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REVIEW

Infiltrating monocytes in liver injury and repair

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Noninfectious liver injury causes many acute and chronic liver diseases around the globe, and particularly in developed nations. Bone marrow-derived monocytes infiltrate the damaged liver tissue and are a critical component of the innate immune response that may drive injury resolution or host death in the short term or chronic inflammation, fibrosis and hepatocellular carcinoma in the long term. Monocytes often play dual roles in liver injury—both perpetuating inflammation and promoting resolution of inflammation and fibrosis. Thus, we will address the role that monocytes play in different experimental forms of noninfectious liver injury; considering in particular the importance of the transition from inflammatory Ly6C^{hi} monocytes to pro-resolution Ly6C^{lo} monocyte-derived macrophages and the consequences of this transition for disease progression and resolution.
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INTRODUCTION

Noninfectious liver injury, including the effects of drugs and diet, is the major cause of liver disease in developed nations. In Western nations, the main cause of acute liver failure is acetaminophen (APAP) overdose, accounting for nearly 50% of acute liver failure cases.[1] Ischemia–reperfusion (IR) injury contributes to up to 10% of early organ failure following liver transplant and can ultimately lead to increased risk of short-term and long-term organ rejection.[2] Chronic damage can also be induced by diet or alcohol. Damage induced by diet results in nonalcoholic fatty liver disease (NAFLD), the most common chronic liver disease in Western nations. Damage induced by alcohol results in alcoholic liver disease (ALD). Both NAFLD and ALD are characterized by fatty liver (steatosis), and with the development of inflammation and fibrosis, these diseases can progress to nonalcoholic steatohepatitis (NASH) and alcoholic steatohepatitis (ASH), respectively. Current studies suggest that between 20 and 30% of the population of the United States and other Western nations is afflicted by fatty liver.[3] Chronic alcohol abuse is a global issue with studies estimating the presence of 140 million alcoholics worldwide.[4] In the United States alone, chronic alcohol consumption contributed to 13 050 deaths in 2006, ~47% of all deaths attributable to chronic liver disease and cirrhosis.[5]

Noninfectious liver injury can induce an immune response that either resolves or results in death of the host, or a chronic immune response that produces repeating cycles of cell death and inflammation. A complex interplay of innate and adaptive cell types, inflammatory signaling molecules and molecular effectors determine these different outcomes. At steady state, the liver is comprised mainly of parenchymal cells, called hepatocytes, and several types of nonparenchymal cells: liver sinusoidal endothelial cells (LSECs), the resident macrophage population called Kupffer cells (KCs), hepatic stellate cells (HSCs), liver dendritic cells (DCs) and intrahepatic lymphocytes dominated by natural killer and natural killer T cell populations. Hepatocytes, which make up ~90% of the total liver mass, comprise only 60–80% of the total cell number in the liver.[6,7] The remainder is made up of the nonparenchymal cell populations. LSECs account for ~50% of the nonparenchymal cells and KCs for ~20%, with the remainder made up by lymphocytes (~25%), hepatic DCs (<1%) and HSCs (<1%).[6] In addition, cells of the myeloid lineage—neutrophils, monocytes, DCs and lymphocytes—are constantly passing through the liver in the bloodstream, setting them up for extravasation into the liver upon detection of inflammatory signals. Upon liver injury, innate myeloid cells—including neutrophils and monocytes—are rapidly recruited to the site of injury.

Monocytes are bone marrow-derived circulating cells that localize to injured and inflamed tissues and differentiate locally into diverse myeloid cell populations, manifesting such functions as phagocytosis, antiviral immunity, antigen presentation, immune suppression and tissue repair. Along with liver-resident cells, monocytes and monocyte-derived macrophages participate in the response to both tissue injury and infection, and in the present review we focus on the way monocytes and monocyte-derived macrophages act in the context of noninfectious liver injury. This is of particular importance as shared features of monocytes, monocyte-derived macrophages and KCs complicate the ability to define the independent roles of each of these populations in the response to injury. This review will attempt to distinguish between these populations to clarify the contribution of monocytes and monocyte-derived macrophages from that of KCs during the course of liver injury, fibrosis and repair. Thus, we will discuss how inflammatory signals are generated by noninfectious stress in the liver, how monocytes are recruited, their differentiation fate and how they impact liver injury and repair. The precise role that monocytes play in the response to insult is an area of active research involving questions such as: What types of monocytes are recruited and what are the signals leading to their recruitment? Do monocytes contribute to initial injury and development of fibrosis or do monocytes contribute to the termination of injury and the regression of fibrosis?

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Do Ly6C^{lo} monocytes derive from Ly6C^{hi} monocytes or are these independent populations? We will address these questions as they pertain to the liver while weaving in results from other systems as they help inform the role of monocytes in liver injury. The recent literature supports the hypothesis that Ly6C^{hi} inflammatory monocytes infiltrate the site of tissue injury, contribute to inflammation and the propagation of fibrosis, and then differentiate into Ly6C^{lo} tissue repair monocyte-derived macrophages that aid in the resolution of injury and fibrosis.

LIVER INJURY, STERILE INFLAMMATION, AND FIBROSIS

The innate immune system has evolved to identify and respond to pathogens through the recognition of conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs); however, innate immune-mediated inflammation also occurs in the absence of pathogen, as with APAP-induced injury or IR injury during organ transplant.[8,9] Termed sterile inflammation, this state is induced by the release of host-derived products, called damage-associated molecular patterns (DAMPs) during tissue damage. DAMPs, which are normally sequestered inside cells, interact with the pattern recognition receptors of the innate immune system and initiate an inflammatory response.

Liver injury is not monolithic, and there are several pathologies that straddle the border between sterile inflammation and pathogen-induced inflammation. One such example is ALD. In ALD, ethanol-induced damage results in the release of various DAMPs; however, ethanol exposure also increases intestinal permeability and results in the leakage of lipopolysaccharide, a bacterial PAMP, from the commensal intestinal flora into the blood supply to the liver. Once in the liver, lipopolysaccharide binds to and activates KCs that then produce inflammatory cytokines that promote hepatocyte damage.[10–12] Although some forms of sterile liver injury may in fact respond solely to DAMPs released from dying cells, many forms of 'sterile' injury are complicated by a response to PAMPs. The response to PAMPs from the normal gut flora as a component of 'sterile' inflammation is increasingly thought to play an important role in pathologies of the liver. The unifying feature of both true sterile inflammation and inflammation involving an additional PAMP component is that they are noninfectious modes of liver injury.

A defining feature of noninfectious liver injury is the death of hepatocytes. In APAP-mediated toxicity, the main mechanism of injury is damage to the endothelial cell microvasculature followed by extensive hepatocyte death in the centrilobular regions of the liver.[13] Historically, there has been controversy as to whether the mode of hepatocyte death is predominantly apoptosis or necrosis, although increasing evidence supports necrosis as the dominant pathway.[13,14] Metabolic disorders such as insulin resistance and obesity also ultimately result in hepatocyte damage via an excess of free fatty acids that contribute to the development of steatosis and NAFLD.[15,16] Progression to NASH occurs with the development of persistent inflammation and increased hepatocyte death, and in some cases leads to cirrhosis.[16] During ASH, tumor necrosis factor-α (TNF-α) released by activated KCs mediates the death of hepatocytes.[17] In both NAFLD/NASH and ALD/ASH, hepatocyte apoptosis plays a large role in the induction of inflammation and the resulting fibrosis, though it is not responsible for all liver injuries.[18–21] Finally, in IR injury following liver transplant, both LSEC and hepatocyte death leads to the release of DAMPs that activate KCs, LSECs and DCs to produce reactive oxygen species and other inflammatory mediators, perpetuating inflammation, although there is disagreement to the extent and mode of death.[22–24]

Acute inflammation is often induced by a single insult, such as IR injury during organ transplant or by APAP overdose. In many cases, acute inflammation is not resolved, particularly in cases of repeated insult and reinjury, and a state of chronic inflammation is established in the liver, such as in NASH or ASH. With enough time, chronic inflammation can progress to fibrosis, cirrhosis and/or hepatocellular carcinoma. As a more complete analysis of liver fibrosis is beyond the scope of this review, we will introduce the main cellular mediators and players, and suggest an excellent recent review by Pellicoro *et al.*[25] for a more in-depth analysis.

In the liver, acute inflammation is characterized by the production of cytokines—interleukin (IL)-6, IL-1β and TNF-α—and chemokines —chemokine (C-C motif) ligand (CCL) and C-X-C motif chemokine (CXCL) family chemokines—in response to liver injury that is largely composed of, but not limited to, hepatocyte death. Traditionally, apoptosis has been considered an inflammatory 'silent' mode of cell death; however, as with necrosis and necroptosis, apoptosis also results in the release of DAMPs.[18] Dying hepatocytes, DAMPs and the release of cytokines and chemokines produced in response to injury results in the activation of KCs and HSCs and their subsequent release of additional proinflammatory mediators. In addition, phagocytosis of apoptotic hepatocytes by KCs and HSCs leads to their activation.[18] Activated KCs produce mediators that can directly induce hepatocyte death (TNF-α, Fas ligand and reactive oxygen species) or indirectly induce hepatocyte death through the recruitment of neutrophils via IL-1β and CXCL2.[26] Production of CCL2 (also known as monocyte chemoattractant protein 1 (MCP-1)) recruits monocytes from the bone marrow to the liver. The influx of neutrophils and monocytes is a hallmark of acute liver injury, and recent literature supports the notion that infiltrating monocytes contribute to the production of inflammatory chemokines, the activation of HSCs and the promotion of fibrosis.[25–27] Activated KCs and HSCs also produce transforming growth factor-β that in turn induces the transdifferentiation of HSCs into the more-activated myofibroblasts that promote deposition of extracellular matrix and collagen, leading to fibrosis of the liver. During inflammatory responses, KCs produce matrix metalloproteinases (MMPs) to degrade collagen and extracellular matrix components that have been deposited, but myofibroblasts counteract MMPs by producing tissue inhibitors of metalloproteinases that degrade MMPs.[25] This cycle of matrix deposition, production of tissue inhibitors of metalloproteinases and inhibition of MMPs leads to scarring of the liver. The cycle is further perpetuated by TNF-α and IL-1β, inflammatory cytokines produced during the response to liver injury that promote myofibroblast survival.[25]

ONTOGENY OF KUPFFER CELLS, MONOCYTE-DERIVED MACROPHAGES AND MONOCYTES

The ontogeny of tissue-resident macrophages, we will consider the case of KCs specifically, has been under considerable debate for years, and remains an active area of research. Studies have shown that long-lived, self-renewing KCs are seeded from embryonic progenitors, although the specific source of these progenitors—yolk sac, fetal liver or embryo—remains controversial.[28–34] In the steady state, KCs maintain without the contribution of circulating bone marrow-derived monocytes.[28,31,32] Following injury, the number of KCs drastically decreases, whereas the number of monocyte-derived macrophages increases in the liver, although whether or not monocyte-derived macrophages ultimately replenish the long-lived KC population has remained an open question.[27,35–39] Although it has been proposed that infiltrating monocytes do not replenish the decreased KC population,

and rather KCs recover through self-renewal, just this year, three groups have shown that infiltrating bone marrow-derived monocytes can replenish the KC population.[38,40–42] Two of these groups further showed that this replacement by bone marrow-derived monocytes resulted in self-renewing macrophages with similar functional and transcriptional profiles to yolk sac-derived KCs—showing that this is yet an evolving area of research.[41,42]

Although it is clear that KCs, monocyte-derived macrophages and monocytes play key roles in liver injury and the progression to fibrosis, the independent contributions of these populations is more difficult to tease apart because of overlapping phenotypic markers, inflammatory products, mechanisms, and ontogeny. Often, monocytes, monocyte-derived macrophages and KCs all fall within the larger umbrella of 'liver macrophage'. Although seminal studies defined dual roles for $CD11b^+$ and $CCR2^+$ cells in liver injury and resolution of injury, the precise roles played by specific populations continues to be debated.[43,44] KCs are well known to contribute to liver injury and, in this review, we consider the role of monocytes and monocyte-derived macrophages in injury, progression to fibrosis and repair in mouse models of sterile liver inflammation including APAP toxicity, carbon tetrachloride (CCl_4)-induced acute injury and fibrosis, as well as in models of NAFLD/NASH and ALD/ASH, and supplemented with data from human studies. We refer to monocytes as the cell population present in the bone marrow, spleen and blood that is recruited to sites of injury whereupon it enters the tissue and differentiates into monocyte-derived macrophages or DCs.[45]

INFILTRATING CELLS: MONOCYTES

Monocytes are bone marrow-derived myeloid cells that circulate in the blood during the steady state as well as maintain a splenic reservoir.[46,47] Upon injury or infection, monocytes are mobilized to the tissue site of insult where they differentiate into monocyte-derived macrophages or DCs. Although there are varying reports on the different subtypes of monocytes in mice, it is generally agreed that there are two main subsets: the classical, inflammatory monocyte and the nonclassical, patrolling (also variably called tissue repair or resident) monocyte.[48–50] These subsets are defined by specific combinations of cell surface molecules and receptors including the chemokine receptors CCR2 and CX_3CR1, the cell surface molecule Ly6C—often referred to as GR1, although it should be noted that GR1 encompasses both the Ly6C and Ly6G epitopes, the latter expressed by neutrophils—as well as CD62L or (L-selectin). Classical monocytes express high levels of CCR2 and Ly6C but low levels of CX_3CR1, whereas nonclassical monocytes express high levels of CX_3CR1 and low levels of CCR2 and Ly6C.[48–50] These monocyte subsets will be referred to as $Ly6C^{hi}$ ($CCR2^+CX_3CR1^{lo}$) and $Ly6C^{lo}$ ($CCR2^-CX_3CR1^{hi}$), respectively. In addition, CD62L is expressed on $Ly6C^+CCR2^+$ monocytes but not on $Ly6C^-CCR2^-$ monocytes.[48,49]

Initially, human monocytes were classified into two subsets, but have since been separated into three functionally and transcriptionally unique subsets with the identification of an intermediate subset.[49,51–57] Surface expression of CD14 and CD16 is used to delineate the three subsets of human monocytes: classical ($CD14^{++}CD16^-$ or $CD14^+CD16^-$), intermediate ($CD14^{++}CD16^+$ or $CD14^+CD16^+$) and nonclassical ($CD14^{+(dim)}CD16^{++}$ or $CD14^{dim}CD16^+$), although a consistent method of identifying the intermediate subset remains to be developed.[58,59] Like mouse classical monocytes, human classical monocytes express high levels of CCR2 and CD62L but low levels of CX_3CR1, and like mouse nonclassical monocytes, human nonclassical monocytes express low levels of CCR2 and CD62L and high levels of CX_3CR1.[48–50,54,60]

The alignment of mouse and human subsets remains a topic of debate, particularly when attempting to compare the human intermediate subset with the mouse subsets. Much of this discord is caused by earlier comparisons of only two monocyte subsets ($CD14^+CD16^-$ and $CD14^+CD16^-$), effectively either excluding or condensing into one of the two populations, the intermediate monocyte population. In addition, differences in gating for the intermediate population add to the variability.[58] On the basis of surface markers as described above, mouse $Ly6C^{hi}$ monocytes align with human $CD14^{++}CD16^-$ monocytes, whereas mouse $Ly6C^{lo}$ monocytes align with human $CD14^+CD16^{++}$ monocytes. Although there is quite a bit of variation in the cytokine production following lipopolysaccharide stimulation, the literature firmly suggests that $CD14^{++}CD16^-$ monocytes are highly phagocytic and that $CD14^+CD16^{++}$ monocytes are weakly phagocytic, further supporting alignment of mouse and human subsets as suggested by surface markers.[51,55–57,61–64] Perhaps most suggestive is that like $Ly6C^{lo}$ mouse monocytes, $CD14^+CD16^{++}$ human monocytes exhibit extensive patrolling behavior.[55] Two transcriptional studies support the notion that $CD14^{++}CD16^-$ monocytes are the human counterparts to the mouse $Ly6C^{hi}$ inflammatory monocytes, whereas the $CD14^+CD16^{++}$ nonclassical monocytes are the human counterparts of the mouse $Ly6C^{lo}$ nonclassical monocytes; however, exact grouping of the intermediate $CD14^{++}CD16^+$ subset is controversial.[55,65] Ingersoll *et al.*[65] grouped monocytes as $CD16^-$ or $CD16^+$, suggesting that both $CD16^+$ human monocyte populations (intermediate and nonclassical) group with $Ly6C^{lo}$ monocytes. A study done in the same year by Cros *et al.*[55] grouped monocytes based on expression levels of CD14, suggesting that both $CD14^+$ populations (classical and intermediate) group with the $Ly6C^{hi}$ monocytes, whereas the $CD14^+CD16^{++}$ nonclassical population groups with the $Ly6C^{lo}$ mouse population. The relation of the human intermediate subset is further complicated by an additional two transcriptional studies that suggest that the intermediate and nonclassical human subsets are more closely related to each other than either are to the classical human subset.[56,57]

Mouse studies have been crucial for elucidating the recruitment patterns of monocytes and the relationship between monocyte subsets. During steady state, $Ly6C^{lo}$ monocytes, or patrolling monocytes, patrol the vasculature via interactions controlled by CX_3CR1 and the integrin LFA-1.[66] Patrolling of the mouse endothelium by human nonclassical monocytes in an LFA-1-dependent manner has also been observed, with CX_3CL1 playing an important role in their arrest and migration.[54,55] Upon injury, predominantly $Ly6C^{hi}$ monocytes are recruited from the bone marrow and spleen to the sites of injury in a CCR2- and CCL2-dependent manner.[27,47,67,68] Human classical monocytes have also been observed to rely on CCL2, as well as CCL3, for migration.[54,60] In steady-state conditions, $Ly6C^{hi}$ monocytes are the precursors for $Ly6C^{lo}$ monocytes, differentiating into the more mature $Ly6C^{lo}$ monocyte subset in both the bone marrow and the blood.[31,46,50] Human studies suggest that classical monocytes differentiate into the intermediate subset before finally differentiating into the nonclassical subset, although this needs to be addressed in further detail before solid conclusions can be drawn.[56,57,69] In contrast to development from $Ly6C^{hi}$ monocytes, it is also possible that $Ly6C^{lo}$ monocytes develop from a distinct precursor in the bone marrow, although transition from $Ly6C^{hi}$ to $Ly6C^{lo}$ within the bone marrow cannot be ruled out.[70] This distinction between classical and nonclassical monocytes is critical when assessing the roles that monocytes play in the inflammatory response to liver injury.

RECRUITMENT OF MONOCYTES TO THE INJURED LIVER

The CCL2/CCR2 axis is important for recruiting Ly6C^{hi} monocytes to the liver following insult. Early studies by Dambach *et al.*[35] show that CCL2 is produced in the liver following APAP-induced liver injury, whereas studies using CCR2$^{-/-}$ and CCR2$^{-/-}$ CCR6$^{-/-}$ mice demonstrated the importance of CCR2 for monocyte/macrophage recruitment to the liver.[27,35,44,71] In humans, CCL2 serum levels are elevated in patients with NAFLD and NASH, as well as with acute liver failure.[72–74] Furthermore, patients with APAP-induced acute liver failure (AALF) show increased expression of CCR2 on their intermediate, but not classical or nonclassical, monocyte subset.[74] Although *in vitro* studies suggest that the CX_3CL1/CX_3CR1 axis recruits human monocytes, the literature in murine liver injury is sparse.[75,76] In mouse models of myocardial infarct and *Listeria monocytogenes* infection of the peritoneum, CX_3CR1 is required for Ly6C^{lo} monocyte recruitment to the injured or infected tissues.[66,77] The seemingly strong dependence on the CCL2/CCR2 axis compared with the CX_3CL1/CX_3CR1 axis for monocyte recruitment to sites of injury may be explained by studies that suggest that Ly6C^{hi} monocytes are initially recruited and then differentiate into Ly6C^{lo} monocytes at the site of injury.[37,38,50,78] In contrast, studies in heart, lung and peritoneum suggest that Ly6C^{hi} and Ly6C^{lo} monocytes are recruited independently and in successive waves to the site of injury, although this does not rule out a role for Ly6C^{hi} differentiation into Ly6C^{lo} in the blood or bone marrow before recruitment to inflamed tissues.[66,77,79]

In addition to the CCL2/CCR2 axis, other liver-specific pathways of monocyte recruitment have been recently elucidated. Recruitment of inflammatory Ly6C^{hi} monocytes to the liver has been found to occur via CCL1/CCR8 interactions in chronic injury.[80] In a mouse model of *L. monocytogenes* infection, intercellular adhesion molecule-1 and CD44 interactions were critical for CCR2^{+}Ly6C^{hi} monocyte recruitment to the liver, whereas CCR2 was only required for emigration out of the bone marrow.[81] Recently, in a mouse model of cholestatic liver injury, the use of agonists against the sphingolipid metabolite S_1PR2 (sphingosine 1-phosphate receptor 2) and S_1PR3 receptors resulted in reduced recruitment of bone marrow-derived monocytes/macrophages to the liver.[82] The role for S_1PR receptors in monocyte trafficking is further supported by a study that showed that S_1PR5 was critical for regulating Ly6C^{lo} monocyte egress from the bone marrow.[83] Finally, recent studies have identified the CCR6 and paired immunoglobulin-like type 2 receptor-α (PILRα) receptors as negative regulators of monocyte recruitment to the liver during inflammation.[84,85]

MONOCYTES AS DRIVERS OF INJURY?

Monocytes play a contradictory role in acute liver injury. Studies in both MCP1$^{-/-}$ and CCR2$^{-/-}$ mice suggest that CCL2-recruited CCR2^{+} monocytes participate in the induction of early injury, showing reduced injury at 24 h but equivalent injury by 48 h, in single-dose CCl_4 models of acute liver injury.[44,86] Most recently, two studies have shown that CCR2^{+} monocytes infiltrate the site of liver injury as early as 8 h following insult and traffic to the site of injury.[39,78] In a model using APAP-induced injury, Tacke and colleagues[39] found that CCR2$^{-/-}$ mice had reduced levels of alanine aminotransferease (ALT), a measure of liver injury, and necrosis 12 h following injury. Furthermore, blockade of CCR2 by a pharmacological inhibitor resulted in reduced levels of ALT and necrosis 12 h following injury, but equivalent injury at 24 and 48 h, supporting the data from the CCl_4 acute liver injury models in which CCR2^{+} monocytes enhance the early phase of liver injury.[39] In contrast, using a model of heat-induced focal necrotic injury, Kubes and colleagues[78] show that lack of CCR2$^{hi}CX_3CR1^{lo}$ monocyte recruitment results in the persistence of dead cells out to 48 h, whereas dead cells are cleared by 24 h when CCR2$^{hi}CX_3CR1^{lo}$ cells are present. In combination with the finding that the transition of CCR2$^{hi}CX_3CR1^{lo}$ to CCR2$^{lo}CX_3CR1^{hi}$ cells within the liver is critical for tissue repair, this study indicates that recruitment of CCR2$^{hi}CX_3CR1^{lo}$ monocytes is more important for repair as opposed to initial injury.[78] In support of this notion, depletion of all blood monocytes by clodronate liposomes resulted in no difference in ALT levels 24 h following CCl_4 injection, suggesting that recruited monocytes do not contribute to the initial stages of liver injury.[27] Although it is possible that the Ly6C^{hi} inflammatory monocytes were recruited directly from the bone marrow, which is partially resistant to the effects of clodronate depletion, this group further showed that CCR2$^{-/-}$ and CCR2$^{-/-}$ CCR6$^{-/-}$ mice exhibited no difference in liver injury at 24 and 48 h following CCl_4 administration, even though infiltrating monocyte levels were decreased.[27,87] Further supporting the lack of a role for monocytes in initial injury are studies using APAP-induced liver injury in which both CCR2$^{-/-}$ and MCP1$^{-/-}$ mice showed no significant differences in ALT levels compared with wild-type mice following APAP administration through 72 h, even though they observed a significant decrease in CD68^{+} (defined as a marker of activation) macrophages at 72 h in CCR2$^{-/-}$ mice.[35]

In humans, serum and liver tissue CCL2 levels are increased in patients with AALF in parallel with an increase in MAC387^{+} infiltrating monocytes/macrophages and Ki67^{+}CD68^{+} resident macrophages in the necrotic areas of the liver.[74] The authors postulate that the anti-inflammatory environment of the liver in AALF patients (increased CCL2, CCL3, IL-6, IL-10 and transforming growth factor-β) suggests that these cells are in fact participating in the resolution of injury; however, the severity of AALF inversely correlated with the number of monocytes in the blood and directly correlated with serum CCL2 levels, suggesting that those patients with poorer outcomes recruited more monocytes to the liver.[74] In line with this conclusion, Mossanen *et al.*[39] show that CCR2^{+} cells are increased in the livers of patients with AALF compared with controls and, furthermore, show that they express the inflammatory marker S100A9, indicating a role in injury.

Collectively, these studies do not clearly define whether or not infiltrating monocytes are involved in promoting acute injury (0–48 h), potentially as a result of variations in the implementation of sterile injury models and as a result of the difficulty of assessing monocytes in human liver samples. For example, in 2012, Galastri *et al.*[88] demonstrated that although CCL2$^{-/-}$ mice on the BALB/c background were protected from chronic damage in a mouse model of NASH, CCL2$^{-/-}$ mice on the C57BL/6J background were not. Clearly, further assessment of infiltrating monocytes, monocyte-derived macrophages, and KCs will be required to conclusively distinguish whether infiltrating monocytes are initially contributing to or inhibiting acute liver injury.

MONOCYTES AS DRIVERS OF FIBROSIS OR RESOLUTION OF FIBROSIS?

Repeated injection of CCl_4 or feeding of specialized diets—high in fat or carbohydrates—are two common models used to induce chronic inflammation and fibrosis within the murine liver. In such models of murine fibrosis, inhibition of the CCL2/CCR2 axis often results in protection from liver inflammation and subsequent fibrosis. In a mouse model of NASH, CCR2$^{-/-}$ mice exhibited less steatosis, infiltrating cells, fibrosis and fewer CD68^{+}Ly6C^{+} monocyte-derived

macrophages compared with wild-type mice.[71] Taking the study one step further, the use of a CCR2 inhibitor in wild-type mice resulted in reduced Ly6C$^+$ infiltrate, liver inflammation and fibrosis.[71] Through the use of clodronate liposomes to deplete KCs, they demonstrate that steatohepatitis, *Ccl2* expression and Ly6C$^+$ monocyte-derived macrophages were all reduced, concluding that CCL2 secretion from KCs is important for the recruitment of CCR2$^+$Ly6C$^+$ monocytes that then contribute to liver injury and fibrosis, a conclusion supported by a recent study of chronic liver injury.[71,89] The idea that Ly6C^{hi} monocytes are contributing to fibrosis is further strengthened by a study from Karlmark *et al.*[27] in which CCR2$^{-/-}$ and CCR2$^{-/-}$ CCR6$^{-/-}$ mice lost their protection from CCl_4-induced liver fibrosis following the adoptive transfer of GR1$^+$ wild-type monocytes. More recently, research from the Tacke research group[90–92] has employed the use of a CCL2 inhibitor, mNOX-E36, to show that Ly6C$^+$ monocyte recruitment did not affect the progression of liver fibrosis but that recruitment of Ly6C$^+$ monocytes during fibrosis regression inhibited the resolution of liver fibrosis in CCl_4-induced and methionine choline-deficient diet-induced models of murine fibrosis. Heymann *et al.*[80] used models of CCl_4-induced fibrosis and bile duct ligation to show that adoptive transfer of wild-type GR1$^+$ monocytes into CCR8$^{-/-}$ mice, which showed reduced ALT levels and measures of fibrosis, resulted in the restoration of fibrosis, further confirming the importance of inflammatory monocytes in the perpetuation of fibrosis. Most recently, Kohyama *et al.*[85] have shown that mice lacking PILRα, which they showed to negatively regulate monocyte recruitment, developed hepatomegaly and liver fibrosis with age that corresponded with an increase in both F4/80^{hi}CD11b$^+$ KCs and F4/80^{int}CD11b^{hi} monocytes in the liver. This suggests that dysregulated recruitment of monocytes in homeostasis can lead to liver fibrosis.

It is important to note that the role of CCR2 may be important not just in monocytes, but also in other liver-resident cell populations. CCR2 was required on bone marrow-derived cells for macrophage recruitment to the liver but it was CCR2 on HSCs, and not bone marrow-derived cells, that was required for the development of fibrosis in mouse models of bile duct ligation and CCl_4-induced fibrosis.[93] It is also possible that infiltrating monocytes and monocyte-derived macrophages activate HSCs that then initiate fibrosis. In a mouse model of CCl4-induced chronic liver injury, CCR2$^{-/-}$ and CCR2$^{-/-}$ CCR6$^{-/-}$ mice exhibited reduced monocyte recruitment, reduced HSC activation and reduced fibrosis, although this protection did not persist upon transfer of wild-type GR1$^+$ monocytes.[27]

Although many studies implicate monocytes, and particularly Ly6C^{hi} monocytes, in the propagation of liver fibrosis, monocytes are also implicated in the resolution of liver fibrosis. Using CCl_4-induced fibrosis, CCR2$^{-/-}$ mice exhibited fewer fibrotic scars and infiltrating F4/80$^+$CD11b$^+$ macrophages—indicating a role for CCR2 in the development of fibrosis.[44] Intriguingly, and in contrast, CCR2$^{-/-}$ mice also showed slower fibrosis regression, suggesting that CCR2 plays a role in both tissue injury and tissue repair.[44] In a model of APAP-induced liver injury, resolution of liver injury (necrotic foci persisting to 48 and 72 h) was delayed in CCR2$^{-/-}$ mice compared with wild-type mice, suggesting that CCR2$^+$ monocytes are important for the resolution of injury.[36] The impact of Ly6C^{lo} monocytes in isolation on the induction and resolution of fibrosis has been evaluated with conflicting results. In models of chronic CCl_4-induced liver fibrosis, Ly6C^{lo} monocyte-derived macrophages were found to be critical for tissue repair, with persisting fibrosis associated with decreased levels of Ly6C^{lo} monocyte-derived macrophages.[37,94] In contrast, a recent study from MacDonald and colleagues[40] using a thioacetamide-induced chronic fibrosis model showed that blockade of macrophage colony-stimulating factor receptor (CSF1R, also known as CD115), the receptor for CSF-1, resulted in decreased Ly6C^{lo} monocyte-derived macrophages in parallel with decreased fibrosis, suggesting that Ly6C^{lo} monocyte-derived macrophages are critical for driving fibrosis. Blockade of CSF1R, however, also results in the depletion of resident KCs, and hence the effects of depleted KCs would need to be disentangled before firm conclusions about the causation of reduced fibrosis can be gleaned from this model. In humans, the intermediate CD14^{++}CD16$^+$ monocyte subset may contribute to fibrosis in chronic liver disease, as it is increased in the livers of patients with ALD and NASH, and produces proinflammatory mediators including TNF-α, IL-6, IL-1β, CCL2, and CCL3.[95]

Finally, a potential confounding factor to consider is the recent description of invading peritoneal macrophages into the liver upon injury.[96] These cells, described as F4/80$^+$CD11b^{hi}CD102$^+$ and GATA6$^+$, were found to infiltrate the liver within 1 h following liver injury in an ATP- and CD44-dependent manner, express markers and genes associated with tissue repair and be absolutely critical for the survival of mice following CCl_4-induced acute liver injury.[96] Furthermore, use of a CCR2$^{RFP/+}$/CX$_3$CR1$^{GFP/+}$ reporter mouse showed that these peritoneal macrophages are indeed distinct from infiltrating monocytes, representing another infiltrating monocyte/macrophage population. Although further studies will need to be conducted to assess how infiltrating peritoneal macrophages and monocytes interface, if at all, as the literature stands, it is quite possible that infiltrating peritoneal macrophages enter the liver at early stages, within 1 h following injury, acting as initial damage control and setting the stage for the subsequent infiltrating monocytes. This is supported by the observations that peritoneal macrophages localize to the center of injury, whereas the later-infiltrating CCR2^{hi}CX$_3$CR1^{lo} monocytes form a ring around the area of injury, indicating distinct functions for each wave of infiltrating cell populations.[78,96]

The split nature of the monocyte response—both promoting fibrosis and resolution of fibrosis—may be because of the different functions of Ly6C^{hi} and Ly6C^{lo} monocytes/monocyte-derived macrophages. Gaining traction in the field is the hypothesis that Ly6C^{hi} monocytes are recruited to the site of insult and drive the initial fibrotic response, but as they differentiate into Ly6C^{lo} monocyte-derived macrophages, their role switches to driving tissue repair and resolution of fibrosis. Such an explanation may explain why CCR2$^{-/-}$ mice have been shown to both promote and resolve fibrosis —the initial influx of CCR2$^+$ (Ly6C^{hi}) monocytes promotes the initial stages of fibrosis, but their differentiation into CCR2$^-$ (Ly6C^{lo}) monocyte-derived macrophages is critical for the resolution of fibrosis.

LY6C^{HI} VERSUS LY6C^{LO} MONOCYTES: DISTINCT POPULATIONS?

The plasticity of inflammatory and tissue repair monocytes remains an area of active research, although many models of liver injury suggest that the tissue repair Ly6C^{lo} monocyte-derived macrophage differentiates from the inflammatory Ly6C^{hi} monocyte/monocyte-derived macrophage. Thus, Sunderkotter *et al.*[50] used clodronate liposomes to deplete blood monocytes and observed that Ly6C^{hi} monocytes repopulated the blood within 2–4 days following depletion, whereas Ly6C^{lo} monocytes did not appear until 7 days following depletion, consistent with the idea that Ly6C^{lo} monocytes derive from Ly6C^{hi} monocytes.

More recently, a series of elegant studies has supported the hypothesis that inflammatory Ly6C^{hi} monocytes transition into tissue

repair Ly6C^{lo} monocyte-derived macrophages at the site of injury. In 2012, Ramachandran *et al.*[37] used a CCl_4-induced model of liver fibrosis and adoptive transfer of CD45.1$^+$Ly6C^{hi} monocytes into wild-type CD45.2$^+$ mice to show that Ly6C^{hi} monocytes recruited by 24 h during fibrogenesis differentiated into Ly6C^{lo} monocyte-derived macrophages, which dominated at 72 h, during maximal scar resolution. Furthermore, elimination of Ly6C^{lo} monocyte-derived macrophages during maximal scar resolution resulted in a persistence of fibrosis, indicating that the Ly6C^{lo} subset were active in tissue repair (they term these 'restorative').[37] In a model of APAP-induced liver injury, adoptive transfer of CD45.1$^+$CX$_3$CR1$^+$ Ly6C^{hi} monocytes into CD45.2$^+$ wild-type recipients demonstrated that Ly6C^{hi} monocytes differentiated into a Ly6C^{lo} subset by 72 h that were cleared by 96 h following liver injury.[38] Finally, an elegant study by Dal-Secco *et al.*[78] used CCR2-RFP and CX$_3$CR1-GFP single and double reporter mice to demonstrate that CCR2^{hi}CX$_3$CR1^{lo} (Ly6C^{hi}) monocytes were initially recruited to the site of liver injury where they encircled the site of injury by 24 h and transitioned into a CCR2^{lo}CX$_3$CR1^{hi} (Ly6C^{lo}) subset that were prevalent at 48 and 72 h. Importantly, this transition relied on IL-10 and IL-4 and inhibition of this transition resulted in reduced clearance of necrotic cells and reduced deposition of collagen within the site of injury.[78] *In vitro* studies of cultured CD14^{++}CD16$^-$ human monocytes showed that they upregulate expression of CD16 following treatment with either IL-10 or transforming growth factor-β, further supporting the idea that classical monocytes can differentiate into more mature subsets.[95] Taken together, these studies strongly suggest that inflammatory Ly6C^{hi} (CCR2^{hi}CX$_3$CR1^{lo}) monocytes infiltrate the liver by 24 h and participate in fibrosis progression until they transition to Ly6C^{lo} (CCR2^{lo}CX$_3$CR1^{hi}) monocyte-derived macrophages that are active in fibrosis resolution.

In contrast to the notion that the Ly6C^{lo} subset only derives from recruited Ly6C^{hi} monocytes at the site of injury is the idea that these monocyte populations are recruited in successive waves. Although this is not supported by studies in the liver, it has been described to occur in the peritoneum, heart and lung.[66,77,79] The paper that defined the patrolling function of GR1$^-$GFP^{hi} (CX$_3$CR1^{hi}) monocytes showed that they were the first responders to *L. monocytogenes* infection of the peritoneum, extravasating from patrolling the endothelium to the site of infection within the first 1–2 h, before GR1$^+$GFP^{lo} (CX$_3$CR1^{lo}) monocytes entered the tissue.[66] In a mouse model of myocardial infarct, inflammatory and phagocytic Ly6C^{hi} monocytes were recruited to injured tissue initially and were followed by the recruitment of Ly6C^{lo} monocytes primed to promote tissue repair.[77] Intriguingly, in both inflamed and noninflamed conditions, GR1^{hi} and GR1^{lo} monocytes were capable of trafficking to the lung tissue independently, but GR1^{hi} monocytes were required to transition to GR1^{lo} monocytes before they could differentiate into lung macrophage.[79] These examples are all unified by recruitment of distinct populations of Ly6C^{lo} and Ly6C^{hi} monocyte populations, but it is apparent that even with distinct recruitment of subsets that there may be significant overlap and plasticity that exists among Ly6C^{hi} and Ly6^{lo} monocytes. Although successive recruitment of distinct populations of monocytes cannot be ruled out in cases of sterile liver injury, the majority of evidence from the liver supports the hypothesis of the Ly6C^{hi} to Ly6C^{lo} transition at the site of injury.

WHAT FACTORS DRIVE THE LY6C^{HI} TO LY6C^{LO} PHENOTYPIC TRANSITION?

Although the CX$_3$CL1/CX$_3$CR1 axis seems to minimally contribute to monocyte recruitment to injured liver in mice, it may play an important role in controlling the transition from Ly6C^{hi} to Ly6C^{lo}. In a model of CCl_4-induced injury, mice lacking CX$_3$CR1 showed increased levels of ALT, transcripts for inflammatory genes and recruitment of inflammatory F4/80$^+$CD68$^+$ macrophages, indicating a protective role for CX$_3$CR1.[97] Intriguingly, the CX$_3$CL1/CX$_3$CR1 axis appeared to promote anti-inflammatory properties in KCs that then suppressed HSC activation, leading to reduced injury and fibrosis.[97] The hepatoprotective role for CX$_3$CL1/CX$_3$CR1 was further delineated by studies from Tacke and colleagues[98] showing that CX$_3$CR1$^{-/-}$ mice developed enhanced fibrosis in models of CCl_4- and bile duct ligation-induced injury. In comparison with control mice, CX$_3$CR1$^{-/-}$ mice had increased numbers of CD11b$^+$F4/80$^+$ monocyte-derived macrophages in the liver and exhibited a more inflammatory phenotype (*Tnf* and *iNOS*), indicating that CX$_3$CR1 is important in driving the transition from Ly6C^{hi} to Ly6C^{lo} phenotypes.[98] Finally, they found that CX$_3$CR1 was also critical for regulating *Bcl2* expression and, therefore, survival of monocytes.[98] In summary, CX$_3$CR1 appears to be required for this transition from Ly6C^{hi}CCR2$^+$CX$_3$CR1^{lo} to Ly6C^{lo}CCR2$^-$CX$_3$CR1^{hi}, and hence without it inflammatory monocytes/macrophages cannot differentiate into the tissue repair phenotype, resulting in the perpetuation of inflammation and fibrosis.

Alternatively, or in addition, CCR8 may also control differentiation of infiltrating monocytes. CCR8$^{-/-}$ mice had fewer CD45$^+$CD11b$^+$ F4/80$^+$CD11c$^-$ cells, termed inflammatory macrophages by Heymann *et al.*,[80] than wild-type mice in a model of CCl_4-induced chronic fibrosis. Intriguingly, CD11b$^+$F4/80$^+$ hepatic macrophages isolated from CCR8$^{-/-}$ mice and stimulated with lipopolysaccharide *in vitro* expressed a more DC-like phenotype (higher expression of major histocompatibility complex class II, CD86 and IL-12) when compared with wild-type macrophages.[80] Although the impact of CCR8 may be indirect, reducing the number of recruited Ly6C^{hi} monocytes and therefore reducing the number of monocytes available to differentiate through different mechanisms, these results indicate that CCR8 may also be directly controlling the differentiation of infiltrating monocytes into inflammatory macrophages and/or inhibiting their differentiation into more DC-like cells.

In addition to chemokine receptors, other potential pathways may contribute to the Ly6C^{hi} to Ly6C^{lo} transition. Using a variety of *in vitro* and *in vivo* methods, Ramachandran *et al.*[37] show that the process of phagocytosis is a key factor in the transition from inflammatory Ly6C^{hi} to restorative Ly6C^{lo} monocyte-derived macrophages. Specifically, phagocytosis of liposomes during the fibrosis resolution phase in CCl_4-injured mice resulted in a reduced amount of Ly6C^{hi} monocyte-derived macrophages and an increased number of Ly6C^{lo} monocyte-derived macrophages that were predominantly liposome positive and correlated with enhanced regression of fibrosis.[37] Concurrent antibody inhibition of IL-10 and IL-4 in a model of acute liver injury resulted in both delayed differentiation of CCR2^{hi}CX$_3$CR1^{lo} to CX$_3$CR1^{hi}CCR2^{lo} cells and removal of necrotic cells, indicating that this transition is important for the resolution of injury.[78] Rantakari *et al.*[94] propose that expression of the scavenger receptor stabilin-1 by monocytes and monocyte-derived macrophages is critical for the resolution of fibrosis, showing that fibrosis is enhanced in stabilin-1$^{-/-}$ mice and mice lacking stabilin-1 in monocytes following repeated CCl_4 injection. Monocytes and macrophages in stabilin-1$^{-/-}$ mice had a more proinflammatory phenotype (*Ccl3*, *Tnf*) and they further show that during the fibrosis resolution phase, stabilin-1$^{-/-}$ mice exhibited an increase in Ly6C^{hi} and a decrease in Ly6C^{lo} monocyte-derived macrophages, indicating that stabilin-1$^{-/-}$ may be involved in this transition required for fibrosis resolution.[94] Blockade of CSF1R resulted in the reduction of

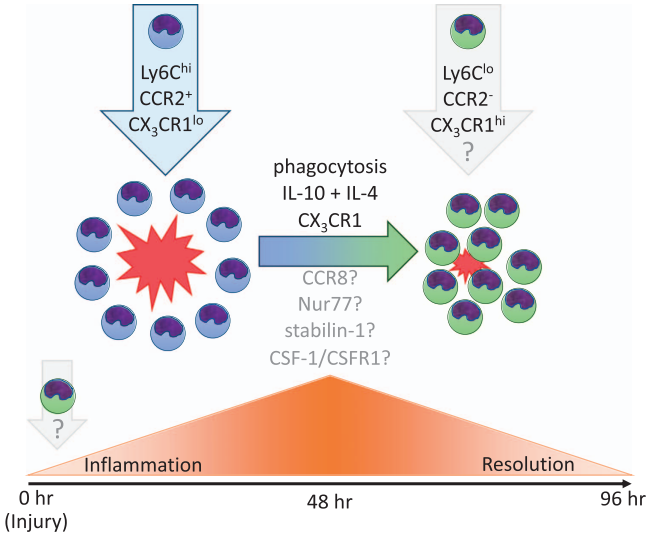

Figure 1 The differentiation of Ly6C^{hi} monocytes in liver injury. Following liver injury, monocytes traffic to the tissue. In the liver, Ly6C^{hi} monocytes (blue arrow) are recruited by CCL2- and CCR2-dependent mechanisms and extravasate from the circulation into the tissue where they surround the site of injury. The Ly6C^{hi}CCR2^{+}CX$_3$CR1^{lo} monocyte-derived macrophages then differentiate into Ly6C^{lo}CCR2^{-}CX$_3$CR1^{hi} monocyte-derived macrophages. This transition relies on a number of mechanisms and interactions (blue to green arrow) including phagocytosis, IL-10 and IL-4, and CX$_3$CR1. The CCR8, CSF1R and stabilin-1 receptors and the transcription factor Nur77 may also play a role in this process. Infiltrating Ly6C^{hi} monocytes and the Ly6C^{hi} monocyte-derived macrophages are inflammatory and contribute to inflammation and the progression of fibrosis, whereas Ly6C^{lo} monocytes and monocyte-derived macrophages exhibit tissue repair and restorative phenotypes, leading to the resolution of fibrosis. Alternate pathways observed in other tissues that may also occur in the liver (gray arrows) are the extravasation of the Ly6C^{lo} monocyte subset into the injured tissue within 1–2 h following injury, and successive recruitment of Ly6C^{hi} and Ly6C^{lo} monocytes that then differentiate into their respective inflammatory and pro-resolution macrophage populations.

GR1^{-} monocytes and an increase in GR1^{+} monocytes in peripheral blood, suggesting that the CSF-1/CSF1R interaction plays a role in the Ly6C^{hi} to Ly6C^{lo} phenotypic switch.[99] In a mouse model, CSF-1 treatment promoted a limited amount of differentiation of infiltrating F4/80^{lo}CD11b^{hi} monocytes into F4/80^{hi}CD11b^{lo} resident macrophages, suggesting that CSF-1/CSF1R may be important in controlling differentiation of monocytes in the liver.[100] In addition, Melino *et al.*[40] show that CSF1R blockade reduced injury in a mouse model of chronic fibrosis in parallel with a reduction in the numbers of Ly6C^{lo} blood monocytes and monocyte-derived macrophages. Finally, the transcription factor Nur77 is critical for the development of Ly6C^{lo} monocytes in the bone marrow and lack of Nur77 results in monocytes with a more proinflammatory phenotype, suggesting that Nur77 may also be important in controlling the phenotypic switch in tissues.[101] Whether or not Nur77 has implications in the differentiation of monocytes recruited to the liver has yet to be determined.

CONCLUSION

Monocytes and monocyte-derived macrophages appear to simultaneously play many distinct roles during tissue injury as drivers of injury, fibrosis and resolution. The plasticity of the monocyte compartment may provide an explanation for these seemingly conflicting roles. Although the data are contradictory on the role that Ly6C^{hi} monocytes play in amplifying the initial insult, infiltrating Ly6C^{hi} monocytes contribute to both the progression and regression of fibrosis, likely through the differentiation into tissue repair/restorative Ly6C^{lo} monocyte-derived macrophages within the injured liver (Figure 1). Specifically, the recruitment of Ly6C^{hi} monocytes initially contributes to driving the initiation of fibrosis, but as the phenotypic switch to the Ly6C^{lo} subset occurs, the functional output transitions to one of repair and resolution of fibrosis. Intriguingly, monocytes do not always appear to contribute to fibrosis resolution, and particularly in models of chronic fibrosis induced by diets high in fats and carbohydrates, monocytes appear to contribute to continuing inflammation and fibrosis. This posits the question of what factors signal the differentiation of Ly6C^{hi} monocytes into the tissue repair Ly6C^{lo} monocytes versus their switch into other inflammatory cell types, such as inflammatory macrophages or DCs. CX$_3$CR1, IL-10, IL-4 and the process of phagocytosis have all been described to participate in this transition of Ly6C^{hi} to Ly6C^{lo}. It is currently unknown whether the absence of differentiation signals or the presence of additional signals drives the transition of Ly6C^{hi} monocytes/monocyte-derived macrophages away from the Ly6C^{lo} subset and toward a more inflammatory cell type. An alternate hypothesis is that continual recruitment of Ly6C^{hi} monocytes from the bone marrow and blood continually propagates inflammation. In tissues, the balance between the Ly6C^{hi} and Ly6C^{lo} subsets could be governed by the rate of the Ly6C^{hi} to Ly6C^{lo} transition, or by *de novo* recruitment of Ly6C^{hi} monocytes or both.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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