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Spatial dimension of macrophage heterogeneity in liver diseases

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ABSTRACT

The structural and cellular organisation of the liver has unique features that define it as both a metabolic and an immunological organ. Noteworthy, liver resident macrophages, named Kupffer cells, represent the most frequent tissue resident macrophage population in the human body. Nonetheless, on acute or chronic tissue injury, Kupffer cells seem rather static and may undergo cell death, while the liver is massively infiltrated by circulating immune cells such as bone marrow-derived macrophages, also termed monocyte-derived macrophages, which drastically alter the hepatic immune landscape. Over the last decade, our knowledge on liver macrophage populations during homeostasis and liver diseases has greatly expanded. This particularly holds true in light of the recent fast-paced technological advances that brought novel dimensions to our knowledge, either in single-cell suspensions, in a two-dimensional plane or a three-dimensional space, or even in time-lapse (intravital) microscopy. This novel understanding goes from unravelling a previously underestimated macrophage diversity (eg, in terms of activation phenotype or cellular origins) to identifying spatially or temporally restricted responses that drive liver disease outcome. This review aims at providing insights into the most recent breakthroughs in our understanding of liver macrophage biology and its roles in liver (patho)physiology, in a four-dimensional perspective.

INTRODUCTION

The liver holds central roles in the organism's metabolism, ranging from energy storage to bile secretion, drug detoxification and protein synthesis. Furthermore, the liver receives two-thirds of its blood supply from the portal vein, originating from the intestines and carrying foodborne nutrients or toxins, as well as microbial products. These vital functions designate the liver as a vividly active metabolic organ which must respond to rapidly changing blood contents and recurring exposure to pathogen-associated molecular patterns (PAMPs) or tissue-damaging toxic compounds, for instance. As a result, hepatocytes, representing the vast majority of liver parenchymal cells, possess a vast array of tunable metabolic functions and may re-enter the cell cycle to compensate for cellular loss resulting from acute or chronic injuries.[1–3]

The liver also possesses alternative regenerative processes that imply the mobilisation of bile duct-associated ductular cells or liver progenitors. Liver regeneration as such is supported by a dynamic extracellular matrix content remodelling, which on sustained injury is mostly conducted by hepatic stellate cell-derived myofibroblasts.[4] Despite possessing tremendous regenerative capacities and metabolic versatility, the hepatic regenerative response is still subject to potential exacerbation on severe or chronic insults, ultimately leading to excessive extracellular matrix deposition (ie, fibrosis) and uncontrolled cellular proliferation, with increased risk of carcinogenesis, metabolic dysregulation and sustained inflammation.

Noteworthy, liver macrophages have been directly implicated oftentimes with both beneficial or detrimental functions in all of the above-mentioned physiological or pathological aspects of liver (patho)physiology.[5] Classically, macrophages have been categorised according to the M1/M2 paradigm. However and as previously reviewed, this now appears to be outdated in light of recent high-end technologies applied to deciphering the liver macrophage universe.[6] Nonetheless, just within the last 5 years, the views on clinical and research implications of liver macrophage diversity kept expanding at a fast pace. Moreover, individual cell spatial localisation is increasingly recognised as a crucial, function-defining parameter that must be integrated into advanced multidimensional analyses. For all these reasons, this review aims at describing the most recent advances in our understanding of liver macrophage diversity and functions, with a particular focus on the novel research angles, namely single cell and spatially resolved analyses.

THE SPECIFICITIES OF THE LIVER MICROENVIRONMENT DRIVING KUPFFER CELL FUNCTIONS

Kupffer cells (KCs) represent the liver resident macrophages, which derive from

embryonic liver precursors. KCs' uniqueness resides in their ability to contribute to the liver metabolic functions, notably by participating in lipid and iron metabolism, and their crucial roles in supporting liver regeneration.[7] In addition, the liver is populated by multiple monocyte and macrophage populations, such as capsular or peritoneal macrophages.[8] However, in most acute and chronic liver diseases, two main populations are drawing a lot of interest due to their complementary functions: the liver resident macrophages (KCs) and the monocyte-derived macrophages (MoMFs). KCs originate from embryonic liver progenitors and are capable of self-renewing.[3 9] They are located within the hepatic blood sinusoids and present long cytoplasmic expansions allowing them to sense blood content changes. KCs are also characterised by potent phagocytic capacities, regarded as the immune system sentinels, able to drive a rapid immune response on tissue injury, lipid exposure or PAMP stimulation, but also making them responsible for detrimental compounds trapping in the liver and making them a privileged port of entry for liver-targeted interventions.[10 11] Nonetheless, KC-driven response is believed to be mostly immune-tolerant, which is regarded as a protective mechanism against excessive immune response towards harmless antigens during homeostasis.[12]

On acute and specific depletion of KCs in mice, MoMFs are recruited to acquire a KC-like phenotype. This generation of 'bone marrow-derived KCs' is driven by multiple signals, such as chemokines, growth factors and adherence proteins, expressed by hepatocytes, liver sinusoidal endothelial cells and hepatic stellate cells.[13] All these events occur within a few hours, defining a 'transient window' of niche availability.[13] During homeostasis, MoMFs patrol the liver through the hepatic sinusoids. However, in most settings of tissue injury, MoMFs originating from the bone marrow are recruited to the liver. MoMFs are characterised by their enormous plasticity, making them a myeloid cell population with a wide range of functions and a broad phenotypic diversity.[14]

SINGLE-CELL RNA SEQUENCING REVEALED MACROPHAGE DIVERSITY

Recent years have seen a sharp increase in single-cell RNA sequencing data generation, mostly allowed by the systematic use of this approach and easier access to reagents, materials and bioinformatics.[15] In the liver in particular, these studies greatly advanced our understanding of cellular diversity and immune cell subpopulation changes under defined healthy or diseased conditions. More notably, single-cell RNA sequencing brought an end to the formerly well-established M1/M2 macrophage paradigm. Indeed, it is now well described that macrophages present a much broader diversity than previously thought in terms of phenotypic activation profiles.[16–19] This diversity has prompted the generation of a macrophage landscape tool that has been appropriately named after the monocyte–macrophage universe or 'MoMac-VERSE'.[18] For instance, TREM2-expressing macrophages were identified in liver cancer.[18] Mouse models identified them as MoMFs. Single-cell sequencing similarly identified TREM2$^+$ CD9$^+$ scar-associated macrophages in human liver cirrhosis, which exhibited a profibrogenic gene signature notably characterised by *TREM2*, *IL1B* and *CCR2*.[17] Multiple studies in mice further established some functions of TREM2$^+$ macrophages in liver disease, and it has been shown that TREM2$^+$ macrophages exert hepatocyte protective functions in non-alcoholic steatohepatitis (NASH) and cholangiopathies, immunosuppressive functions in hepatocellular carcinoma, and even support liver regeneration in murine models of acute (acetaminophen) or chronic (carbon tetrachloride) injury followed by regression.[20–25] This series of studies on TREM2$^+$ macrophages very well illustrates the value of multidimensional analyses for opening novel research avenues by highlighting markers of interest that might not have been identified yet. Nevertheless, they also highlight the important need for functional studies. Furthermore, single-cell sequencing systematically performed on multiple cellular sources helps unravel cell ontogeny and the consequences of a pathology or a model on multiple organs. This strategy revealed, for instance, potent changes in both liver MoMFs and their bone marrow precursors in a model of Western diet-induced liver steatosis.[16] This study further described the broader changes in MoMF phenotypes in this NASH model, which could be mimicked by in vitro free fatty acid stimulation of bone marrow monocytes.[16] Importantly, Western diet-fed mice showed lower injury following acute acetaminophen injection, as compared with normal diet-fed animals. This protective effect was not due to a modified acetaminophen metabolism, but attributed to a reduced myeloid cell-driven inflammatory response to acetaminophen.

Moreover, single-cell transcriptomics coupled with advanced bioinformatics helped in predicting novel cellular interactions notably by using the software tool CellPhoneDB, as well as highlighting macrophage plasticity by RNA velocity studies.[26–30] However, one major caveat of transcriptomic analyses performed on singular cell suspensions is that they lack spatial resolution. Furthermore, the relative frequencies of specific cell populations may be either increased or decreased depending on the method used for sample preparation. This latter pitfall seems to be reduced by the use of single-nucleus sequencing instead of single-cell sequencing.[31] Applying single-nucleus sequencing notably enabled transcriptomic analysis of cells that are generally challenging to isolate from liver tissues, such as cholangiocytes and mesenchymal cells.[31] The limitations regarding the spatial contextualisation, on the other hand, may only be addressed by either using localised tissue microdissections or probe barcoding, or by using alternative approaches such as multiplex immunostaining or in situ sequencing.[32]

SPATIAL LOCALISATION AS A NOVEL PHENOTYPIC MARKER

The liver anatomical location matters for its function in the organism. Also, at a cellular scale, hepatocyte spatial zonation has been well described and shown to be essential for liver metabolic functions.[33 34] This is increasingly recognised as true for liver macrophages as well. Their location within the hepatic lobules appears to be intrinsically linked to KC subpopulation functions. As such, KCs are located within the liver sinusoids and harbour numerous cytoplasmic expansions within the sinusoids, and prolonged through the space of Disse to as far as the hepatocytic cellular layers.[13] As a consequence, KCs are ideally located not only to sense changes occurring in the bloodstream (eg, pathogens, toxins), but also hepatocyte stress. This goes along with their attributed functions in organ immune tolerance and acute response. Interestingly, KCs are also subject to varying spatial distribution in the centrilobular–portal axis. Indeed, Gola *et al*[35] evidenced that murine KCs are specifically accumulating around the portal areas during weaning of offspring mice from their mothers, a phenomenon absent in germ-free mice or in mice with a defect in MyD88 signalling in liver sinusoidal endothelial cells, revealing its dependence on gut-derived microbial antigens. Their manuscript also suggested that KC zonation was crucial to the control of pathogen dissemination. While it is recognised that distinct subpopulations of KCs coexist even at steady state, their differential distribution remains to be demonstrated on a larger scale for in-depth understanding of the functional zonation of KCs, despite a study previously reporting that at least two major KC populations are evenly distributed in overlapping regions.[19]

Contrastingly, MoMFs are circulating through the blood sinusoids and the larger blood vessels, and are of smaller sizes and lower shape complexity, as compared with KCs.[13 36] Noteworthy, a population of macrophages, mostly derived from bone marrow-derived macrophages, seem to home to the vicinity of bile ducts in the healthy liver.[37] This particular localisation may define them as primary responders to bile duct leakage or portal vein-borne pathogens, although they were shown to be less responsive than KCs to Toll-like receptor (TLR) 4 stimulation.[37] In the healthy liver, a small population of these portal area-located macrophages were identified as 'lipid-associated macrophages' (LAMs) due to their similarities with macrophages accumulating during liver steatosis. In the same line, spatial transcriptomics applied to healthy livers revealed a zonation of liver macrophages, with the subpopulations present in the centrilobular areas exhibiting a more proinflammatory phenotype than the macrophages located near the portal areas.[31]

During tissue injury, the overall liver macrophage landscape drastically changes in a disease stage-associated manner, and two-dimensional microscopy has proven itself to be a very efficient tool for assessing this phenomenon at a large scale and on numerous human and animal samples. We and others notably evidenced a switch from a KC-dominant to a MoMF-dominant liver macrophage pool in conditions such as human non-alcoholic fatty liver disease (NAFLD), primary sclerosing cholangitis (PSC) or acetaminophen-induced acute injury mouse models.[38–40] In human NAFLD and cholangiopathies, these changes appear to be particularly localised to the portal areas and to strongly correlate with disease progression markers.[36 37 41–43] This novel research area was well illustrated in an exemplary manuscript by Guilliams *et al*,[37] relying on a combination of proteomic and transcriptomic approaches. In their study, the authors showed evolutionary conserved and spatially restricted hepatic macrophage niches in the healthy liver and could reveal potent changes during steatosis. For instance, LAMs, notably defined as *Gpnmb*$^{+}$*Spp1*$^{+}$ in mouse, accumulated in the centrilobular areas where the steatosis was particularly marked. In line with this, we recently evidenced the accumulation of hepatic IBA1^{+}CD163^{low}CD16^{low} macrophages in the vicinity of CK19^{+} ductular cells in periportal areas, meaning the association of both phenotypic marker expressions and spatial neighbouring, to be predictive of disease progression in large NAFLD and PSC patient cohorts.[36] Of note, CD16 and CD163 are considered as essential functional receptors for macrophage phagocytic functions. In line with earlier studies, these observations demonstrate that the chronically injured liver is populated with MoMFs that harbour reduced phagocytic capacities, as compared with the KCs observed during homeostasis. Altogether, these findings represent robust proofs of an 'immunological scar' that may influence not only liver disease progression, but also organism response to future insults.

MACROPHAGE IN SITU DYNAMICS

In addition to their spatial localisation, another dimension appears crucial for a proper macrophage functional characterisation: time. Real-time imaging technologies drastically changed our vision of immune response and its timing, and this is particularly true for time-lapse in vivo imaging. One of the major studies using this approach demonstrated the rapid and localised recruitment of GATA6^{+} peritoneal macrophages to the site of a local, superficial and sterile heat-induced liver injury in mice.[44] These recruited macrophages showed varying levels of C–C chemokine receptor 2 (CCR2) and chemokine (C-X3-C motif) receptor 1 (CX3CR1) expression, thus highlighting the heterogeneity of recruited macrophages even in acute response. Similarly, liver macrophages do respond in a time-constrained manner to a disruption in liver macrophage homeostasis. On the other hand, KCs appear relatively immobile at steady state and on liver injury.[44] The directed migration of MoMFs towards the site of injury appears crucial for cellular debris clearance and for the initiation of a proper tissue response. Nonetheless, MoMF recruitment, notably through CCR2, may represent a driver of disease progression. This is exemplary shown by earlier studies evidencing a role of CCR2/CCR5 in aggravating mouse phenotypes in

acetaminophen-induced liver injury.[38] Indeed, CCR2-expressing MoMFs were shown to transit through the healthy liver circulation rapidly, but to decelerate and accumulate at sites of active inflammation on injury. Similarly, it was shown that MoMFs transit the mouse liver at an average speed of 8 µm/min at steady state.[13] However, just 24 hours after diphtheria toxin-induced KC depletion in transgenic mice, Ly6C$^+$ MoMFs lost their mobility and started to exhibit prolonged cytoplasmic expansions, as well as to express KC-specific gene signatures, revealing a switch towards a KC-like phenotype.[13] In this model, the authors demonstrated that *Ccr2* deficiency resulted in having four times less MoMFs in the liver, 2 days after KC depletion. Thus, MoMFs' migratory properties represent a target of interest for therapeutic purposes, which may be directly visualised and quantified notably by time-lapse microscopy.

As discussed above, time-lapse in vivo imaging further supported the concept of a relative immobility of KCs in homeostasis and upon acute injury.[13 45] In vivo time-lapse microscopy has indeed been used to visualise platelet clearance by KCs during homeostasis in mice.[46] This approach elegantly showed that clodronate-loaded liposome-mediated KC depletion led to a deficiency in aged platelet removal from the circulation, while platelet desialylation increased their uptake by KC and degradation through the involvement of macrophage galactose lectin.[46] Interestingly, this study also reported that MoMFs do not seem to perform such function. Time-lapse in vivo microscopy also allowed for the visualisation in real time of both KC and MoMF response to triggered acute injury.[45 47] This notably demonstrated an overall reduction in liver macrophage phagocytic capacities during chronic liver injury in mice and humans.[45 48] More specifically, patients with liver cirrhosis showed reduced liver uptake and plasma disappearance of radiolabelled colloid than healthy subjects. This result was obtained by single-photon emission CT combined with CT scan, which allows visualisation of in vivo macrophage abilities in patients. This loss of liver macrophage phagocytosing abilities was particularly high in patients with decompensated cirrhosis, as compared with patients with compensated cirrhosis. Importantly, this observation was associated with increased risks of bacterial infections. This has been attributed to an immunosuppressive phenotype in patients and notably increased programmed cell death ligand 1 (PD-L1) expression. This increased PD-L1 expression was also observed in murine KCs in a 3,5-di ethoxycarbonyl-1,4-dihydrocollidine (DDC) diet model, which was regarded as a response to interferon gamma (IFN-γ).[48] Lastly, the authors demonstrated that a short (24 hours) anti-PD-L1 antibody treatment improved liver macrophage abilities to phagocyte labelled *Escherichia coli* in mice fed a DDC diet for 3 weeks.

Interestingly, time-lapse microscopy is also relevant for in vitro investigations. As such, Liao *et al*[49] reported on the chemotactic effects of sphingosine-1-phosphate (S1P) contained in hepatocyte-derived extracellular vesicles in a microfluidic gradient setting. This mechanism is potentially opening novel research axis for NASH, since hepatocyte S1P expression is increased on palmitic acid exposure in NASH. New technical advances, such as multicellular organoids or perfusable, three-dimensional biochips, are currently being evaluated that aim at facilitating further studies on molecular mechanisms driving the dynamic adaptations of cellular phenotypes based on cellular crosstalk.[50 51] The combination of different organ model systems, for example, gut-on-a-chip connected with liver-on-a-chip, may help decipher molecular mechanisms of interorgan communication.[52]

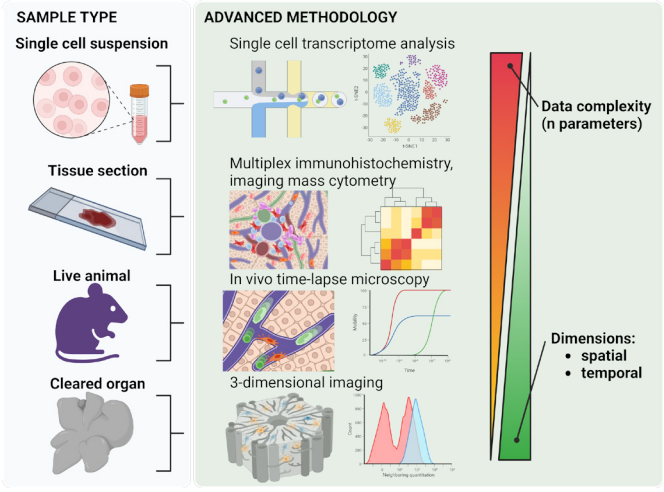

Figure 1 Recent technological breakthroughs greatly advanced our understanding of liver macrophage spatiotemporal diversity. For instance, single-cell suspensions subjected to single-cell transcriptome analysis revealed the simultaneous presence of multiple immune cell phenotypes. Multiplex immunohistochemistry and imaging mass cytometry showed complex and spatially restricted accumulation of defined monocytic populations. In vivo time-lapse microscopy unravelled the timing of the immune response and dynamic macrophage functionality. Organ clearing and three-dimensional imaging further deepened in situ macrophage biology understanding.

CONCLUSIONS

Liver macrophages comprise a heterogeneous population of phagocytes with specific adaptations in their phenotypes to the microenvironment. Therefore, the functional contribution of a macrophage population to liver health and disease progression or regression can only be fully captured if their spatial contextualisation is considered. While two-dimensional (and three-dimensional) static microscopy offers tremendous capacities in terms of number of parameters to be analysed, it lacks dynamic information. On the other hand, in vivo time-lapse microscopy generally does not allow for numerous markers to be analysed simultaneously, and at present mostly focused on a relatively small tissue area and at a low tissue depth (see figure 1). Due to the complexity of the hepatic macrophage landscape and the fast-paced technological breakthroughs that bring novel

Table 1 Hepatic macrophage populations (selection)

Common denomination	Phenotypic marker	Spatially resolved features	Assumed roles in liver biology and pathology	References
Kupffer cells (KCs)	IBA1^{+} CD16^{high} CD163^{high}, VSIG4^{+}, *CD5L*$^{+}$ (human) IBA1^{+} CLEC4F^{+} (mouse)	► Large cytoplasmic expansions. ► Located within the hepatic sinusoids. ► Sessile/low motility. ► Midzonal localisation.	► Aged platelet removal from the circulation. ► Lipid and iron metabolism. ► High phagocytic capacity.	7 36 37 42 46
Monocyte-derived macrophages (MoMFs)	IBA1^{+} CD16^{low} CD163^{low} (human) CCR2^{+} CXCR1^{+} Ly6C^{+} (mouse)	► Smaller size than KCs. ► Round-shaped or oval-shaped. ► Rapidly mobilised to sites of injury or active inflammation.	► Less responsive to TLR4 stimulation than KCs. ► KC replacement on resident macrophage depletion. ► Release of inflammatory and fibrogenic mediators.	16 36 38 42
Lipid-associated macrophages (LAMs)	*Gpnmb*$^{+}$ *Spp1*$^{+}$, lower levels of *Il1b*, *Tnf* and *Il10* during steatosis (mouse)	► Preferentially localised in the portal areas during homeostasis. ► Accumulating pericentrally in steatotic livers.	► Less responsive than KCs to lipopolysaccharides.	37
Non-alcoholic steatosis-associated macrophages	S100A8/A9^{low} (mouse)	–	► Dependent on TLR4 signalling. ► Hepatoprotective roles in acetaminophen-induced liver injury.	16
TREM2-positive macrophages	TREM2^{+}	–	► Hepatoprotective in non-alcoholic steatohepatitis and cholangiopathies. ► Immunosuppressive in liver cancer.	18 20–25
Scar-associated macrophages	TREM2^{+} CD9^{+}, *IL1B* (human)	–	► Profibrogenic in human liver cirrhosis.	17
Peritoneal macrophages	GATA6^{+} CCR2^{+} CX3CR1^{+} (mouse)	► Mostly recruited to the subcapsular areas.	► Mobilised during superficial organ injuries.	8 44

This table summarises monocyte and macrophage denominations across the studies cited in the review article and highlights the overlap in current definitions of subsets. This needs to be considered when comparing functional roles of macrophage subsets identified in different experimental settings.
CCR2, C–C chemokine receptor 2; CD, cluster of differentiation; CLEC4F, C-type lectin domain family 4 member f; CXCR1, chemokine (C-X3-C motif) receptor 1; GATA6, GATA Binding Protein 6; Gpnmb, glycoprotein nonmetastatic melanoma protein B; IBA1, Ionized calcium-binding adaptor molecule 1; IL/Il, interleukin; Ly6C, lymphocyte antigen 6 complex; Spp1, secreted Phosphoprotein 1; TLR4, toll like receptor 4; Tnfa, tumour necrosis factor alpha; TREM2, triggering receptor expressed on myeloid cells 2; VSIG4, V-set and immunoglobulin domain containing 4.

dimensions to macrophage biology studies, consensus on macrophage denominations for deciphering the overarching functions of specific subpopulations would be desirable (see table 1). Further developments are expected in the near future, in particular to allow for multiple omics to be acquired from a singular sample on a large scale and with a high number of parameters (eg, immunostaining combined with in situ messenger RNA sequencing). Technology development and novel technology generalisation will mostly depend on their complexity (ie, both technically and end-to-end data generation) and their accessibility to a broader research community (ie, associated costs and personnel needs). Such data can be expected to revolutionise our understanding on liver macrophage biology, with the aim to develop personalised and targeted interventions in conditions of acute or chronic liver diseases.

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