPH oxidase, an enzyme required for KC oxidative burst, were not protected against APAP injury suggesting that KC-derived oxidant stress is not involved in the injurious APAP process (88).

The role of liver-infiltrating monocytes and their macrophage descendants in acute liver injury has been an area of intense research the last years. Initial studies applying APAP-induced liver injury in CCR2-deficient ($CCR2^{-/-}$) and anti-CCR2 treated mice found increased liver inflammation and delayed tissue regeneration, suggesting that monocytes are crucial for recovery from APAP injury (57, 60). Lack of recruitment of $CCR2^{high}$ $CX3CR1^{low}$ monocytes also results in persistent accumulation of necrotic cells up to 48 h post injury, indicating them crucial for tissue-repair processes (58). Others by using the CCl_4-induced acute liver injury model found that blood monocyte depletion with CCL resulted in comparable ALT levels at 24 h after CCl_4 administration, proposing that recruited monocytes do not contribute to the early stages of injury (59). This group further demonstrated that $CCR2^{-/-}$ mice exhibited comparable liver injury at 24 and 48 h following CCl_4 injection, although liver monocyte numbers were decreased (59). In contrast, others support the idea that monocytes and MoMFs exacerbate liver inflammation. Studies using single-dose CCl_4 models of acute liver injury and MCP1-deficient ($MCP1^{-/-}$) or $CCR2^{-/-}$ animals suggested that CCL2-recruited CCR2+ monocytes contribute to induction of early injury, showing reduced liver injury at 24 h but comparable levels at 48 h (61, 89). In line with this, two recent studies showed that CCR2+ monocytes infiltrate sites of liver injury as early as 8–12 h following insult (53, 58). Using the APAP experimental model, Mossanen et al. found that $CCR2^{-/-}$ mice had reduced ALT levels and necrosis at 12 h after injury (53). Pharmacological inhibition of monocyte infiltration using a CCR2 antagonist early after APAP dose resulted in reduced ALT levels and necrosis at 12 h following injury, but equivalent injury at 24 and 48 h, supporting the data from the CCl_4 model (53). Furthermore, this study revealed that the newly-infiltrated ($Ly6C^{high}$) MoMFs exhibit a pro-inflammatory phenotype, as reflected by their cytokine expression (e.g., TNF-α or IL-1β) profile (53). These results suggest that liver monocytes and MoMFs can produce both pro-inflammatory and repair-associated cytokines, however their function clearly depends on the phase (propagation vs. resolution) of liver injury (53, 90). It is important to note the contradictory evidence on the roles of macrophages in ALF. This may be explained by their functional plasticity during the phases (initiation, propagation, and resolution) of liver inflammation (1, 55, 75, 91). These inconsistencies can also be attributed to the hard distinction of macrophage subsets, especially under inflammatory stress, and the different sensitivity of macrophages to the various experimental depletion techniques applied.

MONOCYTES AND MACROPHAGES AND THE SYSTEMIC IMMUNOPATHOLOGY OF LIVER FAILURE SYNDROMES

Systemic Inflammation

Development of SIRS and CARS in Liver Failure

The systemic inflammatory response syndrome (SIRS) is the constellation of clinical signs, suggestive of immune activation and the presence of inflammation. It is defined by the presence

of any two or more of: fever or hypothermia, tachycardia, tachyopnoea, and white cell response, either leukocytosis, leucopaenia or >10% immature neutrophils (92). SIRS is no longer included in the most recent "Sepsis-3" definition of sepsis, as it was proved to have poor sensitivity and specificity for the identification of patients with infection (93). The lack of specificity is in part because patients with conditions of sterile inflammation like ALF, in which there is a robust innate immune response and high circulating levels of pro-inflammatory cytokines, can exhibit positive SIRS criteria without infection (12). SIRS remains a useful clinical definition for studies investigating immune activation, albeit offering poor discrimination between sterile and sepsis-driven inflammation.

SIRS in ALF is driven by pro-inflammatory cytokine secretion of liver immune cells and is closely associated with the development of extra-hepatic organ dysfunction and adverse outcomes (44). In parallel to the pro-inflammatory response to acute tissue injury, a compensatory anti-inflammatory response syndrome (CARS) develops, as anti-inflammatory cytokines and mediators are concomitantly released from liver macrophages during the initial stages of liver injury (41, 42, 45). CARS is a counter-regulatory, homeostatic mechanism aimed at preventing overwhelming inflammation. Excessive SIRS activation is also hallmark of ACLF. Inflammatory responses in ACLF are imbalanced, ranging from an initial SIRS to subsequent development of a long-lasting CARS (16, 50, 54, 58, 59). ACLF patients are also characterized by high circulating levels of both pro-inflammatory and anti-inflammatory cytokines (22, 50, 63). SIRS and CARS are believed to exert modulatory effects on immune cell effector function in ALF/ACLF, such as monocytes and macrophages, thus contributing to immune dysregulation and defective immune responses to microbial cues (16, 22), as discussed below.

Systemic Immuneparesis

Systemic Immunosuppression in ALF

ALF is a multisystem disorder and immune dysfunction is central to the pathogenesis. Here, the massive hepatic infiltration by myeloid cells is contrasted by immune cell depletion and dysregulation in the systemic circulation (63, 64). From a clinical perspective, it is widely accepted that although the precipitating event of ALF is overwhelming hepatocyte death, mortality occurs due to the profound activation of SIRS and its attendant complications of multi-organ failure, immune dysfunction and recurrent sepsis (12, 94–96). SIRS is driven by pro-inflammatory (e.g., TNF-α, IL-1β, IL-6) cytokine secretion from liver immune cells and is closely associated with the development of extra-hepatic organ dysfunction and adverse outcome in ALF (96). In parallel to the pro-inflammatory response to liver injury, CARS develops as anti-inflammatory cytokines/mediators (e.g., IL-10, SLPI) are concomitantly released from liver macrophages during the initial stages of liver injury (64, 94, 97). Elevated levels of circulating anti-inflammatory cytokines detected in early stages of ALF, such as IL-10, predict poor patient outcome (64, 94, 97). ALF shares striking similarities with systemic sepsis and septic shock with regards to the features of systemic inflammation, multi-organ failure and peripheral immunosuppression, thus may share pathological mechanisms (94, 98). Immunosuppression is considered the predominant driving force for mortality in those patients while accumulating evidence shows that sepsis causes major defects in innate and adaptive immunity, leading to compromised host antimicrobial defense (99, 100). "Septic" monocytes show reduced HLA-DR expression and diminished ability to produce pro-inflammatory cytokines (e.g., TNF-α, IL-6, and IL-12) in response to TLR agonists, findings consistent with endotoxin tolerance (99, 100). In contrast, anti-inflammatory mediator (e.g., IL-10) secretion is unaltered or even augmented (99, 100), and these characteristics may account for the increased patient susceptibility to infections in sepsis (101). Of note, sepsis is one of the most important causes of mortality in ALF patients (12).

In line with these notions, we have provided evidence of peripheral immunosuppression in patients with ALF, also termed "immuneparesis," showing major functional defects in both innate and adaptive arms of immunity (64, 77, 102, 103). A hallmark of ALF is that monocytes are functionally impaired and hyporesponsive to microbial challenge (64, 77, 102). Detailed phenotypic characterization of circulating monocytes *ex vivo* in ALF patients revealed an immunosuppressive phenotype, typified by reduced HLA-DR expression but increased CD163, Tie-2, and MerTK expression (64, 66, 77) **(Figure 3)**. Additionally, ALF monocytes show increased expression of tissue (CCR2, CCR5) and lymph-node (CCR7) homing receptors (64, 66, 77). Functional analyses show that ALF monocytes produce less pro-inflammatory (e.g., IFN-γ, TNF-a, IL-6) cytokines after microbial challenge, through NF-kB pathway inhibition, but have enhanced anti-inflammatory (e.g., SLPI) mediator secretion (64, 66, 77, 104) **(Figure 3)**. Furthermore, monocytes in ALF patients have impaired *E. coli* bacteria uptake and enhanced efferocytosis of apoptotic cells (64, 66, 77, 104).

In support of these findings, we showed that MerTK expression by monocytes/macrophages indicates pro-restorative yet immunosuppressive functions in liver failure syndromes (66, 77). MerTK, a tyrosine-kinase receptor mainly expressed by monocytes/macrophages, is a negative regulator of pro-inflammatory TLR signaling pathway (43). We have shown that MerTK+ monocytes in ALF secrete reduced pro-inflammatory cytokines after microbial challenge (66, 77). Furthermore, transcriptional analyses of MerTK+ monocytes in ALF revealed marked reductions in a number of immune-regulatory pathways, including antigen-processing (e.g., HLA-DRA), TLR and NF-κB signaling (e.g., NFKBIA, NFKBIZ, TLR4), phagocytosis/PRR receptors (e.g., FCGR2A/C, FCGR3A/B), and cytokines (e.g., TNF) (66). These cells have an M2-like skewed profile (e.g., CD163), active MerTK/cytokine down-stream signaling (e.g., IRF3, JAK3) with concomitant down-regulation in cell activation related genes (e.g., NLRP3) (66) **(Figure 3)**. These transcriptomic profile of monocytes is consistent with the impaired monocyte antimicrobial responses (cytokines, phagocytosis) described *ex vivo* in ALF (64, 66, 77, 104). In addition, MerTK+

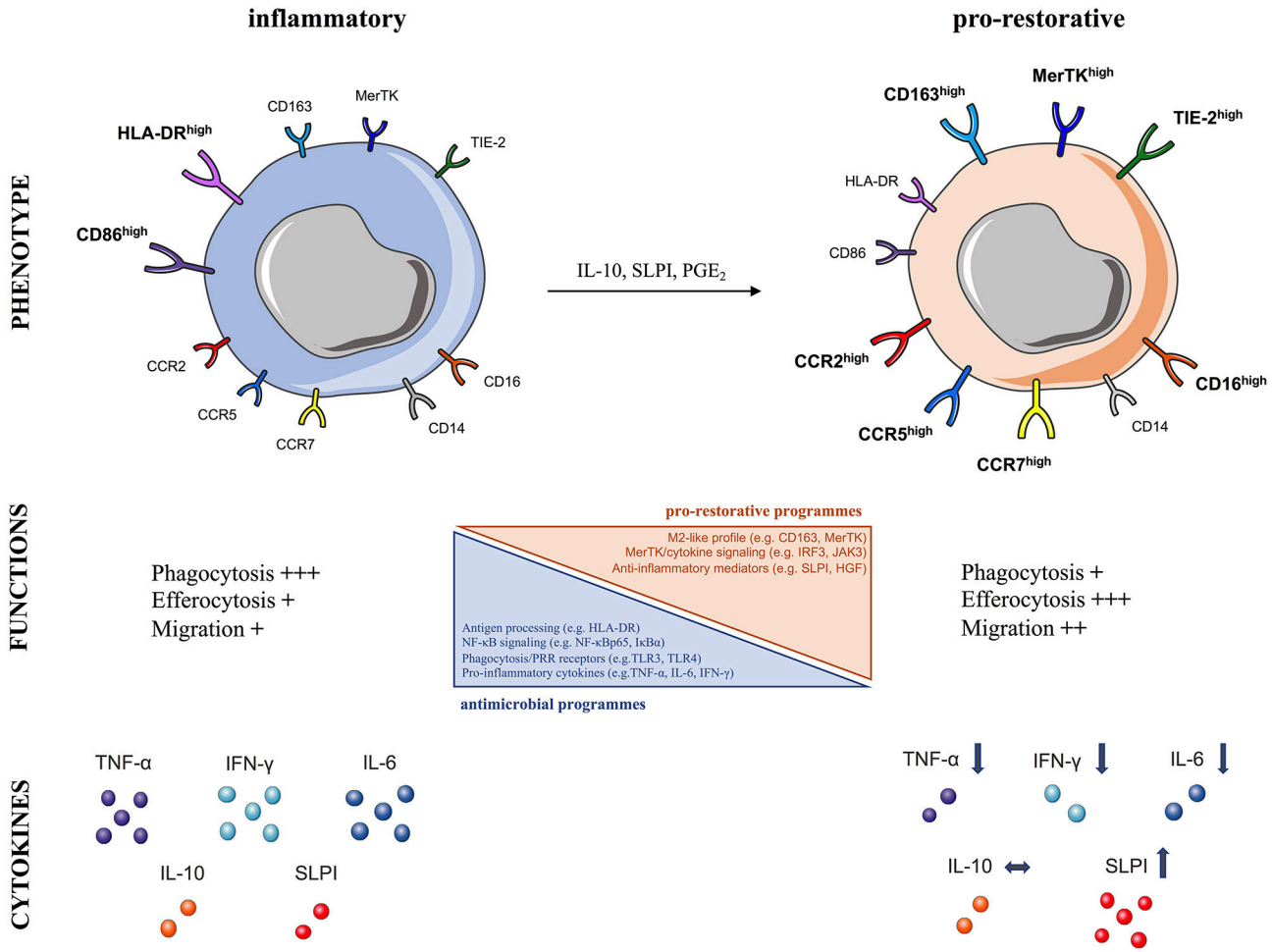

FIGURE 3 | Characteristics of human pro-restorative monocytes and macrophages in acute liver failure syndromes. The schematic summarizes the phenotypic and functional characteristics of **(Left)** steady-state inflammatory and **(Right)** pro-restorative monocytes and macrophages described in acute liver failure syndromes, which can arise in response to micro-environmental cues (IL-10, SLPI, PGE_2), CCR, CC-chemokine receptor; CD, cluster of differentiation; HGF, hepatocyte growth factor; HLA-DR, human leukocyte antigen-DR; IFN-γ, interferon gamma; IL, interleukin; MerTK, Mer Tyrosine Kinase receptor; NF-κB, nuclear factor-κB; PGE2, prostaglandin E2; SLPI, secretory leukocyte protease inhibitor; Tie2, angiopoietin receptor: TNF-α, tumor necrosis factor-alpha; TLR, Toll-like receptor.

monocytes possess enhanced trans-endothelial migratory characteristics which enable them to accumulate in diverse sites of inflammation, so they are found in the liver, lymph nodes, and circulation of patients with ALF (66, 77).

The systemic and hepatic micro-environmental milieu is a critical determinant of monocyte and liver macrophage function during ALF. For instance, *in vitro* incubation of healthy monocytes in the presence of ALF patient derived plasma or liver homogenates induces similar to *ex vivo* monocyte characteristics, described above (55, 64, 66). This work suggests that anti-inflammatory/regenerative mediators (e.g., IL-10, SLPI) that are produced in the inflamed liver, and which serve to dampen pro-inflammatory responses and limit the extent of injury, are of sufficient magnitude to spill-over into the systemic circulation where they reprogram circulating monocytes into an immunosuppressive state (55, 64). This functional impairment renders monocytes less able to respond to secondary infectious stimuli, hence compromises host anti-microbial defense mechanisms and may account for the increased susceptibility to infections and poor outcomes from sepsis encountered in ALF patients (63, 64, 102, 105). It is therefore of great importance to understand in more detail the underlying mechanisms of monocyte suppression in ALF, so that novel immune-based therapies can be developed (55, 106).

Systemic Immunosuppression in ACLF

Immune dysfunction is central to the pathogenesis of ACLF and is postulated to account for its infectious complications and their negative impact on patient survival (28, 77, 107). Immune dysfunction in ACLF is multifactorial. From a pathophysiological perspective, ACLF is a dynamic, multisystem process that involves several defects/abnormalities in cellular and soluble components of the immune system (108, 109). These defects eventually lead to a state of acquired immunodeficiency

(108, 109), impairing the host's antimicrobial responses and thus conferring an increased susceptibility to infections (110, 111). Cellular components involve functionally reprogrammed innate and adaptive immune cells; soluble components include albumin, cytokines, coagulation factors, and the complement system (77, 104, 108, 109). Limitations in the available rodent models of ACLF mean most results are obtained from clinical studies in liver failure patients (10). ACLF's complexity is highlighted by various impairments in different tissue compartments; circulation, gut, peritoneum, liver, and the reticuloendothelial (RES) system, as reviewed with more details elsewhere (108, 109). For instance, increased bacterial translocation of gut-derived organisms to the portal and lymphatic circulations is observed in CLD and ACLF patients, as a consequence of diverse changes; e.g., altered gut microbiota composition and increased intestinal permeability (112). This phenomenon is of pathological significance in ACLF, given that gut-derived PAMPs can perpetually stimulate the immune system (112). Furthermore, pathogen clearance mediated by the RES is reduced proportionally to liver dysfunction severity while reduced hepatic synthesis of innate antimicrobial proteins, such as albumin and complement, thus contributing to decreased bactericidal capacity of phagocytic cells (108, 109).

Inflammatory responses in ACLF are also imbalanced, ranging from initial SIRS activation to subsequent CARS development (77, 104, 107–109). ACLF patients are characterized by upregulated circulating levels of both pro-inflammatory and anti-inflammatory cytokines (11, 28, 77). SIRS and CARS are believed to exert crucial modulatory effects on immune cell effector function in ACLF, such as monocytes/macrophages, resulting in defective immune responses to microbial cues (28, 107). This may explain the dynamic immunosuppressive state differences between patients with stable cirrhosis, acute decompensation and ACLF (77, 104, 108, 109). Work from our group has well-described the monocyte and macrophage dysfunction in ACLF patients (77, 104, 111, 113). The main defects include reduced pro-inflammatory cytokine responses to microbial challenge and impaired antigen-presentation capabilities due to reduced HLA-DR expression (77, 111, 113, 114). These attenuated innate responses are considered a state of refractoriness due to recurrent PAMP exposure and physiological adaptation to counter-regulate inflammatory responses, however this favors development of secondary infections and is associated with increased mortality (77, 108).

In a clinical study led by O'Brien et al. the immunosuppressive lipid mediator prostaglandin E2 (PGE2), that inhibits TLR4 protein expression, was detected at increased levels in plasma derived from patients with ACLF (115). PGE2 was shown to inhibit macrophage pro-inflammatory cytokines in response to LPS and decrease macrophage bacterial killing (115). We have revealed another mechanism explaining immuneparesis in ACLF, that involves the activation of MerTK on monocytes/macrophages in the circulation and tissue sites of inflammation in these patients (77). MerTK overexpression conferred a decreased *ex vivo* response to LPS and closely correlated to levels of immunosuppression, SIRS activation, and disease severity scores in ACLF (77). This study proposed MerTK antagonism as a therapeutic strategy to restore innate responses at later stages of ACLF where prolonged CARS predisposes to infectious complications (77). In the same context, we recently demonstrated an expansion of mononuclear $CD14^{+}HLA\text{-}DR^{-}$ myeloid-derived suppressor cells (M-MDSCs) in the systemic circulation of ACLF patients (104). M-MDSCs in ACLF are highly immunosuppressive: they decrease T cell proliferation, produce less TNF-α following TLR stimulation and have a reduced *E. coli* bacteria phagocytosis (104). M-MDSCs are of great pathological significance in ACLF; given they impair both innate and adaptive responses to microbial agents, they can contribute to the increased frequency of infections encountered in these patients (104). Interestingly, patients with ACLF have a very high short-term occurrence of bacterial and fungal infections (9, 10). Together, these findings suggest that monocyte/macrophage immunosuppression in ACLF develops in parallel to SIRS and this could explain the high risk of nosocomial infections encountered in ACLF patients (77, 108). It is also hypothesized that this immunosuppression might be a regulatory mechanism to limit the monocyte and macrophage responses to elevated amounts of various stimuli (PAMPs, DAMPs, soluble proteins, cytokines and chemokines) (104, 116, 117). However, there is currently no evidence to support this, and future studies are needed to investigate whether the development of systemic inflammation and immunosuppression are related in ACLF (10).

IMMUNOTHERAPEUTIC STRATEGIES TO TREAT LIVER FAILURE

Targeting Liver Macrophages: Opportunities and Challenges

Liver macrophages are central to the pathogenesis of ALF and ACLF driving the initiation, propagation, and resolution of injury and related inflammation. Therefore, they are an attractive target for developing new therapeutic approaches to treat such conditions. First, central pathways regulating their recruitment (chemokines), responses to injurious, or infectious insults (e.g., PRRs, DAMPs, PAMPs, inflammasome activation) and differentiation (effector cytokines, polarization markers) are well-conserved between mice and humans, thus allowing translation from animal experimental models to human diseases (2). Second, a number of drugs with good safety profile approved for liver disease patients (e.g., albumin, glucocorticoids, N-acetylcysteine), including those with ALF/ACLF, are shown to exert great immune-modulatory effects on macrophages (108, 118). Third, the high scavenging capacity of liver macrophages allows their preferential targeting using drug carrier materials (hard-shell microbubbles, liposomes, and polymers) (119). Finally, macrophage-related biomarkers may allow patient selection with favored positive responses (55, 75); increased circulating levels of soluble forms of scavenger receptor CD163 (sCD163) and mannose receptor (sMR) detected in ALF/ACLF patients are proposed as surrogate

markers of macrophage activation and predict mortality (120, 121).

However, the development and application of macrophage-directed therapies to treat liver diseases may face some challenges (2). First, animal models may exhibit quite opposing functions of macrophage subsets depending on the various applied experimental conditions (55, 75). Thus, for any interventions the optimal dosing, timing, subset-specific targeting in relation to the disease stage must be considered. Second, animal models are not fully representative of the mechanistic spectrum of human diseases but reflect only certain aspects of their pathogenesis. For instance, they develop much more rapidly than human diseases which may affect the macrophage adaptations in response to injury. Mice also bear few immunological differences; for example, the murine liver is largely enriched in NKT cells (122). Furthermore, there is greater heterogeneity in patients compared to inbred mouse strains with respect to intrinsic (genetics, sex, age) or extrinsic (microbiota, infections, medications) factors that might influence macrophages. Third, macrophage heterogeneity and their context-specific functions are currently better understood in the mouse rather than the human liver (1). Currently, there is limited access to liver tissue at the various stages of diseases, while technical difficulties in isolating different macrophage populations hamper the detailed characterization of liver macrophage subsets in humans. Despite these challenges, our detailed understanding of the pathways involved in initial liver injury recognition by resident KCs, amplification of responses by monocyte recruitment to the liver and their context-specific differentiation into diverse liver macrophage subsets have offered exciting platforms for developing novel macrophage-directed immunotherapeutic strategies.

Inhibition of Kupffer Cell Activation

At the early phases of ALF and ACLF, immune therapies could be targeted at restricting the profound innate immune activation. The initial recognition of liver injury, that is mainly mediated by resident KCs, triggers inflammatory cascades whose activation can be modulated by several approaches (**Figure 2B**). For instance, the early communication of cellular distress or death from hepatocytes with KCs is through DAMP/PAMP interactions with PRRs and NF-κB signaling and NLRP3 inflammasome activation, so these can be clear targets for immunotherapy. Inhibition of TLR2, TLR3, and TLR4 have been shown to ameliorate liver injury in murine models of APAP hepatotoxicity (19, 123, 124). Furthermore, *in vivo* inhibition of the purinergic receptor P2X7, which acts upstream of the inflammasome and is activated by ATP, has revealed protective effects against APAP injury (38). Another strategy would be to target released DAMPs such as HMGB-1 and histones. Interestingly, HMGB-1 neutralizing antibodies are shown to ameliorate liver injury and reduce bacterial translocation in murine models of paracetamol-induced ALF (21, 32). Further evidence from a rat model of ACLF suggests that blocking HMGB-1 reduces hepatic apoptosis, hepatic inflammatory response and SIRS, thus alleviating inflammation and SIRS in ACLF (125). Hence, pharmacological HMGB-1 blockade in the early stage of human ALF (initiation phase) or ACLF (AD phase) might prevent organ failure so translation of HMGB-1 inhibitors to clinical trials is a potential therapeutic approach (108). Another DAMP, histones, initiate inflammation via TLR2/TLR4 and activation of the inflammasome; animal and human studies showed that targeted anti-histone treatments reduce monocyte pro-inflammatory cytokine production and reduce severity of ALF (36). In the same context, novel drugs targeting inflammasome activation may dampen hepatic pro-inflammatory processes (126).

Increased bacterial translocation, a major contributor to immune dysfunction in ACLF (112), is paralleled by increased gut-derived PAMPs that perpetually stimulate the hepatic innate immune system, magnifying further liver injury (108). Therefore, influencing the gut barrier or the gut microbiome using probiotics or antibiotics could potentially alleviate the pathogenic KC activation (127). Antibiotic agents are widely used drugs for treatment of the infectious complications in ACLF patients (108) while an ongoing study from our group is evaluating the non-absorptive antibiotic Rifaximin that may beneficially influence the gut microbiota to decrease pathologic bacterial translocation. Such therapeutic strategies aimed to limit initial innate immune activation would be most effective if administered very early in disease onset, while levels of DAMPs/PAMPs are high and prior to the propagation phase of liver injury (55). However, this is challenging from a clinical perspective, as the time window for these therapies to be effective is likely to be short. Furthermore, a degree of immune activation is required for resolution of injury, as this initial step leads to recruitment of monocytes and expansion of macrophages at sites of injury, that is associated with effective efferocytosis, pro-restorative functions and facilitation of tissue regeneration (55).

Inhibition of Monocyte Recruitment to the Liver

Inflammatory monocytes amplify and perpetuate inflammation in liver diseases. Their recruitment to the liver is driven by chemokine-chemokine receptor interactions in animal models and patients, with the CCL2–CCR2 and CCL5/CCR5 as the most prominent pathways in ALF and ACLF (53, 57–59, 77). Many available pharmacological strategies can interfere with this signaling including inhibition of chemokines with small molecule inhibitors, monoclonal antibodies against chemokines or receptors and receptor antagonists preventing chemokine binding (128) (**Figure 2B**). For example, the CCR2/CCR5 inhibitor cenicriviroc (CVC) has been recently tested in a randomized, double-blind, phase 2b study including a large cohort of patients with non-alcoholic steatohepatitis (NASH) and liver fibrosis (129). CVC was previously found to block monocyte recruitment to the liver and exert anti-fibrotic effects in experimental models of liver and kidney fibrosis (130). This trial revealed that after 1 year CVC treatment, twice as many subjects achieved improvement in fibrosis and no worsening of steatohepatitis, compared with the placebo (129). CVC might also bear therapeutic potential in ALF or ACLF; Mossanen et al. recently showed that the early pharmacological inhibition of either chemokine CCL2 (by

the inhibitor, mNOX-E36) or CCR2 (by the CCR2/CCR5 inhibitor, CVC) reduced monocyte infiltration and indices of liver injury in murine APAP-induced ALF (53). Importantly, neither the early nor the continuous inhibition of CCR2 hindered repair processes during resolution from injury. Various other CCR2, CCL2, or CCR2/CCR5 inhibitors are currently developed and tested in other inflammatory/metabolic diseases (e.g., type 2 diabetes), clinical trials are needed though to define their efficacy in liver diseases (128). One important finding from the NASH trial was the excellent safety profile of CVC, supporting that inhibiting inflammatory monocytes might not affect essential macrophage immune responses and antimicrobial defense, in a chronic liver disease at least (129).

Promotion of Macrophage Polarization and Differentiation

Macrophages exert key functions in liver injury and therefore there is a theoretical concern that targeting them or inhibiting their recruitment could prove counterproductive. Rather, a therapeutic approach utilizing key mediators to promote an early switch in macrophage function to favor a pro-restorative phenotype and thereby accelerate resolution of injury and hepatocyte regeneration should be considered (55) (**Figure 2B**). Steroids are well-known to promote macrophage anti-inflammatory/resolution responses and therefore their administration might be beneficial in the early phases of liver failure (118). A retrospective analysis of ALF patients did not show an overall survival benefit while transplant-free survival was slightly higher in the steroid-treated group. However, the overall numbers of treated patients in this study were low and steroids were administrated before the onset of ALF in half of the cohort (131). Thus, steroid efficacy in ALF, and their use in ACLF patients (108), is still under debate. As the timing of their administration appears crucial, a prospective trial is suggested to evaluate the steroid effects in the early pro-inflammatory phase of ACLF, so as to limit the initial hepatic inflammation and subsequent SIRS. Steroid usage in the later phases of ACLF, where anti-inflammation prevails, is likely to be detrimental as it may increase predisposition to infection (108).

In contrast to chemokine strategies that reduce monocyte/macrophage recruitment to the liver, there are alternative therapeutic strategies that intentionally cause the opposite, namely augment the macrophage numbers and functions (75) **(Figure 2B)**. Immune dysfunction is common in liver failure syndromes, rendering the patients susceptible to infectious complications. Thus, the effects of hematopoietic growth factors so as to restore immune functions are currently being investigated (132). For instance, granulocyte colony-stimulating factor (G-CSF), produced by macrophages and other immune cells, is shown to reduce disease severity scores, septic episodes and increase survival in a study using G-CSF supplementary for 28 days in the treatment of patients with ACLF (133). This effect may be mediated through mobilization of CD34+ hematopoietic progenitor cells into the liver (133). Furthermore, G-CSF has been shown to improve impaired phagocytosis and bacterial killing by neutrophils *in vitro* and *in vivo* in patients with ALF (134, 135). However, its effects on macrophages are not described. In a similar fashion, the role and therapeutic potential of CSF1 was recently examined in ALF (67). In patients with acetaminophen-induced ALF, low serum CSF1 levels correlated with increased mortality. Exogenous administration of CSF1, in the form of a crystallisable fragment (Fc), promoted hepatic macrophage accumulation in mice (67). CSF1-Fc increased the proliferation of liver-resident KCs and the recruitment of monocytes, promoting their differentiation, which was associated with indicators of increased innate immunity in mice after partial hepatectomy or APAP-induced injury with resident KCs as the main effector cells (67). Thus, patients with impaired macrophage functions such as ALF and ACLF, might benefit from such approach.

Finally, the high scavenging capacity of liver macrophages, especially that of resident KCs, allows their preferential targeting using drug carrier materials such as hard-shell microbubbles, liposomes, and polymers (119). Upon systemic administration in mice, 15–50% of these three prototypic drug delivery systems can be found in the liver, and KCs are the main cellular target for such carriers (119). If particles are functionally endowed with the sugar moiety mannose, the targeting specificity for KCs that carry the mannose scavenging receptor (CD206) can be significantly increased (119). In a proof-of-concept study involving systemic *S. aureus* infection in mice, the liposomal administration of the antibiotic vancomycin efficiently targeted KCs and aided the elimination of a reservoir of *S. aureus* residing within KCs (84). Similarly, the macrophage-targeted delivery of dexamethasone has been shown to reduce fibrosis in mice (136). Taken together, such immunotherapeutic strategies ideally would be able to target liver macrophages without causing a concomitant immunosuppressive effect on circulating monocytes and should allow monocyte-derived macrophage recruitment to sites of tissue inflammation or infection (55). Future studies in mice and humans will provide a better understanding of monocyte and macrophage plasticity in the liver and will hopefully lead to the development of novel therapeutic targets for use in ALF, ACLF, and other inflammatory conditions.

The apparently contradictory aims of some of the described macrophage-targeted approaches highlights the challenges in developing immunotherapeutic strategies for ALF and ACLF. There is an inherent tension between the local hepatic environment, in which promotion of anti-inflammatory programmes is desirable to resolve inflammation and restore tissue integrity, and the systemic immune environment in which enhanced anti-microbial immunity is required. Cellular and humoral components of the response to liver injury, such as MerTK± macrophages and SLPI, which are important in local tissue repair have also been shown to be key drivers of systemic immunosuppression (64, 66, 77, 106). While current experimental tools and models are unable to resolve this paradox, it is hoped that with greater understanding of the inflammation-resolution pathways and the mechanisms of

peripheral immuneparesis, macrophage and monocyte targeted approaches could be developed.

CONCLUSION

The last two decades have seen a great progression in our understanding of acute liver failure syndromes as inflammatory, immune-mediated conditions. The recognition of ACLF as a condition distinct from decompensated chronic liver disease has enhanced interest, research and understanding. In both ALF and ACLF, monocytes and macrophages play a key role in disease pathogenesis; driving local inflammation, tissue repair, and systemic complications. As research reveals greater levels of complexity and diversity of myeloid cell function in acute liver failure syndromes, both the challenges and opportunities of developing targeted immunotherapy are brought into focus.

AUTHOR CONTRIBUTIONS

ET, CA, and LP planned the manuscript. ET and LP wrote the manuscript. ET, KW, MM, and LP reviewed and edited the manuscript.

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Hepatocyte-Derived Lipotoxic Extracellular Vesicle Sphingosine 1-Phosphate Induces Macrophage Chemotaxis

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Background: The pathophysiology of non-alcoholic steatohepatitis involves hepatocyte lipotoxicity due to excess saturated free fatty acids and concomitant proinflammatory macrophage effector responses. These include the infiltration of macrophages into hepatic cords in response to incompletely understood stimuli. Stressed hepatocytes release an increased number of extracellular vesicles (EVs), which are known to participate in intercellular signaling and coordination of the behavior of immune cell populations via their cargo. We hypothesized that hepatocyte-derived lipotoxic EVs that are enriched in sphingosine 1-phosphate (S1P) are effectors of macrophage infiltration in the hepatic microenvironment.

Methods: Lipotoxic EVs were isolated from palmitate treated immortalized mouse hepatocytes and characterized by nanoparticle tracking analysis. Lipotoxic EV sphingolipids were quantified using tandem mass spectrometry. Wildtype and $S1P_1$ receptor knockout bone marrow-derived macrophages were exposed to lipotoxic EV gradients in a microfluidic gradient generator. Macrophage migration toward EV gradients was captured by time-lapse microscopy and analyzed to determine directional migration. Fluorescence-activated cell sorting along with quantitative PCR and immunohistochemistry were utilized to characterize the cell surface expression of $S1P_1$ receptor on intrahepatic leukocytes and hepatic expression of $S1P_1$ receptor, respectively.

Results: Palmitate treatment induced the release of EVs. These EVs were enriched in S1P. Palmitate-induced S1P enriched EVs were chemoattractive to macrophages. EV S1P enrichment depended on the activity of sphingosine kinases 1 and 2, such that, pharmacological inhibition of sphingosine kinases 1 and 2 resulted in a significant reduction in EV S1P cargo without affecting the number of EVs released. When exposed to EVs derived from cells treated with palmitate in the presence of a pharmacologic inhibitor of sphingosine kinases 1 and 2, macrophages displayed diminished chemotactic behavior. To determine receptor-ligand specificity, we tested the migration responses of macrophages genetically deleted in the $S1P_1$ receptor toward lipotoxic EVs. $S1P_1$ receptor knockout macrophages displayed a marked reduction in their chemotactic responses toward lipotoxic palmitate-induced EVs.

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Conclusions: Palmitate-induced lipotoxic EVs are enriched in S1P through sphingosine kinases 1 and 2. S1P-enriched EVs activate persistent and directional macrophage chemotaxis mediated by the $S1P_1$ receptor, a potential signaling axis for macrophage infiltration during hepatic lipotoxicity, and a potential therapeutic target for non-alcoholic steatohepatitis.

Keywords: non-alcoholic steatohepatitis, palmitic acid, sphingosine kinase, sphingolipid signaling, ceramide

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the most frequent chronic liver disease worldwide, is characterized by hepatic steatosis (1). The vast majority of NAFLD patients have hepatic steatosis alone, termed non-alcoholic fatty liver (NAFL). Up to 20% of NAFLD patients develop non-alcoholic steatohepatitis (NASH) characterized by hepatocellular injury including ballooning, inflammation and the potential to develop hepatic fibrosis. The underlying importance of inflammation in NASH progression is well-recognized. In natural history studies with paired liver biopsies over time, it was noted that inflammation was the best predictor of future fibrosis risk (2). Mechanistically, preclinical and human data suggest that hepatocyte lipotoxicity, caused by the presence of excessive saturated free fatty acids (FFAs) and other toxic lipid moieties, is involved in provoking or perpetuating inflammation in NASH (3). While pathologic hepatocyte lipoapoptosis has been shown to contribute to proinflammatory responses in the liver (4), recent studies have focused on signaling events arising from damaged or stressed cells before the onset of cell death, such as extracellular vesicles (EVs) in mediating proinflammatory responses in the injured liver (5, 6).

Two notable features of inflammation in NASH are an increase in infiltrating immune cells and an increase in proinflammatory activation of immune cells (7). Enhanced lobular inflammation due to accumulation of a mixed inflammatory infiltrate that includes proinflammatory macrophages within hepatocyte cords is a diagnostic histologic feature of NASH (8). Many patterns of macrophage accumulation are recognized in NASH including clusters termed microgranulomas, fat droplet containing lipogranulomas, and surrounding ballooned hepatocytes termed "satellitosis" (8). Other cell types including lymphocytes, eosinophils, and neutrophils have also been observed in lobular inflammation. More recent studies have implicated other cells of the innate and adaptive immune system in portal inflammation (CD68, CD3, CD8, CD4, CD20, and neutrophil elastase) (9). The links between lipotoxic hepatocytes and immune cells, specifically, hepatocyte-originating signals that direct inflammatory cell infiltration, are not well-defined. Previous studies have defined the role of soluble mediators, such as the chemokine C-C motif chemokine ligand 2/monocyte chemoattractant protein 1 (CCL2/MCP1) and the receptors C-C motif chemokine receptor (CCR) 2 and 5 in NASH (10). In experimental mouse NASH, pharmacologic inhibition of CCR2 and 5 diminishes the recruitment of proinflammatory macrophages leading to amelioration of liver injury and inflammation (11). In a phase 2b human NASH trial of a dual CCR2/5 inhibitor, improvements in liver histology and fibrosis were reported (12). However, complete resolution of macrophage accumulation and inflammation has not been reported highlighting the need to understand additional macrophage recruitment signals. EVs are important cell-derived mediators of intercellular communication (6, 13, 14). These nanometer-sized vesicles participate in short and long-range signaling under normal and stressed conditions (13, 15, 16). The ability of macrophages to migrate through tissues to sites of injury is fundamental to their function as a part of the host innate immunity and inflammation (17). Whether hepatocyte-derived lipotoxic EVs contribute to macrophage infiltration in the hepatic microenvironment and the signaling mechanisms by which macrophages are recruited under lipotoxic stress conditions, still remains incompletely understood.

Palmitic acid (PA), the most abundant saturated FFA found in humans, which is elevated further in NASH, is a well-defined lipotoxic stressor (18). Hepatocytes treated with PA release an increased number of EVs (6, 19), which is in accordance with current evidence suggesting that circulating EVs are increased in NASH and correlate with macrophage accumulation in the liver (5, 20, 21). We have previously demonstrated that PA induces the release of ceramide-enriched EVs via the *do novo* synthesis of ceramide in the endoplasmic reticulum. Further, these EVs contain elevated levels of sphingosine 1-phosphate (S1P), which others have demonstrated to be important for the formation of a subtype of EVs known as exosomes (5, 22). S1P is a potent, bioactive sphingolipid known to regulate immune cell trafficking; and implicated in liver inflammation in NASH (23–25). However, the role of EV S1P cargo in macrophage recruitment is not well-understood. Formation of S1P occurs through the phosphorylation of sphingosine, a ceramide derivative, by sphingosine kinases (SphK) 1 and 2 (24, 26, 27). Our objective was to determine whether SphK-mediated production of S1P in EVs promotes macrophage recruitment via stimulation of the $S1P_1$ receptor. We demonstrate that SphK1 and SphK2 mediate the formation of S1P in PA-induced EVs, such that resulting EVs are enriched in S1P cargo to stimulate

Abbreviations: BMDM, bone marrow-derived macrophages; EVs, extracellular vesicles; FFA, free fatty acids; HPRT, Hypoxanthine-guanine phosphoribosyl transferase; IMH, immortalized mouse hepatocytes; IRE1α, inositol-requiring enzyme 1 α; LCM, L929 cell-conditioned medium; Non-alcoholic Fatty Liver; NAFLD, Non-alcoholic Fatty Liver Disease; NAFL, NASH, Non-alcoholic Steatohepatitis; PA, Palmitic acid; SphK, sphingosine kinase; SphK1, sphingosine kinase 1; SphK2, sphingosine kinase 2; S1P, sphingosine 1-phosphate; $S1P_1$, sphingosine 1-phosphate receptor 1.

macrophage migration. Furthermore, macrophages express the $S1P_1$ receptor needed for stimulation through the S1P signaling axis. When SphK1 and 2 are inhibited, EV S1P cargo is attenuated and macrophage chemotaxis toward these EVs is reduced. Thus, S1P is an important effector of macrophage chemotaxis, and likely important in this phenomenon *in vivo*. In support of this, the hepatic expression of $S1P_1$ receptor is increased in a murine dietary model of NASH. Our observations provide a potential mechanism for the observed macrophage infiltration in NASH, and identify a potential novel therapeutic target.

MATERIALS AND METHODS

Bone Marrow-Derived Macrophages (BMDM)

This study was carried out in accordance with the recommendations of the public health policy on the humane use and care of laboratory animals. The protocol was approved by the Institutional Care and Animal Use Committee (IACUC) of the Mayo Clinic. Bone marrow-derived macrophages (BMDM) were isolated from hind legs of wild type C57BL/6 mice as previously described by us (28). Once euthanized, the mouse was sprayed with 70% ethanol and the skin was cut open using sterile scissors to expose the hind legs. Cuts were made through the hip and ankle joints to remove each leg. The legs were placed in 70% ethanol for 2–3 min, then placed in phosphate-buffered saline (PBS) on ice. In the tissue culture hood, the leg muscle and epiphyses were removed and bone marrow flushed out onto a petri dish using a syringe and 25 gauge needle containing BMDM media. The BMDM media used for bone marrow differentiation consists of Roswell Park Memorial Institute (RPMI)-1640 supplemented with 20% L929 cell-conditioned medium (LCM), 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL). The flushed media containing bone marrow was drawn through a 23 gauge needle 4–5 times to remove clumps. Bone marrow cells were plated onto 150 mm petri dishes (BD Falcon, Oxford, UK) and incubated at 37°C, 5% CO_2. BMDM media was changed every 2 days on Day 3 and Day 5, and BMDMs were dissociated with Accutase and used in experiments on Day 7.

Immunohistochemistry for $S1P_1$ Receptor

Diet-induced obesity and insulin resistance with concomitant NASH in mice fed a diet high in fat, fructose and cholesterol (FFC) has been well-characterized by us (29, 30). We utilized available archived formalin fixed paraffin embedded liver tissue samples from a previously published, IACUC approved, study where male mice had been fed the FFC diet for 20 weeks for $S1P_1$ receptor immunohistochemistry (30). Diet-induced NASH, liver injury and macrophage-mediated inflammation in this cohort have been previously published. Five μM liver sections were dewaxed and rehydrated through graded alcohols. Antigen retrieval was performed by heating in Tris-EDTA buffer (10 mM Tris 1 mM EDTA, pH 9.0) for 10 min at 95°C. After blocking endogenous peroxidase with 3% H_2O_2 in distilled water for 10 min, the sections were incubated in blocking buffer provided in the Vectastain ABC staining kit (Vector Laboratories), followed by anti-$S1P_1$ receptor antibody (1:100 dilution, Product # 55133-1-1AP, Proteintech) at 4°C in a humidified chamber, overnight. After washing in PBS, slides were incubated with biotin-conjugated secondary antibody, streptavidin conjugated to horseradish peroxidase, and lastly chromogenic substrate per the manufactures instructions (ABC, Vector Laboratories). Sections were dehydrated through graded alcohols and mounted using Permount mounting media (Sigma). Microscopy was performed using the Nikon NIS-Elements advanced research imaging software attached to a Nikon Eclipse TE300 microscope (Nikon, Japan), and the positive areas were captured at 20 × magnification with uniform settings of magnification, light, and exposure time for quantitative image analysis.

$S1P_1$ Receptor Knockout Bone Marrow-Derived Macrophages

Myeloid cell specific knockout mice (KO) mice were generated by crossing mice that express Cre recombinase under the lysozyme 2 promoter (lyzMcre) with mice having the $S1P_1$ exon 2 flanked by loxP recombination sites (The Jackson Laboratory Stock # 019141). The $S1P_1^{loxp}$ mice were generated by Richard Prolia and have been previously described (31). KOs were confirmed by genotyping and quantitative real time PCR (qPCR). BMDM were generated as described above.

Cell Culture

Immortalized mouse hepatocyte (IMH) cell-line has been previously described by us (5). Cells were cultured in Dulbecco's Modified Eagles Media (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS), glucose (4.5 g/L), penicillin (100 units/mL), and streptomycin (100 μg/mL).

Extracellular Vesicle Isolation

IMH cells were grown to 90% confluency in 150 mm tissue culture treated dishes. Cells were washed with PBS twice and treated with treatment media containing 400 μM palmitic acid (PA), 400 μM PA + 2 μM of MP-A08 inhibitor, or vehicle control for 20 h. Treatment media consisted of DMEM supplemented with 5% FBS (exosome-free), prepared by overnight ultracentrifugation at 100,000 × g for 16 h, and 1% bovine serum albumin (fatty acid free). Following the 20-h treatment, the cell culture supernatant was collected and centrifuged at 2,000 × g for 20 min to remove cells and cellular debris. Subsequently, the supernatant was extracted and ultracentrifuged at 10,000 × g for 40 min. Supernatant was again removed and further ultracentrifuged at 100,000 × g for 90 min to pellet EVs. The EV pellet was re-suspended in PBS and spun at 100,000 × g for 90 min again to wash the EVs. Pellets were re-suspended in PBS and characterized by nanoparticle tracking analysis or stored at −80°C until further use.

Caspase 3/7 Assay

IMH cells were plated at a density of 80,000/cm^2, attached for 24 h, and treated as above for 20 h. Following treatment, caspase 3/7 activity was measured using the Apo-ONE homogeneous caspase-3/7 assay (Promega) according to the manufacturer's instructions and previously described by us (32).

Morphologic Assessment of Apoptosis

IMH cells were plated in 6 well plates at a density of 80,000/cm^2, attached for 24 h and treated as above. Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) and fluorescence microscopy were used to count total and apoptotic cells; at least 200 cells were counted per condition. Nuclear changes of chromatin condensation and nuclear fragmentation were considered apoptotic (32).

Sphingosine Kinase Activity

The activity of sphingosine kinases 1 and 2 in cells treated with palmitate with and without the pharmacologic inhibitor, MP-A08, was measured by utilizing a commercially available sphingosine kinase activity assay (Echelon Biosciences). Briefly, IMH cells were plated and treated as described above. Cell lysates were prepared in the provided buffer by freeze thaw followed by sonication. The assay was set up per manufacturer instructions and luminescence normalized to protein content.

Nanoparticle Tracking Analysis

The Nanosight NTA NS300 (Malvern Instruments, UK) was used to characterize EVs through the analysis of light scatter and Brownian motion of each sample (33). EV samples were diluted with PBS accordingly to maintain a concentration of 2E+08 to 8E+08 particles/mL, and a frame rate in between 10 and 40 particles/frame. Each sample was pumped through the observation chamber by a syringe pump at a constant rate of 25 μL/min. For each sample, 3 videos of 30 s were captured and analyzed by the nanoparticle tracking (NTA) software to obtain the concentration of particles (particles/mL) and size (nanometers). The EV concentration is normalized to the number of cells.

Lipidomics

EV sphingolipids were quantified by tandem mass spectrometry at the Mayo Clinic Metabolomics Core Laboratory, as described previously (34). EVs isolated from equal number of cells treated with vehicle, PA, or PA plus MP-A08 were analyzed to quantify changes in sphingolipid content.

CRISPR/Cas9 Gene Editing

SphK2 and SphK1 knockout cell lines for IMH cells were created using the Guide-it CRISPR/Cas9 system (TaKaRa Biotechnology Inc. Japan). The online tool (http://crispr.mit.edu) was used to determine the sgRNA target sequence for mouse SphK2 (5′-TGCGTGCACGCTGCGTCGTCCGG-3′) and mouse SphK1 (5′-ATATATTGCAGTGACGCGTG-3′). Oligonucleotides were annealed and cloned into the pGuide-it Vector (Clontech, Palo. Alto, CA), plasmids isolated, then transfected onto HEK293T cells using Lenti-X Packaging Single Shots (VSV-G) following manufacturer's instructions (Clontech). Virus was harvested at 48 h and filtered through a 0.45 μM pore cellulose acetate filter (Millipore). Virus potency was verified by using Lenti-X GoStix (Clontech), then transferred onto wild type IMH cells for infection. Individual infected cells were flow-sorted into 96-well plates by green and red fluorescence for SphK2 and SphK1, respectively.

RNA Purification, Reverse Transcription-PCR, and Quantitative Real Time PCR

IMH or BMDM cells were washed with PBS, centrifuged, and pellets were suspended in Trizol. Flash-frozen cryo-preserved liver tissue from our previously published study described above was homogenized in Trizol (30). Total RNA was extracted using the Quick-RNA MiniPrep kit (Zymo Research. Irvine, CA), and the RNA yield was assessed by NanoDrop ND1000 (ThermoScientific, Waltham, MA). Reverse transcription of RNA into cDNA was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). PCR amplification was run using the EmeraldAmp MAX Master Mix (TaKaRa) on the Tetrad Thermal Cycler (MJ Research Inc. Watertown, MA). Amplified products were analyzed by QIAxcel Advanced System (Qiagen). Quantitative real time PCR was carried out using the LightCycler 480 SYBR Green I Master Mix (Roche), and run on LightCycler 480 (Roche). Primers used for mouse SphK2 include: forward 5′-TTTACGAGGTGCTGAATGGG-3′ and reverse 5′-ACCGACAACCTGCTCAAAC-3′. Primers for mouse SphK1 include: forward 5′-TGAATGGGCTAATGG AACGG-3′ and reverse 5′-GTCTTCATTAGTCACCTGCT CG-3′. HPRT was used as the house keeping gene for qPCR using primers: forward 5′- TCAGTCAACGGGGGACATAAA-3′ and reverse 5′- GGGGCTGTACTGCTTAACCAG-3′. For $S1P_1$ receptor 5′-ATGGTGTCCACTAGCATCCC-3′ and 5′-CGATGTTCAACTTGCCTGTGTAG-3′ primers were utilized for both qPCR and standard reverse transcription PCR. For standard reverse transcription PCR primers for mouse $S1P_2$: forward 5′-ATGGGCGGCTTATACTCAGAG-3′ and reverse 5′-GCGCAGCACAAGATGATGAT-3′; $S1P_3$: forward 5′-ACTCTCCGGGAACATTACGAT-3′ and reverse 5′-CAAGACGATGAAGCTACAGGTG-3′; $S1P_4$: forward 5′-GTCAGGGACTCGTACCTTCCA-3′ and reverse 5′-GATGCAGCCATACACACGG-3′; $S1P_5$: forward 5′-GCTTTGGTTTGCGCGTGAG-3′ and reverse 5′-GGCGTCCTAAGCAGTTCCAG-3′; and 18S: forward 5′-CGCTTCCTTACCTGGTTGAT-3′ and reverse 5′-GAGCGACCAAAGGAACCATA-3′. PCR products were resolved by capillary electrophoresis using the QIAxcel Advanced System (Qiagen).

Microfluidic 2D Cell Migration Chamber

The microfluidic gradient generator is a 2D cell migration chamber that allows for a passive generation of a stable gradient in space and time. The device itself consists of a piece of PDMS bonded to a glass coverslip, and a network of micro-channels embedded in between (**Supplementary Figure 1**). The microfluidic devices were fabricated using standard soft lithography approaches. The design of the gradient generator was adapted from previous reports (35). The two circular wells on top are inlets for the chemoattractant (RPMI-1640 + EVs) and chemoattractant-free media (RPMI-1640). Immediately after the inlets is a sequence of balance and equilibrium channels, then a network of microchannels that combines and mixes the chemoattractant as they flow through. Each resulting channel

at the end of the network contains a different proportion of the chemoattractant such that the gradient will be established in the cell chamber perpendicular to the direction of flow (34). BMDMs were dissociated with Accutase on Day 7 and reconstituted in complete BMDM media to give a concentration of 1E6 cells/mL. The cells were then seeded into the cell chamber of the gradient generator by backflow through the bottom port. Once the cells were spread throughout the chamber, the device was incubated at 37°C, 5% CO_2 for 1 h to allow the cells to adhere. Subsequently, 200 μL of serum free RPMI-1640 or serum free RPMI-1640+EVs was added to the left and right inlets at the top, respectively. The device was allowed to sit for 10 min for the media to flow through the micro-channels before image capture was begun.

Time-Lapse Microscopy

Following cell seeding and generation of the EV gradient, the microfluidic gradient generator was placed in the stage incubator. Images of macrophage migration in the cell chamber were captured every 15 s for 4 h using the 5x objective, Definite Focus (Zeiss) and the ZEN 2.3 lite software (Zeiss).

Cell Tracking and Analysis

All time-lapse images acquired through the Zen Pro imaging software (Zeiss) were exported as time-lapse videos (100 frames/second). The resulting video file was imported into ImageJ (National Institutes of Health, Bethesda, USA), and cells were tracked using the Manual Tracking plugin (Fabrice Cordelières, Institut Curie, Orsay, France). The resulting cell tracks were analyzed using the Chemotaxis and Migration Tool 2.0 (Ibidi GmbH, Munich, Germany). In each experiment, random migrating cells within frame were manually tracked (10–50 cells). The software was also used to plot cell tracks and calculate measured values, including the migratory persistence (forward migration index), angle of migration relative to the gradient axis, velocity, Euclidean and accumulated distances.

Fluorescence-Activated Cell Sorting (FACS)

Mouse liver was dissociated with the liver dissociation kit, mouse (Miltenyi Biotec, Germany), using the gentleMACS Dissociator (Miltenyi Biotec) following manufacturer's protocols. The liver digest was passed through a 40 μm filter (Falcon 352340; BD Biosciences) to remove large debris and cell clusters. The filtered dissociate was centrifuged at 300 × g for 5 min. The cells were re-suspended in 7 ml 25% Percoll in a 15 ml falcon tube, and 2 ml of 50 % Percoll was slowly laid on the bottom of the same tube with a glass Pasteur pipet. The tube was spun at 2,000 rpm, brakes removed, for 20 min at 4°C. After the tube was carefully removed from the centrifuge, 2–3 ml of debris was removed from the top of the gradient. Using a glass Pasteur pipet, cells from the band in between the middle colored interface and transparent interface below were removed without disturbing the RBC pellet at the bottom. To dilute out the Percoll, cells were transferred to a 50 ml falcon tube, the volume was brought up to 40 ml with SF-RPMI, and spun at 400 × g for 10 min. The supernatant was removed and cells re-suspended in PEB buffer, containing PBS, 0.5% bovine serum albumin (BSA) and 2 mM EDTA. Viable cells were counted on a hemocytometer using Trypan Blue (Gibco). Cells were then incubated with FcR Blocking Reagent (Miltenyi Biotec) for 5 min at 4°C, followed by CD45 conjugated to VioBlue (Miltenyi Biotec), F4/80 conjugated to APC-Cy7 (Biolegend), and mouse $S1P_1$-phycoerythrin (PE) conjugated antibody (RandD Systems) for 10 min at room temperature in the dark. Cells were centrifuged at 400 × g for 6 min at room temperature. Cells were washed once with PEB buffer, and centrifuged for 5 min at 400 × g. The supernatant was removed and cells were re-suspended in 1% paraformaldehyde. Flow cytometry was performed with the MACSQuant Analyzer 10 (Miltenyi Biotec). The stained population was acquired by gating side scattered light and forward scattered light to exclude the dead cell population, gated on CD45 positive and fluorescence minus one controls were used to identify and gate the cell population expressing both F4/80 and $S1P_1$ receptor. F4/80 and $S1P_1$ receptor expressing population is expressed as a percentage of CD45 high intrahepatic leukocytes. Graphs were made in Flowlogic software (Miltenyi Biotec).

Statistical Analysis

Statistical analysis and graphs were made in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA). Mean ± S.E.M. are presented in each graph. The two-tailed student's *t*-test was used to compare groups, and the results were statistically significant when the *p*-value was <0.05. The Rayleigh Test of Uniformity was used to test a circular distribution of cell end points, and was performed using the Chemotaxis and Migration Tool 2.0. Rejection ($p < 0.05$) of the null hypothesis, which states that the distribution of cell end points is uniform, showed that there is sample mean direction of migration.

RESULTS

Macrophages Migrate Directionally and Persistently Toward PA-Induced EVs

Hepatic lipotoxicity, caused by the presence of excessive saturated free fatty acids (FFAs), and its related macrophage recruitment is implicated in NASH pathogenesis (19, 36). Previously, we found that PA promotes stress-induced release of EVs through the ER stress sensor IRE1α (5). To understand recipient cell responses, we asked whether PA-induced lipotoxic EVs could be a mediator of macrophage recruitment into the liver. Proinflammatory intrahepatic macrophages in NASH are derived from bone marrow-derived monocytes; therefore, we used BMDMs in these experiments as a surrogate for monocyte-derived macrophages. BMDMs were exposed to a Veh-EV or PA-EV gradient (EVs isolated from the same number of hepatocytes treated with vehicle or PA) through the microfluidic gradient generator for 4 h. BMDMs migrated directionally (Rayleigh Test $P < 0.05$) when exposed to PA-EVs but not when exposed to Veh-EVs (**Figure 1A**). BMDMs migrated at a higher velocity when exposed to PA-EVs throughout the duration of exposure, and at more acute angles toward PA-EVs (**Figures 1B,C**). A positive

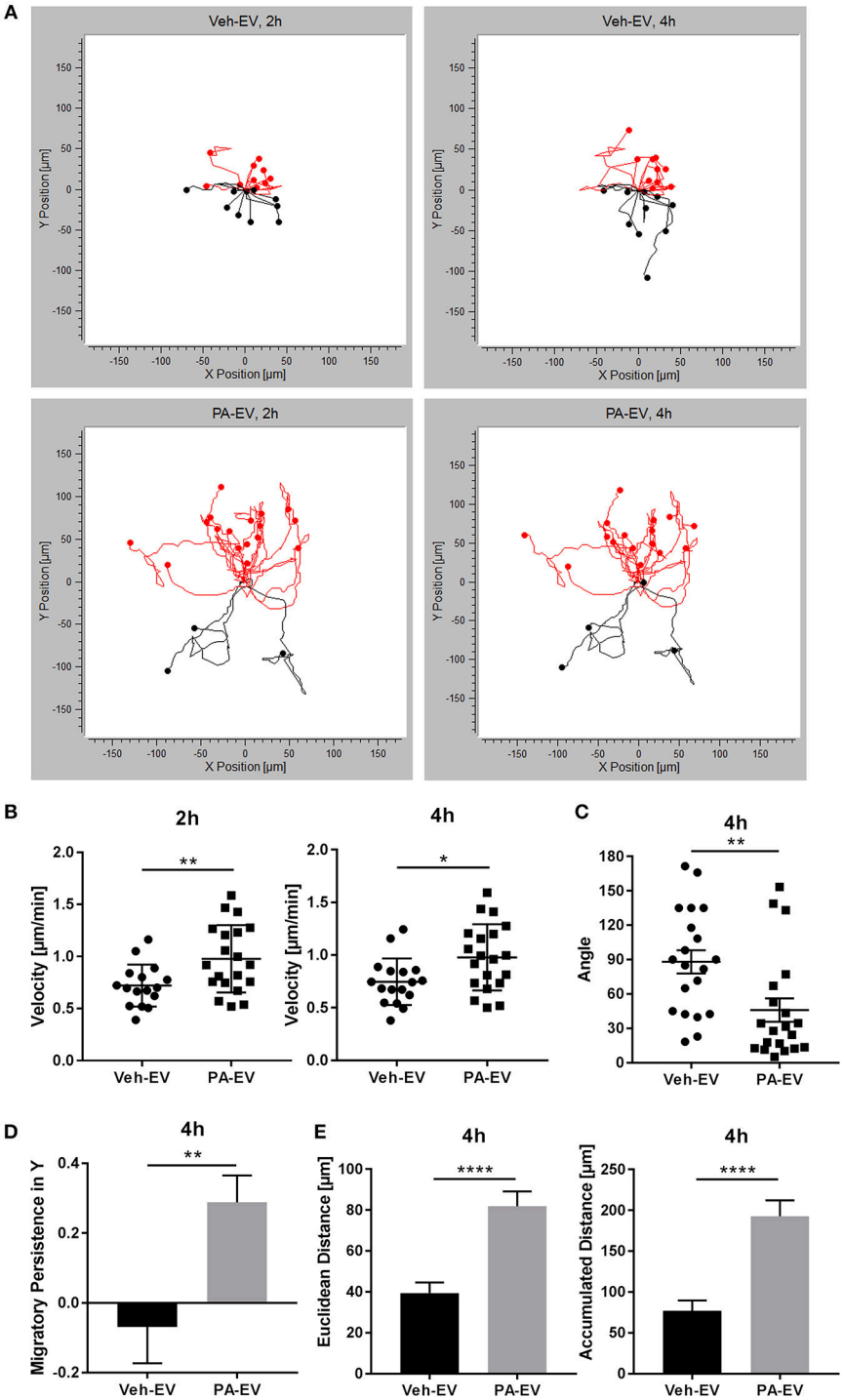

FIGURE 1 | PA-EVs are chemoattractive to macrophages. **(A)** Representative migration plots for 2D chemotaxis assay, showing BMDM migration trajectories of 20 randomly selected cells at 2 and 4 h ($n = 3$). BMDMs were seeded in the microfluidic gradient generator for 1 h, exposed to a Veh-EV or PA-EV gradient for 4 h, and captured by time-lapse microscopy. EVs were isolated from the same number of IMH cells treated with 400 μM PA or vehicle for 20 h. Cells highlighted in red are migrating up toward the positive end of the gradient. The Rayleigh test for uniformity was used to test whether the cell distribution was uniform. **(B)** Migration velocity when BMDMs were exposed to a Veh-EV vs. PA-EV gradient at 2 and 4 h ($n = 3$). **(C)** Angle between the cell's leading edge and gradient axis at 4 h in a Veh-EV vs. PA-EV gradient ($n = 3$). A more acute angle indicates that migration was more consistent with the gradient axis. **(D)** Migratory persistence in Y of BMDMs treated with Veh-EV vs. PA-EV gradient at 4 h ($n = 3$). Strong chemotaxis effects are characterized by higher migratory persistence in Y. **(E)** Euclidean distance (shortest distance between the start and end points) and accumulated distance (total path length) between BMDMs migrating in a Veh-EV or PA-EV gradient for 4 h ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. All error bars are SEM.

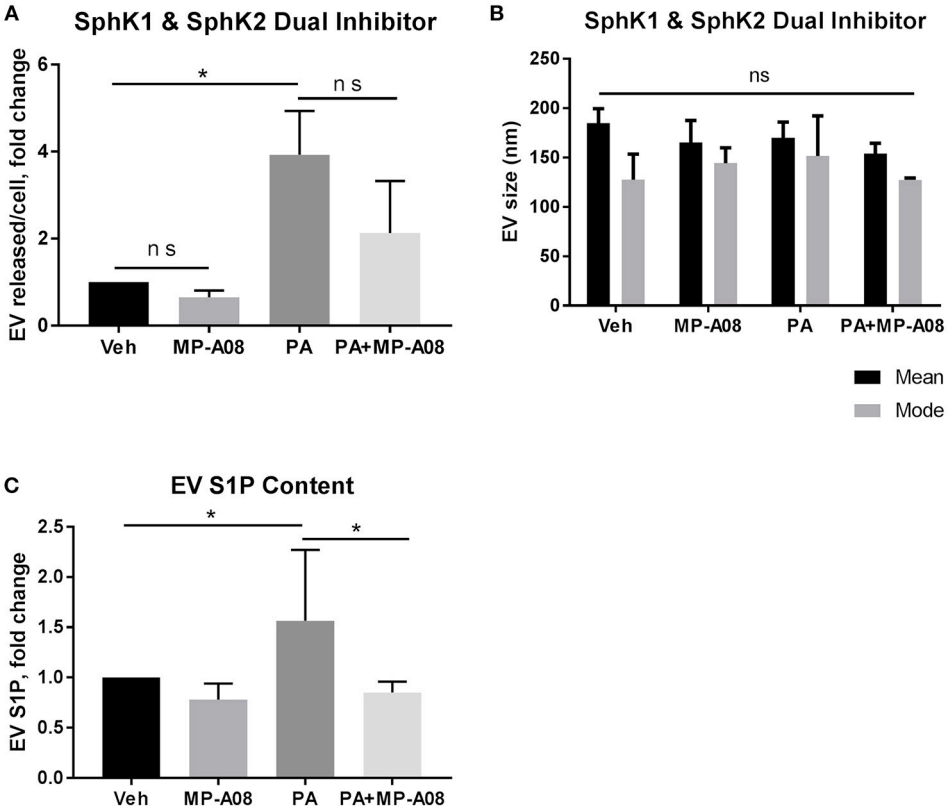

FIGURE 2 | EV S1P is not elevated under SphK1 and SphK2 inhibition. **(A)** IMH cells were treated with 400 μM PA, 400 μM PA + 2 μM MP-A08, vehicle, or vehicle + 2 μM MP-A08 for 20 h. EVs released were isolated by differential ultracentrifugation and characterized by NTA and normalized to cell numbers. Data are pooled from 4 experiments. **(B)** Size (nm, nanometers) of EVs released by IMH cells treated with vehicle, vehicle + 2 μM MP-A08, 400 μM PA, or 400 μM PA + 2 μM MP-A08 for 20 h. Data are pooled from 4 experiments. **(C)** EV S1P content was quantified by tandem mass spectrometry ($n = 2$). *$p < 0.05$, ns, not significant. All error bars are SEM.

migratory persistence in Y [sum of the ratio of displacement along the gradient axis (Y axis) to total accumulated path length of all cell tracks, averaged] indicated that the direction of macrophage migration was toward the positive end of the PA-EV gradient (**Figure 1D**), and they did so over greater distances (**Figure 1E**). Together, these measured values show that macrophages migrate faster, longer, and in a more directionally persistent manner toward PA-EVs.

Inhibition of SphK1 and SphK2 Reduces S1P Levels in PA-Induced EVs

We next examined the EV S1P content and the significance of its formation in the release of PA-induced EVs. S1P is formed through the phosphorylation of sphingosine by SphK1 and SphK2 (24). We attempted to establish a double SphK1 and SphK2 knockout cell line using CRISPR/Cas9 gene editing technology. However, this was not successfully achieved likely due to lethality in the absence of both SphK isoenzymes, similar to observations in mice (37). It is also known that the downregulation of one SphK results in the compensatory upregulation of the other (38). Henceforth, we used a pharmacologic approach in the subsequent experiments. We asked whether SphK1 and SphK2 mediate the formation of S1P in PA-induced EVs by using the dual SphK pharmacologic inhibitor, MP-A08. We treated hepatocytes with vehicle, vehicle + MP-A08, PA, and PA + MP-A08 for 20 h, isolated the EVs released and normalized to cell number. We confirmed the absence of MP-A08-induced apoptosis by the biochemical and morphologic assessment of apoptosis (**Supplementary Figures 2A,B**). In hepatocytes treated with MP-A08, PA, or PA + MP-A08, both caspase 3/7 activity and percent apoptotic nuclei were comparable to vehicle treated cells. The inhibitory effect of MP-A08 was confirmed by measuring sphingosine kinase activity (**Supplementary Figure 2C**). We found almost a 3-fold increase in EV release in PA-treated cells compared to vehicle-treated cells, and no significant difference in EV release when SphK1 and SphK2 were inhibited by MP-A08 (**Figure 2A**). We did not find any difference in vesicle size (nm) between the groups (**Figure 2B**). We next quantified and analyzed the EV S1P content by tandem mass spectrometry. We found that PA-EVs were enriched in S1P cargo compared to those released by vehicle-treated, vehicle + MP-A08-treated and PA + MP-A08-treated cells (**Figure 2C**). Altogether, these data suggest that SphK1 and Sphk2 play a crucial role in the formation of S1P in PA-induced EVs, though the number of EV released is unaffected.

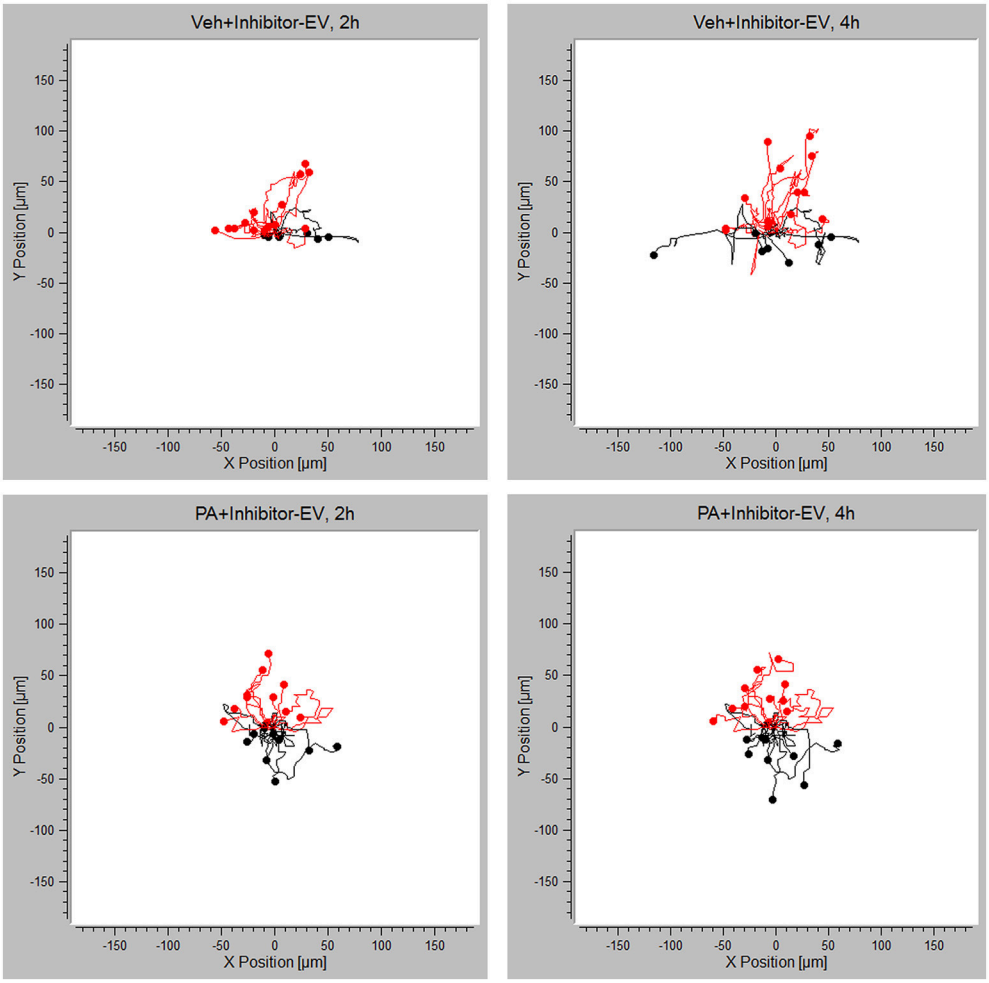

FIGURE 3 | PA-EV chemotactic response is attenuated through inhibition of SphK1 and SphK2. Migration plots of BMDMs migrating in a Veh + MP-A08-EV or PA + MP-A08-EV gradient for 4 h. Plots show migration trajectories of 20 randomly selected cells from each experiment ($n = 3$). The Rayleigh test for uniformity was used to test whether cell distribution was uniform. Cells highlighted in red are migrating up.

Macrophages Are Chemotactic Toward PA-Induced EVs Through the S1P Signaling Axis

Having demonstrated that PA-EVs attract macrophages, and that PA-EVs contain elevated levels of S1P through functional SphK1 and SphK2, we next asked whether SphK mediated formation of S1P plays a role in the chemoattractive effects of PA-EVs. If macrophages are stimulated by the SphK mediated formation of S1P cargo in PA-induced EVs, then the inhibition of both SphK1 & SphK2 should inhibit macrophage migration through impairment of the S1P signaling axis. BMDMs were exposed to 4 different gradients of EVs isolated from Veh-treated, Veh + MP-A08-treated, PA-treated, or PA + MP-A08-treated cells for 4 h. BMDMs did not migrate directionally (Rayleigh Test $P > 0.05$) in either the Veh + MP-A08-EV or PA + MP-A08-EV gradient (**Figure 3**). Migration velocity was significantly reduced toward PA + MP-A08-EVs compared to PA-EVs, similar to the response toward vehicle controls at both 2 and 4 h (**Figure 4A**). We speculate that this is due to the reduction in S1P content by the SphK inhibitor MP-A08. BMDMs in the PA + MP-A08-EV gradient migrated at less acute angles, or less in alignment with the gradient axis compared to those migrating toward PA-EVs (**Figure 4B**). Furthermore, the macrophages did not migrate persistently toward the positive end of the PA + MP-A08-EV gradient (**Figure 4C**), and migration distance was significantly reduced compared to that when exposed to PA-EVs (**Figure 4D**). Taken together, these data suggest that pharmacologic inhibition of SphK1 and SphK2 and the subsequent reduced levels of S1P in PA-EVs diminish their chemoattractive effects toward macrophages. We speculate that S1P mediated recruitment of macrophages is achieved through stimulating the $S1P_1$ receptor on macrophages.

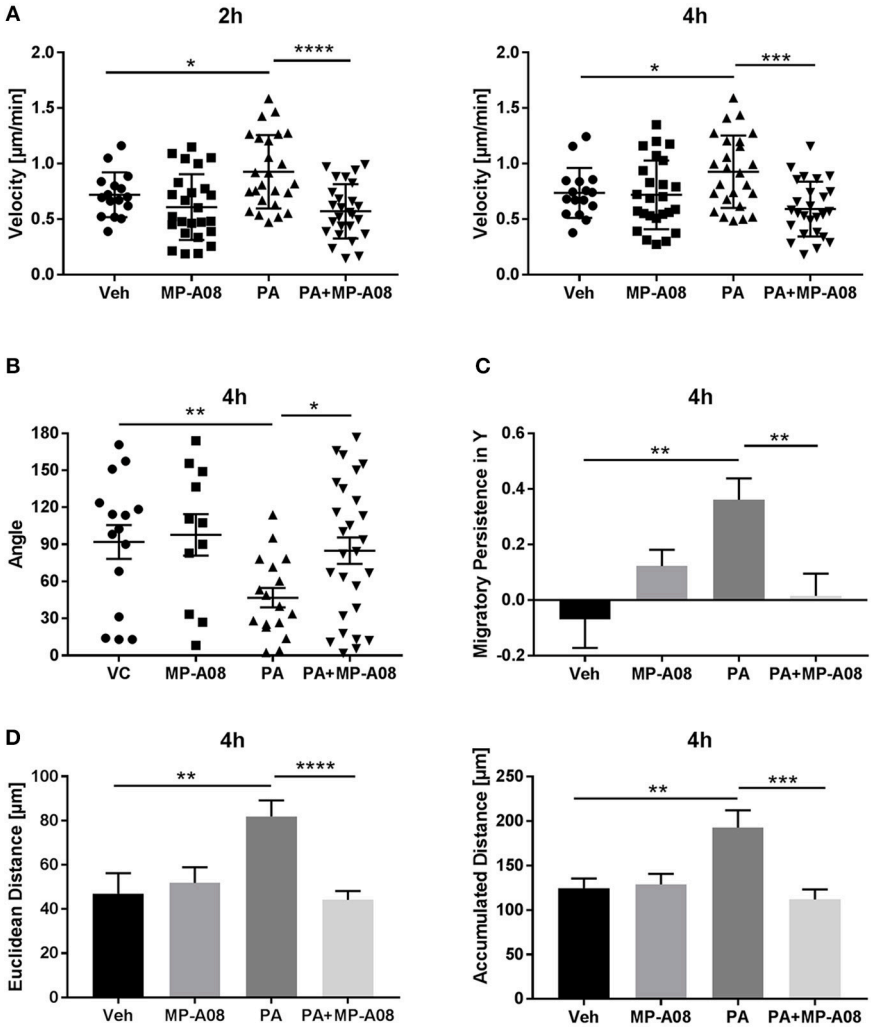

FIGURE 4 | Macrophages migrate persistently and directionally toward S1P-enriched EVs. **(A)** BMDM migration velocity when exposed to Veh-EV, Veh + MP-A08-EV, PA-EV, or PA + MP-A08-EV gradients at 2 and 4 h ($n = 3$). **(B)** The angle between the cell's leading edge and the gradient axis for BMDMs migrating in 4 types of EV gradients for 4 h ($n = 3$). **(C)** Migratory persistence in Y, comparing chemotaxis efficiency of BMDMs migrating in 4 types of EV gradients for 4 h ($n = 3$). **(D)** Comparison of the Euclidean distance and accumulated distance between BMDMs migrating in 4 types of EV gradients for 4 h ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. All error bars are SEM.

Intrahepatic Macrophages Express $S1P_1$ Receptor

As $S1P_1$ receptor is known to be expressed by macrophages (39), we characterized its expression on intrahepatic macrophages in normal mouse livers through FACS analysis. We defined intrahepatic leukocytes as the $CD45^{hi}$ population and further defined the macrophage population as $F4/80^{hi}$. We found that a significant percentage of the $CD45^{hi}$ population expressed both F4/80 and $S1P_1$ receptor (22.5 ± 2% of the $CD45^{hi}$ population, $n = 4$) (**Figures 5A,B**). Having demonstrated that this receptor is expressed by intrahepatic macrophages, we next asked whether it is upregulated in NASH, which would be in keeping with our proposed model. For this we utilized available archived mouse liver RNA and tissue samples from a previously published study (30). In this study mice were fed a diet known to induce obesity, insulin resistance and NASH, thus recapitulate cardinal features of human NASH, as previously reported by us and others (30, 40). Liver injury and macrophage accumulation in these mouse livers have been previously reported (30). In this dietary NASH model we found a significant increase in the hepatic expression of $S1P_1$ receptor by immunohistochemistry (**Figures 5C,D**), in regions of inflammatory cell infiltration. The mRNA abundance in this dietary NASH model was similar to the protein expression observed by immunohistochemistry (**Figure 5E**). Altogether, these data support an increase in $S1P_1$ receptor expression in the liver in NASH, and are consistent with our model that $S1P_1$ receptor expressing macrophages are recruited into the liver in NASH.

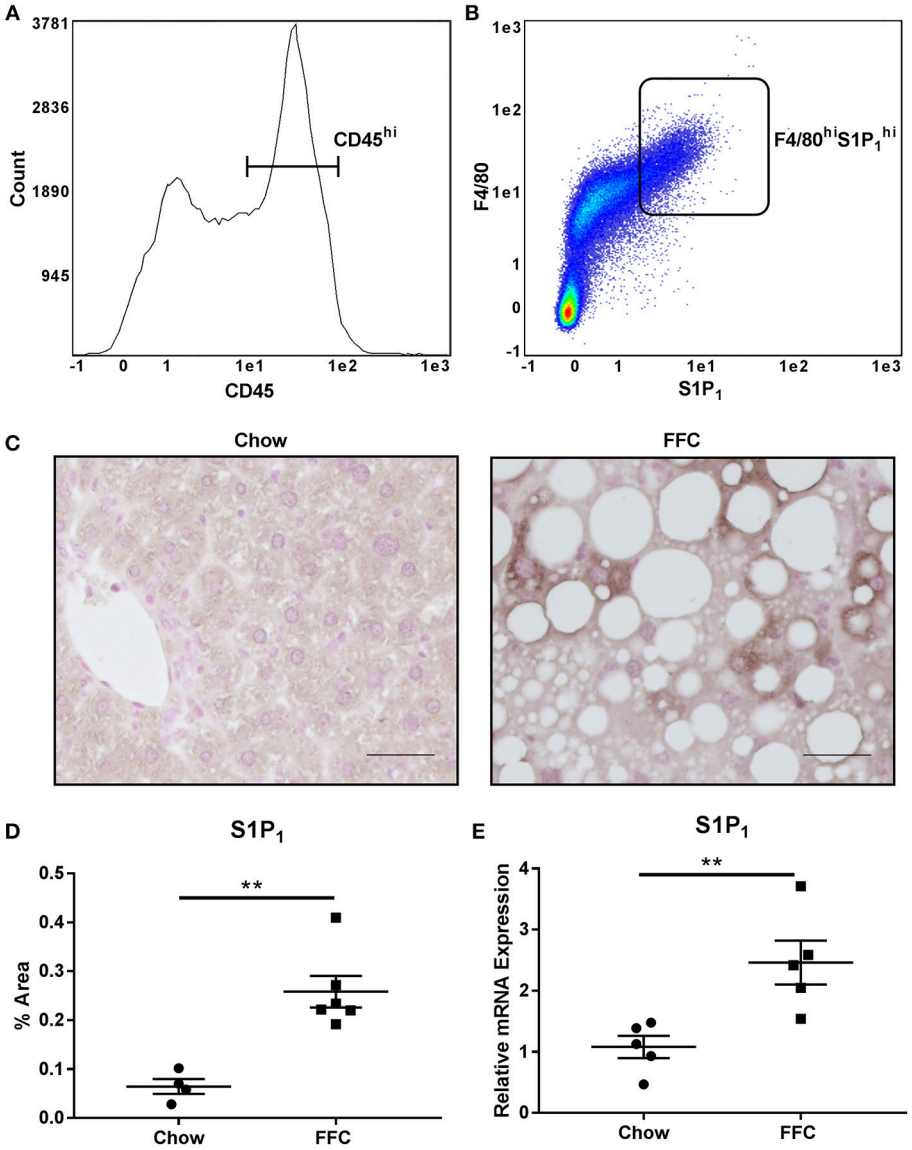

FIGURE 5 | Intrahepatic macrophages express the $S1P_1$ receptor. **(A)** CD45 expression and **(B)** F4/80 and $S1P_1$ receptor expression of $CD45^{hi}$ intrahepatic macrophages was determined in normal mouse livers by FACS analysis. **(C)** $S1P_1$ receptor immunohistochemistry was performed in livers from mice fed a diet high in fat, fructose and cholesterol (FFC) or chow for 20 weeks. Scale bar = 50 μM, $n = 4$ for chow and $n = 6$ for FFC. **(D)** Quantification of immunohistochemistry for $S1P_1$ receptor in chow fed ($n = 4$) and FFC fed ($n = 6$) mice. **(E)** mRNA expression for $S1P_1$ receptor in chow fed ($n = 5$) and FFC fed ($n = 5$) mice. $^{**}p < 0.01$.

Chemotactic Effect Toward PA-EVs Is Attenuated in the Absence of $S1P_1$ Receptor

Having demonstrated the presence of $S1P_1$ receptor on intrahepatic macrophages, we next wanted to confirm whether $S1P_1$ receptor plays a role in macrophage chemotaxis toward S1P in PA-EVs. BMDM $S1P_1$ receptor knockout was confirmed by RT-PCR (**Supplementary Figure 3**); the mRNA expression of S1P receptors 2–5 was unaffected by knockout of $S1P_1$ receptor. $S1P_1$ receptor KO BMDMs were exposed to Veh-EV or PA-EV gradients in the microfluidic gradient generator for 4 h. $S1P_1$ receptor KO BMDMs did not migrate directionally (Rayleigh Test $P > 0.05$) toward PA-EVs compared to WT BMDMs (Rayleigh Test $P<0.05$) when exposed to a PA-EV gradient for 4 h (**Figures 6A,B**). The migration of $S1P_1$ receptor KO BMDMs was similar toward Veh-EVs and PA-EVs and comparable to WT BMDMs migration toward Veh-EVs. This suggests that the $S1P_1$ receptor is crucial in mediating chemotactic effects in macrophages during S1P signaling. This was further confirmed by measuring migration velocity at 2 and 4 h, showing that $S1P_1$

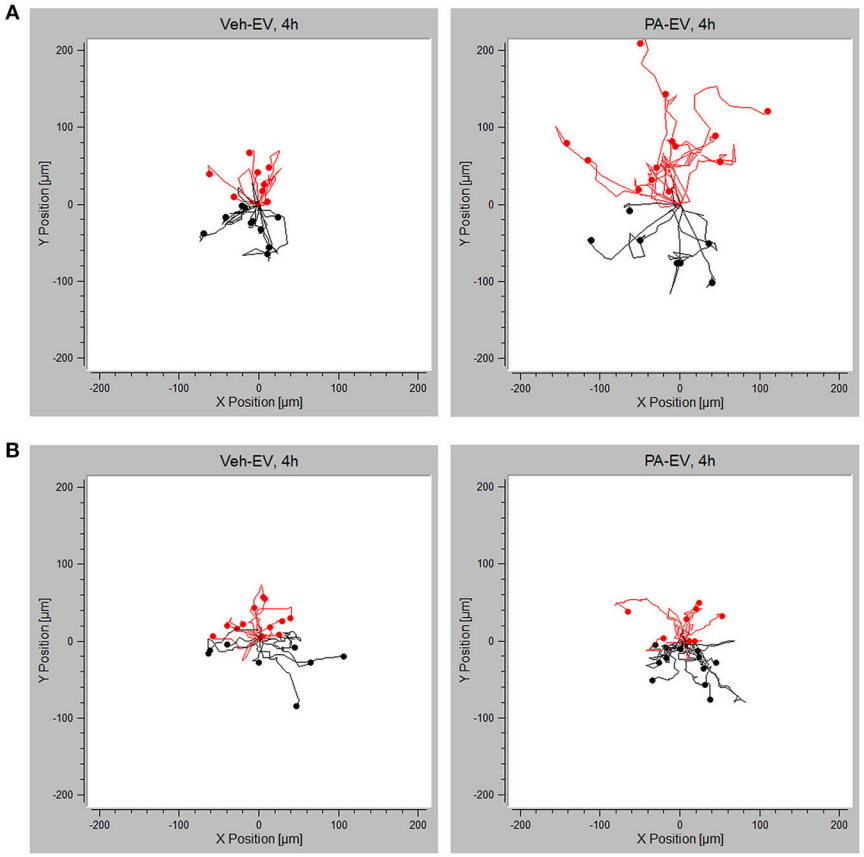

FIGURE 6 | Migration toward PA-EVs is reduced in the absence of the $S1P_1$ receptor. **(A)** Migration plots of WT BMDMs migrating in a Veh-EV and PA-EV gradient for 4 h ($n = 3$). **(B)** Migration plots of $S1P_1$ receptor KO BMDMs migrating in a Veh-EV and PA-EV gradient for 4 h ($n = 2$). Twenty randomly selected cells from each experiment are shown in each plot. The Rayleigh test of uniformity was used to test whether the cell distribution was uniform. Cells highlighted in red are migrating up toward the positive end of the gradient.

receptor KO BMDMs migrate significantly slower toward PA-EVs compared to WT BMDMs (**Figure 7A**). KO BMDMs also migrated at less acute angles, with significantly less persistence, and decreased Euclidean and accumulated distances toward PA-EVs (**Figures 7B–D**). Thus, in the absence of $S1P_1$ receptor, all measured parameters show attenuated migration toward PA-EVs, similar to the migration patterns toward MP-A08 treated EVs. This evidence further confirms the involvement of the $S1P_1$ receptor in S1P signaling in macrophage chemotaxis.

DISCUSSION

Macrophage-mediated sterile inflammation is a salient feature of NASH. The signals that lead to the recruitment of proinflammatory monocyte-derived macrophages such that they infiltrate the hepatic parenchyma are not well-understood. In this paper we report that: (i) lipotoxic hepatocyte-derived extracellular vesicles attract macrophages by chemotaxis; (ii) intrahepatic leukocytes express the sphingosine 1-phosphage 1 ($S1P_1$) receptor; and (iii) macrophage chemotaxis is mediated by the sphingosine 1-phosphate signaling axis. These *in vitro* findings advance our understanding of how lipotoxic hepatocyte-derived extracellular vesicles are proinflammatory and provide an explanation for the infiltration of macrophages into the hepatic cords by responding to EV gradients in the tissue microenvironment.

Lipotoxicity, summarized as accumulation of toxic lipid species with concomitant tissue or cellular injury, is a fundamental observation in obesity-associated disorders such as NASH (3). Several lipid classes have been implicated in lipotoxicity, including saturated free fatty acids, ceramides, lysophosphatidyl choline and free cholesterol (3, 41, 42). Among these lipids, the saturated free fatty acid palmitate, which is most abundant physiologically, and accumulates further in lipotoxic states is best characterized as being lipotoxic (18). Additionally, lipids, that can be synthesized directly from palmitate, such as ceramide via the *de novo* biosynthesis pathway (43), and indirectly such as lysophosphatidyl choline via phospholipase A2 (44), can induce hepatocyte cell death or stress responses. Though rarely observed in standard H&E stained liver biopsies, lipotoxic hepatocyte cell death correlates with features of progressive

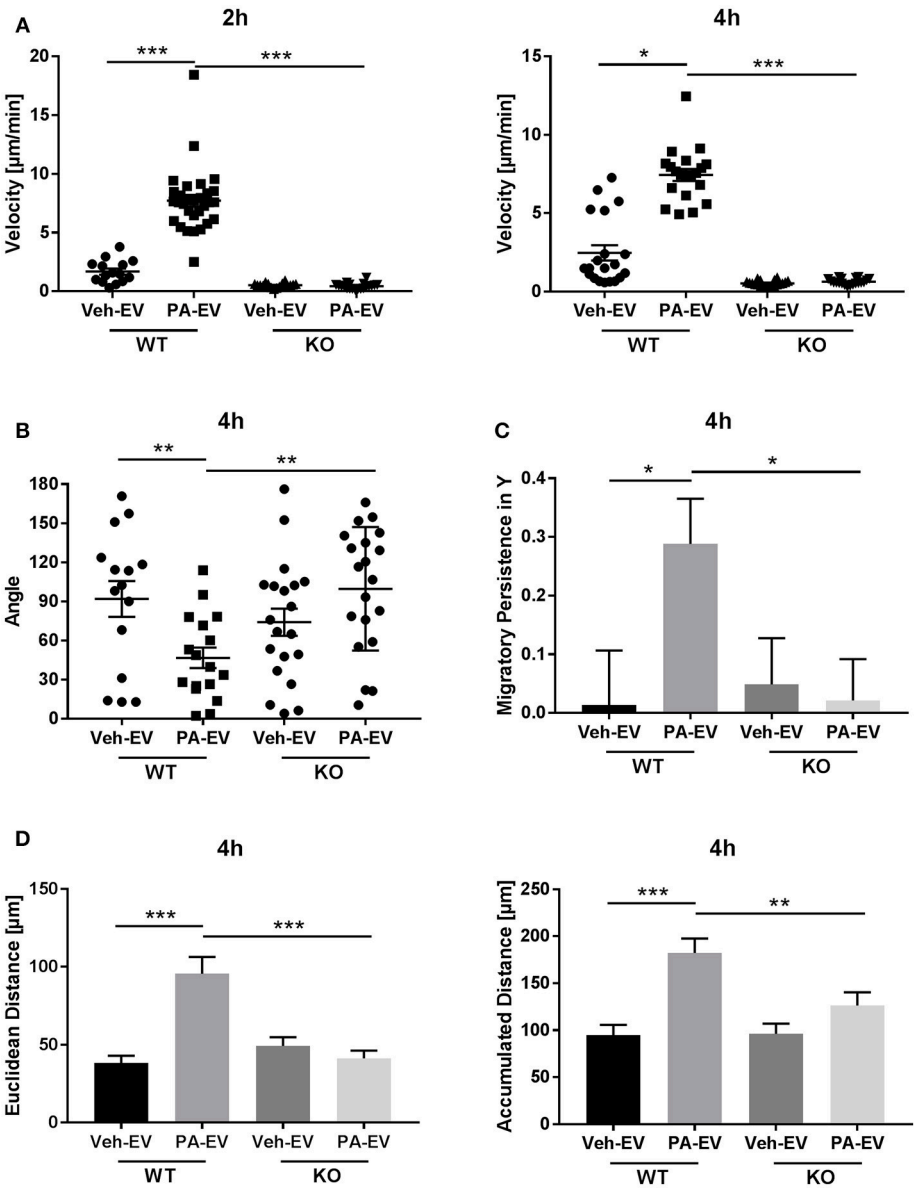

FIGURE 7 | $S1P_1$ receptor KO BMDMs migrate at reduced speeds, distances and persistence toward PA-EVs. **(A)** Migration velocity of WT vs. $S1P_1$ receptor KO BMDMs in Veh-EV and PA-EV gradients at 2 and 4 h ($n = 2$). **(B)** Angle between the cell's leading edge and gradient axis for WT and $S1P_1$ receptor KO BMDMs in Veh-EV and PA-EV gradients for 4 h ($n = 2$). **(C)** Migratory persistence in Y of WT and $S1P_1$ receptor KO BMDMs migrating in Veh-EV and PA-EV gradients for 4 h ($n = 2$). **(D)** Euclidean distance and accumulated distance for WT and $S1P_1$ receptor KO BMDMs migrating in Veh-EV and PA-EV gradients for 4 h ($n = 2$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All error bars are SEM.

NASH (45, 46). However, death is a terminal outcome and there is emerging interest in events that occur in "at-risk" cells prior to the terminal outcome of cell death, which we and others have termed sublethal cellular stress (21). The "at-risk" cells may be defined in many ways including, but not limited to, cells that demonstrate activation of cell stress pathways such as endoplasmic reticulum stress (47), activation of death receptor signaling without cell death (48), secretion of soluble factors, and increasingly, as we demonstrate here, the release of extracellular vesicles (49). EVs are heterogeneous, membrane-enclosed, nanoparticles that most cells secrete into the extracellular space and into circulation (6). EV number and cargo, including lipids, proteins and nucleic acids, changes with disease states and there is immense interest in identifying disease-specific EV signatures, both for their utility as liquid biopsy and as a potentially targetable pathophysiologic mechanism (6, 50, 51). Here we advance the understanding of EV-mediated communication between donor cell, i.e., lipotoxic hepatocytes, and recipient cell, i.e., macrophages.

Proinflammatory activation of the innate immune system, manifest as macrophage infiltration of the hepatic parenchyma,

is linked to key features of NASH including hepatocyte injury, hepatocyte cell death and fibrosis (45, 46). While macrophage activation by itself can lead to secretion of proinflammatory cytokines, death ligands and promote fibrosis, in lipotoxic disorders, where hepatocytes are a key cell that accumulates toxic lipids and lipid accumulation precedes the development of overt inflammation, hepatocyte-to-macrophage signals are important in understanding this key signaling axis. Our laboratory has focused on lipotoxic hepatocyte derived EVs as mediators of this cellular cross talk, and in particular signaling lipid mediators on EVs. We have previously demonstrated that palmitate-treated hepatocytes release ceramide-enriched extracellular vesicles, that these vesicles are also enriched in the ceramide-derived signaling sphingolipid S1P, and that their release is dependent on the endoplasmic reticulum stress sensor, inositol requiring protein 1 alpha (IRE1α) (5). Furthermore, we have recently demonstrated that the release of extracellular vesicles in PA-treated hepatocytes is preserved in the presence of caspase inhibitors, highlighting the potential importance of lipotoxic stress-induced EVs in cellular cross talk with the innate immune system (49). Here we extend our earlier observations by demonstrating persistent and directional migration of macrophages toward an EV S1P gradient. Not only are PA-induced EVs enriched in S1P, PA-stimulated cells release more EVs than vehicle-treated cells raising the possibility that macrophage chemotaxis toward S1P may be occurring toward a higher number of EVs. While this is possible, we show a lack of EV-S1P enrichment in cells treated with sphingosine kinase inhibitor MP-A08 and PA without a significant reduction in EV numbers, suggesting that EV cargo, S1P in this case, is mediating the observed macrophage chemotaxis. Others have reported proinflammatory, proangiogenic and profibrotic EVs in NASH models (52–54). Here, we focused on macrophage chemotaxis toward lipotoxic EVs in order to provide an explanation for macrophage infiltration, as chemotaxis to a site of injury and activation are both important components of macrophage effector responses. Though transwell macrophage migration has been reported by us and others in response to lipotoxic EVs, it does not differentiate between chemokinesis and chemotaxis. This is the first report of true directional chemotaxis of macrophages toward a lipotoxic EV gradient in response to EV S1P. We have previously demonstrated that EV S1P is increased in human and murine NASH (5). Thus, lipotoxic PA-EVs may play a role in macrophage recruitment *in vivo* in addition to the previously defined role of chemokines such as CCL2 and chemokine receptors CCR2 and CCR5 (10).

S1P is a potent bioactive sphingolipid, formed by phosphorylation of sphingosine by two isoenzymes of sphingosine kinase, SphK1 and SphK2. The cellular and subcellular distribution of SphK1 and 2 vary, accounting for distinct roles in the compartmental formation of S1P. Sphingosine, in turn, is formed by the deacylation of ceramide. Studies have shown that S1P is involved in the pathogenesis and progression of NASH, with concurrent upregulation of SphK1 and occasionally SphK2 (24). Both ceramide and S1P are implicated in the formation of intraluminal vesicles (ILVs) in multivesicular bodies and in cargo sorting into intraluminal vesicles (5, 22); however, when we inhibited SphK1 and 2 we did not observe a significant reduction in the number of EVs released by hepatocytes; predictably, EV S1P was significantly reduced to levels comparable to basal levels. S1P is the ligand of a family of 5 G-protein couple receptors, $S1P_1$-$S1P_5$, that regulate downstream signaling to mediate a variety of cellular responses including: immunity, cellular migration, angiogenesis, vascular and cardiac development (55, 56). Of these, $S1P_1$ receptor expression and S1P gradients in the microenvironment are known to influence T lymphocyte trafficking (57), NK cell trafficking (25), dendritic cell trafficking (58). Here, we confirmed the cell surface expression of $S1P_1$ on intrahepatic macrophages (59) and demonstrate that macrophage chemotaxis is attenuated in response to S1P deficient EVs. Our observations of macrophage chemotaxis toward EV-S1P are consistent with earlier reports of macrophage chemotaxis induced by S1P (60). Further, we have demonstrated that the chemotaxis responses of macrophages genetically deficient in $S1P_1$ receptor toward PA-EVs are attenuated, confirming that macrophage chemotaxis toward lipotoxic EVs is mediated by an intact S1P-$S1P_1$ signaling axis. Of the 5 known S1P receptors, $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ are expressed by human macrophages and $S1P_1$, $S1P_2$, and $S1P_4$ by monocytes (39). Our data suggest that the role of $S1P_1$ warrants investigation in human NASH.

Taken together these data provide an *in vitro* mechanism for lipotoxic hepatocyte-derived S1P-enriched EVs in macrophage chemotaxis responses. Recognizing that proinflammatory macrophages are derived from proinflammatory monocytes, we propose a model wherein multiple hepatocyte-derived signals act in concert to mediate macrophage attraction and activation in the microenvironment of NASH livers. Soluble signals such as CCL2 may lead to the egress of proinflammatory monocytes from the bone marrow and their recruitment in to the liver (10, 61); other soluble or EV-mediated signals might home proinflammatory monocytes to the sinusoidal endothelium; and promote adhesion of proinflammatory monocytes to give rise to macrophages which then infiltrate hepatic cords in response to an EV S1P gradient in the local microenvironment. Our data support a key role for S1P-$S1P_1$ in the last process, though we cannot exclude a role in adhesion or homing either. Here we demonstrate that $S1P_1$ receptor expression is increased in mouse livers in a NASH model, and in previous experiments we have demonstrated that pharmacologic inhibition of this signaling axis with FTY720 ameliorates murine NASH (23). However, the macrophage-specific role of S1P signaling has not been examined in this context. In ongoing experiments we are interrogating the *in vivo* role of myeloid cell $S1P_1$ in NASH models, as a corollary to our *in vitro* findings.

AUTHOR CONTRIBUTIONS

C-YL designed and conducted experiments, analyzed and graphed data and wrote the manuscript. MS, YG, and AM designed and conducted experiments. AR designed and

supervised experiments. HM designed and supervised experiments, analyzed data, and wrote the manuscript. All authors reviewed and approved 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.2018.02980/full#supplementary-material

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Regulation of Monocyte-Macrophage Responses in Cirrhosis—Role of Innate Immune Programming and Checkpoint Receptors

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Many aspects of the innate immune system have been studied in cirrhosis, and abnormalities have been described supporting both a pro-inflammatory and anti-inflammatory phenotype of myeloid cells. However, the findings of these studies vary by stage of disease and methodology. The recent description of the syndrome of acute-on-chronic liver failure (ACLF) has refined our understanding of the natural history of cirrhosis. In this context, we review the regulatory mechanisms at play that contribute to the immune abnormalities described in advanced liver disease. Specifically, we review the evidence for epigenetic mechanisms regulating monocyte phenotype, and the role of checkpoint receptors on regulating innate and adaptive immune cell function.

Keywords: cirrhosis, ACLF, ARLD, ALD, alcoholic hepatitis, innate immune cell, myeloid, immune checkpoint

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BACKGROUND

This is an exciting time for the field of immunotherapeutics. Advances in basic science and drug development have progressed our understanding of regulatory mechanisms of both innate and adaptive immune responses, which has directly led to novel immunotherapeutic agents. Moreover, technological advances have allowed unbiased data collection from monocyte-macrophage lineage cells, allowing a deeper understanding of their diversity and plasticity (1). The purpose of this review is to integrate these data and place them within the context of the disease landscape of cirrhosis.

Inflammation, and consequently innate immunity, plays a key role in the development of liver disease at almost every stage. For example, in the early stages, monocyte-macrophage lineage cells play a role in both the development (2, 3) and the resolution (4) of hepatic fibrosis. Understanding and harnessing the mechanism of fibrosis resolution by hepatic macrophages is an area of active translational research, although is beyond the scope of this review. This area has been reviewed recently by Ramachandran et al. (5).

Acute-on-chronic liver failure (ACLF) is a recently defined syndrome, describing an acute clinical deterioration on the background of cirrhosis, characterized by a rapid progression to multi-organ failure and high mortality. The CANONIC study, the largest prospective study of the natural history of cirrhosis, demonstrated that in the majority of cases (60%) a specific trigger for the acute deterioration, such as bacterial infection or acute alcoholic hepatitis, could be identified (6). It was also apparent from this study that dysregulated inflammation is a key feature of the syndrome. The degree of systemic inflammation, determined by leukocyte count and C-reactive protein, was

an independent predictor of the development and prognosis of ACLF. However, alongside these pro-inflammatory responses, immunodeficiency and susceptibility to infection are also features of cirrhosis and ACLF (7, 8). The overarching term for these immune alterations in cirrhosis is cirrhosis-associated immune dysfunction (CAID), although the mechanisms that regulate these diverse and dichotomous immune responses in cirrhosis remain incompletely understood (**Figure 1**). Nevertheless, recent insights into immune pathobiology in cirrhosis, along with general advances in our understanding of regulation of immunity, provide opportunities for novel therapies in cirrhosis. These opportunities will be discussed in more detail in this review.

The lines of evidence describing both features of exaggerated systemic inflammation, as well as immunodeficiency in cirrhosis and ACLF have been recently reviewed elsewhere (7). From a mechanistic perspective, gut bacterial dysbiosis and the translocation of bacterial products to mesenteric lymph nodes and the systemic circulation have been suggested to play a role in the development of these immunological abnormalities in cirrhosis (7) (**Figure 1**). However, a clear association of gut dysbiosis and a specific immune phenotype has not yet been demonstrated. Moreover, most evidence from cirrhotic patients is from single time points, and from varying severities of liver disease, as prospectively collected data delineating the time-course of immune phenotype in cirrhosis and ACLF is currently lacking. As can be seen in **Table 1**, both pro- and anti-inflammatory responses from monocyte-macrophage cells have been described in cirrhosis. Further discussion of the evidence behind these observations is provided below.

CIRCULATING MONOCYTES

Circulating monocytes play an important role in host defense, through initiation and regulation of inflammatory responses (18). In both humans and mice their phenotype can be divided into two main subsets: classical (pro-inflammatory) and non-classical (anti-inflammatory, pro-repair), which are distinguished by surface markers (19, 20). These subsets are primarily separated by their expression of CD14 (the co-receptor for bacterial lipopolysaccharide, LPS) and CD16 (a low affinity type III Fc receptor for IgG). Most circulating monocytes, around 90%, are classical $CD16^-$ monocytes expressing high levels of CD14 ($CD16^-CD14^+$). The remainder $CD16^+$ monocytes are further separated based on the expression of CD14 among $CD16^+CD14^+$ intermediate monocytes and $CD16^+CD14^{lo}$ non-classical monocytes. Similar subsets are found in mice using the Ly6C, CCR2, and CX3CR1 markers, with classical $Ly6C^{hi}CCR2^+CX3CR1^{int}$ monocytes and non-classical $Ly6C^{lo}CCR2^-CX3CR1^{hi}$ monocytes (**Figure 2**).

Conceptually, our understanding of the natural history of cirrhosis has progressed over recent years with description of the syndrome of ACLF, which describes patients with cirrhosis who progress from stable or decompensated cirrhosis to a rapid decline in liver function and extra-hepatic organ failure following a superimposed "hit". As can be seen from **Table 1**, there are few studies that examine immune cell phenotype in this stage of the disease. Monocyte dysfunction has been previously described in ACLF in cross-sectional studies, indicating skewed proportions between monocyte subsets with an increasing prevalence of anti-inflammatory monocytes able to suppress pro-inflammatory innate immune responses correlated with disease severity. Specifically, increased numbers of monocytes expressing the receptor tyrosine kinase Mer (MerTK) have been found in ACLF, associated with reduced pro-inflammatory responses *ex vivo* (17), and similarly, prostaglandin E2 (PGE2) levels have been found to be elevated in ACLF and implicated in the anti-inflammatory monocyte phenotype (16) (**Figure 2**). However, an overarching mechanism for the change in monocyte phenotype in ACLF is currently lacking.

CIRCULATING MONOCYTES RESPOND TO SUPERIMPOSED LIVER INJURY BY ALTERING THEIR PHENOTYPE AND FUNCTION

The superimposed "hit" in ACLF, on the background of cirrhosis, has been suggested to represent an acute liver insult such as gut bacterial translocation, sepsis, alcoholic hepatitis or drug-induced liver injury (DILI), leading to hepatocyte cell death and the release of damage/danger-associated molecular patterns (DAMPs) (8). Therefore, a possible hypothesis for the change in circulating monocyte phenotype in ACLF is that it represents a regulatory response to this superimposed liver injury (**Figure 2**).

The traditional dogma from mouse experiments has been that monocytes sequentially alter their phenotype from classical to non-classical over time, possibly in response to micro-environmental cues. For example, following injury, predominantly $Ly6C^{hi}$ monocytes are recruited from the bone marrow and spleen to sites of injury in a CCR2- and CCL2-dependent manner (21–23). Recent elegant experiments using deuterium labeling in humans, and adoptive transfer experiments in humanized mice, has demonstrated similar transitioning in human monocytes, particularly in response to challenge with bacterial endotoxin (24). The time course for this transition from classical to non-classical phenotype was between 1 and 5 days, with non-classical monocytes persisting for around 12 days, thus demonstrating the importance of time course in determining immune phenotype following infection or injury (**Figure 2**).

The relevance of these observations to liver injury has also been explored in rodent models. In a model of acetaminophen (APAP)-induced liver injury, fate-mapping studies using adoptive transfer of $Ly6C^{hi}CX3CR1^+$ monocytes demonstrated

Abbreviations: ACLF, Acute-on-chronic liver failure; AH, Alcoholic hepatitis; ARLD, Alcohol-related liver disease; CD, Cluster of differentiation; CTLA-4; Cytotoxic T-lymphocyte associated protein 4; DAMP, Damage/Danger-associated molecular pattern; DC, Decompensated cirrhosis; IFN, Interferon; IL, Interleukin; MAIT, Mucosal-associated invariant T cells; NK, Natural killer cells; NKT, Natural killer T cells; PAMP, Pathogen-associated molecular pattern; PD-1, Programmed death 1; PD-L1, Programmed death ligand 1; SC, Stable cirrhosis; TIM-3, T-cell immunoglobulin and mucin domain 3 (also known as Hepatitis A virus cellular receptor 2, HAVCR2); TNF: Tumor necrosis factor

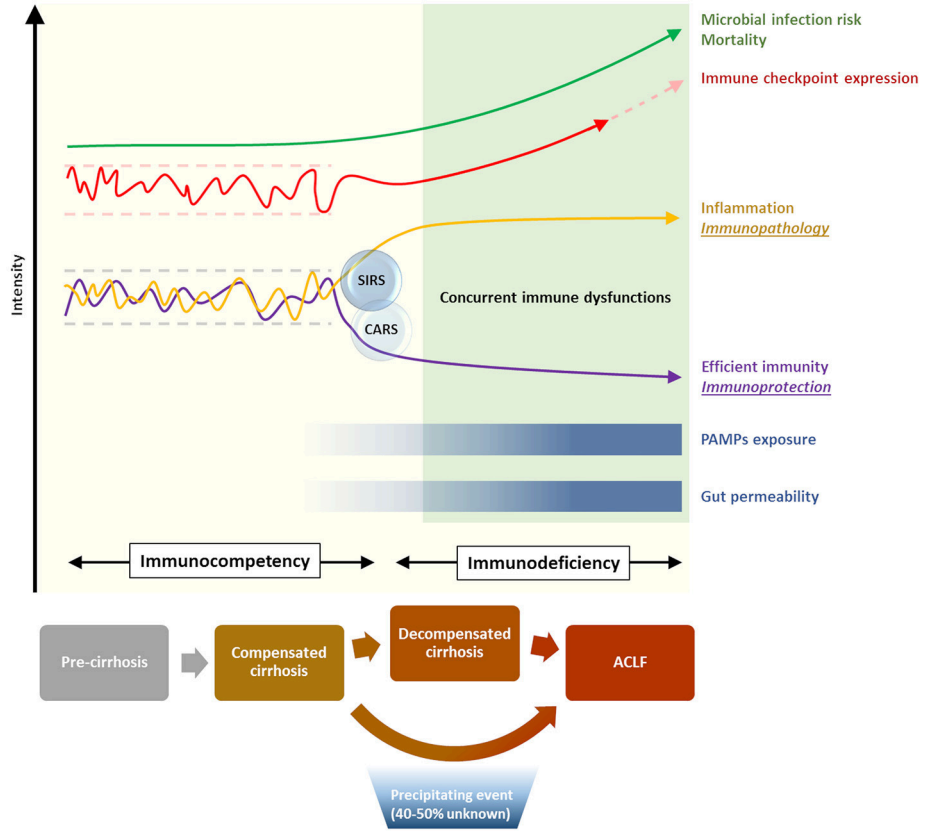

FIGURE 1 | Immunological phenomena associated with progression of cirrhosis. Increasing disease severity is accompanied by the establishment of a skewed immune profile, characterized by concurrent systemic inflammation and deficient immune protection. This state of cirrhosis-associated immune dysfunction (CAID) is crucial in increasing the risk of life-threatening microbial infection, and is in part mediated by a dysregulation of the immune checkpoint network [reviewed in (9)].

that these Ly6C^{hi} monocytes differentiated into a Ly6C^{lo} subset by 72 h following liver injury, and were cleared by 96 h (25). A further study by Dal-Secco and colleagues used CCR2-RFP and CX3CR1-GFP double-reporter mice with a model of sterile liver injury (26). These elegant experiments demonstrated that CCR2^{hi}CX3CR1^{lo} (Ly6C^{hi}) monocytes were initially recruited to the site of liver injury, and over a period of 24 h transitioned into a CCR2^{lo}CX3CR1^{hi} (Ly6C^{lo}) subset that was prevalent for up to 72 h. Therefore, it is clear that liver injury can influence the phenotype of infiltrating cells, but it remains unclear whether this occurs in cirrhosis, and to what extent this influences the phenotype of circulating immune cells. Nevertheless, most studies of monocytes in ACLF have demonstrated that monocyte dysfunction can be induced in healthy monocytes by incubation with ACLF plasma (16, 17) (**Figure 2**). Therefore, liver injury may lead to the subsequent reprogramming of circulating cells as well as infiltrating monocytes to an anti-inflammatory phenotype—this hypothesis merits further attention.

DE NOVO RECRUITMENT OF ANTI-INFLAMMATORY CELLS

A further possible mechanism is the *de novo* recruitment of anti-inflammatory cells. In humans, a recent study in ACLF described an increased population of circulating CD14^{+}CD15^{-}HLA-DR^{-} myeloid-derived suppressor cells, which lead to impaired innate and adaptive immune responses and thus contribute to the anti-inflammatory phenotype of ACLF (27).

Another tier of complexity is the possible infiltration of peritoneal-derived macrophages following liver injury. Recent elegant work from Paul Kubes' lab has demonstrated that these peritoneal cells, described as F4/80^{+}CD11b^{hi}CD102^{+} and GATA6^{+}, were found to relocate to the liver within 1 h following liver injury, to express markers associated with tissue repair, and to be critical for survival of mice following CCl4-induced acute liver injury (28). Furthermore, using CCR2-RFP and CX3CR1-GFP double-reporter mice, it was clear that these peritoneal macrophages are distinct from infiltrating peripheral blood monocytes. Further work is required to see if these cells are present in humans, and to what extent (if any) they can modify systemic immune phenotype.

MONOCYTE REPROGRAMMING AND EPIGENETICS

In a broader sense, the molecular mechanisms of monocyte reprogramming are beginning to be understood and exploited. The concept of "innate immune memory" has arisen over recent

TABLE 1 | Monocyte-macrophages display pro- and anti-inflammatory phenotypes in end-stage liver disease.

Anti-inflammatory phenotype		Pro-inflammatory phenotype	
Monocyte-Macrophage:		**Monocyte-Macrophage:**	
Rimola et al. (10)	*In vivo:* Decreased reticuloendothelial system phagocytosis in cirrhosis (DC) compared to HC, by tracer elimination method. This was associated with increased incidence of bacterial infection.	Albillos et al. (11)	*Ex vivo:* Increased activation markers (HLA-DR and CD80) and intracytoplasmic TNFα expression in monocytes from cirrhosis (DC) compared to HC.
Gomez et al. (12)	*In vivo:* Decreased macrophage-mediated clearance of IgG-coated erythrocytes in cirrhosis (mixed SC and DC). This was associated with increased incidence of bacterial infection.	Tazi et al. (13)	*Ex vivo:* Greater increase in LPS-induced monocyte TLR4 expression and TNFα release from cirrhotic patients compared to HC.
Wasmuth et al. (14)	*Ex vivo:* Decreased monocyte LPS-induced TNFα production and HLA-DR expression in ACLF compared to SC.	Gandoura et al. (15)	*Ex vivo:* Microarray gene expression profiling of PBMCs from ARLD cirrhosis (DC) showed decreased induction of type-1 and type-2 IFN-stimulated genes, compared to HC (see left column).
Gandoura et al. (15)	*Ex vivo:* Microarray gene expression profiling of PBMCs from ARLD cirrhosis (DC) compared to HC, showed increased induction of pro-inflammatory cytokine genes (IL-6, IL-8, TNFα), but decreased induction of type-1 and type-2 IFN-stimulated genes, compared to HC (see right column).		
O'Brien et al. (16)	*Ex vivo:* Plasma from DC and ACLF led to decreased LPS-stimulated TNFα release and bacterial killing when incubated with healthy monocyte-macrophages, compared to plasma from stable cirrhosis.		
Bernsmeier et al. (17)	*Ex vivo:* Decreased monocyte LPS-induced TNFα and IL-6 production in DC and ACLF compared to stable cirrhosis. No change in ROS production.		

years, challenging the dogma that only adaptive immune cells have the capacity for "memory" (29). This concept describes the phenomenon whereby an innate immune cell can mount a qualitatively different response, either exaggerated ("trained immunity") or impaired (tolerance), in response to repeated challenge. As such, it is becoming clear that innate immune cells, particularly monocytes, can be reprogrammed at metabolic, epigenetic, and transcriptional levels (30). In situations with acute excessive inflammation, tolerance acts as a mechanism to dampen the inflammatory response of the host and maintain homeostasis to prevent tissue damage and organ failure (31, 32). Nevertheless, in conditions such as sepsis, chronic inhibitory effects in immune function can also lead to a state of deep and long-lasting immunosuppression associated with a higher risk of secondary infections and a poorer outcome (33).

Epigenetic mechanisms have been implicated in monocyte-macrophage reprograming (30) (**Figure 2**). In response to LPS or upon pathogen exposure, monocytes and macrophages modify their histone acetylation and methylation marks, affecting gene expression patterns upon subsequent stimulation (34). For example, after LPS exposure, the repressive histone modification "H3K9 dimethylation" (H3K9me2) is noted at the promoter regions of IL-1β and TNFα (35, 36). Potential molecular mechanisms include increased expression of histone demethylases and deacetylases following LPS exposure (37, 38), and several of these mechanisms have also been involved in the reprogramming of intracellular metabolic activities affecting the balance between glycolysis and fatty acid oxidation (39, 40) (**Figure 2**). These pathways are potentially targetable: inhibitors of the histone deacetylases sirtuin 1 and 2 (SIRT1/2) have shown efficacy in reversing immune paralysis in mouse models of sepsis (41, 42). Similarly, long non-coding RNAs have been shown to be mediators of a "switch" in monocyte phenotype in sepsis and are also potentially targetable through antisense nucleotide strategies (43). These mechanisms deserve attention in cirrhosis and ACLF.

IMMUNE CHECKPOINTS

A further level of regulation is through interaction with adaptive immune cells and regulation of signaling through immune checkpoint receptors. Immune checkpoints constitute a complex array of regulatory receptors and ligands that are expressed on the surface of both innate and adaptive immune cells. Both co-stimulatory and a relatively larger set of inhibitory checkpoint pathways have been described, and it is the fine balance between all these positive and negative signals that is responsible for the physiological regulation of the fate and direction of ongoing immune responses. The expression of these regulatory

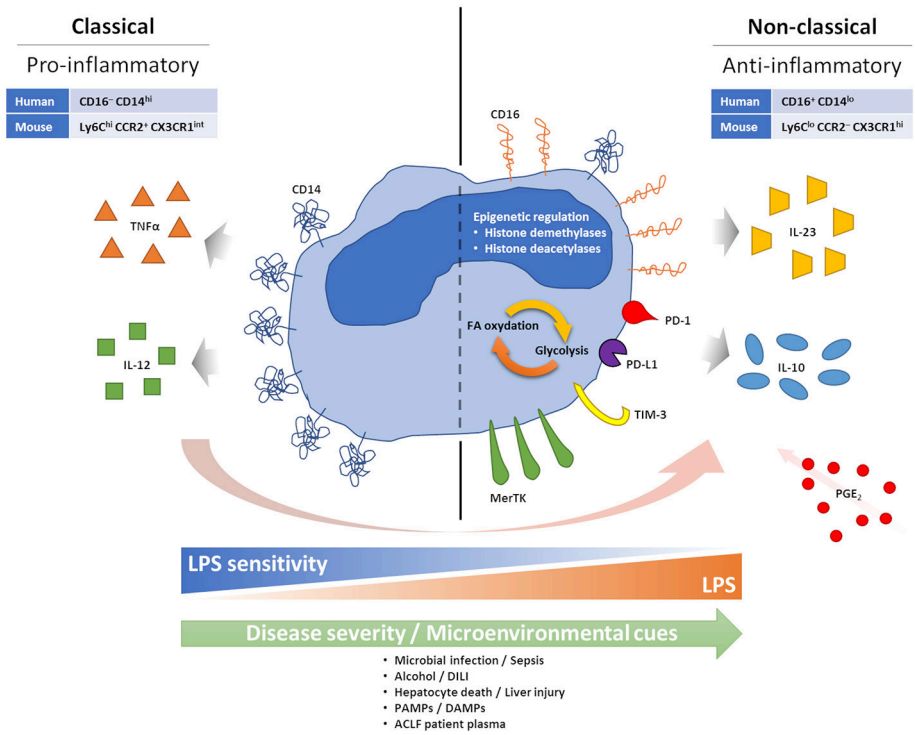

FIGURE 2 | Features of anti-inflammatory monocytes. During acute excessive inflammation monocytes can acquire an anti-inflammatory phenotype. Mechanisms behind this phenomenon are not fully understood, but chronic and intense stimulation with PAMPs and DAMPs, consequent to microbial infection or tissue damage/injury, seems able to facilitate it. PGE2 also seems to play a role in enabling monocyte changes. Anti-inflammatory monocytes appear tolerised to bacterial endotoxin, possibly linked to loss of CD14. High CD16 and MerTK have also been described. Monocyte expression of inhibitory immune checkpoints has been linked to a more suppressive phenotype, with preferential IL-10 production, and worse prognosis in cancer, infections and sepsis. Epigenetic reprogramming, and its effect on intracellular metabolic pathways, are also observed in anti-inflammatory monocytes.

pathways is both anatomically and temporally coordinated in order to facilitate the initiation and the termination of immune responses. However, in situations where the inflammation or the antigenic stimulation persist (such as sepsis, endotoxemia, or chronic infections) inhibitory checkpoints remain upregulated and this overwhelming negative signaling leads to immune cell exhaustion and immunosuppression. Amongst the most characterized inhibitory checkpoints, PD-1 and CTLA4 (with their respective ligands PD-L1 and CD80) have demonstrated to be novel, effective and safe immunotherapeutic targets for cancer, and new monoclonal blocking antibodies for TIM-3 are also currently in clinical development or tested in clinical trials [reviewed in (9)].

Most checkpoint pathways have been first characterized as regulators of T-cell immunity, but it is now clear that their effects are not limited to T cells only. For instance, PD-1 is known to also cause B and NK cell functional suppression [reviewed in (9)], and a study in HIV patients demonstrated that monocytes can express PD-1 upon bacterial exposure. These PD-1^{+} monocytes secrete suppressive IL-10 upon PD-1 engagement (**Figure 2**), and either PD-1 or IL-10 receptor blockade in these patients can reverse adaptive HIV-specific T-cell exhaustion(44) (**Figure 2**). Expression of PD-1 and PD-L1 on monocytes has also been associated with increased mortality in septic patients (45, 46), while expression of TIM-3 on monocytes has been linked to a more aggressive tumor phenotype in gastric cancer patients (47), a reduced pathogen clearance in malaria (48), preferential production of IL-10 and suppression of IFNγ T-cell responses in osteosarcoma patients (49). Furthermore, it has been proposed that expression of TIM-3 on monocytes may be able to shift the balance from IL-12 to IL-23 production and consequently favor type-17 rather than type-1 T-cell responses, driving IL-17-mediated inflammation at the expense of anti-pathogen IFNγ-mediated responses (50, 51) (**Figure 2**). Monocyte expression of TIM-3 is further inducible in response to TLR agonists, including TLR4-mediated LPS stimulation, and this can have a relevant impact in defining the immune milieu in response to bacteria or viruses (50, 51). Importantly, blockade of monocyte TIM-3 seems able to reverse the majority of these regulatory or suppressive effects, supporting the restoration of effective immune responses (48, 49, 51–53)

The above-described altered landscape of immunity in advanced liver disease is influenced by checkpoint receptor expression. A paper published in 2015 by one of the authors (AR) was the first to demonstrate that PD-1 and TIM-3 are key in defining this altered immune landscape, and monocyte hyper-stimulation with gut-derived bacterial LPS was found to be the driving factor for these immune dysfunctions (54). We

observed that adaptive antibacterial T-cell responses in patients with advanced alcohol-related liver disease were prominently skewed toward the production of suppressive IL-10 in response to LPS stimulation, and this was directly correlated with loss of IFNγ production and hyper-expression of PD-1 and TIM-3 on several immune cell subsets, including T, NK, and NKT cells (54), but not—interestingly—innate-like antibacterial T cells (mucosal-associated invariant T cells, or MAIT) (55). Stimulation with LPS dose-dependently induced PD-1, TIM-3 and IL-10 expression, but blockade of TLR4 and CD14 on monocytes completely abolished these effects; furthermore, blocking PD-1 and TIM-3 suppressed IL-10 and restored the production of antibacterial IFNγ, indicating that the immune defects observed in patients with alcohol-related liver disease may be reversible (54). Similar findings have been described in patients with non-alcoholic sepsis and also in mouse models of sepsis-induced endotoxin-driven liver inflammation [reviewed in (9)].

These results indicate that immune checkpoint blockade may be an effective treatment strategy for the restoration of defective antibacterial immunity in patients with end-stage liver disease. Furthermore, the lack of inflammation observed in our study and the good safety profiles of anti-checkpoint monoclonal antibodies currently used in cancer and sepsis suggest that immune checkpoint blockade may be a safe treatment approach also in end-stage liver disease, where conventional treatment options are currently very limited.

CONCLUSION

In conclusion, the innate and adaptive immune systems have many tiers of regulation which have been shown to be dysfunctional in cirrhosis. However, prospectively collected data delineating the time course of immune phenotype by stage of disease in cirrhosis remains scarce. Innate cell reprogramming, through metabolic or epigenetic mechanisms or by targeting checkpoint receptors, remains an attractive area for translational development, although parallel development of reliable immune biomarkers in cirrhosis will be required for immunotherapies to reach their full potential.

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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Compartmentalization of Immune Response and Microbial Translocation in Decompensated Cirrhosis

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Background: Acquired dysfunctional immunity in cirrhosis predisposes patients to frequent bacterial infections, especially spontaneous bacterial peritonitis (SBP), leading to systemic inflammation that is associated with poor outcome. But systemic inflammation can also be found in the absence of a confirmed infection. Detection of bacterial DNA has been investigated as a marker of SBP and as a predictor of prognosis. Data is, however, contradictory. Here we investigated whether levels of IL-6 and IL-8 putatively produced by myeloid cells in ascites are associated with systemic inflammation and whether inflammation depends on the presence of specific bacterial DNA.

Methods and Materials: We enrolled 33 patients with decompensated liver cirrhosis from whom we collected paired samples of blood and ascites. IL-6 and IL-8 were measured in serum samples of all patients using ELISA. In a subset of 10 representative patients, bacterial DNA was extracted from ascites and whole blood, followed by 16S rRNA gene amplicon sequencing.

Results: There were significantly higher levels of IL-6 in ascites fluid compared to blood samples in all patients. Interestingly, IL-6 levels in blood correlated tightly with disease severity and surrogates of systemic inflammation, while IL-6 levels in ascites did not. Moreover, patients with higher blood CRP levels showed greater SBP prevalence compared to patients with lower levels, despite similar positive culture results. Bacterial richness was also significantly higher in ascites compared to the corresponding patient blood. We identified differences in microbial composition and diversity between ascites and blood, but no tight relationship with surrogates of systemic inflammation could be observed.

Discussion: In decompensated cirrhosis, markers of systemic inflammation and microbiota composition seem to be dysregulated in ascites and blood. While a relationship between systemic inflammation and microbiota composition seems to exist in blood, this is not the case for ascites in our hands. These data may suggest compartmentalization of the immune response and interaction of the latter with the microbiota especially in the blood compartment.

Keywords: microbiome, systemic inflammation, myeloid cells, cirrhosis, ascites, cytokines, acute-on-chronic liver failure

INTRODUCTION

Acquired dysfunctional immunity in cirrhosis predisposes patients to frequent bacterial infections contributing to disease progression and may lead to the development of acute-on-chronic liver failure (ACLF) (1). Especially, spontaneous bacterial peritonitis (SBP) is one of the most frequent infections in cirrhosis and therefore a trigger for ACLF (2). Interestingly, SBP is defined by the number of granulocytes in ascites and not by cultural detection of bacteria (3). This is because many patients with SBP show negative bacterial cultivation from ascites, while in many other patients, viable bacteria can be cultivated in the absence of peritonitis, so-called bacterascites (3). Lack of bacterial cultivation in the presence of SBP can be explained by the concept of dormant microbiota, meaning that bacteria can reside inside circulating and resident immune cells but cannot be cultured and hence detected (4).

The source of infection, especially in the case of SBP, is suspected to be bacterial translocation from the gut (2), which is facilitated by impaired intestinal defense and increased intestinal permeability, processes which are correlated with the stage of cirrhosis (5). Not only viable bacteria (1, 6), but also bacterial components broadly termed pathogen-associated molecular patterns (PAMPs) may induce an immune response and aggravate systemic inflammation (2). As a consequence, the presence of circulating bacterial DNA reflects bacterial translocation in cirrhosis. However, the relationship of bacterial DNA with systemic inflammation is still controversially discussed (7, 8). PAMP-associated immune response requires recognition by innate immune cells and is reflected by synthesis and secretion of cytokines. Especially levels of the cytokines IL-6 and IL-8 are closely related to ACLF and clinical outcome (9). At the same time, the antibacterial capacity of myeloid cells and myelopoiesis is impaired during decompensation and ACLF (10, 11). Interestingly, these effects are not only induced by PAMPs (deriving from pathogens), but in general by bacterial-derived molecules, so-called microbe-associated molecular patterns (MAMPs).

Recent data from our group suggest that specific circulating bacteria or MAMPs in blood are associated with myeloid cell dysregulation and systemic inflammation (12). Therefore, a specific microbial DNA may be more crucial than just detecting any bacterial DNA in circulation. In ascites, the detection of bacterial DNA in ascites does not seem to correlate with SBP or clinical signs of infection (13). Therefore, the question remains, whether specific bacteria in ascites lead to myeloid cell activation with subsequent cytokine release and a systemic inflammatory response. To address this question, we investigated whether the representative cytokine (IL-6, IL-8) levels produced by myeloid cells in ascites reflect systemic inflammation. Moreover, we compared the levels of cytokines in paired ascites and blood samples, together with the general bacterial composition and specific species abundances of the microbiota estimated by 16S ribosomal RNA gene sequencing. Our study focused not only on common pathogens but also on the role of MAMPs in activating myeloid cells of the innate immune response. We hypothesize that DNA from specific bacterial species, but not any bacterial DNA, induces an inflammatory response in cirrhotic patients.

MATERIALS AND METHODS

Patient Recruitment and Sample Collection

Ascites and blood were collected in 33 cirrhotic patients scheduled for paracentesis. Paracentesis was performed by international standards (14). The study was performed in accordance with the declaration of Helsinki and approved by the local ethics committee at the University of Bonn (Nr. 121/14). Patients gave their written informed consent.

Microbiological Analysis

Microbiological culture of blood and ascites was performed at the clinical microbiology department at the University of Bonn in accordance with Microbiology Procedures Quality Standards of the German Society for Hygiene and Microbiology (15, 16). Ten milliliters of peripheral whole blood or ascitic fluid were transferred into aerobic, anaerobic and fungal blood culture bottles and incubated for up to 5 days using the Bactex FX blood culture system as described previously (17). SBP was diagnosed based on a leucocyte count in ascites $>250/\mu l$ (3).

Enzyme-Linked Immunosorbent Assay

Ascites and peripheral blood samples (serum, EDTA-plasma and whole-blood) were collected during paracentesis and stored immediately at $-80^{\circ}C$ until further use. Enzyme-linked immunosorbent assay for cytokines IL-6 and IL-8 (DY206 and DY208, respectively; R&D Systems Inc., Minneapolis, MN, USA) were performed according to the manufacturer's protocols. All samples were measured in duplicates.

16S Ribosomal RNA Gene Sequencing

For extraction of total DNA, 100 μl of whole blood or ascitic fluid were used. Samples were mechanically lysed two times for 30 s at 20 Hz in a bead beater (TissueLyser, Qiagen, Hilden, Germany) with 0.1 mm glass beads (MoBio, CA, USA). DNA extraction was performed using the NucleoSpin Blood Kit (Macherey-Nagel, Düren, Germany). Quality and quantity of extracted nucleic acids were controlled by gel electrophoresis. 16S rRNA gene V3-V4 hypervariable regions were amplified and quantified by qPCR, and sequenced using MiSeq technology (Illumina, San Diego, CA, USA) as described previously producing 2 × 300 bp paired-end reads (18, 19).

Bioinformatics and Statistical Analysis

Paired-ends reads were processed in DADA2 version 1.6.0 (20) using the following parameters: "trimLeft = 10, truncLen = (260;230), maxN = 0, maxEE = (2,5), minOverlap = 20, maxMismatch = 1, pool=TRUE." The resulting output was an amplicon sequence variant (ASV) table. The ASVs were mapped against SILVA database (21) release 128. Data analysis was performed in R version 3.5, using phyloseq package (22) version 1.24.2. Genus level relative abundances were generated by combining ASVs annotated with the same genus using phyloseq package. The alpha-diversity measures were calculated based on rarefied data at 6,000 reads/sample. Significant differences in alpha-diversity between ascites and blood samples as well as between samples with different inflammation status were determined using *t*-test.

Beta-diversity was calculated using unweighted and weighted UniFrac measures. Effects of different factors on beta-diversity were determined using Adonis Permutational Multivariate Analysis of Variance (PERMANOVA) test from the vegan package (23) version 2.5.2. For differential abundance analysis, we chose a *t*-test based on the evaluation using DAtest package (24) version 2.7.11, which showed that, compared with different statistical tests, *t*-test has the highest area under the curve (AUC = 0.608) while keeping the false discovery rate (FDR) under 0.05.

Statistical analysis of patient characteristics and clinical measures was performed using SPSS version 24 (IBM, Armonk, NY) and Mann–Whitney test for metric variables and Chi-square test for nominal variables. Receiver operator characteristic curve analysis was used to define a cut-off for CRP and divide the cohort into one group with low CRP profile and one group with high CRP profile. *P*-values were adjusted for multiple testing using Benjamini–Hochberg FDR procedure. An FDR-adjusted *P*-value below 0.05 was considered statistically significant.

RESULTS

General Description of the Cohort

General characteristics of the patients are summarized in **Table 1**. The cohort consists of patients with decompensated liver cirrhosis as reflected by the model for end-stage liver disease (MELD) and Child-Pugh (CHILD) scores, which assess severity of liver disease, prognosis and mortality. The median MELD score of 15 represents a value at which patients would benefit from liver transplantation. The median CHILD score of 10 is associated with a 1-year survival below 50%. Seventy-two percent of the patients (24/33) showed ACLF at baseline.

The median C-reactive protein (CRP) level of 48.8 mg/l in the blood were compatible with bacterial infections (upper limit of normal 5 mg/l), together with findings of SBP in 21% of the patients (**Table 1**). CRP performed well to detect SBP using receiver operator characteristic curve analysis (AUC = 0.901, $P \leq 0.001$). In blood cultures, we detected members of the bacterial genera *Enterococcus, Escherichia*, *Sporosarcina,* and *Staphylococcus* in five patients. Additionally, we detected *Candida* spp. in blood culture of one patient. In ascites, we detected the genera *Enterococcus*, *Serratia,* and *Staphylococcus* in five patients and *Candida* spp. in one patient. None of the patients presented with cultural evidence of bacteria in both, blood and ascites.

There was also evidence of systemic inflammation with elevated levels of cytokines IL-6 (and IL-8, although not statistically significant), which are mainly produced by myeloid cells. The median levels assessed by ELISA assays were 33.53 and 68.78 pg/ml, respectively (**Table 1**). Reported mean levels in healthy individuals are 1.46 and 12.9 pg/ml, respectively, assessed by the same technique (25, 26). Ascites fluid showed higher median levels of both cytokines, with an almost 20-fold increase of median IL-6 levels in ascites compared to blood from the same patient.

Differences in Patients With Systemic Inflammation

In order to decipher the role of systemic inflammation, we divided the cohort into two groups according to their CRP level. A cut-off of 25 mg/l (sensitivity = 1.0, specificity = 0.54 for detection of SBP) was used to divide the cohort into 12 patients with a low CRP profile and 21 patients with a high CRP profile. Patients with the high CRP profile showed higher MELD, CHILD, and Chronic Liver Failure Consortium (CLIF-C)-ACLF scores (**Table 1**; $P = 0.06$, $P = 0.03$, and $P = 0.002$, respectively). SBP was detected in 50% of the patients (7 out of 14) with the high CRP profile, but in none of 12 patients with the low CRP profile ($P = 0.02$). Therefore, the positive predictive value, the probability for patients with high CRP profile to have SBP, is 35%. In contrast, the negative predictive value, the chance for patients with low CRP profile to not have SBP, is 100%. A high negative predictive value in this regards is extremely important since CRP level may be elevated also in absence of infection. Furthermore, patients with the high CRP profile showed a significant increase in blood granulocyte counts ($P = 0.001$), leucocyte counts ($P = 0.004$), and IL-6 levels ($P = 0.002$). However, there was no difference in ascitic cytokine levels between these two groups (**Table 1**). Similarly, IL-6 levels in blood strongly correlate with MELD, CHILD score, and CLIF-C-ACLF score and surrogate markers of systemic inflammation (leucocytes and CRP). However, blood IL-8 levels only slightly reflected disease stage and did not correlate significantly with markers of inflammation (**Figure 1A**). Even though median cytokine levels were higher in ascites than

TABLE 1 | Patient characteristics.

	Parameters	All ($n = 33$)	Low CRP profile ($n = 12$)	High CRP profile ($n = 21$)	*P*
General characteristics	Sex (male/female)	23/10	8/4	15/6	0.78
	Age, years	57 (33–105)	53.5 (40.0–75.0)	60.0 (33.0–105.0)	0.19
	Etiology of cirrhosis (alcohol/viral/other)	18/8/7	8/4/0	10/4/7	0.08
	MELD	15.71 (3.66–39.65)	13.87 (9.79–38.25)	20.50 (3.66–39.65)	0.06
	CHILD	10 (7–15)	9 (7–13)	10 (7–15)	**0.03**
	ACLF (no/yes)	9/24	5/7	4/17	0.16
	CLIF-C-ACLF	57 (39–84)	44 (39–57)	60 (47–84)	**0.002**
	Esophageal varices (absent/small/large)	14/8/11	6/2/4	8/6/7	0.38
	Hepatic encephalopathy (absent/grade 1/grade 2/grade 3/grade 4)	22/4/3/2/2	11/1/0/0/0	11/3/3/2/2	0.20
	Hepatorenal syndrome (no/yes)	14/19	6/6	8/13	0.51
	Spontaneous bacterial peritonitis (no/yes)	26/7	12/0	14/7	**0.02**
	Blood culture (negative/positive/NA)	12/6/15	4/2/6	8/4/9	1.00
	Ascites culture (negative/positive/NA)	24/6/3	11/1/0	13/5/3	0.19
Laboratory blood	Creatinine [mg/dl]	1.40 (0.57–4.68)	1.30 (0.57–2.59)	1.58 (0.86–4.68)	0.10
	Platelets [10^6/l]	120 (7–685)	108 (38–235)	120 (7–685)	0.84
	INR	1.4 (1.0–5.5)	1.35 (1.00–2.10)	1.40 (1.00–5.50)	0.78
	Bilirubin [mg/dl]	2.08 (0.37–45.21)	1.92 (0.75–45.21)	3.20 (0.37–35.41)	0.81
	GGT [U/l]	152 (26–1,139)	139 (33–585)	181 (26–1,139)	0.76
	ALT [U/l]	32 (8–1,050)	49.5 (17.0–269.0)	30.0 (8.0–1050.0)	0.75
	CRP [mg/l]	48.8 (5.7–205.0)	13.55 (5.70–22.20)	60.20 (27.60–205.00)	**0.000**
	Granulocytes [10^6/l]	100 (10–1,070)	40 (10–170)	230 (10–1,070)	**0.001**
	Leucocytes [10^6/l]	9.81 (3.34–31.18)	7.01 (3.60–22.20)	15.09 (3.34–31.18)	**0.004**
	Monocytes [10^3/l]	1.09 (0.24–12.80)	0.86 (0.24–1.90)	1.13 (0.26–12.80)	0.43
	IL6 [pg/ml]	33.53 (3.63–684.84)	10.97 (3.95–236.71)	83.42 (3.63–684.84)	**0.002**
	IL8 [pg/ml]	68.78 (5.67–752.26)	43.76 (15.54–752.26)	77.84 (7.87–1487.36)	0.46
Laboratory ascites	Protein [g/l]	10 (1–44)	9.5 (4.0–19.0)	10.0 (1.0–44.0)	0.65
	Granulocytes [10^6/l]	48 (2–2,333)	48 (7–250)	48 (2–2,333)	0.97
	Leucocytes [10^3/l]	256 (11–3,031)	467 (52–887)	218 (11–3,031)	0.40
	Lymphocytes/Monocytes [10^3/l]	127 (5–740)	300 (39–740)	127 (5–698)	0.15
	IL6 [pg/ml]	642.67 (7.87–1487.36)	596.15 (109.56–1226.54)	653.58 (7.87–1487.36)	0.45
	IL8 [pg/ml]	83.75 (10.91–988.56)	44.91 (15.17–277.70)	95.55 (10.90–988.56)	0.15

Data are medians and (ranges). The results of Mann–Whitney test for metric variables and Chi-square test for nominal variables comparing patients with low CRP profile to those with high CRP profile are shown with P–value (significantly different comparisons are marked in bold).

in blood (**Figure 1B**), ascitic cytokine levels reflected neither disease severity nor systemic inflammation (**Figures 1A,C**). Therefore, ascites cytokine levels did not seem to be affected by systemic inflammation or by the presence of SBP, which was diagnosed in one third of the patients with the high CRP profile (**Figure 1C**).

The rate of positive blood cultures was identical in patients with the low and high CRP profile. There were three positive blood and three positive ascites cultures in patients with a high CRP against two positive blood and one positive ascites cultures in the patients with lower CRP values.

Characterizing the Microbiome in Ascites and Blood

We selected a subset of 10 representative patients (accounting for etiology and inflammation markers in ascites) from the whole cohort and performed 16S rRNA gene sequencing of ascites and blood to identify differences in microbial composition and diversity between ascites and blood. These 10 patients were also

TABLE 2 | Patient characteristics for the subpopulation in which 16S rRNA gene amplicon sequencing was performed in blood and ascites.

	Parameters	Low CRP profile ($n = 6$)	High CRP profile ($n = 4$)	*P*
General characteristics	Sex (male/female)	5/1	2/2	0.26
	Age, years	52.5 (40.0–67.0)	56.0 (33–76)	0.76
	Etiology of cirrhosis (alcohol/viral/other)	5/1/0	3/0/1	0.34
	MELD	10.26 (9.79–38.25)	18.72 (11.21–24.16)	0.17
	CHILD	9.0 (8.0–13.0)	9.5 (8.0–10.0)	0.91
	ACLF (no/yes)	4/2	1/3	0.20
	Esophageal varices (absent/small/large)	3/0/3	2/1/1	0.17
	Hepatic encephalopathy (absent/grade 1/grade 2/grade 3/grade 4)	5/1/0/0	3/1/0/0	0.34
	Hepatorenal syndrome (no/yes)	4/2	2/2	0.60
	Spontaneous bacterial peritonitis (no/yes)	6/0	3/1	0.20
	Blood culture (negative/positive/NA)	2/1/3	0/1/3	0.25
	Ascites culture (negative/positive/NA)	6/0/0	3/1/0	0.20
Laboratory blood	Creatinine [mg/dl]	0.98 (0.57–2.59)	2.27 (1.07–4.68)	0.17
	Platelets [10^6/l]	69.5 (38.0–235.0)	191.5 (123.5–237.5)	0.26
	INR	1.35 (1.00–2.10)	1.20 (1.00–1.20)	0.47
	Bilirubin [mg/dl]	2.78 (0.75–45.21)	1.54 (0.73–7.97)	0.61
	GGT [U/l]	226.0 (152.0–585.0)	149.5 (78.0–1139.0)	0.47
	CRP [mg/l]	14.4 (5.7–22.2)	94.6 (27.6–205.0)	**0.01**
	Leucocytes [10^6/l]	5.33 (3.60–9.07)	7.37 (6.59–17.30)	0.07
	Monocytes [10^3/l]	0.57 (0.24–0.87)	1.10 (0.73–1.17)	0.10
	IL6 [pg/ml]	8.32 (3.95–236.71)	81.66 (19.94–97.29)	0.39
	IL8 [pg/ml]	41.80 (15.54–752.26)	66.61 (25.08–192.48)	0.61
Laboratory ascites	Protein [g/l]	8 (5–17)	21 (10–44)	0.11
	Granulocytes [10^6/l]	18.5 (7–250)	24.0 (10.0–117.0)	0.91
	Leucocytes [10^3/l]	130.5 (52.0–824.0)	163.0 (47.0–480.0)	0.61
	Lymphocytes/Monocytes [10^3/l]	120.0 (39.0–740.0)	139.5 (37.0–363.0)	0.61
	IL6 [pg/ml]	757.19 (109.56–1226.54)	529.64 (7.87–816.83)	0.35
	IL8 [pg/ml]	29.93 (17.46–277.70)	116.82 (43.12–224.85)	0.17

Data are medians and (ranges). The results of Mann–Whitney test for metric variables and Chi-square test for nominal variables comparing patients with low CRP profile to those with high CRP profile are shown with P-value (significantly different comparisons are marked in bold).

divided into two groups based on CRP levels ($n = 4$ for high CRP profile and $n = 6$ for low CRP profile, see **Table 2**). Their disease profiles with regard to stage of liver cirrhosis and inflammatory markers in blood were comparable to those of the respective subgroups and also of the whole cohort.

Microbiota compositions at the phylum level were in line with previously reported bacterial compositions of ascites (27) and peripheral blood (12). The most abundant phylum detected in ascites samples was Proteobacteria (mean relative abundance 77.7%), followed by Firmicutes (9.4%), Actinobacteria (7.9%), Bacteroidetes (3.9%), and Gemmatimonadetes (0.6%) (**Figure 2**). The phylum composition was slightly different in the blood samples, with Proteobacteria accounting for 87.4% on average, while Actinobacteria, Firmicutes, and Bacteroidetes accounted for small proportions (5.3, 3.7, and 3.5, respectively; **Figure 2**). We identified 53 bacterial families in ascites and 48 families in blood samples. Among these, we found a set of six core families detected in all samples in both compartments: *Pseudomonadaceae*, *Oxalobacteriaceae*, *Neisseriaceae*, *Enterobacteriaceae*, *Sphingomonadaceae*, and *Moraxellaceae*. These results were in line with previous studies that reported an increase in potentially pathogenic families such as *Enterobacteriaceae* on the gut microbiome of liver patients and as well a high prevalence in ascites samples (28).

We identified 97 bacterial genera from ascites and 89 genera from blood (**Figure S2**). There were no statistical significant differences between the microbiota compositions of ascites and blood samples at phylum level. At genus level, alpha-diversity indices, such as richness (number of observed genera in a sample) and Shannon diversity (richness as well as evenness of genera in a sample), showed that the ascites microbiome was significantly more diverse than the peripheral blood microbiome ($P < 0.01$; **Figure 3A**).

Translocation of the gut microbiota into the circulatory system and into ascites is increasingly recognized as a driver

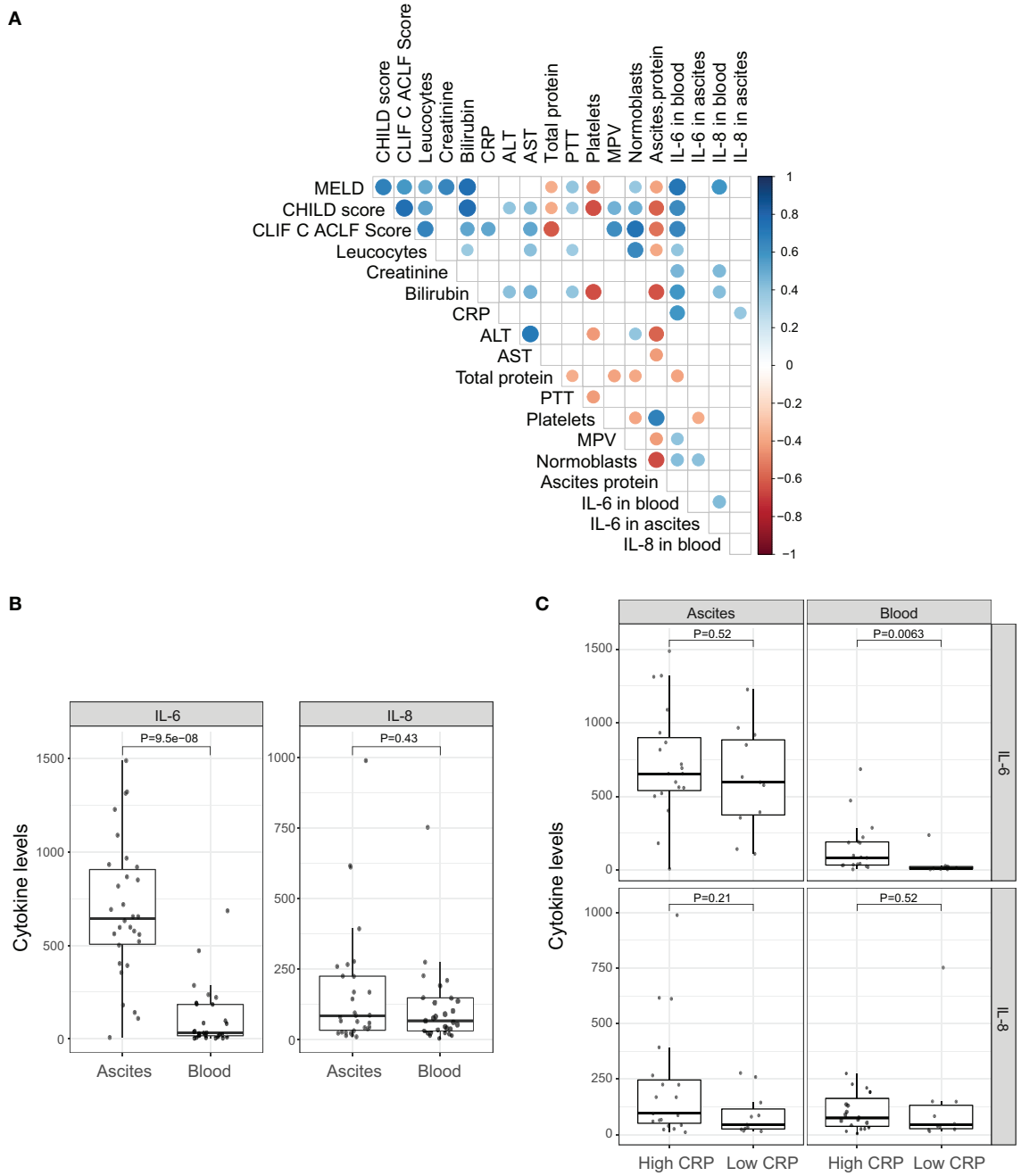

FIGURE 1 | Pro-inflammatory cytokines. **(A)** Correlation of clinical markers for disease progression and systemic inflammation with cytokines IL-6 and IL-8 in blood and ascites. **(B)** Comparison between cytokines levels in ascites and peripheral blood. **(C)** Comparison between cytokines levels in ascites and peripheral blood under different systemic inflammation status (low CRP profile vs. high CRP profile).

of complication in advanced liver disease (27, 29). As we showed above, the levels of pro-inflammatory cytokines (IL-6 and IL-8) were higher in ascites compared to blood, suggesting different drivers of inflammation. To determine the relation of the microbiota with cytokine levels and systemic inflammation, we investigated the differences in the microbiome composition of ascites and blood samples, in patients with low and high CRP levels. CRP levels did not appear to affect microbiota diversity of ascites or blood samples (**Figure 3B**).

Distinct Bacterial Signatures of Ascites and Blood Microbiome

To study the variation in the microbial structure between ascites and peripheral blood samples, we used unweighted

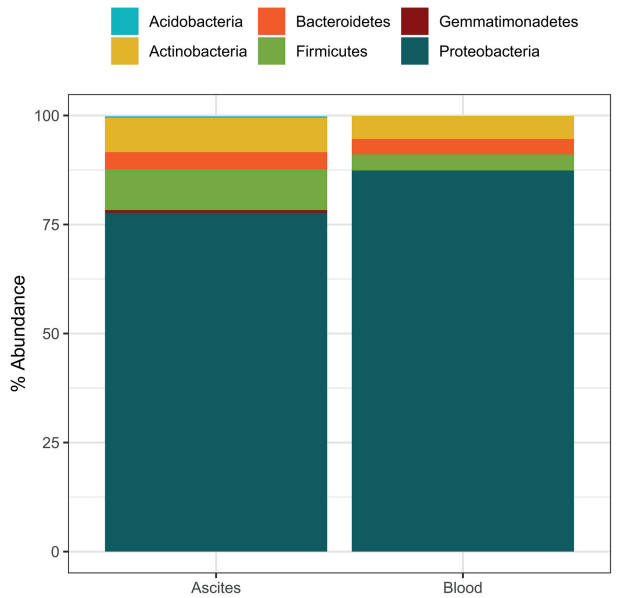

FIGURE 2 | Average microbiome phylum composition in ascites and blood samples ($n = 10$ each).

and weighted UniFrac metrics (30) as measures of the beta-diversity. Unweighted UniFrac uses a phylogenetic tree taking into account only the phylogenetic affiliation of members to evaluate the community structure and diversity, while weighted UniFrac additionally considers the relative abundance of the members of the community. Both measures clearly separated ascites from blood samples (**Figure 4**). The sampling compartment explained 11% of the variance in beta-diversity when using unweighted UniFrac metric (PERMANOVA test, $P = 0.006$), whereas it explained 21.2% of the variance when using weighted UniFrac metric (PERMANOVA test, $P = 0.001$). Additionally, the first principal coordinate (PC1) of microbiome beta-diversity explained 20.4% of the variance in the samples under unweighted UniFrac metric, whereas it explained 34% of the variance in the samples under weighted UniFrac metric (**Figure 4**).

We then investigated specific bacterial signatures that differentiated ascites from peripheral blood samples. We used DAtest (24) to select the most suitable statistical method to identify differentially abundant bacteria. Compared with other statistical methods evaluated by DAtest, *t*-test had the highest power to distinguish between two simulated sample groups based on our cohort, while keeping the false discovery rate of simulated differential taxa under 0.05. Therefore, we performed *t*-tests between the ascites and blood microbiomes using the relative abundances of 99 genera. We found that *Janthinobacterium*, *Serratia,* and *Rugamonas* were significantly enriched in ascites, while *Escherichia/Shigella* and *Pseudomonas* were enriched in blood (**Figure 5**, **Table 3**). These findings were in concordance with our cultivation results, where *Serratia* was detected in ascites culture and *Escherichia* in peripheral blood.

Since the presence of bacteria in blood is generally associated with systemic inflammation, we investigated whether there were differences in the bacterial composition of patients with systemic inflammation (high CRP levels, $n = 4$) and patients without systemic inflammation (low CRP levels, $n = 6$). There was no significant difference between groups, potentially due to limited sample size. We then examined the 5 bacterial genera that were significantly different between ascites and blood samples to determine their relationship with systemic inflammation. Although there were no statistically significant differences between the samples with and without systemic inflammation, *Escherichia/Shigella* and *Janthinobacterium* were more abundant in patients with elevated systemic inflammation, both in ascites and blood samples, while *Pseudomonas* and *Serratia* were increased only in the ascites samples with systemic inflammation (**Figure S1**).

DISCUSSION

The present study demonstrates that the levels of inflammatory cytokines and microbial richness are significantly higher in ascites of patients with decompensated cirrhosis than in their blood compartment. Neither the levels of cytokines nor the overall abundance of bacterial DNA nor microbial richness seemed to be related to the extent of systemic inflammation in these patients.

These findings are particularly striking, since the cell count in circulating blood is several fold higher than in ascites. Still, the levels of inflammatory cytokines were several fold reduced in blood compared to ascites. Moreover, the relationship between the surrogates of systemic inflammation (e.g., leucocyte count, CRP) and severity scores of liver cirrhosis were correlated with the levels of blood cytokines, but no relationship was detected in the same patients in ascites.

A recent study described the role of alcohol consumption for circulating microbiome composition and associated metabolic functions. Even though leucocyte count and endotoxemia increased with the severity of disease in this study, soluble CD14, as a marker for monocyte activation, decreased in severe alcoholic hepatitis underlining the dysregulation of the immune response (31).

Overall, these results may suggest a dysregulation in myeloid cell function in cirrhosis with an excess of cytokine synthesis in ascites. This does, however, not result in at least the two main markers of systemic inflammation investigated here. Although rather descriptive, our study suggests compartmentalization of the immune response in this setting. This compartmental dysregulation may occur as a response to MAMPs, e.g., bacterial DNA.

The reason for this compartmentalization of the immune response remains elusive. Granulocytes, as one of the main producers of cytokines, besides macrophages and monocytes, but crucial for the diagnosis of SBP, were similar or lower in ascites samples than blood samples, although levels of IL-6 and IL-8 were many times higher in ascites. This suggests an external source of these cytokines and inflammation in

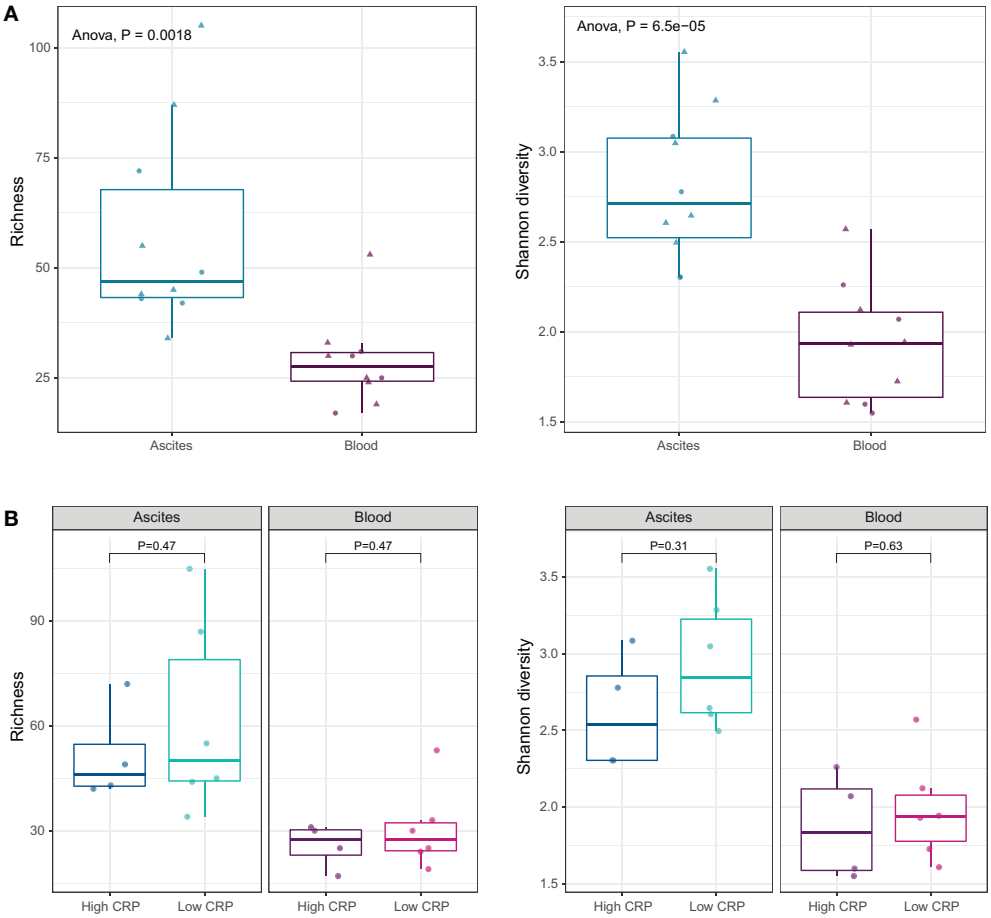

FIGURE 3 | (A) Alpha diversity indices at genus level. Ascites microbiota has higher richness and diversity compared to peripheral blood microbiome. **(B)** Alpha diversity indices under different systemic inflammation status. The inflammation status does not affect the microbial diversity of the ascites or blood samples.

ascites. Bacterial translocation from the gut is suspected to be the primary source of infection in SBP (2). However, bacteria not associated to the gut can also be found in ascites, suggesting other additional sources of infection (27). Bacterial translocation has been associated with impaired immune response and decompensation of liver cirrhosis, but the efforts to elaborate simple biomarkers such as detection and cut-off levels of bacterial DNA have shown discrepant results. Such discrepancies highlight that the relationship of presence or amount of bacterial DNA is not a strong predictor for decompensation and systemic inflammation in these patients, and probably also does not explain compartmentalization of the immune response. However, recent studies show an association of bacterial DNA levels in ascites and survival (32, 33).

Therefore, we have characterized the microbiome of matched ascites and blood samples from patients with decompensated liver cirrhosis. While ascites and blood harbored the same phyla with marginally different proportions, they showed distinct compositions at genus level. Genus alpha-diversity represented by richness and Shannon's diversity was significantly higher in ascites than in blood. Depending on the choice of the beta-diversity measure, 11% (unweighted UniFrac), or 21.2% (weighted UniFrac) of the variance in beta-diversity was dependent on the sampling compartment. These results suggest that the phylogeny of the members as well as their relative abundance have a significant yet cumulative effect in explaining the differences between ascites and blood microbial communities.

Culture-positive organisms *per se* do not seem to trigger systemic inflammation assessed by two representative cytokines (9) in patients with cirrhosis as demonstrated by the blood and ascites culture results in the two sub cohorts stratified by their CRP profile. Therefore, we hypothesize that abundance of specific genera must play a prominent role in the induction of systemic inflammation in cirrhosis. This hypothesis is supported by other studies where specific bacterial species correlate with clinical measures (27, 31).

We also identified five bacterial genera that were significantly different between ascites and blood samples, despite the small cohort size. Some of these genera were also cultivated from additional samples of the respective compartments,

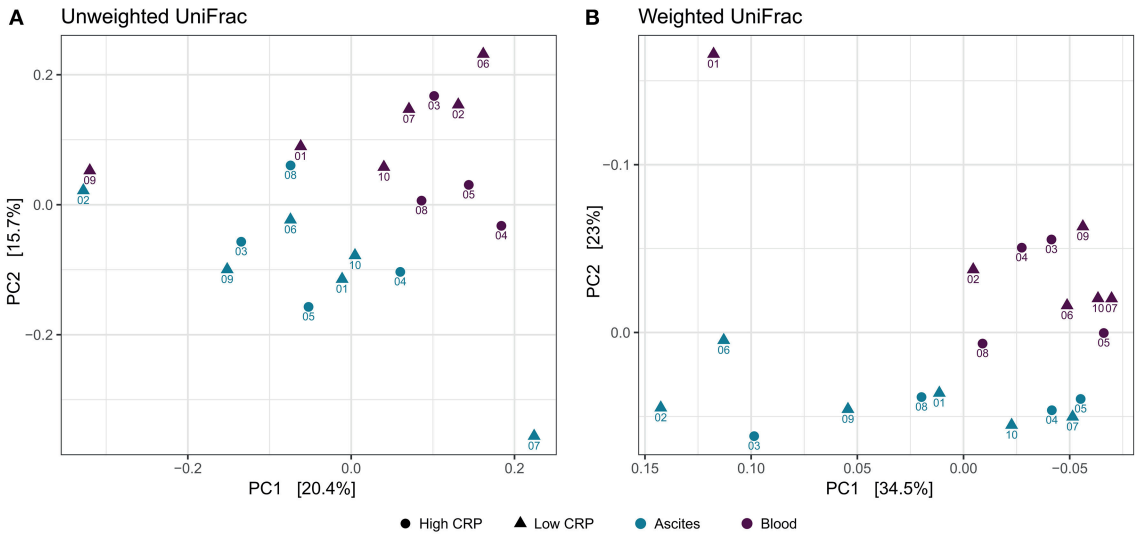

FIGURE 4 | Beta-diversity analysis. Principal coordinate analysis shows clear separation between ascites and peripheral blood samples, but samples do not cluster based on inflammation status. **(A)** Unweighted UniFrac **(B)** Weighted UniFrac.

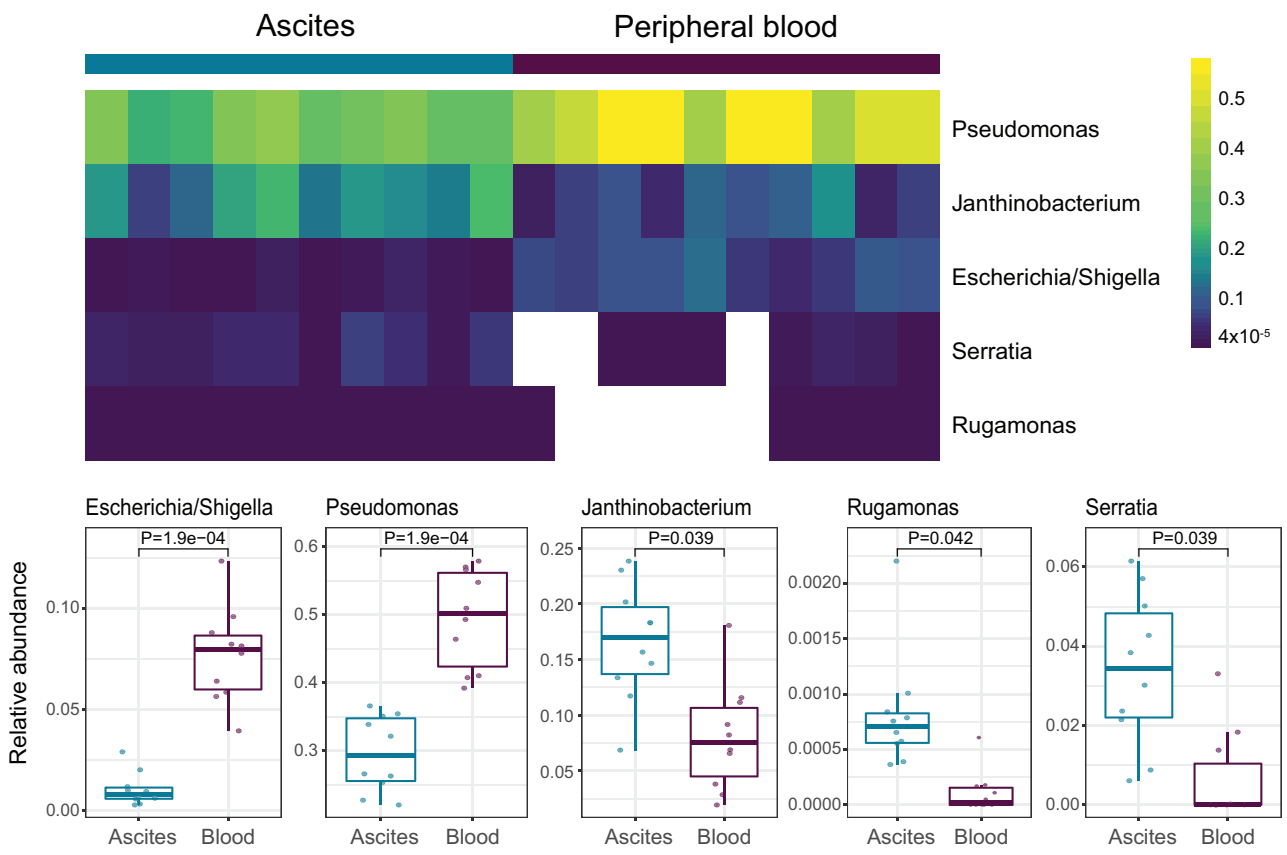

FIGURE 5 | Genera differentially abundant among ascites and blood samples.

TABLE 3 | Differentially abundant genera between ascites and blood.

Kingdom	Phylum	Class	Order	Family	Genus	Padj	Sig.	log2FC	Dir
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Shigella	0.00019243	***	2.75	Blood
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.00019243	***	0.74	Blood
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	0.038589035	*	−1.04	Ascites
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	0.038589035	*	−2.21	Ascites
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Rugamonas	0.041897934	*	−0.71	Ascites

Padj, adjusted P-value; Sig., significance level; $^{***}P < 0.001$, $^{*}P < 0.05$; Dir, direction of enrichment; log2FC, log2 of fold change.

confirming the viability of at least some of the bacteria reported in our study. Even though high CRP levels were associated with SBP, we did not see a significant difference in microbiome composition between high and low CRP samples, possibly due to reduced statistical power. However, the genera *Escherichia/Shigella* and *Janthinobacterium* showed higher abundance in blood samples of patients with systemic inflammation. *Escherichia* is already known for its pathogenic capabilities via lipopolysaccharides binding to Toll-like receptor 4, especially in individuals with dysfunctional immune response. *Janthinobacterium*, another Gram-negative bacterium, has not been described in relation to bacteremia previously, but could also induce inflammation via the same mechanism. On the other hand, while relative abundance of *Escherichia* was decreased in ascites samples, *Janthinobacterium* was increased. We speculate that the compartmentalization of immune dysfunction might be the reason behind the different behavior of these two bacteria, both of which are Gram-negative, in the different compartments.

In decompensated cirrhosis, representative markers of systemic inflammation and the microbiota seem to be dysregulated in ascites and blood. While a relationship between systemic inflammation and the microbiota seems to exist in blood, this is not found in ascites. These data may suggest the compartmentalization of the immune response and its interaction with the microbiome is especially important and evident in blood, while in ascites not found in our hands.

AUTHOR CONTRIBUTIONS

CA-S, RS, MA, and JT: conceptualization and methodology. CA-S, RS, AP, MA, and JT: formal analysis. CA-S, RS, AP, FM, FU, and PR: investigation. FM, FU, PR, and EL: resources. BL: data curation. CA-S, RS, EL, BL, JC, MV, MA, and JT: writing—original draft. CA-S, RS, MA, and JT: visualization. MA and JT: supervision.

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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.2019.00069/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Causes and Consequences of Innate Immune Dysfunction in Cirrhosis

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Liver cirrhosis is an increasing health burden and public health concern. Regardless of etiology, patients with cirrhosis are at risk of a range of life-threatening complications, including the development of infections, which are associated with high morbidity and mortality and frequent hospital admissions. The term Cirrhosis-Associated Immune Dysfunction (CAID) refers to a dynamic spectrum of immunological perturbations that develop in patients with cirrhosis, which are intimately linked to the underlying liver disease, and negatively correlated with prognosis. At the two extremes of the CAID spectrum are systemic inflammation, which can exacerbate clinical manifestations of cirrhosis such as hemodynamic derangement and kidney injury; and immunodeficiency, which contributes to the high rate of infection in patients with decompensated cirrhosis. Innate immune cells, in particular monocytes/macrophages and neutrophils, are pivotal effector and target cells in CAID. This review focuses on the pathophysiological mechanisms leading to impaired innate immune function in cirrhosis. Knowledge of the phenotypic manifestation and pathophysiological mechanisms of cirrhosis associated immunosuppression may lead to immune targeted therapies to reduce susceptibility to infection in patients with cirrhosis, and better biomarkers for risk stratification, and assessment of efficacy of novel immunotherapies.

Keywords: innate immunity, decompensated chirrosis, infection, monocyte, ascites

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INTRODUCTION

Chronic liver injury, most commonly caused by viral infection, alcohol or liver fat accumulation associated with features of the metabolic syndrome, causes activation of resident, and infiltrating immune cells, leading to progressive inflammation and liver fibrosis. Advanced liver fibrosis (cirrhosis) disrupts the normal architecture and function of the liver. Cirrhosis typically has an asymptomatic phase (compensated cirrhosis) followed by a rapidly progressive phase (decompensated cirrhosis) signaled by development of complications of portal hypertension and liver dysfunction (ascites, variceal bleeding, hepatic encephalopathy), which can progress to multi-organ failure known as acute on chronic liver failure (ACLF). Cirrhosis is associated with both systemic inflammation (elevated steady state immune cell activation and circulating inflammatory mediators), which can exacerbate clinical manifestations of cirrhosis such as hemodynamic derangement and kidney injury, and, as disease progresses, immunosuppression, and impaired antimicrobial function, which is associated with increased susceptibility to infection. The immunodeficient state is most marked in the setting of ACLF, which resembles the

immunopathology of sepsis, with an initial systemic inflammatory response (cytokine storm) leading to a compensatory anti-inflammatory response that impairs resistance to infection (1). The dynamic spectrum of immunological perturbations that develop in patients with cirrhosis is referred to as cirrhosis associated immune dysfunction (CAID) (2). This review focuses on the pathogenesis and features of innate immune dysfunction that develop in advanced cirrhosis, its role in susceptibility to infection, and recently trialed therapeutic approaches with the potential to alleviate the significant mortality and morbidity associated with infections in these patients.

BURDEN AND PATHOGENESIS OF INFECTION IN PATIENTS WITH DECOMPENSATED CIRRHOSIS

Patients with cirrhosis are susceptible to a range of complications, with infections being one of the most clinically important problems, associated with a marked reduction in life expectancy. The rate of bacterial infections is as high as 34% per year in patients with advanced cirrhosis—-in 30–50% of cirrhotic patients, infection is the cause of hospital admission, and a further 15–35% develop nosocomial infections, as compared with 5–7% of the general population (3, 4). Bacterial infections can lead to further decompensation, including variceal hemorrhage and hepatorenal syndrome, and are the main precipitant of ACLF. They account for a 4-fold increase in mortality in hospitalized patients, and a post-infection mortality rate of 28% at 1 month and 63% at 1 year (5, 6). The most common infections in cirrhotic patients are spontaneous bacterial peritonitis (SBP), which alone accounts for more than 30% of infections, urinary tract infections, pneumonia, and soft tissue infections (3, 4, 6).

The majority of infections in patients with cirrhosis are caused by Gram-negative bacteria of intestinal origin, although Gram-positive infections have been associated with severe sepsis in cirrhotic patients in intensive care units (7). Pathological translocation of intestinal bacteria to extraintestinal sites is thus implicated as a major pathogenic mechanism in the development of these infections, especially SBP and bacteremia's. In the early stages of cirrhosis, bacterial translocation contributes to systemic inflammation, with chronic stimulation of immune cells, and elevated levels of circulating inflammatory mediators; which can exacerbate hemodynamic derangements in these patients, lead to further decompensation, and cause tissue damage (2). This chronic, sub-clinical immune activation ultimately leads to immunosuppression, which increases susceptibility to infections, including those due to Gram-positive or bacteria of non-intestinal origin. Overall, the most common Gram negative bacterial infections identified in patients with cirrhosis include *Enterobacteriaceae* such as *Escherichia coli* and *Klebsiella Pneumoniae*, whilst *Staphylococcus aureus,* and *Enterococci* are among the most common Gram positive infections (8, 9). The epidemiology of infections in cirrhosis continues to change with increasing prevalence of multidrug resistant organisms (29–38% increase from 2011 to 2017) (6, 8).

PATHOGENESIS OF CIRRHOSIS ASSOCIATED INNATE IMMUNE DYSFUNCTION

Innate immune cells, principally macrophages, monocytes and neutrophils, detect tissue damage, and invading microorganisms, and orchestrate tissue healing and eradication of infection. During chronic liver disease progression they are critical mediators of liver and systemic inflammation, initially responding to so-called damage-associated molecular patterns (DAMPs) released from injured liver cells to initiate and drive progression of fibrosis through activation of hepatic stellate cells and perpetuation of inflammation (10). Systemic perturbations in circulating monocytes and inflammatory cytokines have been reported in pre-cirrhotic and early cirrhotic disease, potentially triggered by ongoing liver inflammation as monocytes traffic through the liver, and, due to the size of the liver, the release of DAMPs and other soluble mediators into the circulation providing constant inflammatory stimuli. Advanced cirrhosis, particularly decompensated disease, is associated with increasing perturbations in gut homeostasis, including microbiome alterations, reduced motility, small intestinal bacterial overgrowth, and increased gut permeability (11); which together lead to increased systemic exposure to gut microbes and microbial products providing further chronic stimulation of innate immune cells. This increase in microbial products may be an important step in the switch from the predominantly proinflammatory immune dysfunction observed in early cirrhosis to a predominant hyporesponsive, immunodeficient phenotype observed in decompensated disease. The inflammatory and immunodeficient phenotypes of cirrhosis associated immune dysfunction are thus the extremes of a dynamic spectrum, both caused by chronic liver inflammation and its systemic consequences.

Mechanistically, the development of this immunodeficient state is driven by innate immune memory–an adaptive property of innate immune cells, particularly monocytes/macrophages whereby prior exposure to microbes or microbial products dictates subsequent responses to stimulation. Whereas, monocyte priming with fungal products typically leads to enhanced inflammatory cytokine production upon re-stimulation (trained immunity), priming with bacterial products, especially lipopolysaccharide (LPS), through toll like receptor (TLR) 4 dampens subsequent responses to stimulation (tolerance) (12, 13). This functional reprogramming is mediated by alterations in intracellular signaling, cellular metabolism and epigenetic changes. LPS tolerance is suggested to be the fundamental mechanism responsible for the immunoparalysis that occurs after Gram negative sepsis, many features of which are also observed in patients with advanced cirrhosis. *In vivo*, innate immune cells are chronically exposed to a dynamic spectrum of stimuli, including microbial products, as the clinical manifestations of disease, especially portal hypertension, worsen. Innate immune function in these patients thus reflects the integration of many stimuli over time. Genetic factors also contribute to increased infection risk in patients with cirrhosis.

Common polymorphisms in genes encoding key innate immune pattern recognition receptors including TLR2, TLR4, TLR9, CD14, and NOD2 have been associated with the development of infections in some (14–16) but not all (17) studies. Two polymorphisms in the IL-1 pathway (IL1B and IL1RA) were recently reported to be associated with reduced circulating levels of inflammatory mediators in patients with ACLF (18). As many as 80% of inducible gene expressed by activate human monocytes show heritable variation in their level of expression (19). Such variation has been strongly linked to genetic susceptibility to inflammatory bowel disease (20), which could in turn impact on disordered gut permeability.

IMMUNODEFICIENCY IN ADVANCED CIRRHOSIS

Liver Immunodeficiency

The liver is an essential organ contributing to immune system homeostasis and defense against infection and is central to both ends of the CAID spectrum. Underlying liver disease drives systemic inflammation, whilst progressive loss of surveillance and synthetic function contributes to hepatic immunodeficiency, and consequently reduced systemic resistance to infection. The liver is the first organ in contact with bacteria and bacterial products originating from the gut via the portal vein, functioning as a filter for gut-derived bacteria that escape surveillance by gut immune cells. Liver resident macrophages, the largest population of tissue macrophages with direct access to the blood stream, are critical for surveillance of blood borne infections, avoiding the systemic spread of microbes and microbial antigens (10). Impaired liver macrophage phagocytic capacity *in vivo* has been demonstrated in patients with cirrhosis (largely of alcoholic etiology) compared to healthy controls, which correlated with liver disease severity, subsequent development of infection and mortality (21, 22). By contrast, the phagocytic capacity of liver macrophages isolated from cirrhotic patients and cultured *ex vivo* did not differ significantly from controls (23). Aside from intact microrganisms, liver resident macrophages contribute to clearance of senescent blood cells (erythrocytes, platelets and neutrophils) and numerous bioactive molecules (including haemopoietic growth factors, hormones (e.g., insulin, glucocorticoids and parathyroid hormone) cytokines and chemokines, food and luminal antigens, toxins (both microbial and xenobiotic), clotting factors/fibrin, lipoproteins, prostanoids, and immune complexes) (24). Liver spillover and increased levels of these endocytic substrates into the circulation contributes to a systemic pathogenic milieu.

Increased circulating levels of agents that are normally cleared by the liver is accompanied by the loss of products normally produced by the liver, including factors that comprise the humoral component of innate immunity. Both hepatocytes and liver macrophages synthesize soluble factors, such as complement and soluble pattern recognition receptors (e.g., lipopolysaccharide binding protein, soluble CD14) that are essential for effective systemic immune responses and resistance to infection. In response to inflammation, the liver produces acute phase proteins, such as Mannose Binding Lectin (MBL), C-reactive protein (CRP), hepcidin, fibrinogen, and proteinase inhibitors, which participate in innate immune responses and control tissue damage and repair. Reduced production of complement and other proteins such as ficolins and MBL that contribute to bacterial recognition and killing, is commonly observed in cirrhotic patients and linked to the development of infections and other clinical outcomes (25–30). Reduced production of CRP, which is routinely used as a biomarker of infection in the general population, in response to bacterial infection has also been reported in patients with cirrhosis (3, 31). Serum bactericidal activity is impaired in cirrhotic patients, and correlated with low complement levels (25). One of the most common blood abnormalities associated with liver disease is thrombocytopenia, which may be a consequence of both dysregulated platelet clearance and decreased production of thrombopoietin. Platelets and the clotting cascade are an important component of bacterial recognition. The mechanisms and the impacts of platelet deficiency, on pathogen clearance have been reviewed recently (10, 32).

While the extent of and mechanisms underlying liver macrophage dysfunction in cirrhotic patients have not been widely investigated, it was recently demonstrated in mice that gut bacterial translocation induced type 1 interferon expression in the liver, conditioning myeloid cells to produce high levels of IL-10 upon Listeria infection, impairing antibacterial immunity and leading to a loss of infection control (33). A prominent liver interferon signature was also observed in patients with cirrhosis, and liver myeloid cells showed increased IL-10 production after bacterial infection (33). In cirrhosis, systemic spread of gut-derived bacteria and bacterial products is also facilitated by portosystemic shunts, which form as a result of portal hypertension and divert portal blood from the liver to the systemic circulation.

Circulating Innate Immune Cells

Circulating innate immune cells play a key role in resistance to blood borne infections, which are common patients with cirrhosis (34), and are also recruited to tissues to effectively combat infections. Although the impact of immune phenotype and function observed *ex vivo* on bacterial clearance function *in vivo* is difficult to investigate in patients with chronic liver disease, Ashare et al. took advantage of the transient bacteremia that occurs following tooth-brushing, and demonstrated prolonged bacteremia in patients with cirrhosis compared to healthy controls and patients with pulmonary disease, which correlated with disease severity (Child Turcotte Pugh (CTP) and Model for End Stage Liver Disease (MELD) scores) (35). This acutely impaired bacterial clearance suggests functional impairments in circulating neutrophils and/or monocytes, the principal innate immune effector cells, *in vivo*.

Neutrophil Phenotype and Function in Cirrhosis

Neutrophils are a key component of the innate immune system that play vital roles in defense against infection but have also been shown to contribute to the development of

systemic inflammation and organ failure in sepsis. Circulating neutrophils exhibit limited microbicidal activity in the steady state; they require priming by endogenous inflammatory mediators or microbial agents to exert maximal functionality, with upregulation of endothelial adhesion molecules required for recruitment to damaged or infected tissues. Key neutrophil antimicrobial activities include extrusion of neutrophil extracellular traps, phagocytosis, production of reactive oxygen species (ROS, termed the respiratory burst), and the release of antimicrobial proteases and other mediators via degranulation.

The most consistent findings in relation to circulating neutrophil function in patients with cirrhosis are diminished phagocytic capacity and/or elevated ROS production at steady state (36–42), which correlate with serum pro-inflammatory cytokines and markers of bacterial translocation, increase with disease severity measures (36, 38, 41), and correlate with the development of infections and mortality (38, 41). In a prospective study of 62 patients, baseline neutrophil phagocytic capacity and resting ROS production predicted the development of infection, organ dysfunction and mortality at 90 days and 1 year (41). Notwithstanding the elevated resting ROS production; impaired neutrophil killing of intracellular bacteria (43) and reduced capacity to mount an augmented oxidative burst response is observed in patients with cirrhosis in general (43, 44) and in patients with active infections (36). In patients with decompensated alcoholic cirrhosis, neutrophil antimicrobial activity and microbicidal protein release was severely impaired. Bactericidal activity could be restored *ex vivo* by the TLR7/8 agonist CL097, which potentiated signaling through the formyl peptide receptor and myeloperoxidase release (45). Garfia et al. reported selective impairment of Phospholipase C-, but not Calcium/Protein Kinase C-dependent activation of the burst response in patients with cirrhosis (44), whilst Rajkovic et found depleted intracellular glutathione in cirrhotic patient neutrophils, suggesting reduced ability to detoxify ROS and resulting oxidant stress may contribute to impaired neutrophil function (43). Impaired neutrophil phagocytosis in cirrhosis has also been associated with reduced responsiveness to Tuftsin, a natural tetrapeptide that can stimulate neutrophil phagocytosis (42).

Several studies have demonstrated that serum/plasma from patients with cirrhosis can transmit the elevated ROS and impaired phagocytosis phenotype to healthy neutrophils (38, 46–48). In one study, depletion of serum LPS restored neutrophil dysfunction (38), suggesting impairments may be reversible. However, serum inhibitory factors have not associated with neutrophil impairment in all cirrhotic cohorts (42, 43).

Impaired neutrophil endothelial adhesion and chemotaxis *in vitro* (46, 49, 50) as well as mobilization to an aseptic inflammatory site *in vivo* (skin blister) (48, 49), have also been demonstrated in patients with cirrhosis. Impaired neutrophil migration and phagocytosis of heat-killed *E. coli in vivo* in patients with cirrhosis was further reduced in patients with previous episodes of bacterial infection compared to non-infected patients (49). In this study, circulating neutrophil complement receptor 3 (CR3), an integrin that mediates neutrophil endothelial adhesion and binding to complement-opsonised bacteria, was elevated in cirrhotic patients compared to healthy controls, but reduced in patients with a previous bacterial infection. Granulocyte colony stimulating factor (G-CSF) treatment increased transendothelial migration of neutrophils from cirrhotic patients *in vitro*, suggesting this factor may be able to increase neutrophil recruitment to infected sites (50). Elevated expression of other neutrophil adhesion molecules, including LSEL [CD62L (50)] and ITGAM (CD11B) (37), which may contribute to alterations in neutrophil recruitment *in vivo*, have also been reported.

Together, the available evidence supports the conclusion that neutrophils in patients with cirrhosis are chronically activated, exhibiting high resting ROS production but are impaired in their ability to traffic to sites of infection and to mount effective antimicrobial responses. These perturbations are linked to persistent *in vivo* stimulation with microbial products or other inflammatory mediators, and the degree of neutrophil dysfunction increases with disease severity, and likely contributes to increased susceptibility to infection. Cirrhosis-associated neutrophil dysfunction may be reversible as several studies have reported improved function *in vitro* with interventions such as LPS-depletion (38), TLR7/8 agonism (45, 50), G-CSF (50), and GM-CSF treatment (46). Notably, most, though not all, investigations of neutrophil function in cirrhosis have been conducted in patients with alcoholic liver disease. Alcohol consumption is known to elevate neutrophil ROS production and impair phagocytosis, even in people without chronic liver disease (51), and alcohol use is a significant contributor to gut permeability and bacterial translocation in cirrhotic patients (52).

Monocyte Phenotype and Function in Cirrhosis

In addition to augmenting tissue macrophage pools via recruitment to inflammatory sites, circulating monocytes are important innate immune effector cells in their own right, and also initiate and regulate the development of adaptive immunity, via antigen presentation and the production of immunoregulatory cytokines. Although there is clearly evidence of increased monocyte infiltration of the diseased liver (10), there is little evidence of a profound monocytosis in liver disease; most focus has been on changes in function and differentiation. Human blood monocytes have been broadly classified into 3 subsets based on CD14 and CD16 expression; "classical" CD14high/CD16- monocytes (comprising ∼80% of peripheral blood monocytes) that express high levels of chemokine (C-C motif) receptor (CCR)2, non-classical CD14+CD16+ monocytes, which preferentially express the chemokine (C-X3-C motif) receptor (CX3CR)1 and intermediate CD14HighCD16+ monocytes. Functionally, classical monocytes exhibit strong phagocytic capacity, whilst non-classical monocyte subsets, in particular the intermediate subset, have been designated pro-inflammatory (53, 54). Detailed transcriptome and enhancer profiling of these "subsets" indicated that they represent a differentiation series, in which the intermediate population expresses intermediate levels of every transcript that distinguishes classical and non-classical monocytes (55).

Alterations in monocyte subsets, in particular an increase in intermediate and/or non-classical monocytes, are frequently observed in infectious and inflammatory diseases, and have been associated with clinical outcomes (53). Modest elevations in non-classical monocytes, that have limited phagocytic capacity and high production of inflammatory cytokines, have been reported even in early stages of chronic liver disease in some (56, 57), but not all (58) studies, with the most prominent perturbations suggested to occur in patients with cirrhosis. CD16+ inflammatory monocytes have also been suggested to preferentially accumulate in the liver (56, 57).

Monocyte surface marker expression, especially microbial pattern recognition receptors (since increased activation by LPS and other microbial stimuli is postulated to be responsible for high pro-inflammatory cytokine levels in cirrhosis) has been investigated in a number of studies, with inconsistent results. Xing et al. reported higher monocyte expression of TLR4 (that recognizes LPS from Gram-negative bacteria, among other ligands) and TNF production in patients with hepatitis B viral infection (HBV) and ACLF compared to healthy controls (59). By contrast, selective down-regulation of TLR4-dependent immune responses, which was restored by Gram-negative-targeted antibiotic therapy, was proposed to contribute to impaired monocyte function and infection risk in cirrhosis, suggesting a role for Gram negative gut microbes in suppressing peripheral TLR4 responses (60). Other studies suggest a stimulatory role for Gram-positive bacterial components in monocyte dysfunction in cirrhosis. Riordan et al. reported that monocyte TLR2, but not TLR4, expression was increased in patients with cirrhosis, and significantly correlated with serum TNF, whilst *in vitro* TNF production in response to a Gram-positive bacterial infection was blunted (61). Manigold et al. also reported elevated TLR2, but reduced TLR4 expression, in cirrhotic patients, which strongly correlated with serum levels of LPS (62). In addition to TLR expression, reduced monocyte expression of CCR2 (a key chemokine receptor for monocyte recruitment to infected or inflamed tissue sites) has been reported, regardless of disease stage (57, 58), and we found elevated expression of the adhesion receptor LSEL (CD62L) in patients with decompensated cirrhosis (58).

The most consistent finding with regard to monocyte phenotype in cirrhosis is a reduction in HLA-DR expression (58, 63–66), although increased expression has also been reported (57, 67). Reduced HLA-DR expression, which likely compromises antigen presentation and the development of adaptive immune responses, is one of the hallmarks of LPS tolerance, the immunosuppressed state in which monocytes become refractory to further stimulation with LPS and other microbial stimuli. Monocyte HLA-DR expression appears to diminish with advancing disease. A step-wise reduction in HLA-DR expression was observed in HBV-infected patients with non-cirrhotic chronic liver disease, compensated cirrhosis and ACLF (59). HLA-DR expression was modestly reduced in our cohort of patients with NAFLD, and significantly reduced in patients with HCV, with the most pronounced reduction in patients with decompensated cirrhosis (58). In ACLF, expansion of HLA-DRLow monocytes was associated with poor clinical outcomes, with significantly lower HLA-DR expression in patients who died compared to those who survived (68). Similarly, the percentage of HLA-DR+ monocytes was significantly reduced patients with decompensated cirrhosis, both with and without ACLF (with alcohol as the commonest etiology in both cohorts), compared to healthy controls; and low HLA-DR expression was associated with death from ACLF (65). In a longitudinal study of critically ill cirrhotic patients, low HLA-DR expression at baseline was associated with mortality, and reduced further over time in non-survivors, compared to stable or increasing expression in survivors (69), suggesting impaired HLA-DR expression is reversible. Longitudinal alterations in HLA-DR were a stronger predictor of survival than measures of liver insufficiency (MELD score) or organ failure (Sequential Organ Failure Assessment (SOFA) score). There is limited evidence of a functional consequence of reduced HLA-DR, but one study reported attenuated antigen-specific T cell responses in cirrhotic patients, associated with a high frequency of CD14+/HLADR- monocytes (64). Nevertheless, *ex vivo* monocyte-derived dendritic cells from cirrhotic patients had a similar capacity to upregulate co-stimulatory molecules and stimulate expansion of antigen-specific T cells compared to healthy controls, further suggesting recovery of HLA-DR expression is possible (70).

Monocyte production of pro-inflammatory cytokines in response to microbial activation is key to innate immune defense against infection, as these cytokines enhance antimicrobial functions. Reduced monocyte production of pro-inflammatory cytokines (in particular TNF and IL-6) in response to LPS stimulation is another hallmark of the LPS tolerant immunosuppressed state in sepsis and other severe inflammatory conditions. In early chronic liver disease as well as compensated and decompensated cirrhosis, elevated spontaneous and/or LPS-induced monocyte TNF production have been identified (57, 67, 68). Impaired inflammatory cytokine (TNF, IL-6, IL-1, IL-12) production only becomes apparent in late stage ACLF (68). Similarly, plasma from patients with acutely decompensated, but not compensated, cirrhosis suppressed LPS-induced TNF production by healthy monocytes (71). This suppressive effect was mediated by increased plasma Prostaglandin E2 (PGE2) in acutely decompensated cirrhosis related to low levels of albumin, which regulates PGE2 bioavailability (71). In our cohort of patients with chronic liver disease, LPS-induced TNF production was not impaired in patients with decompensated cirrhosis overall, but was significantly reduced in patients who died during 6 months follow up and correlated with time to death (58).

Monocyte bactericidal capacity depends on effective phagocytosis and the action of phagolysosomal enzymes as well as the production of ROS. Although monocyte bacterial killing has not been directly investigated in chronic liver disease or cirrhosis, there is some evidence that monocyte antimicrobial functions are impaired in these patients. Elevated resting ROS production, and reduced ability to respond to microbial challenge with augmented ROS production (oxidative burst), correlated with advancing disease, has been reported (30). In patients with HCV or NAFLD, we found monocyte capacity to mount an oxidative burst response was significantly reduced in patients with cirrhosis as well as in patients with chronic liver disease but

without advanced fibrosis (58). By contrast Bruns et al. reported impaired neutrophil but not monocyte oxidative burst capacity in cirrhotic patients (predominantly alcoholic liver disease) (36). Monocyte phagocytic capacity was severely impaired in patients with ACLF, related to the expansion of a poorly phagocytic HLA-DRLow immature myeloid population (68). We found monocyte phagocytic capacity for *E. coli* bioparticles was negatively correlated with ALT, suggesting a relationship with liver injury and inflammation, but was not significantly impaired in patients with compensated or decompensated cirrhosis (58). Like cytokine production, impaired phagocytic capacity may be a late event in the course of decompensated disease.

Together, the available evidence suggests that chronically activated monocytes contribute to the systemic inflammatory state in patients with chronic liver disease by production of inflammatory cytokines, but significant impairment in monocyte function, especially reduced HLA-DR expression and stimulated cytokine production, progressively occurs in decompensated cirrhosis, and is associated with clinical events and outcomes. Although the pathophysiological mechanisms underlying features of monocyte suppression have not been elucidated, high levels of LPS and anti-inflammatory cytokines (such as IL-10) and reduced levels of interferon γ have been suggested to contribute (63). Given the diversity of bioactive molecules that spillover into the circulation from the diseased liver, it seems unlikely that any single stimulus is entirely responsible. Both HLA-DR expression and cytokine expression may be reversible, however; interferon γ therapy restored monocyte function and resulted in clearance of sepsis in a small cohort of critically ill patients (72).

Peritoneal Innate Immune Phenotype and Function in Cirrhosis

Ascites is the most common complication of decompensated cirrhosis, and SBP is the most common infection in these patients, with a prevalence of up to 50% in patients admitted to hospital, and mortality of ∼30% (3, 4). SBP is considered to be mainly caused by gut-derived microbes, as a result of increased intestinal permeability and bacterial translocation. The main identifiable causes of SBP are Gram negative, enteric microbes (especially *E. coli*) and Gram-positive Streptococci. However, SBP is diagnosed by an elevated ascites fluid neutrophil count (>250/ml) because the rate of culture negativity in the diagnostic microbiological analysis of ascites samples is high (up to 60% (11)), even where there are clinical signs of infection, necessitating empirical antibiotic treatment. The efficacy of currently recommended empirical antibiotic therapy was reported to be 83% in community-acquired infections, but low in health-care acquired infections (40%) (73), highlighting the need to identify adjunct approaches to prevent infections in this high-risk population. Unlike other tissues, including the liver, ascites fluid is relatively accessible, facilitating investigations into innate immune function at this key site of infection. It should be noted, however, that ascites fluid and the cells it contains only comprise part of the picture, as the omentum also plays essential roles in peritoneal defense (74).

The presence of microbial products, including LPS, bacterial peptides and, particularly, bacterial DNA in ascites fluid, even in the absence of overt infection, is a consistent finding (75–80). The presence of bacterial DNA was associated with similar cytokine profiles in ascites fluid to that seen in the presence of active infection (81). These findings suggest peritoneal immune cells, like circulating cells, in patients with decompensated cirrhosis are chronically exposed to microbes and/or microbial products. Separate studies revealed an association between low peritoneal macrophage HLA-DR expression and the presence of bacteria in both uninfected and infected patients (76, 82), the latter study also demonstrating resolution with antibiotic treatment. Ascites macrophage HLA-DR expression did not correlate with circulating monocyte HLA-DR (76), suggesting, not surprisingly, that the peritoneal microenvironment shapes macrophage phenotype and function. The underlying etiology of cirrhosis significantly affected ascitic immune cell subsets and cytokine levels, with lower leukocyte numbers and higher inflammatory cytokine levels in patients with alcoholic cirrhosis compared to HCV (83). Other studies have identified further differences between ascites macrophages and donor-matched circulating monocytes, including relatively increased HLA-DR and cytokine (TNF, IL-6, IL-10) expression and stronger responses to e*x vivo* LPS and Candida stimulation in ascites macrophages, whereas monocytes produced significantly more IL-12 and IL-1β (84, 85).

Both factors present in the peritoneal microenvironment and cell intrinsic differences may contribute to the differences observed between peritoneal and circulating monocytes/macrophages. Murine peritoneal macrophages can be subdivided into mature resident cells, which are capable of self-replication, and monocyte-derived macrophages which slowly replace the resident population and acquire a differentiated phenotype (86). The mature resident peritoneal macrophages have a unique gene expression profile, including high expression of genes encoding phagocytic receptors (e.g., Vsig4, Timd4, and Marco) (87). Similarly, two phenotypically and functionally distinct macrophage populations can be identified in human patient ascites fluid; a highly phagocytic population that expresses high levels of CD14, HLA-DR, and Complement Receptor for Immunoglobulin (CRIg, encoded by the VSIG4 gene) and resemble murine resident peritoneal macrophages, and a poorly phagocytic population, which express CCR2 and resemble murine peritoneal monocyte-derived macrophages (88). The relative proportions of CRIgHi and CCR2+ macrophages varied between patients, and within patients over time. A high proportion of CRIgHi macrophages and macrophage phagocytic capacity significantly correlated with lower disease severity (MELD score) (88). A similar relationship between monocyte-derived populations and lower disease severity was also inferred based on CD14 (85), which is more highly-expressed on the CRIgHi population. The reduced ascites macrophage CD14 and HLA-DR expression and low macrophage phagocytic capacity reported in patients with SBP compared to sterile ascites (82) probably reflects dilution by the CD14Low monocyte-derived macrophage population in the setting of infection. Compared to peritoneal macrophages

from non-cirrhotic controls, ascites macrophages from cirrhotic patients without overt infection had a higher oxidative burst response to microbial stimulation, and lower phagocytic capacity (89).

In addition to being a biomarker of bacterial translocation, bacterial DNA in ascites fluid is also an innate immune stimulus. Because a variety of pathogens use the cytosol for replication, it is under surveillance by DNA-sensing pattern recognition receptors, including the Absent in Melanoma 2 (AIM2) inflammasome, that induces a broad inflammatory response, and the cGAS-STING axis, that drives antiviral immunity by inducing type 1 interferons. Lozano-Ruiz et al. reported elevated expression of AIM2, which specifically induces inflammasome formation in response to double stranded DNA, and constitutive caspase 1 activation and IL-1β production in blood and ascites fluid macrophages from patients with decompensated cirrhosis, in the absence of infection, which correlated with disease severity (CTP score) (90). Bacterial DNA-stimulated inflammasome activation *ex vivo* did not require a priming signal, suggesting ascites macrophages were pre-activated i*n vivo*. Although, suggesting a role for AIM2, these data are also consistent with DNA-dependent activation of the NLRP3 inflammasome, downstream of the cGAS-STING pathway that has recently been reported (91).

Neutrophils are also a significant cellular component of ascites fluid in patients with cirrhosis, especially in the presence of infection, and they mirror some of the systemic deficiencies discussed above. Ascites neutrophil oxidative burst capacity in response to Phorbol Myristate Acetate (PMA) stimulation was significantly impaired in patients with SBP compared to sterile ascites, and this resolved with antibiotic treatment in patients with culture-negative, but not culture-positive, SBP (82). The respiratory burst in neutrophils from patients with cirrhotic ascites was impaired compared to malignant ascites and inhibitory factors were identified in ascites fluid independent of complement factor 3 [C3, which is known to be deficient in cirrhotic ascites fluid (92, 93) and predispose to SBP (94)]. Engelmann et al. (95) compared blood and peritoneal neutrophil function in patients with decompensated cirrhosis and non-cirrhotic controls and found impaired phagocytosis and oxidative burst activity upon *E. coli* challenge in cirrhotic ascites compared to peripheral blood, whereas non-cirrhotic ascites had higher phagocytic activity but equally suppressed oxidative burst (52). Ascites neutrophil function could be partially restored after incubation with autologous plasma, underscoring the role of inhibitory factors in ascites fluid in impaired neutrophil function.

In addition to cellular antimicrobial function, ascites fluid has been reported to have poor opsonising activity (29, 96), with low levels of C3 reported in particular (92, 93), that have also been associated with the development of infection at this site.

BIOMARKERS OF IMMUNE DYSFUNCTION WITH IN CIRRHOSIS

Since the transition from innate immune activation to dysfunction seems to be a key pathophysiological mechanism contributing to the development of infection, immune function-based biomarkers may have utility for risk stratification, identification of patients who may benefit from immunotherapy, and assessing outcomes of therapeutic interventions in patients with cirrhosis. Although perturbations in pro- or anti-inflammatory biomarkers have been associated with clinical outcomes in decompensated cirrhosis (97–99), these do not necessarily reflect changes in functional immunity and direct assessment of cellular function may be required. Functional innate immune responses, including increased resting neutrophil ROS production and phagocytic capacity, have been associated with the development of infections and survival (38, 41), suggesting potential utility as indicators of susceptibility, but they may be difficult to translate into clinical practice. Monocyte HLA-DR expression appears to diminish progressively with advancing disease, and longitudinal changes in monocyte HLA-DR expression were strongly associated with mortality (mostly from sepsis) in critically ill patients with cirrhosis, as has been demonstrated in non-cirrhotic sepsis (69). The utililty of monocyte HLA-DR expression as a biomarker of infection risk at earlier disease stages remains to be clarified. The development of standardized methods of flow-cytometric assessment of HLA-DR expression may facilitate translation to the clinic in the future (100). In an integrated approach to assessing innate and adaptive immune function, reduced interferon production in response to combined innate and adaptive immune stimulation with the TLR7 agonist R848 and anti-CD3 (Quantiferon platform) was associated with the development of infections in patients with decompensated cirrhosis (101). With further validation Quantiferon may be a promising biomarker in cirrhosis, as this platform has been successfully adapted for clinical use (for the detection of latent tuberculosis infection).

IMMUNOTHERAPEUTIC APPROACHES TO REDUCE SUSCEPTIBILITY TO INFECTION IN CIRRHOSIS

Since gut bacterial translocation is considered to be the main source of infection in patients with decompensated cirrhosis, infection prevention is mainly based on selective intestinal decontamination using orally administered poorly absorbed antibiotics such as norfloxacin (targeting Gram-negative *Enterobacteriacea* and *Enterococci*). Antibiotic prophylaxis carries the risk of development of antibiotic resistance, limiting therapy and increasing morbidity and mortality (102). Prophylaxis is thus limited to patients at very high risk of developing infections, defined by clinical criteria (3). A variety of novel adjunct approaches to antibiotic prophylaxis have been explored, including strategies targeting intestinal bacterial overgrowth and/or abnormal gut microbiota (non-selective intestinal decontamination (Rifaximin), probiotics, fecal microbiota transplantation), intestinal motility (prokinetics, beta-blockers) and barrier function (bile acids, beta-blockers) (102). Strategies to improve intestinal homeostasis would be anticipated to beneficially impact systemic immune function, via reducing chronic stimulation that leads to immunosuppression

as well as reducing exposure to potentially infectious organisms. Many antibiotics, including those used prophylactically in patients with decompensated cirrhosis, also exert direct effects on the immune system, although the underlying mechanisms are not well-defined (103).

In addition to gut-targeted approaches, there is also emerging interest in immunotherapeutic approaches to reduce susceptibility to infection in decompensated cirrhosis. However, given the co-existing spectrum of inflammation and immunodeficiency in cirrhosis, timing of immunotherapy appears critical. In the setting of ACLF, therapies to limit hepatic and systemic inflammation that contribute to multiorgan failure may be relevant in the early stages of decompensation, whereas immune boosting approaches may be beneficial in later stages where there is prolonged immunosuppression and high risk of secondary infections. Immunotherapeutic approaches for the treatment and prevention of infection in ACLF have recently been comprehensively reviewed (104). The potential of prophylactic immunotherapy to prevent infections, which are a major precipitant of ACLF, in relatively stable decompensated cirrhosis warrants further investigation. The literature reviewed herein suggests significant immunosuppression is only present in a subset of patients with "stable" decompensated cirrhosis; those at risk of poor outcomes, including infections. Identification and application of appropriate therapies requires better understanding and improved assessment of immune function to identify patients most likely to benefit from immunotherapy. Two strategies that have been the subject of recent clinical trials, albumin and granulocyte colony stimulating factor treatment, are discussed below.

Albumin Supplementation

Albumin is a 67 kDa protein synthesized exclusively in the liver and released to the intravascular compartment. It has multiple functions such as binding and transport of substances, regulation of endothelial function, antioxidant and scavenging properties and regulation of inflammatory responses (105). In chronic liver disease, serum albumin level is reduced due to defective synthesis and altered structurally and functionally due to post-transcriptional modification, hindering its ability to perform its physiological functions (106, 107). Albumin modifications that affect its binding capacity have been reported in cirrhotic patients, and correlated with liver disease severity measures, the development of complications and survival (108). Oxidized serum albumin, that is commonly elevated in patients with cirrhosis, was also recently shown to directly trigger an inflammatory response in peripheral blood mononuclear cells (97). Intravenous albumin is commonly used to regulate blood volume in patients with decompensated cirrhosis, and has been proven to prevent renal failure (109). Patients with low serum or ascitic fluid albumin have also been shown to be at increased risk of infection (110) but the immunoregulatory effects of albumin administration are still unclear. A large (n = 1,818) randomized controlled trial in patients with severe sepsis found no improvement in outcomes in patients administered albumin in addition to a crystalloid volume expander compare to crystalloid solution alone, however this study did not achieve the target serum albumin level of >30 g/L (111).

Patients with decompensated cirrhosis were recently demonstrated to have high levels of plasma PGE2, which impaired LPS-stimulated TNF production in healthy monocytes *ex vivo*. Monocyte function could be restored by albumin supplementation via reducing PGE2 bioavailability (71). On the basis of this finding, albumin supplementation has been investigated as an immunomodulatory therapy in decompensated cirrhosis. In a recent single arm feasibility study (n = 79) daily administration of 20% human albumin solution to hospitalized patients with ACLF and low serum albumin levels for 14 days (or until discharge or death) increased serum albumin to the target level of >30 g/L [since albumin < 30 g/L was the only clinical characteristic that predicted suppressed monocyte responses in *in vitro* studies (71)] without significant safety concerns (112). Patient plasma significantly suppressed macrophage LPS-stimulated TNF production compared to healthy plasma, and restoration of serum albumin to >30 g/L failed to alter plasma PGE2 levels, but did increase albumin PGE2-binding capacity and restore TNF production to similar levels observed in healthy plasma (113). A randomized controlled trial of albumin administration to reduce infections in ACLF has commenced recruitment. In another study, albumin co-administration for 3 days following SBP diagnosis (50 ml 20% Human Albumin Solution) only modestly increased serum albumin, but significantly reduced serum and ascitic fluid cytokines (IL-6 and TNF) and LPS, compared to patients receiving antibiotic therapy alone (n = 15 per group) (114). Albumin treatment also reduced ascites fluid TNF in patients without SBP (114). In addition to short-term administration to acutely decompensated patients, the efficacy of long-term albumin administration in patients with ascites has recently been investigated in a randomized, multi-site trial (115). Four hundred forty patients with decompensated cirrhosis (uncomplicated ascites) received standard medical care or standard care plus albumin (40 g twice weekly for 2 weeks then weekly for up to 18 months). Overall 18-month survival was significantly higher in patients who received albumin compared to standard care (115). Given its excellent safety profile and these promising results, albumin appears to have potential as a prophylactic treatment in patients with decompensated cirrhosis, possibly targeted to patients with demonstrable immunosuppression, however the optimum dose and treatment duration remains to be clarified, with consideration of the cost-effectiveness of therapy and the availability of human albumin in hospitals.

Granulocyte Colony Stimulating Factor (G-CSF) and Stem Cell Therapy

G-CSF, a potent cytokine that mobilizes CD34+ haematopoietic stem cells from the bone marrow, and autologous stem cell therapy have been investigated for their potential to improve outcomes in patients with decompensated cirrhosis, including ACLF. Mobilization or delivery of hematopoietic stems cells, which have the capacity to differentiate into multiple cell lineages, is postulated to promote liver regeneration. A renewed

supply of leukocytes may also overcome the immune paralysis that contributes to the development of infections. G-CSF may also improve the function of existing immune cells, as G-CSF improved neutrophil transendothelial migration *in vitro* (50). Several randomized controlled trials (RCTs) of G-CSF therapy have been conducted in patients with cirrhosis. Most employ a dose regime established to mobilize HSC from the bone marrow (∼5 daily doses of 5 ug/kg), though some studies continued to dose weekly for an extended period. One RCT in patients with ACLF demonstrated significant improvement in liver disease severity measures, reduced development of multi-organ failure and other complications (including sepsis) and a >2-fold 60-day survival rate after 30 days G-CSF therapy (n = 23–24 per group). G-CSF therapy significantly increased peripheral leukocyte counts and hepatic CD34+ cells (116). G-CSF treatment was further shown to increase circulating and intrahepatic myeloid dendritic cells, CD4 and CD8 T cells; and to reduce CD8 T cell IFNγ production (117). Although IFNγ is a key factor known to restore monocyte immunosuppression *in vitro* and in sepsis (72), innate immune phenotype and function were not investigated. In this cohort, G-CSF was administered prior to the onset of sepsis and the authors suggested that early identification of patients and commencement of G-CSF treatment could prevent the development of sepsis and multiorgan failure (116). G-CSF therapy was also demonstrated to improve liver function and 3-month survival, with significantly fewer patients dying of sepsis in the treatment compared to the control group, in patients with HBV-associated ACLF (n = 27–28 per group) (118). In another study, treatment of patients with decompensated cirrhosis with G-CSF and erythropoietin for 1 month reduced liver disease severity (CTP score) and improved survival at 12 months follow-up compared to placebo, and significantly fewer patients in the treatment arm developed sepsis during follow up (n = 26–29 per group) (119).

An RCT conducted in a cohort of decompensated cirrhotic patients (excluding ACLF, n = 21–23 per group) assessed the impact of 3 monthly G-CSF administration for 1 year, with and without Growth hormone (GH, postulated to promote liver regeneration), compared to standard care. Transplant free survival at 12 months was significantly improved in both groups of G-CSF-treated patients, associated with decreased measures of liver disease severity and a significant reduction in the odds of developing a bacterial infection (including ∼88%

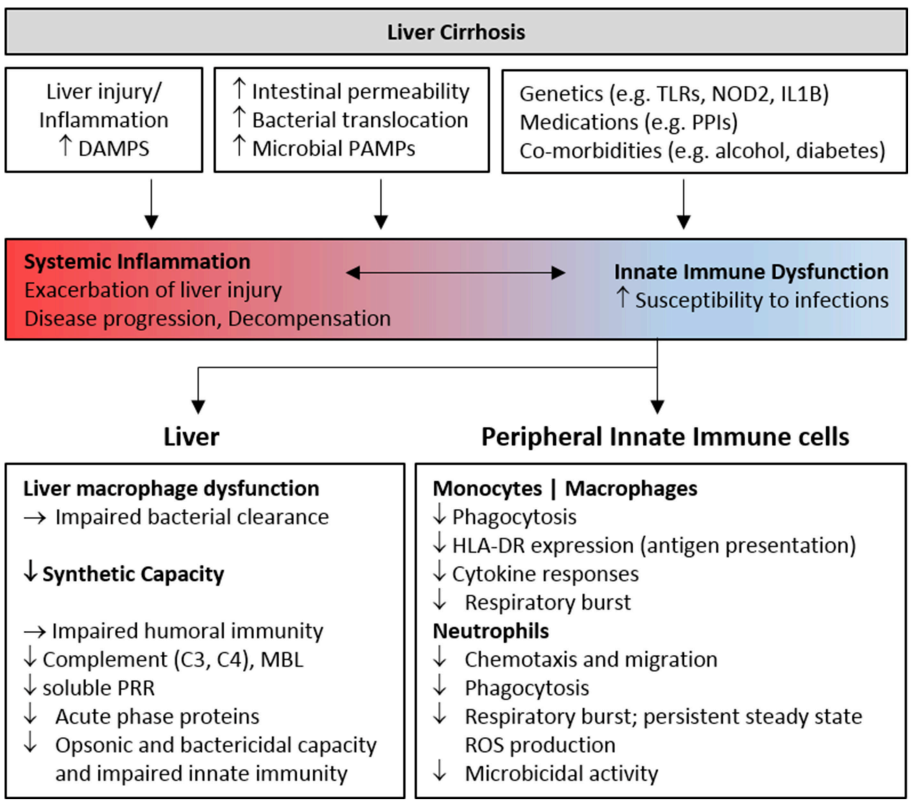

FIGURE 1 | Features of innate immune deficiency in advanced cirrhosis. Host and microbial inflammatory mediators resulting from liver injury and gut bacterial translocation initially activate innate immune cells, contributing to inflammation and the development, and progression of liver cirrhosis. Chronic stimulation progressively perturbs innate immune sensing and surveillance functions, contributing to infection risk in patients with decompensated cirrhosis. Genetic and environmental factors also contribute to immune dysfunction. The features of cellular and humoral innate immune deficiency commonly identified in cohorts of patients with advanced cirrhosis are summarized. DAMP, Damage Associated Molecular Pattern; PAMP, Pathogen Associated Molecular Pattern; PRR, Pattern Recognition Receptor; PPI, Proton Pump Inhibitor; MBL, Mannose Binding Lectin; TLR, Toll-like receptor.

reduction in the odds of developing sepsis). GH treatment did not show any additional benefit in reducing infections or in survival (120). In contrast to these promising results, G-CSF treatment for 5 consecutive days or G-CSF treatment followed by 3 doses of autologous CD133+ hematopoietic stem cells in patients with compensated cirrhosis did not improve liver disease severity (MELD score, the primary outcome), and there was evidence to suggest G-CSF treatment, with or without stem cells, was associated with an increased frequency of adverse events compared to standard care (n = 26–28 per group) (121). In a study by Sharma et al. patients with non-viral decompensated cirrhosis received a hepatic artery infusion of autologous CD34+ hematopoietic stem cells (mobilized by 3 consecutive days G-CSF treatment) or standard care (n = 22–23 per group). Stem cell therapy significantly increased serum albumin levels at 1 month but this was not sustained at 3 months, and there was no difference in survival between the treatment arms (122). Overall, G-CSF and stem cell therapy appear to be promising approaches to improve liver function and reduce the burden of infections in patients with ACLF, but may not be applicable in non-ACLF decompensated cirrhosis as efficacy has not been consistently demonstrated and one study raised safety concerns.

CONCLUSION

Infections are very common in patients with decompensated cirrhosis, they precipitate further decompensation and multi-organ failure (ACLF) and are therefore associated with high morbidity and mortality. Prevention of bacterial infections in these patients is crucial to improve outcomes, however current approaches are limited to antibiotic therapy, which is associated with antibiotic resistance and increased infections with non-classical pathogens (6, 8, 9). The identification of risk factors contributing to the development of infections in this vulnerable population, including impaired immune resistance to infection, may provide novel adjunct therapeutic approaches to minimize the burden of infections, improve outcomes and minimize antibiotic resistance. With regards to innate immunity, the first line of defense against infection, whilst chronic activation is apparent in pre-cirrhotic and compensated cirrhotic disease, functional impairment becomes progressively apparent in decompensated cirrhosis, potentially associated with increased gut permeability and exposure to gut-derived microbes and microbial products. The most commonly identified features of innate immune dysfunction in decompensated cirrhosis are summarized in **Figure 1**. Whilst defects in core immunological functions that contribute to infection resistance (phagocytosis, ROS production, cytokine production) have consistently been reported, actual killing capacity for relevant pathogens has rarely been investigated. This is potentially important as it was recently demonstrated in mice that reduced cytokine production as a result of microbial product-induced tolerance did not correlate with bacterial killing *in vivo* (123). Moreover, increased understanding of other antimicrobial functions (e.g., autophagy), may lead to novel therapeutic approaches.

Increased resting neutrophil ROS production and reduced monocyte HLA-DR expression with disease progression have been consistently linked to outcomes and may aid in risk stratification or provide useful biomarkers of therapeutic efficacy, at least in research settings. However, identification of universal features of cirrhosis associated immunodeficiency, and the clinical stage at which they occur, is hampered by the dynamic interplay between factors driving systemic inflammation and immunosuppression in cirrhosis, and the variability in patient cohorts that have been studied. Many studies do not distinguish between compensated and decompensated cirrhosis, whilst others focus exclusively on patients with ACLF. In addition to disease stage, medications (e.g., steroids, proton pump inhibitors, non-selective beta blockers), underlying liver disease etiology and associated lifestyle factors also impact intestinal permeability and immune function (e.g., diabetes, alcohol). Immunotherapeutic approaches to prevent infections in patients with decompensated cirrhosis are only beginning to be explored, and trials to date have only reported short term outcomes. Given its excellent safety profile and the recent preliminary evidence for efficacy, albumin administration is a promising strategy. Improved understanding of the pathophysiology and natural history of the spectrum of immune activation and immunodeficiency in the course of decompensated cirrhosis may enable the identification of targeted preventative immunotherapies in the future.

AUTHOR CONTRIBUTIONS

KI wrote the paper. IR contributed to literature review and writing. EP and DH contributed to writing and critically reviewed the paper.

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Corrigendum: Causes and Consequences of Innate Immune Dysfunction in Cirrhosis

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Addressing Profiles of Systemic Inflammation Across the Different Clinical Phenotypes of Acutely Decompensated Cirrhosis

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Background: Patients with acutely decompensated cirrhosis (AD) may or may not develop acute-on-chronic liver failure (ACLF). ACLF is characterized by high-grade systemic inflammation, organ failures (OF) and high short-term mortality. Although patients with AD cirrhosis exhibit distinct clinical phenotypes at baseline, they have low short-term mortality, unless ACLF develops during follow-up. Because little is known about the association of profile of systemic inflammation with clinical phenotypes of patients with AD cirrhosis, we aimed to investigate a battery of markers of systemic inflammation in these patients.

Methods: Upon hospital admission baseline plasma levels of 15 markers (cytokines, chemokines, and oxidized albumin) were measured in 40 healthy controls, 39 compensated cirrhosis, 342 AD cirrhosis, and 161 ACLF. According to EASL-CLIF

criteria, AD cirrhosis was divided into three distinct clinical phenotypes (AD-1: Creatinine<1.5, no HE, no OF; AD-2: creatinine 1.5–2, and or HE grade I/II, no OF; AD-3: Creatinine<1.5, no HE, non-renal OF).

Results: Most markers were slightly abnormal in compensated cirrhosis, but markedly increased in AD. Patients with ACLF exhibited the largest number of abnormal markers, indicating "full-blown" systemic inflammation (all markers). AD-patients exhibited distinct systemic inflammation profiles across three different clinical phenotypes. In each phenotype, activation of systemic inflammation was only partial (30% of the markers). Mortality related to each clinical AD-phenotype was significantly lower than mortality associated with ACLF ($p < 0.0001$ by gray test). Among AD-patients baseline systemic inflammation (especially IL-8, IL-6, IL-1ra, HNA2 independently associated) was more intense in those who had poor 28-day outcomes (ACLF, death) than those who did not experience these outcomes.

Conclusions: Although AD-patients exhibit distinct profiles of systemic inflammation depending on their clinical phenotypes, all these patients have only partial activation of systemic inflammation. However, those with the most extended baseline systemic inflammation had the highest the risk of ACLF development and death.

Keywords: acute decompensation, cirrhosis, signature, ACLF, organ failure, organ dysfunction

INTRODUCTION

Natural history of patients with acutely decompensated (AD) cirrhosis may be complicated by acute-on-chronic liver failure (ACLF) (1). ACLF, which has been intensively investigated during the recent years, is characterized by the presence of organ failure(s) (OFs) and high short-term mortality (1–4). The diagnosis of OFs is based on the CLIF-C OF scoring system which assesses the deterioration in the function of the six major organ systems, including liver, kidney, coagulation, brain, circulation, and respiration (1). ACLF is recognized when patients have either a single renal failure; moderate renal dysfunction (creatinine between 1.5 and 1.9 mg/dl) and/or cerebral dysfunction (grade I and II hepatic encephalopathy) in combination with any isolated non-renal OF; or two OFs or more (1). ACLF is also characterized by the presence of high-grade systemic inflammation. Many biomarkers of systemic inflammation are elevated in ACLF, and associated with outcome (5–12).

Unlike patients with ACLF, patients with AD have low short-term mortality (1). AD-patients without ACLF at hospital admission may present three distinct clinical phenotypes which do no overlap (1). The first phenotype (hereafter called AD-1) includes patients without any single OF, who have serum creatinine of <1.5 mg/dL and do not have hepatic encephalopathy (HE). The second phenotype (AD-2) includes patients with isolated renal dysfunction and/or HE I or II, but without any associated single non-renal OF. Finally, the third phenotype (AD-3) includes patients with a single non-renal OF without any kidney dysfunction. Although it is known that some AD-patients without ACLF at hospital admission can subsequently develop ACLF and die (1), the baseline profile of systemic inflammation in these patients developing or not ACLF during short-term follow-up is unknown. Also the profiles of systemic inflammation across the three distinct clinical phenotypes have not been investigated. Expanding our knowledge about the profile of systemic inflammation associated with each clinical phenotype should deliver not only insights into the pathogenesis of ACLF, and also provide clinical tools for stratification of patients and therapy (e.g., anti-TNF, G-CSF).

We hypothesized that each of the three distinct clinical phenotypes which compose the group of patients with AD cirrhosis who were free of ACLF may have a distinctive baseline inflammatory profile. In addition, we wondered whether, among the patients with AD cirrhosis without ACLF, the baseline inflammatory profile was able to distinguish those who will develop ACLF during follow-up from who will not, as well as differentiate those who will die from those who will remain alive. To address these hypotheses, we investigated a battery of markers of systemic inflammation in a large cohort of 582 individuals including healthy controls, patients with compensated cirrhosis

Abbreviations: ACLF, acute-on-chronic liver failure; AD, acute decompensation; ADH, antidiuretic hormone; ALT, alanine aminotransferase; BUN, blood urea nitrogen; BT, bacterial translocation; CHE, cholinesterase; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HE, hepatic encephalopathy; HNA2, human non-mercaptalbumin-2; HRS, hepatorenal syndrome; HPLC, high performance liquid chromatography; IL, interleukin; IL-1ra, IL-1 receptor antagonist; INFγ, interferon gamma; INR, international normalized ratio; IP-10 (CXCL10), 10kDa interferon gamma-induced protein (C-X-C-motif chemokine 10); MCP-1 (CCL2), monocyte chemotactic protein 1 (C-C-motif chemokine 2); MELD, model for end-stage liver disease; MIP-1β, macrophage inflammatory protein 1-beta; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; SD, standard deviation; SEM, standard error of the mean; SI, systemic inflammation; TNFα, tumor necrosis factor alpha.

without prior decompensation, patients with AD who were free of ACLF, and patients with ACLF.

PATIENTS AND METHODS

Patients

In all patients, presence of cirrhosis was diagnosed either by unequivocal signs in imaging, presence of complications of portal hypertension or development of AD and/or ACLF. This study analyzed a total of 582 individuals, of whom 542 were patients with cirrhosis. Three hundred and forty-two of these had been enrolled in the CANONIC study and were selected because they had AD cirrhosis but no ACLF at enrollment (1). These 342 patients were compared to 39 patients with compensated cirrhosis who had never presented an episode of decompensation, and 40 healthy volunteers as negative controls. Moreover, 161 patients with ACLF (95 ACLF grade 1, 66 patients with ACLF grade 2) enrolled in the CANONIC study were selected to serve as positive controls, since those patients have an extensive elevation of all systemic inflammation markers. The selection of the CANONIC study patients was based on the availability of blood samples within the first 2 days after enrollment from patients under intensive surveillance during hospitalization (5). All patients gave their written informed consent. Each center obtained the ethics approval from the local ethics committee for the CANONIC study (1, 5).

Definition of AD Cirrhosis, OF, and ACLF

AD of cirrhosis was defined according to criteria established by the CANONIC study (1). Briefly, it includes acute development of large ascites, hepatic encephalopathy, gastrointestinal hemorrhage, bacterial infection, or any combination of these (1).

Individual OFs were diagnosed according to the CLIF-C OF score (1). Liver failure was defined by serum bilirubin of 12 mg/dl or more, kidney failure by creatinine of 2 mg/dl or more (or renal replacement therapy), coagulation failure by INR of 2.5 or more. Circulatory failure was diagnosed when vasopressors were used, and respiratory failure when the patient received mechanical ventilation (not due to HE-induced coma) or PaO_2/FiO_2 was 200 or lower. Finally, cerebral failure was defined as HE grade III and IV (1).

As mentioned earlier, three distinct phenotypes characterized of patients with AD without ACLF at admission, and ACLF was defined according to criteria established by the CANONIC study (1).

Data Collection

Healthy controls were recruited among 45–65 year-old medical and non-medical staff from the Hospital Clinic, while patients with compensated cirrhosis were recruited from the University Hospital Bologna, University Hospital Padova and Royal Free Hospital London and the data at baseline were recorded. Data from the CANONIC study patients were obtained as previously described (1, 5). Briefly, data from previous medical history, physical examination, and laboratory parameters were recorded at baseline, including etiology, previous episodes of acute decompensation, potential precipitating events and reason for hospitalization. Moreover, close 28-day follow-up data were collected according to the CANONIC protocol (1). Finally, information on liver transplantation, mortality and causes of death were obtained on day 28, and at 3 and 6 months and 1 year after enrollment.

Sample Collection and Analysis of biomarkers

The baseline blood samples were obtained in Vacutainer EDTA tubes at the time of enrollment in the study and/or within the first 2 days after enrolment in the study (48 h of hospital admission). Samples at the last assessment could be obtained in 132 patients. In all cases, blood was rapidly centrifuged at 4°C and the plasma frozen at −80°C until analysis.

We measured TNF-α, IL-6, IL-8, MCP-1, IP-10, MIP-1β, G-CSF, GM-CSF, IL-10, IL-1ra, INFγ, IL-17A, IL-7, and eotaxin in 25 μl of plasma using a multiplexed bead-based immunoassay (Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore, Darmstadt, Germany) on a Luminex 100 Bioanalyzer (Luminex Corp., Austin, TX). The readouts were analyzed with the standard version of the Milliplex Analyst software (Merck Millipore). A five-parameter logistic regression model was used to create standard curves (pg/mL) and to calculate the concentration of each sample. Finally, the levels of irreversibly oxidized albumin (HNA2) were assessed by high performance liquid chromatography (5) as marker of systemic oxidative stress. The levels of systemic inflammation markers in patients with ACLF have been published previously (5).

Statistical Analysis

Plasma levels were above detection limits in most patients. In healthy subjects and patients with values of cytokines or any other measurement below the detection limit, the threshold of detection was assigned as the determined value. Results are presented as frequencies and percentages for categorical variables, means and SDs for normally distributed continuous variables and medians with interquartile range for not normally distributed continuous variables. Hierarchical clustering analysis was performed using the GP plot package from R software. Intensity of inflammation was evaluated according to the relationship between the set of cytokines in different combinations stratifying for different groups of patients. In univariate statistical comparisons, Chi-square test was used for categorical variables, Student's *t*-test or ANOVA for normal continuous variables and Mann-Whitney *U*-test or Kruskal-Wallis test for non-normal continuous variables. Multiple-testing was corrected by the Bonferroni correction (corrected *p*-value for 15 markers $0.05/15 = 0.003$). To assess the strength of the association between each marker and ACLF, logistic regression models were performed. Factors showing a clinically and statistically significant association to the outcome in univariate analyses were selected for the initial model. The final models were fitted using a stepwise forward method based on likelihood ratios with the same significance level ($p < 0.05$) for entering and dropping variables. The proportional hazards model for competing risks proposed by Fine and Gray was used to identify independent predictors of mortality as previously

described (1). This model was chosen to account for liver transplantation as an event "competing" with mortality. Variables with a skewed distribution were log-transformed for statistical analyses and graphical comparisons. A p- ≤ 0.05 was considered statistically significant. Analyses were done with SPSS V. 23.0, SAS V.9.4, and R V.3.4.2 statistical packages.

RESULTS

General Characteristics of the Patients

This study investigated 15 markers of systemic inflammation and oxidative stress in 342 AD-patients but without ACLF at admission. These were compared to the levels measured in 161 patients admitted to the hospital with ACLF grade 1 or 2, 39 patients with compensated cirrhosis and no prior decompensation episode, and 40 healthy controls (**Supplementary Tables 1**,**2**). The reason for selecting only patients with ACLF grade 1 or 2 was to exclude severely diseased patients who had three OFs or more, since the enormous elevation of inflammatory markers in these patients may make difficult the comparison of their profile of systemic inflammation with that of patients with AD and without ACLF.

Importantly, our patients with compensated cirrhosis had never experienced any decompensation, despite the fact that these patients were at risk of developing it. Briefly, these patients had a mean value of 37.8 kPa (21.4–49.7 kPa) measured by Fibroscan® (Echosense, France) and median platelet count of 108 x 10^9/L (72–159 × 10^9/L), surrogates suggesting the presence of clinical significant portal hypertension (13). Moreover, in 18 (46%) patients, esophageal varices were already diagnosed. Of note, levels of systemic inflammation markers were only moderately altered in patients with compensated cirrhosis compared to healthy controls (**Supplementary Table 1**), indicating the absence of significant systemic inflammation in most of these patients. Of note, patients with compensated cirrhosis were analyzed only in a cross-sectional manner, precluding any assessment of the development of AD disease in these patients (**Supplementary Table 1**).

While the demography was similar, there were important, but expected between-group differences, with the most abnormal values being observed in the ACLF group (**Supplementary Table 2**).

Markers of Systemic Inflammation According to the Three Clinical Phenotypes in AD Patients

The profile of systemic inflammation markers significantly differed across the three phenotypes of AD without ACLF (AD-1, AD-2, and AD-3; **Figure 1**, **Table 1** depicting median values). Interestingly, lower levels of TNF-α (OR, 0.52; 95%-CI, 0.34–0.79), eotaxin (OR, 0.57; 95% CI, 0.38–0.86) and HNA2 (OR,

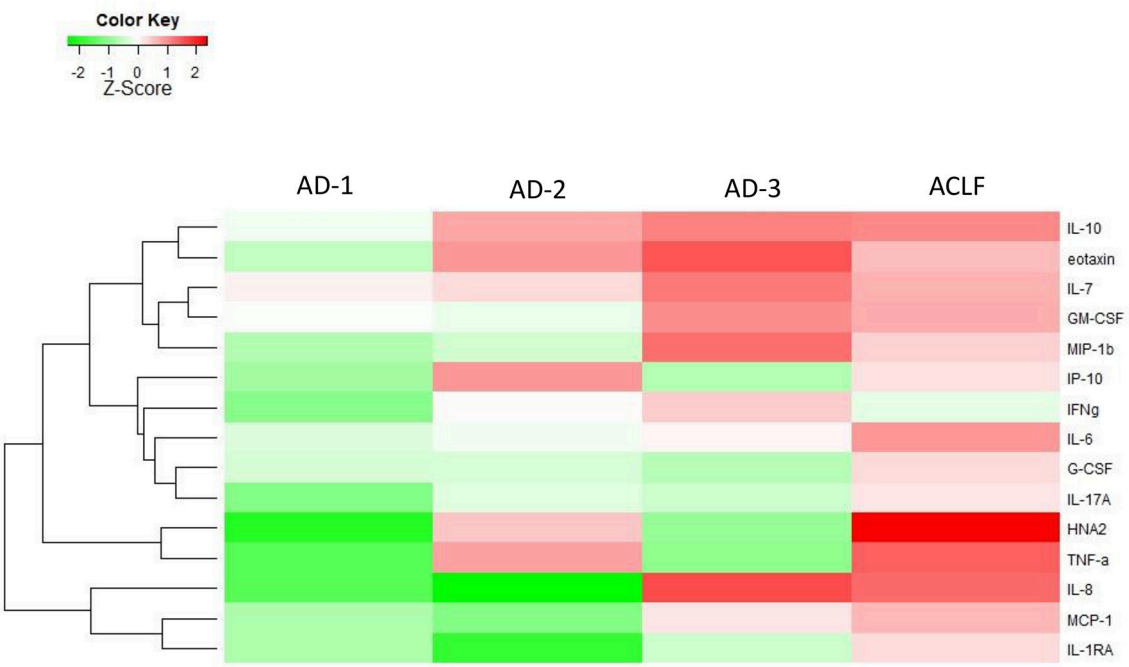

FIGURE 1 | Heat-map highlighting medians of the levels of the different biomarkers of systemic inflammation in patients with acutely decompensated (AD) cirrhosis (with and without ACLF). The patients with "ACLF-free" AD cirrhosis were stratified into three phenotypes. The first phenotype (AD-1) included patients without any single OF, who have serum creatinine of <1.5 mg/dL and do not have hepatic encephalopathy. The second phenotype (AD-2) included patients with isolated renal dysfunction and/or cerebral dysfunction, i.e., without any associated single non-renal, non-cerebral OF. The third phenotype (AD-3) included patients with a single non-renal OF, without any kidney dysfunction. The magnitude of the levels is color-coded and the clustering for each marker with the rest of the markers is shown to the left of the heat-map.

TABLE 1 | Clinical characteristics, routine laboratory tests, and inflammatory mediators, all at enrollment, across the three distinct phenotypes of patients with acutely decompensated (AD) cirrhosis who were free of ACLF (AD-1, AD-2, AD-3), and the group of patients with ACLF.

Variable	AD-1 (*N* = 155)	AD-2 (*N* = 121)	AD-3 (*N* = 66)	ACLF (*N* = 161)	*P*-value
CLINICAL CHARACTERISTICS					
Age—year	57.6 ± 11.90	59.2 ± 10.35	51.7 ± 11.25	57.3 ± 11.45	< 0.001
Male gender—no./total no. (%)	99/155 (63.9)	83/121 (68.6)	45/66 (68.2)	108/161 (67.1)	0.843
Mean arterial pressure—mm Hg	84.1 ± 10.87	83.1 ± 13.02	84.9 ± 10.63	80.8 ± 13.40	0.050
PRECIPITATING EVENTS—NO./TOTAL NO. (%)					
Alcohol consumption	19/144 (13.2)	7/112 (6.3)	14/59 (23.7)	27/150 (18.0)	0.008
Mortality at 90 days	22/155 (14.2)	21/121 (17.4)	12/66 (18.2)	59/161 (36.6)	< 0.001
ETIOLOGY OF CIRRHOSIS—NO./TOTAL NO. (%)					
Alcoholic	66/145 (45.5)	55/113 (48.7)	36/63 (57.1)	90/152 (59.)	0.080
HCV	40/145 (27.6)	28/113 (24.8)	10/63 (15.9)	27/152 (17.8)	0.109
Alcohol ± HCV	11/145 (7.6)	13/113 (11.5)	5/63 (7.9)	17/152 (11.2)	0.623
Others	28/145 (19.3)	17/113 (15.0)	12/63 (19.0)	18/152 (11.8)	0.300
MEDIAN VALUES FOR ROUTINE LABORATORY TESTS (IQR)					
Serum albumin—g/dl	2.9 (2.50–3.25)	2.9 (2.60–3.30)	2.8 (2.40–3.10)	3.0 (2.40–3.40)	0.157
Serum bilirubin—mg/d	2.5 (1.49–5.18)	2.8 (1.56–5.20)	13.7 (4.90–22.30)	6.1 (2.00–14.37)	< 0.001
Serum creatinine—mg/dl	0.8 (0.66–1.00)	1.5 (0.98–1.70)	0.8 (0.70–1.05)	2.2 (0.98–3.04)	< 0.001
C-reactive protein—mg/L	16.7 (6.80–41.40)	17.9 (6.50–43.00)	19.9 (6.50–34.00)	25.0 (9.70–50.40)	0.163
International Normalized Ratio	1.5 (1.27–1.73)	1.5 (1.27–1.66)	1.7 (1.39–2.20)	1.7 (1.37–2.30)	< 0.001
Platelets— $\times 10^9$/L	95.0 (63.00–131.00)	84.5 (53.50–139.50)	81.0 (43.00–135.00)	76.0 (53.00–121.00)	0.094
White-cell count— $\times 1\ 0^9$/L	6.5 (4.38–9.88)	6.1 (4.60–7.90)	6.6 (4.08–10.72)	8.0 (5.30–12.20)	< 0.001
MEDIAN VALUES FOR INFLAMMATORY MEDIATORS (IQR)					
TNF-α—pg/ml	17.9 (13.43–26.64)	24.2 (17.88–34.23)	19.9 (13.05–29.26)	29.0 (17.38–42.83)	< 0.001
IL-6—pg/ml	21.2 (11.71–44.47)	25.0 (12.75–54.20)	24.6 (14.49–47.12)	36.7 (13.79–106.83)	< 0.001
IL-8—pg/ml)	35.9 (19.45–75.22)	37.3 (21.84–63.59)	78.4 (41.69–220.92)	84.5 (38.64–165.10)	< 0.001
MCP-1—pg/ml	316.8 (235.9–395.7)	323.1 (209.49–455.92)	372.2 (254.60–494.73)	410.3 (293.88–690.00)	< 0.001
IP-10—pg/ml	904.6 (530.4–1499.0)	1200.0 (627.0–2255.0)	950.3 (639.9–1718.0)	1147.0 (651.2–2123.0)	0.022
MIP-1ß—pg/ml	20.1 (13.55–37.43)	22.6 (12.93–34.30)	30.2 (16.88–45.41)	26.2 (17.89–42.55)	0.002
G-CSF—pg/ml	22.6 (12.32–54.85)	24.0 (11.19–52.24)	23.0 (12.80–46.65)	30.5 (13.85–81.63)	0.186
GM-CSF—pg/ml	5.0 (2.28–9.08)	4.5 (1.75–10.98)	7.5 (3.68–15.87)	6.8 (3.47–15.97)	0.003
IL-10—pg/ml	3.0 (0.90–9.36)	4.0 (1.37–13.07)	5.3 (1.88–13.39)	7.2 (1.90–25.78)	< 0.001
IL1-ra—pg/ml	11.9 (5.40–25.90)	10.4 (4.52–23.03)	13.4 (6.14–40.97)	18.7 (8.56–50.48)	< 0.001
IFNγ—pg/ml	4.4 (1.65–19.38)	6.7 (2.38–24.29)	7.8 (2.82–18.85)	6.0 (2.32–23.12)	0.107
Eotaxin—pg/ml	103.7 (68.43–152.08)	120.4 (90.48–162.49)	137.0 (94.67–173.76)	123.5 (86.55–177.21)	0.008
IL-17A—pg/ml	3.1 (1.37–13.10)	4.2 (2.03–10.00)	3.8 (1.75–11.31)	4.8 (1.62–14.90)	0.376
IL-7—pg/ml	2.5 (0.85–7.46)	2.7 (1.13–11.74)	4.3 (1.38–8.80)	3.5 (1.62–11.07)	0.135
HNA2—%	4.2 (2.35–8.07)	6.9 (3.79–10.99)	5.6 (3.07–8.85)	11.0 (6.25–15.15)	< 0.001

Patients with acutely decompensated cirrhosis were classified as having the AD-1, AD-2, or AD-3 phenotype or having ACLF according to the EASL-CLIF Consortium criteria (1, 2). Data are shown as means ± SD or median (range). P-values were calculated by unpaired Students' t-test or Kruskall-Wallis test where appropriate.

HCV denotes hepatitis C virus; IQR interquartile range; TNF, umor necrosis factor; IL, interleukin; MCP-1, monocyte chemotactic protein 1; IP-10, 10 kDa interferon gamma-induced protein; MIP-1ß, macrophage inflammatory protein 1-beta; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1ra, interleukin-1 receptor antagonist protein; IFN, interferon; HNA2, human non-mercaptalbumin 2.

0.64; 95% CI, 0.45–0.91) were independently associated with AD-1, while higher levels of TNF-α (OR, 3.25; 95% CI, 2.00–5.28) and HNA2 (OR, 1.75; 95% CI, 1.20–2.55) but lower levels of IL-8 (OR, 0.67; 95% CI, 0.53–0.85) were independently associated with AD-2 (renal and/or cerebral dysfunction, **Supplementary Table 3**).

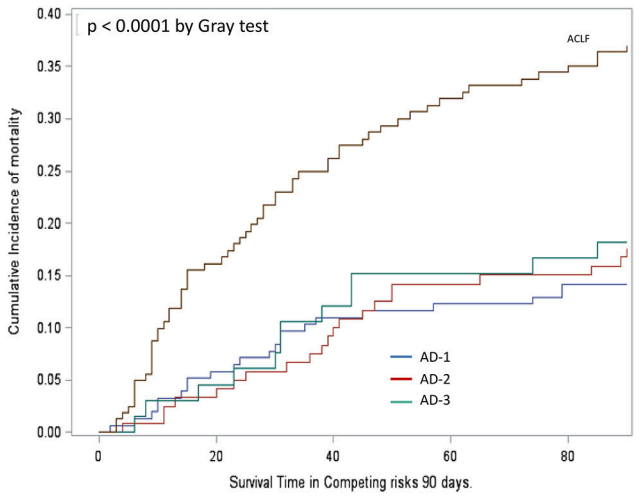

FIGURE 2 | Cumulative incidence function assessing survival in patients' groups analyzed in **Figure 1**. Mortality was significantly higher in patients with ACLF than in those without, irrespective of their phenotype, AD-1, AD-2, or AD-3 (Gray's test $p < 0.0001$). Mortality did not significantly differ between the three phenotypes AD-1, AD-2, and AD-3. For definitions of these phenotypes, see **Figure 1** legend.

By contrast, higher levels of IL-8 (OR, 2.30; 95% CI, 1.72–3.06) and lower levels of G-CSF (OR, 0.78; 95% CI, 0.64–0.94) were independently associated with isolated non-renal OF (AD-3, **Supplementary Table 3**). Importantly, all these results were independent of presence of infection, since we could not find any association of those markers with the presence of infection in the respective stratification of the patients, while IL-6 was independently associated with infection in the entire cohort (OR, 1.36; 95%-CI 1.13–1.65; $p = 0.01$).

Interestingly, the pattern of elevated markers for patients in AD-2 and AD-3 were opposite to each other, i.e., markers that were elevated in AD-2 were lower in AD-3 and vice-versa (**Figure 1**). The addition of elevated markers in AD-2 with the elevated markers in AD-3, recapitulated the profile of systemic inflammation seen in ACLF (**Figure 1**).

Importantly, not only the distribution of elevated biomarkers, but also the quantitative changes in their levels defined their affiliation to either AD-1, AD-2, or AD-3 (**Figure 1**, **Table 1**). Another interesting finding was that patients with ACLF did not show the highest levels of the single markers, but the highest number of elevated markers (**Figure 1**), suggesting a "full-blown" systemic inflammation in this group of patients and a rather attenuated systemic inflammation in the groups of patients without ACLF.

Another important observation was that despite the significant differences between the severity and profile of systemic inflammation markers across the three clinical phenotypes of "ACLF-free" AD cirrhosis, the cumulative incidence of death by 90 days, was similar irrespective

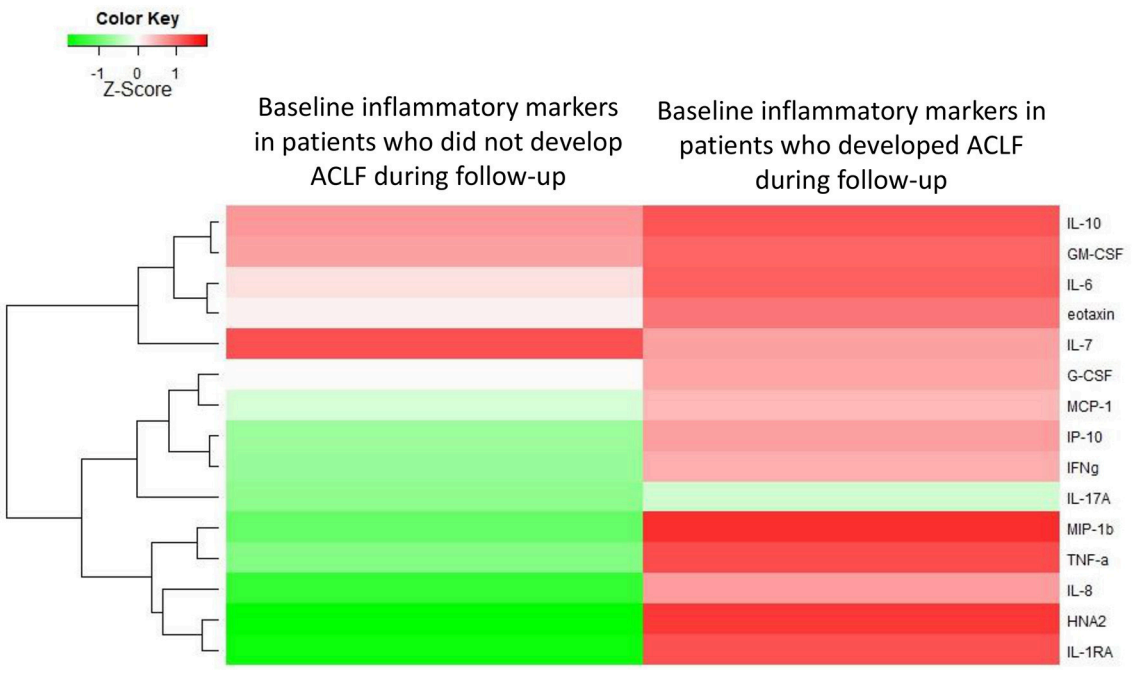

FIGURE 3 | Heat-map showing the median levels of systemic inflammation markers at enrollment of patients with acutely decompensated cirrhosis who were free of ACLF. For the comparison, patients were divided into two groups according to their outcome (i.e., development of ACLF or not, during 28 days of follow-up). The magnitude of the levels is color-coded and the clustering for each marker with the rest of the markers is shown to the left of the heat-map.

of the phenotype (**Figure 2**). In contrast, the "full-blown" systemic inflammation observed in patients with ACLF was associated with increased cumulative incidence of death by 90 days (**Figure 2**).

Predicting ACLF Development Using Baseline Systemic Inflammation Profiles

Next, we asked whether among AD-patients without ACLF at admission, the baseline systemic inflammation profile differed between those who will subsequently develop ACLF relative to those who will not develop this syndrome. Among the 342 patients with AD at admission, 57 developed ACLF within 28 days after admission. Importantly, baseline levels of systemic inflammation markers were significantly higher among patients who subsequently developed ACLF than among those who remained free of ACLF during the 28-day follow-up (**Figure 3**, **Table 2**). Therefore, in AD-patients without ACLF at admission, the development of ACLF can be predicted using the baseline profile of systemic inflammation-related markers.

When observing the magnitude of specific markers among patients with AD cirrhosis who were free of ACLF on admission, we saw that higher baseline levels of IL-6 (OR, 1.43; 95% CI, 1.04–1.96; $p = 0.03$), IL-1ra (OR, 1.46; 95%-CI 1.10–1.93; $p = 0.009$) and HNA2 (OR, 2.84; 95%-CI 1.52–5.34; $p = 0.001$) were independently associated with development of ACLF within 28 days.

Baseline Profiles Predicting Survival in Patients With "ACLF-free" AD cirrhosis

Among AD-patients without ACLF at hospital admission 55 died and 28 received a liver transplant. The baseline levels of several markers were significantly higher in patients who subsequently died than in those patients who survived (**Supplementary Table 4**; **Figure 4**). In particular, TNF-α, IL-6, IL-8, IL-10, eotaxin, IL-17A, IL-7, and HNA2 were higher in patients who died (**Supplementary Table 4**). Nevertheless, only IL-8 and HNA2 were independently associated with mortality in the patients with AD at baseline (**Table 3**).

DISCUSSION

This study offers a homogeneous classification way in the heterogeneous population of patients with acutely decompensated cirrhosis, which is related to ACLF development and death. Beyond the large sample size, our study provides novel information by showing that each of the three distinct clinical phenotypes, which compose the group of patients with AD cirrhosis who were free of ACLF had a distinctive baseline inflammatory profile. A second aspect of the novelty in our results is that, among the patients with AD cirrhosis without ACLF, the baseline inflammatory profile was able to distinguish those who will develop ACLF during follow-up from who will not. Finally, we showed that among the patients with AD cirrhosis without ACLF, the baseline inflammatory profile was different between those who will die at short-term and those who will survive.

This novel point of view is demonstrated in four major findings of the present study discussed in the following. The first was that inflammatory markers were only slightly altered in patients with compensated cirrhosis and no prior episode of decompensation. This finding is surprising and interesting considering that many of these patients had clinical significant portal hypertension, as assessed either by the presence of esophageal varices and/or high liver stiffness and low platelets (15). By contrast, most inflammatory mediators were markedly increased in patients admitted to hospital with AD (with or without ACLF). Indeed, this observation is of importance since it shows that severe systemic inflammation and acute decompensation of cirrhosis are concomitant processes, as proposed in the so-called "Systemic Inflammation Hypothesis" (16). This novel finding is probably a result of the careful review of the medical history of the patients included in the compensated control group, excluding any patients with compensated cirrhosis who had prior history of AD episodes. Although it remains unclear which of these processes (acute decompensation or severe systemic inflammation) occurs first, it is tempting to assume that systemic inflammation is a prerequisite for the development of AD cirrhosis. In any case, our findings suggest that systemic inflammation may serve to classify the stage of disease in patients with cirrhosis.

The second important observation was that patients with AD but without ACLF at admission had a very heterogeneous profile of circulating inflammatory mediators. There were three distinct clinical phenotypes (AD-1, AD-2, and AD-3) characterizing those AD patients; each phenotype being associated with distinct profile of systemic inflammation, irrespective of the fact that infection was present or not. The patients hospitalized with AD cirrhosis and neither OF, renal dysfunction nor cerebral dysfunction (AD-1 phenotype), had very mild systemic inflammation, while the patients with an isolated non-renal OF (AD-3 phenotype), and those with isolated renal and/or cerebral dysfunction (AD-2 phenotype) had a higher number of markedly increased markers of systemic inflammation. Moreover, our results obtained in patients with "ACLF-free" AD cirrhosis, suggest a potential explanation for the systemic inflammation signature of ACLF, which can be seen as a result of continuum of activation of systemic inflammation. Indeed, according to the EASL-CLIF consortium definition, the combination of any single non-renal, non-cerebral OF with renal and/or cerebral dysfunction defines ACLF grade 1. While some markers of inflammation were elevated in patients with AD-3 phenotype, other markers were elevated in patients with AD-2 phenotype. As suggested by **Figure 1**, the profile of systemic inflammation in ACLF could be seen as merging of the inflammatory profile of the AD-2 phenotype and that of the AD-3 phenotype. It could be argued that the division of ACLF-free AD cirrhosis into three phenotypes was arbitrary, one phenotype (AD-2) being more severe than the two others. However, several features do not support this contention. First, the rationale for dividing this group of patients into three distinct phenotypes was based on clinical evidence provided by the CANONIC study (1). Second, the results of the present study now provide a biological support to this distinction by showing that each clinical phenotype was

TABLE 2 | Baseline characteristics of 342 patients with acutely decompensated cirrhosis who were free of ACLF at enrollment, divided into two groups according to the development or the absence of development of ACLF during 28 days of follow-up.

Characteristic	Without ACLF development (*N* = 285)	With ACLF development (*N* = 57)	*P*–value
Age— year	56.7 ± 11.63	58.5 ± 11.00	0.2847
Male gender — no./total no. (%)	191/285 (67.0)	36/57 (63.2)	0.5734
Mean arterial pressure — mm Hg	83.8 ± 11.84	84.2 ± 10.46	0.8259
PRECIPITATING EVENTS — NO./TOTAL NO. (%)			
Alcohol consumption	33/265 (12.5)	7/50 (14.0)	0.7631
Mortality 90 days	29/285 (10.2)	26/57 (45.6)	< 0.0001
ETIOLOGY OF CIRRHOSIS — NO./TOTAL NO. (%)			
Alcoholic	128/269 (47.6)	29/52 (55.8)	0.2797
HCV	66/269 (24.5)	12/52 (23.1)	0.8224
Alcohol + HCV	26/269 (9.7)	3/52 (5.8)	0.5957
Others	49/269 (18.2)	8/52 (15.4)	0.6248
MEDIAN VALUES FOR LABORATORY VARIABLES (IQR)			
Serum albumin—g/dl	2.9 (2.60–3.30)	2.7 (2.19–3.01)	0.0049
Serum bilirubin—mg/d	3.0 (1.60–6.70)	4.4 (1.69–9.06)	0.0963
Serum creatinine—mg/dl	0.9 (0.70–1.32)	1.1 (0.80–1.40)	0.1285
C-reactive protein—mg/L	16.2 (6.00–39.60)	25.8 (10.00–45.60)	0.0887
International Normalized Ratio	1.5 (1.27–1.70)	1.7 (1.42–1.94)	0.0004
Platelets—x10^9/L	87.0 (57.00–139.00)	94.5 (57.50–119.50)	0.9581
White-cell count—x10^9/L	6.1 (4.08–9.03)	7.3 (5.04–10.53)	0.0146
MEDIAN VALUES FOR INFLAMMATORY MEDIATORS (IQR)			
TNF-α—pg/ml	19.5 (14.14–27.37)	26.7 (17.69–36.52)	< 0.001
IL-6—pg/ml	21.2 (11.27–41.32)	34.3 (19.85–105.54)	< 0.001
IL-8—(pg/ml)	37.3 (20.45–76.41)	58.7 (40.89–108.34)	< 0.001
MCP-1—pg/ml	318.0 (228.03–436.02)	360.0 (276.53–586.47)	0.013
IP-10—pg/ml	965.2 (557.62–1676.00)	1272.0 (760.31–2150.00)	0.053
MIP-1ß—pg/ml	20.1 (13.14–33.55)	36.9 (23.88–56.10)	< 0.001
G-CSF—pg/ml	22.6 (11.19–49.93)	31.0 (15.86–69.19)	0.048
GM-CSF—pg/ml	4.7 (1.96–9.48)	10.7 (3.76–20.05)	< 0.001
IL-10—pg/ml	3.4 (1.12–9.15)	8.0 (2.45–26.42)	0.002
IL1-ra—pg/ml	9.9 (4.72–22.47)	24.0 (12.81–62.11)	< 0.001
IFNγ—pg/ml	5.5 (2.00–18.11)	9.2 (3.20–31.34)	0.013
Eotaxin—pg/ml	110.4 (80.76–155.42)	135.8 (94.97–186.19)	0.008
IL-17A—pg/ml	3.7 (1.57–10.25)	3.4 (1.98–24.37)	0.214
IL-7—pg/ml	2.6 (0.99–8.50)	5.1 (1.88–14.52)	0.032
HNA2—%	4.5 (2.50–8.82)	8.1 (4.89–9.95)	< 0.001

Patients with acutely decompensated cirrhosis were classified as being free of ACLF or having ACLF according to the EASL-CLIF Consortium criteria (1, 14). Data are shown as means ± SD or median (range). P-values were calculated by unpaired Students' t-test or Man-Whitney U-test where appropriate.

HCV, hepatitis C virus; IQR, interquartile range; TNF, umor necrosis factor; IL, interleukin; MCP-1, monocyte chemotactic protein 1; IP-10, 10 kDa interferon gamma-induced protein; MIP-1ß, macrophage inflammatory protein 1-beta; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1ra, interleukin-1 receptor antagonist protein; IFN, interferon; HNA2, human non-mercaptalbumin 2.

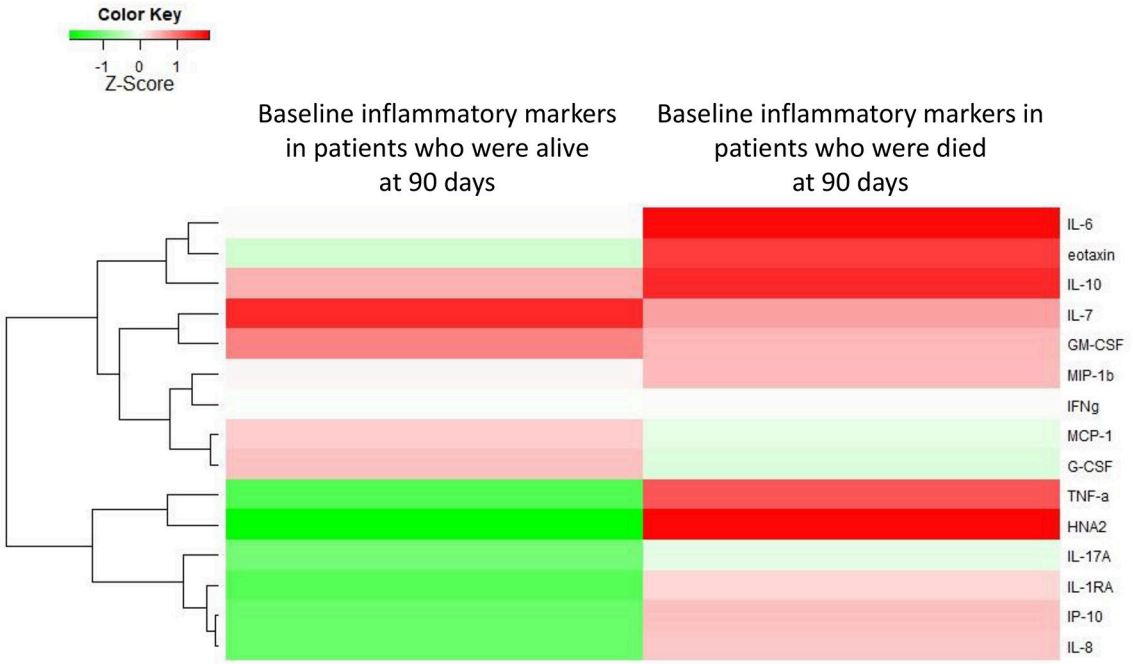

FIGURE 4 | Heat-map showing the median levels of systemic inflammation markers at enrollment of patients with acutely decompensated cirrhosis who were free of ACLF. For the comparison, patients were divided into two groups according to their outcome (i.e., occurrence of death or not during 90 days of follow-up). The magnitude of the levels is color-coded and the clustering for each marker with the rest of the markers is shown to the left of the heat-map.

TABLE 3 | Hazard ratios for death at 90 days in univariate and multivariable analyses of inflammatory mediators assessed at enrolment of 342 patients with acutely decompensated cirrhosis who were free of ACLF.

Inflammatory mediator	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% IC	*P*-value	Hazard ratio	95% IC	*P*-value
Tumor necrosis factor-α	1.650	(1.056–2.578)	0.028	–	–	–
Interleukin-6	1.389	(1.094–1.764)	0.007	–	–	–
Interleukin-8	1.521	(1.256–1.842)	<0.001	1.608	(1.304–1.982)	<0.001
Eotaxin	2.765	(1.392–5.492)	0.004	–	–	–
Interleukin-17A	1.217	(1.033–1.434)	0.019	–	–	–
Interleukin-7	1.221	(1.047–1.422)	0.011	–	–	–
Human non-mercaptalbumin 2	2.116	(1.456–3.074)	<0.001	2.237	(1.506–3.323)	<0.001

The final models were fitted using a stepwise forward method based on likelihood ratios with the same significance level ($p < 0.05$) for entering and dropping variables. IC denotes confidence interval.

associated with a specific inflammatory profile. Third, none of these inflammatory profiles was as intense as the profile found in patients with ACLF. It was also interesting that, although marked differences in systemic inflammation profiles existed between the three clinical phenotypes of "ACLF-free" AD cirrhosis, there were no significant differences in survival between these three phenotypes. Together these findings indicate that the division into 3 phenotypes was not arbitrary, and more importantly did not underestimate the severity of one phenotype, in particular of the AD-2 phenotype. Our data are novel and very important, indicating that not a maximum level of a specific biomarker, but rather the extension (number of elevated markers) of systemic inflammation, such as that observed in ACLF, must be reached to determine increased mortality. Yet further studies are required to refine the risk assessment in these phenotypes.

There were, however, some differences in the pattern of systemic inflammation across the three clinical phenotypes of "ACLF-free" AD cirrhosis. For example, the presence of an isolated renal and/or cerebral dysfunction was independently associated with high TNF-α levels, while an isolated single non-renal OF was associated with low TNF-α levels. The reasons for these between-group differences in TNF-α expression are unclear but may explain some interesting observations of prior studies. Thus, large-scale trials in severe alcoholic hepatitis showed that anti-TNF approaches (e.g., pentoxifylline) might not work in patients with severe disease and liver failure, but had positive

effects in the presence of renal failure (17, 18). Pentoxifylline has also been shown to improve outcomes in patients with alcoholic hepatitis and hepato-renal syndrome (19, 20).

In our study, some markers (i.e., TNF-alpha, HNA2, and IP-10) were elevated in AD-2 patients and lower in AD-3 patients, features, which could seem counterintuitive. We have no clear explanation for these differences, which contribute to the fact that each clinical phenotype has a specific inflammatory profile. We can only speculate on the pathophysiological consequences of differences in some marker levels. For example, TNF-alpha is known to protect the liver by stimulating liver regeneration (21). Therefore, increased TNF-alpha levels in AD-2 may be involved in the absence of liver failure in this group, and, conversely, low TNF-alpha levels could favor the development of liver failure in AD-3 patients. Obviously, future studies are needed. Surprisingly, TNF-alpha and MCP-1 levels were both lower in patients with compensated cirrhosis than in healthy subjects. We do not have clear explanations for these differences, only hypotheses. As mentioned earlier, TNF-alpha is known to stimulate liver regeneration. Therefore, low TNF-alpha levels in patients with compensated cirrhosis may reflect an insufficient TNF-alpha production in the liver, playing a role in subclinical liver failure in these patients. Regarding MCP-1, one should have in mind that this chemokine is produced by damaged tissues to attract monocytes whose function, once migrated, is to restore tissue homeostasis. Therefore, low MCP-1 levels in compensated cirrhosis may result in defective tissue homeostasis.

A third highly relevant finding was the observation that patients with AD cirrhosis who were free of ACLF at enrollment but subsequently developed ACLF within 28 days, had significantly higher baseline levels of inflammatory mediators. Moreover, these patients showed a distinct signature of systemic inflammation, relative to those who did not develop ACLF. These findings reveal that systemic inflammation precedes the development of ACLF, suggesting a cause-to-effect relationship. Importantly, in our study, higher IL-6 levels independently predict ACLF development, a finding which is consistent with previous results showing that elevated IL-6 levels were strongly associated with ACLF and its progression (5). Moreover, higher IL-1ra levels were independently associated with development of ACLF, which is fully in line with previous data demonstrating that polymorphisms of IL-1ra predispose to ACLF (22). Finally, HNA2, a marker for oxidative stress, was independently associated with ACLF development (5, 23). This latter finding calls for an important discussion not only on the pathogenesis of ACLF, but also on the prophylactic treatment since albumin is a potent immune modulator involved in reducing oxidative stress. In fact, there is strong evidence that albumin administration during an episode of spontaneous bacterial peritonitis prevents type I HRS—which represents a special form of ACLF—and improves survival (24). This has also recently been confirmed in the ANSWER trial, a randomized controlled trial in almost 400 patients, showing that long-term weekly albumin administration reduces the incidence of organ failure and thereby improves overall survival in decompensated cirrhotic patients (25). Further studies (e.g., PRECIOSA, NCT03451292) are underway.

Finally, in patients with "ACLF-free" AD cirrhosis, the extension of systemic inflammation at baseline was associated with 90-day mortality. The independent predictors of death were higher levels of IL-8 and HNA2 suggesting that decreasing the levels of these two inflammation-related markers may be an objective for future therapies aiming to increase survival in the group of patients with AD who are at high risk of death. Of note, among patients with AD at enrollment, those who will die had lower G-CSF levels than those who will survive. These patients might benefit from G-CSF therapy as recently shown in patients with ACLF (26).

Although the present study tested a large number of patients and a large number of systemic inflammation mediators, it has its limitations. The concept of this study is to observe systemic inflammation associated with AD cirrhosis (with and without ACLF) without taking into account specific events that could have precipitated the acute decompensation of cirrhosis (e.g., data about use of anti-inflammatory drugs, although unlikely since contraindicated in these patients). Moreover, there might be inter-center heterogeneity in the diagnosis of portal hypertension, although each liver unit participating in the CANONIC study had expertise in the diagnosis and treatment of complications of cirrhosis, therefore limiting the inter-center heterogeneity. Finally, this study did not aim to elaborate on a specific score, but rather to offer pathophysiologic insight into the role of systemic inflammation in patients with AD. Future studies are needed to further elaborate the specific events in AD cirrhosis.

In conclusion, baseline inflammatory markers exhibit no or slight abnormalities in compensated cirrhosis, while in "ACLF-free" AD cirrhosis their profile was heterogeneous, being markedly elevated in those who developed ACLF during follow up. Moreover, among patients with AD cirrhosis who were free of ACLF, this study showed a specific baseline profile of circulating inflammatory mediators in patients who died during follow-up.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JT, PG, RJ, AG, MB, PA, MP, RM, JC, and VA: study concept and design; JT, AlA, CP, ET, JA-Q, CD, JF-G, SP, PC, KO, JM, ES, WL, MC, TW, CS, RG, TG, MR, and AdG: acquisition of data; JT, AlA, CP, JF-G, KO, JM, RPM, WL, AdG, MP, RM, JC, and VA: analysis and interpretation of data; JT, AlA, CP, AG, RM, JC, and VA: drafting of the manuscript; JT, AlA, CP, JA-Q, CD, SP, PC, MP, and JC: statistical analysis; All authors: critical revision of the manuscript regarding important intellectual content; JT and VA: funding recipient; ET, RoS, SP, RPM, WL, TG, RB, AgA, SZ, VV, FS, FN, CA, AdG, PG, RJ, TS, AG, RuS, MB, PA, RM, JA-Q, and VA: administrative, technical and material support; JT, JF-G, PC, RPM, WL, TG, RB, AgA, SZ, VV, FS, FN, CA, AdG, PG, RJ, TS, AG, RuS, MB, PA, RM, JA, and VA: study supervision.

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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.2019.00476/full#supplementary-material

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frontiers in Immunology

MINI REVIEW
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Targeting Myeloid-Derived Cells: New Frontiers in the Treatment of Non-alcoholic and Alcoholic Liver Disease

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Non-alcoholic fatty liver disease (NAFLD) and Alcoholic Liver Disease (ALD) are major causes of liver-related morbidity and mortality and constitute important causes of liver transplantation. The spectrum of the liver disease is wide and includes isolated steatosis, steatohepatitis, and cirrhosis. The treatment of NAFLD and ALD remains, however, an unmet need, and therefore it is a public health priority to develop effective treatments for these diseases. Alcoholic and non-alcoholic liver disease share common complex pathogenetic pathways that involve different organs and systems beyond the liver, including the gut, the adipose tissue, and the immune system, which cross-talk to generate damage. Myeloid-derived cells have been widely studied in the setting of NAFLD and ALD and are implicated at different levels in the onset and progression of this disease. Among these cells, monocytes and macrophages have been found to be involved in the induction of inflammation and in the progression to fibrosis, both in animal models and clinical studies and they have become interesting potential targets for the treatment of both NAFLD and ALD. The different mechanisms by which these cells can be targeted include modulation of Kupffer cell activation, monocyte recruitment in the liver and macrophage polarization and differentiation. Evidence from preclinical studies and clinical trials (some of them already in phase II and III) have shown encouraging results in ameliorating steatohepatitis, fibrosis, and the metabolic profile, individuating promising candidates for the pharmacological treatment of these diseases. The currently available results of myeloid-derived cells targeted treatments in NAFLD and ALD are covered in this review.

Keywords: myeloid-derived cells, NAFLD (non-alcoholic fatty liver disease), ALD (alcoholic liver disease), treatment, liver immunology

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INTRODUCTION

Fatty liver represents a wide spectrum of disease encompassing stages ranging from isolated steatosis to steatohepatitis and it can be accompanied by different grades of fibrosis up to cirrhosis with all its complications, including hepatocellular carcinoma. The onset of fatty liver can occur in the presence or in absence of excessive alcohol consumption. The cut-off of a daily alcohol consumption $\geq$30 g for men and $\geq$20 g for women (1) is used to differentiate alcoholic vs.

non-alcoholic fatty liver disease. Therefore, the presence of fatty liver identifies non-alcoholic fatty liver disease (NALFD) in the absence of excessive alcohol consumption, and alcoholic liver disease (ALD), in the presence of excessive alcohol consumption. The presence at liver histology of steatosis, as well as both lobular inflammation and hepatocyte ballooning specifically identifies steatohepatitis [respectively, non-alcoholic steatohepatitis (NASH) and alcoholic steatohepatitis (ASH), depending on whether or not there is an association with excessive alcohol consumption] (2) (**Figure 1**).

Although they share similar histologic characteristics, these two entities have different peculiarities. NAFLD can be associated to metabolic impairment and to cardiovascular disease and is considered the hepatic expression of the metabolic syndrome (3, 4) (**Table 1**).

ALD, instead, is specifically related to alcohol-induced damage, including alcoholic hepatitis (AH), which represents a severe type of ASH, usually associated with more severe clinical course and histological lesions (5).

This dichotomy is, however, not always so unequivocal and appears—at least in part—arbitrary, given that patients consuming moderate amounts of alcohol may also have metabolic risk factors that predispose them to NAFLD and these metabolic factors seem to have a higher impact on the occurrence of steatosis and fibrosis (6).

The prevalence of NAFLD in the Western adult population is 25–30%, and even higher in populations with risk factors such as obesity or diabetes (7). About 20% of heavy drinkers develop fatty liver (8) and 35–40% of patients with chronic excessive alcohol abuse develop alcoholic hepatitis (AH) (9). ALD and NAFLD, respectively, represent the second and the third cause of liver transplantation and NAFLD has been estimated to become the primary cause of liver transplantation in the next decades (10).

Given the burden of these diseases, understanding the complex underlying mechanisms and the crosstalk between the different organs involved in the pathogenesis of NAFLD and ALD (and specifically of steatohepatitis) has been a research priority in the last decade, also in order to identify possible therapeutic targets.

The pathogenesis of NAFLD is complex and implicates the crosstalk between different metabolically active sites. Initially, the so called "two hits" hypothesis was proposed: insulin resistance, the "first hit," leads to hepatic triglyceride accumulation (steatosis) and is followed by a "second hit" driven by, amongst others, oxidative stress, which in turn favors the development of steatohepatitis and fibrosis (11). Subsequent research has transformed this model into a "multiple parallel hits" hypothesis in which a number of different processes involving various organs such as adipose tissue, gut and muscle contribute to a cascade of inflammation, fibrosis and eventually tumorigenesis. In this setting, endoplasmic reticulum stress, cytokines, adipokines, and immunity are emerging drivers of liver damage (12).

The pathogenesis of ALD largely relates to the direct toxic effects of alcohol and its intermediate metabolite acetaldehyde. Together, these agents induce oxidative stress, mitochondrial damage, lipogenesis, hepatic fat accumulation—through increased influx of free fatty acids originating from the adipose tissue and gut-derived chylomicrons—, malnutrition, and leakage of endotoxins from the gut. Subsequently, these processes will result in the activation of a myriad of immune cells [including Kupffer cells (KC)] and the secretion of proinflammatory cytokines (13). [For an extensive review about the pathogenesis of, respectively, NAFLD and ALD see (13–16)].

Moreover, the liver itself displays immune properties, and can be viewed as an "immunological organ" (14, 17, 18). Many immune cell populations have been studied and have been implicated in the pathogenesis of fatty liver (both alcohol and non-alcohol related) and may act as treatment targets (**Figure 2**).

Currently, no drugs are approved for the treatment of fatty liver, constituting an unmet medical need and a public health priority. Concerning possible treatment targets for fatty liver, several considerations should be noted. Firstly, a candidate target to block one or more pathways involved in the pathogenesis of the disease should be identified. Secondly, the "aim" of the treatment, i.e., reduction of either disease activity (i.e., steatohepatitis) or fibrosis progression, should be determined. Currently, the most desired outcome is still under debate. In the setting of NAFLD, while fibrosis has been identified as the most important predictor of both liver- and non-liver-related adverse outcomes [including overall and liver-related mortality (with a decline in prognosis from F2 onwards)], steatohepatitis is considered the driving force of these outcomes (19). This dichotomy, however, seems to be rather artificial, given that different pathways overlap and fibrosis progression is probably to be considered a marker of long-standing disease activity (and therefore a driver of the outcome). Considering ALD—aside from the fact that the cornerstone of any therapeutic intervention is alcohol abstinence—the same general concepts described above are true (20). Moreover, AH, steatosis, fibrosis itself, and especially alcoholic steatohepatitis are all independent predictive factors of fibrosis progression (1, 21).

MYELOID-DERIVED CELLS

Among the myeloid-derived cells (**Table 2**), monocytes and macrophages play an important role in the onset of both fatty liver and fibrosis. The liver harbors about 80% of all macrophages of the body and is also patrolled by other myeloid cells (such as blood monocytes), which scan the liver vasculature and eventually infiltrate into the liver. Monocyte-derived cells can develop into liver dendritic cells or monocyte-derived macrophages, the former being mainly responsible for antigen presentation of small or soluble structures to adaptive immune cells and the latter acting as primary filter cells, constantly removing particles from the circulation. KC are resident macrophages that belong to the reticuloendothelial system in the liver, which constitutes a primary line of defense against invading microorganisms, functions as a sensor for altered tissue integrity and largely contributes to maintain tissue homeostasis by contributing to the anti-inflammatory micromilieu as well as directly inducing tolerance in passenger leukocytes patrolling the sinusoidal system (22).

Traditionally, macrophages were categorized dichotomically in either pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes. These cells, however, display a broad spectrum

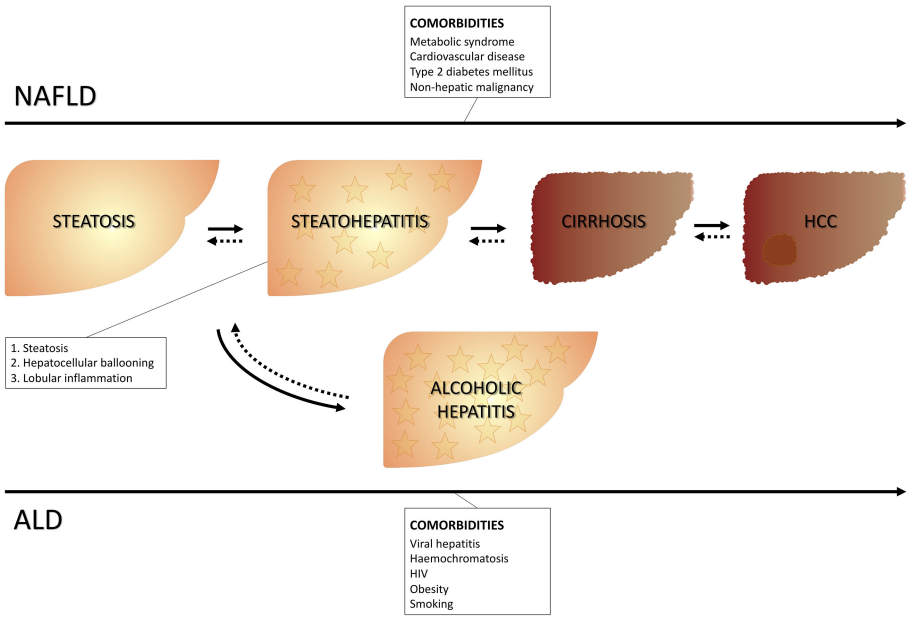

FIGURE 1 | Clinical course of NAFLD and ALD. NAFLD, Non-alcoholic liver disease; ALD, Alcoholic liver disease.

TABLE 1 | Definition of metabolic syndrome (3, 4).

Required	Waist circumference ≥94/80 cm for men/women
Number of abnormalities	≥2 of
Hypertension	≥130/85 mmHg or treatment for hypertension
Fasting glucose	≥100 mg/dl or treatment for type 2 diabetes
Triglycerides	>150 mg/dl or treatment for dyslipidaemia
HDL cholesterol	<40/50 mg/dl for men/women or treatment for dyslipidaemia

of activation states in which macrophages often perform multiple functions and simultaneously express "M1" and "M2" markers (23).

Macrophages critically influence not only liver inflammation but also metabolic impairment (namely insulin resistance) in metabolic disorders and alcoholic liver disease (24). KC have an essential role in liver fibrosis in mouse models of ASH and NASH, propagating hepatic inflammation via tumor necrosis factor (TNF) and leukocyte recruitment via intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (25). On the contrary, predominance of M2-polarized, interleukin (IL)-10-expressing KC, promoting M1 macrophage apoptosis and hepatocyte senescence, is protective in both experimental ALD or NAFLD models (26). An M1-prone profile has been associated not only with liver injury in NASH patients but also with metabolic impairment (insulin resistance and visceral fat deposition) and with portal hypertension in NASH patients (27). Expanded $CD11c^+$ $CD206^+$ and C-C chemokine receptor-2 ($CCR2^+$) macrophage populations in visceral adipose tissue and a higher production of pro-inflammatory cytokines have been observed in NASH patients (28). Moreover, transcription of pro-inflammatory pathways in adipose tissue corresponds to progressive histologic impairment in NASH patients. Central molecules identified in these pathways are IL-8, C-C chemokine ligand-2 (CCL-2), JUN-B, and IL-6, all of which are involved in inflammation (28). $CCR2^+$ monocyte-derived macrophages are recruited to the liver (but also to the adipose tissue or atherosclerotic plaques) in metabolic disorders (29), making this pathway an attractive target for inflammatory therapies in NASH.

Monocytes and macrophages have indeed become interesting potential targets for the treatment of NAFLD and ALD. The different mechanisms by which these cells can be targeted include modulation of KC activation, monocyte recruitment in the liver and macrophage polarization and differentiation (30). Evidence from preclinical studies and clinical trials (some of them already in phase II and III) have shown encouraging results in ameliorating steatohepatitis, fibrosis, and the metabolic profile, individuating promising therapeutic candidates.

Granulocytes are also implicated in the onset of fatty liver and steatohepatitis (14). Neutrophils are involved in adipose tissue inflammation, in the induction of insulin resistance and in the progression to steatohepatitis (31–34). There is some evidence that eosinophils, basophils and mast cells may be associated to metabolic impairment, while mast-cell infiltration may also promote liver fibrosis (35–38). Dendritic cells are professional antigen presenting cells that are implicated in the induction of central and peripheral immunological tolerance, in the regulation of the T-cell immune responses, and act as sentinel cells of innate immunity in the recognition of microbial pathogens. These cells are associated to hepatic fibro-inflammatory injury (39, 40).

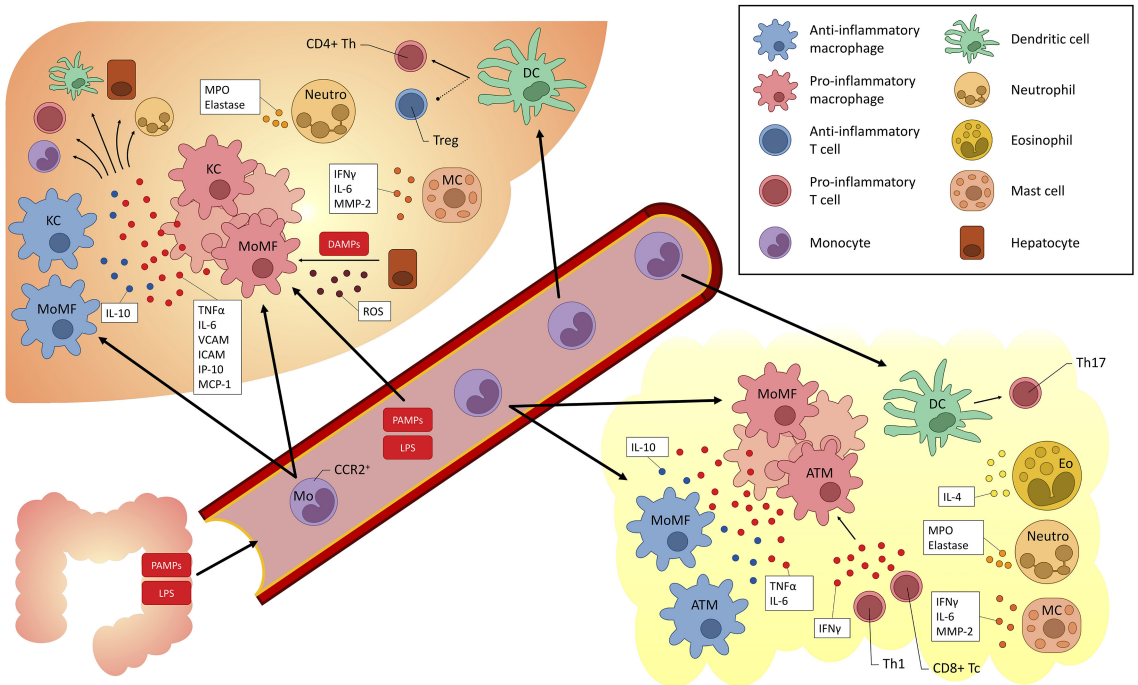

FIGURE 2 | Overview of myeloid-derived cells implicated in fatty liver (alcoholic and non-alcoholic) and their cross-talk with other cells. KC, Kupffer cells; DC, dendritic cells; Eo, eosinophils; Neutro, neutrophils; MC, mast cells; Th, T helper; LPS, lipopolysaccharide; TLR, Toll-like receptor; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; ROS, reactive oxygen species; Tregs, T regulatory cells; MoMF, monocyte-derived macrophages; ATM, adipose tissue macrophages; MPO, myeloperoxidase; MMP, matrix metalloproteinase; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; IP, interferon gamma-induced protein; MCP, Monocyte chemoattractant protein; PAMP, pathogen-associated molecular pattern; DAMP, danger-associated molecular pattern; CCR, C-C chemokine receptor; ROS, reactive oxygen species.

Considering all these mechanisms, myeloid derived-cells are candidate novel targets for the treatment of NAFLD (41) and ALD (**Table 3**).

DRUGS IN DEVELOPMENT FOR NASH TREATMENT

Cenicriviroc

Cenicriviroc is a CCR2-CCR5 dual antagonist. CCR2 and CCR5 play an important role in macrophage recruitment and polarization (42, 73). CCR2-CCR5 blockade showed anti-inflammatory and anti-fibrotic effects in preclinical models (73–75) and clinical studies (42, 76, 77). The year 1 analysis of a large 2-years phase-2 trial (42) showed a significant decrease in systemic inflammation but could not show a significant improvement in the activity of steatohepatitis and its components (except for ballooning) as assessed by histology. Although the primary endpoint of hepatic histological improvement in NASH Activity Score (NAS) (2) (more than 2 points and no worsening of fibrosis stage) was hence not met, the study did show a significant benefit of cenicriviroc over placebo in terms of regression of fibrosis and amelioration of grade-2 ballooning at histology. As mentioned, the drug was also effective in attenuating the inflammatory signaling. Cenicriviroc was able to induce the reduction of circulating markers of systemic inflammation (such as high-sensitivity C-reactive protein, IL-6, IL-1ß, and fibrinogen) and soluble cluster of differentiation-14 (a marker of monocyte activation) and induced an increase in CCL-2 and CCL-4, confirming potent CCR2-CCR5 blockade. These findings are consistent with previous studies including those conducted in HIV patients (74, 77, 78). Currently, the drug is further being investigated as anti-fibrotic agent in a phase-3 trial with reduction of fibrosis as the primary endpoint.

Selonsertib

Selonsertib is an apoptosis signal-regulating kinase-1 (ASK-1) inhibitor. ASK-1 is a ubiquitously expressed serine/threonine kinase, which is activated by oxidative stress to promote hepatocellular apoptosis, inflammation and fibrosis, via downstream phosphorylation of p38 and Jun N-terminal kinases (JNK). Both p38 and JNK have well-characterized roles, not only in hepatocytes but also in other cell types, including macrophages (79–81). KC are indeed activated, among others, by p38 and JNK and blocking the inflammatory signaling pathways of KC was shown to reduce inflammation and fibrogenesis in NASH (81). Therefore, it is plausible that Selonsertib also interferes with macrophage activation (43). Selonsertib was tested in a small 6-months trial in combination with or without Simtuzumab in an anti-fibrotic strategy. Selonsertib was superior to placebo (Simtuzumab was considered a placebo given that

TABLE 2 | Summary of the impairment of myeloid-derived cells in NAFLD and ALD.

	NAFLD	ALD
Monocytes	- Differentiation into tissue resident macrophages - Differentiation in DC	- Differentiation into tissue resident macrophages - Differentiation in DC
Macrophages/KC	- M1 enhancement - Imbalance of lipogenesis - Increased LPS/TLR4-mediated signaling - Increased TNF-α, IL-1β, IFN-γ, IL-6 - Fibrosis stimulation	- M1 enhancement - Increased LPS/TLR4-mediated signaling - Increased TNF-α, IL-1β, ROS
DC	- Altered CD8/CD4 ratio - Decreased Treg infiltration - Increased inflammation	- Increased cytokine secretion via TLRs - Increased TNF-α, IFN-γ
Neutrophils	- Liver infiltration - Progression to steatohepatitis (MPO)	- Liver infiltration - Increased TNF-α
Eosinophils	- Increased Th2-type cytokines - Increased M2 polarization	- Increased Th2-type cytokines - Increased M2 polarization

NAFLD, Non-alcoholic liver disease; ALD, alcoholic liver disease; DC, dendritic cells; IFN, interferon; IL, interleukin; KC, Kupffer cells; LPS, lipopolysaccharide; NAFLD, non-alcoholic fatty liver disease; ROS, reactive oxygen species; Th2, T helper 2; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg, T regulatory cell; MPO, myeloperoxidase.

other Simtuzumab trials appeared negative) in terms of fibrosis regression, without an effect on steatohepatitis or on the metabolic features. Selonsertib is tested in 2 Phase-3 trials, one in F4 and one in F3 patients (43). The trial in F4 patients was recently reported to be negative on the pre-specified week 48 primary endpoint of a ≥ 1-stage histologic improvement in fibrosis without worsening of NASH. Selonsertib was generally well-tolerated and safety results were consistent with prior studies. The trial was discontinued. The trial in patients with F3 is still ongoing (82).

Peroxisome Proliferator-Activated Receptors (PPAR) Agonists

PPARs are ligand-activated nuclear receptors that function as master regulators in adipose tissue and the liver, controlling insulin sensitivity, glucose and lipid metabolism, inflammation and fibrogenesis (83, 84). There are three isoforms of PPARs. The PPARα isoform is highly expressed in hepatocytes and controls fatty acid transport and β-oxidation and dampens the inflammatory response (47). The PPARγ isoform is highly expressed in adipose tissue; its activation promotes adipocyte differentiation, increases glucose uptake and triglyceride storage (hence reducing free fatty acid flux to the liver), and increases secretion of the anti-inflammatory cytokines like adiponectin. The PPARδ isoform contributes to the regulation of glucose and lipid metabolism. Of note, PPARδ exerts an anti-inflammatory effect in the liver by skewing M2 polarization of KC and decreases the expression of inflammasome components [nucleotide oligomerization domain-like receptor family, pyrin domain containing-3 (NLRP-3), caspase-1, and IL-1] under stimulus of saturated fatty acids and lipopolysaccharides. PPARs also interact with hepatic stellate cells (HSC) to regulate fibrosis: PPARγ and PPARδ are expressed at various levels in HSC, which contribute to liver fibrosis, while PPARγ holds HSC in a quiescent non-fibrogenic state (46).

PPARα agonists like fibrates failed to show a histological benefit in NASH (85). However, recent data showed that PPARα expression is inversely correlated to the severity of NASH and that NASH improvement is associated with increased PPARα expression, giving rationale to a PPARα-targeted treatment despite the negative data with fibrates (86). Several multi- or pan-agonists are in development and, by means of the δ isoform, are likely to act on macrophages. Elafibranor, a dual PPARα-δ agonist, was able to induce resolution of NASH without worsening of fibrosis in significantly more patients compared to placebo if baseline NASH was sufficiently severe. Moreover, it was shown to reduce fibrosis in those patients that responded to treatment (45). Additionally, it improved serum lipids and glycaemic control, reducing the calculated overall cardiovascular risk (45). Elafibranor is now in phase-3 and the first part of the cohort needed for the interim analysis has recently been fully recruited.

Lanifibranor is a next-generation pan-PPAR agonist. In different preclinical models of NASH, Lanifibranor induced an improvement of liver histology (including an anti-fibrogenic effect) and of the metabolic profile (ameliorated insulin sensitivity, body weight, adiposity index and serum triglycerides). Moreover, Lanifibranor inhibited the expression of pro-fibrotic and inflammasome-related genes while increasing the expression of β-oxidation-related and fatty acid desaturation-related genes in both the methionine/choline-deficient diet (MCDD) and in the foz/foz model. Additionally, in the foz/foz model it showed a reduced macrophage recruitment (46). Lanifibranor is currently being evaluated in a phase-2 study.

Farnesoid X Receptor (FXR) Agonist

FXR plays an important role in bile acid metabolism, but also impacts on several metabolic, and fibrogenic pathways (55). Obeticholic acid (OCA) is a potent bile acid FXR agonist already licensed for the treatment of primary biliary cholangitis and under investigation in the setting of NASH. Preclinical studies have shown that OCA also targets KC, as shown by the dose-dependent inhibition of TNF-α and bacterial lipopolysaccharide (LPS)-stimulated expression of monocyte chemoattractant protein-1 (MCP-1) in KC (57). Moreover, this effect of OCA on KC translates in a decrease of not only pro-inflammatory cytokines, but also of anti-inflammatory cytokines, such as IL-10. In a phase-2 study OCA showed a significant response—defined as a NAS reduction of ≥2 points–compared to placebo, as well as a beneficial effect on fibrosis

TABLE 3 | Summary of the treatments in development for NAFLD (#) and ALD (§).

Compound	Classification	Effect	Mechanisms of action
Cenicriviroc #	CCR2-CCR5 dual antagonist	Fibrosis regression Improvement of grade-2 ballooning (42)	Reduction high-sensitivity C-reactive protein, IL-6, IL-1ß, and fibrinogen Reduction of monocyte activation through CCR2-CCR5 blockade (42)
Selonsertib #,§	ASK-1 inhibitor	Fibrosis regression No effect on steatohepatitis No effect on metabolic parameters (43)	Reduction p38 and JNK phosphorylation (44) Inflammatory signaling pathways blockade Macrophage activation impairment (43)
Elafibranor #	Dual PPARα-δ agonist	Resolution of NASH without worsening of fibrosis Regression of fibrosis (if response to treatment) Improvement of serum lipids Improvement of glycaemic control Reduction of calculated overall cardiovascular risk (45)	PPARα: *Control of fatty acid transport and β-oxidation* Dampening of inflammatory response (47, 48) PPARδ: *M2 polarization of KC* (49)
Lanifibranor	Pan-PPAR agonist	*Improvement of liver histology* *Anti-fibrogenic effect* *Improvement of insulin sensitivity and serum triglycerides* *Improvement of body weight and adiposity index* (46)	*Decreased expression of inflammasome components* (50) PPARγ: *Promotion of adipocyte differentiation* (51) Increase of glucose uptake and reduction of triglycerides (52, 53) *Increase of anti-inflammatory cytokines* (54)
Obeticolic acid #,§	Bile acid FXR agonist	Improvement of fibrosis Improvement of steatohepatitis (55, 56) Decrease of HDL No improvement of glycaemic control (55)	*Targets KC* *Decrease of TNF-α and LPS* *Decrease of MCP-1 and IL-10* (57)
BAR501 #	GPBAR-1 agonist	*Reduction of steatosis* *Reduction of inflammation* *Improvement of fibrosis* (58, 59)	*Release of GLP-1* *Modulation of macrophage phenotype* (58, 59)
BI 1467335 #	VAP-1 inhibitor	*Reduction of liver injury* (60)	*Reduction of leucocyte infiltration in the liver during fibrogenesis* (60)
Tipelukast #	Leukotriene receptor antagonist	*Anti-inflammatory and anti-fibrotic properties* *Decrease of serum triglycerides* (61)	*Down-regulation of inflammation-related genes (including CCR2 and MCP-1)* (61)
JKB-121 #	TLR-4 receptor antagonist	*Prevention of LPS-induced inflammatory liver injury in MCDD model* No benefit on human liver disease (62)	*Stimulation of KC activation* (62)
Emricasan #,§	Pan-caspase inhibitor	*Effective in preclinical models of liver injury (including NAFLD and ALD)* (63) Decrease of transaminases in viral hepatitis (64)	*Interference with the signaling cascade of the NLRP-3 inflammasome* (63)
GR-MD-02 #	Galectin-3 inhibitor	Reduction of portal pressure Reduction of occurrence of esophageal varices (65)	*Interference with fibrogenesis* (66)
HepaStem #	Liver-derived mesenchymal stem cells	Reduction in NAS and fibrosis in mouse model of NASH (67, 68)	*Inhibition DC differentiation* *Inhibition of TNF-α production* *Promotion hepatocyte regeneration* (67)
G-CSF §	G-CSF	Mobilization of hematopoietic stem cells Improvement of liver function and survival in AH	Stimulation of neutrophil function Mobilization of hematopoietic stem cells Induction of liver regeneration (69–71)
DUR-928 §	Small molecule epigenetic regulator	*Anti-fibrotic and anti-inflammatory properties* (72)	*Reduction of MCP-1 and TNF-α* (72)

NAFLD, Non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ALD, Alcoholic liver disease; AH, alcoholic hepatitis; CCR, C-C chemokine receptor; ASK, apoptosis signal-regulating kinase; PPAR, peroxisome proliferator-activated receptor; FXR, farnesoid X receptor; GPBAR, G protein-coupled bile acid receptor; VAP, Vascular adhesion protein-1; TLR, Toll-like receptor; G-CSF, Granulocyte-colony stimulating factor; IL, interleukin; KC, Kupffer cells; TNF, tumor necrosis factor; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; GLP, glucagon-like peptide; NLRP, nucleotide oligomerization domain-like receptor family pyrin domain containing protein; DC, dendritic cells. Preclinical data are indicated in cursive; HDL, high-density lipoprotein; MCDD, methionine/choline- deficient diet.

(which was a secondary study endpoint). These results were, however, associated with a decrease in high-density lipoprotein (HDL) levels and a lack of improvement of glycaemic control (87). At this moment, the study has proceeded to a phase 3 study. The recently released interim analysis showed that in the primary efficacy analysis (Intent-to-Treat), once-daily OCA 25 mg met the primary endpoint of fibrosis improvement (≥1 stage) with no worsening of NASH. Moreover, a greater proportion of patients treated with OCA compared to placebo achieved the primary endpoint of NASH resolution with no worsening of liver fibrosis, although statistical significance was not reached (56).

G Protein-Coupled Bile Acid Receptor-1 (GPBAR-1) Agonist

GPBAR-1 is a G-protein coupled receptor, activated by secondary bile acids. GPBAR-1 is expressed in various cells types in the intestine, the adipose tissues and non-parenchymal liver cells, particularly KC. The activation of this receptor in the intestine causes the release of glucagon-like peptide-1 (GLP-1). Moreover, this receptor is highly expressed by monocytes and macrophages and its activation counter-regulates the innate immune response in the intestine and liver. Activation of GPBAR-1 is also able to modulate the macrophage phenotype from a classically activated (M1) to an alternatively activated (M2) phenotype. BAR501 is a non-bile acid, selective GPBAR-1 ligand that has been shown effective in reducing steatosis, inflammation and fibrosis in preclinical models of NASH (58, 59) and is currently under development for the treatment of NASH.

Vascular Adhesion Protein-1 (VAP-1) Inhibitors

The semicarbazide-sensitive amine oxidase (SSAO)/VAP-1 is a homodimeric glycoprotein adhesion molecule that is widely expressed in the vascular system. During inflammation this complex facilitates leukocyte recruitment through its SSAO component and its activation promotes liver inflammation and fibrosis. Moreover, its soluble variant showed a correlation with NAFLD severity in humans. BI1467335 is an oral small molecule SSAO/VAP-1 inhibitor that was shown effective in reducing liver injury in rodents. VAP-1 inhibition blunted leucocyte (including macrophages and other myeloid cells) infiltration in the liver during fibrogenesis (60). A phase-2 clinical trial in patients with NASH was started in 2017 (88).

Tipelukast

Tipelukast, also known as MN-001, is an orally bioavailable small molecule leukotriene receptor antagonist. The molecule was shown to be anti-fibrotic and anti-inflammatory in preclinical models and exerts these effects through several mechanisms, including: leukotriene (LT) receptor antagonism, inhibition of phosphodiesterases (PDE) (mainly 3 and 4), and inhibition of 5-lipoxygenase (5-LO). It has also been shown to down-regulate expression of genes that promote inflammation, including CCR2 and MCP-1. A phase-2 open-label study to evaluate the effectiveness, safety, tolerability and pharmacokinetics of tipelukast, including its effects on HDL function and serum triglyceride levels in patients with NASH/NAFLD and hypertriglyceridemia, is ongoing (89). The interim analysis showed a significant decrease of serum triglycerides, which was a primary endpoint (61).

Toll-Like Receptor-4 (TLR-4) Receptor Antagonist

JKB-121 is a long-acting small molecule that functions as a TLR-4 receptor antagonist. TLRs are expressed by KC and are able to stimulate their activation upon infectious and non-infectious threats in order to induce a immunogenic T-cell response (90). It has been shown that JKB-121 prevents LPS-induced inflammatory liver injury in a MCDD rat model of NAFLD. Although the preclinical data were promising and were based on a solid rationale, the results of a phase-2 study failed to show a beneficial effect on liver disease (62).

Caspase Inhibitors

Inhibition of caspases attenuates inflammatory and apoptotic processes by interfering with the signaling cascade of the NLRP-3 inflammasome, which was shown to be activated in KC in preclinical models of NASH and ALD (63). Emricasan, a pan-caspase inhibitor, was shown to be effective in lowering transaminase levels and attenuating fibrosis in a preclinical animal model (91). Interestingly, this molecule was already shown to decrease transaminase levels in chronic hepatitis C patients (64). The compound is currently in phase 2 for the treatment of NASH.

Galectin-3 Inhibitor

Galectin-3 is a protein expressed predominantly in immune cells that recognizes and binds to galactose residues and is an essential protein in liver fibrogenesis (66). GR-MD-02 is a galectin-3 inhibitor that is currently undergoing a phase-2b trial in NASH patients with fibrosis/cirrhosis. The interim analysis of this study (65) suggests a clinical improvement in cirrhotic patients: significant decrease in portal pressure and a reduction in the development of newly formed esophageal varices.

Cell-Based Therapy

Another frontier in NASH treatment is cell-based therapy, which is currently given full consideration for application in clinical trials. HepaStem are liver-derived mesenchymal stem cells (MSC) with regenerative, anti-fibrotic, and anti-inflammatory potential. MSC can affect monocyte and DC recruitment, differentiation, maturation and function (92, 93). HepaStem have been shown to inhibit T-cell proliferation and activation as well as DC differentiation, maturation and production of TNF-α *in vitro* and can promote hepatocyte regeneration by inhibiting HSC (67). In a mouse model of NASH HepaStem were shown to induce a reduction in NAS and fibrosis (67, 68). In humans, a phase-2 trial is ongoing in patients with acute liver failure (94).

DRUGS IN DEVELOPMENT FOR ALD TREATMENT

Macrophages are potential targets for the treatment of ALD (18). It is a well-established concept that alcohol abstinence is the cornerstone in the treatment of ALD. Alcohol abstinence *per se* can influence macrophage function in terms of cytokine production (95) and phenotype switching (96). Anti-inflammatory treatments targeting macrophage function, such as treatment with corticosteroids and pentoxyfyllin (a phosphodiesterase inhibitor) have long been evaluated for ALD (18). Corticosteroids constitute the standard treatment of severe AH and pentoxyfillin can be used for this indication in those patients with contraindications to corticosteroids (3, 20). In contrast, anti-TNFα antibodies did not show effectiveness in

the treatment of AH and yielded a higher probability of severe infections and a higher mortality (97). Macrophages, however, remain a candidate target for the treatment of ALD, particularly AH, its most severe form. Combining biologicals, small-molecule drugs and anti-oxidant therapies targeting macrophage function and phenotype could provide an added therapeutic benefit (5). Therefore, new drugs targeting macrophages are currently being evaluated in clinical trials (**Table 3**).

Selonsertib

Besides the ongoing studies in NAFLD patients, mentioned above, the ASK-1 inhibitor Selonsertib is also currently under investigation in the setting of AH. As previously reported, the downstream effect of ASK-1 inhibition would likely also affect macrophage activation (43). A phase-2 study comparing the effect of Selonsertib with prednisolone compared to prednisolone alone in AH has recently completed recruitment.

FXR Agonists

The FXR agonist OCA is another molecule in development for both NAFLD and ALD. A phase-2 double-blind, placebo-controlled trial of OCA in patients with moderate to severe AH is currently ongoing to evaluate a possible reduction in Model For End-Stage Liver Disease (MELD) score as a measure of effectiveness, as well as the incidence of serious adverse events during treatment.

Granulocyte Colony-Stimulating Factor (G-CSF)

This cytokine is a potent stimulus of neutrophil function and is able to mobilize hematopoietic stem cells and induce liver regeneration. G-CSF was safe and effective in the mobilization of hematopoietic stem cells and improved liver function and survival in patients with severe alcoholic hepatitis in small trials (69–71). These encouraging results need to be confirmed in larger studies (98).

Caspase Inhibitors

As mentioned above, inhibition of caspases attenuates inflammatory and apoptotic processes by interfering with the signaling cascade of the NLRP-3 inflammasome, which was shown to be activated in KC in both mouse models of ALD and a human cohort (99–101). Moreover, alcohol exposure was shown to cause hepatocytes to release extracellular vesicles in a caspase-dependent manner to elicit apoptosis and macrophage activation (102). Based on the positive data in NAFLD, Emricasan, a pan-caspase inhibitor, has also been evaluated in the setting of ALD. A phase-2 clinical trial concluded that Child Pugh A and B cirrhotic patients with a baseline MELD $\geq$15 showed significantly improved liver function compared to placebo (103).

Small Molecule Epigenetic Regulators

DUR-928 is an endogenous, orally bio-available small molecule that modulates the activity of various nuclear receptors that play an important regulatory role in lipid homeostasis, inflammation and cell survival. It has been demonstrated in mice models of NASH that this molecule exerts anti-fibrotic and anti-inflammatory effects and is able to reduce hepatic transcripts of TNF-α and MCP-1 in a dose-dependent manner (72). DUR-928 is currently being investigated in a phase-2, open-label, dose-escalation study in AH.

CONCLUSIONS

Fatty liver and steatohepatitis (alcoholic and non-alcoholic) constitute a spectrum of highly prevalent liver conditions with a possibly unfavorable outcome, for which the treatment is an unmet medical need. A plethora of clinical trials, many of which acting on inflammatory processes, has been set up in an attempt to resolve this issue. Myeloid-derived cells are promising candidate targets in the pharmacological treatment of these diseases. The results of the phase-3 trials are expected by 2020 and will likely change the scene in the treatment of these diseases.

AUTHOR CONTRIBUTIONS

LV and SF conceived the paper. LV and SF wrote the paper with contribution of MVH and JW. LV and MVH designed the figures. SF supervised the paper. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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The Role of Myeloid-Derived Cells in the Progression of Liver Disease

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Control of homeostasis and rapid response to tissue damage in the liver is orchestrated by crosstalk between resident and infiltrating inflammatory cells. A crucial role for myeloid cells during hepatic injury and repair has emerged where resident Kupffer cells, circulating monocytes, macrophages, dendritic cells and neutrophils control local tissue inflammation and regenerative function to maintain tissue architecture. Studies in humans and rodents have revealed a heterogeneous population of myeloid cells that respond to the local environment by either promoting regeneration or driving the inflammatory processes that can lead to hepatitis, fibrogenesis, and the development of cirrhosis and malignancy. Such plasticity of myeloid cell responses presents unique challenges for therapeutic intervention strategies and a greater understanding of the underlying mechanisms is needed. Here we review the role of myeloid cells in the establishment and progression of liver disease and highlight key pathways that have become the focus for current and future therapeutic strategies.

Keywords: hepatitis (general), hepatocellular carcinoma, cirrhosis, fibrosis, myeloid derived suppressor cell (MDSC), neutrophil (PMN), macrophage, circulating monocytes

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INTRODUCTION

Myeloid cells arise from the common myeloid precursor and give rise to monocytes, dendritic cells and macrophages, and granulocytes. Myeloid cell functions include the recognition, ingestion and degradation of cellular debris, foreign material or pathogens, subsequent control of inflammatory responses, and maintenance of tissue architecture. There is increasing evidence implicating granulocytes in liver homeostasis and disease but this review will focus mainly on monocytes and macrophages. Macrophages are a diverse, heterogeneous population derived from short-lived, but plastic, precursor monocyte populations. Monocytes are rapidly recruited to sites of injury and their functions are imprinted in the bone marrow, whereas macrophages tend to be long-lived and tissue-resident, where their functions are dictated by environmental cues (1). A highly coordinated pathway of monocyte recruitment and subsequent imprinting of macrophage "identities," the mechanisms of which are only now beginning to be understood, controls local tissue inflammatory and regenerative functions and is critical in maintaining tissue architecture (2–4). Extensive rodent and human studies have demonstrated key roles for monocytes and macrophages in the establishment, progression and regression of liver disease including a critical role in directing tissue regeneration (5–7). This fine balance of pro- and anti-inflammatory mediators is crucial to determining the path of disease progression, and understanding how myeloid cells contribute to

injury and repair will enable the rational design of novel therapies. In this review we summarize the identities and roles of myeloid cell populations in the liver, and describe approaches that are being developed to reduce inflammation through targeting this innate immune cell population.

HEPATIC MONOCYTE AND MACROPHAGE POPULATIONS

Kupffer Cells

The liver contains a population of self-renewing resident macrophages, termed Kupffer cells (KC), derived from yolk sac-derived progenitor cells (8, 9) or hematopoietic stem cells (10). In mice KC phenotype is thought to be controlled by a specific set of transcription factors including ID3 and ZEB2 through progenitor cell development and maintenance of the expression of LXRα permitting replenishment of the KC niche by progenitors from the circulation (2, 11–13) (**Figure 1**, **Table 1**). They are non-migratory, being retained in the sinusoids where they maintain a tolerogenic environment despite the presence of low levels of food particles and bacterial antigens delivered from the gut via the portal vein (4, 14, 15). This is achieved through highly effective phagocytic and scavenging mechanisms triggered by toll-like receptor (TLR) signaling and scavenger receptors such as CD36, scavenger receptor-A and galectin-3 (16, 17). Their expression of high levels of pattern recognition receptors (PRRs) allows macrophages to respond to a wide range of danger-associated molecular patterns (DAMPs) released during tissue injury, such as high mobility group protein B1 (HMGB1), ATP, uric acid, DNA fragments and cholesterol crystals (5) and pathogen-associated molecular patterns (PAMPs, such as lipopolysaccharide and flagellin) released from microbes. Activation of PRRs leads to the formation of the inflammasome (18) via multi-protein complexes including the NOD-, LRR- and pyrin domain-containing 3 (NLRP3) (19). Formation of the inflammasome promotes the release of potent signaling molecules including IL-1β, PGE_2, HMGB1, TNF-α, and IL-17, driving inflammation and fibrosis (20–24). KC from both mouse and human liver also secrete the anti-inflammatory cytokine IL-10 (25–27), express low levels of MHC class II and co-stimulatory molecules combined with high levels of the T-cell inhibitory molecule PDL-1 (27). This makes them unable to fully activate T cell effector function but rather to promote the development of regulatory T-cells (Treg). This is further enhanced through their secretion of PGE_2 (28) and upregulation of the indolamine 2,3-dioxygenase pathway which promotes immune cell tolerance (29).

Murine KC have been well characterized under homeostatic conditions and in experimental models of hepatic injury, where they express $CD11b^+F4/80^{++}CD68^+CD11c^{+/-}CLEC4F^+TIM4^+$ in addition to TLR4, TLR9, and CRIg, but are negative for the chemokine receptor CX_3CR1. Recent advances in proteomic analysis has revealed circadian regulation of not only KC numbers in uninjured mouse liver, but also components of the immune response pathway which peak during the daytime, including Tlr4, Myd88, Irak4, and Tak1 (30). Human KC are less well described but can be identified through expression of $CD68^+CD14^+TLR4^+$ and lack of CX_3CR1.

Infiltrating Monocytes

Circulating monocytes are actively recruited to the liver, guided by adhesion molecules and chemokine gradients generated at the sinusoidal endothelial interface (see below). In mice bone marrow derived myeloid cells expressing high levels of Ly6C and CCR2 rapidly infiltrate tissue and are associated with the expression of pattern recognition receptors (PRR) and inflammatory cytokines ($CD11b^+CCR2^+CX_3CR1^+CD43^-$). In contrast $Ly6C^{low}$ monocytes from the spleen express a broad range of scavenger receptors and exhibit a patrolling behavior that may enable the engulfment of apoptotic cells ($CD11b^+CCR2^-CX_3CR1^{++}CD43^+$) (27, 31–36). In humans there is no discriminatory expression of Ly6C and monocytes are classified according to the expression of CD14 and CD16 giving rise to classical ($CD14^{++}CD16^-$), intermediate ($CD14^+CD16^+$), and non-classical ($CD14^-CD16^+$) populations. Gene expression profiling of these subsets has determined that $CD14^{++}CD16^-$ monocytes resemble murine $Ly6C^{high}$ infiltrative cells and $CD14^-CD16^+$ monocytes more closely align with the patrolling $Ly6C^{low}$ population (31). Potent immunomodulatory myeloid derived suppressor cells (MDSC) are also present in both murine and human liver tissue. MDSCs are a heterogeneous population of cells which express markers shared with other immune cell populations ($CD11b^+Ly6C^+$ in mice, $CD14^+HLA\text{-}DR^{+/-}CD33^+$ in humans), therefore identification is usually confirmed by means of a T-cell suppression assay (37). MDSC suppress immune responses through production of arginase 1 (Arg1), inducible nitric oxide synthase (iNOS) and generation of reactive oxygen species (ROS), or secretion of IL-10 (38).

RECRUITMENT FROM THE CIRCULATION AND DIFFERENTIATION IN TISSUE

Damage to tissue results in an upregulation of adhesion molecules on liver sinusoidal endothelium (LSEC) and the secretion of chemokines, cytokines and other bioactive molecules that promote immune cell recruitment [reviewed in (39)]. Circulating $CCR2^+$ monocytes are recruited in response to local CCL2, released primarily by hepatic stellate cells (HSC) (40, 41), or through the CCR8/CCL1 and CXCR3/CXCL10 axes (42–44). In humans the migration of $CD14^-CD16^+$ monocytes is promoted through activation of CX_3CR1 by endothelial CX_3CL1, a transmembrane chemokine that is expressed at high levels during inflammation (45). Intermediate $CD14^+CD16^+$ monocyte populations are enriched in the diseased liver (46), partly due to their increased propensity when compared with other monocytes to migrate across LSEC. These cells exhibit high phagocytic activity and secrete pro-inflammatory and fibrogenic mediators (47). Bidirectional migration of monocytes affects the local balance of inflammatory and anti-inflammatory cells. Pro-inflammatory $CD14^-CD16^+$ subsets undergo reverse migration from tissue back into the circulation via across LSEC from

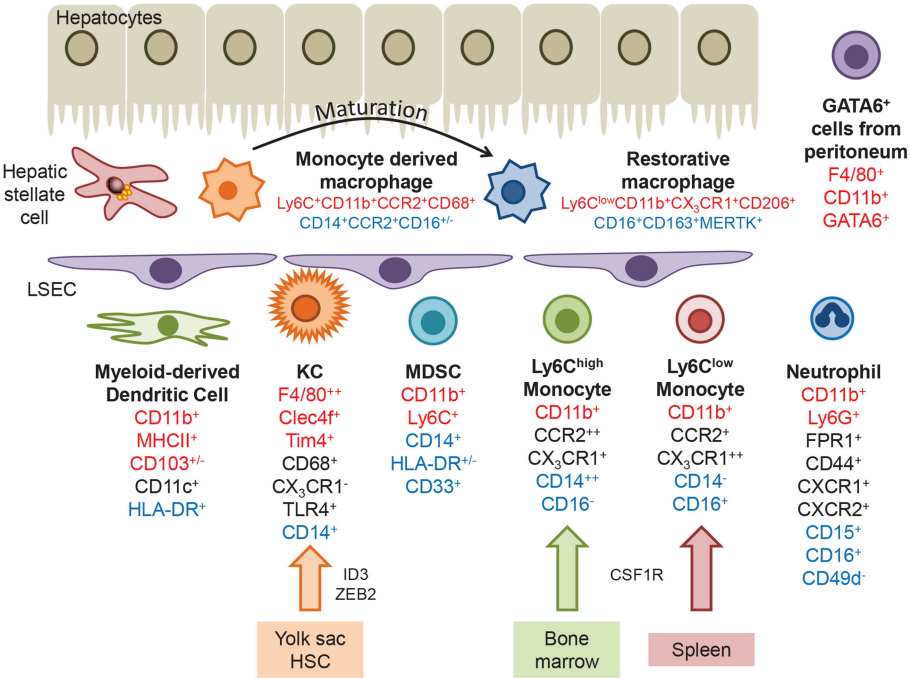

FIGURE 1 | Myeloid populations present within the liver. Kupffer cells are derived from the yolk sac or hematopoietic stem cells, under the control of transcription factors such as ID3 and ZEB2. Infiltrating monocytes originating in the bone marrow or spleen express the chemokine receptors CCR2 and CX_3CR1, and can differentiate into monocyte-derived dendritic cells. Following injury monocytes undergo transendothelial migration across LSEC and differentiate into monocyte derived macrophages, which can mature into a more restorative phenotype or replenish the KC pool. A $GATA6^+$ macrophage population that migrates from the peritoneum during hepatic injury has been identified in mice. Markers that identify hepatic macrophages in mice, humans or are common to both are highlighted in red, blue and black text, respectively.

where they may contribute to systemic inflammatory responses, whereas anti-inflammatory cells remain in the tissue where they suppress T-cells and promote endotoxin tolerance (48). A phenotypic switch in macrophage phenotype is observed during acute liver injury in humans where MAC387 (S100A9) can be used to identify circulation-derived macrophages in contrast to $CD68^+$ resident populations (49). Infiltrating monocytes also undergo local intrahepatic differentiation into anti-inflammatory MDSC following injury via contact-dependent mechanisms such as communication with hepatic stellate cells (HSC), or interaction with soluble mediators such as catalase (50, 51).

In mouse models of sterile injury $CCR2^+Ly6C^{high}$ monocytes form rings to demarcate the extent of injury (14), and subsequently mature into $Ly6C^{low}$ monocytes that promote the resolution of injury and fibrosis (52). These "pro-restorative" macrophages exhibited a phenotype distinct from the classical M1 (pro-inflammatory) or M2 (pro-resolution) dichotomy with increased expression of genes that promote tissue restoration including matrix metalloproteinases (MMPs), growth factors, and phagocytosis-related genes. Murine monocytes can also take unconventional routes into liver tissue. In a model of sterile liver injury GATA6-positive macrophages ($CD11b^+F4/80^+Gata6^+$) originating in the peritoneal compartment were observed within the hepatic compartment at a very early stage of tissue damage (53). These cells migrate directly across the mesothelium, dependent on adenosine triphosphate and the adhesion molecule CD44. The contribution of these cells to disease pathogenesis is currently unknown.

Thus, local polarization of myeloid cell populations, and recruitment of macrophages from other sites, has important implications in disease pathogenesis where the balance of pro-/anti- inflammatory mediators and fibrogenic responses dictates the course of the disease.

RESPONSE TO ACUTE AND CHRONIC LIVER DISEASE

Acute Liver Disease (Such as Acetaminophen Overdose, Acute Viral, or Alcoholic Hepatitis)

Acute liver failure (ALF) is associated with high mortality and toxic liver injury in response to overdose of drugs such as acetaminophen is a more common cause of ALF than immune-mediated injury arising from acute viral hepatitis (54, 55). Much of what we know of macrophage function during early disease is derived from experimental models of acute liver injury in rodents such as carbon tetrachloride toxicity (hepatocyte necrosis) (56), bacterial infection (57),

TABLE 1 | Myeloid cell populations in humans and mice.

Myeloid population	Murine markers	Human markers	Role
Myeloid-derived dendritic cell	CD11b$^+$ MHCII$^+$ CD11c$^+$ CD103$^{+/-}$	CD11c$^+$ HLA-DR$^+$	Tolerogenic in nature; Upon injury may adopt an inflammatory phenotype; Functional role in liver disease ill-defined
Kupffer cells	CD11b$^+$ CD68$^+$ F4/80^{++} CLEC4f$^+$ TIM4$^+$ CX_3CR1^- TLR4$^+$ TLR9$^+$ CRIg$^+$	CD68$^+$ CD14$^+$ TLR4$^+$ CX_3CR1^-	Promote tolerance under steady-state conditions to restrict immune response against food-borne antigens; Activated during tissue damage; main source of cytokines / chemokines governing local inflammation
Myeloid derived suppressor cells	CD11b$^+$ Ly6C$^+$	CD14$^+$ HLA-DR$^{+/-}$ CD33$^+$	Immunosuppressive; Facilitate HCC growth by dampening T-cell activity
Monocyte derived macrophage	CD11b$^+$ Ly6C$^{+/-}$ F4/80$^{+/-}$ CCR2$^+$ CX_3CR1^+ CD64$^+$	CD14$^+$ CCR2$^+$ CD16$^{+/-}$	*See subsets below*
Inflammatory macrophage	Ly6C^{high} CD11b$^+$ CCR2^{++} CX_3CR1^+ iNOS$^+$ TNF$^+$	CD14^{++} CD16$^-$ CLEC5A$^+$ S100A9$^+$	Pro-inflammatory, massively recruited during liver injury; elicits tissue damage; drive fibrogenesis by maintaining inflammation and activating fibrosis effector cells; can undergo phenotypic switch to restorative macrophages
Pro-resolution macrophage	Ly6C^{low} CD11b$^+$ CCR2$^+$ CX_3CR1^{++} CD206$^+$ MMP9$^+$ MMP12$^+$	CD14$^-$ CD16$^+$ CD163$^+$ CCR2$^+$ CX_3CR1^{++} Stabilin-1$^+$ (MERTK$^+$)	Anti-inflammatory; restorative function in liver fibrosis; promote tissue repair after acute damage; in humans CD16$^+$ rather linked to fibrosis progression
Neutrophils	CD11b+ Ly6G+ Fpr1+ CD44$^+$ CXCR1$^+$ CXCR2$^+$	CD15$^+$ CD16$^+$ CD49d$^-$ FPR1$^+$ CD44$^+$ CXCR1$^+$ CXCR2$^+$	Ambiguous role in liver injury; functional role likely context-dependent; putatively profibrogenic in steatohepatitis
Peritoneal infiltrating cells	CD11b$^+$ F4/80$^+$ GATA6$^+$	Unknown	Currently not known

concanavalin A (T cell mediated hepatocyte destruction) (58), ischemia-reperfusion (I-R) injury (59), sterile injury (14), and viral infection (60). These models show that extensive hepatocyte damage mediated by heat/toxin/immune-mediated killing releases DAMPs such as HMGB1 and nuclear DNA which are sensed by KC leading to the release of cytokines and chemokines, creating an environment that drives the recruitment of inflammatory macrophage subsets (5). In the absence of persistent injury, tissue repair is initiated by the maturation of pro-inflammatory populations to a more restorative phenotype, associated with anti-inflammatory and pro-angiogenic responses (52) (**Figure 2**).

In acetaminophen (APAP) induced liver injury, perhaps the best described rodent model of ALF, KC respond to tissue damage through the rapid release of cytokines and chemokines including IL-1β, TNF-α, CCL2, and CCL5 (61). Initially the numbers of KC are reduced (<24 h) and early injury is associated with high numbers of infiltrating Ly6C^{high} monocytes which produce proinflammatory cytokines such as TNF-α and IL-1β and chemokines such as CCL2 and CCL5 (62–65). Evidence that these early entrants drive tissue injury comes from data showing that (i) infiltration of these cells during acetaminophen-induced injury can be reduced through blockade of CCR2-mediated recruitment (mNOX-E36, a CCL2 inhibitor, or cenicriviroc, a CCR2/CCR5 dual inhibitor) and (ii) that adoptive transfer of bone marrow monocytes exacerbated tissue damage (66). Initiation of repair and control of inflammation is mediated following a phenotypic switch in hepatic macrophages toward a pro-resolution, hepatoprotective subset expressing IL-10, IL-4 and IL-13 (67–69). This maturation event is dependent on colony stimulating factor 1 (CSF1) and secretory leukocyte protease inhibitor (SLPI) in areas of hepatic necrosis (70–72). The emergence of CCR2^{low}CX$_3$CR1^{high} cells is also associated with the expression of vascular endothelial growth factor A (VEGF-A) which promotes repair of the vascular architecture, and increased phagocytic capacity to remove dead and dying cells (72–74). In murine models this reparative pathway can be disrupted via modulation of CCR2 signaling or depletion of macrophages through treatment with liposomal clodronate, indicating that both tissue resident and infiltrating myeloid cell populations orchestrate repair (62, 63, 75).

Similar findings have been described in patients with ALF. Clusters of CCR2$^+$ macrophages are seen in patients with APAP-induced liver failure (66) and increased serum CCL2 levels are associated with an unfavorable prognosis (49). A pro-resolution population of MerTK$^+$HLA-DR^{high} cells has been identified in circulatory and tissue compartments of patients with ALF (72, 76). Analysis of these macrophages determined that they secreted anti-inflammatory mediators and exhibited reduced responses to bacterial challenge, consistent with an anti-inflammatory immune tolerant function. This is supported by the fact that APAP-treated *Mer* knockout animals exhibited persistent liver injury and inflammation associated with a defect in efferocytosis (72).

The pathways involved in other acute injury settings also result in activation of KC following hepatocyte damage mediated by T-cells (concanavalin A), oxidative stress (I-R), heat (sterile injury), or virus induced apoptosis (hepatitis viruses). During viral infection of humans KC increase in number and drive the infiltration of other immune cell populations through the production of inflammatory cytokines such as IL-1β, IL-18, and TNF-α (77–80). KC expression of IL-6, IFN-γ, reactive oxygen species, FAS ligand, granzyme B and TRAIL has been shown to inhibit hepatitis C (HCV) replication, and induces apoptosis of infected hepatocytes (81, 82). Triggering of KC responses arises as a result of engulfment of hepatitis B viral particles (leading to production of IL-18 and NK cell stimulation) (83)

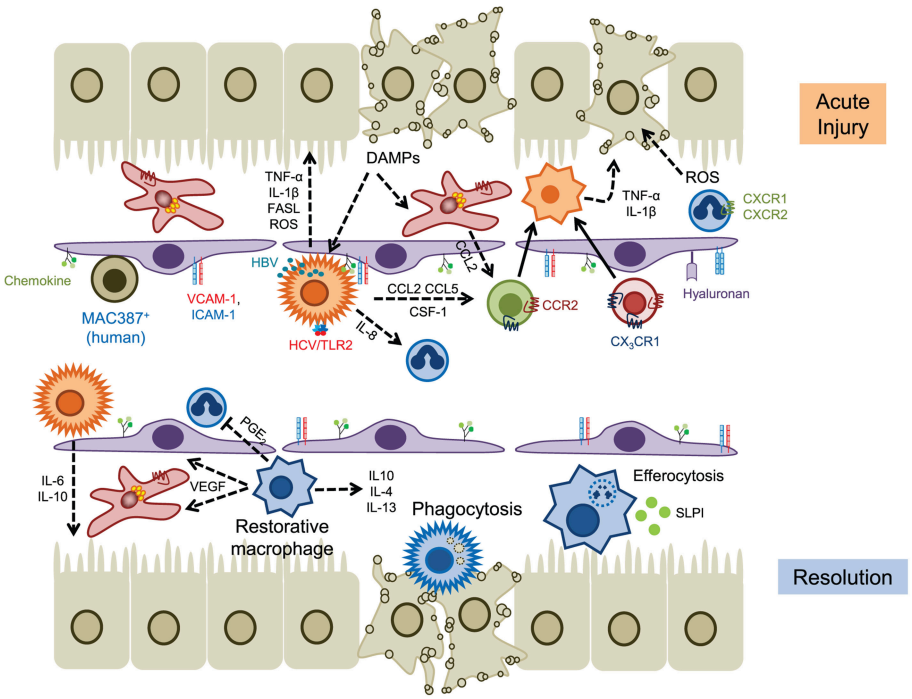

FIGURE 2 | The role of myeloid cells in acute liver injury. Hepatocyte cell death releases DAMPs that activate KC and hepatic stellate cells, leading to the release of chemokines such as CCL2 and IL-8 (CXCL8) that drives the recruitment of myeloid cells into local areas of inflammation (top). Neutrophils are recruited via CD44 and hyaluronan and generate ROS that promotes hepatocyte death, whereas infiltrating monocytes (and KCs) secrete proinflammatory cytokines such as TNFα and IL-1β. Hepatic viruses can also stimulate KC through internalization (HBV) or binding to TLR2 (HCV). Homeostasis is restored through the action of restorative macrophages (matured by phagocytosis) that secrete anti-inflammatory cytokines and promote angiogenesis, and secretion of IL-6 and IL-10 by KC (lower panel). Infiltrating neutrophils are removed through efferocytosis mediated by MERTK^{+} macrophages and SLPI. Solid lines indicate cell migration, dashed lines represent the secretion of soluble mediators.

or via TLR2 signaling and formation of the inflammasome, with concomitant secretion of IL-18 and IL-1β, in the case of HCV (84, 85). Conversely in the setting of chronic hepatitis B viral infection the immune response is impaired through release of IL-10 (86), reduced IL-12 expression (87) or T-cell exhaustion (88) mediated by TLR2 signaling on KCs, via upregulation of galectin-9 expression driving further immune cell exhaustion following engagement with Tim-3 (89), or through increased expression of the inhibitory ligand PDL1 (90). An excess of hepatitis B virus antigen can also dampen TLR responses which contribute to viral evasion of innate and adaptive immune responses (91). This is thought to occur through suppression of proinflammatory cytokines and expression of tolerogenic mediators (IL-10 in particular) reminiscent of the tolerogenic effects of LPS, although the signaling pathways mediating this effect may be distinct.

Chronic Liver Disease and Contribution to Fibrosis

A prolonged cycle of iterative bursts of tissue damage and inflammation underlies chronic liver disease leading to fibrogenesis and ultimately in some cases cirrhosis. A proportion of patients will develop hepatocellular carcinoma on the background of continuing inflammation and fibrogenesis (92). The incidence of non-alcoholic fatty liver disease (NAFLD) and alcohol related liver disease (ARLD) has increased rapidly in recent years and following advances in the treatment of chronic viral hepatitis, attention is now switching to treating these increasingly common chronic conditions (93) (**Figure 3**).

NAFLD is a spectrum of disease ranging from simple steatosis (fatty liver) to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis (with or without malignancy). The underlying pathology is driven by dysregulation of lipid metabolism and accumulation of lipid in hepatocytes. It is a systemic disease where dysregulated inflammation in adipose, and liver tissue and changes in the gut microbiome all drive the production of inflammatory mediators such as cytokines and chemokines (94). In patients with NAFLD enlarged and aggregated KC populations are seen in the liver and their presence correlates with the severity of the disease (95).

This is consistent with observations in diet-induced murine models of NAFLD where KC activation leads to triglyceride accumulation and production of proinflammatory cytokines such as TNF-α (96, 97). Murine hepatic macrophages can also receive activation signals from lipid-stimulated hepatocyte-derived extracellular vesicles via tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2, also known as DR5) and receptor-interacting protein kinase 1 (98), and

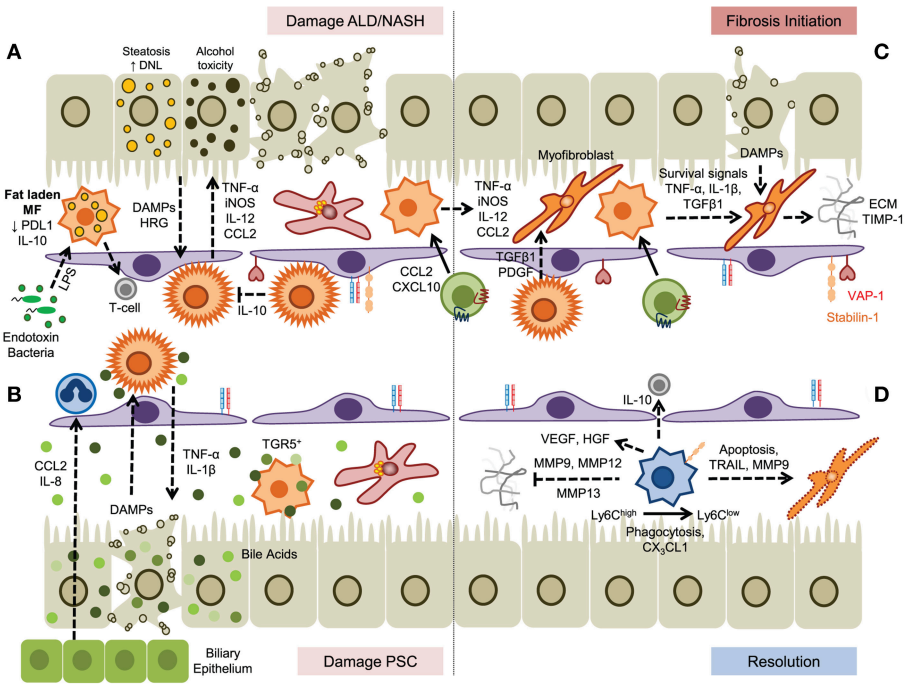

FIGURE 3 | A dual role for myeloid cells in the establishment and resolution of chronic liver disease. **(A)** Hepatocyte damage driven by steatosis or alcohol toxicity activates KC which secrete proinflammatory cytokines that drive disease progression and promotes infiltration of myeloid cells. In steatotic livers fat laden macrophages exhibit impaired endotoxin responses but may prime T-cell mediated immunity. **(B)** Cholangiocyte-derived chemokines promote recruitment of hepatic neutrophils and subsequent damage to hepatocytes promotes further inflammation. Bile acids promote KC inflammasome formation; however this can be suppressed through binding of bile salts to TGR5 expressed by monocyte-derived macrophages. **(C)** Secretion of soluble factors by KC and monocyte-derived macrophages promotes fibrosis through the activation and differentiation of hepatic stellate cells, promoting survival of myofibroblasts and the generation of extracellular matrix proteins. **(D)** Resolution of fibrosis is mediated by Ly6C^{low} macrophages, generated from Ly6C^{high} precursors, by degradation of ECM by matrix metalloproteinases, induced apoptosis of hepatic stellate cells and myofibroblasts, and secretion of anti-inflammatory cytokines.

obese mice also show reduced expression of the glucocorticoid-induced leucine zipper (GILZ) in macrophages associated with a proinflammatory phenotype (99). In this context the development of steatohepatitis arises from chronic inflammation associated with an influx of Ly6C^{+} monocytes that enhances the proinflammatory environment through activation of liver resident cell populations (100, 101). These infiltrating monocyte subsets are recruited via chemokine receptor pairs such as CCR2-CCL2 (40, 102) and CXCR3-CXCL10 (44), or atypical adhesion molecules including vascular adhesion protein-1 and scavenger receptors (103).

Intestinal dysbiosis and hepatocyte apoptosis contribute to the inflammatory response via DAMP- and PAMP-mediated pathways respectively (104, 105) associated with increased expression and activation of receptors such as *TLR4* and *TLR9* in both humans and murine models of NASH (106). Changes in the microbiome can have complex effects on the liver altering metabolic response through the production of metabolites that enter the liver via the portal vein as well as through bacterial products such as LPS, and in the presence of a leaky gut intact bacteria are taken up by KC (107). KC also regulate anti-inflammatory responses through secretion of IL-10. In addition to its more general anti-inflammatory properties KC derived IL-10 also induces the apoptosis of proinflammatory KC allowing KC to self-regulate toward a more tolerogenic environment (108). The induction of a pro-resolution M2 KC phenotype is dependent on activation of RORα and KLF4, and provision of an activator of RORα (JC1-40) improved the symptoms of NASH in a high fat diet murine model suggesting that KC polarization is a viable therapeutic strategy (109). Immune checkpoint proteins such as Tim-3 have been detected on a range of macrophage subsets in murine models of NASH. The presence of TIM-3 limits steatohepatitis by controlling ROS induced activation of NOX2 and the NLRP3 inflammasome and secretion of IL-1β and IL-18 (110). Thus, therapeutic strategies could look at promoting the recruitment or differentiation of TIM-3 macrophages to shift the local environment toward resolution and suppression of inflammation.

Similar mechanisms of disease progression have been described for ARLD, where metabolism of alcohol in the liver drives hepatocyte cell death. In rodent models of ARLD such as the Lieber-DeCarli diet hepatic macrophages become activated to produce TNF-α, IL-6, CCL2 and ROS (111, 112) and depletion of macrophage populations with either $GdCl_3$ or liposomal clodronate attenuated alcohol-induced liver inflammation (111, 113). Expression of myeloid NADPH oxidase, specifically the catalytic subunit gp91^{phox}, contributes to the pathogenesis of murine ARLD by driving a switch between

pro-inflammatory and pro-resolution macrophage populations. Thus, gp91^{phox}-deficient animals show an increased ratio of Ly6C^{high}/Ly6C^{low} intrahepatic macrophages and a diminished capacity for efferocytosis (114). Clustering of myeloid cells close to portal tracts is observed in ARLD patients (115) associated with increased levels of cytokines (IL-6, IL-8, IL-18), chemokines and macrophage activation markers that correlate with outcome and severity of disease (116–119). Gut permeability is increased in patients with ARLD leading to high levels of endotoxin in the liver resulting in a greater sensitivity of circulating monocytes from these patients to LPS (120, 121); a phenomenon also reported for resident KC isolated from alcohol-fed mice where increased sensitivity to endotoxin promoted expression of TNF-α and CCL2 (122, 123).

The role of macrophages in cholestatic diseases such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) is not well described. Accumulation of perisinusoidal hepatic macrophages in human tissue is reported in PSC but not in PBC (124) and increased infiltration of CD68$^+$/CCR2$^+$ cells was observed at later stages of disease in PSC including both CD206$^+$ (anti-inflammatory) and iNOS$^+$ (pro-inflammatory) macrophages (125). In diseases associated with cholestasis dysregulated bile acid production and excretion by cholangiocytes directly affects macrophage function and differentiation although the effects are complex. Although in mice hydrophobic bile acids have been reported to promote the formation of macrophage inflammasomes and IL-1β secretion (126, 127) other studies report activation of anti-inflammatory pathways in human macrophages by taurolithocholic acid through a PKA-mediated increase in IL-10 (128). Mice lacking the bile acid transporter *Mdr2* (*Abcb4*) develop hepatobiliary inflammation and fibrosis with some, but not all, features of PSC including an accumulation of peribiliary, proinflammatory macrophages recruited in response to cholangiocyte secretion of IL-8 and CCL2. Pharmacological treatment of mice with the CCR2/CCR5 antagonist cenicriviroc attenuated macrophage infiltration and liver injury consistent with an effector role for macrophages (125) and other rodent models have shown that the G-protein-coupled bile acid receptor, *Gpbar1* (TGR5) is expressed by macrophages to sense and respond to bile acids (129, 130). Activation of murine TGR5 leads to PKA-induced ubiquitination of NLRP3, acting as a brake on inflammasome activation (131) and dampening cytokine responses (129). In a murine model of colitis treatment with the TGR5 agonist BAR501 reduced the trafficking of Ly6C^{high} monocytes into the intestinal mucosa, reduced the expression of inflammatory genes (*Tnfa*, *Ifng*, *Il1b*, *Il6,* and *Ccl2*), and induced a regulatory T-cell environment through the production of IL-10 and TGF-β (132). The therapeutic potential of other TGR5 agonists such as 6α-ethyl-23(S)-methyl-cholic acid (6-EMCA, INT-777) are currently being explored in cholestatic liver disease (126, 133).

NEUTROPHIL MEDIATED LIVER INJURY

Neutrophils are derived from bone marrow and are released into the peripheral circulation where they play an important role in host defense and tissue healing (134), characterized by a high phagocytic capacity, the production of antimicrobial molecules and ability to shape immune responses (134, 135). The identities of neutrophil subsets and their functions are not clearly defined, with much of our knowledge arising from murine models. As a result their important roles in liver homeostasis and disease are only beginning to be understood (136, 137).

Neutrophil recruitment from the circulation into the liver is independent of selectins (138) and in many conditions is also independent of α2 integrin and ICAM-1 (139). Instead, neutrophils use CD44 to bind hyaluronan (HA) on LSEC and respond to chemokine ligands of CXCR2. A signaling network of TLR2, S100A9 and CXCL2 was shown to be necessary for neutrophil recruitment in a chronic model of liver injury in the mouse (140), while activation of TLR4 on LSEC was sufficient to induce the deposition of serum-associated hyaluronan-associated protein within the hepatic sinusoids which promoted CD44-dependent neutrophil migration in a murine model of endotoxemia (141). In sterile rodent injury models such as local thermal injury, HA-CD44 driven recruitment is less important and neutrophils use αMβ2 (Mac-1) binding to ICAM-1 (21). This pathway plays little role in septic injury because IL-10 leads to a loss of cell surface αMβ2 (142). Invading neutrophils in septic injury tend to arrest soon after infiltrating the tissue, whereas in sterile injury these cells migrate toward the focus of damage and adopt a swarming behavior which restricts neutrophil motility to within the boundary of the injury. This behavior is amplified by leukotriene B4 (LTB4) produced by the first invading neutrophils (21, 143). In sterile injury neutrophil recruitment can be promoted by ATP release from necrotic hepatocytes leading to activation of the inflammasome, and presentation of ligands for CXCR2 on the surface of the hepatic sinusoids (21). Alternatively N-formyl peptides released from dying and dead hepatocytes are detected by the formylated peptide receptor-1 on neutrophils which guide them toward the site of injury (21, 144, 145). This enables neutrophils to prioritize their responses to chemoattractant gradients that arise directly from damaged tissue over competing signals from chemokines or LTB4 and remain within the boundaries of the necrotized tissue (146–149). Live cell imaging in mice identified a non-muscle myosin II protein that was essential for neutrophil trafficking, demonstrating that myosin heavy chain 9 (Myh9) was localized in branching lamellipodia and in the uropod where it may enable fast neutrophil migration (150).

During acute liver injury, neutrophils use the receptor for advanced glycation end products (RAGE) to respond to HMGB1 released by necrotic hepatocytes (151). However, this pathway also contributes to sepsis through diminished bacterial killing by neutrophils and reduced NADPH oxidase activation (152). Neutrophils form extracellular traps by a process known as NETosis to enhance antibacterial defenses [reviewed in (153)]. Defects in NET formation have been linked to impaired efferocytosis and contribute to liver injury and sepsis in models of liver disease (154). In murine models of chronic liver disease neutrophils drive hepatocellular damage but are also associated with mechanisms of tissue repair. Myeloperoxidase secreted by neutrophils drives oxidative damage and contributes to the

development of NASH in mice (155) and increased levels of myeloperoxidase activity have been detected in patients with NASH (156). The development of obesity-related inflammation in patients with NASH also correlated with an increase in the ratio of neutrophil elastase to its inhibitor α1-antitrypsin, although the ratio reduced as the disease progressed to fibrosis (157). Conversely murine neutrophils can alleviate fibrosis through secretion of MMP8 and MMP9 (158), and depletion of neutrophils improved liver function in a diet-induced model of NASH (159). Following resolution of tissue damage in sterile injury neutrophils migrate out of the tissue and back into the vasculature and, following passage through the lungs where they upregulate CXCR4, return to the bone marrow where they undergo apoptosis (160). It is not currently known if this process contributes to the pathogenesis of other hepatic diseases.

The Contribution of Dendritic Cells to the Development of Liver Disease

In contrast to hepatic macrophages liver dendritic cells are scarce and mostly scattered in the portal region where they capture antigens delivered by via the portal vein (161). Dendritic cells can also translocate from blood to lymph via the hepatic sinusoids to concentrate in regional perihepatic lymph nodes (162). Hepatic dendritic cells comprise plasmacytoid DCs (pDCs) and classical (myeloid) DCs (cDCs) which express high levels of MHC-Class II molecules (e.g., HLA-DR) but are negative for other hematopoietic lineage markers (163). In general hepatic DCs are tolerogenic and inherently anti-inflammatory, but they can gain pro-inflammatory properties in the setting of chronic liver injury (164, 165). Plasmacytoid DCs identified as $lin^{-}CD11c^{int}MHC\text{-}II^{int}PDCA\text{-}1^{+}Siglec\text{-}H^{+}$ in some respects resemble B-cells and represent the most abundant subset in the murine liver under steady-state conditions (163, 166). Human pDCs are characterized by BDCA-2 and CD123 expression, but occur less frequently than in mice (167). This cell population responds to TLR7/8 ligands and mediate antiviral immunity by secreting type I interferons such as IFN-α but are less potent T-cell inductors. Classical DCs comprise two subtypes: cross-presenting $lin^{-}CD11c^{+}CD11b^{-}CD103^{+}CX_3CR1^{-}$ DCs (mainly interacting with $CD8^{+}$ T-cells via MHC-I) and conventional $lin^{-}CD11c^{+}CD11b^{+}CD103^{-}CX_3CR1^{+}$ DCs (presenting MHC-II bound antigens to $CD4^{+}$ T-cells) which correspond to human CD141 (BDCA-3) and CD1c (BDCA-1) DCs respectively (61, 163). In human liver $CD1c^{+}$ DCs prevail in contrast to mice (168). Another recent nomenclature differentiates hepatic DCs based on lipid content with high-lipid liver DCs inducing robust T-cell activation and cytokine secretion whereas low-lipid DC promote immune tolerance in both mice and humans (169).

Several factors contribute to the tolerogenic nature of hepatic DCs. When compared to splenic DCs, hepatic DCs were shown to be relatively immature (less CD40, CD80, CD86, CD83) with a reduced capacity to cross-present antigen to T-cells. Human DCs secrete high levels of IL-10, but less IL12p70 upon LPS-exposure (165) thereby contributing to endotoxin tolerance in the healthy liver (170). According to some reports hepatic DCs also predominantly induce regulatory and IL-4 secreting T-cells (171). During homeostasis low levels of circulating LPS trigger the expression of indoleamine-2,3-dioxygenase in human pDCs which catalyzes the production of immunoregulatory metabolites (172, 173). Following CpG stimulation murine pDC also fail to release abundant class I interferons owing to high NOD2 expression (174). Interestingly, circulating DCs that cross the hepatic sinusoids to reach the afferent lymphatics are educated by the hepatic microenvironment to adopt a regulatory phenotype, emphasizing the inherent tolerogenic phenotype of the hepatic niche (175).

Compared to other myeloid cells such as macrophages and monocytes the role of DCs in the initiation and progression of liver diseases is poorly defined. After switching from a regulatory to a proinflammatory state hepatic DCs can exacerbate acute liver injury in certain murine models (176, 177) whereas in human fatty liver disease there is emerging evidence they are protective by removing cellular debris and restricting DAMP driven activation of innate effector $CD8^{+}$ T-cells (178). $CD103^{+}$ DCs might be central to this response as *Batf3* deficient mice that lack CD103 displayed a more aggressive course in an experimental model of NASH (179). The failure to clear HCV has been associated with a reduced capacity of pDCs to secrete antiviral IFN-α and their ability to stimulate inhibitory T-cell receptors such as PD-1, TIM-3, and CTLA-4 (180–182). Thus, impaired DC activation in HCV infection might favor T-cell unresponsiveness leading to viral immune escape and persistence. DCs are not the only APCs within the liver. The liver's unique metabolic functions and constant exposure to gut antigens and gut-derived microbial products has resulted in a complex system for regulating immune responses in which DCs, endothelial cells and stromal cells may all contribute to presenting antigens and maintaining immune homeostasis (175).

Some studies have suggested that DCs may play a role in driving fibrogenesis beyond their ability to activate immune responses. However, the data are not compelling and most evidence points to DCs being largely dispensable for the progression of fibrosis. Although the expansion of $CD11b^{+}$ DCs has been observed during hepatic fibrogenesis in mice (183, 184), DCs are thought to promote resolution rather than progression of fibrosis. For example depletion of DCs during the regression phase of murine liver fibrosis significantly impairs tissue repair whereas *in vivo* expansion or adoptive transfer of purified DCs enhanced fibrosis reversal. This pro-resolution effect was mediated by MMP9 activity and clearance of activated hepatic stellate cells (185). Moreover, due to their anti-angiogenic properties DCs can counteract the profibrotic effect of VEGF mainly by expressing the VEGF receptor 1 (sFLT1) thus reducing the bioavailability of VEGF (186).

Role of Macrophages in Fibrosis Progression and Resolution

Liver fibrogenesis was previously regarded as a unidirectional process with little chance of resolution once scar tissue has formed. However, evidence now shows that even advanced fibrosis and in some circumstances cirrhosis are at least partially

reversible if the cause of liver injury can be eliminated (187). This concept has been demonstrated in both experimental models of chronic liver injury (188–190) and in human liver disease (191). For example in humans successful treatment of chronic viral hepatitis can lead to a marked improvement in liver architecture indicating that the liver has the potential for regeneration and remodeling of scar tissue. A landmark study by Marcellin et al. demonstrated that following 5 years of treatment of chronic Hepatitis B infection with tenofovir disoproxil fumarate, cirrhosis could be reverted in 74% of cases (192).

Kupffer cells and infiltrating monocyte-derived macrophages are crucially involved in this process of tissue remodeling (**Figure 3**) and it is clear that hepatic macrophages can play context-dependent fibrogenic and fibrolytic roles due to their heterogeneity and plasticity. For example hepatocyte-derived HRG, a non-inflammasome activating factor contributing to KC stimulation, favors a profibrotic phenotype of murine hepatic macrophages. This was demonstrated in HRG-deficient knockout mice where liver fibrosis was significantly attenuated in diet and toxin-induced models of liver injury (193). Similarly in humans interleukin-34 and macrophage colony-stimulating factor (M-CSF) promote a profibrotic phenotype in hepatic macrophages in the setting of chronic viral hepatitis (194). KC neutralize circulating endotoxins during homeostasis and release anti-inflammatory mediators such as IL-10 during low-level lipopolysaccharide (LPS) exposure (26). However, dysbiosis and translocation of gut bacteria and bacterial products due to intestinal barrier dysfunction result in the excessive presence of PAMPs within the hepatic microvasculature that reach the liver via the portal vein (195) which, in murine models, drives inflammasome activation of profibrotic hepatic stellate cells (196, 197). In a recent publication the cell-specific innate immune receptor triggering receptor expressed on myeloid cells-1 (TREM-1) was reported to promote hepatic inflammation and fibrosis in mice and humans (198), and inhibition of TREM-1 in mice ameliorated inflammation and macrophage and neutrophil activation in a mouse model of ARLD (199).

Chemokines released by KCs shapes the subsequent phase of hepatic inflammation. The CXC chemokines CXCL1, CXCL2, CXCL8 attract neutrophils whereas CCL2 is the major chemokine that governs influx of bone-marrow-derived monocytes (5). Hepatic stellate cells are another important source of CCL2 and there is a bidirectional relationship between pro-fibrotic effector cells and hepatic macrophages (197) as shown by the ability of HSC to respond to CCL2 and CCL5 produced by hepatic macrophages. Bone-marrow chimeric mice were used to show that activation of CCR2 and CCR5 in HSC drives fibrogenesis through stimulation of HSC migration and collagen production, whereas CCR1 acts solely on monocytes/macrophages (200–202). In humans macrophages exposed to HCV serum synthesize CCL5 and activate hepatic stellate cell confirming the murine data (203). CCL3 deficient mice display reduced HSC proliferation and migration and attenuated fibrogenesis (204). Secretion of CCL3 was shown to be dependent on the expression of the scavenger receptor Stabilin-1 by a specific macrophage population, and genetic deletion of *Stab1* led to diminished anti-fibrotic responses in diet and toxin-induced murine models of liver disease (205).

The release of CCL2 during early hepatic injury in mice augments the intrahepatic pool of macrophages by selectively attracting bone-marrow derived inflammatory $CCR2^{+}CX_3CR1^{low}Ly6C^{high}$ monocytes but not $CCR2^{-}CX_3CR1^{high}Ly6C^{low}$ counterparts (56). The expansion of hepatic macrophages is maintained during iterative episodes of liver injury that drive fibrogenesis (56) and CCR2 directed inhibition of monocyte recruitment during liver injury in murine models reduces liver scarring (56, 200, 206, 207). Targeting CCR2 with either the small molecule inhibitor cenicriviroc (208–210) or the L-enantiomeric RNA oligonucleotide mNOX-E36 (211) achieved similar results. In line with this data from the phase 2b Centaur trial revealed that treatment with cenicriviroc reduces fibrosis in patients with NASH after 1 year of treatment (212). Despite these advances, the profibrotic role of monocytic CCR2 is not fully understood following recent studies demonstrating that CCR2 expressed by monocytes/macrophages is dispensable for liver fibrogenesis (200). This was confirmed following further studies in mice which revealed that CCR1 (which binds CCL3 and CCL4), CCR8 (which binds mainly CCL1) and CCR9 which binds CCL25 are also involved in recruiting monocytes to the site of hepatic injury during fibrogenesis (42, 201, 213).

Both monocyte-derived macrophages and Kupffer cells promote fibrogenesis by secreting TGF-β and galectin-3, which drive transdifferentiation of HSCs into matrix secreting myofibroblasts (56, 214–216). Hepatic macrophages are also implicated in the survival and activation of HSC through secretion of IL-1β and TNF-α [in a NF-κB activation-dependent fashion (183)] or via IL-4 and IL-13 secretion in Th2-dominated rodent injury models such as parasitic infections (217). Oncostatin M (OSM) might also function as a potent regulator of hepatic macrophage/HSC interaction by enhancing the expression of profibrotic and mitogenic genes such as TGF-β and PDGF in bone-marrow derived infiltrating macrophages, with macrophage-depleted livers being largely protected from OSM-induced fibrosis (218). Interestingly, the profibrotic effect of KC-secreted TGF-β is retained following inhibition of CCL2-dependent monocyte in experimental steatohepatitis (102) which might impede the effectiveness of CCL2/CCR2 based therapies to treat liver fibrogenesis.

The first reports of hepatic macrophages driving fibrosis resolution were published alongside data describing the profibrogenic nature of Kupffer cells and monocyte-derived macrophages in liver injury. This apparent paradox was clarified when Duffield and colleagues reported a dual role for macrophages during different phases of chronic liver injury in mice. They used a CCl_4 model to show that mice in which hepatic macrophages were selectively depleted exhibited less matrix deposition at advanced stages of fibrogenesis but more fibrosis when macrophages were depleted during the resolution phase (219). These data suggested the existence of distinct macrophage populations within the liver that fulfill opposing functions according to the disease stage. A subsequent study by

the same group confirmed this by reporting the accumulation of macrophages around scar fibers during the resolution phase that were capable of degrading ECM through expression of matrix metalloproteinase protein 13 (MMP13) (220). These scar-associated macrophages are also equipped with MMP9, MMP12 and TRAIL, and contribute to the disruption of scar tissue and induction of fibroblast apoptosis (187). However, these studies did not identify which hepatic macrophage population gave rise to this profibrolytic subset. One of the first lines of evidence that monocyte-derived macrophages might be responsible stems from a paper showing that CCR2 deficiency is protective during fibrogenesis but hinders scar removal during the regression phase following cessation of CCl_4 challenge in rodents. The putative mechanism is a balance between levels of tissue inhibitor of metalloproteinase-1 (TIMP1) and MMP1 and MMP13 mRNA, in the liver (207). This study also determined that profibrotic and pro-resolution macrophages share the same precursor cells; a hypothesis supported by a seminal paper in 2012 showing that in mice $Ly6C^{high}$ inflammatory macrophages undergo a phenotypic switch to an anti-inflammatory and anti-fibrotic "restorative" $CD11b^{high}F4/80^{int}Ly6C^{low}$ subtype (52). The accumulation of $Ly6C^{low}$ macrophages producing matrilytic MMPs peaked at the maximum point of fibrosis resolution whereas the early phase of liver parenchyma damage is dominated by freshly recruited inflammatory $CCR2^{+}Ly6C^{high}$ macrophages. Phagocytosis of cell debris drives macrophage transdifferentiation toward a restorative $Ly6C^{low}$ phenotype (52). The concept of a context dependent hepatic macrophage plasticity was demonstrated using sterile liver inflammation models in which $CCR2^{high}CX_3CR1^{low}$ macrophages accumulate early after focal tissue injury in a ring-like structure and then give rise to a reparative $CCR2^{low}CX_3CR1^{high}$ phenotype which facilitate wound repair (14). There is also evidence that *in situ* reprogramming of infiltrating macrophages from a profibrotic to an antifibrotic subset is controlled by the CX_3CR1/CX_3CL1 axis, which promotes macrophage survival and imprints an anti-inflammatory state. Consequently, CX_3CR1 knockout mice display enhanced tissue damage and fibrosis after bile duct ligation and CCl_4 exposure (221, 222). Though circulating $Gr1^{low}$ ($Ly6C^{low}$) CX_3CR1^{+} monocytes show patrolling behavior in blood stream (34) there is no data so far to support the idea that these cells are directly recruited to the inflamed liver and thereby perpetuate fibrosis resolution. Never the less, this cannot be excluded since extravasation of CX_3CR1^{+} monocytes into affected organs has been demonstrated in models of myocardial infarction and lung injury (223, 224).

Translating findings from rodent models into patients is not straightforward. Most importantly—as outlined above—human liver macrophage subsets lack well-defined surface marker patterns that allow for distinction of resident Kupffer cells from infiltrating monocytes. For example $CD68^{+}$ which is deemed to be a macrophage marker in mice can also be detected on circulating monocytes in human, and although gene profiles show overlap between murine $Ly6C^{high}$ and "classical" human $CD14^{++}CD16^{-}$ monocytes and murine $Ly6C^{low}$ and "non-classical" human $CD14^{+}CD16^{++}$ monocytes (31) there are clear functional differences. In addition, it is difficult to integrate the "intermediate" $CD14^{++}CD16^{+}$ subset into the murine nomenclature (225). In general, $CD16^{+}$ monocytes are enriched in the liver in comparison to peripheral blood even under steady state conditions (46), with increased numbers being observed in patients with cirrhosis (47). $CD14^{+}CD16^{-}$ cells can acquire CD16 expression under the influence of soluble factors present in the diseased liver such as IL-10 and TGF-β, and $CD16^{+}$ monocytes display higher phagocytic capacity and can secrete both pro- and anti-inflammatory cytokines upon LPS stimulation thus resembling both $Ly6C^{low}$ and $Ly6C^{high}$ monocytes/macrophages in mice. Of note, $CD16^{+}$ but not $CD16^{-}$ monocytes can directly activate human HSC (47). Rodent models also lack the highly-crosslinked scar tissue observed for patients with advanced fibrosis, and as a result macrophage-mediated resolution of fibrosis is accelerated in murine models compared to humans. Therefore, further research is warranted to better define disease-specific characteristics of hepatic monocyte and macrophage subsets in human diseases. Despite these differences there are some striking parallels between mice and humans. For example, the CCL2/CCR2 axis plays a similar role in fibrosis and macrophage infiltration to the inflamed liver in mice and humans, and TREM-1 is emerging as an additional shared marker.

MALIGNANCY

Myeloid cell subsets are linked to virtually all steps in the natural course of tumor formation and spreading in the liver. Extensive research has shown a prominent role for tumor associated macrophages (TAM) and MDSC in the development of hepatocellular carcinoma (HCC), an archetypical inflammatory tumor in which chronic inflammation drives liver cancer pathogenesis, invasion, and metastasis (226). In the context of liver carcinogenesis monocytes/macrophages exert dualistic functions in a stage-dependent manner with $CCR2^{+}$ CCL2-responsive monocytes promoting tumor surveillance through the elimination of senescent premalignant hepatocytes in healthy livers (**Figure 4**), whereas in established tumors monocytes/macrophages are reprogrammed to silence NK cells resulting in tumor growth (227, 228). Tumor associated macrophages derived from infiltrating monocytes are a dominant cellular component of human tumor stroma with increased density of TAMs in peritumoral margins being closely linked to poor prognosis (229, 230). Consistent with this the absence of macrophages correlated with improved patient survival (231). In both humans and murine models TAMs drive tumorigenesis by sustaining inflammatory pathways mediated through secretion of cytokines such as TNF-α and IL-6 that suppress hepatocyte apoptosis and induce proliferation in response to tissue damage (232–234). The surface receptor TREM-1 expressed on myeloid cells governs the secretion of proinflammatory mediators and engagement of TREM-1 in hepatic macrophages has been shown to trigger carcinogenesis (235). *Trem1*-deficient mice treated with diethylnitrosamine (DEN) were protected from malignancy due to attenuation of cytokine secretion (e.g., IL-6, IL-1β, TNF, CCL2)

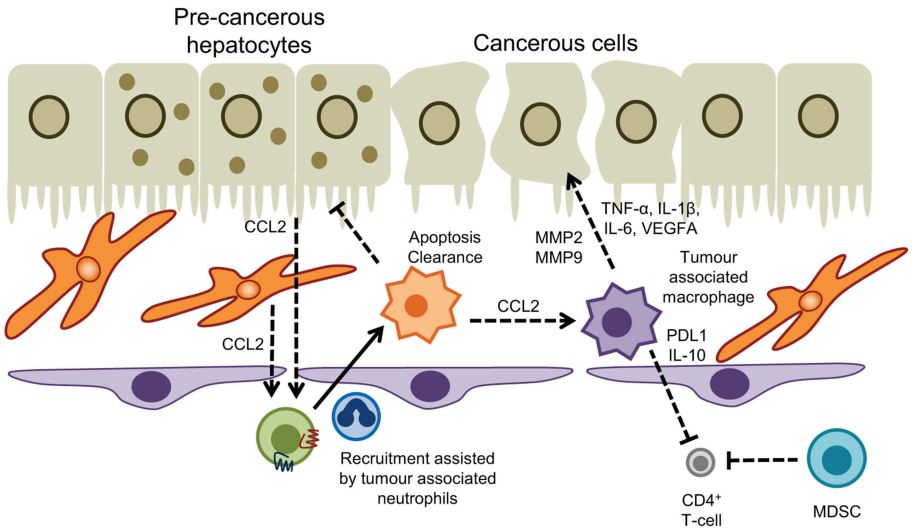

FIGURE 4 | Myeloid cells in hepatic malignancy. Tumor associated macrophages promote cancer cell proliferation and neoangiogenesis, and act in concert with myeloid-derived suppressor cells to dampen T-cell immunosurveillance. Conversely recruitment of monocytes into the tumor microenvironment driven by CCL2 produced by hepatocytes and hepatic stellate cells, and interactions with tumor associated neutrophils promotes apoptosis and clearance of pre-cancerous hepatocytes to prevent HCC.

and ablation of inflammatory signaling pathways (p38, ERK1/2, JNK, MAPK, and NF-κB) in KC (235).

The inflammatory environment present in chronic liver injury facilitates the recruitment and retention of monocyte-derived TAMs which promote tumorigenesis in a MMP2/MMP9 dependent fashion. This was demonstrated by comparing the seeding of injected HCC cells in healthy livers with CCl_4 preconditioned livers where an alternatively-activated macrophage population (M2-like) were enriched in the tumor environment (236). Infiltrating TAMs are frequently reported to resemble alternatively-activated macrophages (237–239) although the dichotomous approach of M1/M2 polarization does not fully reflect the entire spectrum and heterogeneity of tissue macrophages. Nevertheless, reversal of M2-like polarization in experimental HCC has yielded promising results in containing tumor progression (240) with TIM-3 and Wnt ligands identified as critical drivers of alternative activation of TAMs and HCC growth (239, 241). In patients, total immune cell infiltration into HCC correlated with M1-like macrophage populations and a more favorable prognosis (242).

The CCL2/CCR2 axis is a promising novel target in HCC therapy. Antagonism of CCR2 by the compound RDC018 not only reduced TAM infiltration but also restored anti-tumor immune response and ameliorated HCC outcome in murine models of HCC (243). Tumor associated neutrophils (TAN) provide an important source of CCL2 in HCC and can act synergistically with TAM to support liver tumor progression (244). In humans neutrophil extracellular traps can also promote inflammation and development of HCC on the background of NASH, driven by the presence of free fatty acids (245); however our understanding of the precise role played by neutrophils in liver cancer remains elusive. One striking feature of TAM is the induction of an immune suppressive microenvironment that disrupts anti-tumor immunity. For example, release of regulatory cytokines such as TGF-β and IL-10 by TAM impair Th1 and cytotoxic T-cells but promote regulatory T cells and Th2 activity all of which facilitate tumor growth. TAMs also express high levels of PDL1, galectin-9, and indoleamine-pyrrole 2,3-dioxygenase (IDO) that foster T-cell exhaustion and prevent effective anti-tumor immune response (241). In HCC the expression of PDL1 by TAMs correlated with increased tumor burden and the intensity of the protein was associated with high mortality and reduced survival (246). MDSC share many mechanisms with TAM to protect from HCC-targeted T-cell activity, and the net effect of MDSCs in HCC nodules and peritumoral stroma is progression of the tumor (241). Furthermore, MDSC reduce the tissue availability of arginine and cysteine, which are essential for T-cell proliferation and impede NK cell cytotoxicity and development via NKp30 receptor (241).

RATIONAL DESIGN OF THERAPEUTIC STRATEGIES TARGETING MYELOID POPULATIONS

There is a major unmet need for effective therapies to prevent or reverse liver fibrosis particularly in the context of a major increase in fatty liver disease and the continuing high prevalence of alcoholic cirrhosis (247). Macrophages have the dual potential to serve as therapeutic targets and as treatment vehicles for inflammation-induced liver fibrosis and carcinogenesis (248). In principle, macrophages can be targeted at different stages

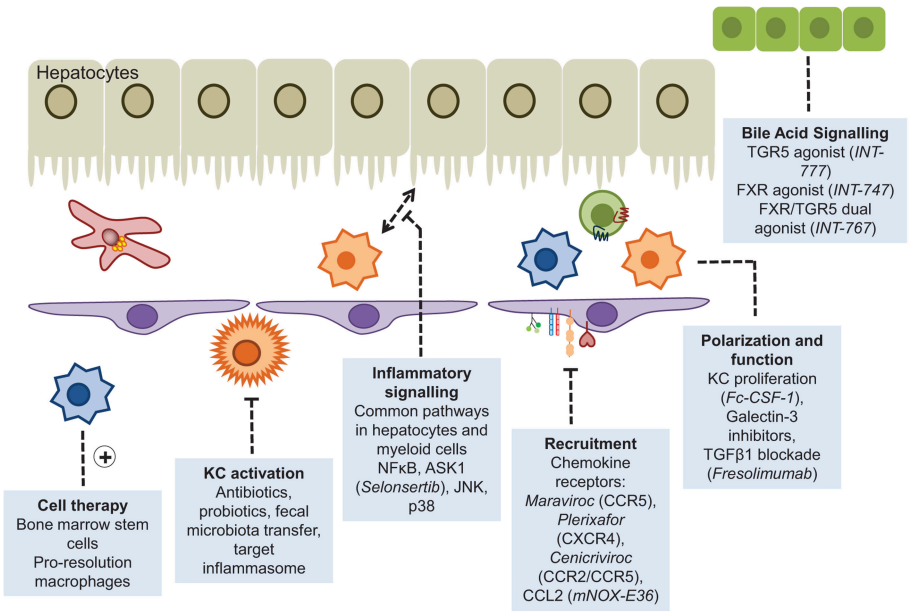

FIGURE 5 | Myeloid cells as therapeutic targets. Approaches that have been adopted to enhance or diminish the role of myeloid cells in liver disease include disruption of the recruitment cascade or inflammatory signaling pathways, and augmented pro-resolution responses through cellular infusions of stem cells or the provision of agonists driving macrophage polarization.

of disease and different subsets of monocyte macrophages can be targeted. Such strategies include (i) attenuation of Kupffer cell activation by anti-inflammatory compounds; (ii) inhibition of macrophage precursor cell (i.e., monocyte) recruitment to the injured liver; (iii) manipulation of macrophage polarization and differentiation to facilitate transition toward a restorative reparative phenotype (iv) infusion of beneficial pro-restorative macrophages (248) (**Figure 5**). Interference with chemokine pathways to restrict influx of inflammatory monocytes is one of the most advanced approaches. As stated earlier, the CCR2/CCR5 antagonist Cenicriviroc has entered phase 2b clinical trials with promising results reported after 12 months treatment of NASH-related fibrosis (212). The current options in targeting macrophages in the context of liver disease have recently been comprehensively summarized (248). Adoptive cell therapy using hematopoietic stem cells or macrophages is an approach that is attracting increasing interest. The first studies reporting efficacy of bone marrow cell transfer in murine models of liver fibrosis were published almost 15 years ago when injection of bone marrow cells was shown to cause MMP9-dependent reduction in ECM deposition in response to CCl_4 (249). In a study by Thomas et al. in 2011 bone-marrow derived macrophages (BMM) were prepared *in vitro* by stimulation with CSF-1 and subsequently injected into the portal vein of mice with long-term CCl_4 induced fibrosis. The infused cells did not conform to the M1/M2 paradigm but expressed IL-10, TWEAK, and MMP13, which are known to suppress inflammation and to promote cell regeneration and fibrolysis. Treatment significantly reduced liver scarring by promoting myofibroblasts apoptosis, MMP-induced degradation of ECM and by stimulating liver regeneration. In contradistinction, non-purified whole bone marrow cells increased liver fiber content (250). Similar results were obtained in another study showing that IL-10 producing $CD11b^{+}Gr1^{+}$ myeloid cells account for the tissue remodeling effect of bone marrow transplantation in liver fibrosis (251). BMM also ameliorate oxidative stress and reduce production of the potent profibrotic cytokine IL-13 (252). Interestingly, macrophages derived from pluripotent embryonic stem cells exhibit comparable antifibrotic effects to BMM though these cells tend to resemble resident Kupffer cells rather than infiltrating macrophages (253). A contributing factor to the success of bone marrow derived macrophage transplantation in liver fibrosis could be activation of the sphingosine-1-phosphate receptor (S1PR) that critically controls BMM motility (254). Mice treated with FTY720 which triggers S1PR internalization retained infused $c\text{-}kit^{+}/sca1^{+}/lin^{-}$ hematopoietic stem cells in the liver due to a failure of the cells to egress into the draining lymph. This was associated with reduced scarring in methionine-choline-deficient diet fed and CCl_4 treated mice (255). Further studies are needed to dissect whether S1P/S1PR antagonism also augments the antifibrotic effects of transplanted BMM. Despite the promising experimental findings human cell therapy trials in advanced clinical cirrhosis have so far proven disappointing. The REALISTIC trial tested the efficacy of G-CSF mobilized and autologous infusions of $CD133^{+}$ stem cell therapy in cirrhosis but failed to show any improvement in liver function with more complications in the treatment group (256). This is perhaps unsurprising given that resolution will only occur if the right cells are infused into the right microenvironment at the right disease

stage. This requires the design of more sophisticated precision medicine trials. Such studies are underway (257, 258) but we are only at the start of understanding macrophage therapy for liver diseases.

AUTHOR CONTRIBUTIONS

CW, HZ wrote parts of the manuscript. DA edited and finalized the manuscript.

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Dysfunctional Immune Response in Acute-on-Chronic Liver Failure: It Takes Two to Tango

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Acute-on-chronic liver failure (ACLF) is characterized by the acute decompensation of cirrhosis associated with organ failure and high short-term mortality. The key event in the pathogenesis is a dysfunctional immune response arising from exacerbation of the two main immunological alterations already present in cirrhosis: **systemic inflammation** and **immune cell paralysis**. High-grade systemic inflammation due to predominant activation and dysregulation of the innate immune response leads to the massive release of cytokines. Recognition of acutely increased pathogen and damage-associated molecular patterns by specific receptors underlies its pathogenesis and contributes to tissue damage and organ failure. In addition, an inappropriate compensatory anti-inflammatory response over the course of ACLF, along with the exhaustion and dysfunction of both the innate and adaptive immune systems, leads to functional immune cell paralysis. This entails a high risk of infection and contributes to a poor prognosis. Therapeutic approaches seeking to counteract the immune alterations present in ACLF are currently under investigation.

Keywords: ACFL, systemic inflammation, cirrhosis-associated immune dysfunction, immune paralysis, liver, cirrhosis

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INTRODUCTION

Cirrhosis is characterized by progressive fibrosis, portal hypertension, and liver failure. It comprises two consecutive but potentially reversible stages: compensated and decompensated cirrhosis. Cirrhosis also features a progressively dysfunctional immune response that encompasses systemic inflammation (SI) and immunodeficiency. The term cirrhosis-associated immune dysfunction (CAID) (1) defines the spectrum of immune system alterations present in cirrhosis, which induce extrahepatic clinical manifestations and a higher susceptibility to bacterial infection.

Over the course of compensated or decompensated cirrhosis, an acute precipitating event, such as bacterial infection, may challenge liver homeostasis producing a syndrome called acute-on-chronic liver failure (ACLF). ACLF is characterized by acute decompensation of cirrhosis, hepatic and/or extrahepatic organ failure and high short-term mortality (2). The activation of an inadequate immune response to the triggering event is key to its pathogenesis and involves the highest grade of SI and immunodeficiency. This review is an overview of the major immune

alterations that occur in ACLF, considering the specific circumstances of patients with end-stage liver disease.

ACLF: CONCEPT AND DEFINITION

The most widely accepted definition of ACLF arises from the results of a large, prospective, multicenter European project, the CANONIC study (2). According to its results, ACLF is defined in terms of its three main features:

1. *Acute decompensation* of cirrhosis, which refers to the acute development or significant worsening of ascites, encephalopathy, gastro-intestinal bleeding or any combination of these in a patient with cirrhosis. ACLF may occur at any stage from compensated to decompensated cirrhosis, and can be triggered by hepatic or extrahepatic factors (2).
2. *Organ failure*, including both the liver and extrahepatic organs. Organ failure is the diagnostic hallmark of ACLF. The kidney is the most commonly failing organ (55.8%) followed by the liver (43.6%) (2). According to the affected organ and number of organs failing, ACLF severity is graded as three stages (ACLF 1-3).
3. *High short-term mortality*, 28-day mortality is poor and correlates with severity, ranging from 23 to 74% in patients with ACLF grades 1 to 3, respectively (2).

IMMUNE DYSFUNCTION IN ACLF

The key immune pathogenic events in ACLF are exacerbated SI and worsening of the defective effector immune response already present in cirrhosis (**Figure 1**). Both are general mechanisms leading to organ failure in other clinical scenarios, such as sepsis, in otherwise immunocompetent subjects (3). In cirrhosis, however, systemic inflammation and immune cell anergy give rise to more severe consequences and a worse prognosis due to preexisting immune dysfunction and liver damage.

Cirrhosis-Associated Immune Dysfunction

Cirrhosis, independently of stage and etiology, is associated with a spectrum of distinctive abnormalities of the immune system that result in SI and acquired immunodeficiency, collectively termed CAID (1). The severity of CAID parallels the cirrhosis stage, and can worsen acutely as the consequence of incidental events, such as bacterial infection or hepatic inflammation (i.e., alcoholic hepatitis). The immune system phenotype of patients with decompensated cirrhosis and, to a lesser extent, those with compensated disease, features low-grade systemic inflammation associated with the increased expression of activation antigens and production of pro-inflammatory cytokines by the activated circulating and tissue-resident immune cells. At this stage, the effector immune response against pathogens is not yet fully compromised. Further, the most severe immune disturbances in cirrhosis are found in patients with ACLF who present an immune system phenotype characterized by high-grade systemic inflammation and severe immunodeficiency. CAID exemplifies the dynamic nature and plasticity of the immune response, as it can switch from a predominantly low-grade pro-inflammatory phenotype to another response involving massive systemic inflammation and marked immune cell anergy, and vice versa.

Systemic Inflammation in ACLF

Evidence of SI in ACLF

Inflammation is triggered by various noxa as a host response designed to restore tissue homeostasis. In cirrhosis, the greatest inflammatory responses are those associated with bacterial challenge or massive hepatocyte damage targeted at eliminating bacteria and promoting tissue repair. Eventually, immune activation becomes massive and results in an overwhelming production of pro-inflammatory cytokines, which leads to tissue damage and organ failure. Evidence of high-grade SI in ACLF is supported by increased plasma levels of pro-inflammatory (IL-6 and TNFα) and anti-inflammatory cytokines (IL-10 and IL-1ra) (4), soluble markers of macrophage activation (sCD163 and mannose receptor) (5), C-reactive protein, and circulating numbers of white blood cells (2). These biomarkers are moderately increased in patients with acutely decompensated cirrhosis without organ failure, but markedly augmented in those with ACLF, which indicates more severe inflammation in the latter, similar to the degree observed in patients with sepsis admitted to intensive care units (6, 7). Such increased levels of inflammatory markers closely correlates with the number of failing organs, and therefore, with severity and prognosis (8). Additionally, the best characterized triggers of ACLF (bacterial infection and alcoholic hepatitis) are recognized inductors of the inflammatory response, and SI is even present when no identifiable precipitating factors are found at presentation. Together these findings suggest that inflammation plays a major pathogenic role in ACLF.

Mechanisms of Systemic Inflammation

A. **General mechanisms of SI in cirrhosis**

Infection and tissue injury are the classically known inducers of inflammation. Bacterial pathogens of a given class present sets of conserved molecular patterns (PAMPs) that can be recognized by pattern recognition receptors (PRRs), such as Toll- (TLRs) and NOD-like receptors (NLRs) (9). Recognition of PAMPs by PRRs activates intracellular signaling pathways leading to cytokine production and activation and recruitment of neutrophils. However, inflammation in the absence of pathogens (sterile inflammation) also occurs, as PRRs may recognize molecules released by dead or injured cells. These molecules (damage-associated molecular patterns or DAMPs) are intracellular factors usually invisible to the host immune system, thus preventing pathological inflammation and autoimmunity (10).

In cirrhosis, low-grade systemic inflammation is prompted by increased intestinal barrier permeability due to loosening of the tight-junctions, higher bacterial transcytosis, and a reduction in the mediators that limit contact between bacteria and intestinal microvilli. In addition, bacterial overgrowth and dysbiosis promote translocation of bacteria and PAMPs, such as LPS, from the intestinal lumen to the mesenteric lymph nodes and gut-associated lymphoid tissue. The capacity of the

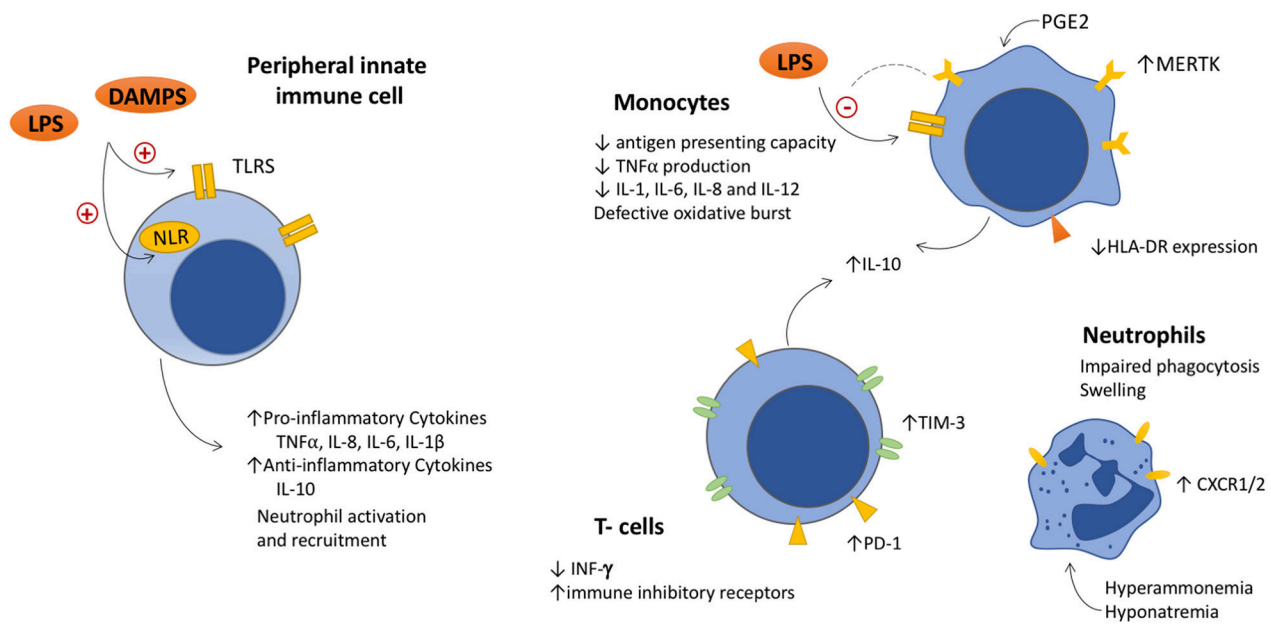

FIGURE 1 | The dysfunctional immune response in ACLF is featured by high-grade systemic inflammation and immune cell paralysis. **(A)** High-grade systemic inflammation. Activation of Toll like and Nod-like receptors in peripheral monocytes and dendritic cells by increased DAMPs and PAMPs, leads to the overproduction of pro-inflammatory and anti-inflammatory cytokines, as well as to the activation and recruitment of neutrophils to the site of injury. Detrimental effects due to the excessive immune cell activation (immunopathology), contribute to tissue damage, and organ failure, which also generates more DAMPs and perpetuates immune activation. **(B)** Immune cell paralysis. As ACLF progresses, (i) an excessive compensatory anti-inflammatory response, (ii) the exhaustion of the immune effectors, and (iii) metabolic and neuroendocrine disturbances (increased PGE2, hyperammonemia, and hyponatremia), lead to an acquired immunodeficiency that involves the innate and the adaptive responses. IL-10 seems to play a pivotal role in the pathogenesis of the immune cell paralysis as it modulates NF-kβ activity decreasing TNFα, IL-1, IL-6, IL-8, and IL-12 secretion by monocytes. IL-10 inversely correlates with levels of immune inhibitory receptors in monocytes (MERTK), lymphocytes (PD-1 and TIM-3), and INF-γ production, which contributes to the immune cell dysfunction. Decreased HLA-DR expression in monocytes impairs their antigen presenting role and TNFα production in response to LPS stimulation. Additionally, neutrophils show high levels of CXCR1/CXCR2 receptors, which contributes to hepatocyte death through early apoptosis and necrosis. LPS, Lipopolysaccharides; DAMPs, damage associated molecular patterns; TLR, Toll like receptors; NRL, nod like receptors; TNFα: Tumor necrosis factor α; IL, interleukin; PGE2, Prostaglandin E2; MERTK, tyrosine-protein kinase MER; IFN-γ, interferon-γ; TIM-3, T-cell immunoglobulin and mucin domain 3; PD-1, Programmed cell death-1; CXCR1/2, chemokine receptor1/2.

lymph nodes to eliminate intestinal bacteria may be eventually overcome, allowing gut-derived PAMPs to spread, reach the liver, and activate proinflammatory signaling pathways. Moreover, DAMPS released from chronically injured or dead hepatocytes, such as high mobility group box 1, are also recognized by PRRs contributing to persistent SI in cirrhosis (11). The analysis of the microbiota in portal vein as in other different locations (liver outflow, central venous blood, and peripheral venous blood), has recently shown that the microbial composition does not differ significantly among the circulatory compartments. Cytokine levels in serum directly correlated with the abundance of blood microbiome genera, providing further evidence of the association between circulating microbiome and inflammation in cirrhosis (12).

B. Specific mechanisms active in ACLF

ACLF is characterized by an extreme exacerbation of the low-grade SI already present in cirrhosis. Cytokines involved in the innate immune response are significantly up-regulated, suggesting the predominant pathogenic involvement of the innate arm of the immune system. In addition, different cytokine profiles according to the precipitating event have been observed: thus, IL-8 is significantly higher in alcoholic patients, while TNFα, IL-6, and IL-1ra are mostly increased when bacterial infection is the trigger (4).

Sepsis-induced ACLF

Bacterial infection is the most frequent identifiable trigger of ACLF (30%). The most common infections are spontaneous bacterial peritonitis, sepsis and pneumonia (2). Of note, during early infection stages, cirrhotic patients display higher levels of pro-inflammatory cytokines as compared to non-cirrhotic ones (13). In sepsis-induced ACLF, bacteria, and PAMPs are recognized by PRRs, which results in activation of TLR and NLR signaling pathways. In turn, this increases the activity of transcription factors involved in the production of pro-inflammatory cytokines and the activation of immune cells. TLR4 is activated in response to bacterial LPS through a recognition receptor complex that involves the co-receptors CD14 and MD-2. Downstream TLR4, LPS may activate two different

circuits: MyD88-dependent or independent signaling pathways. The MyD88-dependent pathway involves nuclear translocation of NF-κB (14). This transcription factor induces the release of pro-inflammatory cytokines such as TNFα, IL-6, and IL-1β in response to infection, and, interestingly, NF-κB has also been implicated in the pathogenesis of several liver diseases, including viral hepatitis, steatohepatitis, and hepatocellular carcinoma. In contrast, the MyD88-independent pathway leads to phosphorylation of the interleukin regulatory factor three, which increases the production of type-I interferon. LPS may be also recognized in the cytoplasmic compartment by the pro-inflammatory caspases 4 and 5. Activation of NLR by caspases results in up regulation of pro-inflammatory cytokines, such as IL-1β, which are then released to recruit and activate additional inflammatory cells (15).

Alcohol-induced ACLF

Excessive alcohol consumption induces SI through both sterile and non-sterile mechanisms. First, alcohol alters the amount and composition of the gut microbiota. In addition, alcohol metabolism directly affects the intestinal epithelial barrier, disrupting the integrity, and altering the expression of tight junction proteins. Dysbiosis and increased intestinal permeability promote translocation of bacteria and PAMPs, which activates proinflammatory immune receptors leading to increased production of cytokines and the activation and recruitment of neutrophils to the liver (11).

In addition, sterile stimuli also contribute to inflammation. Ethanol promotes the release of mitochondrial cytochrome c and increased expression of the Fas ligand. This leads to hepatocyte apoptosis through caspase-3 dependent pathways (16). Further, oxidative stress and hepatocyte reactive oxygen species production mediate alcohol-induced liver injury, increasing the activity of cytochrome P-450 2E1 (17). The subsequent mitochondrial damage and TNF-α overproduction induce endoplasmic reticulum–dependent apoptosis and lipid synthesis up-regulation (18).

The above mechanisms are critically exacerbated in alcoholic hepatitis, which is the second most frequent identifiable trigger of ACLF (20%) (2). Extensive damage to the hepatocytes leads to cell death thus generating more DAMPs, which amplifies and perpetuates the inflammatory response.

Immune System Cell Paralysis in ACLF

Despite exacerbated SI, patients with ACLF paradoxically feature a compromised effective immune response against pathogens which increases the risk of bacterial infection (19). This was documented in the CANONIC study in which the rate of bacterial infections increased in steps according to ACLF grade (2). An excessive compensatory anti-inflammatory response along with exhaustion and dysregulation of immune effector cells, underlie the pathogenesis of this paralysis (19). Compensatory anti-inflammatory response syndrome (CARS) has been described in patients with high grade SI due to sepsis, trauma, burns, or tissue injury (20). CARS is characterized by lymphocyte anergy and reduced numbers due to apoptosis (21), decreased cytokine production and HLA receptors upon monocyte stimulation (22), and increased expression of anti-inflammatory cytokines such as IL-10 (23). Most of these alterations have been, accordingly, described in patients with ACLF, as described below.

Evidence

In ACLF, the inadequate immune cell response against pathogens affects both the innate and adaptive arms of the immune system. Circulating **monocytes** in patients with ACLF show decreased HLA-DR expression, which impairs antigen presenting capacity and TNFα production in response to LPS stimulation (19). The clinical relevance of these findings was supported by a link observed between reduced monocyte HLA-DR expression and survival in critically ill cirrhotic patients (24). Additionally, **neutrophils** in alcoholic hepatitis-related ACLF show reduced phagocytic capacity and high expression levels of CXCR1/CXCR2 receptors, which contributes to hepatocyte death through early apoptosis and necrosis (25), and is associated with a greater risk of infection, organ failure and mortality. Interestingly, neutrophil dysfunction was reversed by *ex vivo* removal or neutralization of the endotoxin (26). In addition, the inefficient immune cell response of ACLF is not limited to the innate arm but extends to the T lymphocyte compartment, whose cells feature the increased expression of suppressor receptors (27).

Mechanisms

The exact mechanisms underlying immune cell paralysis are not fully understood, although several events contribute to its pathogenesis: (i) an excessive inhibitory immunoregulatory response triggered to counteract the massive SI, (ii) the exhaustion of effector immune system cells subjected to persistent chronic stimuli of enteric origin, and (iii), the dysfunction of immune effectors related to the metabolic and neuroendocrine abnormalities associated with hepatic insufficiency.

i) As in other critical pathological scenarios such as sepsis, in ACLF, CARS responses lead to systemic deactivation of the immune system in an attempt to rescue homeostasis from an excessive inflammatory state. IL-10, primarily produced by monocytes and, to a lesser extent, lymphocytes, is the main anti-inflammatory cytokine involved in CARS (28). IL-10 modulates NF-kB activity and decreases TNFα, IL-1, IL-6, IL-8, and IL-12 secretion by monocytes (29). It also reduces the production of reactive oxygen intermediates, platelet activating factors and chemokines (28). The magnitude of CARS on hospital admission, measured by increased levels of IL-10, has been shown to predict a poor outcome in patients with ACLF (28). In murine models of liver fibrosis (bile duct ligation and CCl4), translocation of gut microbiota induces the overexpression of IFN-I in the liver and IL-10 in myeloid cells, which consequently impairs the antibacterial ability of myeloid cells (30). Monocytes from patients with ACLF display elevated frequencies of interleukin IL-10-producing cells, reduced human leucocyte antigen DR isotype expression and impaired phagocytic and oxidative burst capacity. This immunotolerant phenotype

of monocytes/macrophages in ACLF may be partially restored by the metabolic reprograming of the cells using a pharmacological inhibitor of glutamine synthetase (31).

Additionally, increased numbers of monocytes and macrophages expressing MER receptor tyrosine kinase (MERTK) have been documented in patients with ACLF. MERTK is involved in down-regulation of innate immune responses aimed at resolving inflammation (32). Activation of MERK in monocytes inhibits TLR activation and proinflammatory cytokine production. The number of MERK+ cells in ACLF has been found to correlate with disease severity and inflammation. Of note, the *ex vivo* addition of an inhibitor of MERTK, was able to rescue the production of inflammatory cytokines upon LPS stimulation (33).

ii) The exhaustion of immune system cells exposed to persistent antigen and inflammatory signals also occurs in other conditions such as acute and chronic infections or cancer (34). In ACLF, this exhaustion is due to chronic and exacerbated translocation of bacteria and PAMPs from a leaky gut (35), as well as increased DAMPs released from injured hepatocytes. In fact, bowel decontamination with antibiotics was observed to normalize the activation state, restore phagocytosis, and increase TNFα production upon LPS stimulation of intestinal dendritic cells in a cirrhotic rat model of bacterial translocation (35). In patients with alcoholic hepatitis-related ACLF, LPS-mediated activation of TLRs induces pronounced impairment of neutrophil function (phagocytosis, and oxidative burst), associated with the reduced production of interferon-gamma by T cells mediated by increased IL-10 production (27). In addition, T-cells from these patients express higher levels of immune inhibitory receptors (specifically, programmed cell death-1, and T-cell immunoglobulin and mucin domain). Blockade of these receptors with specific antibodies was able to restore the antimicrobial activities of neutrophils and T cells (27). Of note, all these events take place in a T-cell compartment that is already retracted in cirrhosis due to defective thymopoiesis and increased activation-driven cell death (36). Monocyte oxidative burst in alcoholic hepatitis is also impaired due to reduced NADPH oxidase expression. A defective oxidative burst has been shown to predict the development of infections and death in this context (37).

iii) Finally, metabolic and neuroendocrine abnormalities subsequent to severe liver insufficiency can impair immune cell functions. In this regard, prostaglandin E2, which appears elevated in patients with acute decompensation of cirrhosis (38), contributes to monocyte dysfunction by inhibiting NADPH oxidase-mediated bacterial killing (39). Prostaglandin E2 induced immunosuppression was antagonized by indomethacin and by the binding effect of albumin. Accordingly, decreased levels of serum albumin (below 30 mg/ml) predicted susceptibility to infection (38). Other distinctive metabolic features of liver failure, such as hyperammonemia and hyponatremia, contribute to the immune dysfunction of cirrhosis. Hyponatremia acts synergistically with ammonia to cause neutrophil swelling and impaired phagocytosis, an effect abrogated by p38 mitogen-activated protein kinase signaling inhibition (40). SI also activates tryptophan metabolism increasing its degradation via the kynurenine pathway. Under physiological conditions, kynurenine metabolites are synthesized in the liver by local enzymes, however, in the setting of SI and hepatic insufficiency, there is a predominant extrahepatic metabolism. Kynurenine pathway activity is markedly increased in cirrhotic patients with ACLF, and a high baseline activation independently predicts mortality in this situation (41). In fact, in ACLF, kynurenine metabolites seem to contribute to systemic circulatory dysfunction by increasing nitric oxide production and oxidative stress, immunosuppression by increasing IL-10 release, and brain dysfunction.

MECHANISMS OF ORGAN FAILURE IN ACLF

ACLF is defined by the presence of hepatic and/or extrahepatic organ failure, which differentiates this syndrome from acute decompensation of cirrhosis. Despite early detection and intervention on the precipitant events, the dysfunctional immune response leads to further organ failure, which is mainly driven by the massive systemic inflammatory response (**Figure 2**).

A. General mechanisms of organ failure in high-grade SI

The mechanisms underlying organ failure are related not only to the hemodynamic derangement, but also to cell dysfunction and cell death (apoptosis and necrosis) induced by the excessive inflammatory response (42). The term "immunopathology" refers to the collateral damage of the activated immune cells and derived factors on other cells and tissues resulting in organ failure. The degree of immunopathology correlates with the magnitude, duration, and type of the immune response. In this regard, effector responses including IFNγ-activated macrophages, recruited neutrophils, cytotoxic lymphocytes or T helper 17 cells are related to a high immune-mediated tissue damage and consequently to an increased risk of organ failure (43). In ACLF, significantly higher levels of cytokines such as IL-6 or IL-8, among others, are found (4). IL-6 is critical for lymphocytes T helper 17 responses, which contribute to neutrophil production and activation. On the other hand, IL-8 induces neutrophil chemotaxis and phagocytosis. Recruited neutrophils release reactive oxygen species and proteolytic enzymes contributing to immunopathology and cell death.

In addition, it has been suggested that cirrhosis is associated with a decreased capacity of tolerance to infections (44). Tolerance aims to reduce the negative impact of infection on host fitness, decreasing the susceptibility to tissue damage caused by the pathogens, and the immune response (45). In fact, cirrhotic livers are abnormally sensitive to *in vivo* LPS-induced TNFα-mediated apoptosis, due to an altered unfolded protein response, which might

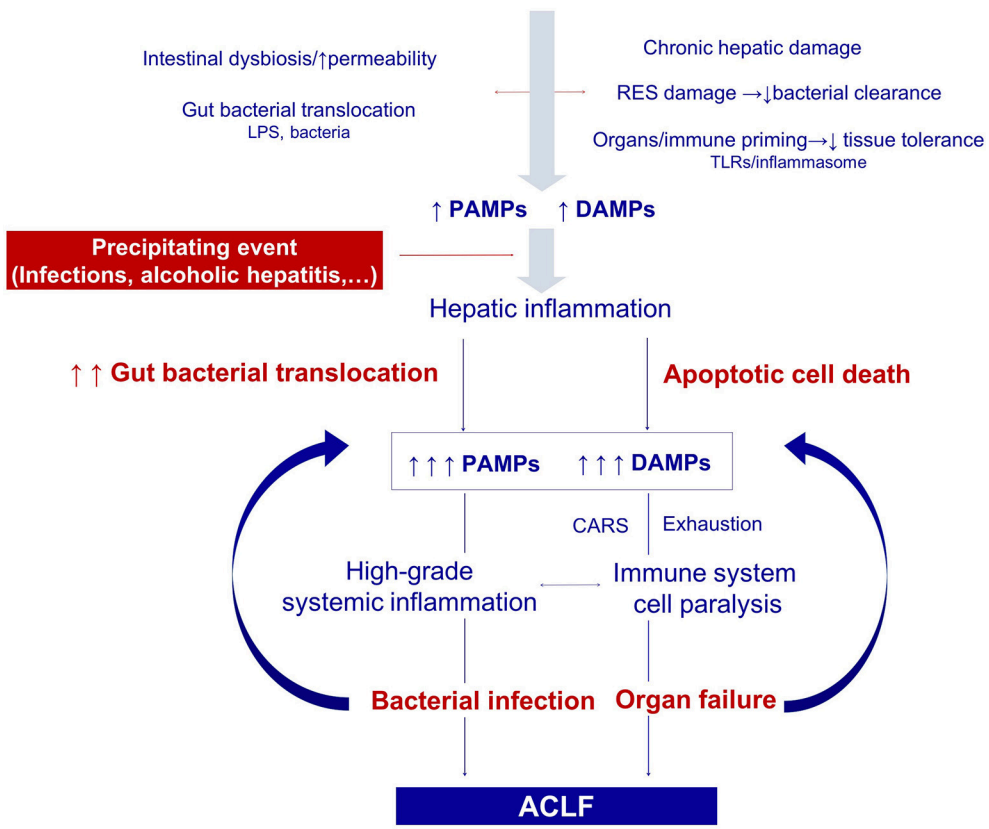

FIGURE 2 | Pathogenesis of the organ failure in ACLF. Architectural changes of the liver due to cirrhosis, derange the surveillance role of the hepatic reticuloendothelial system (RES), and contribute to impaired endotoxin and bacterial clearance. The progressive damage to the hepatocytes impairs the synthesis of proteins involved in the innate immune response. In addition, gut dysbiosis and increased permeability exacerbate bacterial translocation and priming of the immune system cells leading to low-grade systemic inflammation. Monocytes and macrophages persistently exposed to low levels of endotoxin eventually become unresponsive to further endotoxin challenge. Additionally, the loss of the protective-tissue intrinsic mechanisms of tolerance in the cirrhotic liver results in a severe outcome after any noxa. In this scenario, sterile or infectious acute events induce hepatic inflammation and necrosis, and thus generate more PAMPs and DAMPs. Activation of pattern recognition receptors by increased PAMPs and DAMPs triggers a massive release of cytokines and hepatocyte death by immune mediated apoptosis and necrosis. Organ failure is not only related to the collateral effects of the immune response, but also to the hemodynamic derangement present in ACLF. In parallel, an excessive compensatory anti-inflammatory response trying to counteract the high-grade systemic inflammation, and the exhaustion and dysfunction of key innate and adaptive immune system cells, lead to a functional immune cell paralysis, which increases the risk of infections. Therapies targeting the elements of the altered immune response or the factors leading to it are promising in ACLF, including those aiming to correct gut dysbiosis and reduce bacterial translocation, to reduce high-grade systemic inflammation, and to improve the immune cell dysfunction. RES, Reticuloendothelial system; DAMPs, damage associated molecular patterns; PAMPs, pathogen associated molecular patterns; LPS, Lipopolysaccharides; PRRs, pattern recognition receptors; TLR-4, Toll like receptor 4; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; CAR, compensatory anti-inflammatory response.

contribute to liver damage under bacterial challenge in cirrhosis (44). In agreement with the latter experimental findings, the lack of tolerance seems to be more marked in those patients with ACLF without previous acute decompensations (2), and correlates with an increased mortality in this group.

Tissue hypoperfusion and hypoxia secondary to microthrombi formation, blood maldistribution, tissue edema, and decreased perfusion pressure as a consequence of nitric oxide overproduction, are key to the pathogenesis of organ failure. Excessive nitric oxide also impairs mitochondrial function leading to altered cell oxygen consumption and ATP synthesis. Neutrophil recruitment to injured tissues may have a detrimental effect through the production of lysosomal enzymes and superoxide free radicals (46). Finally, inflammation promotes tissue factor release, which in turn triggers the extrinsic coagulation cascade and production of activated thrombin leading to microthrombi formation and eventually disseminated intravascular coagulation.

B. Specific mechanisms of organ failure in ACLF

Portal hypertension in cirrhosis induces profound hemodynamic derangement characterized by splanchnic arterial vasodilation and hyperdynamic circulation with

high cardiac output and low systemic vascular resistance. In ACLF, cytokine-induced overproduction of nitric oxide and reactive oxygen species intensifies arterial vasodilation, decreases mean arterial pressure and impairs left ventricular function, contributing to the hemodynamic deterioration (47).

Reduced arterial pressure and activation of neurohumoral compensatory mechanisms lead to renal arterial vasoconstriction and prompt kidney hypoperfusion. Hemodynamic derangement, but also ischemic acute tubular necrosis due to intense renal capillary leukocyte infiltration, microthrombosis, cell apoptosis, and mitochondrial injury contribute to acute kidney failure in ACLF (48). The relevance of mitochondrial dysfunction in the pathogenesis of sepsis-induced renal failure is increasingly recognized. During sepsis, several mitochondrial functions, key for cellular homeostasis, are altered. In particular, oxidative phosphorylation and ATP production decrease, while reactive oxygen species production and apoptosis augment (49). Affected mitochondria also release DAMPs, such as mitochondrial DNA, which further contribute to amplify the immune response (50). The involvement of inflammatory mechanisms rather than systemic circulatory dysfunction in renal failure in ACLF explains the lower efficacy of terlipressin and albumin in this context as compared to decompensated cirrhosis (51).

Hepatocytes are protected from TNFα-induced apoptosis through activation of NF-kβ-dependent anti-apoptotic mechanisms (52). However, in cirrhotic rats, hepatocyte endoplasmic reticulum stress prevents the translation of these antiapoptotic messenger RNAs into proteins (44). Under these circumstances of failure of protective tissue-intrinsic mechanisms, it is likely that the response of the liver to a precipitant event can be particularly severe. This contributes to liver injury and failure upon LPS-mediated production of TNFα. Additionally, LPS engagement to PRRs is related to hepatocyte necrosis and neutrophil infiltration induced by endothelin 1. Treatment with a non-selective endothelin receptor antagonist has been observed to decrease intrahepatic neutrophil infiltration and increase *in vivo* survival in endotoxin-challenged cirrhotic rats (53).

Collectively, these lines of evidence support the immunopathological mechanisms underlying organ failure in cirrhosis and ACLF.

THERAPEUTIC STRATEGIES TARGETING THE IMMUNE SYSTEM IN ACLF

Currently, the treatment of ACLF is based on organ support and correction of the precipitating events when possible. However, the potential reversibility of the immune alterations, at least in *ex vivo* models, suggests promising therapeutic targets for patients with ACLF.

Therapies Targeting the Gut

Therapies targeting gut microbiota may potentially improve hemodynamic derangement and immune cell dysfunction in ACLF. Intestinal decontamination with non-absorbable antibiotics has been shown to ameliorate systemic vascular nitric oxide production, inflammation, and hemodynamic alterations in experimental models and in human cirrhosis (35, 54–57). As described above, bowel decontamination was also able to normalize dendritic cell dysfunction and increase TNF-α production in experimental cirrhosis (35). Currently, clinical trials are further investigating the efficacy of simvastatin and rifaximin in patients with decompensated cirrhosis to prevent ACLF, reduce complications and hospital readmissions, and improve cost-effectiveness, quality-of-life and survival (LIVERHOPE project, EU H2020).

A different approach targeting the gut involves the use of poorly absorbable, adsorptive materials, such as synthetic adsorptive nanoporous carbons that bind gut-derived toxins and bacterial products. A novel synthetic activated carbon (Yaq-001) has shown promising results in rats with biliary cirrhosis (58). Clinical trials using Yaq-001 are ongoing as part of the European Commission Horizon 2020 program (carbalive.eu).

Therapies Targeting High-Grade Inflammation

Preclinical studies have shown that treatment with the IL1 receptor antagonist anakinra reduces liver inflammation and neutrophil infiltration, improving hepatocyte regeneration, and recovery in mouse models of ACLF (59). Conversely, IL-22 has been shown to protect and repair liver injury in mice with alcoholic hepatitis, targeting signal transducer, and activator of transcription 3 (60). The antiapoptotic, proliferative, and antimicrobial effects of IL-22 are potentially beneficial for patients with ACLF. The safety and efficacy of this molecule is currently being tested in patients with alcoholic hepatitis (ClinicalTrials.gov ID: NCT02655510).

Targeting inflammation and apoptosis mediated by caspases has yielded promising results in chronic hepatitis C and non-alcoholic fatty liver disease (61, 62). In ACLF, the role of pan-caspase inhibitors (such as Emricasan) has also been explored. Unfortunately, clinical benefits assessed by severity of liver disease (MELD or CLIF-C ACLF score) or mortality, has not been demonstrated in this context so far (63).

Therapies Targeting the Immune Cell Paralysis

The *in vitro* rescue of cell function using immune targeted strategies, and recapitulation of immune paralysis using serum from ACLF patients, suggest that cell reprograming leading to immunodeficiency may be reversible, and provide evidence for new therapeutic targets.

Albumin has not only shown hemodynamic effects as a plasma expander but has also proven immune functions. The latter arise from its capacity to bind and inactivate pro-inflammatory molecules such as LPS and other bacterial products, reactive oxygen and nitrogen species, and prostaglandins (64), thus

supporting its potential therapeutic effects in patients with ACLF (65).

Granulocyte colony-stimulating factor (G-CSF) promotes mobilization of bone marrow stem cells, which has been shown to promote hepatic regeneration and restore neutrophil function (66). In patients with ACLF, treatment with G-CSF increased the number of CD34(+) stem cells, and was significantly associated with higher survival rates (67, 68). Currently, several clinical trials are underway to confirm G-CSF efficacy and safety in this context (ClinicalTrials.gov ID: NCT01383460, NCT02788240, NCT03162419, and NCT02669680). Interestingly, it has been suggested that liver-derived mesenchymal stem cells produce higher levels of pro-angiogenic, anti-inflammatory, and anti-apoptotic cytokines than stem cells derived from the bone marrow (69). The safety and efficacy of these cells in ACLF are currently under investigation (ClinicalTrials.gov ID: NCT02946554).

CONCLUSION

Massive SI and immune cell paralysis associated with ACLF represent the extreme severity of CAID in response to an infectious or sterile challenge. The severe immune disturbance plays a pivotal role in the pathogenesis of the distinctive features of ACLF: organ failure and bacterial infection susceptibility. Excessive SI in ACLF results from the massive activation and dysfunction of an innate immune system challenged by increased PAMPs and DAMPs. SI leads to cell and tissue immunopathology contributing to hepatic and extrahepatic organ failure. Concomitantly, the course of ACLF is associated with a disproportionate compensatory anti-inflammatory response along with exhaustion and dysregulation of the innate and adaptive arms of the immune system, with the subsequent functional immune paralysis that confers a higher risk of infections and further ACLF progression. Therapies aimed at preventing the development of these immune disturbances in ACLF are novel promising strategies to improve survival in these patients.

AUTHOR CONTRIBUTIONS

The manuscript was drafted by RM-M and AA. MA-M and AA critically reviewed the manuscript. All the authors approved the final draft for submission. The guarantor of the manuscript is AA.

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MINI REVIEW
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Review of Defective NADPH Oxidase Activity and Myeloperoxidase Release in Neutrophils From Patients With Cirrhosis

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Patients with decompensated cirrhosis are highly susceptible to develop bacterial infections and these can trigger multiorgan failure associated with high in-hospital mortality. Neutrophils from patients with decompensated cirrhosis exhibit marked alterations that may explain the susceptibility of these patients to develop bacterial infections. These neutrophil alterations include marked defects in intracellular signaling pathways involving serine/threonine kinases such as protein kinase B (AKT), p38-mitogen-activated protein kinase (MAPK), and the MAP kinases1/2; activation of the NADPH oxidase complex; myeloperoxidase (MPO) release; and bactericidal activity of neutrophils stimulated by the bacterial peptide formyl-Methionine-Leucine-Phenylalanine (fMLF). Impaired activity of the NADPH oxidase 2 (NOX2) complex is also related to reduced levels of expression of its major components through post-transcriptional mechanisms. In addition, the catalytic NOX2 component gp91^{phox} is subject to degradation by elastase highly present in patients' plasma. A defect in the protein kinase B (AKT) and p38 MAPK-mediated signaling pathways may explain the decrease in phosphorylation of p47^{phox} (an important component of the NADPH oxidase complex) and MPO release, in response to neutrophil stimulation by fMLF. Most of these alterations are reversible *ex vivo* with TLR7/8 agonists (CL097, R848), raising the possibility that these agonists might be used in the future to restore neutrophil antibacterial functions in patients with cirrhosis.

Keywords: liver disease, infection, neutrophil dysfunction, granule exocytosis, serine-threonine protein kinases

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INTRODUCTION

The natural history of cirrhosis, the most common chronic liver disease, is characterized by episodes of acute decompensation (e.g., development of ascites, gastrointestinal hemorrhage, or hepatic encephalopathy) (1). Patients with acutely decompensated cirrhosis are usually admitted to the hospital (1). Of these, 70% have traditional acute decompensation do not exhibit any organ dysfunctions or failures and have a 28-day mortality rate of <5%. The 30% remaining patients have acute-on-chronic liver failure (ACLF) which is defined by the presence of organ failures and a 28-day mortality rate ranging from 20 to 80% or more, depending on the number of failing

organs (1). Patients with cirrhosis are highly susceptible to develop acute bacterial infection, which is the most common trigger of traditional acute decompensation and ACLF (1, 2). Studies have shown that neutrophils from patients with cirrhosis exhibit *ex vivo* defective adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) (3–5) and of myeloperoxidase (MPO) exocytosis (4), which both may contribute to the susceptibility to infection in patients with cirrhosis. Before summarizing our knowledge about the defective neutrophil functions in cirrhosis, it is important to have some general information on NADPH oxidase activity and MPO release in neutrophils.

NADPH OXIDASE ACTIVATION AND MPO RELEASE IN NEUTROPHILS FROM THE GENERAL POPULATION

Almost 90% of granulocytes in peripheral blood are composed of neutrophils which represent the first line of cellular defense against bacterial infections and play an important role in innate immunity and inflammation. Circulating neutrophils are the first to arrive at a site of infection, and they stay for only a short time (the first 24 h), most of them undergoing cell death in the inflamed tissue as a consequence of their antibacterial effector functions (6). Phagocytosis of bacteria at the infection site activates neutrophil functions, such as the release of proteases, bactericidal peptides and reactive oxygen species (ROS) (7, 8). ROS production is initiated by the generation of superoxide anion ($O_2^{-\cdot}$) by the NADPH oxidase. In the phagosomes, $O_2^{-\cdot}$ reacts with protons to form hydrogen peroxide (H_2O_2), which is used by myeloperoxidase (MPO, an azurophilic [or primary] granule lumen protein) to produce the highly bactericidal ROS, hypochlorous acid. The rapid increase in oxygen and glucose consumption, together with ROS overproduction during neutrophil NADPH oxidase activation, is known as "respiratory burst" (RB). NADPH oxidase is a multicomponent protein (see below); an inherited defect in the expression of one of these components results in a rare disease called chronic granulomatous disease, which is characterized by a defect in ROS production in phagocytes and an increased susceptibility to recurrent bacterial and fungal infections (7). On the other hand, excessive neutrophil ROS production can cause tissue damage (7, 8). The importance of effective MPO release is highlighted by the findings in *MPO*-knockout mice of increased prevalence of infections, prolonged inflammation, and shorter survival (9, 10).

NADPH Oxidase Activity

In its active state the NADPH oxidase is a multiprotein complex comprising the catalytic core flavocytochrome b_{558} heterodimer consisting in two associated transmembrane proteins, $gp91^{phox}$ (i.e., cytochrome b-245 heavy chain, commonly called NOX2) and $p22^{phox}$ (i.e., cytochrome b-245 light chain), and four proteins recruited from the cytosol, including $p67^{phox}$ (i.e., neutrophil cytosol factor 2), $p47^{phox}$ (i.e., neutrophil cytosol factor 1), $p40^{phox}$ (i.e., neutrophil cytosol factor 4), and Rac2 (7). The oxidase is fully activated when cytosolic and membrane proteins are assembled into a complex, which makes $gp91^{phox}$ able to use cytosolic NADPH to produce $O_2^{-\cdot}$ (7, 8, 11).

Different molecules can activate neutrophil NADPH oxidase including the bacterial peptide formyl-Met-Leu-Phe (fMLF), the complement fragment C5a, opsonized bacteria, opsonized zymosan and chemical agents such as calcium ionophores and the protein kinase C (PKC) activator, phorbol-myristate acetate (PMA) [reviewed in (11)]. FMLF, engages the surface formyl peptide receptor fPR1, a G-protein-coupled receptor, to activate several intracellular phospholipases, protein tyrosine kinases, serine/threonine kinases, including PKC isoforms, protein kinases B and B beta (hereafter called AKT1and AKT2, respectively), mammalian target of rapamycin (mTOR), and mitogen-activated protein kinases (MAPK), which include p38-MAPK and MAPK 1 (hereafter called ERK2) and MAPK 3 (hereafter called ERK1) (**Figure 1A**). Serine/threonine kinases phosphorylate the components of the NADPH oxidase (**Figure 1A**) at sites which are detailed in **Table 1** and contribute to the assembly of the complex and $O_2^{-\cdot}$ production. Of note, it has recently been shown that during the first hour of their *ex vivo* fMLF stimulation of neutrophils from healthy subjects, these cells release the protease elastase (contained in azurophil granules and specific [or secondary] granules) in the extracellular milieu to induce degradation of transmembrane $gp91^{phox}$ (5). This degradation is followed by that of $p22^{phox}$, which is an elastase-independent process, and might be a consequence of $gp91^{phox}$ degradation that would render $p22^{phox}$ unstable and degradable by intracellular proteases (5). The two cytosolic components of the NADPH oxidase complex, $p47^{phox}$ and $p40^{phox}$ are not affected by fMLF-induced elastase release (5).

MPO Release

In freshly isolated neutrophils from healthy subjects, fMLF also induces MPO exocytosis from primary granules (4, 12), which is an important part of the oxygen-dependent antibacterial arsenal (8, 12). Other toxic mediators stored in these primary granules are also released such as elastase and defensins. Stimulation of neutrophils triggers also the degranulation of two other cytosolic compartments, secondary and tertiary granules which contain common mediators (gelatinase, lysozyme, and ß2-microglobuline) and specific mediators, i.e., lactoferrin and acetyltransferase, respectively. The membrane of secondary and tertiary granules contains high amounts of $gp91^{phox}$ (NOX2) and the fMLF receptor fPR1. Thus, during the process of MPO release by neutrophils stimulated with bacteria or bacterial peptides, the fusion of granular membranes to plasma membrane and phagosomes bring new pools of NOX2 and fPR1, which contribute to increase ROS production and bacterial killing. Experiments using selective pharmacological antagonists for phosphorylation of AKT, p38-MAPK and ERK1/2 showed that phosphorylation of AKT, p38-MAPK, but not that of ERK1/2, was involved in MPO exocytosis (4). Moreover, these pharmacological experiments have shown that p38-MAPK

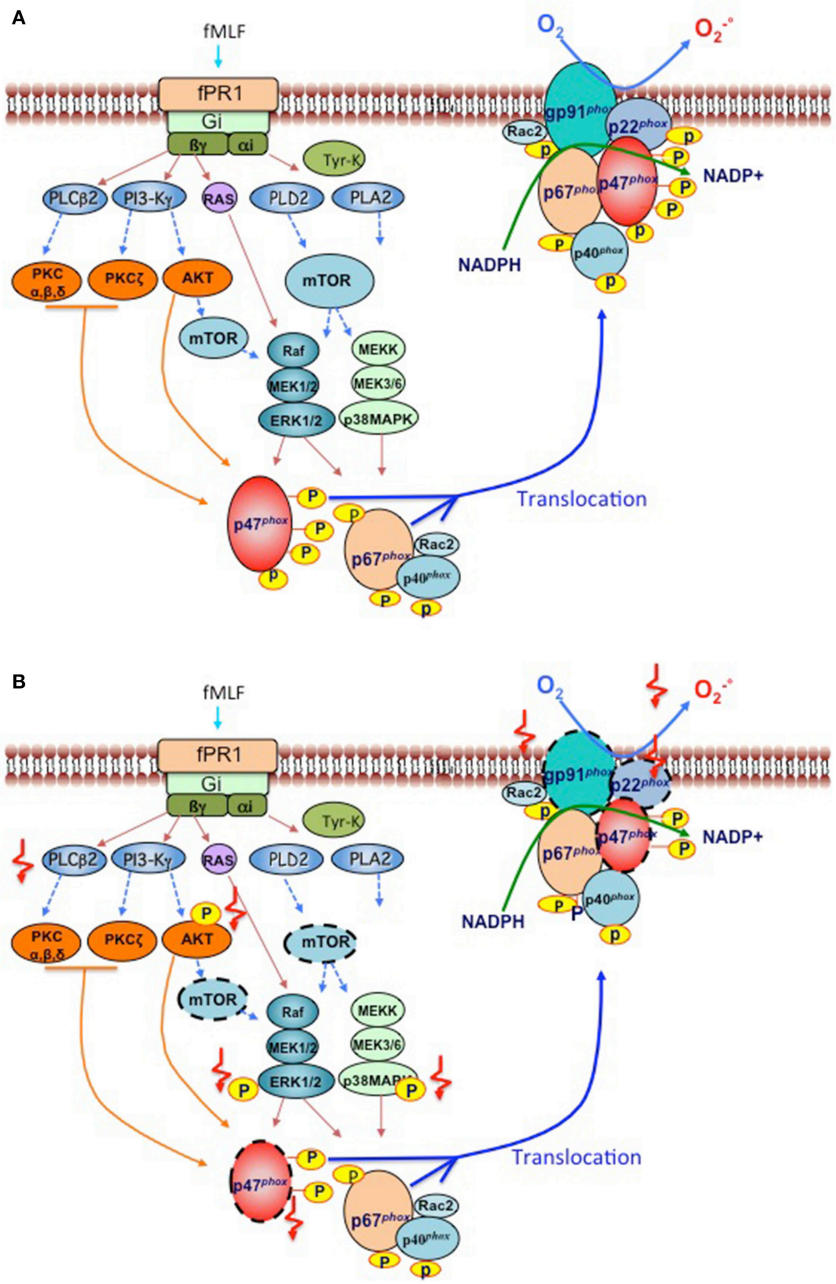

FIGURE 1 | Signaling pathways involved in phosphorylation and activation of the NADPH oxidase induced by bacterial peptides in human neutrophils from respectively "healthy subjects" and "cirrhotic patients". **(A)** Healthy subjects. The binding of the bacterial formylated peptide fMet-leu-Phe (fMLF) to its Gi-protein-coupled receptor fPR1, triggers the activation of various major early signaling effectors such as phospholipase C (PLCß2), Phospholipase D (PLD2), Phospholipase A (PLA2), Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-Kγ), tyrosine kinases, and the small G-protein Ras. Second messengers produced by phospholipases stimulate various protein kinases, such as protein kinase C (PKC) isoforms, protein kinase B (AKT1/2), mammalian target of rapamycin (mTOR), which in turn activates two major families of mitogen-activated protein kinases (MAPKs), including ERK1/2 and p38-MAPK. PKCs and MAPKs phosphorylate cytosolic components of the NADPH oxidase ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$) which allows their translocation to the plasma membrane, together with the small G protein Rac2, to activate a cytochrome b, constituted with the $gp91^{phox}$ (also known as NOX2) and its partner $p22^{phox}$. The activated NOX2 reduces oxygen to superoxide ($O_2^{-\cdot}$) at the expense of NADPH. **(B)** Patients with cirrhosis. Two types of deficiencies have been identified in neutrophils of cirrhotic patients stimulated *in vitro* by bacterial peptides; those which decrease the activation/phosphorylation of signaling effectors such as AKT, MAP-Kinase ERK1/2, and p38-MAP kinase, $p47^{phox}$; and PLCβ2 activity (indicated by) and those which decrease the expression of protein effectors (indicated by a dashed outline,) especially for mTOR, $gp91^{phox}$, $p22^{phox}$, and $p47^{phox}$. These alterations lead to a deficient production of superoxide anion by $gp91^{phox}$.

TABLE 1 | The different components of the NADPH complex, their site(s) of phosphorylation, phosphorylating serine/threonine kinases, and effects of phosphorylation on the NADPH complex (7, 8, 11).

Gene symbol	Usual name of the protein subunit	Recommended name of the protein subunit	Site(s) of protein phosphorylation: serine/threonine kinase(s) involved	Effects of phosphorylation on the NADPH complex
CYBB	gp91^{phox} (also known as NOX2)	Cytochrome b-245 heavy chain	Ser486: Protein kinase C (PKC) isoforms	Phosphorylation promotes the catalytic activity and assembly of the NADPH oxidase complex
CYBA	p22^{phox}	Cytochrome b-245 light chain	Thr147: Conventional PKC	Phosphorylation promotes NADPH oxidase complex assembly and activation.
NCF1	p47^{phox}	Neutrophil cytosol factor 1	Between Ser303 and Ser379: PKC isoforms Ser304, Ser328: AKT Ser345: p38-MAPK, ERK1/2	Required for complex assembly and activation
NCF4	p40^{phox}	Neutrophil cytosol factor 4	Thr154 and Ser315: PKC	Phosphorylation at Thr154 is required for NADPH oxidase complex assembly and activation at the phagosome
NCF2	p67^{phox}	Neutrophil cytosol factor 2	Thr233: p38-MAPK, ERK1/2, PKC	Unknown

is activated downstream to AKT through mechanisms that remain elusive.

DEFECTIVE RESPONSE TO FMLF IN NEUTROPHILS FROM PATIENTS WITH DECOMPENSATED CIRRHOSIS

Freshly isolated blood neutrophils from patients with decompensated alcoholic cirrhosis stimulated *ex vivo* with fMLF exhibit a marked defect in $O_2^{-\cdot}$ production (i.e., decreased NADPH oxidase activity), RB, and MPO exocytosis, and accordingly reduced bactericidal activity (3–5). These results confirm PN dysfunctions reported by other groups also showing impaired phagocytic activities (13–22). However, in some studies, basal ROS production by patient neutrophils was increased, despite defective bactericidal activity, which suggests a primed and or pre-activated state of patient neutrophils (17–19, 23, 24). We confirmed this paradoxical situation with some of our cirrhotic patients. However, the increased basal ROS production remained low which questions about its relevancy in antibacterial activity, in contrast to the high and rapid production of ROS induced by fMLF which leads to bacterial killing (4, 5). Because patients' neutrophils have decreased baseline protein expression of gp91^{phox}, p22^{phox}, and p47^{phox} (5), these decreased expressions likely contribute to defective fMLF-induced NADPH oxidase activation in these cells (summarized in the **Figure 1B**). The finding that here is no simultaneous decrease in the baseline mRNA levels of *CYBB* (encoding gp91^{phox}, or NOX2), *CYBA* (encoding p22^{phox}), and *NCF1* (encoding p47^{phox}) in patients' neutrophils (5), suggests that decreased expression of the corresponding proteins is related to posttranscriptional mechanisms. Abnormally high plasma levels of elastase are found in patients with decompensated cirrhosis (25). Neutrophils from healthy subjects exposed to plasma from patients or purified elastase, but not neutrophils exposed to plasma from healthy subjects, exhibit a decrease in gp91^{phox} expression which can be prevented by the neutrophil elastase inhibitor, NEI) (5). These findings, together the finding that elastase released by fMLF-stimulated neutrophils from healthy subjects causes gp91^{phox} degradation (see above), suggest that high plasma elastase levels in patients may explain that their neutrophils have low baseline expression of neutrophil gp91^{phox} and p22^{phox}. Since increased extracellular elastase levels do not affect p47^{phox} expression in neutrophils from healthy subjects (see above), high plasma elastase levels cannot explain decreased baseline expression of p47^{phox} in neutrophils from patients with decompensated cirrhosis. The mTOR protein complex 1 (known as mTORC1) promotes protein synthesis by regulating translation of several mRNAs into proteins (26) including gp91^{phox} (5). Because neutrophils from patients with decompensated cirrhosis exhibit decreased mTOR protein (5), this has been suggested to result in reduced translation of *NCF1* into p47^{phox} in these cells as well-translation of gp91^{phox} (5).

In addition to these alterations in protein expression of the NADPH oxidase complex, defects in signaling pathways have been shown in neutrophils from patients with decompensated alcoholic cirrhosis. Following fMLF stimulation, neutrophils from patients have decreased phosphorylation (i.e., activation) of AKTs, p38-MAPK, and ERK1/2 with no changes in the expression levels of corresponding unphosphorylated proteins and in the expression of the formyl peptide receptor [(3, 4), **Figure 1B**]. Together these findings suggest the existence

of an important defect in signaling pathway, somewhere between the surface receptor and effector proteins. Alterations in the G proteins which are coupled to the formyl peptide receptor have been suggested to exist in neutrophils from patients with cirrhosis based on the impaired phospholipase C (PLC) activity in response to fMLF and fluoride, a G protein activator (16).

Neutrophil dysfunctions associated with cirrhosis are caused by intrinsic cellular alterations because they persist after cell washing (13). Neutrophils from healthy subjects exhibit decreased fMLF-induced MPO exocytosis when they are cultured with plasma from patients but not with plasma from healthy subjects (4), which reveals the presence of cell-permeant inhibitors in patients' plasma. Neutrophil dysfunctions have been shown to be reversible after removal of endotoxins (i.e., lipolysaccharide) from patients' plasma (17).

The defect in signaling pathways in patients' neutrophils may have several functional consequences. Decreased phosphorylation of AKT and p38-MAPK is associated with a defect in MPO exocytosis in patients' neutrophils (4) indicating a decrease in exocytosis of primary granules. In addition, activated AKT and p38-MAPK both phosphorylate a main component of NADPH oxidase, $p47^{phox}$, at Ser304/Ser328 and Ser 345, respectively (**Table 1**). Therefore, defective activation of AKT and p38-MAPK may contribute to the defect in NADPH oxidase activity in patients' neutrophils. Finally, the defect in ERK1/2 phosphorylation may contribute to decrease NADPH oxidase activity, because ERK1/2 activation accounts for 30% of ROS production by fMLF-stimulated neutrophils from healthy subjects (4). Unlike ROS production, the MPO exocytosis induced by fMLF does not appear to be regulated by ERK1/2 (4, 27), but via the p38 MAPK, AKT (4), and PLC/calcium signaling pathways (12). These later are impaired in neutrophils from cirrhotic patients with may contribute to the defective MPO release (4, 16).

Interestingly, the defects in $O_2^{-\cdot}$ production, MPO release, decreased phosphorylation of AKT, p38 MAPK, and bactericidal activity in patients' neutrophils can be reversed *ex vivo* by treatment of patients' neutrophils with toll-like receptor 7/8 (TLR7/8) agonists (4, 5). This treatment also stimulates a rapid *CYBB* transcription and translation into $gp91^{phox}$ which is inhibited by rapamycin, indicating a mTOR-dependent process. These findings suggest that TLR7/8 agonists might be used in the future to restore neutrophil functions in patients with decompensated alcoholic cirrhosis. In support to this assumption, the TLR7/8 agonist R848 (Resiquimod) was recently shown to restore the impaired production of ROS in whole blood of cirrhotic rats and improve the survival of cirrhotic rats infected by bacteria (28). In this murine model of cirrhosis treated with R848, both neutrophils and mononuclear leukocytes (monocytes and lymphocytes) were modified as indicated by a decreased amount of these cells in the blood of ∼40–50%, although modifications of their immune function remain not known.

In patients with decompensated cirrhosis, various defects have also been described in peripheral blood mononuclear cells (PBMCs), notably an impaired expression of genes induced by interferon type 1 (29), a decrease in antigenic presentation by lymphocytes and monocycte/dentritic cells and a decrease in lymphocyte proliferation (30). The effects of TLR7/8 agonists on these adaptive immunity cells in the context of cirrhosis are not known.

However, *in vitro* models, TLR7/8 agonists are particularly effective in inducing robust immune responses (31) including TNFα production in monocytes, IL12 production in human adult and newborn monocytes, INFα production in the dentridic cells, and the activation of T-regulatory cells. Because of their potent immunostimulatory properties, the TLR7/8 agonists are used in non-clinical and clinical studies as vaccine adjuvants (32). TLR 7/8 agonists are also evaluated for a variety of indications in clinical studies and animal models to treat various viral infections and skin cancer (31).

AREAS OF FUTURE RESEARCH

Studies should be performed in patients with cirrhosis to address several questions including the mechanisms for acquired defective signaling pathways in response to fMLF. In particular, the role of LPS should be investigated because it has been shown to be present in the plasma of patients with cirrhosis who did not have ongoing infection by Gram-negative bacteria (33) and because LPS may have effects on neutrophil ROS production (5). In addition, the responses to other potential stimuli for neutrophil activation should investigated in patients' neutrophils.

The phenomenon of neutrophil extracellular traps (NETs, which are mainly composed of DNA that is released from neutrophils upon pathogen encounter) has been considered an alternative to other nonlytic (apoptosis) or lytic (pyroptosis, necroptosis) cell death and called NETosis (6). NETs can entrap bacteria, fungi, and even viruses and contribute to antimicrobial defense. NETosis relies on the presence of the major neutrophil serine protease elastase, MPO and active NADPH oxidase (8). Therefore, NETosis would not be expected to occur in neutrophils from patients with cirrhosis which have defect in NADPH oxidase and in MPO exocytosis. Because defective NETosis might contribute to the fact that patients' neutrophils have decreased ability to kill bacteria, NETosis should be investigated in these cells.

It will be also important to investigate, in patients with cirrhosis, the phenotype of circulating neutrophils according to the severity of the disease identifying cell-surface markers and transcriptome profile, and investigating their ability to leave circulation toward tissues.

Finally, the efficacy and safety of TLR7/8 agonists should be further investigated *in vivo* preclinical experiments performed in mouse models of chronic liver disease.

CONCLUSIONS

Neutrophils from patients with decompensated cirrhosis exhibit marked alterations that may explain the susceptibility

of these patients to develop bacterial infections. These neutrophil alterations include marked defects in fMLF-induced activity of the NADPH oxidase complex, MPO release, and bactericidal activity. Decreased activity of the NADPH oxidase complex is related to reduced levels of expression of its major components through post-transcriptional mechanisms. A defect in the AKT/p38 MAPK signaling pathway may explain the decrease in phosphorylation of $p47^{phox}$ (an important component of the NADPH oxidase complex) and MPO release, in response to fMLF stimulation. Most of these alterations are reversible *ex vivo* with TLR7/8 agonists.

AUTHOR CONTRIBUTIONS

RM and AP wrote the manuscript. AP and VA provided critical revision of the manuscript for important intellectual content. AP drew the figures.

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