Review

The Multifaced Roles of Hepatic Macrophages in MASLD

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Abstract: Metabolic dysfunction-associated steatotic liver disease (MASLD) is becoming the leading cause of chronic liver disease-related morbidity and mortality globally. It encompasses a spectrum from metabolic dysfunction-associated steatotic liver (MASL) to metabolic dysfunction-associated steatohepatitis (MASH), potentially advancing to cirrhosis and hepatocellular carcinoma. While the pathogenesis of MASLD is complicated and not yet completely elucidated, hepatic macrophages, including liver resident Kupffer cells (Res-KCs) and recruited circulating monocyte-derived macrophages (MoDMs), play a pivotal role in its initiation and progression. Recent advancements in single-cell RNA sequencing have unveiled significant heterogeneity among macrophages and their diverse contributions to MASLD progression. This review delineates the origin and surface markers of hepatic macrophages, emphasizing their multifaceted roles in the pathogenesis of MASLD in steatosis, inflammation and fibrosis. Furthermore, we delve into the latest advancements in pharmacological treatment strategies for patients with MASLD.

Keywords: metabolic dysfunction-associated steatotic liver disease; macrophage; resident Kupffer cell; monocyte-derived macrophage; therapy

1. Introduction

MASLD is considered as a multi-system metabolic dysregulation, closely associated with other metabolic diseases. Meta-analysis has shown that nearly 70% of patients with MASLD are overweight or obese [1], and more than 20% are diagnosed with type 2 diabetes mellitus (T2DM) [2]. With the prevailing epidemic of obesity and metabolic syndrome, MASLD has become the most prevalent chronic liver disease globally, affecting over 30% of the population [3,4]. MASLD has two histological categories: metabolic dysfunction-associated steatotic liver (MASL) and its inflammatory and severe form metabolic dysfunction-associated steatohepatitis (MASH), which poses a higher risk of progressing to end-stage liver diseases, including cirrhosis, liver failure and hepatocellular carcinoma. Although MASLD represents a substantial global clinical and economic burden, only one drug (Resmetirom, thyroid hormone receptor-β agonist) has been recently approved by the US Food and Drug Administration (FDA) in conjunction with diet and exercise for treating patients of MASH with moderate to advanced liver fibrosis. Current management options primarily refer to lifestyle interventions with some drugs approved for improving metabolic comorbidities such as obesity and T2DM [5].

The precise pathogenesis of MASLD remains incompletely understood, although insulin resistance, endoplasmic reticulum oxidative stress, and mitochondrial damage are recognized as significant contributing factors. Recent studies have indicated the critical roles of hepatic macrophages in MASLD development [6]. Proinflammatory cytokines, lipotoxicity and dysbiosis trigger the activation of resident Kupffer cells (Res-KCs), which further recruit peripheral monocytes into the liver, exacerbating hepatic inflammation and promoting the activation of hepatic stellate cells (HSCs), ultimately leading to fibrosis. Given the crucial roles of hepatic macrophages in MASLD, this review summarizes their origins, heterogeneity, diverse roles in MASLD development and the novel treatment strategies. Notably, the emerging new terminologies, including MASLD, MASL and MASH, were recently proposed by three authoritative pan-national liver associations to replace 'non-alcoholic fatty liver disease' (NAFLD), 'non-alcoholic fatty liver' (NAFL) and 'non-alcoholic steatohepatitis' (NASH), respectively [7,8]. This nomenclature modification aims to reduce stigma, enhance disease awareness, and involve a broader range of patients. A retrospective study found that about 99% of individuals meeting the

NAFLD criteria also met MASLD criteria [9,10], providing a solid foundation for referencing previous research findings on NAFLD to the realm of MASLD.

2. Macrophages in the Liver Homeostasis

The liver, an essential metabolic and immunological organ, comprises various cell types. Hepatic cells are divided into parenchymal cells, i.e., hepatocytes, and non-parenchymal cells, which include macrophages, HSCs, cholangiocytes, liver sinusoidal endothelial cells (LSECs), T cells, B cells and granulocytes [11,12]. The liver harbors the most abundant population of macrophages among all solid organs of the body, highlighting that liver macrophages perform critical roles in maintaining homeostasis within the liver and throughout the whole body [13]. Specifically, macrophages not only exert the function of immunological tolerance and classical phagocytoses such as removing damaged erythrocytes and aged platelets, but also contribute to regulating lipid metabolism to adapt to dynamic, systematic lipid levels [14–16].

2.1. The Origins of Hepatic Macrophages

Researchers have progressively unravelled the developmental ontology of macrophages by utilizing various techniques like fate-mapping experiments and parabiosis experiments, revealing the existence of two distinct lineages of hematopoietic cells in the liver [17]. These two subsets include tissue-resident macrophages, known as Res-KCs in the liver, and monocyte-derived macrophages (MoDMs) from bone marrow [18,19].

Res-KCs are predominant in maintaining liver homeostasis, constituting approximately 80% and 90% of the total hepatic macrophages pool in humans and mice, respectively [20,21]. Erythro-myeloid progenitors (EMPs) are generated in the yolk sac (YS) at around embryonic day 8.5 (E8.5) of mice, representing the first wave of definitive hematopoiesis. EMPs develop into YS macrophages and c-*Myb*$^{+}$ EMPs at E9.5. The former develop independently of transcription factor myb, and are released into postnatal tissues as resident macrophages [22]. The latter migrate to the fetal liver and differentiate into Colony-Stimulating Factor-1 Receptor^{+} (CSF1R, also known as CD115) progenitors, which at E12.5 give rise to fetal monocytes at E12.5 that enter into various tissues including the liver [23,24]. After monocytes migrating into the liver, the transcription factor inhibitor of DNA binding 3 (ID3) is responsible for guiding their differentiation process towards the specialized hepatic KCs [25]. Therefore, the embryo-derived macrophages, commonly called Res-KCs in the liver, are generated through these two approaches, resulting in the presence of abundant resident macrophages with embryonic origin in the liver before birth. On the other hand, hematopoietic stem cells are derived from hemogenic endothelium of large arteries at around E10.5, known as the second wave of the definitive hematopoiesis [26]. Subsequently, these cells migrate to the fetal liver where they expand rapidly before transferring to bone marrow to further generate circulating monocytes at around E17.5 [27]. These monocytes are usually considered as immature precursors of tissue macrophage [28]. Under steady conditions, Res-KCs are sustained through self-renewal without reliance on circulating monocytes [29–31]. However, in the presence of inflammation or injury in the liver, circulating monocytes can be rapidly recruited into the liver. Following a sequential interaction with HSCs, LSECs, and hepatocytes in the space of Disse, those monocytes imprint their identity and gradually mature into tissue-like macrophages, known as MoDMs [32].

The ontogeny of macrophages in humans remains inadequately explored due to a shortage of samples. However, insights from human multi-omics data indicate that the human hepatic macrophages pool also has mixed embryonic and monocyte-derived cells [33].

2.2. Surface Markers to Distinguish Hepatic Macrophage Subpopulations

The cellular origin of hepatic macrophages has been proposed to have a great impact on their gene expression profiles [34]. Accordingly, Res-KCs and MoDMs can be distinguished from each other based on their diverse surface markers in both mice and humans.

In mice, Res-KCs express the pan-macrophage markers F4/80$^{\text{high (hi)}}$ [35] and CD11b$^{\text{intermediate (int)/low (lo)}}$, as well as their specific markers such as C-type lectin domain family 4 member F (Clec4F) [36], V-set immunoglobulin-domain-containing 4 (Vsig4, also known as CRIg) [37,38], and T cell immunoglobulin and mucin domain containing 4 (Timd4) [20,39], while MoDMs can be identified by F4/80$^{\text{int/lo}}$, CD11b$^{\text{hi}}$, as well as the monocytes progenitor markers like lymphocyte antigen 6 complex locus C (Ly6C) [40], CC chemokine receptor 2 (CCR2), and CX_3C chemokine receptor 1 (CX_3CR1) [41]. Commonly, by the use of flow cytometry technology, Res-KCs and MoDMs are separately described as F4/80$^{\text{hi}}$CD11b$^{\text{int/lo}}$Clec4F^{+}Vsig4^{+}Timd4^{+} and F4/80$^{\text{int/lo}}$CD11b$^{\text{hi}}$Ly6C^{+}CCR2^{+}CX_3CR1^{+}subsets, respectively. Using single-cell RNA sequencing (scRNA-seq) technique on mice in a steady state, Res-KCs can be further divided into two KC populations exhibiting distinct

gene and protein expression signatures, a major endothelial cell-selective adhesion molecule ESAM^{-}CD206^{lo} subpopulation (named KC1, up to 85%) and a minor ESAM^{+}CD206^{hi} subpopulation (named KC2) [42]. These two subpopulations share the common core markers of Res-KCs, while KC2 additionally expresses a set of markers including CD206, CD31 and ESAM, which previously thought to be restricted to LSECs. In addition, Krenkel O et al. observed three subclusters of MoDMs, i.e., MoDM I-III, in different metabolic states of mice, with distinctive gene expression profiles to characterize their potential roles [43]. MoDM I had a high expression of marker genes associated with oxidative stress, notably the extracellular matrix protein fibronectin 1 (*Fn1*), microsomal glutathione S-transferase 1 (*Mgst1*) and methionine sulfoxide reductase B1 (*Msrb1*). MoDM II displayed fewer marker genes including Chitinase 3-Like 1 (*Chil1*), indicating its origin in the bone marrow. MoDM III showed a high expression of *IL-1β*, representing an inflammatory activated state. Interestingly, the abundance of MoDMs I was relatively unchangeable in mice fed either a western diet (WD) or chow diet (CD), while MoDM II and MoDM III were dramatically increased in mice fed WD as compared with those fed a CD. Moreover, following diphtheria toxin (DT) administration to deplete Res-KCs, MoDMs were found to gradually adopt an F4/80^{hi}CD11b^{int} characteristic comparable to Res-KCs, and the expression of Timd4 in MoDMs was also increased [44]. This phenomenon can be elucidated that under circumstances of the deletion or reduction of Res-KCs, MoDMs differentiate into near-identical Res-KCs populations in order to adapt to their available niche of macrophage [17], implying the necessity for dedicated studies to identify more accurate markers to discern macrophages from varied origins.

Compared with findings from the preclinical mouse model, human hepatic macrophages' subpopulations are relatively less characterized due to the difficulty of sample collection. In a depth-analysis of data from patients with chronic liver diseases, Zimmermann HW et al. revealed that the level of CXCR1 was associated with hepatic macrophage accumulation and the proinflammatory CD16^{+}MoDMs were remarkedly increased [45]. And a recent study demonstrated that hepatic macrophages can be branched into CD68^{+}MARCO^{+} and CD68^{+}MARCO^{-} subsets, transcriptionally similar to Res-KCs and MoDMs identified in mice [21]. Further human studies are urgent to update the comprehension of human hepatic macrophages in future.

2.3. Functions of Hepatic Macrophages

Macrophages have an asymmetrical distribution within the hepatic architecture, playing vital roles in immune protection and systematic metabolism. Increasing evidence suggests that macrophages, as ideal sensors, detect and respond to local environmental signals such as pathogens, metabolites, and unfit cells via a range of receptors such as Vsig4 and CD36 [37,38,46]. In addition, macrophages and other cells within the liver such as HSCs and LESCs, might constitute cell-cell circuits to perform the essential homeostatic liver function [32,47].

By the mean of multiparameter confocal imaging, the zonation of Res-KCs can be observed within the liver sinusoids, with a preference for periportal lobular regions [47], where the portal venous blood is rich in gut-derived microbial products and metabolites. The close spatial proximity with periportal space enables Res-KCs to sense hepatic microenvironment and maintain hepatic immunological and metabolic homeostasis. As an important component of the mononuclear phagocyte system, Res-KCs exhibit strong phagocytic activity and play a key role in removing cellular debris, aged platelets and blood-borne pathogens, thus significantly contributing to maintaining hepatic homeostasis [48,49]. These phagocytic functions rely on the specific presence of distinct fragment crystallizable and scavenger receptors. For instance, Vsig4, highly expressed on Res-KCs, is required for the rapid removal of pathogens by Res-KCs [38]. Recent research has indicated that the MYD88-dependent signalling from LSECs orchestrates macrophage localization in the liver to achieve the optimal host defense for preventing bacterial dissemination and sepsis [32,47]. Moreover, Res-KCs maintain hepatic immune tolerance by diverse mechanisms. Under the normal state, Res-KCs only express low levels of antigen-presenting molecules such as major histocompatibility complex class II (MHC II), B7-1 and B7-2, failing to induce a protective immune response. On the other hand, Res-KCs can mitigate T cells overactivation and reduce the secretion of proinflammatory cytokines such as IL-2, interferon-γ, and TNF-α through the upregulation of programmed death ligand 1 and the release of prostaglandins (PGs) like PGE_2 and 15d-PGJ_2 [15,50–52]. In addition, Res-KCs produce quantities of immunoregulatory cytokines including the anti-inflammatory IL-10, which promotes proinflammatory M1 macrophage apoptosis and hepatocyte senescence [50,53,54].

Recent scRNA-seq data has demonstrated that a subset of CD206^{hi}ESAM^{+}Res-KCs exhibit a pronounced metabolic signature with the high expression of genes involved in carbohydrate and lipid metabolisms, particularly in relation to fatty acid processing through CD36 [42]. In addition, Res-KCs control cholesterol metabolism via cholesterol ester transfer protein (CETP) to facilitate the transfer of cholesteryl esters from high-density lipoproteins (HDL) to very-low-density lipoprotein (VLDL) [16,55]. Notably, Res-KCs also highly express ATP

binding cassette (ABC) A1 and ABCG1 to regulate cholesterol efflux [56]. Besides, Res-KCs maintain body iron homeostasis, through scavenger receptors sensitive to polyinosinic acid and phosphatidylserine to clear the aged or stressed red blood cells as well as their vesicles [57,58], and suppression of hepcidin expression to increase the serum iron level in the context of decreased hepatic iron stores and increased iron utilization [59,60].

MoDMs, as a minority of hepatic macrophages in healthy liver, are mainly localized in the portal triad. Like Res-KCs, MoDMs actively participate in regulating both lipid and iron metabolism [57,61]. However, only 32% MoDMs isolated from mice with MASLD were positive for the uptake of oxidized low-density lipoprotein (oxLDL, rich in cholesterol), but Res-KCs presented a greatly higher uptake rate of up to 98% [62]. Meanwhile, DT-treated $CD207^{DTR}$ mice (deletion of Res-KCs) showed a decrease in triglycerides (TGs) storage, highlighting a notable divergence in lipid processing between Res-KCs and MoDMs [61]. Notably, MoDMs engaged in erythrophagocytosis only during the period of elevated iron recycling demand, and diminished as the demand wanes [57]. Indeed, other key roles of MoDMs in MASLD are mostly represented after being recruited to the liver, triggered mainly through the axis of CCR2/CCL2 in response to liver injury.

Interestingly, a recent investigation revealed a third type of hepatic macrophages within the hepatic capsule during the steady state in mice. With dendritic morphology, these MHC II^+ capsular macrophages (LCMs) express macrophage markers including F4/80, CD64, CX_3CR1 and CSF1R, indicating their origin from bone marrow and replenishable by circulating monocytes [42]. Besides, LCMs expressing low or negative expression of Ly6C and Timd4 enables to distinguish them from MoDMs [63]. Functionally, LCMs can prevent peritoneal pathogens from entering the liver via mediating neutrophil recruitment [63]. Apart from that, other specific identities of LCMs remain to be explored at steady state and diseases.

Nowadays, emerging multi-omics techniques facilitate the identification of distinct markers to delineate hepatic macrophage subgroups and dissect their roles with greater precision. However, given the significant heterogeneity of hepatic macrophages in their origins, phenotypic characteristics and genetic profiles, the following challenge remains. First, macrophage subsets are identified through various genes among studies, resulting in the existence of diverse subsets even with similar functions. Moreover, most findings are obtained from preclinical animal models, predominantly mice, underscoring the critical need to translate these outcomes into large-scale human validation studies.

3. Roles of Hepatic Macrophages in MASLD Development

As shown in Figure 1, Res-KCs are only marginally replaced by MoDMs under steady conditions. Nevertheless, in the context of various liver pathologies such as MASLD/MASH, fibrosis, and cirrhosis, the composition of hepatic macrophages is substantially remodeled. In response to the increased loss of Res-KCs, $Ly6C^{hi}$ circulating monocytes are rapidly recruited into the liver, resulting in the augmented presence of MoDMs in the liver [44,64]. Diverse hepatic macrophage subsets play multifaceted roles in the progression of MASLD.

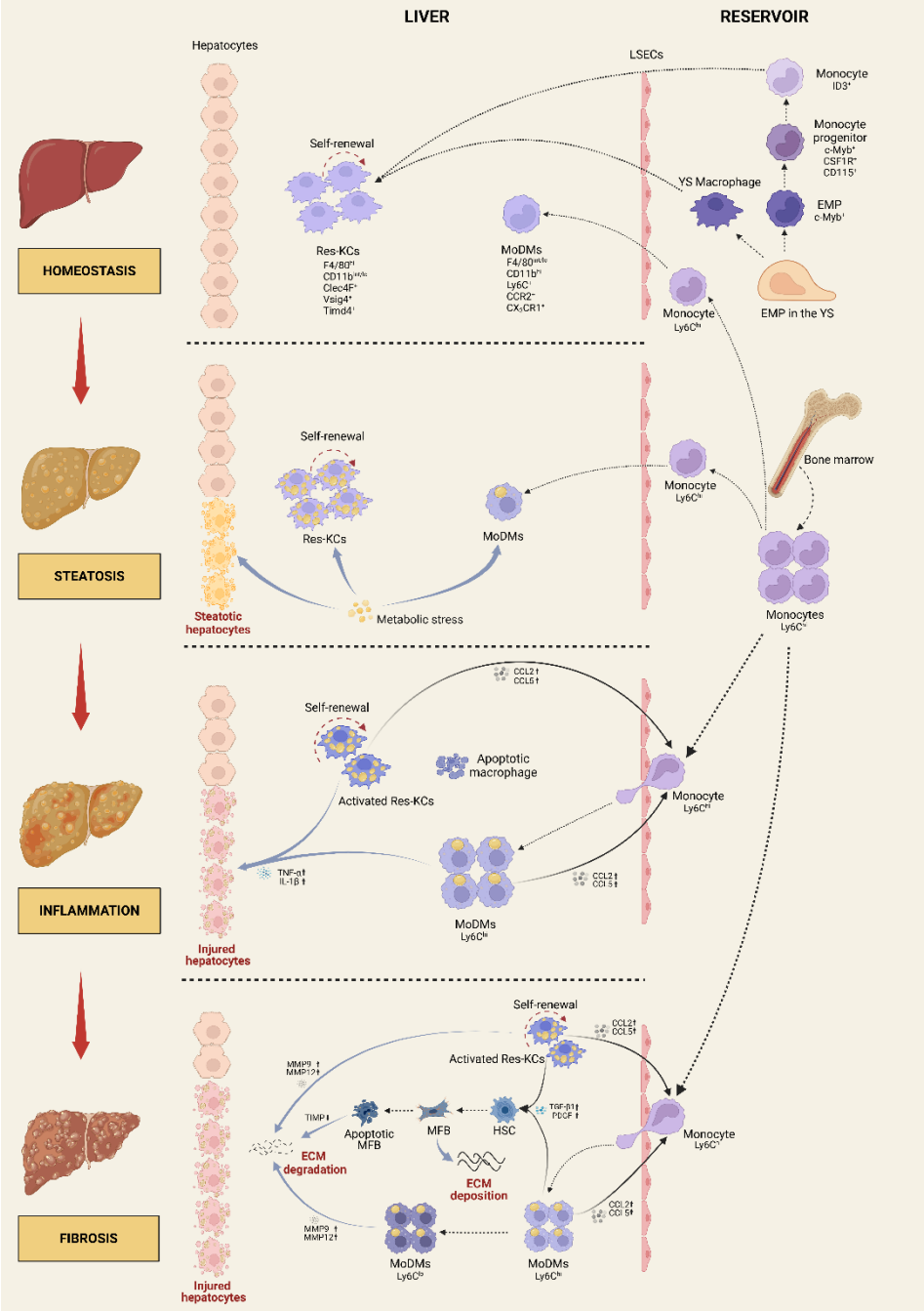

Figure 1. The origin and roles of hepatic macrophages in homeostasis and different stages of MASLD. There are two macrophage subsets including Res-KCs and MoDMs in liver, with different origins. Res-KCs, derived from the embryonic YS, originate from YS macrophages and monocyte progenitors, while MoDMs develop from bone marrow-hematopoietic stem cells. In homeostasis, Res-KCs are the predominant hepatic macrophage population and sustained by self-renewal with the lower contribution of MoDMs to hepatic macrophage pool. In the context of hepatic steatosis, mainly induced by metabolic stress such as lipotoxicity, both Res-KCs and MoDMs engulf the excessive modified lipids, becoming the fat-laden macrophages with impaired lipid process ability. With the progression of MASLD, increased Ly6C^{hi}monocytes are recruited into the liver and differentiate into Ly6C^{hi}MoDMs in response to the impaired self-renewal capacity of Res-KCs in MASH. Notably, the activated Res-KCs and Ly6C^{hi}MoDMs further express proinflammatory cytokines and chemokines such as TNF-α and IL-1β to perpetuate liver damage. In hepatic fibrosis, hepatic macrophages have a different role in fibrosis progression and resolution. First, those activated Res-KCs and Ly6C^{hi}MoDMs express TGF-β and PDGF to promote the survival and activation of HSCs. These activated HSCs further differentiate into MFBs which produce a substantial of ECMs to disrupt hepatic architecture and make fibrotic scar. In addition, profibrotic Ly6C^{hi}MoDMs are capable of differentiation into reparative Ly6C^{lo}MoDMs. Ly6C^{lo}MoDMs and activated Res-KCs upregulate the expression of MMPs, and apoptotic MFBs reduce the production of TIMP to promote ECM degradation and fibrosis resolution. Res-KC, resident Kupffer cell; EMP, Erythro-myeloid progenitor; YS, yolk sac; MoDM, monocyte-derived

macrophage; CCL2, C-C chemokine ligand 2; LSEC, liver sinusoidal endothelial cells; TNF, tumor necrosis factor; IL, interleukin; TGF, transforming growth factor; PDGF, platelet-derived growth factor; HSC, hepatic stellate cell; MFB, myofibroblasts; ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; int, intermediate; lo, low.

3.1. Roles in Steatosis and Lipid Accumulation

As mentioned above, MAFL, the process of bland steatosis, is the early stage of MASLD in most cases, characterized as excessive intrahepatic lipids (mainly TGs) accumulation, which is primarily located in zone 3 (peri-central) of the liver acinus [65,66]. Multiple risk factors, including overnutrition, lack of exercise, gut dysbiosis and genetic factors, can induce steatotic liver. Specifically, these lipids accumulation in the liver is attributable to increased uptake of fatty acids (FAs) originating from adipose tissue lipolysis and daily diet, enhanced de novo FAs synthesis in the liver, and decreased lipid clearance through FAs oxidation [65].

Recent studies have reported that Res-KCs impact steatosis via multiple approaches. First, Res-KCs can take up and store modified lipoproteins primarily via CD36 and Macrophage scavenger receptor 1 (MSR1) pathways [67,68]. Compared to the normal state, lipid storage within macrophages increases twofold during hepatic steatosis, leading to the formation of large lipid droplets in the cytoplasm of Res-KCs, commonly called fat-laden KCs. Those fat-laden KCs show dysregulated lipid trafficking and synthesis accompanied by upregulated expression of lipogenesis-related genes such as diacylglycerol O-acyltransferase 1 (*Dgat1*) and Stearoyl-CoA Desaturase 1 (*Scd1*) [69]. Furthermore, Res-KCs polarize towards proinflammatory M1 phenotype in steatosis and the polarized state leads to hepatocyte TG accumulation through increased FAs esterification and decreased FAs oxidation [70]. When subjected to gadolinium chloride ($GdCl_3$) to deplete Res-KCs, rats exhibited improved histological manifestations associated with hepatic steatosis and insulin resistance upon feeding a high-fat diet (HFD) [70]. It is noteworthy that hepatic macrophages serve as the primary source of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), both of which contribute to the development of steatosis [71]. Stienstra R et al. demonstrated that Res-KCs in obese mice promoted steatosis by releasing IL-1β to suppress peroxisome proliferator-activated receptor α (PPARα) activity and expression via upregulating nuclear factor(NF)-κB subunit p50 and p65, potentially leading to Res-KCs activation and tissue factor matrix metalloproteinases-9 (MMP-9) production in the liver [71,72]. By contrast, Clementi AH et al. showed Res-KCs ablation in obese mice increased hepatic TG accumulation and decreased insulin sensitivity [73], suggesting that Res-KCs partially protect steatosis and glycemic control in the context of obesity. In addition, recent research has shown that Clec4F$^+$KCs could directly inhibit the hepatocellular lipogenic process by secreting exosomes containing miR-690 to interact with hepatocytes and maintain lipid homeostasis [74]. These contrary effects imply the multifaceted functions of hepatic macrophages in different metabolic conditions.

The roles of MoDMs in steatosis are rather obscure. Findings from the majority of studies indicate that newly infiltrated monocytes were rarely observed during simple steatosis, but significantly increased in the liver of MASH cases [75,76]. Leroux A et al. found no notable difference in recruited macrophages determined by flow cytometry between mice fed CD and those fed HFD without any indication of liver inflammation [69]. Furthermore, Westerbacka J et al. described that the level of CD68, a marker highly expressed by human monocytes and tissue macrophages, showed no discrepancy between MAFL patients and the general population [77]. These studies indicate that in steatosis, the composition of hepatic macrophages is relatively stable, and the number of MoDM is unaltered. However, another clinical study by using liver biopsies revealed that portal infiltrating macrophages were observed in MASLD patients at the stage of steatosis alone before the occurrence of inflammation and fibrosis [78]. The exact role of MoDMs in the development of steatotic liver disease is unknown, and more studies are needed by using liver biopsies or single-cell analysis approach to explore their effects on regulating metabolism or immunity in the step of simple steatosis.

Intriguingly, some researchers support that hepatic steatosis may be a protective process against excessive FFA induced hepatic oxidative stress and liver damage [79], as most of patients with simple steatosis did not develop into severe liver injury and remain stable for decades [80,81]. Although some studies showed that steatosis could decrease greatly and even vanish in advanced fibrosis and cirrhosis [82], the majority of studies have observed that patients with severe steatosis progress to lobular inflammation gradually, leading to activated immune cells and exacerbating hepatic injury [83,84].

3.2. Roles in Hepatic Inflammation

MASH is a severe form of MASLD, characterized by significant steatosis, lobular inflammation and hepatocyte ballooning with various extents of fibrosis [85]. Commonly, liver inflammation is recognized as a

hallmark of MASH, involved in the initiation and progression of steatohepatitis. Once the proinflammatory microenvironment is triggered by long-term lipid-mediated stress, hepatocytes death and other diverse intrahepatic or extrahepatic factors, a large body of $Ly6C^{hi}CCR2^{+}$monocytes that potentially give rise to MoDMs flow into the liver, causing a dramatic shift in the composition of hepatic macrophages. Fate-mapping studies revealed that recruited monocytes could account for up to 60% of the total number of macrophages in MASH mice and exceed 75% in patients with MASH [44,61,76].

Res-KCs are considered to be the early responders in the initiation of steatohepatitis. A significant finding is that the self-renewal capacity of Res-KCs is impaired in MASH, as evidenced by the reduction of $Timd4^{+}$-mature Res-KCs in mice fed a HFD for 16 weeks [61,86]. This can be partially attributed to an altered local microenvironment, where Res-KCs could engulf greater lipids, leading to imbalanced reactive oxygen species (ROS) and abundant cell damage in response to the AMLN diet (containing high fat, high fructose, and high cholesterol), a commonly used diet to induce MASH in rodents [20]. In fact, it is proposed that the loss of Res-KCs is a crucial signal for MoDM influx [61]. Furthermore, Res-KCs sense the disturbances of the hepatic local microenvironment and release of proinflammatory cytokines like IL-6, TNF-α and IL-1β, along with chemokines such as CCL2, CCL3 and CCL5 [87]. These signals stimulate lipid accumulation in hepatocytes, peripheral monocyte influx and HSCs fibrogenesis, collectively contributing to the MASH progression [74,88,89]. Growing evidence highlights the important role of the CCR2/CCL2 axis in recruiting circulating monocytes during MASH [40], related to the upregulated expression of CCL2 and CCR2 predominantly in activated Res-KCs and $Ly6C^{hi}$ inflammatory monocytes, respectively [90,91]. Genetic deficiency and pharmacological inhibition of CCR2 in mice have diminished recruited monocytes in the liver, along with decreased hepatic steatosis, inflammation and fibrosis [92,93]. The interaction between the availability of empty macrophage niche and CCL2 signaling enables peripheral monocytes to migrate into the liver, aiming to maintain the normal number of hepatic macrophages in MASH [29,61,94].

As compared with Res-KCs, MoDMs display an increased proinflammatory phenotype and produce more TNF-α during the progression of MASH, despite their limited lipid storage and phagocytosis capabilities [29,61,95]. Recent studies have indicated that MoDMs acquire the capacity to transition into monocyte-derived KCs (MoKCs) and ultimately resemble Res-KCs in mice following DT administration to delete Res-KCs [20,32,44]. Consistently, many genes viewed as Res-KCs' markers, such as *Clec4f* and *Timd4*, which are commonly downregulated in Res-KCs, are gradually expressed by MoDMs during MASH progression [61]. These findings raise the question of whether MoKCs can perform bona fide functions of Res-KCs and how to promote macrophage phenotype shift. Strikingly, a key study showed that combining endogenous LXR ligands, notch ligand delta-like protein 4 (DLL4), and transforming growth factor-β (TGF-β) ligands facilitate the differentiation of monocytes towards Res-KC phenotypes [96]. Another important point to note is that the timing and magnitude of monocytes infiltrating into liver vary significantly among different MASH models. For example, mice fed a HFCD display abundant monocyte infiltration to establish MASH model for at least 16 weeks, while mice fed a methionine- and choline-deficient (MCD) diet for 4 weeks show severe hepatic inflammation [97,98]. Thus, it is important to develop preclinical models that closely mimic the progression of MASLD in humans, enabling a detailed investigation into the timing of Res-KCs apoptosis and $Ly6C^{hi}$ monocytes influx to the liver to accurately describe the functions of macrophages in MASH transition.

3.3. Roles in Hepatic Fibrogenesis and Liver Injury

Fibrosis is a significant predictor of liver-related morbidity and mortality, especially in individuals at advanced stage (F3–F4) of fibrosis or cirrhosis [99]. The formation of fibrotic liver can be concluded as excessive extracellular matrix (ECM) production and deposition in the space of Disse, which are mainly attributed to the overactivated HSCs transdifferentiating into myofibroblasts (MFBs) with high expression of α-smooth muscle actin(α-SMA) [100]. MFBs produce a substantial ECM with an altered composition, mainly consisting of crosslinked collagens type I and III, which are more difficult to degrade [101,102]. The imbalance between production and degradation of ECM result in their constant accumulation in the liver, significantly disrupting hepatic architecture and exacerbating liver damage. Additionally, a recent study showed that portal fibroblasts, hepatic mesothelial cells, and BM-derived fibrocytes were also the originators of MFBs [103,104]. Among various modulated signals associated with fibrosis, macrophages play distinct roles in liver fibrosis progression and resolution. On one hand, deletion of infiltrating MoDMs at the onset of fibrosis results in reduced HSC activation and restricted extent of fibrosis [105]. In contrast, loss of MoDMs during fibrotic resolution worsened hepatic fibrosis and led to the failure of matrix degradation [105].

During the fibrosis progression, activated Res-KCs release the chemokine CCL2 to attract circulating profibrogenic $CCR2^+$monocytes in the early stage of liver injury. In *Ccr2*$^{-/-}$ transgenic mice [40,106] or those mice being treated with pharmacological CCR2/CCR5 antagonist cenicriviroc (CVC) [93], diminished $CCR2^+$ monocyte infiltration in the liver led to fewer MFBs, thus reducing hepatic fibrosis. In addition, multiple cell types, including infiltrating monocytes, damaged hepatocytes, and activated Res-KCs, can secrete abundant cytokines to activate HSCs. To date, TGF-β1 (the most abundant isoform of TGF-β in the liver), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) are acknowledged as key profibrotic factors [107]. In an experiment with rats exhibiting persistent liver fibrosis induced by dimethylnitrosamine, blocking TGF-β1 signaling showed promise in ameliorating liver fibrosis [108]. Furthermore, activated HSCs themselves also produce TGF-β, leading to ongoing fibrogenesis [109]. Another non-negligible fact is that a series of proinflammatory cytokines such as TNF-α, IL-6, IL-1β and IL-17, mainly generated by hepatic macrophages including both Res-KCs and MoDMs, are involved in amplifying and exacerbating liver fibrosis [110,111]. Interestingly, TNF and IL-1 do not directly promote HSCs activation but prolong the survival of HSCs by stimulating NF-κB activity [112]. Recent scRNA-seq data has identified a subset of $CD9^+TREM2^+$scar-associated macrophages (SAMs) in fibrotic niches, with the expression of secreted phosphoprotein-1 (SPP1), fatty acid binding protein5 (FABP5), TNF and CD63, presenting a pro-fibrogenic activity [21,113,114]. SAMs, originating from circulating monocytes, increase with MASLD activity and positively correlate with fibrosis severity. While a study showed that $CD9^+TREM2^+$SAMs could promote the expression of fibrillar collagen and proliferation of HSCs [115], another study suggested that the $TREM2^+$ recruited macrophages prevented aggravated steatohepatitis and fibrosis [94], indicating their complex roles both in pro-fibrogenesis and anti-fibrogenesis during fibrosis development. Of note, hepatic macrophages mediate fibrosis resolution in multiple ways. During the regression of liver fibrosis, the increased proportion of restorative MoDM with $Ly6C^{lo}$ phenotype and Res-KCs enhances the expression of MMP-9 and MMP-12, growth factors (insulin-like growth factor 1 and hepatocyte growth factor) and phagocytosis-related genes (Glycoprotein non-metastatic melanoma protein b and *Marco*) [116,117]. Specifically, $Ly6C^{lo}$ MoDMs mainly upregulate the expression of MMP-9 to degrade type IV collagens, while Res-KCs mainly express MMP-12 to degrade soluble and insoluble elastin [117–119]. The apoptosis of activated HSCs during liver fibrosis regression leads to a reduction of tissue inhibitor of metalloproteinase (TIMPs), consequently facilitating ECM degradation [120]. Additionally, it has been demonstrated that the activated macrophages enable to secrete milk fat globule epidermal growth factor 8 (Mfge8) to bind and target collagen for internal uptake and degradation [121]. Given the fact that the balance between profibrogenic $Ly6C^{hi}$ MoDMs and reparative $Ly6C^{lo}$ MoDMs is crucial in determining the severity of fibrosis, an in-depth understanding of macrophage phenotypic transition and its underlying mechanisms hold great importance in liver fibrosis investigations. The emerging targeted therapeutic strategies that aim at reprogramming macrophages with restorative $Ly6C^{lo}$ phenotype or inducing activated HSCs apoptosis have the potential to prevent even reverse the progression of fibrosis.

Crucially, hepatic macrophages do not exist in isolation but collaborate with surrounding cells to enable liver function. For instance, in the context of metabolic stress, the disturbed hepatocytes generate ROS to induce the activation of macrophages and secrete proinflammatory extracellular vesicles to recruit peripheral monocytes into liver [122]. In turn, hepatic macrophages can also exacerbate hepatocyte damage through releasing proinflammatory cytokines, i.e., IL-1β [20,71]. During the development of MASH, the fat-laden macrophages recruit diverse lymphocyte subsets, such as $CD8^+$T cells, through secreting cytokines like IL-6, IL-12 and IL-23 [69]. The activated $CD8^+$T cells in obese MASH mice could directly lead to hepatocyte death and monocyte accumulation in the liver to amplify liver inflammation and activate HSCs to worsen fibrogenesis [123]. While existing literature has highlighted the significance of crosstalk between macrophages and other immune cells in MASLD, few studies have elaborated on the contribution of such interplay to the development of metabolic diseases such as MASLD. Further studies investigating the cell-cell interactions are crucial for comprehensively understanding the involvement of hepatic macrophages in the pathogenesis of MASLD.

4. Therapeutics Targeting Hepatic Macrophages in MASLD

Considering the increasing burden of MASLD around the world, there is an urgency to explore new therapeutic approaches to slow or even reverse the progression of MASLD. The central roles of hepatic macrophages in different stages of MASLD, particularly in the progression of inflammation and fibrosis, prompt them to be an attractive target for the treatment of MASLD. Here, as shown in Table 1, we summarize several strategies of targeting or affecting hepatic macrophages to treat MASLD in preclinical studies and clinical trials.

Table 1. Current status of Drug Development Targeting or Affecting Macrophages in MASLD. CCR, C-C chemokine receptor; GLP1R, glucagon-like peptide 1 receptor; ASK, apoptosis signal-regulating kinase; FXR, farnesoid X receptor; PPAR, peroxisome proliferator-activated receptor; DHCR, dehydrocholesterol reductase; MASH, metabolic dysfunction-associated steatohepatitis; NAS, non-alcoholic fatty liver disease activity score; T2DM, type 2 diabetes mellitus; MetS, metabolic syndrome; BMI, body mass index; MASLD, metabolic dysfunction-associated fatty liver disease; HFCD, high fat high cholesterol diet.

Drugs	Mechanism	Phase	Population	Main results	Reference
Cenicriviroc	Dual CCR2/CCR5 antagonist	Phase 2b	289 MASH patients with higher disease activity (NAS ≥ 4), liver fibrosis (F1–F3) and metabolic dysfunction (T2DM or MetS)	Improvement in liver fibrosis (150 mg, once-daily, $p = 0.02$), no notable changes in NAS score	Friedman SL et al., 2018 [124]
		Phase 3	1778 patients with MASH and liver fibrosis (F2–F3)	No efficacy for treating liver fibrosis	Anstee QM et al., 2024 [125]
Belapectin (GR-MD-02)	Galectin-3 inhibitor	Phase 1	31 patients with biopsy-proven MASH and bridging hepatic fibrosis (F3)	Significant reduction in Fibro Test scores in patients with high dose (8 mg/kg, once-weekly, $p = 0.005$)	Harrison SA et al., 2016 [126]
		Phase 2b	162 patients with MASH associated cirrhosis	No significant change in liver histology and the incidence of cirrhosis	Chalasani N et al., 2020 [127]
Anti-CD163-dexamethasone	Delivery corticoid to macrophages	Preclinical	MASH model of rat fed a high-fructose diet	Strong reduction in liver inflammation, hepatocyte ballooning, fibrosis, and glycogen deposition (0.02 mg/kg, three times per week, $p < 0.01$)	Svendsen P et al., 2017 [128]
Liraglutide	GLP-1R agonist	Phase 2	52 patients with MASH and BMI ≥ 25 kg/m^2	Definite histological improvement and resolution of MASH (1.8 mg, once-daily, $p = 0.019$)	Armstrong MJ et al., 2016 [129]
Semaglutide	GLP-1R agonist	Phase 2	320 patients with MASH and liver fibrosis (F1-F3)	Significant resolution of MASH (0.4mg, once-daily, $p < 0.001$), but no improvement in fibrosis stage	Newsome PN et al., 2021 [130]
		Phase 3	1200 patients with non-cirrhotic MASH	Ongoing	
Selonsertib	ASK1 inhibitor	Phase 2	72 patients with MASH and liver fibrosis (F2–F3)	Resolution of liver fibrosis in patients with MASH and moderate to severe fibrosis (6mg, once-daily, $p < 0.05$)	Loomba R et al., 2018 [131]
		Phase 3	808 patients with MASH and fibrosis (F3); 883 MASH patients with compensated fibrosis (F4)	Pre-terminated due to lack of efficacy in fibrosis regression	Harrison SA et al., 2020 [132]
Obeticholic acid	FXR agonist	Phase 2	283 MASLD patients with higher disease activity (NAS ≥ 4) and non-cirrhotic	Improvement in histological features of MASH (25 mg, once-daily, $p = 0.0002$)	Neuschwander-Tetri BA et al., 2015 [133]
		Phase 3	1968 adult patients with MASH, NAS ≥ 4 and liver fibrosis (F1–F3)	Significant improvement in fibrosis and NAS score in patients with high dose (25 mg, once-daily, $p = 0.0002$) from interim report	Younossi ZM et al., 2019 [134]
Lanifibranor	Pan-PPAR agonist	Phase 2b	247 patients with MASH and noncirrhotic	Great resolution of MASH (800mg, once-daily, $p = 0.07$;1200mg, once-daily, $p = 0.007$)	Francque SM et al., 2021 [135]

		Phase 3	1000 patients with MASH and liver fibrosis (F2–F3)	Ongoing	
Elafibranor	Dual PPARα/δ agonist	Phase 3	2157 MASH patients with fibrosis (F1–F4)	Failed to achieve MASH resolution	
SH42	DHCR24 inhibitor	Preclinical	MASH model of mice fed a HFCD	Improvement in hepatic steatosis, inflammation and liver collagen content (0.5 mg, three times per week, $p < 0.01$)	Zhou E et al., 2023 [136]

Given that peripheral monocyte recruitment is an important indicator of MASLD progression, inhibiting this process provides a promising therapeutic strategy for MASLD patients. The intervention of chemokines signaling such as CCL2/CCR2, CCL5/CCR5 and CCL1/CCR8, efficiently hurdle the inflammatory immune cell infiltration into the liver. In mice fed with a MCD diet, the dual CCR2/CCR5 antagonist CVC significantly inhibited the infiltration of $Ly6C^{hi}$ monocytes, concurrently ameliorating histological MASH activity and hepatic fibrosis [93]. Other chemokine receptor antagonists such as CCR5 antagonist maraviroc and chemokine ligand antagonist such as CCL2 antagonist mNOX-E36 showed a beneficial effect on hepatic steatosis development in mice [137,138]. In contrast to promising outcomes obtained from rodent studies, chemo-attractive pathway inhibitors for treating MASH in humans have insufficient efficacy. A phase 2b study (NCT02217475) of 289 MASH patients showed that CVC had great effects on ameliorating liver fibrosis but without notable improvement in NAFLD activity score [124]. However, the phase 3 trial (NCT03028740) did not demonstrate the anti-fibrotic effects and other meaningful benefits such as alanine aminotransferase, aspartate aminotransferase and high-sensitivity C reactive protein, for patients with MASH and fibrosis (at the stage of F2–F3) under the same dose of CVC [125]. It is noted that at present the clinical applications of chemokine receptor antagonists are limited to specific indications. For instance, maraviroc is approved by the FDA only for managing human immunodeficiency virus infection [139].

An alternative strategy targeting macrophages is to use an inhibitor of galectin-3 belapectin (GR-MD-02). In patients with MASH, the level of galectin-3 protein increases, with the highest secretion by macrophages. Inhibition of galectin-3 improved histopathological features with a significant reduction in fibrosis in a murine model of MASH [140]. The phase 1 study, which recruited 31 patients with biopsy-proven MASH with advanced fibrosis (NCT01899859) showed that a high dose of belapectin (8mg/Kg) could decrease Fibro Test scores while maintaining good safety and tolerability [126]. However, the phase 2b study including 162 patients with MASH cirrhosis (NCT02462967) did not find any significant improvement in portal hypertension or fibrosis [127].

In addition, hepatic macrophages express varieties of scavenger receptors, suggesting the possibility of a delivery drug tool directing at macrophages with fewer systematic adverse events. Dexamethasone conjugate targeting the CD163 receptor in macrophages strongly reduces liver inflammation, hepatocyte ballooning, fibrosis and glycogen deposition in rats fed a high-fructose diet [128]. However, such targeted drug delivery approach in clinical application to treat MASLD is still scarce and requires more investigations.

At present, glucagon-like peptide-1 receptor agonists (GLP-1RAs) are mainly used for treating T2DM and obesity owing to their ability to enhance insulin secretion and suppress appetite to induce weight loss. Given that MASLD patients accompanied by other metabolic diseases such as T2DM are more likely to progress to the development of end-stage liver diseases [141], great attention has been paid to glucagon-like peptide-1 receptor agonists (GLP-1RAs) as an emerging treatment for MASLD. Preclinical data suggested that GLP-1RA exendin-4 reduced hepatic lipid content and inflammation, accompanied by less monocyte recruitment in a human-like mouse model [142]. Liraglutide decreased inflammatory and fibrogenic markers (Galectin-3, Collagen type I alpha 1, α-SMA) of macrophages and HSCs in MASH mice [143]. In the phase 2 trial, semaglutide (NCT02970942) and liraglutide (NCT01237119) resulted in significant resolution of MASH [129,130]. Also, liraglutide showed superiority in preventing fibrosis worsening. A phase 3 clinical study is underway to evaluate the effects of semaglutide on MASH resolution and fibrosis improvement in patients with noncirrhotic MASH (NCT04822181).

The inflammatory pathway apoptosis signal-regulating kinase 1 (ASK1) plays an important role in activating c-Jun N-terminal kinase (JNK) and p38 signal cascades. In the context of MASH, ASK pathway is activated among macrophages and HSCs, in turn releasing proinflammatory cytokines like IL-1β, and exacerbating hepatic inflammation [144,145]. ASK1 inhibitor selonsertib could reduce monocyte infiltration and improve histopathological features in MCD-induced MASH mice [146]. Although inhibition of ASK1 has shown an encouraging effect in the phase 2 trial including 72 patients with MASH and fibrosis (F2–F3) [131], the subsequent phase 3 trials (NCT03053050 and NCT03053063) failed to reach the primary endpoint of improving fibrosis in patients with MASH and advanced fibrosis (F3–F4) [132].

Increasing strategies focus on stimulating nuclear receptors like farnesoid X receptor (FXR, known as bile acid receptor), PPARs, LXR, to regulate hepatic lipid and glucose metabolism while alleviating inflammation simultaneously. FXR agonist GW4064 reduced the expression of proinflammatory cytokines in macrophages of MASLD mice [147]. Furthermore, INT-767, a dual FXR/Takeda G-protein-coupled receptor 5 (TGR5) agonist, coordinated the phenotype between monocytes and macrophages towards $Ly6C^{lo}$ restorative type with increased production of IL-10 both in vitro and in vivo [148]. The FXR agonist obeticholic acid (OCA) improved liver histology based on the data from phase 2 clinical trials and the completed phase 3 interim results in non-cirrhosis MASH patients (NCT01265498 and NCT02548351) [133,134]. However, the MASH resolution endpoint is not met from the 3-phase interim results, and its final results are eager to be posted. Of note, another phase 3 trial evaluating the efficacy of OCA in patients with MASH-associated cirrhosis (NCT03439254) did not show any improvement in fibrosis.

PPARs (3 isotypes, described as-α, -δ, -γ) are also widely studied therapeutic targets of MASLD for their pleiotropic actions on systematic metabolism and immune cells. Experiments using different murine models of MASH suggested that pan-PPAR agonist lanifibranor ameliorated all histopathological features as a result of a synergistic combination of different effects of single PPAR agonists [149]. Specifically, those beneficial effects are mostly attributed to PPARα activation in hepatocytes, PPARδ activation in macrophages, and PPARγ activation in HSCs [149,150]. Another study showed that PPARγ agonist rosiglitazone reduces proinflammatory $CD11c^{+}$macrophages by inhibiting the NF-κB pathway in mice fed HFD [151]. However, PPARγ agonists are being seriously considered due to concerns on weight gain and osteoporosis, as well as a potential risk of bladder cancer in the T2DM population [152,153]. In a phase 2b study involving 247 patients with MASH (NCT03008070), lanifibranor demonstrated great MASH resolution within a mere 24-week therapy, instilling a sense of assurance for the ongoing phase 3 study (NCT04849728) [135]. However, the phase 3 trial of a selective alternative agonist of PPARα/δ, elafibranor in 2157 MASH patients with fibrosis (NCT02704403) was prematurely terminated due to lack of efficacy in achieving MASH resolution.

In addition, LXRs (2 isotypes, described as-α, -β), crucial regulators of lipid metabolism and immune responses, are becoming attractive targets for metabolic diseases. Currently, most LXRs agonists are limited to be applied for treating MASLD as they concurrently activate sterol regulatory element-binding proteins (SREBPs) to stimulate lipogenesis in hepatocytes, resulting in hepatic steatosis and hypertriglyceridemia [154]. Fortunately, a recent study has found that endogenous ligand desmosterol activates LXR but also can inhibit the expression of SREBPs target genes in macrophages[155]. Additionally, SH42 as the inhibitor of D24-dehydrocholesterol reductase (DHCR24), an important enzyme to convert desmosterol into cholesterol in distal cholesterol biosynthesis, increased the level of desmosterol both in the liver and plasma, improved hepatic steatosis and reduced immune cell infiltration without increasing circulating lipids. These effects disappeared in $LXR\alpha^{-/-}$ mice, suggesting that the therapeutic benefits of DHCR24 inhibitor on hepatic steatosis and inflammation strictly depend on LXRα activation [136]. It is highly anticipated that increasing endogenous desmosterol by DHCR24 inhibition will be used in clinical evaluation to treat MASH patients.

As mentioned above, diverse drugs have shown promising results in MASH animal models, while clinical trials have not yielded satisfactory outcomes in MASH patients. The underwhelming results may be attributed to the limited sample size of clinical trials, uniform disease stages of patients with MASLD, as well as obvious discrepancies among different species. Therefore, it is imperative to broaden the scope of MASLD patient cohorts across different disease stages and develop human liver organoid or humanized mouse models to recapitulate the complexity of hepatic microenvironment. It should be noted that current pharmacological therapies primarily target managing metabolic syndrome-related comorbidities including T2DM, hypertension and dyslipidemia [156]. There is an urgent need to develop personalized strategies for improving hepatic inflammation and fibrosis.

5. Conclusions

MASLD, being the main cause of end-stage liver disease, is increasingly becoming an important worldwide health problem. Preclinical and clinical studies provide convincing evidence that hepatic macrophages play a key role in MASLD occurrence and progression. Classically, hepatic macrophages can divide into Res-KCs deriving from embryos and MoDMs deriving from bone marrow. Recent advances in biomedical techniques such as scRNA-seq do not only facilitate deep insight into the heterogeneity of macrophages in their origins, phenotypes and functions, but also enhance the comprehension of their varied roles in pathogenic mechanisms of MASLD. The precise delineation of macrophage subsets through the specific surface markers is likely to help determine the disease stage of MASLD patients and devise tailored therapeutic strategies for hindering disease progression. Furthermore, a profound understanding of macrophage heterogeneity fosters the development of novel

medications that selectively target the disease-promoting macrophage populations without affecting the homeostatic macrophages or other cell types, thereby paving the way for developing safe and effective therapeutic interventions for MASLD patients. Another promising therapeutic approach is expected to the transformation of immature proinflammatory macrophages into mature anti-inflammatory macrophages by ideal drugs. Nevertheless, the process of dynamic phenotypic shift in recruiting monocytes within the liver and those key determinants in different metabolic and immune microenvironment is unclear. Future studies are needed to focus on how to modify macrophages as a specific anti-inflammatory phenotype during MASLD progression to maximize the promising therapeutic outcomes.

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