

## REVIEW

## The Multifaceted Roles of Macrophages in NAFLD Pathogenesis

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## SUMMARY

Distinct macrophage populations are key drivers in promoting but also in attenuating disease progression during all states of nonalcoholic fatty liver disease pathogenesis, making them an attractive therapeutic target. This review provides an overview of the broad spectrum of functionally diverse macrophage phenotypes in nonalcoholic fatty liver disease and related systemic metabolic diseases. The focus was placed on pathogenic relationships and mechanistic interactions of different macrophage populations, as well as on possible therapeutic approaches targeting their (mal) function.

Nonalcoholic fatty liver disease (NAFLD) is the liver manifestation of the metabolic syndrome. NAFLD constitutes a spectrum of pathologies ranging from simple hepatic steatosis (nonalcoholic fatty liver) to the more progressive form of steatohepatitis and fibrosis, which can culminate in liver cirrhosis and hepatocellular carcinoma. Macrophages play multiple roles in the context of NAFLD pathogenesis by regulating inflammatory responses and metabolic homeostasis in the liver and thereby may represent an attractive therapeutic target. Advances in high-resolution methods have highlighted the extraordinary heterogeneity and plasticity of hepatic macrophage populations and activation states thereof. Harmful/disease-promoting as well as beneficial/restorative macrophage phenotypes co-exist and are dynamically regulated, thus this complexity must be taken into consideration in strategies concerning therapeutic targeting. Macrophage heterogeneity in NAFLD includes their distinct ontogeny (embryonic Kupffer cells vs bone marrow-/monocyte-derived macrophages) as well as their functional phenotype, for example, inflammatory phagocytes, lipid- and scar-associated macrophages, or restorative macrophages. Here, we discuss the multifaceted role of macrophages in the pathogenesis of NAFLD in steatosis, steatohepatitis, and transition to fibrosis and hepatocellular carcinoma, focusing on both their beneficial and maladaptive functions at different disease stages. We also highlight the systemic aspect of metabolic dysregulation and illustrate the contribution of macrophages in the reciprocal crosstalk between organs and compartments (eg, the gut-liver axis, adipose tissue, and cardiohepatic metabolic interactions). Furthermore, we discuss the current state of development of pharmacologic treatment options targeting macrophage biology. (*Cell Mol*

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Nonalcoholic fatty liver disease (NAFLD) is the most frequent chronic liver disease in the world, with a prevalence of 25% to 30% in Western societies.<sup>1</sup> NAFLD can progress to diseases associated with poor outcome, such as liver cirrhosis and hepatocellular carcinoma, placing an increasing burden on health care systems.<sup>1</sup> NAFLD is considered the hepatic manifestation of the metabolic syndrome because it is strongly associated with obesity, type 2 diabetes, dyslipidemia, and cardiovascular disease.<sup>2</sup> The collective term *NAFLD* encompasses various phenotypes of metabolic liver disease, ranging from simple fat accumulation termed *hepatic steatosis* or *nonalcoholic fatty liver*, to nonalcoholic steatohepatitis (NASH), displaying additional lobular inflammation and hepatocellular damage. In NASH, a proinflammatory hepatic microenvironment stimulates fibrogenic processes leading to liver fibrosis and, eventually, cirrhosis.<sup>3</sup> The only curative therapy for end-stage liver disease and the early stages of hepatocellular carcinoma is liver transplantation. Because of the increasing prevalence and lack of diagnostic and therapeutic strategies, NAFLD is likely to become the leading indication for liver transplantation in the near future.<sup>4</sup>

The pathogenic processes of NAFLD are incompletely understood. Within the liver, the disruption of the finely regulated interplay between hepatocytes, hepatic stellate cells, endothelial cells, and various immune cell subtypes, driven by secreted cytokines and mediators, can result in a proinflammatory, profibrotic, and protumorigenic hepatic

**Abbreviations used in this paper:** CCL, chemokine C-C motif ligand; CX3CR1, fractalkine receptor; GLP-1, glucagon-like peptide 1; GPNMB, glycoprotein NMB; IL, interleukin; Ly6C, lymphocyte antigen-6C; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PD-L1, Programmed cell death ligand-1; PPAR, peroxisome proliferator-activated receptor; scRNA-seq, single-cell RNA sequencing; TIM4, T-cell membrane protein 4; TNF, tumor necrosis factor; TREM2, triggering receptor expressed on myeloid cells 2.

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microenvironment.<sup>5</sup> In addition, extrahepatic factors such as nutritional intake, gut dysbiosis, and systemic inflammation, as well as genetic disposition influence the disease processes in the liver.<sup>5</sup>

Macrophages are innate immune cells that are abundant as resident cells in almost all organs of the body.<sup>6</sup> They exert key functions in tissue development and homeostasis but also may contribute to pathogenesis of various diseases. Over the past decade, high-resolution methods, such as single-cell RNA sequencing (scRNA-seq), spatial proteogenomics, fate-mapping experiments, and intravital microscopy substantially have increased our knowledge of macrophage origin, activation, and function.<sup>7</sup> However, these major advances also have raised many new questions as the impressive complexity of macrophage phenotypes, plasticity, and functional regulation in different organ-specific microenvironments becomes increasingly apparent (Figure 1).

Sophisticated fate-mapping experiments have highlighted the existence of embryonically derived, long-living, and self-sustaining resident macrophage populations in almost all organs, referred to as Kupffer cells in the liver. In addition to these resident phagocytes, the liver also contains infiltrated bone marrow-derived monocytes and macrophages (termed *monocyte-derived macrophages*).<sup>8–10</sup> Under homeostatic conditions, resident macrophages exert highly specialized housekeeping functions regulated by the niche-specific microenvironment in which they reside.<sup>11,12</sup> Moreover, in the context of disease, the classic dichotomous concept of a proinflammatory M1 and a restorative M2 polarization state has been replaced by a more dynamic view that takes into account the polyfunctional nature and exceptional plasticity of macrophages.<sup>13</sup> scRNA-seq of whole human livers has partially elucidated the complexity of co-existing proinflammatory and restorative macrophage phenotypes in the homeostatic steady state.<sup>14</sup>

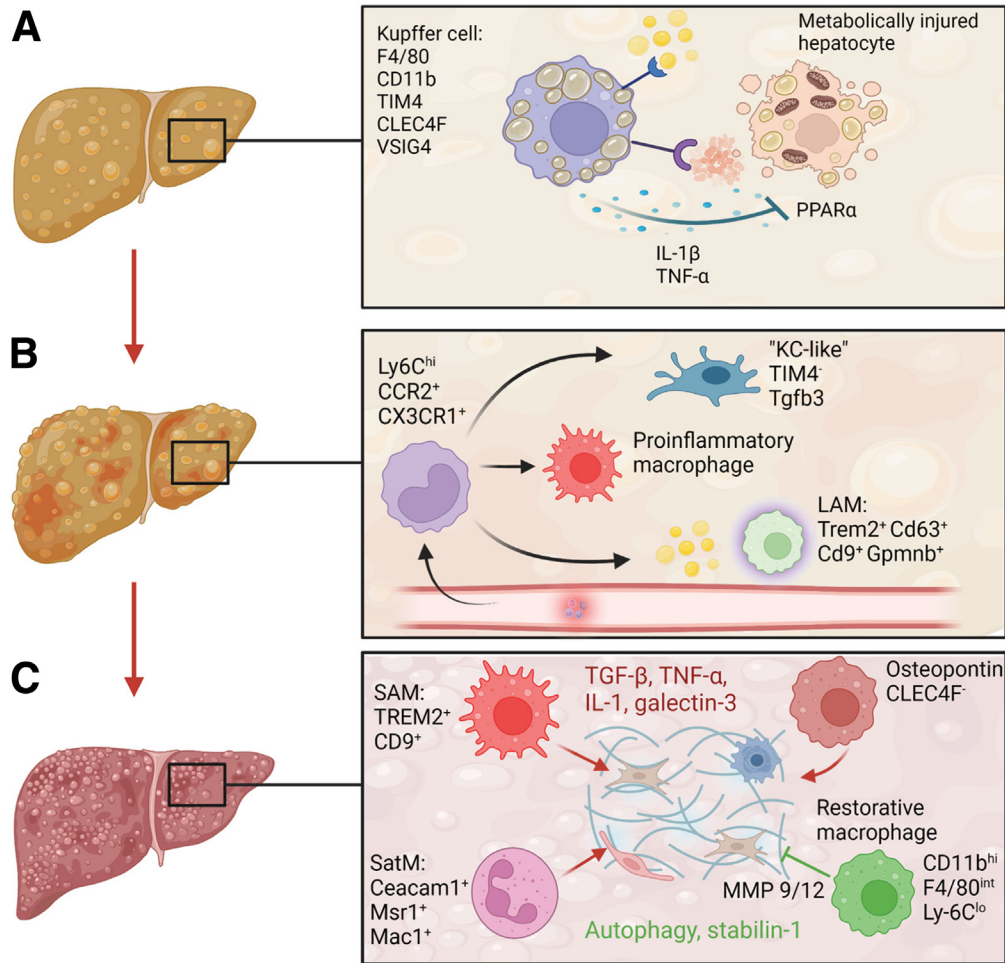
Given their complex regulation in homeostasis, it is not surprising that macrophages play a diverse role in various diseases,<sup>6,9</sup> including the development of NAFLD, progression to NASH, fibrosis, and end-stage liver disease, as well as in the evolution of associated complications such as hepatocellular carcinoma. In fact, the increase in inflammatory macrophage populations and their spatially distinct accumulation in portal fields and close to (proliferating) bile ducts are a key characteristic of progressive chronic liver diseases, including NAFLD/NASH, but also cholangiopathies or alcohol-associated hepatitis. The exceptional function of macrophage populations in both driving and terminating inflammation, as well as their involvement in local and systemic metabolic regulation, makes them an attractive but challenging therapeutic target. In this review, we summarize the contribution of macrophages to different stages of metabolic liver disease and provide an outlook on open questions and potential therapeutic targets.

## Macrophages Regulate Lipid Metabolism in Steatosis and Play a Role in the Initiation of Steatohepatitis

Hepatic steatosis is defined histologically by an accumulation of fat, mainly triglycerides, in more than 5% of

hepatocytes.<sup>15</sup> Interestingly, there seems to be a nonlinear relationship between simple steatosis and lipotoxicity, the main trigger for the transition to the inflammatory phenotype of steatohepatitis. It has not been determined conclusively whether patients with severe steatosis develop full-blown steatohepatitis more rapidly than subjects with a lesser steatosis burden.<sup>15</sup> Lipotoxicity occurs when the liver is stressed by an excessive accumulation of fatty acids in hepatocytes, by which the capacity to use, store, and export them as triglycerides is exceeded.<sup>16</sup> This leads to endoplasmic reticulum and oxidative stress, hepatocellular senescence, and lipopoptosis.<sup>3,16</sup> Steatotic hepatocytes secrete cytokines and chemokines, such as C-C motif chemokine ligand (CCL) 2 or C-X-C motif chemokine ligand (CXCL) 10, and extracellular vesicles, which in turn activate nonparenchymal cell types such as hepatic stellate cells, liver sinusoidal endothelial cells, and liver macrophages.<sup>16</sup> Another pathway to Kupffer cell activation in NAFLD is mediated by the potent proinflammatory effect of free cholesterol.<sup>17</sup> Macrophages are equipped with pattern recognition receptors, promoting proinflammatory signaling, and scavenger receptors, involved in lipid uptake and phagocytosis. In the context of NAFLD, much attention has been focused on the scavenger receptor A and the fatty acid transporter CD36, which mediate the uptake of modified low-density lipoprotein.<sup>18,19</sup> Activated, enlarged, fat-laden Kupffer cells show impaired lipid metabolism and recruit other leukocytes owing to their inflammatory phenotype.<sup>20</sup> Interestingly, this pathogenic process involves similar receptors and displays resemblance to foam cell-driven atherosclerotic plaque formation,<sup>21</sup> underscoring the systemic nature of metabolic diseases.

During homeostasis, bona fide Kupffer cells, defined as F4/80<sup>hi</sup>CD11b<sup>int</sup> cells, express T-cell immunoglobulin mucin (TIM) 4, C-Type Lectin Domain Family 4 Member F (CLEC4F), and V-set and immunoglobulin domain containing 4 (VSIG4) in mice,<sup>8,9</sup> whereas monocyte-derived macrophages tend to have a CD11b<sup>hi</sup>F4/80<sup>int</sup> phenotype and express CX3C motif chemokine receptor (CX3CR) 1 and C-C chemokine receptor type 2 (CCR2).<sup>9</sup> Depletion of Kupffer cells in mice fed a high-fat diet reduced hepatic steatosis and inflammation.<sup>22</sup> Activated Kupffer cells secrete proinflammatory cytokines such as interleukin (IL)1 $\beta$  and tumor necrosis factor (TNF), which inhibit genes involved in hepatocyte lipid metabolism via the peroxisome proliferator-activated receptor (PPAR)- $\alpha$  pathway, thereby promoting hepatocyte steatosis.<sup>23,24</sup> Interestingly, the onset of inflammation may occur at early stages of the disease. Liver samples of obese patients already displayed a gene expression pattern of low-grade inflammation before showing histologic abnormalities.<sup>25</sup> Conversely, fat-laden macrophages also can adopt an anti-inflammatory phenotype after the uptake of apoptotic steatotic hepatocytes.<sup>26</sup> Moreover, using single-cell transcriptome analyses and fate-mapping approaches, Blériot et al<sup>27</sup> recently showed that in addition to the major CD206<sup>lo</sup>ESAM<sup>-</sup> Kupffer cell population, a secondary CD206<sup>hi</sup>ESAM<sup>+</sup> subpopulation also is present in healthy and obese mice and is involved in the regulation of lipid metabolism, particularly fatty acid metabolism, via its expression of CD36. Hence, the involvement of Kupffer cells/macrophages in the



**Figure 1. Macrophages foster progression from nonalcoholic fatty liver to NASH and fibrosis.** (A) Kupffer cells are activated upon ingestion of apoptotic fat-laden hepatocytes and free cholesterol, triggering proinflammatory activation. (B) The inflammatory microenvironment recruits monocytes that differentiate in heterogeneous monocyte-derived macrophage populations (Kupffer cell-like, inflammatory macrophages, and lipid-associated macrophages [LAMs]) based on signals from the environment. (C) Macrophage populations shape both profibrotic (red) and antifibrotic (green) processes within the fibrotic niche. Important phenotypic markers of the macrophage populations identified in mouse models are shown in the figure. CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; CLEC4F, C-Type Lectin Domain Family 4 Member F; Mac1, macrophage-1 antigen; MMP, matrix metalloproteinase; Msr1, macrophage scavenger receptor 1; SAM, scar-associated macrophages; SatM, segregated-nucleus-containing atypical monocytes; Tgfb3, transforming growth factor  $\beta$ ; VSIG4, V-set and immunoglobulin domain containing 4. (Figure was created with BioRender).

regulation of metabolic processes and modulation of lobular inflammation is complex.

## Macrophages in the Transition to Steatohepatitis

Once the proinflammatory milieu is established, a dramatic shift in macrophage composition occurs. Although the number of embryonically derived  $TIM4^+$  mature Kupffer cells decreases as a result of apoptosis and impaired self-renewal, macrophages derived from lymphocyte antigen 6 (Ly6) $C^{hi}CCR2$ -expressing monocytes accumulate in the liver. Specifically, recruited monocytes undergo distinct differentiation programs depending on the signals they receive from the environment and primarily develop into  $TIM4^+$  macrophages.<sup>28–30</sup> Monocyte-derived macrophages show

predominantly immature proinflammatory phenotypes that exacerbate liver injury and drive disease progression.<sup>31</sup> Accordingly,  $Ccr2^{-/-}$  mice, which lack monocyte recruited macrophages, challenged in dietary NASH models showed less steatosis, inflammatory cell infiltration, and fibrosis.<sup>29</sup>

An elegant study deciphered the replenishment of Kupffer cells during NASH. Using a  $Ccr2^{-/-}$ -based chimeric mouse model, Tran et al<sup>30</sup> showed that monocyte-derived macrophages also can adopt a Kupffer cell-like phenotype to repopulate the accessible niche in NASH. Interestingly, these monocyte-derived Kupffer cells did not promote hepatic triglyceride storage as efficiently as their embryonic-derived counterparts. However, after disease regression they were able to develop a fully mature Kupffer cell phenotype. These results are important because they strongly suggest a functional difference between Kupffer

cells of different origins, at least in the context of NAFLD. Transcriptome and epigenome analyses in human and mouse NASH livers showed that Kupffer cells lose their epigenetic identity by altering their transcriptional signature and up-regulating a NASH-specific enhancer profile with activation of the *AFT3*, *CD9*, *Arhgap22*, and *Tgfb3* loci.<sup>32</sup>

## Lipid-Associated Macrophages

The NASH microenvironment is a highly interconnected network of autocrine and paracrine intrahepatic crosstalk between different cell types.<sup>33</sup> This microenvironment and its specific microanatomic niches shape the chromatin landscape of myeloid cells, associated with reduced Kupffer cell survival and an increase of monocyte-recruited macrophages, and emergence of a scar-associated macrophage phenotype.<sup>32</sup> A subpopulation of TIM4<sup>+</sup> monocyte-derived macrophages gives rise to lipid-associated macrophages; this designation arises from a characteristic lipid metabolism-related genetic signature of these macrophages and from their localization in the vicinity of sites of steatosis in crown like structures.<sup>28</sup> Lipid-associated macrophages are heterogeneous and can be divided into a transitional CX3CR1<sup>+</sup>CCR2<sup>+</sup> lipid-associated macrophage subset, and Trem2<sup>+</sup> Cd63<sup>+</sup> Cd9<sup>+</sup> Gpmb<sup>+</sup> classic lipid-associated macrophages.<sup>28</sup>

Classic lipid-associated macrophages are not specific to the liver but are an integral part of adipose tissue and can be found in various organs and conditions.<sup>34</sup> Indeed, liver macrophages during obesity,<sup>34</sup> aortic macrophages in atherosclerosis,<sup>35</sup> and even disease-associated microglia in the brain of mice and human beings with neurodegenerative diseases<sup>36</sup> display a conserved lipid-associated, macrophage-related gene expression signature. Their function is linked strongly with the expression of triggering receptor expressed on myeloid cells 2 (TREM2), which mediates lipid uptake and metabolism; lipid-associated macrophages in fat thereby prevent adipocyte hypertrophy, adipose tissue dysfunction, and inflammation, as well as systemic metabolic dysregulation.<sup>34</sup> Consistently, TREM2 deficiency promotes fat accumulation, dyslipidemia, and glucose intolerance in diet-induced obesity.<sup>34</sup> Constitutive TREM2 deficiency accelerates the progression of NASH in a high-fat diet mouse model. The secretion of exosomes by *Trem2*<sup>-/-</sup> macrophages has been suggested to disrupt hepatocyte mitochondrial function, although how exosomes specifically target hepatocytes has not been resolved.<sup>37</sup> In contrast, the deletion of TREM2 in hematopoietic cells, which also leads to the deletion of TREM2 in monocyte-derived macrophages, protects from steatohepatitis and liver fibrosis in mice.<sup>38</sup>

Recently, Williams et al<sup>12</sup> created a liver cell atlas by combining proteogenomic and spatial information to characterize parenchymal and immune cells in healthy and diseased mouse and human livers.<sup>39</sup> Interestingly, the investigators found that lipid-associated macrophages present even in healthy livers, where they reside in close proximity to bile ducts. In liver samples with steatosis in greater than 10% of hepatocytes, lipid-associated macrophages not only increased in number but also relocated to the pericentral steatotic areas.<sup>12</sup> Comparison of the transcriptional signatures of lipid-associated macrophages between healthy and obese mice

showed down-regulation of IL1 $\beta$ , TNF, and IL10 upon high-fat diet feeding.<sup>12</sup> This suggests that lipid-associated macrophages may switch toward an anti-inflammatory phenotype as an adaptation to protect against NAFLD progression.<sup>38</sup>

## Presence of Hepatic Crown-Like Structures Indicates Progression of Disease

Phagocytosis of apoptotic steatotic hepatocytes and uptake of free cholesterol is a key characteristic of macrophages in NAFLD.<sup>17</sup> However, lipid-laden hepatocytes can swell considerably in size, which makes classic phagocytosis impossible.<sup>40</sup> To circumvent this, lipid-associated macrophages may encapsulate dying enlarged hepatocytes by forming multinucleated aggregates through cell membrane fusion and secrete lysosomal contents into these hydrolytic synapses.<sup>40,41</sup> These aggregates also occur in visceral adipose tissue and atheromatous plaques.<sup>42</sup> In metabolic liver disease, these macrophage aggregates surrounding dying hepatocytes are referred to as *hepatic crown-like structures*. In fat, crown-like structure formation may prevent adipocyte hypertrophy, massive adipocyte cell death, and preserve systemic lipid homeostasis and tissue integrity.<sup>34</sup> TREM2 signaling also appears to play a critical role in crown like structure formation because deletion of *Trem2* abolished macrophage recruitment to enlarged adipocytes and the emergence of crown-like structures.<sup>34</sup> In the liver, it was proposed that hepatic crown-like structure-forming monocyte-derived macrophages have a unique transitional CX3CR1<sup>+</sup>CCR2<sup>+</sup> lipid-associated macrophage phenotype term that is distinct from classic Trem2<sup>+</sup> Cd63<sup>+</sup> Cd9<sup>+</sup> Gpmb<sup>+</sup> lipid-associated macrophages.<sup>28</sup> Importantly, it also has been claimed that hepatic crown-like structures are a significant source of inflammation.<sup>43</sup> A specific population of CD11c<sup>+</sup> macrophages involved in the formation of hepatic crown-like structures is thought to drive pathogenesis and fibrosis.<sup>44</sup> In any case, the presence of hepatic crown-like structures indicates an advanced stage of disease because their number correlates positively with the extent of fibrosis and is associated with transition from simple steatosis to steatohepatitis.<sup>42,43</sup> However, further studies are needed to elucidate the heterogeneity of macrophages with respect to their protective and progressive effects on hepatic crown-like structure formation and liver disease.

## The Fibrotic Niche Harbors Heterogeneously Regulated Macrophages

Liver fibrosis is a key feature in advanced stages of NASH and is associated closely with its prognosis.<sup>45</sup> Approximately 30% of NASH patients are at risk for progressive fibrosis, which can lead to cirrhosis and portal hypertension, impaired organ function, and hepatocellular carcinoma.<sup>46</sup> The severity of fibrosis is the most important predictor of liver-related morbidity and mortality.<sup>46,47</sup> The fibrotic niche defines the complex interplay between nonparenchymal immune cells, endothelial cells, and mesenchymal cells, including hepatic stellate cells and myofibroblasts.<sup>48</sup>

Macrophages have been implicated as regulators of liver fibrosis.<sup>49</sup> Their plasticity and heterogeneous involvement in profibrotic and healing processes becomes evident in macrophage-depletion models. Depletion of macrophages in early stages of fibrosis results in reduced scar formation and fewer myofibroblasts, whereas macrophage depletion during recovery causes failure of matrix degradation in the carbon tetrachloride mouse fibrosis model.<sup>50</sup> In a genome-wide association study examining the effects of genetic variations on liver fibrosis progression, the strongest association was found with a single-nucleotide polymorphism in the *MERTK* gene, which is part of the core macrophage signature and is involved in phagocytic clearance of apoptotic cells.<sup>51,52</sup>

Even though a profibrogenic subpopulation of Kupffer cell-derived CD11c<sup>+</sup> macrophages has been described,<sup>44</sup> RNA sequencing analysis showed that monocyte-derived macrophages rather than Kupffer cells up-regulate growth factors and cytokines associated with fibrosis progression,<sup>53</sup> suggesting a predominant role of monocyte-derived macrophages in hepatic scar formation. In line with this, *Ccr2*<sup>-/-</sup> mice show lower numbers of monocyte-derived macrophages and less fibrosis.<sup>54</sup>

However, monocyte-derived macrophages of the fibrotic niche are phenotypically and functionally heterogeneous. scRNA-seq showed a scar-associated profibrogenic TREM2+CD9+ macrophage population.<sup>48</sup> The transcriptomic signature of scar-associated macrophages included several genes regulating scar-producing myofibroblasts, such as *IL1 $\beta$* , *SPPI1*, *LGALS3*, *CCR2*, and *TNFSF12*.<sup>48</sup> Interestingly, they share substantial similarities with lipid-associated macrophages found in healthy and steatotic livers.<sup>12,28</sup> Another study identified a Ceacam1+Msr1+Ly6C-F4/80-Mac1+ monocyte population that was termed *segregated-nucleus-containing atypical monocytes*, based on its segmented nuclear shape and cytoplasmic granules. This population is regulated by *CCAAT/enhancer binding protein  $\beta$*  and is profibrogenic.<sup>55</sup> In addition, a profibrogenic population of osteopontin-expressing CLEC4F- monocyte-derived macrophages was described.<sup>56</sup> Although it is not clear how these different macrophage subpopulations relate to each other exactly, the relevance of functionally and phenotypically heterogeneous macrophage populations is supported by clinical studies showing increased levels of osteopontin, monocyte chemoattractant protein (CCL2), and IL8 in serum as well as transcriptional up-regulation of these genes in liver samples of patients with NASH fibrosis.<sup>57</sup>

Macrophages also are important regulators of fibrosis regression and restoration of tissue integrity because they degrade the extracellular matrix and have immunoregulatory functions. Depletion of CX3CR1<sup>+</sup>CCR2<sup>+</sup> lipid-associated macrophages resulted in reduced hepatic crown-like structure formation but increased liver fibrosis.<sup>28</sup> Furthermore, a CD11b<sup>hi</sup> F4/80<sup>int</sup>Ly-6C<sup>lo</sup> monocyte-derived macrophage subset expressing high levels of matrix metalloproteinase has been described in liver tissue with pronounced fibrosis. This population arises from a phenotype switch of inflammatory recruited monocytes upon engulfment of cellular debris.<sup>58</sup>

The fibrotic niche can be understood only by considering the interactions between macrophages, endothelial cells, and collagen-producing mesenchymal cells. Macrophage-derived transforming growth factor  $\beta$ , TNF, IL1 $\beta$ , and galectin-3 are important profibrotic signals that activate the nuclear factor- $\kappa$ B pathway in hepatic stellate cells, inducing collagen production and promoting their survival.<sup>49,59</sup> Yes-associated protein signaling in macrophages mediates the crosstalk to a fibrogenic vascular endothelial cell subset.<sup>60</sup> Moreover, autophagy<sup>61</sup> and the scavenger receptor stabilin-1<sup>62</sup> have been identified as crucial pathways in restorative macrophages (Figure 1). Together, the molecular mechanisms by which macrophages regulate fibrotic processes represent an active field of research.

## Tumor-Associated Macrophages Emerge in the Transition From NASH to Hepatocellular Carcinoma

In addition to liver cirrhosis, the development of hepatocellular carcinoma represents a critical complication of NAFLD/NASH with poor prognosis. Hepatocellular carcinoma is the fourth leading cause of cancer death worldwide,<sup>63</sup> and NAFLD is the fastest-growing cause of hepatocellular carcinoma.<sup>64</sup> NAFLD patients may develop hepatocellular carcinoma even without existing cirrhosis.<sup>65</sup> Potentially curative treatment options include liver transplantation at early stages, surgical resection, and locoregional therapies (eg, transarterial chemoembolization or radiofrequency ablation).<sup>66</sup> The advent of immunotherapy not only highlights the critical role of the immune system in hepatocarcinogenesis, but also greatly has expanded the available treatment options.<sup>66</sup> Nevertheless, treatment success is limited in inflammation-driven tumors such as hepatocellular carcinoma owing to high resistance and disease recurrence, illustrating the need to develop additional therapeutic approaches and biomarkers for early detection and therapy monitoring.<sup>67</sup>

Hepatocellular carcinoma is a cancer driven by inflammation and fibrogenesis. Patients with hepatocellular carcinoma have an increase in proinflammatory cytokines, such as IL8, IL13, CCL3, CCL4, and CCL5, as well as an increased number of activated blood monocytes compared with patients with NASH.<sup>68</sup> The increase of inflammatory mediators in the blood is associated with tumor recurrence after surgery.<sup>68,69</sup> CCL2 is highly expressed in liver samples from patients with hepatocellular carcinoma and predicts poor outcome. Furthermore, treatment with a CCR2 antagonist inhibits tumor growth and metastasis and improves survival in a hepatocellular carcinoma mouse model.<sup>70</sup> The proinflammatory and profibrotic hepatic microenvironment featuring activated macrophages promotes malignant degeneration of hepatocytes and fosters the development and progression of hepatocellular carcinoma.<sup>71</sup> Macrophages are involved in the clearance of premalignant senescent hepatocytes at early stages of hepatocellular carcinoma,<sup>72</sup> but progression is characterized by the breakdown of this immune surveillance.<sup>64</sup>

Monocyte-derived macrophages accumulating at the hepatocellular carcinoma tissue develop into tumor-associated macrophages and adopt a heterogenous phenotype.<sup>71</sup> Their transcriptional signature is characterized by high expression of *TREM2*, *GPNMB*, *SLC40A1*, *APOE*, *C1QA*, and *C1QB*, and, interestingly, resembles not only the lipid-associated macrophage signature,<sup>34</sup> but also the tumor-associated macrophage signature in lung cancer.<sup>73,74</sup> Importantly, lipid-associated macrophages have been implicated in different cancers as immune-suppressive, tumor-promoting macrophages.<sup>75,76</sup>

The frequency of hepatocellular carcinoma infiltrating tumor-associated macrophages is an independent predictor of poor overall and disease-free survival.<sup>77</sup> The mechanisms by which the tumor reprograms macrophages for its own benefit are poorly understood. In any case, proinflammatory phenotypes that drive hepatocellular carcinoma progression and immunosuppressive phenotypes that promote tumor evasion of immune surveillance appear to co-exist. This is reflected in the co-expression of the full spectrum of M1- and M2-related genes within the tumor-associated macrophage population.<sup>73</sup> In hepatocellular carcinoma, *Gpnmb*<sup>-/-</sup> macrophages produced lower levels of TNF upon Toll-like-receptor stimulation, suggesting a proinflammatory role of transmembrane glycoprotein NMB (GPNMB).<sup>73</sup> Simultaneously, tumor-associated macrophages suppress the anti-tumor immune response of cytotoxic T cells and natural killer cells and lead to immunosuppression via activation of regulatory T cells.<sup>78</sup> Although GPNMB expression seems to be associated with a proinflammatory phenotype in hepatocellular carcinoma, GPNMB<sup>+</sup> TREM2<sup>+</sup> lipid-associated macrophages appear to play a rather protective role in steatohepatitis. It is not clear to what extent these populations are related and reflect extensive macrophage plasticity. Thus, further characterization in this context is necessary to understand the full picture of macrophage contributions to hepatocellular carcinoma progression.

One signaling pathway that has attracted much attention is the immunosuppressive programmed cell death protein (ligand) 1 PD-1–PD-L1 interaction.<sup>79</sup> After activation by tumor cells, infiltrating monocytes express high levels of PD-L1, and this expression is maintained by autocrine secretion of TNF and IL10.<sup>80</sup> PD-L1 expression in monocytes increases with disease progression and is associated with a high mortality rate in patients with hepatocellular carcinoma.<sup>80</sup> Mechanistically, PD-L1 signaling promotes suppression of CD8<sup>+</sup> cytotoxic immunity against tumor cells.<sup>81</sup> Two antibodies directed against PD-1, nivolumab and pembrolizumab, have been widely used for immunotherapy of hepatocellular carcinoma,<sup>82,83</sup> although the primary end point, prolongation of survival, was not met in phase III clinical trials.<sup>84,85</sup> The anti-PD-L1 antibody atezolizumab in combination with bevacizumab (anti-vascular endothelial growth factor) resulted in improved overall and progression-free survival in patients with advanced hepatocellular carcinoma, making it the current first-line systemic therapy.<sup>86</sup> Despite impressive progress in this field, response rates of these so-called

immune checkpoint inhibitors in inflammation-driven tumors, including hepatocellular carcinoma, remain rather low.<sup>87</sup> In fact, some clinical data indicate that patients with NASH-driven hepatocellular carcinoma may respond less well to immunotherapies compared with other etiologies.<sup>88</sup> The mechanisms underlying tumor cell resistance to immunotherapies are poorly understood, but hold great potential for improving treatment efficacy in the future. Even though treatment resistance to immune checkpoint inhibitors has been attributed mainly to T-cell anergy,<sup>89</sup> there also is evidence that different macrophage populations are involved.<sup>90</sup>

## Peripheral Macrophages Impact Liver Disease

Because NAFLD represents the hepatic manifestation of the metabolic syndrome, other diseases such as obesity, type 2 diabetes, and cardiovascular diseases are linked closely with this condition. To develop appropriate prevention strategies and treatment options, it is necessary to understand how different functional compartments cooperate across organs (Figure 2). Advances in high-resolution analysis techniques have made this possible. For example, whole-transcriptomic analysis has shown that lipid-associated macrophages found in NAFLD livers are related closely to those in adipose tissue and also to foam cell-forming macrophages in atherosclerosis.<sup>34,35</sup> In addition, the systemic nature of metabolic diseases is illustrated by extensive cytokine-mediated crosstalk between organs.

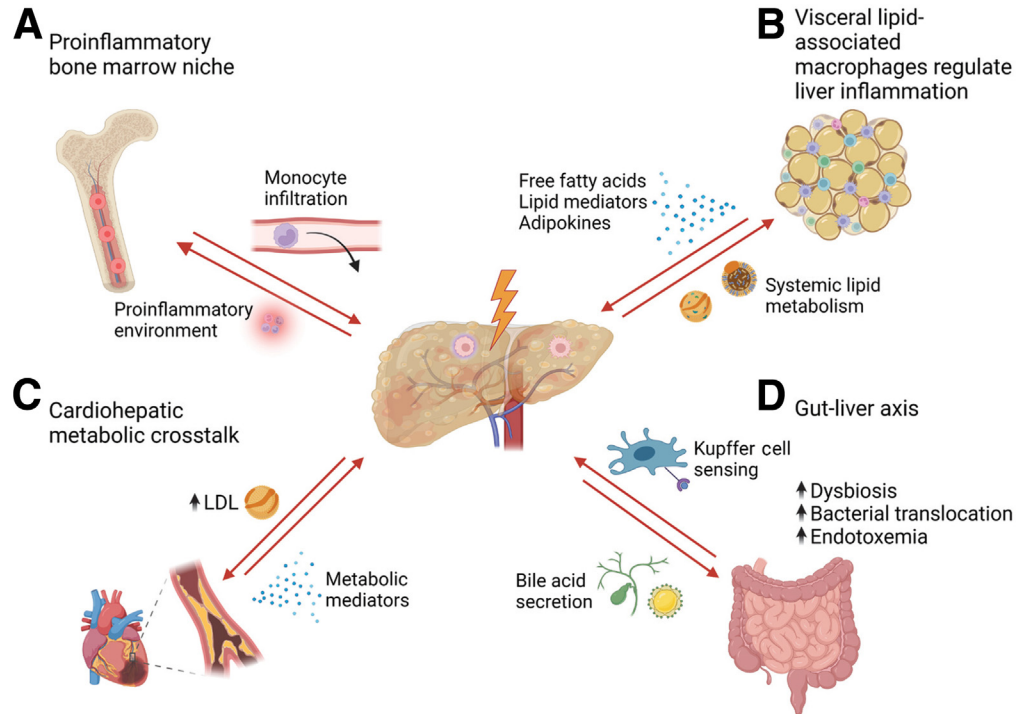
In this context, several lines of evidence indicate bidirectional communication between disturbed organs and the bone marrow niche.<sup>91–94</sup> The bone marrow microenvironment senses stress-related and inflammatory signals deriving from the rest of the body, and promotes expansion and differentiation of hematopoietic stem and progenitor cells, thereby enhancing myelopoiesis and mobilization of immune cells into the bloodstream.<sup>91,93,94</sup> This phenomenon also has been described in a mouse model of steatohepatitis, in which scRNA-seq showed that the myeloid compartment in the liver and myeloid progenitor cells in the bone marrow adopt a common unique inflammatory phenotype.<sup>92</sup>

Another important crosstalk exists between visceral adipose tissue and dysfunctional organs. Transplantation of visceral adipose tissue from obese mice to lean low-density lipoprotein-receptor-deficient mice resulted in increased hepatic neutrophil and macrophage accumulation that exacerbated steatohepatitis upon feeding a high-fat diet compared with transplantation of lean adipose tissue.<sup>95</sup> Interestingly, this phenotype was partially abrogated by depletion of lipid-associated macrophages in adipose tissue before transplantation. This suggests an important role of lipid-associated macrophages in the visceral adipose tissue in regulating systemic metabolism.

NAFLD patients are at high risk of developing cardiovascular disease.<sup>96</sup> Of note, cardiovascular disease is the leading cause of death in patients with NAFLD, even ahead of liver-related complications.<sup>97</sup> The liver–heart axis implies the recognition of metabolites as signaling effectors

### Figure 2. Hepatic inflammation and organ cross-talk.

Stress-induced signals from the liver shape metabolic processes and immune cell phenotypes in remote organs and vice versa. (A) NAFLD is associated with a proinflammatory signature of progenitor cells in the bone marrow. (B) Lipid-associated macrophages in visceral adipose tissue that promote liver inflammation. (C) Cardiovascular disease is related to alteration of hepatic lipid metabolism. (D) Intestinal bacterial translocation and endotoxemia promote liver inflammation in NAFLD. LDL, low-density lipoprotein. (Figure was created with BioRender)



between the heart and liver.<sup>98</sup> In a mouse model of hypertrophic cardiomyopathy, alterations in lipid metabolism also have been described in the liver.<sup>99</sup> Alterations in hepatic lipid metabolism contribute to dyslipidemia and an increase in low-density lipoprotein, which subsequently promotes foam cell formation and atherogenesis. Whether and how liver macrophages also contribute to the cardiohepatic crosstalk is not fully understood.

Regarding organ interactions in liver disease, much attention has been focused on the gut–liver axis, although many studies are correlational.<sup>100</sup> Epidemiologic data on human microbiome profiles suggest a contribution of dysbiosis and impaired intestinal barrier function to the progression of NAFLD and other metabolic diseases.<sup>101–103</sup> Susceptibility to gut leakiness could be the reason for increased endotoxemia owing to translocation of bacterial components and microbially derived metabolites.<sup>104</sup> Recently, increased levels of microbially produced ethanol in the portal venous circulation have been associated with progression of NAFLD.<sup>105</sup> This pathophysiological relationship, in addition to altered lipid metabolism, is reflected in the multiple hit hypothesis.<sup>106</sup> The lamina propria of the intestine harbors large numbers of CX3CR1<sup>+</sup> macrophages. These are involved in maintaining a tolerogenic microenvironment to commensal bacteria and regulate intestinal barrier function by stimulating the proliferation of epithelial progenitor cells.<sup>107</sup> High-resolution intravital microscopy has highlighted their perivascular location, where they form tight junctions and provide an excellent second barrier underneath the epithelium.<sup>108</sup> Lamina propria macrophages scavenged fluorescently labeled bacteria administered into

the intestinal lumen. Any disruption of this macrophage barrier, including in a dysbiosis mouse model, resulted in increased translocation of bacteria.<sup>108</sup> As a consequence, macrophages in the liver become exposed to bacteria, endotoxin, and bacterial metabolites, which contribute to an inflammatory macrophage phenotype.<sup>109</sup> In the intestine of patients with liver cirrhosis, the presence of activated CD14<sup>+</sup> Trem1<sup>+</sup> inducible nitric oxide synthase<sup>+</sup> macrophages releasing IL6 and nitric oxide has been described, which is associated with intestinal barrier dysfunction.<sup>110</sup> Moreover, the phagocytic capacity of hepatic macrophages is impaired in patients with cirrhosis, contributing to the clinical observation of an increased susceptibility to infections.<sup>111</sup> Taken together, these findings suggest an important interplay between intestinal macrophages, microbiota, and gut epithelial integrity with pathogenic mechanisms related to disease progression in the liver.

### Opportunity and Hurdles in Targeting Macrophages for Therapy

In consideration of the increasing burden of the metabolic epidemic on health care systems, new therapeutic approaches urgently are needed. Current pharmacologic treatment for patients with NASH and fibrosis, who are at high risk for disease progression,<sup>112,113</sup> is limited because no drugs have been approved by the US Food and Drug Administration or the European Medicines Agency. The crucial involvement of macrophages in all stages of disease, as well as their role in inflammation and metabolic regulation, makes them an attractive and amenable therapeutic

target. In the past decade, several promising approaches have found their way into preclinical and clinical evaluation.<sup>114,115</sup> Although some clinical trials still are ongoing, some late-stage clinical trials, unfortunately, have failed (Table 1).

The most prominent example is probably the dual CCR2/CCR5 antagonist cenicriviroc. Inhibition of monocyte recruitment to the liver showed potent anti-inflammatory and antifibrotic effects in preclinical animal models.<sup>53,116</sup> The phase 2b clinical trial involving 289 patients showed no improvement of NASH activity, but a significant improvement in fibrosis after 1 year of therapy.<sup>117</sup> However, this antifibrotic efficacy was not sustained over 2 years of treatment with cenicriviroc compared with placebo.<sup>118</sup> The subsequent phase 3 clinical study was terminated owing to a lack of efficacy based on a preplanned interim analysis (NCT03028740). Belapectin (GR-MD-02), an inhibitor of galectin-3, also expressed on macrophages, showed no significant effect on fibrosis, NASH activity, or liver-related outcomes in a randomized phase 2b study (NCT02462967).<sup>119</sup> Selonsertib, the selective inhibitor of apoptosis signal-regulating kinase 1, which promotes secretion of proinflammatory cytokines in macrophages,<sup>120</sup> did not reach the primary end point of fibrosis improvement in the phase III STELLAR trials (NCT03053050 and NCT03053063).<sup>121</sup>

More encouraging data exist for the farnesoid X-receptor agonist obeticholic acid, which currently is being evaluated in an ongoing phase III trial. This synthetic bile acid analogue significantly regulates hepatic lipid and glucose metabolism. Studies in mice suggest that it furthermore may exert anti-inflammatory effects by inhibiting nuclear factor- $\kappa$ B signaling and promoting phenotype switching to restorative Ly6C<sup>lo</sup> macrophages.<sup>122,123</sup> The phase II clinical trial using obeticholic acid in NASH patients showed convincing improvement in histologic features (NCT01265498).<sup>124</sup>

Interim results from the ongoing phase III trial showed improvement in histologic fibrosis in 23% of patients treated with 25 mg obeticholic acid daily compared with 12% in the placebo group.<sup>125</sup> Final data on corresponding clinical outcomes are eagerly awaited. Of note, the interim analysis of another phase 3 trial evaluating obeticholic acid in patients with NASH-associated liver cirrhosis (NCT03439254) failed to show a benefit of obeticholic acid in this patient population.

Increased attention has been paid to the beneficial effects of glucagon-like peptide-1 (GLP-1)receptor agonists on the progression of NASH.<sup>126</sup> These drugs have found widespread use for the treatment of obesity as well as type 2 diabetes owing to their blood glucose-lowering effect by inducing insulin release in pancreatic beta cells and their strong appetite-suppressive effects. The GLP-1/GLP-1-receptor signaling cascade has been reported to foster M2-like polarization of macrophages.<sup>127</sup> However, the main actions of GLP-1 or GLP-1/glucose-dependent insulinotropic polypeptide (GIP)-receptor agonists on macrophages are likely to be indirect and rather owing to favoring a metabolically beneficial environment upon weight reduction. Phase II clinical trials also have shown significant improvement in NAFLD resolution for liraglutide and semaglutide (NCT02970942 and NCT01237119).<sup>128,129</sup> In particular, liraglutide showed encouraging superiority over placebo. Thirty-nine percent of treated patients met the primary end point of NASH resolution, compared with 9% in the control group.<sup>128</sup> Phase III clinical studies currently are underway (NCT04822181 and NCT02654665).

Another antidiabetic drug that has shown a beneficial effect on the resolution of NAFLD is the PPAR- $\gamma$ -receptor agonist pioglitazone.<sup>130,131</sup> PPAR- $\gamma$  is a transcription factor that activates expression of M2-like anti-inflammatory genes in macrophages and its activation has been shown to restore a high-fat diet-induced imbalance of M1/M2 polarization of Kupffer cells.<sup>132,133</sup> The use of pioglitazone

**Table 1.** Current Status of Drug Development Affecting or Targeting Macrophages in Nonalcoholic Steatohepatitis (Selected Compounds and Trials)

Drug	Mechanism	Clinical evaluation
<b>Cenicriviroc</b>	CCR2/CCR5 inhibitor	Failed phase 3 (NCT03028740)
<b>Belapectin (GR-MD-02)</b>	Galectin-3 inhibitor	Failed phase 2b (NCT02462967)
<b>Selonsertib</b>	ASK1 inhibitor	Failed phase 3 (NCT03053050/NCT03053063)
<b>Obeticholic acid</b>	Farnesoid X-receptor agonist	Successful in phase 2 (NCT01265498) Failed phase 3 in NASH cirrhosis (NCT03439254) Phase 3 in NASH fibrosis ongoing (NCT02548351)
<b>Liraglutide/semaglutide</b>	GLP-1-receptor agonist	Successful in phase 2 (NCT02970942/NCT01237119) Phase 3 ongoing (NCT04822181/NCT02654665)
<b>Pioglitazone</b>	PPAR- $\gamma$ agonist	Evaluated for patients with concurrent type 2 diabetes
<b>Elafibranor</b>	PPAR- $\alpha/\delta$ agonist	Failed phase 3 (NCT02704403)
<b>Lanifibranor</b>	Pan PPAR- $\alpha/\gamma/\delta$ agonist	Successful in phase 2 (NCT03008070) Phase 3 ongoing (NCT04849728)
<b>Resmetirom</b>	THR- $\beta$ agonist	Successful in phase 2 (NCT02912260) Phase 3 ongoing (NCT03900429)

ASK1, apoptosis signal-regulating kinase 1; GLP-1, glucagon-like peptide-1; GR-MD-02, belapectin; THR, thyroid hormone receptor.



in subjects with NAFLD is advocated in the guidelines published by the National Institute for Health and Care Excellence and the American Association for the Study of liver diseases under certain circumstances.<sup>112</sup> The European Association for the Study of the Liver guideline is more cautious and recommends that pioglitazone may be considered as a treatment for diabetes with concurrent NAFLD.<sup>134</sup> Increasing evidence of a higher risk of bladder cancer in patients treated with pioglitazone also may prompt cautious use.<sup>135</sup> As an alternative drug, the PPAR- $\alpha/\delta$  agonist elafibranor showed good results in pre-clinical studies but did not meet the predefined efficacy end point in phase III clinical studies (NCT02704403). In contrast, lanifibranor is a pan-PPAR agonist acting on all 3 PPAR isoforms.<sup>136</sup> In preclinical NASH models, lanifibranor potently improved NASH and fibrosis because it acts on hepatocytes (mainly PPAR- $\alpha$  in mice), macrophages (mainly PPAR- $\delta$ ), and hepatic stellate cells (mainly PPAR- $\gamma$  in mice) simultaneously.<sup>137</sup> A phase 2b clinical trial in patients with NASH and fibrosis showed beneficial treatment effects after only 24 weeks of therapy,<sup>138</sup> mandating the further investigation in a large phase III trial.

Encouraging results also have been reported in clinical trials evaluating the thyroid hormone receptor- $\beta$ -agonist resmetirom in patients with NASH.<sup>139</sup> Although its protective effects are mediated primarily through regulation of hepatic lipid metabolism, thyroid receptors also have been reported to promote anti-inflammatory polarization of macrophages.<sup>140</sup> Preliminary data from the ongoing phase 3 study suggest a significant reduction of liver steatosis after 52 weeks of treatment. Additional readouts and histologic results from the phase 3 trial are awaited (NCT03900429).

There are a number of molecular targets in macrophages whose manipulation in preclinical studies have suggested pharmacologic benefit, such as macrophage scavenger receptor 1,<sup>18</sup> Toll-like receptor 4,<sup>141</sup> TREM2,<sup>37</sup> and the transcription factor X-box binding protein 1 (XBP1).<sup>142</sup> It will be interesting to see which approaches make it into clinical evaluation.

In summary, the development of pharmacologic therapeutic options for NAFLD has seen both encouraging and disappointing results. Of note, translation of animal models to human beings often fails, increasing the need for better models and alternative approaches. From a macrophage perspective, not only identifying important cellular (subsets, organ-specific phagocytes) and molecular (eg, pathways of inflammatory or fibrogenic activation) targets is relevant, but also developing new, safe, and specific drug delivery systems such as hard-shell microbubbles, liposomes, and polymers, in which the phagocytic activity of macrophages could be harnessed for targeted drug administration.<sup>114,143</sup> Moreover, macrophage-based adoptive cell transfer therapies, which currently are being evaluated in early clinical phase and tested in different preclinical models, hold great promise.<sup>144</sup> A combination of reductionist basic science and translational approaches will be required to elucidate mechanisms behind dysregulated macrophages and identify novel potential therapeutic interventions. Until then, the mainstay of therapy will be lifestyle intervention, including adjusted caloric intake and physical activity.<sup>145</sup> In fact, lifestyle modification has been shown not

only to reduce intrahepatic lipid content but even to influence monocyte and macrophage activation positively.<sup>146,147</sup>

## Soluble CD163, Soluble TREM2, and Sialic Acid-Binding Immunoglobulin-Like Lectin-7 Are Potential Macrophage-Related Biomarkers

Pharmacologic treatment is desirable in patients with NASH and fibrosis and in patients at high risk for progressive disease.<sup>113</sup> However, it is difficult to predict the risk of transition from simple steatosis to more progressive forms including fibrosis and steatohepatitis. Liver biopsy is the gold standard for the diagnosis of present liver fibrosis, but because of its invasiveness is not suitable as a regular follow-up examination. Biomarkers are needed for early detection and disease monitoring in NAFLD. Because of the stage-specific contributions of macrophages to NAFLD progression and their increase during progression, macrophage-related molecules have been suggested as potential circulating biomarkers.<sup>115</sup> One potential marker is the hemoglobin-haptoglobin scavenger receptor CD163, which is located on the surface of Kupffer cells and released into blood circulation after activation.<sup>148</sup> The soluble form of CD163 has shown promise as a biomarker in various liver diseases, including hepatocellular carcinoma.<sup>148</sup> It correlates with the degree of steatosis, but does not predict the severity of fibrosis.<sup>149</sup> Although soluble CD163 was not associated with hepatocellular carcinoma size and number, it appears to be useful as a surveillance biomarker because it was associated independently with lower overall survival and cancer progression.<sup>148</sup> Another potential biomarker is sialic acid-binding immunoglobulin-like lectin-7, which is expressed by Kupffer cells. Recently, sialic acid-binding immunoglobulin-like lectin-7 was shown to be an independent diagnostic marker with high specificity for advanced fibrosis in patients with NAFLD.<sup>150</sup> Based on the recurrent observation that TREM2+ macrophages accumulate in the fibrotic septa of patients with NASH and cirrhosis,<sup>12</sup> systemic levels of soluble TREM2 have been suggested as a potential biomarker in NAFLD. Indeed, plasma soluble TREM2 levels mirror NASH severity (no steatosis – steatosis – NASH – fibrosis) in human patients, suggesting that this macrophage-related receptor is a valuable biomarker in NAFLD patients.<sup>38</sup> The use of macrophage-derived biomarkers for screening, identification, and disease monitoring in NAFLD needs further (prospective) investigation in broader patient populations.

## Future Perspective and Challenges

Different manifestations and numerous parallel pathophysiological metabolic and inflammatory processes make NAFLD an extremely complex clinical condition. The heterogeneously regulated macrophage populations reflect this complexity. Multiple macrophage populations co-exist at different stages in metabolically injured livers, promoting or inhibiting disease progression, and their relationship to each

other as well as their crosstalk to further cells in the liver environment still are insufficiently understood. The tremendous advance in biomedical research techniques allows us now to reclassify macrophage subsets based on the transcriptomic and proteomic profile, their spatial organization, their dynamic behavior, as well as based on their functional capacity with respect to disease stage.<sup>5</sup> Advances in bioinformatics and data science will allow integration of knowledge on extrahepatic signals (gut, adipose tissue) as well as on patient-specific features (nutrition, genetics, comorbidities), giving rise to the expectation that tailored targeting to modify macrophages as a central immune regulator during NAFLD progression may be a realistic goal.

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#### Author contributions

Joscha Vonderlin conceived, designed, and wrote the manuscript; Triantafyllos Chavakis and Michael Sieweke provided intellectual input and edited the manuscript; and Frank Tacke contributed to the conception, provided intellectual input, and edited the manuscript.

#### Conflicts of interest

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