

## REVIEW

# Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies

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Macrophages represent a major cell type of innate immunity and have emerged as a critical player and therapeutic target in many chronic inflammatory diseases. Hepatic macrophages consist of Kupffer cells, which are originated from the fetal yolk-sack, and infiltrated bone marrow-derived monocytes/macrophages. Hepatic macrophages play a central role in maintaining homeostasis of the liver and in the pathogenesis of liver injury, making them an attractive therapeutic target for liver diseases. However, the various populations of hepatic macrophages display different phenotypes and exert distinct functions. Thus, more research is required to better understand these cells to guide the development of macrophage-based therapeutic interventions. This review article will summarize the current knowledge on the origins and composition of hepatic macrophages, their functions in maintaining hepatic homeostasis, and their involvement in both promoting and resolving liver inflammation, injury, and fibrosis. Finally, the current strategies being developed to target hepatic macrophages for the treatment of liver diseases will be reviewed.

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## INTRODUCTION

Macrophages, also termed phagocytes, are myeloid immune cells that are widely distributed throughout the tissues in the body. They recognize, ingest, and degrade cellular debris, foreign material, or pathogens and exert a central function in orchestrating inflammatory processes.<sup>1</sup> Hepatic macrophages hold central positions in maintaining homeostasis in the liver as well as in the pathogenesis of acute or chronic liver injury. This makes them in principle an attractive target for novel therapies for liver diseases. However, macrophages exert a wide range of different functions in the liver and consist of functionally opposing cellular subsets, thus rendering the development of macrophage-based interventional strategies quite challenging so far.<sup>2</sup>

Intensive research on animal models of liver injury and on samples from patients with liver diseases has elucidated the complex heterogeneity of macrophages in the liver. Monocytes (the circulating precursors) and macrophages in the liver can be distinguished based on their origin, their migratory behavior, their differentiation, certain marker expression, and, most importantly, their functions during homeostasis and disease

(Table 1). However, these characteristics cannot be considered static, as macrophages are extraordinarily versatile cells. For instance, macrophages respond to environmental signals from tissue in a highly diversified manner, leading a wide spectrum of different 'polarization states' *in vitro* and *in vivo*.<sup>3,4</sup> Liver-infiltrating pro-inflammatory macrophages can rapidly develop into restorative, tissue-repairing cells in case of cessation of liver injury.<sup>5</sup> In this review article, we will summarize the current knowledge on the role of hepatic macrophages in maintaining homeostasis, promoting and resolving inflammation, repairing tissue, and causing fibrosis progression or regression. With a better understanding of the heterogeneity and functional diversity of these cells, macrophage-directed therapies may become a promising future option for the treatment of liver diseases.

## ORIGIN AND COMPOSITION OF HEPATIC MACROPHAGES

One of the most important paradigm shifts in macrophage biology during recent years referred to the origin of tissue macrophages, including macrophages in the liver.<sup>1</sup> While it has been long believed that tissue macrophages originate from

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**Table 1 Macrophage heterogeneity in healthy and diseased liver**

Origin of hepatic macrophages	
Kupffer cells	Monocyte-derived macrophages
Liver-resident, stationary, derived from a local stem cell seeded during embryogenesis	Derived from infiltrating blood monocytes, migratory, diverse functional properties depending on microenvironment
Polarization of hepatic macrophages	
M1 macrophage polarization	M2 macrophage polarization
Pro-inflammatory, anti-tumoral, induced by LPS or IFN $\gamma$ , key cytokines TNF, IL1 $\beta$ , and IL-12, linked to Th1 T cells	Anti-inflammatory, immune suppressive, pro-tumoral, induced by IL-4 and IL-13, key cytokine IL-10, linked to Th2 T cells
Subsets of circulating monocytes	
Ly-6C <sup>high</sup> monocytes	Ly-6C <sup>low</sup> monocytes
Inflammatory mouse monocyte subset, rapid recruitment to sites of inflammation, likely human counterpart: CD14 <sup>high</sup> monocytes	Mature mouse monocyte subset with a characteristic patrolling pattern, likely human counterpart: CD14 <sup>dim</sup> CD16 <sup>++</sup> monocytes
Functional macrophage subsets regarding liver immunology	
Tolerogenic macrophages	Immunogenic macrophages
Mostly Kupffer cells during homeostasis, express MHC-II, PDL-1, IL-10, and induce regulatory T cells	Likely derived from infiltrating monocytes, express MHC-II and costimulatory molecules (CD80, CD86), induce T cell responses
Functional macrophage subsets regarding liver disease	
Inflammatory macrophages	Restorative macrophages
Promote liver inflammation and fibrosis, activation via TLR, release of inflammatory cytokines (TNF, IL1 $\beta$ ) and chemokines (CCL2), pro-fibrogenic (via TGF- $\beta$ )	Promote resolution of inflammation (anti-inflammatory cytokines) and fibrosis (matrix metalloproteinases MMP-9, MMP-12, MMP-13), Ly-6C <sup>low</sup> expression in mice, post-phagocytic

Important dichotomies that have been introduced to describe the heterogeneity of monocyte and macrophage subsets in the liver.

circulating adult blood monocytes, it is now clear that many resident tissue macrophages are established during embryonic development and persist independent from blood monocytes in homeostasis.<sup>6</sup> This dichotomy of macrophages is prototypically found in the liver. The liver harbors about 80% of all macrophages of the body and is furthermore patrolled by blood monocytes.<sup>2</sup> The circulating blood monocytes can principally infiltrate the liver and give rise to monocyte-derived macrophages, but this is characteristic to liver injury.<sup>7</sup> During homeostasis, the macrophages in the liver predominantly self-renew from resident stem cells that originated from the fetal yolk-sack.<sup>8–10</sup> Most researchers call these hepatic macrophages Kupffer cells in order to distinguish the tissue-resident macrophages from infiltrating (monocyte-/bone marrow-derived) cells.<sup>2</sup> In mouse models, innovative fate mapping strategies, multi-marker flow-cytometric phenotyping and intravital microscopy are used to distinguish Kupffer cells from monocyte-derived macrophages.<sup>6,11</sup> However, no single marker is currently able to definitely discriminate these populations. In human liver, CD68 has been proposed as an indicator, but non-exclusive marker for Kupffer cells.<sup>12</sup> More recently, CD163L was proposed as a marker for tissue-resident macrophages, while CLEC5A might identify monocyte-derived pro-inflammatory macrophages.<sup>13</sup>

Blood monocytes, the circulating precursors for macrophages and dendritic cells, consist of subtypes as well, which are characterized by different Ly-6C (Gr-1) expression levels in

mice. Ly-6C<sup>hi</sup> monocytes express the chemokine receptor CCR2 and rapidly infiltrate tissue upon injury, while Ly-6C<sup>low</sup> monocytes express higher levels of CX3CR1 and show a patrolling behavior *in vivo*.<sup>14</sup> The human counterparts of these monocyte subsets are termed classical (CD14<sup>++</sup>CD16<sup>-</sup>), intermediate (CD14<sup>+</sup>CD16<sup>+</sup>), and non-classical (CD14<sup>dim</sup>CD16<sup>++</sup>) monocytes.<sup>15</sup> Intravital microscopy experiments in mice suggest that the Ly-6C<sup>low</sup> monocytes crawl along the hepatic endothelium,<sup>16</sup> possibly to orchestrate the disposal of apoptotic cells or other cellular debris.<sup>17</sup> Such a crawling migratory behavior was also observed for classical and non-classical but not intermediate human monocytes on endothelial monolayers *in vitro*, indicating subtype-specific human monocyte migratory behavior patterns with distinct adhesion molecule dependence.<sup>18</sup>

Upon acute or chronic hepatic injury in mice, Ly-6C<sup>hi</sup> blood monocytes are massively recruited to the liver and predominate the macrophage pool.<sup>7</sup> These monocyte-derived macrophages can form ring-like structures around defined areas of injury<sup>5</sup> or accumulate around periportal regions in cases of widespread liver damage.<sup>19</sup> Although these monocyte-derived macrophages initially exert pro-inflammatory and pro-fibrogenic actions, they can differentiate into Ly-6C<sup>low</sup> monocyte-derived macrophages<sup>5</sup>, which may foster tissue repair and injury resolution.<sup>20,21</sup> The massive infiltration of monocytes is also a hallmark feature of acute and chronic liver injury in humans.<sup>12,22,23</sup>

## HEPATIC MACROPHAGE FUNCTIONS IN HOMEOSTASIS

Kupffer cells belong to the reticuloendothelial system in the liver, a highly dynamic and complex network, which constitutes a primary line of defense against invading microorganisms, functions as a sensor for altered tissue integrity and largely contributes to the upkeep of tissue homeostasis.<sup>24</sup> Because the liver is constantly exposed to antigens from the intestine as well as low levels of bacterial endotoxins, many mechanisms suppress an 'accidental' activation of the immune system. Kupffer cells play a major role in maintaining immunological tolerance in the liver and in providing an anti-inflammatory microenvironment during homeostasis.<sup>24</sup> For instance, Kupffer cells secrete the anti-inflammatory cytokine interleukin (IL)-10 in mouse<sup>11</sup> as well as in human liver.<sup>25</sup> Although Kupffer cells express lower levels of MHC class II molecules than classical dendritic cells, they are able to interact with T cells. However, unlike dendritic cells, Kupffer cells favor the development of regulatory T cells, thereby promoting immune tolerance. In a mouse model using the systemic delivery of particulate antigen, Kupffer cells interact with T cells in an antigen-specific manner, expand IL-10 expressing regulatory T cells, and are able to establish a tissue-protective systemic tolerance against these antigens.<sup>11</sup>

Several mechanisms have been identified that contribute to the tolerogenic features of Kupffer cells. On the one hand, Kupffer cells express relatively high levels of the T-cell inhibitory molecule PDL-1 in healthy liver, but low levels of co-stimulatory molecules.<sup>11</sup> Moreover, when Kupffer cells are purified from healthy liver, they can induce regulatory T cells *in vitro* via the secretion of prostaglandins PGE2 and 15d-PGJ2.<sup>26</sup> The pretreatment of Kupffer cells with IFN- $\gamma$  upregulates the enzyme indoleamine-2,3-dioxygenase (IDO) that appears essential for the induction of a suppressive T-cell response.<sup>27</sup>

Kupffer cells not only interact with T cells, but can also interact with many cellular components in the liver. For instance, Kupffer cells can initiate the recruitment of monocytes to the liver in case of injury, which is an important prerequisite for liver regeneration,<sup>28</sup> and they also cross-talk with hepatic stellate cells (HSCs).<sup>29</sup> However, due to their rather stationary behavior, Kupffer cells are not optimally suited to migrate to sites of injury or to interact with dynamic processes such as angiogenesis. Infiltrating monocytes, on the other hand, are motile and better suited to interact with the vasculature, promoting an ordered vascular growth during liver regeneration<sup>30</sup> or during chronic injury.<sup>31</sup> Nevertheless, although infiltrating monocyte-derived macrophages can resemble the phenotype of Kupffer cells quite rapidly after virus-induced inflammation<sup>32</sup> or after acetaminophen (APAP)-induced hepatotoxicity,<sup>33</sup> they remain functionally distinct.

## HEPATIC MACROPHAGE FUNCTIONS IN ACUTE LIVER INJURY

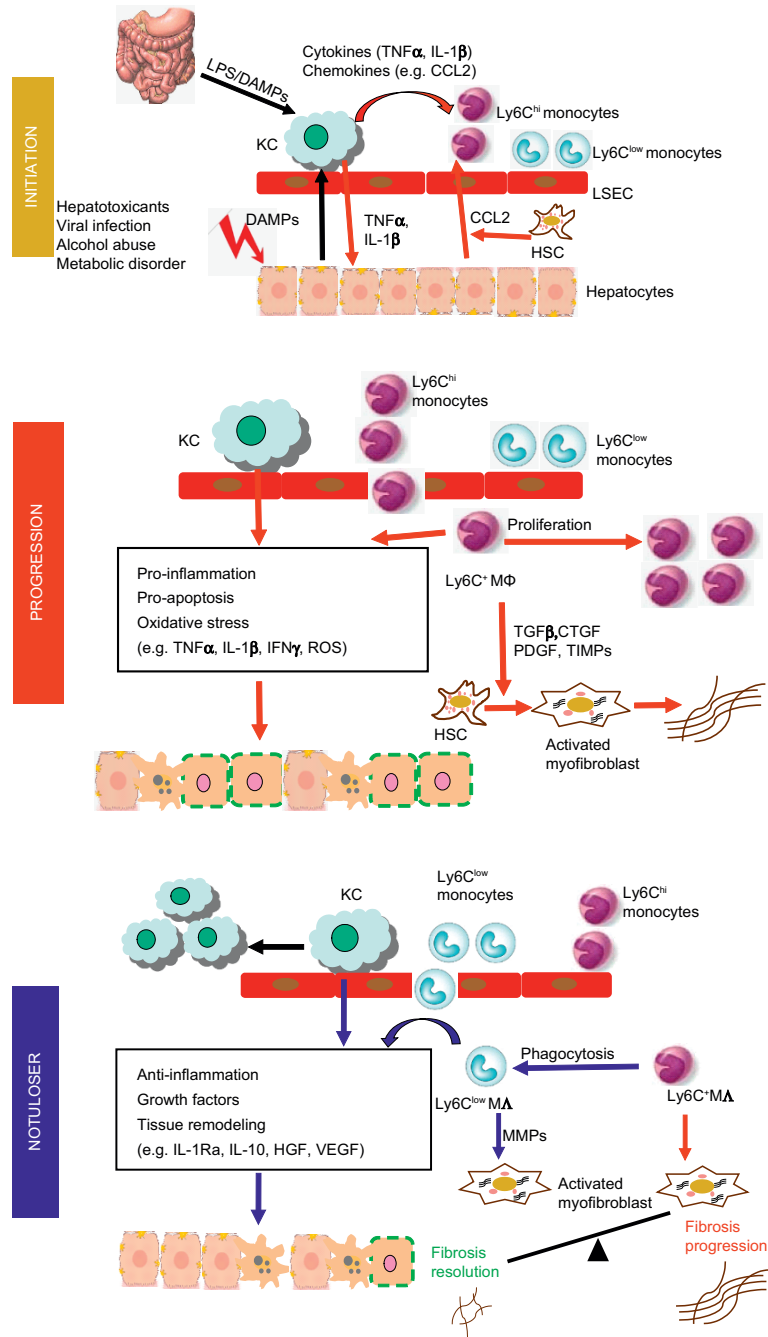
During liver injury, resident Kupffer cells are important in the initial response to injury by rapidly producing cytokines and chemokines, including IL-1 $\beta$ , tumor necrosis factor (TNF) $\alpha$ ,

CCL2, and CCL5, resulting in the recruitment of other immune cells, such as monocytes. Both acute and chronic liver injuries are characterized by a dramatic expansion of the hepatic macrophage population owing to the massive influx of monocytes into the liver (Figure 1). For example, in mouse models of CCl<sub>4</sub>-induced liver fibrosis, the infiltration of Ly-6C<sup>hi</sup> monocytes contributes to a threefold to fivefold expansion of hepatic macrophages.<sup>7</sup> Consistent with the distinct chemokine receptor and adhesion molecule expression patterns on Ly-6C<sup>hi</sup> and Ly-6C<sup>low</sup> monocytes,<sup>15</sup> the infiltrated monocytes are dominated by the Ly-6C<sup>hi</sup> subset. After transmigration into the liver, the Ly-6C<sup>hi</sup> monocytes differentiate into Ly-6C<sup>+</sup> macrophages, which are thought to exhibit pro-inflammatory and pro-fibrogenic functions (Figure 1). Accumulating evidence suggests that the tissue micro-environmental factors during disease conditions could promote the switch of Ly-6C<sup>+</sup> macrophages into Ly-6C<sup>low</sup> macrophages (Figure 1). To therapeutically target hepatic macrophages in acute and chronic liver diseases, it is important to understand the respective roles of resident Kupffer cells and infiltrated Ly-6C<sup>hi</sup> and Ly-6C<sup>low</sup> macrophages in various types of liver diseases. The different models of acute liver injury, as reviewed in this article, yielded interesting context-dependent functions of the hepatic macrophage subsets in either propagating or limiting the immune response in acute liver disease.

### Acetaminophen hepatotoxicity

Overdose APAP can cause severe liver damage with the potential to progress to liver failure, which accounts for nearly half of the liver failure cases in the USA and a significant portion in Europe. APAP-induced direct damage to hepatocytes triggers the activation of resident Kupffer cells and the massive recruitment of circulating monocytes in mice (Figure 1).<sup>33,34</sup> It was observed that the number of resident Kupffer cells reduced significantly by 24 h after APAP challenge and began to recover by 72 h. Although it is clear now that Kupffer cells are established prenatally by yolk-sack progenitor cells,<sup>9,10</sup> it remains a question whether circulating monocytes contribute to the replenishment of Kupffer cells during liver injury. In the model of APAP-induced liver injury (AILI), the replenishment of Kupffer cells is mainly achieved by self-renewal rather than from infiltrating monocytes.<sup>33</sup> Consistent with this finding, it has been reported that Kupffer cells expand from resident progenitor cells during parasite infection where Th2 immunity dominates.<sup>35</sup>

Hepatic macrophages were initially thought to contribute to AILI through their production of pro-inflammatory cytokines and mediators, such as TNF $\alpha$  and IL-1 $\beta$ .<sup>36,37</sup> However, macrophages are also the main source of IL-10, IL-4, and IL-13, all of which can protect against AILI.<sup>38-40</sup> In line with a protective role of Kupffer cells, depletion of these cells was shown to exacerbate AILI.<sup>41,42</sup> Aside from activating Kupffer cells, APAP challenge causes the recruitment of circulating monocytes in a CCR2-dependent manner.<sup>34</sup> A more recent study further separated the infiltrating macrophages into Ly-6C<sup>hi</sup> monocytes, which are recruited into the liver in a CCR2-dependent fashion,



**Figure 1** Involvement of hepatic macrophage populations during the initiation, progression and resolution of liver injury in mouse models. In the initial phase, hepatocyte damage triggers the release of damage-associated molecular pattern molecules (DAMPs) which stimulate Kupffer cell (KC) activation. Endotoxin from the gut–liver axis may additionally endorse Kupffer cell activation. Activated Kupffer cells secrete pro-inflammatory cytokines, including IL-1 $\beta$  and TNF $\alpha$ , which contribute to hepatocyte injury. Hepatocytes, stellate cells and Kupffer cells secrete chemokines such as CCL2 that promotes the recruitment of Ly-6C<sup>hi</sup> monocytes into the liver, where they develop into Ly-6C<sup>+</sup> macrophages. These cells promote the progression of liver injury by secreting pro-inflammatory cytokines and producing ROS. During chronic injury, Ly-6C<sup>+</sup> macrophages also trigger HSC activation and promote myofibroblast production of extracellular matrix through releasing pro-fibrotic mediators, such as tumor growth factor (TGF)- $\beta$ , connective tissue growth factor (CTGF), PDGF, and tissue inhibitor of matrix metalloproteinase (TIMPs). Phagocytosis of dead cells initiates the resolution of inflammation and tissue restoration. Upon phagocytosis or other resolution stimuli, the Ly-6C<sup>hi</sup> macrophages switch to Ly-6C<sup>low</sup> macrophages that exhibit a restorative phenotype. The Ly-6C<sup>low</sup> macrophages, as well as Kupffer cells produce anti-inflammatory mediators, such as IL-1 receptor antagonist (IL-1Ra) and IL-10. They release growth factors, including hepatocyte growth factor (HGF) and VEGF. Moreover, the Ly-6C<sup>low</sup> macrophages produce matrix metalloproteinases (MMP-9, -12, -13) and promote the degradation of excessive extracellular matrix proteins. Overall, the phenotype and functional heterogeneity of hepatic macrophages play critical roles in determining the balance between the mechanisms of progression and resolution of tissue injury during both acute and chronic liver injuries.

and Ly-6C<sup>low</sup> monocyte-derived macrophages (Figure 1).<sup>33</sup> CCR2<sup>-/-</sup> mice or anti-CCR2 antibody-treated mice exhibit delayed recovery from AILI, suggesting that the infiltrated macrophages may contribute to tissue repair after acute injury.<sup>33,34</sup> In the combined absence of Kupffer cells and infiltrating macrophages, tissue repair is much more significantly impaired, further supporting a protective function of hepatic macrophages in AILI.<sup>21</sup> Moreover, a recent study in mice indicated that the augmentation of both, Kupffer cells and monocyte-derived macrophages, via macrophage-colony stimulating factor (CSF1) might hold beneficial therapeutic potential in AILI by facilitating hepatocyte regeneration and restoring innate immune functions.<sup>43</sup>

### Concanavalin A-induced hepatitis

Concanavalin A (ConA) is a plant lectin that acts as a T cell mitogen and causes T cell-mediated hepatotoxicity. Thus, ConA-induced liver injury in mice represents a commonly used animal model of autoimmune liver disease. Aside from stimulating T cells, ConA also activates liver resident Kupffer cells to produce TNF $\alpha$  and IFN $\gamma$ , which are key factors that cause hepatocyte damage.<sup>44,45</sup> Kupffer cells also facilitate a Th1 immune response, thereby contributing to ConA hepatotoxicity<sup>46</sup>. Moreover, Kupffer cells and regulatory T cells produce IL-10 upon repeated exposure to ConA and cause immune tolerance.<sup>47,48</sup> The data emphasize the relevance of Kupffer cell–T cell interactions for immune homeostasis as well as for autoimmunity.

### Ischemia/reperfusion-induced liver injury

Ischemia/reperfusion (I/R)-induced liver injury may occur in a number of clinical settings such as trauma, shock, liver surgery, and liver transplantation<sup>49</sup>. During I/R injury, Kupffer cells are activated to produce reactive oxygen species (ROS) and secrete TNF $\alpha$ , IL-1 $\beta$ , and chemokines, which contribute to hepatocyte death, endothelial damage and recruitment, and activation of leukocytes<sup>50</sup>. Consistent with a pathological role of Kupffer cells, depletion of these cells attenuates I/R liver injury.<sup>51–54</sup> Interestingly, some studies have reported a protective function of Kupffer cells during I/R. The protection appears to be mediated by the release of anti-inflammatory IL-10 and production of antioxidant hemoxygenase by Kupffer cells.<sup>54–56</sup> Monocyte-derived macrophages are recruited into the liver at the later phase of ischemia and they are involved in sustaining the tissue inflammation and destruction.<sup>57</sup>

### Liver injury as result of parasite infection

Infection by the protozoan parasite *Entamoeba histolytica* causes hepatocyte damage in focal areas leading to amebic liver abscess (ALA). Selective depletion of Kupffer cells using liposome-entrapped clodronate or inhibition of monocyte infiltration using CCR2<sup>-/-</sup> mice revealed that Kupffer cells and inflammatory Ly-6C<sup>hi</sup> monocytes, through producing TNF $\alpha$ , are main effector cells responsible for liver destruction during ALA.<sup>58</sup> *Trypanosoma congolense* infection causes inflammatory response syndrome (SIRS) that is initiated by liver

injury. C57BL/6 mice are tolerant to *T. congolense* infection-induced SIRS because of the accumulation of Ly-6C<sup>low</sup> macrophages in the liver. The Ly-6C<sup>low</sup> macrophages protect against liver injury by secreting IL-10 and inhibiting hepatocyte death caused by TNF $\alpha$  that is released by Ly-6C<sup>+</sup> inflammatory monocyte. Moreover, the Ly-6C<sup>low</sup> macrophages promote the differentiation of the Ly-6C<sup>+</sup> monocytes into anti-inflammatory macrophages.<sup>59</sup> These data suggest that enhancing the accumulation and/or functionality of the Ly-6C<sup>low</sup> macrophages may be an effective strategy to complement anti-microbial medication in treating liver injury caused by parasite infection.

## HEPATIC MACROPHAGE FUNCTIONS IN CHRONIC LIVER INJURY

Chronic liver diseases can be caused by multiple etiologies such as alcohol abuse, viral infection, and autoimmune and metabolic disorders. Regardless of the etiology, the disease progresses from inflammation to liver fibrosis/cirrhosis and to hepatocellular carcinoma (HCC). Evidence from clinical and animal studies suggests that hepatic macrophages play important roles in all stages of chronic liver disease.

### Alcoholic liver disease

Alcohol abuse is a leading cause of chronic liver disease that affects millions of people worldwide. Alcoholic liver disease (ALD) can eventually lead to liver cirrhosis and HCC. Clinical observations in patients with alcoholic hepatitis and fibrosis support the involvement of macrophages in the disease progression. The number of macrophages in the liver is increased in the early stage of fatty liver as well as the later stages of hepatitis and cirrhosis.<sup>60</sup>

It has been reported that hepatic levels of macrophage inflammatory genes are upregulated in alcohol-related cirrhotic patients.<sup>61</sup> A recent study of liver samples from patients with alcoholic steatohepatitis demonstrates the presence of macrophages expressing receptors and cytokines associated with both M1 and M2 macrophages, suggesting the co-existence of different types of hepatic macrophages.<sup>62</sup> Indicators of macrophage activation, such as neopterin and leukocyte function associated antigen 3, as well as cytokines such as IL-6, IL-8, IL-18, and chemokines are elevated in ALD patients.<sup>63–66</sup> Moreover, circulating monocytes from ALD patients spontaneously produce TNF $\alpha$  and are more sensitive to lipopolysaccharide (LPS) stimulation.<sup>67,68</sup> Animal models of ALD have demonstrated that acute and chronic ethanol administrations are associated with the activation of hepatic macrophages, which produce TNF $\alpha$ , IL-6, CCL2, and ROS.<sup>69,70</sup> Depletion of macrophages by gadolinium chloride (GdCl<sub>3</sub>) or clodronate liposomes attenuated alcohol-induced liver inflammation.<sup>70,71</sup> Resident Kupffer cells from alcohol-fed mice are sensitized to endotoxin and exhibit increased responses to LPS, resulting in elevated production of TNF $\alpha$  and CCL2 (Figure 1).<sup>72,73</sup> A recent study also demonstrated that chronic alcohol feeding causes the accumulation of hepatic infiltrating monocytes/macrophages in mice.<sup>74</sup> The infiltrated macrophages consist

of two subsets with differential expression levels of Ly-6C (Ly-6C<sup>+</sup> and Ly-6C<sup>low</sup>) and distinct genetic profiles. The Ly-6C<sup>+</sup> cells exhibit a pro-inflammatory tissue-damaging phenotype; in contrast, the Ly-6C<sup>low</sup> cells exhibit an anti-inflammatory and tissue protective phenotype.<sup>74</sup> Furthermore, upon phagocytosis of apoptotic hepatocytes, Ly-6C<sup>+</sup> macrophages appears to switch to Ly-6C<sup>low</sup> macrophages (Figure 1).<sup>74</sup> Although macrophages have emerged as a critical player in ALD, directly targeting some of their detrimental functions appears challenging in clinical practice; for instance, anti-TNF antibody application in severe alcoholic hepatitis did not translate into improved clinical outcome in patients.<sup>75</sup> Further understanding their heterogeneity and functional diversity is therefore warranted for therapeutically targeting these cells.

### Viral hepatitis

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections can result in chronic liver disease with increased risk for liver fibrosis/cirrhosis, hepatic failure, and HCC.<sup>76,77</sup> Evidence suggests that hepatic macrophages play an important role in viral hepatitis. Because it is difficult to distinguish Kupffer cells from infiltrating macrophages, studies in this area often refer to hepatic macrophages as Kupffer cells and do not properly take macrophage heterogeneity into account when describing their functions. Evidence suggests that hepatic macrophages ('Kupffer cells') have a beneficial anti-viral effect in the early phase after infection, but appear to play a role in suppressing anti-viral immunity during chronic infection. While only few studies show HBV binding to Kupffer cells and causing activation, HCV and its proteins have been shown to activate Kupffer cells.<sup>78–80</sup> In HCV infection, Kupffer cells increase in number<sup>81,82</sup> and expression levels of activation markers such as CD163 and CD33.<sup>83,84</sup> Moreover, they produce pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-18, and TNF $\alpha$ .<sup>78–80,85</sup> Among these cytokines, Kupffer cell-derived TNF $\alpha$  promotes HCV entry of hepatoma cells;<sup>86</sup> in contrast, IL-6, IL-1 $\beta$  and IFN $\gamma$  inhibit HCV replication,<sup>87–89</sup> suggesting that Kupffer cells play diverse roles in modulating anti-viral activities by producing a variety of cytokines. Kupffer cells also express TRAIL, Fas-ligand, granzyme B, perforin, and ROS, which directly cause cytotoxicity of infected hepatocytes.<sup>90–92</sup> On the contrary to their anti-viral effects, Kupffer cells produce immunomodulatory mediators, such as IL-10, TGF $\beta$ , galectin-9, PD-L1, and PD-L2, during chronic HBV and HCV infection.<sup>78,94–97</sup> These factors can suppress anti-viral immunity through inhibiting T cell function or inducing T cell death. Aside from their involvement in modulating anti-viral immunity, Kupffer cells are thought to be involved in the fibrosis development during chronic viral infection. Although the causative role has not been demonstrated, the number of CD14<sup>+</sup>CD68<sup>+</sup> Kupffer cells is increased in patients with viral hepatitis,<sup>96</sup> and Kupffer cells are known to produce pro-fibrogenic factors.<sup>98</sup> In summary, there is growing appreciation of hepatic macrophages in viral hepatitis. The multifaceted or even opposing roles of these cells may be attributed to not only resident Kupffer cells but also infiltrating macrophages. Future research of the functional dif-

ferences and respective contributions of the different populations of hepatic macrophages is warranted.

### Liver fibrosis

Evidence suggests that Kupffer cells can activate HSCs through the production of profibrotic cytokines TGF $\beta$  and platelet-derived growth factor (PDGF)<sup>99</sup>. On the other hand, Kupffer cells also express multiple matrix metalloproteinases (MMP-9, -12 and -13) that promote extracellular matrix degradation and thus favor the resolution of fibrosis.<sup>100,101</sup> More recent studies that distinguish hepatic macrophages using various cell surface markers reveal the critical roles of infiltrated macrophages in orchestrating liver fibrosis development and resolution (Figure 1).<sup>7,20,102</sup> Using CD11b-diphtheria toxin receptor (DTR) transgenic mice, researchers have demonstrated that targeted deletion of infiltrated macrophages during fibrosis development ameliorates fibrosis; however, depletion of these macrophages during the resolution phase exacerbates fibrosis.<sup>20,102</sup> The pro- and anti-fibrotic functions of hepatic macrophages may be explained by the fact that different subsets of infiltrated macrophages play opposite roles in liver fibrosis. During the early phase of tissue injury, the recruited Ly-6C<sup>hi</sup> monocytes differentiate into pro-inflammatory macrophages, and they directly interact with HSCs to promote fibrosis through the production of TGF $\beta$  (Figure 1).<sup>7</sup> The CCL2-CCR2 pathway plays a critical role in hepatic recruitment of the Ly-6C<sup>hi</sup> monocytes. Both genetic deletion of CCR2 in CCR2<sup>-/-</sup> mice and pharmacological inhibition of CCL2 by the RNA-aptamer mNOX-E36 result in attenuation of liver fibrosis.<sup>7,31,103</sup> These findings support a pro-fibrotic function of the Ly-6C<sup>hi</sup> macrophages. A recent study demonstrates that phagocytosis of cellular debris facilitates the phenotypic switch of the Ly-6C<sup>hi</sup> macrophages to the Ly-6C<sup>low</sup> subset.<sup>20</sup> Two lines of evidence suggest that the Ly-6C<sup>low</sup> macrophage subset is the primary source of MMPs and plays a critical role in orchestrating fibrosis resolution.<sup>20</sup> First, the depletion of this population caused a failure in scar remodeling.<sup>20</sup> Second, treatment of mice with the CCL2 inhibitor, mNOX-E36, caused a significant increase of the Ly-6C<sup>low</sup> macrophages and accelerated the regression of experimental liver fibrosis in mice caused by carbon tetrachloride or methionine-choline-deficient diet.<sup>103</sup> The finding further supports for the anti-fibrotic function of the Ly-6C<sup>low</sup> macrophages.

### Hepatocellular carcinoma

HCC is the most common type of primary liver cancer and the third leading cause of cancer-related death in the world.<sup>104</sup> In HCC, tumor-associated macrophages play important roles in tumor growth, angiogenesis, and metastasis through the production of a number of growth factors (PDGF $\beta$ , vascular endothelial growth factor (VEGF), TGF $\beta$ , and EGFR ligands), cytokines (IL-6, TNF $\alpha$ , and IL-10), chemokines (CCL17, CCL22, CCL24, CXCL12, and IL-8), as well as other soluble factors (MMPs, osteopontin, and cyclooxygenase-2).<sup>104</sup> In diethylnitrosamine-induced HCC in mice, induced HCC, pro-inflammatory activation of Kupffer cells during the early

stages of chemical-induced carcinogenesis is important in tumor development.<sup>105,106</sup> Once the primary tumor is established, the liver infiltrated macrophages appear to play a more prominent role than Kupffer cells in HCC progression.<sup>106</sup> HCC-associated hepatic macrophages are often identified by immunohistochemical detection of CD68<sup>+</sup> or CD14<sup>+</sup> cells, and cell number correlates with HCC progression and reduced survival.<sup>107–110</sup> Targeting macrophages may be an effective approach in therapy. The majority of tumor cells in HCC express high levels of glypican-3, which is a member of the glypican family of heparin-sulfate proteoglycans and it promotes the recruitment of macrophages into the tumors. Antibodies that target glypican-3 have been investigated in phase I clinical trials of advanced HCC with promising results.<sup>111,112</sup> Zoledronic acid, which is used to prevent skeletal complications associated with bone metastases, has also shown anti-tumor effect through targeting tumor-associated macrophages as it is phagocytosed by macrophages and induces apoptosis of the cells.<sup>113,114</sup> The combination therapy of zoledronic acid and sorafenib to treat advanced HCC is being evaluated in phase II studies (NCT01259193).

## STRATEGIES FOR THERAPEUTIC TARGETING OF HEPATIC MACROPHAGES

The essential roles of liver macrophages for maintaining tissue homeostasis, for initiating inflammatory responses in injury, as well as for promoting the progression, but also regression of liver disease emphasize that these processes are prime targets for novel therapeutic approaches. Several interventional concepts have been validated in experimental animal models and are currently being translated into clinical scenarios (see Table 2). However, quite a few obstacles hamper the direct translation from mouse models to human disease, including the relatively poor definition of hepatic macrophage hetero-

geneity and subset-specific functions in human liver, the important contribution of etiology-specific factors (such as bile acids in cholangiopathies, lipids, and lipid products in non-alcoholic fatty liver diseases, adaptive immunity in viral hepatitis) to macrophage activation in patients with distinct types of liver diseases as well as the variable and slow-going course of disease progression in patients.<sup>2</sup>

Some of the promising strategies for therapeutic targeting of hepatic macrophages include (see Table 2):

Dampening Kupffer cell activation, e.g., by modifying the gut–liver axis;

Inhibiting inflammatory monocyte recruitment to injured liver, e.g., by targeting chemokine pathways;

Shaping hepatic macrophage function, e.g., by either influencing their polarization or inhibiting inflammatory effector molecules;

Augmenting the differentiation to restorative macrophages, e.g., by providing phagocytic stimuli.

### Dampening Kupffer cell activation via the gut–liver axis

In animal models as well as in patients with chronic liver diseases, increased levels of endotoxins reaching the liver via the portal vein accelerate hepatic inflammation and fibrosis, emphasizing the relevance of gut–liver interactions.<sup>115</sup> In fact, patients with liver cirrhosis show characteristic alterations of their gut microbiota, with an enrichment of potentially more invasive bacterial strains<sup>116</sup>. The toll-like receptor (TLR)-dependent activation of inflammatory pathways in Kupffer cells and stellate cells can be effectively inhibited by intestinal decontamination via broad-spectrum antibiotics in mouse models, which reduces the progression of liver fibrosis and also hepatocarcinogenesis within inflamed livers.<sup>29,117</sup> Similarly, commensal microbiota, as compared to germ-free gut conditions, is protective in mouse models of liver fibrosis, empha-

**Table 2 Strategies for therapeutic targeting of hepatic macrophages (derived from experimental models of chronic liver injury)**

Target	Possible approach (selected from experimental models)	Reference(s)
Dampening Kupffer cell activation by influencing the gut–liver axis	<ul style="list-style-type: none"> <li>restoration of the normal microbiome, by application of probiotics, antibiotics or fecal microbiota transfer;</li> <li>sequestering of deoxycholic acid (or other bile acids associated formation oxidative stress and DNA damage);</li> <li>application of compounds that lead to elevated tightness of the intestinal barrier.</li> </ul>	29,115,117,118
Inhibition of (Ly-6C <sup>+</sup> ) inflammatory monocyte recruitment to the liver	<ul style="list-style-type: none"> <li>pharmacological antagonism of CCL2 (MCP-1), e.g., by RNA aptamer molecules;</li> <li>pharmacological inhibition of CCR2 and/or of other related chemokine receptors like CCR1/CCR5 (e.g., cenicriviroc).</li> </ul>	103,120
Modulating hepatic macrophage polarization and function	<ul style="list-style-type: none"> <li>nanoparticles influencing hepatic macrophage polarization;</li> <li>Delivery of “polarizing” drugs (e.g., dexamethasone) to hepatic macrophages;</li> <li>Neutralization of inflammatory effector cytokines like TNF and IL-1;</li> <li>Inhibition of the pro-inflammatory mediator Galectin-3.</li> </ul>	123,126,128,129,131,132
Augmentation of restorative hepatic macrophages	<ul style="list-style-type: none"> <li>Application of IL-4 to force local proliferation of tissue-remodeling macrophages;</li> <li>Autologous cell transfer of <i>in vitro</i> matured and polarized macrophages or of hematopoietic precursors;</li> <li>Inhibition of inflammatory monocyte influx during the regression of fibrosis;</li> <li>Injecting apoptotic cells or PS-containing liposomes to accelerate the differentiation to Ly-6C<sup>low</sup> macrophages.</li> </ul>	20,120,135,136

izing the relevance of intestinal homeostasis for preventing inflammatory Kupffer cell activation.<sup>118</sup> Therapeutic interventions aiming at restoring the normal gut microbiome, such as probiotics, antibiotics, fecal microbiota transfer, and sequestration of deoxycholic or other bile acids, might significantly dampen Kupffer cell activation in the liver.

### Inhibiting inflammatory monocyte recruitment to injured liver

The infiltration of inflammatory monocytes into the liver is critically regulated by chemokines, which appears to be an attractive target for therapeutic interventions. The chemokine CCL2 and its receptor CCR2 represent the key pathway for monocyte accumulation in injured livers in mice and men.<sup>119</sup> The therapeutic potential for targeting this pathway has been exemplarily demonstrated by inhibiting CCL2 using anRNA-aptamer molecule, termed mNOX-E36, in mouse models of fibrosis. Antagonizing CCL2 ameliorated steatohepatitis, reduced hepatic angiogenesis, and accelerated regression of liver fibrosis in mice.<sup>31,103,120</sup> An oral inhibitor of CCR2 and CCR5, cenicriviroc, is currently being tested in a phase 2b clinical trial in patients with non-alcoholic steatohepatitis and fibrosis (Centaur trial, NCT02217475). An alternative (or complementary) strategy could target hematopoietic growth factors that affect monocytopoiesis, their release into the circulation and their accumulation at sites on inflammation. In patients with chronic HCV infection, high levels of macrophage colony stimulating factor (M-CSF) are associated with pro-inflammatory properties of hepatic macrophages,<sup>121</sup> indicating that inhibiting M-CSF might hold therapeutic potential in fibrosis. On the other hand, it needs to be considered that patients with advanced stages of liver cirrhosis are jeopardized by severe immune suppression ('immune-paralysis'), and the augmentation of myeloid cell numbers and function by administration of G-CSF has beneficial effects in this group of patients.<sup>122</sup>

### Shaping hepatic macrophage function

It is undoubtedly clear that hepatic macrophages, like macrophages in other tissues, can adapt their phenotype to different microenvironmental cues in the liver.<sup>2,4</sup> Identifying the critical factors as well as the transcriptional networks that are important in promoting the differentiation will lead to novel therapeutic strategies. Many strategies to shape the functional polarization of macrophages or inhibit their key effector molecules have been suggested. For instance, hepatic macrophages can be very well targeted by nanoparticle-based drug carriers, due to the efficient scavenger function of macrophages in the liver.<sup>123</sup> However, the type of particles (e.g., liposomes, polymers, micelles) or modifications of their surface chemistry (e.g., peptide modifications, sugar moieties) can already have immune-modulatory effects on hepatic macrophages. For instance, silica nanoparticles by itself can trigger the release of inflammatory cytokines like TNF or IL-1 $\beta$  by Kupffer cells,<sup>124</sup> while peptide-modified gold nanorods can polarize hepatic macrophages towards the M1 phenotype,<sup>125</sup> in both

instances, mice exposed to these nanoparticles are more susceptible to liver injury. On the other hand, the delivery of dexamethasone to liver macrophages via liposomal constructs can ameliorate experimental liver injury<sup>126</sup> by mechanisms that are well preserved between mouse and human macrophages.<sup>127</sup> The specific delivery of anti-inflammatory drugs to hepatic macrophages or even subsets could be potentially further improved by innovative carrier systems, such as mannosylated albumin<sup>128</sup> or mannose-modified trimethyl chitosan-cysteine (MTC) conjugated nanoparticles.<sup>129</sup> An alternative (or complementary) strategy could target effector mediators released by hepatic macrophages, e.g., inhibit TNF or deliver IL-10.<sup>130</sup> However, the broad systemic TNF inhibition by the anti-TNF antibody infliximab carries significant risks, as revealed from studies in patients with acute alcoholic hepatitis that had increased rates of bacterial infections.<sup>75</sup> Thus, more specific inflammatory mediators are likely better pharmacological targets. Inhibitors against Galectin-3, a protein with pleiotropic functions that is highly upregulated in hepatic macrophages in liver disease models,<sup>131,132</sup> are currently being explored in early phase clinical trials.

### Augmenting the differentiation to restorative macrophages

A striking observation from mouse models of liver fibrosis regression was the existence of restorative macrophages that develop from Ly-6C<sup>+</sup> monocyte-derived macrophages in conditions of injury cessation.<sup>20</sup> Not all of the molecular factors promoting the differentiation to restorative macrophages have been identified so far,<sup>133</sup> but phagocytosis appears to be a natural trigger for this process.<sup>20</sup> Shifting the hepatic microenvironment from inflammation to resolution as well as augmenting restorative differentiation pathways in macrophages by delivering phagocytic stimuli (liposomes, apoptotic cells) appear as promising avenues for macrophage-directed therapies especially in chronic liver diseases.<sup>134</sup> Moreover, therapeutic approaches could also include the transfer of autologous 'beneficial macrophages.' The injection of *ex vivo* differentiated macrophages was advantageous in a mouse model of liver fibrosis.<sup>135</sup> This concept is currently being tested in the REpeated AutoLogous Infusions of STem cells in Cirrhosis trial (REALISTIC) in patients with liver cirrhosis, comparing G-CSF injections alone or followed by repeated infusions of hematopoietic stem cells to standard conservative management.<sup>136</sup>

Taken together, the rapid progress in understanding macrophage heterogeneity in acute and chronic liver diseases gives rise to the expectation that we will soon be able to translate these findings into novel therapies in clinical practice.

### COMPETING INTEREST

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