Review

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Posted Date: 12 April 2024

doi: 10.20944/preprints202404.0819.v1

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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 vital 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 potential therapeutic targets in this area.

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

1. Introduction

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. MASH has the potential to progress to cirrhosis and ultimately to hepatocellular carcinoma (HCC) [2,3]. Approximately a quarter of the global population is now thought to have MASLD, with the estimated global prevalence of MASH ranging from 3% to 5% [2,4,5]. Additionally, MASLD has emerged as a major cause of end-stage liver failure and is rapidly becoming the leading cause of liver transplantation in the United States [6]. Age, gender,

 doi:10.20944/preprints202404.0819.v1

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 [7–9]. 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 the 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]. They contribute significantly to liver homeostasis, injury, and repair, exhibiting diverse subpopulations that dynamically change in health and disease [19]. In MASLD, macrophages are pivotal in driving inflammation, fibrosis progression, and regression, thus influencing the disease's pathogenesis and progression [3,20,21].

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. Over the past 5 years, there has been rapid expansion in understanding the clinical and research implications of liver macrophage diversity in MASLD. 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 potential therapeutic targets of macrophages in this field.

2. General Overview of Macrophages

Macrophages, a distinct type of cells with unique ability to clear up foreign bodies 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 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 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.

Macrophages are motile and their migrations towards the site of infection and inflammation are critical for their role as effector cells in innate immunity. They express a diverse array of surface receptors facilitating interaction with the host-derived and foreign ligands. These receptors enable

 doi:10.20944/preprints202404.0819.v1

macrophages to sense their environment and perform various functions. Typical examples include phosphatidylserine recognition receptors for apoptotic cells 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. Their adapt to their environment, displaying specialized functions. Despite being fully differentiated; resident macrophages exhibit high plasticity and undergo phenotypic reprogramming in response to changing tissue microenvironment [25,32]. In a healthy state, resident macrophages balance response to foreign particles while minimizing tissue damage. They patrol tissues, phagocytose cellular debris, clean the surroundings, and facilitate tissue repair. Resident macrophages play diverse roles throughout an organism's life span, including involvement in osteoclast and bone remodeling, erythropoiesis, brain development, and lung homeostasis during early developmental stages [33–37]. Their absence can lead to developmental abnormalities in organs like mammary glands, pancreas, and kidneys [38]. For instance, in mice lacking biologically functional colony stimulating factor 1 (CSF-1), the absence of osteoclasts, which are resident macrophages crucial for bone remodeling, leads to the development of osteoporosis—a condition characterized by weakened and brittle bones [39]. In specialized bone marrow areas called erythroblastic islands, macrophages support the production of red blood cells by maintaining cell interactions between erythroblasts and macrophages [40–42] and facilitate erythropoiesis by phagocytizing extruded erythroblast nuclei and supplying iron to erythroid progenitors [43,44]. Microglia, the tissue resident macrophages of the brain and spinal cord, play essential roles in central nervous system (CNS) development, homeostasis, and diseases [45,46]. Alveolar macrophages, found in lungs, help maintain lung function by clearing inhaled dust [47].

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) [48]. Comprising approximately 15% of the total liver cell population, KCs represent 80-90% of all tissue-resident macrophages in the body [49]. Within the liver lobules, 43% of Kupffer cells are distributed in the periportal area, 28% in the midzonal, and 29% in the centrilobular area [50]. KCs are located within the lumen of liver sinusoids with proximity to the sinusoidal endothelial cells that form the blood vessel walls. Despite being considered fixed resident cells, evidence shows that they can exhibit some degree of mobility along sinusoidal walls, either with or against the direction of blood flow [51].

Differential expressions of cell surface markers enable the distinction between KCs and MDMs. In mice, KCs are identified as $CD11b^{low}$, $F4/80^{high}$ and $Clec4F^{+}$, while MDMs are defined as $CD11b^{+}$, $F4/80^{intermediate}$, $Ly6C^{+}$, and colony stimulating factor 1 receptor $(CSF1R)^{+}$ [52–55]. 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 model of liver diseases [56]. Fate mapping experiment revealed that $CSF1R^{+}$ 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 steady state independent of MDMs [27,62]. The advent of single-cell RNA-Seq has facilitated the characterization of human hepatic macrophages, classifying them into three distinct subgroups: $CD68^{+}MARCO^{+}$ KCs, $CD68^{+}MARCO^{-}$ macrophages, and $CD14^{+}$ monocytes [63,64]. The $CD68^{+}MARCO^{+}$ KCs contribute to immunotolerance, while the latter two exhibit a proinflammatory.

 doi:10.20944/preprints202404.0819.v1

KCs act 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 involved several mechanisms. KCs present antigens to promote the arrest of CD4 T-cells and expand interleukin-10-producing regulatory T cells, fostering tolerogenic immunity [65]. In addition, KCs express lower levels of Major Histocompatibility Complex (MHC) class II and other costimulatory molecules like B7-1 and B7-2, resulting in weaker T cell activation compared to dendritic cells [66]. Moreover, KCs generate prostaglandins like PGE2 and 15d-PGJ2 that can suppress dendritic cell-mediated activation antigen-specific T cells [66].

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

In diet-induced disease models of MASLD, the hepatic macrophage numbers increase significantly with the feeding period [67]. The infiltrated proinflammatory $CD45^{+}/CD11b^{+}/F4/80^{intermediate}$ MDMs population in the liver of obese mice nearly doubled compared to lean control [68]. Importantly, diet not only increases the infiltration of MDMs but also disrupts the balance of pro and anti-inflammatory macrophages in the liver [69]. 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 hepatocellular carcinoma (HCC) [70,71]. As KCs number depletes, MDMs infiltrate the liver [72]. Chemokines, small heparin-binding proteins regulating cell trafficking, 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, monocyte/macrophages are the major source [73,74]. CCL2 exerts its effects through its cognate receptor CCR2, whose expression is restricted to certain cell types, including monocytes [75]. Studies in both ob/ob and HFD-fed obese mice models have shown a positive association between hepatic expression of both Ccl2 and Ccr2 and body weight. In study by Morinaga et al. [76], both KCs and MDMs were fluorescently labeled and evaluated in a high-fat diet-fed MASH mice model. The MDMs 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, evident from the significantly elevated expression of CCL2, while MDMs displayed significantly higher expression of CCR2, leading to the conclusion that MDMs contribute to the severity of inflammation and hepatic insulin resistance in MASLD [76]. This group also demonstrated that blocking of the infiltration of MDMs using CCR2 antagonists ameliorated steatohepatitis and fibrosis [77].

Similarly, an increase in macrophage numbers has been observed in liver samples from MASH patients. A retrospective study analyzing liver biopsies from young MASLD patients revealed elevated numbers of $CD68^{+}$ KCs, with higher levels correlating with MASLD severity [78]. Another study showed that the presence of enlarged KCs with significantly elevated phagocytic activity in the hepatic sinusoids closely associates with transformed hepatic stellate cells and hepatic oval cells during MASH, signifies their strong involvement in disease progression [79]. In addition, a significant increase in the number of $CCR2^{+}$ MDMs in human liver samples from MASH patients is strongly correlated with the severity of MASH and fibrosis [76]. Furthermore, an increase in portal macrophage numbers has been observed in individuals with MASL [80]. As the disease progression towards MASH, portal inflammatory infiltrates accompany elevated expression of proinflammatory cytokines IL1B and tumor necrosis factor (TNF) [80]. The presence of increased CD11c-positive macrophages surrounding hepatocytes containing large lipid droplets, forming aggregates known as hepatic crown-like structures, correlates with hepatocytes death and the development of fibrosis in human patients with MASH [81,82]. These aggregates are considered important sources of

 doi:10.20944/preprints202404.0819.v1

inflammation and fibrosis because of their intactness and close association with activated fibroblasts and 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 the de novo lipogenesis, exacerbates oxidative stress and lipid peroxidation in MASLD, thereby aggravating liver inflammation and fibrosis and accelerating MASH progression [83]. When co-culture 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 [84]. Saturated fatty acids, such as lauric acid (C12:0) and palmitic acis (C16:0), induce the expression of inflammatory markers, including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and IL-1α in Raw 264.7 mouse macrophage cell line through the activation of toll like receptor (TLR) 4 and NF-κB pathway [85]. On the contrary, unsaturated fatty acids inhibit the NF-κB pathway, thus impeding the saturated fatty acids-mediated COX-2 expression in Raw 264.7 cells. In addition, palmitic acid activates TLR2 in THP-1 human monocytic cell line, inducing inflammasome-mediated-IL-1β production, suggesting that dietary fat can activate monocytes and promote peripheral inflammation in MASLD [86]. Furthermore, Kim et al. has demonstrated that infiltrating macrophages, rather than resident macrophages, generate reactive oxygen species (ROS) in response to palmitate through the dynamin-mediated TLR4- NADPH oxidase 2 (NOX2) axis. Their study also showed that mice lacking Nox2 are resistant to high-fat diet-induced hepatic steatosis and insulin resistance [87].

The disruption of hepatic cholesterol homeostasis and the accumulation of free cholesterol in hepatocytes are linked to the pathogenesis of MASH [88]. Loannou et al. described the presence of free cholesterol in the hepatocytes of MASH patients and diet-induced MASH mice model. They proposed that the aggregation and activation of KCs in crown-like structures around lipid droplets containing cholesterol crystals are significant indicators of the progression from simple steatosis to MASH [89]. In addition, KCs within the crown-like structures stained positively for NLRP3 and activated caspase 1, indicating a mechanistic link between KC activation exposure to cholesterol [90].

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 [91]. 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 TNFα [92]. Additionally, extracellular vesicles (EV) released from hepatocytes, particularly in response to palmitic acid treatment, contain factors like tumor necrosis factor-related apoptosis-inducing ligand [93]. These EVs induce the expression of IL-1β and IL-6 in mouse bone marrow-derived macrophages.

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 [94]. 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 [95]. In addition, triglycerides enhance LPS-mediated expression of proinflammatory mediators such as inducible iNOS, TNFα, IL-1β, and IL-6 in rat KCs, compared to LPS stimulation alone [96]. Inhibition of the NF-κB pathway significantly reduces the potentiating effect of triglycerides on iNOS expression by KCs [96]. Electron microscopic analysis of KCs from high-fat diet-fed mice reveals intracellular lipid droplet accumulation [97]. 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.

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6. Classical M1/M2 Macrophage Paradigm and MASLD Development

The dynamic heterogeneity and reprogramming of macrophages contribute significantly to the pathogenesis and progression of various liver diseases. 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 an 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 M-1 like macrophages capable of generating nitric oxide (NO) to kill parasites. One the contrary, Th2 cytokines, including IL-4 and IL-10, suppress the activation of M-1 like macrophages, and these M2-like macrophages exhibit elevated arginine metabolism [98,99]. Although the M1/M2 classification oversimplifies the intricate in vivo response, 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.

6.1. Mechanisms Control Macrophage Polarization

6.1.1. TLR and NF-κB

Bacterial endotoxin LPS activates TLR4 expressed 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 [100–102]. 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 [103]. Jing et al., using 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 [104]. In a separate study, Xiang et al. showed that olean-28,13β-olide 2 (NZ) 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 [105]. Similarly, Lu et al. demonstrated that quercetin, a natural flavonoid compound, inhibits LPS-mediated M1 macrophage polarization via the NF-κB and IRF5 signaling [106]. Together, these findings suggest that the macrophage-specific TLR4/NF-κB signaling axis plays an important role in M1 like proinflammatory macrophage polarization.

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 [107,108]. Interferons and TLR signaling polarizes macrophages towards M1 like proinflammatory phenotype via STAT1 signaling, whereas IL-4 and IL-13 tilt macrophages towards M2 like anti-inflammatory phenotype through STAT6 signaling pathways [109]. 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 towards M2 subtype through the JAK1/STAT6 pathway [110]. 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 M2 to M1 subtype [111].

6.1.3. Transforming Growth Factor Beta (TGF-β) and SMAD

Upon TGF-β binding to the TGF-β receptor complex, the receptor complex activation triggers Smads (Smad2 or 3)-mediated or non-Smad signaling pathways to regulate gene expression [112].

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Growth differentiation factor 3 (GDF3), a member of the TGF-β superfamily, can modulate the inflammatory response by influencing macrophage inflammatory phenotypes. GDF3 phosphorylates and activates Smad2/Smad3, inhibiting NLRP3 expression in macrophages and directing macrophage polarization toward the M2 phenotype [113]. Similarly, it has been shown that quercetin-mediated inhibition of the TGF-β1-smad2/3 pathway constrains M2 polarization [106].

6.1.4. Peroxisome Proliferator-Activated Receptor γ

PPARγ belongs to PPAR nuclear receptors family and is mainly expressed in adipose tissue, hematopoietic cells, and the large intestine. As a transcription factor, PPARγ plays a crucial role in glucose and lipid metabolism, anti-inflammatory responses, and the regulation of oxidative stress [114]. Studies have underscored the important association between PPARγ activation and macrophage polarization [115,116]. PPARγ regulates inflammatory processes by directly interacting with NF-κB, resulting in NF-κB degradation [117]. Activation of PPAR-γ by a PPARγ agonist in Raw264.7 macrophages shifts lipid-mediated macrophages polarization from the M1 to M2 phenotype through the interaction between PPAR-γ and NF-κB p65 [118]. Similarly, PPARγ activation promotes native human monocytes toward an anti-inflammatory M2 phenotype [119]. Using mice with macrophage-specific deletion of PPARγ, it was shown that PPARγ is essential for the maturation of alternatively activated macrophage [120]. These results suggest that PPAR-γ is a master regulator M2 polarization of macrophages.

6.1.5. MicroRNAs (miRNAs) and Other Mechanisms

MicroRNAs (miRNAs) have garnered significant interest due to their pivotal roles in macrophages polarization by regulating various signaling pathways [121]. For example, miR-221-3p and MiR-1246 facilitate alternative macrophage polarization, with mechanistical involvement in modulating JAK3/STAT3 and NF-κB signaling pathways [111,122]. Exosomal vesicles derived from adipocytes delivered miR-34a into macrophages, repressing kruppel-like factor 4 (Klf4) expression and consequently inhibiting M2 like macrophage polarization [123]. Beyond these mechanisms, additional signaling pathways, including notch signaling, mammalian target of rapamycin (mTOR) signaling, PI3K/Akt signaling, and JNK/c-Myc signaling pathways, have been identified to play roles in macrophage polarization [124–127].

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 and type 2 diabetes. An initial study using a methionine-choline-deficient (MCD) diet in C57BL/6 and Balb/c mice has demonstrated that C57BL/6 mice with M1 bias displayed elevated liver steatosis and lobular inflammation compared to the Balb/c mice with M2 bias [128]. Further research corroborated that high-fat diet-fed BALB/c mice displayed increased M2 KC polarization, leading to the apoptosis of M1 KCs and mitigating liver steatosis and hepatocyte death compared to C57BL6/J mice, with in vitro experiments demonstrating that M2 macrophages induce M1 apoptosis via IL10-mediated arginase activation [129]. Additional studies found that high-fat diet enriched in polyunsaturated fatty acids promotes the alternative M2 macrophage activation and improves metabolic disturbances [129,130]. The activation of M2 KCs by PPARδ promotes and ameliorates obesity-induced insulin resistance [131]. Histidine-rich glycoprotein (HRGP) is an α2-plasma glycoprotein and 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 [132]. In MCD diet or carbon tetrachloride (CCl_4)-induced chronic liver injury model, macrophages polarization was tipped towards M2 in mice lacking HRGP, attenuating liver injury and fibrosis [133].

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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. Interestingly, the expression of anti-inflammatory macrophage markers such as IL-10, dectin-1, and epidermal growth factor-like module containing mucin-like hormone receptor 1 (EMR1) are also induced in MASH, suggesting a reparative role of M2 macrophages following tissue injury, which may contribute to fibrosis development [81,134]. 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 [135,136]. The scavenger receptor CD163 is considered as 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 [137]. 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) [55]. During the early stages of MASLD, KCs engage in lipid storage, compromising their ability for self-renewal [138]. Consequently, embryonic KCs are gradually lost and replaced by MDMs lacking Timd4 expression [139,140]. Mulder K et al. integrated 41 mononuclear phagocytes scRNA-seq datasets to compile a comprehensive monocytes-macrophage-focused compendium, revealing a diverse array of specialized cell subsets distributed across multiple tissues [141]. 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 [141]. 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 [142–144]. Several studies in mice have elucidated various roles of $TREM2^+$ macrophages in liver disease, demonstrating protective functions for hepatocytes in MASLD and cholangiopathies [145,146], immunosuppressive roles in hepatocellular carcinoma, and contribution to supporting liver regeneration in both acute and chronic murine injury models [143,147]. 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 [148].

Similarly, transcriptional profiles obtained from scRNA-seq analysis of parenchymal and non-parenchymal cells in human livers unveils distinct subsets of hepatic macrophages [63]. The first subset, $CD68^+MARCO^-$ macrophages exhibit characteristics of pro-inflammatory macrophages with 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 of $MARCO^+$ $TIMD4^-$ KCs observed in livers from cirrhosis patients [149].

These studies collectively suggest that macrophages exhibit a wider spectrum of phenotypic activation profiles during MASLD development than previously recognized. The integration of

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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 intertwined with macrophage function. In the past decade, the 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 and revealed spatially specific responses that influence liver disease progression [150–152].

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) [140]. Unlike KCs, MDMs are characterized by their smaller size and circulate through the sinusoids [140]. Their 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. 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 the weaning in mice, murine KCs tend to cluster around periportal regions, influenced by MYD88-dependent signaling activation in liver sinusoidal endothelial cells triggered by gut-derived bacteria, suggesting the significance of KC zonation in controlling pathogen dissemination [153]. In addition, spatial transcriptomics of healthy liver tissue unveils non-inflammatory macrophage genes and signatures in periportal regions and inflammatory counterparts closer to the central vein [154].

During liver injury, the hepatic macrophage landscape undergoes significant changes associated with disease stages. For instance, through conditional depletion of liver KCs, researchers demonstrate that Ly6C-high monocytes, when recruited, could replenish the KC pool with the recruited monocytes differentiating into F4/80+ monocyte-derived KCs and their subsequent CSF1R-dependent proliferation reaching the steady-state KC density by day 6 after depletion, a process occurring throughout the entire liver parenchyma without restriction to a particular hepatic zone [140]. In contrast, during MASLD development, 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 [155]. Additionally, they found that KC development crucially depends on their cross-talk with hepatic stellate cells via the ALK1-BMP9/10 axis [155]. Aligned with this finding, another study that utilized human liver samples form MASLD and primary sclerosing cholangitis patients combined RNA-seq and imaging cytometry, revealing intense aggregation of $IBA1^{+}CD16^{low}CD163^{low}$ MDM-derived macrophage in nonparenchymal areas, exhibiting a distinct spatial proximity to $CK19^{+}$ ductular cells in periportal areas [156]. Furthermore, they find that the loss of hepatocytes and increased ductular reaction tightly correlates with the accumulation of $IBA1^{+}CD163^{low}$ MDMs, serving as a prominent immunological feature that underscores the progression of MASLD, primary sclerosing cholangitis, primary biliary cholangitis, and alcoholic hepatitis.

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 [157,158]. Depletion of resident KCs in rats using gadolinium chloride has shown a protective role against diet-induced alterations in hepatic lipid metabolism and insulin sensitivity [159]. In monocytes/macrophages and KCs, the glucocorticoid receptor (GR) and glucocorticoid induced leucine zipper (GILZ) axis is involved in a variety of inflammatory processes, significantly contributing to the pathogenesis of diet-induced liver inflammation. Knockdown of Gilz renders KCs more susceptible to LPS, and transgenic mice

 doi:10.20944/preprints202404.0819.v1

overexpressing macrophage-specific Gilz significantly reduce obesity-induced liver inflammation [160].

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 [161]. 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 [137]. Galectin-3, a member of the endogenous lectin family with the ability to bind to terminal galactose residues in glycoproteins, has been extensively studied for its role in acute inflammation. It modulates immune cell adhesion and migration, cytokine production, phagocytosis, and immune cells survival [162–165]. Traber et al. investigated two galectin-3 binding carbohydrate drugs to treat MASH in a high-fat diet-fed mouse model. They reported that galectin-3 targeting drugs ameliorated MASH pathologies, suggesting a mechanistical involvement of macrophages [166].

10. Conclusions

Hepatic macrophages are a crucial cellular component of the liver and play vital roles in liver function, contributing significantly to tissue homeostasis and swift responses during pathophysiological conditions. While significant progress has been made in recent decades regarding the role of macrophages in MASLD, the functional activation of macrophages in the disease remains inadequately understood. Despite the identification of various proteins and signaling pathways implicated in macrophage activation, 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. 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, ultimately facilitating the development of personalized and targeted interventions for MASLD and other liver diseases.

Author Contributions: F.A. performed the literature search and wrote the first draft. N.E., E.J. and Y.Z. edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received funding from National Institutes of Health grants R01DK119131 and K22CA184146. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The author declares no conflict of interest.

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:

 doi:10.20944/preprints202404.0819.v1

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: Nonalcoholic 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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