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Review

Understanding Macrophage Complexity in Metabolic Dysfunction-Associated Steatotic Liver Disease: Transitioning from the M1/M2 Paradigm to Spatial Dynamics

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Abstract: Metabolic dysfunction-associated steatotic liver disease (MASLD) encompasses metabolic dysfunction-associated fatty liver (MASL) and metabolic dysfunction-associated steatohepatitis (MASH), with MASH posing a risk of progression to cirrhosis and hepatocellular carcinoma (HCC). The global prevalence of MASLD is estimated at approximately a quarter of the population, with significant healthcare costs and implications for liver transplantation. The pathogenesis of MASLD involves intrahepatic liver cells, extrahepatic components, and immunological aspects, particularly the involvement of macrophages. Hepatic macrophages are a crucial cellular component of the liver and play important roles in liver function, contributing significantly to tissue homeostasis and swift responses during pathophysiological conditions. Recent advancements in technology have revealed the remarkable heterogeneity and plasticity of hepatic macrophage populations and their activation states in MASLD, challenging traditional classification methods like the M1/M2 paradigm and highlighting the coexistence of harmful and beneficial macrophage phenotypes that are dynamically regulated during MASLD progression. This complexity underscores the importance of considering macrophage heterogeneity in therapeutic targeting strategies, including their distinct ontogeny and functional phenotypes. This review provides an overview of macrophage involvement in MASLD progression, combining traditional paradigms with recent insights from single-cell analysis and spatial dynamics. It also addresses unresolved questions and challenges in this area.

Keywords: macrophages; diversity; Kupffer cell; monocyte-derived macrophage; spatial dynamics; metabolic dysfunction-associated steatotic liver disease (MASLD)

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1. Introduction

The accumulation of excess fat in the liver exceeding 5% can result in steatotic liver disease (SLD). Among various etiologies of steatosis, individuals with minimal or no alcohol consumption were previously diagnosed with a condition known as nonalcoholic fatty liver disease (NAFLD). NAFLD is now referred to as metabolic dysfunction-associated steatotic liver disease (MASLD), encompassing patients with hepatic steatosis and at least one of five cardiometabolic risk factors [1]. MASLD includes two histological subtypes: metabolic dysfunction-associated fatty liver (MASL), a relatively mild form characterized by fat accumulation or steatosis in the liver, and metabolic dysfunction-associated steatohepatitis (MASH), a progressive form accompanied by steatosis, inflammation, hepatocyte death, and fibrosis. A meta-analysis of PubMed/MEDLINE data spanning from 1989 to 2015, focusing on terms related to the epidemiology and progression of MASLD, revealed that approximately over 25% of the global population is now thought to have MASLD, with estimated global prevalence rates for MASH ranging from 1.5% to 6.45% [2]. A recent study using dynamic Markov modeling to assess the burden of MASLD-related disease predicted that by 2030, the total MASLD population will increase, reaching a prevalence of

28.4% [3]. MASLD has the potential to progress to cirrhosis and ultimately to hepatocellular carcinoma (HCC) [4,5]. MASLD is now identified as the primary etiology contributing to the incidence of HCC in the US, and the prevalent HCC cases associated with MASLD are estimated to rise, with an anticipated increase from 10,820 in 2016 to 24,860 cases in 2030 [3]. Age, gender, ethnicity, and metabolic conditions like diabetes and obesity are recognized as major risk factors for MASL and MASH. In addition, genetic and environmental factors add to the complexity of MASLD [6–8]. Additionally, MASLD is rapidly becoming the leading cause of liver transplantation in the United States [9]. The economic burden of MASLD is enormous, with the healthcare costs for MASLD patients significantly exceeding those for patients with similar comorbidities but without MASLD [10,11].

Researchers have extensively studied the pathogenesis and progression of MASLD, recognizing the involvement of both intrahepatic liver parenchymal and nonparenchymal cells and extrahepatic components in disease development [12]. One conceptual framework used to explain the progression from MASL to MASH is the 'two-hit' hypothesis. This hypothesis proposes that dysregulated hepatic lipid accumulation constitutes the initial hit, while oxidative, metabolic, and cytokine stresses represent the second hit [13,14]. In addition, a 'three-hit' hypothesis suggests that in MASLD, oxidative stress diminishes hepatocyte proliferation, prompting alternative regeneration pathways involving hepatic progenitor cells, with fibrosis progression dependent on the efficiency of hepatocyte regeneration, thus implicating impaired progenitor cell proliferation and cell death as the 'third hit' in MASLD progression [15–17]. Recently, there has been significant attention on immunological aspects in MASLD progression, particularly the involvement of macrophages. Hepatic macrophages are key players in innate immunity and a crucial cellular component of the liver, with the ratio of hepatocytes to macrophages ranging from 5:1 to 2.5:1 [18]. Macrophages contribute significantly to liver homeostasis, injury, and repair, exhibiting diverse subpopulations that dynamically change in health and disease [19]. In MASLD, macrophages can drive inflammation, fibrosis progression, and regression, thus influencing the disease's pathogenesis and progression [5,20,21].

Traditionally, macrophages have been classified based on the M1/M2 paradigm, but recent advancements in high-end technologies have rendered this classification outdated, particularly in the context of deciphering the liver macrophage landscape. There has been a rapid expansion in understanding the clinical and research implications of liver macrophage diversity in MASLD over the past 5 years. Additionally, the spatial localization of individual cells is increasingly recognized as a crucial parameter defining their function, necessitating integration into advanced multidimensional analyses. In this review, we summarize the role of macrophages in various stages of MASLD, encompassing the traditional M1/M2 paradigm alongside recent insights from single-cell and spatially resolved analyses. Furthermore, we offer perspectives on unresolved questions and challenges in this field.

2. General Overview of Macrophages

Macrophages, a distinct type of cells with a unique ability to clear up foreign bodies such as bacteria, viruses, debris, and other particles through a process known as phagocytosis, were discovered by a Russian-born zoologist and microbiologist Ilya (Elie) Metchnikoff nearly 140 years ago [22]. Metchnikoff's groundbreaking discovery earned him the Nobel Prize in Physiology and Medicine in 1908. Macrophages are integral components of the mononuclear phagocytic system (MPS), a classification introduced by Furth et al. to encompass phagocytic mononuclear cells, which consist of immature monocytic cells along with their bone-marrow precursors, peripheral blood monocytes, and tissue-resident macrophages [23]. Lineage studies examining gene expression profiles and recruitment dynamics of various tissue macrophages have uncovered distinct developmental origins of these cells. This includes embryonic-derived and monocyte-derived macrophages, and these lineages remain independent of each other throughout adulthood [23–28]. As the effector cells of the innate immune system, macrophages play a crucial role as the body's primary line of defense. They not only identify and eliminate pathogens but also engage in

communication with a specialized defense mechanism known as the adaptive or acquired immune system [29].

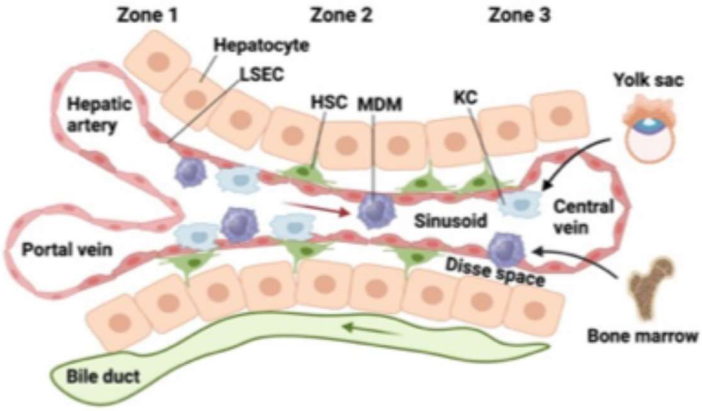
Macrophages are ubiquitous across various tissues in the body, serving crucial functions throughout an organism's life, from developmental stages to maintaining homeostasis and influencing the pathophysiology of diseases. They are motile cells, and their migrations toward the sites of infection and inflammation are critical for their role as effector cells in innate immunity. This migration is mediated by the expression of a diverse array of surface receptors on macrophages, which facilitate their interaction with foreign ligands. These receptors enable macrophages to sense their environment and perform various functions. Typical examples include phosphatidylserine recognition receptors for apoptotic cell removal, complement receptors for clearing opsonized necrotic cells and altered-self molecules, and pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), nod-like receptors (NLRs), sensors for intracellular DNA and RNA, c-type lectin, and scavenger receptors. This PRR family allows macrophages to recognize and bind directly to pathogens and their products, initiating processes like inflammasome activation [30,31].

Resident macrophages are crucial components of tissues, contributing to organ development and maintaining homeostasis. They adapt to their environment, displaying specialized functions. Despite being fully differentiated, resident macrophages exhibit high plasticity and undergo phenotypic reprogramming in response to a changing tissue microenvironment [25,32]. In a healthy state, resident macrophages balance the response to foreign particles while minimizing tissue damage. They patrol tissues, phagocytose cellular debris, clean the surroundings, and facilitate tissue repair. Consequently, resident macrophages play essential roles throughout an organism's lifespan, including promoting ductal branching, angiogenesis, vascular remodeling, osteoclast and bone remodeling, erythropoiesis, brain development, and lung homeostasis during early developmental stages [33–37]. For instance, in tissues such as the mammary glands, pancreas, and kidneys, macrophages are essential for clearing apoptotic epithelial cells, regulating cell proliferation during lumen formation, and secreting growth factors and cytokines that promote tissue remodeling and ductal branching. Their absence can result in ductal branching abnormalities in these organs [38]. In mice with null mutations in the colony-stimulating factor 1 (*Csf1*) gene, which leads to the absence of macrophages, various developmental abnormalities occur, such as atrophic mammary glands, reduced mass of insulin-producing β-cells, impaired pancreatic cell proliferation, and compromised kidney function [39,40]. Additionally, the absence of osteoclasts, which are resident macrophages crucial for bone remodeling, leads to the development of osteopetrosis [41]. In specialized bone marrow areas called erythroblastic islands, macrophages support the production of red blood cells by maintaining cell interactions between erythroblasts and macrophages [42,43] and facilitate erythropoiesis by phagocytizing extruded erythroblast nuclei and supplying iron to erythroid progenitors [44,45]. Microglia, the tissue-resident macrophages of the brain and spinal cord play essential roles in central nervous system (CNS) development, homeostasis, and diseases [46,47]. Alveolar macrophages, found in the lungs, help maintain lung function by clearing inhaled dust [48].

3. Hepatic Macrophages: Type, Origin, and Function

Hepatic macrophages consist of two major types: resident macrophages and infiltrated monocyte-derived macrophages (MDM) that rapidly emerge during injury. The resident hepatic macrophages were initially discovered by Karl Wilhelm von Kupffer, a Baltic German anatomist, and are now known as Kupffer cells (KCs) [49]. Comprising approximately 15% of the total liver cell population, KCs represent 80–90% of all tissue-resident macrophages in the body [50]. Within liver lobules, 43% of Kupffer cells are distributed in the periportal area, 28% in the midzonal area, and 29% in the centrilobular area [51]. KCs are located within the lumen of liver sinusoids with proximity to the liver sinusoidal endothelial cells (LSECs) that form the blood vessel walls (Figure 1). Despite

being considered fixed resident cells, evidence shows that KCs can exhibit some degree of mobility along sinusoidal walls, either with or against the direction of blood flow [52].



Cell type	Origin	Human	Mouse
KC	Yolk sac	F4/80^{High}, CD206^{+}, CD163^{+}, CD68^{+}, Marco^{+}	IBA^{+}, CD16^{High}, CD163^{High}, VSIG4^{+}, CD11b^{low}, F4/80^{high}, Clec4F^{+}
MDM	Bone marrow	CD14^{+}, CD16^{+}, CCR2^{+}, CD68^{+}, Marco^{-}	IBA^{+}, CD16^{low}, CD163^{low}, CD11b^{+}, CCR2^{+}, F4/80^{intermediate}, Ly6C^{+}, and CSF1R^{+}

Figure 1. Schematic overview of hepatic sinusoid and macrophages. The liver is divided into three zones: the areas around the hepatic arteries and portal veins are known as zone 1, those near the central vein are zone 3, and the cells in between are referred to as zone 2. Oxygen-rich blood from the hepatic artery combines with nutrient-rich blood from the portal vein and flows along the sinusoids toward the central vein (red arrow). Meanwhile, bile flows from zone 3 to zone 1, collected by the bile ducts (green arrow). Hepatic macrophages consist primarily of two distinct subtypes: liver resident Kupffer cells (KC), originating from yolk sac, and monocyte-derived macrophages (MDM), from the bone marrow (black arrow). KCs and MDMs can be differentiated by their distinct cell surface markers. Located near liver sinusoidal endothelial cells (LSECs) along the hepatic sinusoids, KCs and MDMs play an important role in influencing the activity of hepatic stellate cells (HSCs) and hepatocytes.

Differential expressions of cell surface markers enable the distinction between KCs and MDMs. In mice, KCs are identified as IBA^{+}, CD16^{High}, CD163^{High}, VSIG4^{+}, CD11b^{low}, F4/80^{high}, and Clec4F^{+}, while MDMs are defined as IBA^{+}, CD16^{low}, CD163^{low}, CD11b^{+}, CCR2^{+}, F4/80^{intermediate}, Ly6C^{+}, and colony stimulating factor 1 receptor (CSF1R)$^{+}$ [53–56] (Figure 1). A fate mapping experiment revealed that erythro-myeloid progenitors (EMPs), originating in the yolk-sac at embryonic day E8.5 and subsequently colonizing the fetal liver at E10.5, give rise to KCs during embryonic development [57,58]. In one-year-old mice, most KCs are of embryonic origin, with some derived from hematopoietic stem cells [58,59]. The average half-life of mouse KCs is 12.4 days, while in rats, their lifespan extends from several weeks to months [60,61]. KCs are self-renewing and can proliferate into mature cells, making their replenishment in a steady state independent of MDMs [27,62]. Bone marrow progenitor cells, defined as CX3CR1^{+}CD117^{+}Lin^{-}, differentiate into MDMs, which can further be subdivided into Ly6C^{low} or Ly6C^{high} MDMs in mouse models of liver diseases [63].

Studies on mice have provided considerable insights into the tissue residence and replenishment of macrophages in human livers. Remarkably, recent single-cell RNA sequencing of human fetal and adult livers has identified distinct clusters of macrophages, showing some transcriptional overlap with hepatic KCs and infiltrating MDMs defined in mice [64–69]. For instance, a study using single-cell RNA-Seq to analyze transcriptional profiles of parenchymal and non-parenchymal cells from fresh hepatic tissues of five healthy human livers identified two distinct subgroups of intrahepatic CD68^{+} macrophages based on the expression of the macrophage receptor with collagenous structure (MARCO): CD68^{+}MARCO^{+} KCs and CD68^{+}MARCO^{-} macrophages [64]. This study found that MARCO is expressed exclusively in non-inflammatory KCs, with CD68^{+}MARCO^{+} cells concentrated in periportal areas contributing to immunotolerance. In contrast, CD68^{+}MARCO^{-} macrophages exhibit a

proinflammatory transcriptional profile like that of recruited macrophages found in mouse livers [64].

Unlike mouse KCs, the half-life of human KCs is not precisely defined. Estimates from animal studies and limited human data suggest that the turnover rate of human KCs may be on the order of several months to years, varying depending on factors such as age, health status, and environmental conditions. A recent study using human leukocyte antigen (HLA)-mismatched liver allografts to distinguish donor liver-resident KCs from recipient infiltrating MDMs showed that although allografts were rapidly infiltrated by recipient monocytes that underwent partial reprogramming to macrophages, a small residual pool of donor cells, including KCs, persisted in the allografts for over a decade [70]. This study revealed remarkably long-lived KCs in human livers. Further research is needed to provide a more precise estimation of the average half-life of human KCs.

KCs serve as the liver's primary defense against pathogens and antigens from the gastrointestinal tract. To maintain tissue homeostasis, the liver fosters an anti-inflammatory microenvironment, promoting immunological tolerance to prevent unnecessary immune responses against food-derived antigens and bacterial products from the portal vein. Among liver cells, KCs play a central role in scavenging circulating antigens. Their immunological tolerance function involves several mechanisms, including presenting antigens to promote the arrest of CD4 T-cells and the expansion of interleukin-10-producing regulatory T cells, fostering tolerogenic immunity [71]. In addition, KCs express lower levels of Major Histocompatibility Complex (MHC) class II and costimulatory molecules such as B7-1 and B7-2, leading to weaker T cell activation compared to dendritic cells [72]. Moreover, KCs generate prostaglandins like PGE2 and 15d-PGJ2, which can suppress the dendritic cell-mediated activation of antigen-specific T cells [72].

4. Macrophage Accumulation in MASLD: Insights from Animal Models and Human Studies

In diet-induced mouse models of MASLD, hepatic macrophage numbers increase significantly with the feeding period [73]. For instance, the infiltrated proinflammatory $CD45^+/CD11b^+/F4/80^{intermediate}$ MDMs population in the liver of obese mice nearly doubled compared to a lean control [74]. Importantly, diet not only increases the infiltration of MDMs but also disrupts the balance of pro and anti-inflammatory macrophages in the liver [75]. Therefore, characterizing the heterogeneity of hepatic macrophage subpopulations is crucial for advancing our understanding of MASLD.

Resident KCs are the predominant hepatic macrophages in the healthy liver, but their numbers have been reported to be reduced in MASH and MASH-associated HCC [76,77]. As the KC number depletes, MDMs infiltrate the liver [78]. Chemokines are small heparin-binding proteins regulating cell trafficking and play a crucial role in this process. Monocyte chemoattractant protein-1 (MCP-1/CCL2), a member of the C-C chemokine family and a potent chemoattractant for monocytes, controls the migration and infiltration of MDMs. While various cell types produce CCL2, monocytes/macrophages are the major source [79,80]. CCL2 exerts its effects through its cognate receptor CCR2, whose expression is restricted to certain cell types, including monocytes [81]. Studies in both ob/ob and HFD-fed obese mice models have shown a positive association between hepatic expression of Ccl2 and Ccr2 and body weight. In a study by Morinaga et al. [82], both KCs and MDMs were fluorescently labeled and evaluated in a high-fat diet-fed MASH mice model. They found that the MDM population in obese mice was approximately six times higher in number and more proinflammatory compared to MDMs from lean mice. This study demonstrated that KCs played a vital role in recruiting MDMs, which is evident from the significantly elevated expression of CCL2, while MDMs displayed significantly higher expression of CCR2 [82]. This group also demonstrated that blocking the infiltration of MDMs using CCR2 antagonists ameliorated steatohepatitis and fibrosis [83].

Similarly, increased macrophage numbers have been reported in liver samples from MASH patients. A retrospective study analyzing liver biopsies from young MASLD patients

has revealed elevated numbers of $CD68^+$ KCs, with higher levels correlating with MASLD severity [84]. Another study discovered the presence of enlarged KCs with significantly elevated phagocytic activity in the hepatic sinusoids [85]. These enlarged KCs are closely associated with transformed hepatic stellate cells and oval cells during MASH development. In addition, a significantly increased number of $CCR2^+$ MDMs in human liver samples is strongly correlated with the severity of MASH and fibrosis [82]. Furthermore, an increased number of portal macrophages with elevated expression of proinflammatory cytokines IL1B and tumor necrosis factor (TNF) has been observed in patients with MASL progression to MASH [86]. Similar to findings in mouse studies, an increased presence of CD11c-positive macrophages surrounding hepatocytes with large lipid droplets, forming aggregates known as hepatic crown-like structures, correlates with hepatocyte death and fibrosis development in human MASH patients [87,88]. These aggregates are important sources of inflammation and fibrosis due to their intact structure and close association with activated fibroblasts for collagen deposition.

5. Stimuli Trigger Hepatic Macrophage Activation during MASLD Development

Multiple stimuli, such as fatty acids, cholesterol, damage-associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs), trigger macrophage activation in MASLD. The heightened influx of fatty acids into the liver, along with de novo lipogenesis, exacerbates oxidative stress and lipid peroxidation in MASLD [89]. When co-cultured with macrophages, steatotic hepatocytes release pro-inflammatory cytokines such as TNFα, MCP-1, interleukin 6 (IL-6), and IL-18, which can activate the macrophages [90]. Saturated fatty acids, such as lauric acid (C12:0) and palmitic acid (C16:0), induce the expression of inflammatory markers, including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and IL-1α in a mouse macrophage cell line Raw 264.7 cells through the activation of TLR4 and NF-κB pathway [91]. On the contrary, unsaturated fatty acids inhibit the NF-κB pathway, thus impeding saturated fatty acid-mediated COX-2 expression in Raw 264.7 cells. In addition, palmitic acid activates TLR2 in a human monocytic cell line THP-1 cells, inducing inflammasome-mediated-IL-1β production [92]. Saturated fatty acids activate macrophages and promote inflammation, while unsaturated fatty acids inhibit it. This has been demonstrated not only in vitro but also in in vivo mouse studies and patients with MASLD. Using dietary mouse models, Kim et al. found that palmitate stimulates reactive oxygen species (ROS) production in $CD11b^+F4/80^{low}$ infiltrating macrophages rather than resident macrophages [93]. This occurs through the direct binding of palmitate to a monomeric TLR4-MD2 complex, triggering endocytosis of TLR4 and NADPH oxidase 2 (NOX2), leading to pro-interleukin-1β expression in macrophages [93]. Consequently, mice lacking *Nox2* are resistant to high-fat diet-induced MASLD development [93,94]. Consistent with this observation, increased serum levels of soluble NOX2-derived peptides and NOX2-generated oxidative stress have been found to be associated with the severity of liver steatosis in MASLD patients [95].

The disruption of hepatic cholesterol homeostasis and accumulation of free cholesterol in hepatocytes are linked to the pathogenesis of MASH [96]. Ioannou et al. described the presence of free cholesterol in the hepatocytes of MASH patients and diet-induced MASH mice model [97]. They proposed that the aggregation and activation of KCs in 'crown-like structures' containing cholesterol crystals around lipid droplets, similar to those previously described in inflamed visceral adipose tissue, are significant indicators of the progression of disease from simple steatosis to MASH [97]. To test this hypothesis, they fed c57bl/6J mice a 15% high-fat diet for 6 months, supplemented with various amounts of dietary cholesterol ranging from 0% to 1%. They revealed that increasing cholesterol led to cholesterol loading in the liver but not in adipose tissues, inducing MASH at a threshold dietary cholesterol concentration of 0.5%, whereas mice on lower-cholesterol diets developed only MASL [98]. Additionally, KCs surrounded dead hepatocytes and processed cholesterol crystal-containing lipid droplets, possibly via lysosomal exocytosis, forming the 'crown-like structures'. These macrophages stained positively for NLRP3

inflammasome and activated caspase 1 [98], likely due to the phagocytosis of cholesterol crystals by macrophages leading to lysosomal swelling and the release of cathepsin B, a lysosomal protease, which activates the NLRP3 inflammasome, a mechanism similar to that responsible for atherosclerosis pathogenesis [99,100].

In addition, oxidative damage to cellular proteins, lipids, and DNA in hepatocytes generates oxidation-specific epitopes, acting as DAMPs, which interact with macrophage-expressed PRRs such as CD36 and TLR4, thereby initiating various immune responses [101]. For instance, in vitro studies demonstrated that mouse KCs can engulf apoptotic bodies from UV-treated mouse hepatocytes, triggering the production of Fas ligand and tumor necrosis factor α (TNFα) [102]. Extracellular vesicles (EVs) are membrane vesicles derived from various types of cells containing biologically active molecules such as RNAs, proteins, and lipids. EVs released from primary mouse hepatocytes, particularly in response to palmitic acid treatment, contain factors like TNF-related apoptosis-inducing ligands [103]. These EVs induce the expression of IL-1β and IL-6 in mouse bone marrow-derived macrophages. In contrast, EVs from primary rat LSECs suppressed the expression of inflammatory genes in LPS-treated KCs [104]. However, this anti-inflammatory effect was diminished when LSECs were exposed to free fatty acids (FFAs), indicating that EVs from LSECs are important in regulating macrophage activation.

Furthermore, PAMPs such as lipopolysaccharide (LPS) and microbial nucleotides act as danger signals recognized by PRRs on macrophages, triggering inflammatory responses via intracellular signaling pathways [105]. In MASLD, there is an increased influx of gut-derived microbial products into the liver due to changes in gut microorganisms and increased intestinal permeability. This leads to elevated TLRs-mediated immune signaling, contributing to liver inflammation and fibrogenesis [106]. In addition, triglycerides enhance the LPS-mediated expression of proinflammatory mediators such as inducible iNOS, TNFα, IL-1β, and IL-6 in rat KCs, compared to LPS stimulation alone [107]. The inhibition of the NF-κB pathway significantly reduces the potentiating effect of triglycerides on iNOS expression by KCs [107]. Electron microscopic analysis of KCs from high-fat diet-fed mice reveals intracellular lipid droplet accumulation [108]. These fat-laden KCs generate significantly high levels of proinflammatory cytokines and chemokines in response to LPS compared to KCs from chow diet-fed mice.

6. Classical M1/M2 Macrophage Paradigm in MASLD Development

The dynamic heterogeneity and reprogramming of macrophages contribute significantly to disease pathogenesis and progression. An important aspect of this macrophage adaptability is evident in the differentiation of macrophages into either classically activated M1, characterized by a pro-inflammatory profile, or alternatively activated M2 macrophages, displaying anti-inflammatory and pro-fibrogenic phenotypes (73,77). This classification is rooted in their origins from the Th1 strains (C57BL/6, B10D2) or Th2 strains (BALB/c, DBA/2), respectively. Macrophages activated in response to IFN-γ differentiate into M1-like macrophages capable of generating nitric oxide (NO) to kill parasites. On the contrary, Th2 cytokines, including IL-4 and IL-10, suppress the activation of M1-like macrophages, and these M2-like macrophages exhibit elevated arginine metabolism [109,110]. Although the M1/M2 classification oversimplifies the intricate in vivo responses of macrophages, it is widely recognized that the differentiation of macrophages into distinct pro-inflammatory or anti-inflammatory phenotypes profoundly influences host defense and the pathogenesis of various liver diseases. The key mechanisms regulating macrophage polarization are discussed below and illustrated in Figure 2.

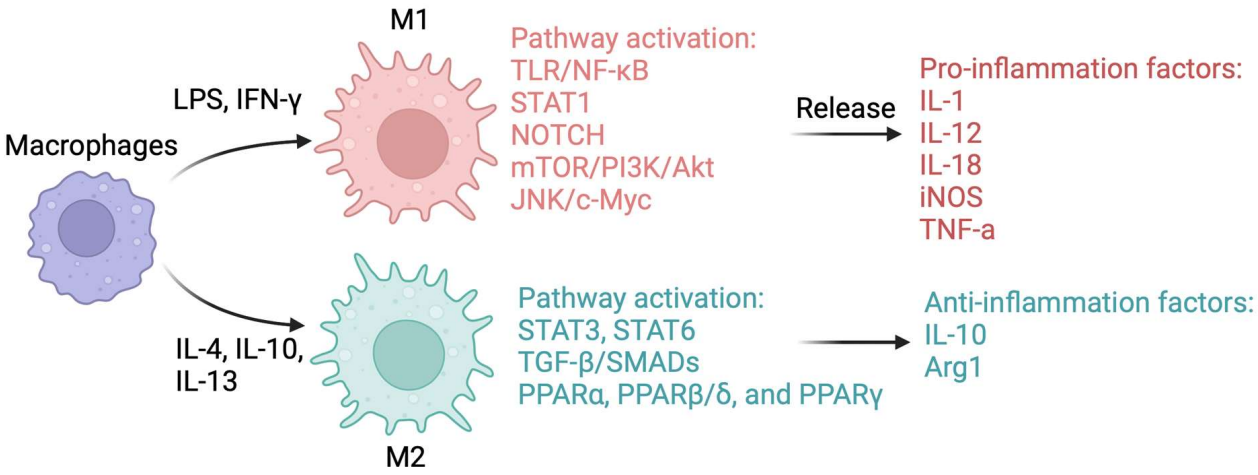


Figure 2. Schematic overview of macrophage M1 and M2 polarization. Macrophages can polarize into two distinct phenotypes depending on the microenvironmental stimuli they encounter. M1 macrophages, induced by LPS and IFN-γ, activate pathways such as TLR/NF-κB, STAT1, NOTCH, mTOR/PI3K/Akt, and JNK/c-Myc, leading to the release of pro-inflammatory factors like IL-1, IL-12, IL-18, iNOS, and TNF-α. In contrast, M2 macrophages, induced by IL-4, IL-10, and IL-13, promote anti-inflammatory activity through pathways like STAT3/6, TGF-β/SMADs, and PPAR (α, β/δ, and γ), expressing IL-10 and arginase 1.

6.1. Mechanisms Control Macrophage Polarization

6.1.1. TLR and NF-κB

Bacterial endotoxin LPS activates TLR4 on macrophages, triggering proinflammatory reactions crucial for eliminating invading bacteria. Beyond LPS, endogenous DAMPs, including high-mobility group box protein 1 (HMGB1) and hyaluronic acid, are released during tissue injury, activating TLR4 to facilitate tissue repair [111–113]. TLR4 activation leads to NF-κB activation through the myeloid differentiation factor 88 (MyD88)-dependent pathways or interferon regulatory factor (IRF) 3, promoting the expression of proinflammatory factors [114]. Studies have shown that the loss-of-function mutations or deletions of *Tlr4* or *MyD88* protect mice against diet-induced inflammation in adipose tissue and liver, accompanied by altered macrophage polarization [115–117]. In contrast, drugs or compounds inhibiting TLR4/NF-κB signaling can repress M1-like proinflammatory macrophage polarization. For example, Jing et al., using mouse macrophage cell line Raw264.7 cells and primary peritoneal macrophages, demonstrated that berberine, a competitive inhibitor of TLR4, disrupts the TLR4/MyD88/NFκB signaling pathway, interfering with LPS-mediated proinflammatory M1-like macrophage polarization [118]. In a separate study, Xiang et al. showed that olean-28,13β-olide 2 (NZ), a newly synthesized derivative of oleanolic acid, inhibited LPS-mediated generation of proinflammatory cytokines in Raw264.7 cells through the suppression of TLR-NF-κB signaling, downregulation of NLRP3 expression, and inhibition of caspase-1 activation [119]. Similarly, Lu et al. demonstrated that quercetin, a natural flavonoid compound, inhibits LPS-mediated M1 macrophage polarization via the NF-κB and IRF5 signaling [120]. These findings were further supported by in vivo studies showing that berberine and quercetin can inhibit inflammation and prevent metabolic disorders such as MASLD and type 2 diabetes by improving insulin resistance, lipid metabolism, and liver enzymes [121–125]. Together, these findings suggest that the TLR4/NF-κB signaling axis plays an important role in M1-like proinflammatory macrophage polarization, and targeting this pathway holds promise for improving MASLD.

6.1.2. Signal Transducer and Activator of Transcription (STAT)

The STAT family, comprising seven structurally similar and highly conserved members, including STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6, are recognized as

important regulators of macrophage polarization [126,127]. Interferons and TLR signaling polarize macrophages toward the M1-like proinflammatory phenotype via STAT1 signaling, whereas IL-4 and IL-13 tilt macrophages toward the M2-like anti-inflammatory phenotype through STAT6 signaling pathways [128]. In a study with J774 murine macrophages, Haydar et al. demonstrated that azithromycin promotes M2-like macrophage polarization by inhibiting STAT1 and NF-κB signaling pathways. Another study by He et al. showed that IL-4 skews macrophage toward the M2 subtype through the JAK1/STAT6 pathway [129]. In addition, STAT3 plays a determinative role in M2 polarization, as the suppression of JAK3/STAT3 by miR-221-3p promotes the shift of macrophage polarization from the M2 to M1 subtype [130].

6.1.3. Transforming Growth Factor Beta (TGF-β) and Suppressor of Mothers against Decapentaplegic (SMAD)

Upon TGF-β binding to the TGF-β receptor complex, the receptor complex activation triggers Smads (Smad2 or 3)-mediated pathways to regulate gene expression [131]. Studies have revealed the important role of the TGF-β/Smads signaling pathway in modulating M2 macrophage polarization. For instance, growth differentiation factor 3 (GDF3), a member of the TGF-β superfamily, can phosphorylate and activate Smad2/Smad3, inhibiting NLRP3 expression in macrophages and directing macrophage polarization toward the M2 phenotype [132]. The flavonoid compound quercetin can regulate macrophage polarization and reduce kidney fibrosis by antagonizing the TGF-β1/smad2/3 pathway [120]. This inhibitory effect was also observed in a mouse model of asthma-induced airway inflammation, where quercetin treatment reduced airway inflammation response and lung fibrosis by downregulating the TGF-β1/Smad pathway [133].

6.1.4. Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are a subfamily of nuclear receptors consisting of three members: PPARα, PPARβ/δ, and PPARγ. Their transcriptional activity is mediated by PPAR: retinoid X receptor (RXR) heterodimers, which bind to specific DNA sequence elements called PPREs in the regulatory regions of their target genes. PPARs regulate the expression of genes involved in various functions, including lipid and carbohydrate metabolism, cell proliferation and differentiation, sexual dimorphism, and immune response [134]. PPARα, PPARβ/δ, and PPARγ have tissue-specific but partially overlapping expression patterns. PPARα is highly expressed in tissues that perform significant fatty acid catabolism, such as brown adipose tissue, liver, heart, kidney, and intestine. PPARβ/δ functions prominently in the skin, gut, placenta, skeletal and heart muscles, adipose tissue, and brain. PPARγ exists in two isoforms, PPARγ1 and PPARγ2, which differ at their N termini. PPARγ1 has a broad expression pattern, including the gut, brain, vascular cells, and immune cells, while PPARγ2 is predominantly found in adipose tissues.

All three PPAR isotypes exhibit a common anti-inflammatory function, primarily inhibiting inflammation through transcriptional repression of inflammatory genes. This mechanism involves the activation of PPARs by ligands that bind to key regulators of inflammation, such as NF-kB, activator protein 1 (AP-1), nuclear factor of activated T cells (NFAT), and STAT, resulting in stabilization of corepressor complexes at the promoters of inflammatory genes, thereby repressing their transcription and reducing inflammation [134]. In addition, PPARα has been shown to upregulate the expression of IκB, which prevents the nuclear translocation and activation of NF-κB [135]. Moreover, PPARγ has been shown to directly interact with NF-κB p65, resulting in NF-κB p65 degradation [136].

Studies have underscored the important association between PPAR activation and macrophage polarization [137–139]. For example, PPARα promotes M2 macrophage polarization by interacting with dual specificity phosphatase 1 (DUSP1), which can alleviate cardiomyocyte injury in a macrophage–cardiomyocyte co-culture system [140]. Additionally, delivery of PPARα via lentiviral particles attenuates sepsis-induced myocardial injury in a cecal ligation and puncture mouse model [140]. Activation of PPAR-γ in

Raw264.7 macrophages by a PPARγ agonist shifts lipid-mediated macrophage polarization from the M1 to M2 phenotype through its interaction with NF-κB p65 [141]. Similarly, PPARγ activation promotes native human monocytes toward an anti-inflammatory M2 phenotype [142]. Furthermore, a mouse with macrophage-specific deletion of PPARγ impairs the maturation of the M2 macrophage [143]. Eosinophil-derived IL-4 and IL-13 are crucial for maintaining adipose M2 macrophages, which requires PPARβ/δ and PPARγ [144]. These in vitro and in vivo results suggest that PPARs are master regulators of M2 macrophage polarization.

6.1.5. MicroRNAs (miRNAs) and Other Mechanisms

MicroRNAs (miRNAs) have garnered significant interest due to their important roles in macrophage polarization by regulating various signaling pathways [145]. For example, miR-221-3p and miR-1246 facilitate alternative macrophage polarization through modulating JAK3/STAT3 and NF-κB signaling pathways [130,146]. Exosomal vesicles derived from adipocytes delivered miR-34a into macrophages, repressing kruppel-like factor 4 (Klf4) expression and consequently inhibiting M2-like macrophage polarization [147].

Beyond these mechanisms, additional signaling pathways, including Notch signaling, mammalian target of rapamycin (mTOR) signaling, phosphoinositide 3-kinase (PI3K)/Ak strain transforming (Akt), and Jun NH2-terminal kinase (JNK)/c-Myc signaling pathways, have been identified to play roles in macrophage polarization [148–151]. Singla et al. have shown that during M1 macrophage differentiation, the Notch 1 receptor is upregulated, and the activation of Notch signaling promotes THP-1 human monocytes toward M1 macrophage polarization [148]. Conversely, interference with Notch signaling impairs M1 and enhances M2 macrophage polarization [148]. The important role of Notch signaling in macrophage polarization was further supported by an in vivo study showing that myeloid *Notch1* deficiency facilitates M2 macrophage polarization by repressing YAP signaling in an acute liver injury mouse model induced by lipopolysaccharide/D-galactosamine [152]. This study suggests that targeting the macrophage Notch1-YAP circuit could be an effective strategy for treating liver inflammation-related diseases.

The mTOR pathway is a key nutrient/energy sensor that controls cellular metabolism to maintain cellular homeostasis. Dysregulation of mTOR signaling has been implicated in interfering with macrophage polarization and function [153]. This is supported by numerous in vitro and in vivo studies. Mice with myeloid-specific deletion of *Tsc1*, leading to constitutive activation of mTOR complex 1 (mTORC1), exhibit increased susceptibility to sepsis, spontaneous development of inflammatory disorders, and reduced IL-4-induced M2 macrophage polarization, which is accompanied by increased activities of JNK and Ras along with reduced activities of Akt and C/EBPβ [149,154,155]. In line with these studies, myeloid-specific deletion of *Raptor*, which leads to mTORC1 deficiency, protects mice against obesity-induced inflammation and insulin resistance and decreases atherosclerosis development [156,157].

6.2. Macrophage Polarization in Early Stage of MASLD

Macrophages with pro-inflammatory phenotypes exacerbate early MASLD severity, while those with anti-inflammatory characteristics contribute beneficially to MASLD initiation. Maina et al., using a methionine-choline-deficient (MCD) diet in C57BL/6 and Balb/c mice, demonstrated that C57BL/6 mice with M1 bias displayed elevated liver steatosis and lobular inflammation compared to Balb/c mice with M2 bias [158]. Further research corroborated that high-fat diet-fed BALB/c mice displayed increased KCs M2 polarization compared to C57BL6/J mice, leading to the apoptosis of M1 KCs via IL10-mediated arginase activation and mitigating liver steatosis and hepatocyte death [159]. Additional studies found that a high-fat diet enriched in polyunsaturated fatty acids promotes alternative M2 macrophage activation and improves metabolic disturbances [159,160]. The activation of M2 KCs by PPARδ promotes and ameliorates obesity-induced insulin resistance [161]. Histidine-rich glycoprotein (HRGP) is an α2-plasma glycoprotein and is mainly produced

by liver parenchymal cells in mammals. Liver-derived HRGP has been shown to promote the polarization of M1 macrophages and inhibit M2 polarization in both tumor and inflammatory environments [162]. Consequently, macrophage polarization was tipped toward M2 in mice lacking HRGP, attenuating liver injury and fibrosis induced by MCD diet or carbon tetrachloride (CCl_4) [163].

6.3. Macrophage Polarization in Advanced Stage of MASLD

Liver biopsies from patients with MASH reveal an increase in proinflammatory myeloperoxidase-positive KCs along with elevated expression of the proinflammatory marker IL-6 [164]. Interestingly, the expression of anti-inflammatory macrophage markers such as IL-10 and dectin-1 is also induced in MASH, suggesting a reparative role of M2 macrophages following tissue injury, which may contribute to fibrosis development [87,164]. Type 2 immunity is characterized by increased levels of cytokines such as IL-4, IL-5, IL-9, and IL-13. Blocking anti-inflammatory type 2 TGFβ and IL-13 signaling has been shown to protect against high-fat diet-induced liver fibrosis in mice [165,166]. The scavenger receptor CD163 is considered a marker for anti-inflammatory macrophages. Interestingly, targeting CD163 in KCs and other M2 macrophages with an anti-CD163-IgG-dexamethasone conjugate has been shown to improve MASH pathologies, including hepatic inflammation, hepatocyte ballooning, fibrosis, and glycogen deposition in a rat model of fructose-induced MASH [167]. These findings underscore the important role of M2 macrophage activation in MASLD progression.

7. Revealing the Dynamic Landscape of Hepatic Macrophages in MASLD: Heterogeneity and Plasticity

The widespread use of single-cell RNA sequencing (scRNA-seq) has greatly enhanced our comprehension of cellular diversity and changes in macrophage subpopulations under specific healthy or diseased conditions, surpassing the traditional M1/M2 macrophage paradigm. In the normal mouse liver, KCs are identified using markers such as F4/80, CLEC4F, and T cell immunoglobulin and mucin domain-containing 4 (Timd4) [56]. During the early stages of MASLD, KCs engage in lipid storage, compromising their ability for self-renewal [168]. Consequently, embryonic KCs are gradually lost and replaced by MDMs lacking Timd4 expression [169,170]. Mulder K et al. integrated 41 mononuclear phagocyte scRNA-seq datasets to compile a comprehensive monocyte–macrophage-focused compendium, revealing a diverse array of specialized cell subsets distributed across multiple tissues [171]. They identified three conserved macrophage populations across tissues, namely, TREM2, IL4I1, and HES1, suggesting that TREM2 and IL4I1 macrophages could be predominantly derived from monocytes, whereas HES1 macrophages bear an embryonic signature [171]. TREM2 macrophages were initially studied in the context of brain disorders and neurodegeneration; however, recent evidence has revealed their presence not only in the brain but also in adipose tissue, liver, and different types of tumors, indicating a potential immunoregulation role in these contexts [172–174]. Several studies in mice have elucidated the various roles of $TREM2^+$ macrophages in liver disease, demonstrating their protective functions for hepatocytes in MASLD and cholangiopathies [175,176], immunosuppressive roles in hepatocellular carcinoma, and contribution to supporting liver regeneration in both acute and chronic murine injury models [173,177]. In another study using scRNA-seq, researchers delineated the functional phenotypes of myeloid cells and liver macrophages throughout the progression of MASH, revealing significant alterations in both liver MDMs and their bone marrow precursors, as indicated by the downregulation of the inflammatory marker calprotectin [178].

Similarly, the transcriptional profiles obtained from an scRNA-seq analysis of parenchymal and non-parenchymal cells in human livers unveil distinct subsets of hepatic macrophages [64]. The first subset, $CD68^+MARCO^-$ macrophages, exhibits characteristics of pro-inflammatory macrophages with an enriched expression of LYZ, CSTA, and CD74. The second subset, $CD68^+MARCO^+$ macrophages, are identified as

KCs and expressed genes associated with immune tolerance, including CD5L, MARCO, VSIG4, CD163, MAF, VCAM1, and KLF4. Furthermore, two distinct populations of $MARCO^+$ KCs are distinguished by the expression of TIMD4, with a selective reduction in $MARCO^+$ $TIMD4^-$ KCs observed in the livers of cirrhosis patients [68].

These studies collectively suggest that macrophages exhibit a wider spectrum of phenotypic activation profiles during MASLD development than previously recognized. The integration of single-cell transcriptomics with advanced bioinformatics enables the prediction of novel cellular interactions and macrophage plasticity throughout MASLD progression.

8. Unraveling the Complexity of Hepatic Macrophages in MASLD: Insights into Spatial Dynamics

The localization of liver macrophages within hepatic lobules is closely linked to their function. KCs are not restricted to blood vessels but extend into the perisinusoidal space of Disse, where they interact closely with hepatocytes and hepatic stellate cells (HSCs) [170]. Unlike KCs, MDMs are characterized by their smaller size and circulate through the sinusoids [170]. The MDMs' specific localization around the periportal area suggests that these cells may serve as primary responders to events such as bile duct leakage or the presence of pathogens in the portal vein. Over the past decade, technological advancements such as single-cell analysis and in situ expression measurements of landmark genes have significantly deepened our comprehension of liver macrophage populations during homeostasis and disease. These technologies have revealed spatially specific responses that influence liver disease progression [179–181]. For instance, a 2022 study presented a comprehensive spatial proteogenomic single-cell atlas of the immune cell landscape in healthy and obese human and mouse livers, identifying three distinct macrophage populations in the homeostatic liver, with KCs being the most prevalent [182].

Hepatocyte zonation for metabolic functions is well-known, and recent studies indicate similar spatial variability in macrophages along the centrilobular–portal axis. For instance, during weaning in mice, KCs tend to cluster around periportal regions, influenced by the activation of liver sinusoidal endothelial cells triggered by MYD88-dependent signaling from gut-derived bacteria, suggesting the significance of KC zonation in controlling pathogen dissemination [183]. In addition, spatial transcriptomics of healthy human liver tissues unveils non-inflammatory macrophage genes and signatures in periportal regions and inflammatory counterparts closer to the central vein [184]. During liver injury, the hepatic macrophage landscape undergoes significant changes associated with disease stages. In a study using a mouse model with conditional depletion of liver KCs, researchers demonstrate that Ly6C-high monocytes, when recruited, could differentiate into $F4/80^+$ KCs to replenish the KC pool [170]. Their subsequent CSF1R-dependent proliferation reaches the steady-state KC density by day 6 after depletion [170].

Consistent changes in the spatial dynamics of immune cell subsets have been observed during the progression of human MASLD. A summary of studies utilizing scRNAseq, snRNAseq, and spatial transcriptomics on normal human livers and livers from MASLD patients is provided in Table 1 [64–66,68,182,184–194]. For instance, researchers combining single-cell and -nucleus sequencing with spatial mapping have revealed distinct and evolutionarily conserved, spatially restricted hepatic macrophage niches, such as $Gpnmb^+$ $Spp1^+$ lipid-associated macrophages (LAMs) in the centrilobular areas where steatosis occurs [182]. This study also found that KC development crucially depends on their cross-talk with HSCs via an activin receptor-like kinase (ALK1)–bone morphogenic protein (BMP) 9/10 axis [182]. Another study using human liver samples from patients with MASLD and primary sclerosing cholangitis revealed intense aggregation of $IBA1^+$ $CD16^{low}$ $CD163^{low}$ MDM-derived macrophage, exhibiting distinct spatial proximity to $CK19^+$ ductular cells in periportal areas [195]. Additionally, the accumulation of $IBA1^+$ $CD163^{low}$ MDMs tightly correlates with the loss of hepatocytes and increased ductular reaction during the progression of MASLD, primary sclerosing cholangitis, primary biliary cholangitis, and alcoholic hepatitis [195,196].

Table 1. Studies on scRNAseq, snRNAseq, and spatial transcriptomics conducted on normal human livers and livers from MASLD patients.

Method	Tissues	Database Accession	Publications
scRNAseq	Fetal livers (10.5- and 17.5-week gestation) and healthy liver tissues from partial hepatectomy of patients with primary or secondary liver tumors or benign liver diseases (n = 3)	GSE81252 and GSE96981	[185]
scRNAseq	Livers from donors for liver transplantation (n = 5)	GSE115469	[64]
scRNAseq	Non-diseased liver tissues from patients who underwent liver resections for colorectal cancer metastasis or cholangiocarcinoma (n = 6)	GSE124395	[66]
scRNAseq	Non-parenchymal cells from healthy livers (n = 5) and cirrhotic livers (two MASLD, two ALD, one PBC)	GSE136103	[68]
snRNAseq	Tumor-free liver tissue from a partial hepatectomy of a patient with colon cancer and hepatic metastasis (n = 1)	EBI BioStudies S-BSST324	[186]
scRNAseq	Liver CD45$^+$ immune cells from healthy livers (n = 3)	GSE125188	[65]
scRNAseq	Liver CD45$^+$ immune cells from steatosis (n = 4) and MASH livers (n = 3)	GSE159977	[187]
scRNAseq; Spatial transcriptomics	Fetal livers (8- and 17-week gestation)	GSE167096	[188]
scRNAseq	Liver tissues from donors (n = 2)	GSE158723	[189]
scRNAseq	Healthy livers from donors (n = 6)	EBI BioStudies E-MTAB-10553	[190]
scRNAseq; snRNAseq; Spatial transcriptomics	Healthy livers from donors for liver transplantation (n = 4)	GSE185477	[184]
snRNAseq	Healthy livers (n = 2), MASLD cirrhotic livers (n = 2), HCC (n = 2), and adjacent cirrhotic livers (n = 2)	GSE174748 and GSE212047	[191]
scRNAseq; snRNAseq; CITE-seq	Healthy (n = 14), >10% steatosis with no fibrosis (n = 5)	GSE192742	[182]
scRNAseq	Healthy (n = 1) and cirrhotic MASH liver (n = 1)	GSE190487 and GSM5724573	[192]
snRNAseq	Normal livers (non-tumor tissue from liver metastasis resections) (n = 3) and MASH livers (n = 9)	GSE212837	[193]
snRNAseq	Healthy livers (n = 3) and MASH livers (n = 3)	GSE189600	[194]

Fibrotic liver disease causes significant changes in vascular architecture. KCs, located within liver sinusoids, primarily filter bacteria-rich portal blood flow. To understand how fibrotic remodeling of the vascular architecture affects the KC compartment, a study using high-resolution intravital microscopy (IVM) in a mouse model of liver fibrosis induced by CCl_4 revealed that increased collagen deposition and collateral vessel growth around sinusoids cause sinusoid-resident KCs to lose their identity and function due to diminished contact with parenchymal cells [197]. This study found that MDMs are recruited and formed multinucleated KC-like syncytia within these collateral vessels. These KC-like syncytia displayed enhanced bacterial capture ability and are also observed in human liver cirrhosis from different etiologies, including cholestatic liver disease, viral hepatitis, alcoholic hepatitis, and MASLD [197]. Taken together, these studies revealed significant changes in the spatial dynamics of macrophage subsets in MASLD and other chronic liver diseases. Future studies will focus on fully understanding how macrophage heterogeneity evolves throughout liver disease progression.

9. Targeting Macrophages for the Treatment of MASLD

Macrophages have emerged as important therapeutic targets in MASLD. The impact of Kupffer cells on steatosis, to some extent, is regulated by IL-1beta-mediated suppression of PPARα expression and activity, a master regulator of fatty acid oxidation in the liver [198]. A 2010 study showed that the depletion of resident KCs in rats using gadolinium chloride

had a protective role against diet-induced alterations in hepatic lipid metabolism and insulin sensitivity [199].

In monocytes/macrophages and KCs, the glucocorticoid receptor (GR) and glucocorticoid-induced leucine zipper (GILZ) axis is involved in a variety of inflammatory processes, contributing to the pathogenesis of liver inflammation. The knockdown of Gilz renders KCs more susceptible to LPS, and transgenic mice overexpressing macrophage-specific Gilz significantly reduce obesity-induced liver inflammation [200]. Dexamethasone, a potent glucocorticoid widely used to treat diseases including multiple sclerosis, allergies, cerebral edema, inflammation, and shock, has been explored in the context of MASLD treatment. Svendsen et al. demonstrated that a low dose of an anti-CD163-IgG-dexamethasone conjugate, specifically targeting CD163 receptors on KCs and alternatively activated macrophages, significantly reduces high-fructose diet-induced MASH-like pathologies, including hepatocyte ballooning, hepatic inflammation, and fibrosis in rats, without apparent systemic side effects [167].

Therapies targeting or inhibiting pro-fibrotic macrophages have been evaluated in various clinical trials. Drugs like CCR2/CCR5 inhibitors (e.g., cenicriviroc) and galectin-3 antagonists (e.g., GR-MD-02) have shown potential in reducing fibrosis in MASLD [201,202]. For instance, in the CENTAUR trial, year 1 data for cenicriviroc, a CCR2/CCR5 antagonist, revealed fibrosis improvement in 20% of patients without affecting steatohepatitis compared to a placebo, though continued fibrosis improvement was not observed by the end of year 2 [201,203]. Galectin-3 is a member of the endogenous lectin family with the ability to bind to terminal galactose residues in glycoproteins. It modulates immune cell adhesion and migration, cytokine production, phagocytosis, and immune cell survival [204–207]. Mice with Gal-3 deficiency are protected from dietary-induced MASH [208,209], which led to the development of galectin-3 inhibitors as a treatment strategy for MASH. Belapectin (GR-MD-02), a soluble and physiologically compatible polysaccharide derived from a natural plant compound consisting of oligosaccharide chains with galactose residues that specifically bind to galectin-3, holds promise for the treatment of MASH with advanced fibrosis. Traber et al. studied GR-MD-02 in a high-fat diet-fed mouse MASH model and found that it reduced Gal-3 expression in liver macrophages and ameliorated MASH progression [210]. A phase 1 study demonstrated that belapectin is safe and well tolerated at single and multiple doses in patients with well-characterized MASH and advanced fibrosis but not cirrhosis [202]. While a phase 2b trial of belapectin in patients with MASH cirrhosis and portal hypertension conducted throughout 36 centers in the USA did not meet its primary endpoint, a sub-analysis excluding patients with esophageal varices showed that belapectin at 2 mg/kg reduced hepatic vein pressure gradient and the development of new varices [211]. This results in an adaptive phase 2B-3 study of belapectin currently being initiated to evaluate its safety and efficacy among MASH cirrhosis patients without esophageal varices [212].

10. Conclusions and Challenges

Hepatic macrophages are essential cellular components of the liver, playing critical roles in maintaining tissue homeostasis and facilitating rapid responses to pathophysiological conditions. Despite significant advances in understanding the biology and role of macrophages in MASLD over recent decades, their heterogeneity and complexity in the disease remain inadequately understood. Most studies have relied on cell surface markers, such as clusters of differentiation, to assess immune cell phenotyping and define cell subpopulations, often assuming specific functions based on marker expression. However, there remains a substantial gap between marker expression and the presumed functions, with limited evidence to demonstrate that macrophages expressing these markers actually perform the associated functions or that cells lacking these markers are functionally inactive.

Developing personalized, macrophage-targeted interventions for MASH treatment continues to present significant challenges for several reasons. Many studies of the hepatic immune response have been conducted in vitro using mouse macrophage cell lines like Raw264.7 or human monocyte cell lines like THP-1, which may not accurately reflect the

in vivo responses of hepatic macrophages. This is particularly important, considering that KCs interact with their microenvironment to shape the hepatic cellular landscape and modulate liver function. Additionally, small animal models often fail to reliably translate to human clinical trials. For instance, studies have shown significant differences in gene expression between mouse and human livers in fatty liver disease [213,214]. Guillot et al. reported that monocytic macrophages are key drivers of MASH progression, with marked differences in the spatial localization of recruited monocytes between human MASLD and diet-induced obesity-MASH mouse models [195]. They found that in mouse models of MASH, monocyte-derived macrophages accumulate throughout the liver parenchyma, whereas in human MASH, these macrophages predominantly expand in the portal area, a feature shared by various chronic human liver diseases, including MASLD [195]. These findings underscore the limited predictive value of mouse models for human outcomes. As a result, compounds like the galectin-3 inhibitor belapectin, which shows promise in preclinical mouse models, often fail to demonstrate robust efficacy in improving fibrosis in MASH patients [211]. However, while human studies are more clinically relevant, they also face challenges due to the variability in macrophage populations and druggable targets among patients. Recent research reveals considerable variation in macrophage-related gene and protein expression in MASLD patients, highlighting the importance of accounting for individual differences within the hepatic microenvironment to develop effective treatments [215].

Additionally, the complexity of liver macrophage biology adds further challenges to treatment development. Although various proteins and signaling pathways implicated in macrophage activation in MASLD have been identified, a comprehensive understanding of the coordinated regulatory mechanisms is still lacking. A more detailed examination of how macrophage phenotypes evolve over time could provide insights into their specific roles during distinct phases of the disease, from early steatosis to advanced fibrosis. Liver macrophages represent a heterogeneous population of phagocytes with specific adaptations in their phenotypes to the microenvironment. Therefore, fully capturing the functional contribution of a macrophage population to disease progression or regression in MASLD requires consideration of their spatial contextualization [216]. Given the complexity of the hepatic macrophage landscape and the rapid technological advancements in macrophage biology studies, reaching a consensus on macrophage denominations to decipher the overarching functions of specific subpopulations would be advantageous. Further developments are expected to enable the acquisition of multiple omics data from a single sample on a large scale and with a high number of parameters, such as immunostaining combined with in situ messenger RNA sequencing. These advancements hold the potential to revolutionize our understanding of liver macrophage biology in MASLD.

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Abbreviations

ARG1: Arginase 1; BMDMs: Bone Marrow-Derived Macrophages; CCL2: Chemokine (C-C Motif) Ligand 2; COL1A1: Collagen Type 1 Alpha 1; COL1A2: Collagen Type 1 Alpha 2; DAMP: Damage-Associated Molecular Patterns; HCC: hepatocellular carcinoma; HSC: hepatic stellate cell;

IFNg: Interferon Gamma; IL: interleukin; KC: Kupffer cell; LPS: lipopolysaccharide; MAPK: Mitogen-Activated Protein Kinase; MASH: metabolic dysfunction-associated steatohepatitis; MASL: metabolic dysfunction-associated steatotic liver; MASLD: metabolic dysfunction-associated steatotic liver disease; MCD: Methionine Choline Deficient; MDM: Monocyte Derived Macrophage; NAFL: non-alcoholic fatty liver; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; NF-κB: Nuclear Factor Kappa B; NLRP3: NLR Family Pyrin Domain Containing 3; NOS2: Nitric Oxide Synthase 2; NPC: non-parenchymal cell; PA: palmitic acid; PPAR: Peroxisome Proliferator-Activated Receptor; ROS: reactive oxygen species; SLD: steatotic liver disease; T2DM: Type II Diabetes Mellitus; TGF-b: Transforming Growth Factor Beta; TLR: Toll-like receptor; TNFa: Tumor Necrosis Factor Alpha.

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